European Journal of Clinical Nutrition (2016) **70,** 10–16

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ORIGINAL ARTICLE

A combination of isolated phytochemicals and botanical extracts lowers diastolic blood pressure in a randomized controlled trial of hypertensive subjects

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BACKGROUND/OBJECTIVES: Isolated phytochemicals have been shown to reduce blood pressure; however, combinations of phytochemicals have rarely been tested in humans. We hypothesized that a combination of extracts from grape seed and skin (330 mg), green tea (100 mg), resveratrol (60 mg) and a blend of quercetin, ginkgo biloba and bilberry (60 mg) would reduce blood pressure (BP) in hypertensive subjects.

SUBJECTS/METHODS: Eighteen individuals meeting BP requirements (⩾130 mm Hg systolic or ⩾ 85 mm Hg diastolic) and criteria for metabolic syndrome were enrolled in a double-blinded, placebo-controlled, crossover trial (ClinicalTrials.gov, NCT01106170). The 28-day placebo and supplement arms were separated by a 2-week washout period, and 14 -h daytime ambulatory BP was assessed at baseline and at the end point of each arm.

RESULTS: BP was not altered after placebo. After supplement treatment, diastolic pressure was reduced by 4.4 mm Hg ($P = 0.024$, 95% CI, 0.6–8.1), systolic pressure was unchanged and mean arterial pressure trended ($P = 0.052$) toward reduction. Serum angiotensin-converting enzyme activity was similar between placebo and supplement arms, but urinary nitrate and nitrite concentrations were significantly increased ($P = 0.022$) after supplementation. Human aortic endothelial cells treated with metabolites of the polyphenols used in the human supplement trial had a significant increase ($P = 0.005$) in insulin-stimulated eNOS phosphorylation and greater ($P < 0.001$) accumulation of nitrates/nitrites.

CONCLUSIONS: Our clinical and *in vitro* data support the theory that this combination of polyphenols reduced diastolic pressure by potentiating eNOS activation and nitric oxide production. Such supplements may have clinical relevance as stand-alone or adjunct therapy to help reduce BP.

European Journal of Clinical Nutrition (2016) **70,** 10–16; doi:10.1038/ejcn.2015.88; published online 10 June 2015

INTRODUCTION

In the United States, hypertension is the most prevalent form of cardiovascular disease (CVD), affecting an estimated 76 400 000, or roughly one in three, Americans.[1] Further, the World Health Organization estimates that nearly 1 billion people have hypertension.[2] This condition increases the risk of left-ventricular hypertrophy, heart attack, stroke and heart failure.[3] In this era of advanced health care it is alarming that the number of deaths caused by hypertension-related heart disease remains high, with a reported ~36 000 American deaths in 2009.[4] Hypertension is also a common feature of metabolic syndrome, characterized by the presence of three or more of the following criteria: fasting glucose ⩾ 100 mg/dl; waist circumference ⩾ 102 cm in men or ⩾ 88 cm in women; blood pressure >130/85 mm Hg (systolic/diastolic); triglyceride levels ⩾ 150 mg/dl; and high-density lipoprotein ⩽ 40 mg/dl in men or ⩽ 50 in women.[5] Approximately 34% of all adults in the United States over age 20 meet these criteria and are at higher risk for mortality due to CVD and stroke.[6–8]

Given the epidemic numbers of hypertensive individuals there is a great interest in efficacious diet and lifestyle-based interventions to lower blood pressure. Many studies have examined the efficacy of various botanically derived phytochemicals to reduce blood pressure. For example, grape seed extract reduces blood pressure in spontaneously hypertensive rats fed normal and high-salt diets.[9] In humans both 150 and 300 mg/day grape seed extract can lower blood pressure,[10] and a meta-analysis examining nine randomized controlled human trials reported significant reductions in systolic pressure.[11] Supplementary green tea polyphenols lowered blood pressure in stroke-prone hypertensive rats[12] and angiotensin II-infused rats.[13] Green tea supplements are also reported to reduce blood pressure in healthy humans,[14] insulin-resistant adults[15] and in subjects with pre-hypertension.[16] Resveratrol, a polyphenol found in grape skin, lowers arterial pressure in hypertensive[17,18] and obese rat models.[19] Quercetin, a flavonol found in onions and apples, can also alleviate hypertension in pressure-overloaded rats[20] and spontaneously hypertensive rats.[21] Finally, quercetin supplemented (150–730 mg/d) to humans with metabolic syndrome and/or high blood pressure reduces systolic and diastolic blood pressure by ~3–7 mm Hg.[22,23]

As data from both animals and humans indicate that individual polyphenolic compounds reduce blood pressure, we hypothesized that a combination of polyphenolic agents may also be an effective intervention. To test our hypothesis we conducted a double-blinded, placebo-controlled trial, in which a supplement containing isolated polyphenols and botanical extracts was administered to hypertensive subjects. The primary outcome measure assessed in this study was ambulatory blood

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Received 9 October 2014; revised 11 March 2015; accepted 2 May 2015; published online 10 June 2015

pressure, and secondary outcome measures included serum angiotensin-converting enzyme (ACE) activity and urinary metabolites of nitric oxide. Parallel studies were conducted in human aortic endothelial cells (HAECs) in culture treated with metabolites of the polyphenolic combination used in our human studies. We report here that a 4-week supplementation period of this combination significantly reduced diastolic blood pressure compared with placebo, and present evidence supporting that the responsible mechanism may be increased activation of endothelial nitric oxide synthase and greater nitric oxide production.

PATIENTS AND METHODS

Participants and recruitment criteria

This study was approved by the University of Utah Institutional Review Board and conformed to the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects; it has been registered in Clinical Trials.gov (NCT01106170), and adhered to updated 2010 COSORT guidelines.[24] Written informed consent was obtained from each participant prior to enrollment in this placebo-controlled, crossover study to determine the efficacy of the phytochemical supplement to reduce blood pressure. Subjects were screened for eligibility at the University of Utah Nutrition Clinic. Blood pressure during screening was measured using an Omron random zero automated blood pressure cuff as previously described.[22] All volunteers had to meet 3 of 5 metabolic syndrome inclusion criteria, and exclusion criteria summarized in Table 1. Participants were recruited from the greater Salt Lake City area via flyers, public health screenings, advertisements (radio, the Internet, public transportation) and by word of mouth. Twenty-nine participants were originally recruited; 18 completed all experimental protocols and were included in the final analysis (Figure 1, Table 2). The most common reasons cited for volunteers leaving the study during or after the first intervention arm were health problems, moving out of state, inability to get to the clinic due to transportation problems and loss of interest in continuing participation.

Supplement and placebo

We tested a proