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A green tea-containing starch confection increases plasma catechins without protecting against postprandial impairments in vascular function in normoglycemic adults^{†,‡}

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Abstract

Postprandial hyperglycemia (PPH) increases cardiovascular disease risk regardless of glucose intolerance by transiently impairing vascular endothelial function (VEF) by limiting nitric oxide bioavailability in an oxidative stress-dependent manner. Preclinical studies show that green tea catechins attenuate PPH by inhibiting starch digestion. We hypothesized that a starch-based confection containing catechin-rich green tea extract (GTE) would limit PPH-mediated impairments in VEF in normoglycemic adults. We formulated a unique GTE confection and then conducted a double-blind, randomized, controlled, crossover study in healthy men ($n = 15$; 25.3 ± 1.0 years; 22.4 ± 1.8 kg m⁻²) in which they ingested starch confections (50 g carbohydrate) formulated with or without GTE (1 g) prior to evaluating sensory characteristics of confections and plasma glucose, biomarkers of lipid peroxidation and nitric oxide homeostasis, and brachial artery flow-mediated dilation (FMD) at 30 min intervals for 3 h. Sensory evaluation of confections indicated acceptable consumer appeal and an inability to distinguish between confections regardless of GTE. Plasma catechins concentrations increased following ingestion of the GTE confection. However, plasma glucose peaked at 60 min ($P < 0.05$) following confection ingestion and was unaffected throughout the postprandial period by the GTE confection ($P > 0.05$). FMD was significantly decreased only at 60 min regardless of confections containing GTE. Also at 60 min, both confections similarly increased plasma malondialdehyde while decreasing arginine and increasing asymmetric dimethylarginine/arginine. The successfully formulated GTE-containing confection effectively delivered catechins, but without mitigating PPH-mediated impairments in VEF in association with oxidative stress that likely limits nitric oxide bioavailability.

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Introduction

Cardiovascular disease (CVD) is the leading cause of death globally, contributing to 17.3 million deaths annually with an estimated \$47 trillion in medical costs projected by 2030.¹ Although numerous factors contribute to CVD, postprandial hyperglycemia (PPH) has been suggested to be an early, and better predictor of future CVD compared with fasting glucose.² The mechanism by which PPH increases CVD risk is under active investigation, but postprandial excursions in blood glucose are implicated in increasing oxidative stress responses that impair nitric oxide (NO•) homeostasis and induce vascular endothelial dysfunction (VED).³

VED, which is characterized by impairments of the vascular endothelium in response to stimuli that promote vasodilation, is well-associated with traditional CVD risk factors (*e.g.* aging, smoking, hypercholesterolemia, hypertension, diabetes).^{4–8} Brachial artery flow-mediated dilation (FMD) is a validated, non-invasive ultrasound-based approach that assesses vascular endothelial function (VEF) in humans.³ FMD responses have prognostic value in predicting future CVD events,⁹ and are augmented by lifestyle changes that regulate CVD risk.³ We and others have demonstrated that PPH following an oral glucose challenge impairs postprandial VEF.³ Specifically, postprandial glycemia is inversely correlated to FMD in normoglycemic adults¹⁰ or those afflicted by metabolic syndrome.¹¹ PPH-mediated impairments in vascular function are also accompanied by increases in postprandial lipid peroxidation and the ratio of asymmetric dimethylarginine relative arginine (ADMA/ARG),^{10,11} an index that predicts NO• status.³ Of importance, ARG, the amino acid required for NO• biosynthesis, decreases whereas ADMA, a competitive inhibitor of endothelial nitric oxide synthase (eNOS), increases in an oxidative stress-dependent manner.^{3,12}

The involvement of PPH-mediated oxidative stress in impairing VEF supports dietary strategies that reduce CVD risk directly by attenuating postprandial glycemic excursions or indirectly by ameliorating pathologic insults downstream of PPH. Although chronic ingestion of green tea (*Camellia sinensis*; 5 servings per d) is associated with a lower risk of CVD-related mortality in older Japanese adults,¹³ the mechanism underlying this cardioprotective activity has not been fully defined.¹⁴ Green tea is rich in catechins, including its predominant catechin epigallocatechin gallate (EGCG), and epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) that are present to a lesser extent.¹⁵ Findings from experimental models support green tea catechins in regulating macronutrient digestion and absorption, energy metabolism, and exerting antioxidant activity,^{15–17} and suggest that their acute ingestion may limit PPH-mediated increases in oxidative stress leading to VED.

Evidence *in vitro* and from experimental models support that green tea may be effective in reducing PPH. Studies in mice show that co-ingestion of EGCG with corn starch, but not maltose, sucrose, or glucose, attenuated PPH without affecting serum insulin or small intestinal glucose transporter expression.¹⁸ Enzyme kinetic studies in a cell-free system further show that EGCG and EGC inhibit pancreatic α -amylase activity.¹⁸ However, EGCG was more effective and exhibited noncompetitive inhibition on α -amylase at concentrations likely occurring in the small intestinal lumen following green tea ingestion.¹⁸ However,

green tea catechin-mediated regulation of PPH in humans has not been well translated, especially in relation to VED. The potential benefits of green tea on PPH-mediated VED may also be limited in westernized countries since its consumption is lower than from Asian countries.¹⁹ However, Americans consume up to 24% of their daily energy from snack foods.²⁰ This suggests a convenient approach to potentially mitigate PPH by formulating a green tea-containing snack food that effectively delivers catechins while also attenuating PPH by limiting pancreatic α -amylase-dependent hydrolysis of starch. We therefore conducted the present study to formulate a starch-based confection containing green tea extract (GTE) having sensory attributes that would be acceptable to consumers. We then performed a randomized, double-blind, placebo-controlled intervention study to investigate the extent to which a novel GTE-containing starch-based confection would mitigate PPH-mediated impairments in VEF occurring in response to a starch-based confection.

Methods and materials

Materials

All solvents were of HPLC-grade or higher and were purchased from Thermo Scientific (Pittsburgh, PA) as were the following chemicals: butylated hydroxytoluene (BHT), diethylenetriamine pentaacetate (DTPA), monomethylarginine, *ortho*-phthalaldehyde (OPA), 3-mercaptopropionic acid, boric acid, potassium phosphate monobasic anhydrous, and trichloroacetic acid (TCA). The following were from Sigma (St Louis, MO, USA): caffeine, EC, ECG, EGCG, phosphate buffered saline (PBS), 2-thiobarbituric acid (TBA). Decaffeinated powdered GTE containing 82% (w/w) total catechins (63% EGCG, 18% EGC, 10% ECG, and 9% EC) as verified by HPLC-UV²¹ was from Taiyo International (90LB Sunphenon; Minneapolis, MN).

Formulation of confections

A sugar-free confection containing a substantial quantity of dent corn starch (32% wet basis) was required to meet the objectives of this study. Indeed, confections were specifically formulated in this study to examine the putative glucose-lowering activities of GTE thought to be mediated through the inhibitory effects of EGCG on pancreatic α -amylase activity.¹⁸ Confections were formulated (Table 1) using corn starch (73 : 27 amylopectin : amylose) with the goal of delivering 1 g decaffeinated GTE for each 50 g of starch. The quantity of starch was selected based on data suggesting this dietary carbohydrate intake would elicit acute hyperglycemic responses that induce postprandial impairments in vascular function.^{22,23} Decaffeinated GTE was specifically used to examine the inhibitory activities of its catechins on acute hyperglycemia while minimizing the confounding effects of caffeine on vascular reactivity.²⁴ EGCG-rich GTE was selected to recapitulate studies in mice showing that EGCG attenuates postprandial glycemic responses and *in vitro* indicating that EGCG inhibits α -amylase activity.¹⁸ GTE at 1 g was chosen to deliver the equivalent of 5 servings (120 mL per serving) of freshly brewed green tea consistent with epidemiological studies suggesting that this intake level reduces the risk of CVD-related mortality by 26%.¹³ Confections were prepared by mixing corn starch with distilled water, erythritol, glycerin, guar gum, and citric acid (Table 1). Apple flavoring was used to mask the confections and ensure treatment blinding. The mixture was stirred (90 °C; 65–68° Brix) to form a gel, and

then rapidly cooled to 40 °C using an ice bath while homogeneously mixing GTE into the gel prior to pressing into 12 edible cubes of approximately 2 cm³. After curing overnight, confections were packaged in airtight amber bags, stored under ambient conditions and administered to participants within 72 h. Small portions of each confection batch were also stored at -80 °C to assess long-term catechin stability. Catechin content of confections were analyzed as described below.

Differential scanning calorimetry (DSC)

Since water comprised only ~48% of the formulation, a differential scanning calorimeter equipped with a refrigerated cooling system (Model Q100; TA Instruments, New Castle, MA) and nitrogen gas for purging the DSC chamber was used to verify completeness of cornstarch gelation. Samples ($n = 3$ replicates) were each collected from three separate batches of the confection slurry mixture prior to and after preparation of the confections as well as from the center of prepared confections cured for 72 h. Samples (15–20 mg) were analyzed in hermetically sealed stainless steel pans (PerkinElmer; Boston, MA) with an empty pan used as a reference. DSC calibration was performed with indium at a 5 °C min⁻¹ ramp. Samples were cooled to -50 °C and isothermally held for 5 min prior to being heated to 150 °C at a 5 °C min⁻¹.²⁵ Heat flow was recorded in W g⁻¹ and plotted against temperature. Transitions identified from DSC thermogram were analyzed using Universal Analysis (TA Instruments, New Castle). The starch melting peak was recorded as endothermic heat flow around 60–80 °C. The starch melt in the slurries regardless of GTE formulation was observed between 70–90 °C (Fig. 1A and B) whereas no transition was observed in the finished confections (Fig. 1C and D).

Postprandial clinical study

This study protocol was approved by the Institutional Review Board at The Ohio State University, and participants provided written informed consent before enrolling in the study. Healthy men ($n = 15$) were enrolled based on age (18–30 years), body mass index <25 kg m⁻², resting blood pressure <140/90 mmHg, non-smoking status, non-use of dietary supplements (>2 months), non-use of any medications, participation in <5 h per week of exercise, alcohol consumption <2 drinks per d, and fasting blood chemistries (glucose, total cholesterol, HDL-C, triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN)) within normal clinical reference ranges.²⁶ In a randomized, crossover, double-blind design, participants arrived to the study center after an overnight fast (10 h) to ingest a confection containing 50 g starch and 0 or 1 g of decaffeinated GTE (Table 1) on two occasions separated by 7 d. Eight of the 15 participants received the GTE-containing confection in the first arm of the crossover trial. Blood was collected from the ante-cubital vein into evacuated blood collection tubes containing EDTA or sodium heparin immediately prior to (0 min) and at 30 min intervals for 3 h following ingestion of each test confection. Brachial artery FMD, as described below, was measured at these same time intervals to assess vascular function. Plasma was separated by centrifugation (1500g, 15 min 4 °C), snap-frozen in liquid nitrogen, and stored at -80 °C until analysis.

Sensory analysis

Participants completed a sensory analysis of each test confection during the initial 10 min of each trial. After their initial fasting blood sample was collected, sensory tests were administered to participants in a private room while seated in a reclining hospital bed positioned at 90° under fluorescent lighting. Participants received 12 confection cubes (~2 cm³ each) distributed between two air tight plastic soufflé cups (160 mL) that were labeled with a random 3-digit alphanumeric code. The tests administered for the sensory evaluation were modified to accommodate the requirements of the randomized, crossover study design, with samples being presented using a serial monadic protocol. Participants were instructed to rinse their mouths with cold tap water before initiating the sensory evaluation and prior to evaluating each sensory attribute.

Paper surveys designed with a 9-point hedonic scale (1 = dislike extremely to 9 = like extremely) were administered to assess acceptability of the confection, overall likability, aroma, flavor, sweetness, bitterness, and hardness.²⁷ A 5-point just-about-right scale (JAR; 1 = much too weak to 5 = much too strong) assessed flavor (sweet, sour, and bitter), aroma (fruit and off-odor), texture (hardness, stickiness, and chewiness), and quantity (serving size and size of confection). Descriptive analysis was also performed using a horizontal line scale (15 cm) that evaluated the following attributes from 1–10 (1 = slightly to 10 very): green color, sweetness, sourness, bitterness, hardness, stickiness, and chewiness. Finally, participants indicated their ideal intensity for each attribute using the following terminology to orient their senses: celery (1) and grass (10) for green color; grape (1) and honey (10) for sweetness; banana (1) and lemon (10) for sourness; spinach (1) and coffee (10) for bitterness; blue cheese (1) and cheddar cheese (10) for hardness; cream cheese (1) and caramel (10) for stickiness; white bread (1) and raisin (10) for chewiness.

Dietary control and analysis

Participants were instructed by a registered dietitian to abstain from tea, caffeine, alcohol, and exercise for 3 d prior to each trial while consuming a diet providing 50–60% of energy from carbohydrate to ensure reliable physiologic responses to each confection. They were also instructed to complete a 3 d food record, which was verified by the dietitian for accuracy. Participants were then instructed to replicate their diet for the second phase of the crossover study. Energy and nutrient intakes were assessed using the Nutrition Data System for Research dietary analysis software (University of Minnesota; Version 2014; Minneapolis, MN).

Plasma chemistries and markers of oxidative stress and NO[•] homeostasis

Plasma glucose, triglyceride, total cholesterol, HDL-C, ALT, AST, BUN and creatinine were measured using commercially available spectrophotometric kits in accordance with the manufacturer's instructions (Pointe Scientific, Canton, MI). Plasma vitamin E (as α - and γ -tocopherol) was measured by LC-MS as we described.²⁸ In brief, plasma was saponified and extracted with hexane before drying under nitrogen gas. Samples were then reconstituted in methanol : ethanol before injecting on the LC-MS and quantifying α - and γ -tocopherols using selective ion monitoring following negative ionization using an electro-spray ionization source.

Plasma malondialdehyde (MDA), a marker of lipid peroxidation, was measured by HPLC-FL as we described.¹⁰ In brief, plasma was saponified, proteins were then precipitated with TCA, and the resulting supernatant following centrifugation was derivatized with TBA reagent before extracting the MDA-TBA adduct with butanol. The extract was injected on a HPLC-FL system set to 532/553 nm (excitation/emission) and separation was performed at 1.0 mL min⁻¹ using a Luna C₁₈ column (250 × 4.6 mm i.d., 5 µm; Phenomenex; Torrance, CA) and mobile phase consisting of 50 : 50 methanol and 25 mmol L⁻¹ phosphate buffer (pH 6.5).

ARG and ADMA, critical regulators of NO• homeostasis, were measured by HPLC-FL as we described.¹⁰ In brief, plasma was mixed with PBS and methylmonoarginine (2.0 µM; internal standard) before solid-phase extraction on a polymeric cation-exchange column using ammonia : water : methanol (10 : 40 : 50). After drying the eluent under nitrogen gas, the residue was dissolved in water for derivatization with OPA. HPLC separation was performed using a Kinetex XB-C18 100A column (50 × 3.0 mm, 2.6 µm; Phenomenex; Torrance, CA) at 1.1 mL min⁻¹ using 50 mmol L⁻¹ potassium phosphate buffer (pH 6.5) and 6.5% acetonitrile as the mobile phase. Analysis was performed at 340/455 nm (excitation/emission) with the detector programmed to increase in sensitivity after ARG eluted at 3.3 min in order to detect ADMA.

Green tea catechins

Plasma catechins (EGCG, EGC, ECG, EC) were extracted as described,²⁹ with minor modifications. In brief, 200 µL plasma was mixed with 20 µL ascorbic acid (20% w/v) containing 2.5 mM DTPA, 200 µL sodium acetate buffer (1 mol L⁻¹, pH 5.4), and 40 µL hydrolysis solution containing glucuronidase (25 U) and sulfase (76 U). Following 45 min incubation (37 °C) in a shaking water bath, samples were chilled rapidly, and subsequently centrifuged (3000g, 5 min, 4 °C). Following extraction three times with ethyl acetate, the extract was evaporated under nitrogen gas, reconstituted in 30% acetonitrile containing 2% (v/v) formic acid, and filtered with a 0.22 µm filter. The filtrate was then analyzed by HPLC as described,²¹ with minor modification to use an Ultimate 3000 HPLC-ECD system (Thermo Scientific, Sunnyvale, CA) equipped with a quaternary solvent delivery module (LPG-3400BM), a refrigerated auto-sampler (WPS-3000) maintained at 4 °C, and two dual-channel coulometric analytical cells (6011 RS Ultra) programmed to 50, 75, 400, and 450 mV. Separation was performed at 0.9 mL min⁻¹ using an Xterra RP C₁₈ column (100 × 3.9 mm, 3.5 µm; Waters, Milford, MA) with the following binary gradient consisting of mobile phase A (899 : 100 : 1 water : acetonitrile : formic acid) and mobile phase B (699 : 300 : 1 water : acetonitrile : formic acid): linear gradient from 1–99% B from 0–7 min before returning to initial conditions from 7–9 min, and re-equilibrating from 9–13 min.

For confections, 1 g of solid that was pulverized under liquid nitrogen was mixed with 70% acetone containing 0.1% formic acid and subjected to vortexing and sonicating (Vibra-Cell; Sonics & Materials Inc.; Newtown, CT) for 30 s each. Following centrifugation (1500g, 5 min, 4 °C), the supernatant was collected and the remaining solids were subjected to acetone extraction two additional times. The extract was filtered (0.22 µm) and the eluent was analyzed by HPLC-UV using the above separation conditions with detection performed at

280 nm. Based on the analyzed content of powdered GTE, we expected the confection would provide 515 mg EGCG, 148 mg EGC, 80 mg ECG, 76 mg EC, and 8.8 mg caffeine. HPLC analysis indicated that 82–99% of the expected catechin and caffeine content was recovered following their extraction from the confection (Table 2). Storage at -80°C for 6 months did not affect catechin content (data not shown).

Brachial artery flow-mediated dilation

High-frequency ultrasound was performed to assess brachial artery FMD in a manner that best reflects NO-dependent vaso-dilation,³⁰ as we detailed previously.^{28,31} In brief, an inflatable blood pressure cuff was placed on the forearm distal to the ante-cubital fossa and the brachial artery was imaged by a cardiac ultrasound system. Resting brachial artery diameter was collected for 30 heartbeats prior to rapidly inflating the blood pressure cuff (200 mmHg) to occlude blood flow for 5 min. After rapidly releasing the cuff, post-occlusion brachial artery diameter was assessed for 300 heartbeats. Electrocardiogram-gated ultrasound images were analyzed using edge-detection software to assess peak post-occlusion artery diameter relative to that of pre-occlusion. Brachial artery FMD is expressed as the percent change from baseline: $[\text{post-occlusion peak diameter (mm)} - \text{baseline diameter (mm)}] / \text{baseline diameter (mm)} \times 100$. FMD responses from each participant were obtained and analyzed by the same technician in a blinded manner.

Statistical methods

Data were analyzed by GraphPad version 6.0 (GraphPad Software; La Jolla, CA). All analyses were considered statistically significant at $P < 0.05$. Sensory attributes of the confections were compared using the nonparametric Wilcoxon matched pairs signed rank test and these data are reported as median $\pm$ inter-quartile range. Radars analysis comparing parametric attributes of the confections prepared with or without GTE compared to participants' self-described ideal confection were examined by 1-way RM ANOVA with Bonferroni's *post hoc* test.

No human studies have investigated GTE on PPH-mediated impairments in VEF. However, our studies indicate that post-prandial excursions in blood glucose are highly correlated with decreases in FMD responses.^{10,23} A power calculation was performed using postprandial glycemic and FMD responses ($\text{AUC}_{0-180 \text{ min}}$) to determine that 12 participants would be needed to detect an estimated 20% treatment difference in these variables (80% power; $P < 0.05$). We therefore enrolled 15 participants to improve statistical power. Statistical analyses examining changes in variables throughout the 3 h postprandial period were performed using 2-way RM ANOVA to evaluate main and interactive effects of GTE and time. $\text{AUC}_{0-180 \text{ min}}$ for each variable was calculated using the trapezoidal rule, and between trial differences in $\text{AUC}_{0-180 \text{ min}}$ were examined using a paired *t*-test. Plasma catechin pharmacokinetic parameters (maximal concentration, C_{max} ; time to maximal concentration, T_{max}) were assessed using PK Solutions (version 2.0; Summit Research Services; Montrose, CO). Correlations between study variables were assessed by linear regression. All data from the postprandial clinical study are reported as means $\pm$ SEM.

Results

Participants

A total of 15 healthy participants were enrolled and completed the study without any adverse events during the sensory assessment of confections or the crossover clinical study.

Participants were normotensive, and considered healthy on the basis of anthropometric and plasma lipids, liver and renal function tests within normal clinical limits (Table 3).²⁶

Sensory analysis of test confections

Based on hedonic scoring, overall liking, aroma, flavor, sweetness, and bitterness were above neutral regardless of confections containing GTE and no differences were observed between confections for any of these sensory attributes ($P=0.36$ – 0.99 ; Fig. 2A). Of importance, participants were unable to ascertain a difference in the bitterness of confections regardless of formulation with GTE nor could they correctly identify the confection containing GTE (data not shown; $P>0.05$).

JAR assessment suggested that participants perceived that the confection serving size (156 g or 157 g for starch and GTE-starch confections, respectively), was too large despite being provided as 12 cubes of approximately 2 cm³ each (Fig. 2B). Individual cubes were also perceived as being too large (Fig. 2B). Assessment parameters for flavor (sweet, sour, and bitter), aroma (fruit and off-odor), and texture (hardness, stickiness, and chewiness) suggested that confections were reasonably well-received by participants regardless of GTE. Radar analysis indicated that the GTE-containing confection was more sticky than the starch confection ($P=0.004$), although neither confection was different from participants' self-defined ideal confection ($P=0.22$; Fig. 2C). Similarly, the GTE-containing confection was described as more chewy than the starch confection ($P=0.033$), but neither confection was different from an ideal confection ($P>0.05$). None of the other attributes assessed (green color, sweetness, sourness, bitterness, hardness) were different between test confections nor did they differ from the ideal confection regardless of GTE ($P=0.06$ – 0.73).

Plasma catechins

Consistent with instruction to participants to abstain from catechin-containing foods, plasma catechins were below detection limits at baseline (0 min) in both trials. Plasma catechins also were not detected in participants following ingestion of confections formulated without GTE (data not shown). Consistent with our goal of formulating a confection that facilitates oral delivery of catechins, data show that ingestion of confections formulated with GTE increased plasma EGCG (Fig. 3A) by 30 min. Plasma concentrations also remained elevated throughout the 3 h postprandial period with no time-dependent differences from 30–180 min. Similarly, plasma EGC, ECG, and EC all increased postprandially without any time-dependent differences between 30–180 min (Fig. 3A). Time to maximal concentration ($T_{\max}$) was 150 ± 14.1 min for EGCG, 117 ± 17.6 min for EGC, 150 ± 15.8 min for ECG, and 132 ± 17.4 min for EC. Maximal plasma concentrations ($C_{\max}$) of EGCG, EGC, ECG, and EC were 1.68 ± 0.27 , 1.32 ± 0.52 , 0.45 ± 0.11 and 0.55 ± 0.23 $\mu\text{mol L}^{-1}$, respectively. Plasma AUC_{0–180 min} of the catechins followed a similar response (Fig. 3B). Thus, the

starch-based confection facilitated catechin bioavailability with the same relative catechin profile as that of the GTE used in formulating the confection (Table 2).

PPH and vascular function were unaffected by GTE-containing confections

Consistent with the successful formulation of a starch-based confection that delivers green tea catechins (Fig. 3), our randomized crossover study examined their potential bioactivity in mitigating PPH-mediated impairments in VEF. Evaluation of participants' dietary records indicated their compliance to the dietary regimen as evidenced by no significant between-trial differences in energy or nutrient intakes ($P > 0.05$) as well as participants consuming 53–55% of energy from dietary carbohydrate as instructed (ESI Table 1[†]).

Fasting plasma glucose concentrations were not significantly different between trials at baseline (0 min) ($P > 0.05$; Fig. 4A). Ingestion of starch-based confections increased postprandial plasma glucose concentrations ($P < 0.0001$), which occurred without any significant GTE or GTE $\times$ time interactive effects ($P = 0.30$ and 0.87 , respectively). Regardless of GTE content, both confections increased plasma glucose at 30–90 min compared to baseline ($P < 0.05$) with maximal concentrations occurring at 60 min, and returning to levels no different from baseline by 120 min ($P > 0.05$). Data were also transformed to calculate changes in plasma glucose concentrations relative to baseline concentrations to consider between-participants heterogeneity of postprandial responses (Fig. 4B). Similar to untransformed data, a significant effect for time ($P < 0.0001$) indicated an increase in the change of plasma from 30–90 min relative to baseline ($P < 0.05$) whereas no GTE or GTE $\times$ time effects ($P = 0.30$ – 0.94) were observed. Moreover, AUC_{0–180 min} of plasma glucose between test confections indicated no differences for untransformed (starch, 981 ± 28 vs. starch + GTE, 998 ± 29 mmol L⁻¹ min⁻¹; $P = 0.71$) or transformed data (starch, 256 ± 24 vs. starch + GTE, 259 ± 20 mmol L⁻¹ min⁻¹; $P = 0.71$).

Baseline brachial artery FMD responses were not different between trials ($P > 0.05$; Fig. 5). Consistent with studies demonstrating that PPH transiently impairs vascular function,³ we observed a significant decrease in brachial artery FMD following test confection ingestion regardless of examining untransformed data (Fig. 5A) or data that was transformed to visualize the change in FMD responses relative to baseline (Fig. 5B). Decrease in FMD following test confection ingestion occurred without any significant GTE or GTE $\times$ time effects (Fig. 5A and B), with *post hoc* analysis indicating a significant lowering of FMD only at 60 min ($P < 0.05$) and a return to baseline levels by 90 min ($P > 0.05$). There were also no differences between trials for FMD AUC_{0–180 min} (starch, 653 ± 66 vs. starch + GTE, $614 \pm 65\%$ min; $P = 0.27$) or FMD AUC_{0–180 min} (starch, 526 ± 30 vs. starch + GTE, $517 \pm 33\%$ min; $P = 0.81$).

Finally, in considering the large between-subject heterogeneity in postprandial green tea catechin concentrations (Fig. 3), we hypothesized that their concentrations would be inversely correlated to PPH and impairments in VEF. To the contrary, no significant pairwise correlations ($P > 0.05$) were observed between the AUC_{0–180 min} of plasma glucose or FMD and any of the plasma catechins. Similarly, no significant correlations ($P > 0.05$) were

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observed between plasma catechin concentrations at 60 min and time-matched plasma glucose or FMD responses.

Starch-based confection mediated alterations in postprandial vascular health

Because GTE-containing confections had no apparent effect on the primary outcome variables of plasma glucose and brachial artery FMD, and physiological effects on glycemia and FMD were largely limited to 60 min postprandially, all subsequent analysis on biomarkers mediating vascular health were performed at 60 min. To test the hypothesis that PPH-mediated impairments in VEF occurred in an oxidative stress-dependent manner that lowers NO[•] bioavailability,³ we examined the lipid peroxidation marker MDA. At baseline (0 min), plasma MDA was not different between trials (Fig. 6A). However, ingestion of confections significantly increased plasma MDA by ~24% ($P < 0.05$) regardless of confections containing GTE ($P > 0.05$). Plasma concentrations of ARG, the amino acid required for NO[•] biosynthesis,³ decreased by 9% ($P < 0.05$; Fig. 6B) following confection ingestion without any GTE or GTE $\times$ time effects ($P > 0.05$). We also considered that oxidative stress would increase accumulation of ADMA, which is expected to competitively inhibit ARG binding to nitric oxide synthase to limit NO[•] bioavailability.³ Although there was a significant time effect ($P = 0.04$), *post hoc* analysis indicated that plasma ADMA only significantly increased in response to the test confection that was devoid of GTE (Fig. 6C). However, upon calculating ADMA/ARG to better consider NO[•] status, it was observed that both test confections regardless of GTE significantly increased ADMA/ARG to a similar extent by ~18% ($P < 0.05$; Fig. 6D).

Discussion

Consistent with our hypothesis, this investigation demonstrates that a GTE-containing starch-based confection can be formulated successfully to serve as a catechin delivery system while blinding sensory attributes of green tea and providing reasonable consumer acceptance. However, contrary to our hypothesis, acute ingestion of a GTE-containing confection by healthy adults was unable to mitigate postprandial impairments in VEF in association with PPH otherwise resulting from a starch-based confection providing 50 g of carbohydrate. Indeed, similar to PPH-mediated VED occurring following glucose ingestion,¹⁰ the starch-based confections increased postprandial lipid peroxidation and ADMA/ARG, thereby providing continued support that PPH impairs vascular function in an oxidative stress-dependent manner that limits NO[•] bioavailability.

Green tea (5 servings per d) lowers the risk of CVD-related mortality by 26%.³² Its polyphenolic catechins are thought to mediate its cardioprotective activities, although most studies have focused on EGCG because of its greatest abundance in green tea. The mechanisms by which catechins potentially lower CVD risk are numerous, and potentially involve anti-oxidant, antiinflammatory, hypolipidemic, anti-obesity, and insulin sensitizing activities as extensively reviewed.^{33–35} In adults, the consumption of green tea in the absence of other foodstuffs increases postprandial FMD responses.^{36,37} This is further supported by a meta-analysis showing that green or black tea consumption significantly improves endothelial-dependent vasodilation.³⁸ However, recent preclinical studies provide

novel evidence that co-ingesting green tea catechins with starch limits PPH by inhibiting pancreatic α -amylase activity.¹⁸ Although EGCG was more potent, it and the nongallated catechin EGC inhibited α -amylase activity *in vitro*. In mice, ingestion of EGCG with corn starch, but not other carbohydrates lacking an α -1,6-glycosidic linkage (*i.e.* maltose, glucose, or sucrose) attenuated PPH without affecting expression of intestinal GLUT or serum insulin to support that EGCG inhibits α -amylase activity *in vivo*. However, the extent to which green tea protects against starch-mediated PPH in humans has not been investigated. This is of public health importance consistent with prospective studies indicating that 2 h blood glucose following an oral glucose tolerance test better predicts CVD-related mortality compared with fasting blood glucose regardless of the presence of impaired glucose tolerance.²

Our work acknowledges that green tea is consumed to a lesser extent by Americans compared with Asians.²⁰ Thus, we formulated a GTE-containing snack food consistent with Americans consuming a substantial proportion of their daily energy from snacks foods²⁰ while providing a novel food-based delivery system for catechins similar to our earlier work in which a strawberry-based confection was developed to deliver ellagitannins.³⁹ To test our hypothesis that GTE would mitigate PPH-induced VED in an α -amylase-dependent manner, the GTE-containing confection was formulated with dent corn starch having a specific amylopectin : amylose profile (73 : 27) enriched with GTE to provide a single solid delivery vehicle rather than separately administering GTE and starch. A concern in developing the formulation was heat-mediated degradation of catechins consistent with studies showing 10–15% loss of catechins upon heating at 98 °C for 15 min.⁴⁰ Our efforts to minimize thermal exposure (60 °C to 40 °C in <5 min) afforded marginal benefit based on our findings indicating 82–99% recovery of each catechin. Nonetheless, and despite our rigid constraints to formulate a sugar-free confection containing substantial amounts of starch, sensory studies showed that confections had indistinguishable sensory characteristics regardless of GTE, were well-tolerated, and had reasonable consumer acceptance with the caveat that participants found the confection size and confection portion size to be too large. Likewise, other critical hurdles (*i.e.* syneresis, gel fracturing, molding) for achieving an acceptable product for clinical trials were addressed by using guar gum, erythritol, and glycerin to plasticize the rigid starch gel whereas the bitterness of GTE was effectively masked by using citric acid, non-nutritive sweeteners, and apple flavoring.

With an acceptable starch-based confection developed to contain 1 g GTE, an intake closely equivalent to the catechin content of 5 servings of green tea,¹⁵ HPLC analysis was performed to examine the extent to which the confection would facilitate oral delivery of catechins while potentially protecting against PPH-mediated VED. We demonstrate that the four major GTE catechins (EGCG, EGC, ECG, EC) appeared in plasma by 30 min postprandially following the ingestion of the GTE-containing confection. Similar to the distribution of catechins incorporated into the confection, plasma catechin concentrations closely matched their relative abundance in the confection. Consistent with the relatively high intake of catechins, participants' plasma catechin concentrations were also higher than those from studies examining catechin bioavailability following a single, freshly brewed serving of green tea.^{41,42} Nonetheless, our studies were limited to examining acute GTE ingestion and further work is warranted to assess dose–response relationships and chronic

consumption of the GTE confection on catechin bioavailability, consistent with repeated daily dosing improving polyphenol bioavailability compared with its acute ingestion.⁴³

Although the catechins from the confection were absorbed, evidence from this study indicates that incorporating GTE into the starch-based confection affords no apparent protection against PPH-mediated VED. This is despite evidence from a meta-analysis indicating that tea consumption improves endothelial-dependent vasodilation³⁸ at doses (2–3 cups) comparable to that used in the present study (5 cups). Thus, the lack of benefit in the present study is likely attributed to an interactive effect with starch whereas prior studies³⁸ show that FMD responses are improved when tea is consumed in the absence of co-ingesting carbohydrate. Indeed, the present findings were contrary to our hypothesis, which was based on evidence suggesting that catechins inhibit the activity of α -amylase through hydrogen bonding and hydrophobic interactions with this enzyme.⁴⁴ Specifically, EGCG and ECG were shown to have strong affinity towards the pancreatic α -amylase catalytic site by their interaction with 12–13 amino acid residues.⁴⁵ Liu *et al.*⁴⁶ also showed a 50% decrease in α -amylase activity when starch and green tea catechins (10%, w/w relative to starch) were incubated *in vitro*. Work by Forester *et al.*¹⁸ showed that α -amylase activity *in vitro* was inhibited by 34% at 20 μ M, a concentration that is achievable in the intestinal lumen. This was corroborated by studies in mice showing EGCG attenuates corn starch (5 g kg⁻¹)-mediated increases in blood glucose. However, the EGCG concentration in the small intestinal contents following oral gavage of corn starch and EGCG (100 mg kg⁻¹) was estimated at 972–2065 μ M.¹⁸ That this dietary EGCG level in mice greatly exceeded the more physiologically-relevant dose used in our clinical study (~10 mg EGCG per kg) likely explains these discrepant findings. Alternatively, the carbohydrate content of the starch-based confections may have been too high, although 50 g of starch used in the present study was deemed to be necessary to induce a sufficient glycemic response²² in order to test our hypothesis.

Consistent with the lack of attenuation in PPH by the GTE confection, FMD responses were significantly decreased at 60 min postprandially and coincided with peak plasma glucose concentrations. The relation between PPH and transient impairments in vascular function is well-established in clinical studies, with evidence supporting that hyperglycemia induces oxidative stress responses, likely originating from the mitochondria and/or inflammatory cell activation, that impair NO[•] bioavailability to the vascular endothelium.³ Indeed, the lipid peroxidation biomarker MDA increased at 60 min post-prandially regardless of confection type. This also coincided with decreased plasma ARG, and increased ADMA and ADMA/ARG. As we reviewed,³ oxidative stress increases arginase-dependent catabolism of ARG while also increasing protein methyltransferases activity to yield ADMA synthesis from ARG. Oxidative stress also inhibits dimethylarginine dimethylaminohydrolase activity, which limits ADMA detoxification and results in its accumulation. Consequently, ARG depletion may limit eNOS-mediated NO[•] biosynthesis in addition to ADMA competitively inhibiting ARG binding to eNOS, thus resulting in reduced NO[•] bioavailability. Indeed, our present findings are in agreement with others showing that a glucose challenge¹⁰ or carbohydrate-rich rice milk¹¹ transiently impairs VEF consistent with PPH increasing MDA and the ratio of ADMA/ARG in normoglycemic or metabolic syndrome adults.

However, a difference between the present and earlier studies is that the observed increase in plasma glucose and decrease in FMD occurred to a lesser extent following starch ingestion relative to those responses following the ingestion of glucose¹⁰ or rice milk.¹¹ Indeed, a 75 g glucose challenge increased plasma glucose by ~2.5 mmol L⁻¹ and decreased FMD by ~3.5% whereas the present study providing starch confections (50 g) shows a ~1 mmol L⁻¹ increase in plasma glucose with a corresponding ~0.5% decrease in FMD. Not only is an attenuated glycemic response by starch ingestion expected to be associated with lower CVD risk,² findings from several meta-analyses also suggest a 10–13% greater risk of future CVD events for each 1% decrease in FMD.^{9,47,48} Thus, reducing the glycemic load independent of GTE in the present study was partly effective in mitigating PPH-mediated VED compared to ingesting simple carbohydrate.¹⁰

In conclusion, this study demonstrates the successful formulation of a GTE-containing starch confection having the ability to deliver catechins as a snack food with acceptable consumer appeal that can serve as potential alternative to a conventional brewed tea matrix. Although our translational study was designed based on the favorable outcomes of preclinical studies,^{18,45,46} it showed a lack of effect of GTE in mitigating PPH-mediated VED in normoglycemic young adults. Thus, studies are warranted to consider the potential vasoprotective activities of GTE confections in populations with underlying VED, and/or glucose intolerance, who may be more responsive to improvements in endothelial-dependent vasodilation. Consideration should also be given to optimize the delivery of catechins to minimize postprandial glycemic excursions while simultaneously addressing the snacking preferences of Westernized consumers. In addition, the availability of a convenient delivery system lends itself for assessing the chronic benefit of increased green tea catechin intakes through functional snack foods that potentially lower CVD risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Differential scanning calorimeter thermograms of starch slurries formulated with or without GTE (A–B) and cured for 72 h (C–D). Insets of slurries at 65–90 °C show distinct amylopectin melt peaks regardless of GTE (A–B) and the absence of these enthalpic peaks in finished confections following a 72 h cure (C–D).

Fig. 2.

(A) Hedonic assessment of starch and green tea extract (GTE)-containing starch confections. (B) Penalty score derived from Just-About-Right scores of confections where the percent of the consumer panel that fell into each category was plotted against their mean drops. (C) Radar plot of confection attributes collected during descriptive analysis where intensity was rated from 1 to 10, with 10 having the highest intensity.

Fig. 3. Postprandial plasma concentrations (A) and area under the curve (AUC)_{0-180 min} and maximal concentrations (C_{max}) (B) of catechins following the ingestion of a green tea extract-containing starch-based confection in normoglycemic young adults. Data are means $\pm$ SEM, $n = 15$.

Fig. 4. Postprandial plasma glucose concentrations (A) and change in plasma glucose from 0 min (B) following the ingestion of starch-based confections formulated with or without green tea extract (GTE) in normoglycemic, young adults. Group means not sharing a common superscript are significantly different. Analysis was performed by 2-way ANOVA with Bonferroni's post-test to evaluate main and interactive effects of time and GTE. Data not sharing a common superscript are significantly different for both trials across the time course ($P < 0.05$). Data are means $\pm$ SEM, $n = 15$.

Fig. 5. Postprandial brachial artery flow-mediated dilation (FMD) (A) and changes FMD responses from 0 min (B) following the ingestion of starch-based confections formulated with or without green tea extract (GTE) in normoglycemic, young adults. * $P < 0.05$ relative to 0 min for both trials. Analysis was performed by 2-way ANOVA with Bonferroni's post-test to evaluate main and interactive effects of time and GTE. Data are means $\pm$ SEM, $n = 15$.

Fig. 6. Plasma concentrations of MDA (A), ARG (B), ADMA (C), and the ratio of ADMA/ARG (D) at 60 min following the ingestion of starch-based confections formulated with or without GTE in normoglycemic, young adults. * $P < 0.05$ relative to 0 min. Analysis was performed by 2-way ANOVA with Bonferroni's post-test to evaluate main and interactive effects of time and GTE. Data are means $\pm$ SEM, $n = 15$. Abbreviations: ARG, arginine; ADMA, asymmetric dimethylarginine; GTE, green tea extract; MDA, malondialdehyde.

Table 1

Formulation of starch-based confections prepared with or without decaffeinated green tea extract (GTE)

Ingredient (Vendor)	Starch		Starch + GTE	
	Mass (g)	Wet basis (%)	Mass (g)	Wet basis (%)
Water	75	48.1	75	47.7
Corn starch (Tate & Lyle PLC, London, UK)	50	32.1	50	31.8
Erythritol (Now Foods, Bloomingdale, IL)	15	9.6	15	9.6
Apple flavoring (Kraft-Heinz, Pittsburgh, PA)	2	1.3	2	1.4
Guar gum (Bob's Red Mill, Milwaukie, OR)	3	1.9	3	1.9
Glycerin (McKesson, San Francisco, CA)	10	6.4	10	6.4
Citric acid (Tate & Lyle PLC, London, UK)	1	0.6	1	0.6
GTE ^a	–	–	1	0.6
Total	156	100	157	100

^a Powdered decaffeinated GTE (90LB Sunphenon; Taiyo International, Minneapolis, MN) contained 82% (w/w) total catechins, with 63% epigallocatechin gallate, 18% epigallocatechin gallate, and 9% epicatechin as determined by HPLC-UV.²¹

Table 2

Recovery of green tea catechins and caffeine from a starch-based confection (50 g starch) formulated with 1 g powdered GTE^{a,b,c}

	Expected content (mg)	Measured content (mg)	Recovery (%)
EGCG	515	489.1 ± 7.2	95.0 ± 1.4
EGC	148	144.5 ± 2.0	97.4 ± 1.4
ECG	80	65.7 ± 1.0	82.2 ± 1.4
EC	76	75.5 ± 1.1	99.4 ± 1.5
Caffeine	8.8	7.4 ± 0.1	83.8 ± 1.1

^a Powdered GTE contained 82% (w/w) total catechins. Catechin and caffeine content of GTE and confections were determined by HPLC-UV as described under Materials and methods.

^b Recovery was calculated by dividing the measured analyte relative to its expected quantity × 100, $n = 5$ independent experiments.

^c Abbreviations: EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; GTE, green tea extract.

Table 3Baseline characteristics of participants enrolled in the crossover study^a

Parameter	Value
Age (y)	25.3 ± 1.0
Body mass index (kg m ⁻²)	22.4 ± 1.8
Waist circumference (cm)	82.9 ± 5.5
Systolic blood pressure (mmHg)	119.1 ± 8.1
Diastolic blood pressure (mmHg)	75.9 ± 7.9
Plasma glucose (mmol L ⁻¹)	4.98 ± 0.32
Plasma triglyceride (mmol L ⁻¹)	0.99 ± 0.40
Plasma total cholesterol (mmol L ⁻¹)	4.13 ± 0.54
Plasma HDL-C (mmol L ⁻¹)	1.39 ± 0.30
Plasma alanine aminotransferase (U L ⁻¹)	12.3 ± 6.9
Plasma aspartate aminotransferase (U L ⁻¹)	11.6 ± 4.3
Plasma blood urea nitrogen (mmol L ⁻¹)	4.75 ± 1.25
Plasma α-tocopherol (μmol L ⁻¹)	25.2 ± 0.96
Plasma γ-tocopherol (μmol L ⁻¹)	1.65 ± 0.13

^aMeans ± SEM, *n* = 15.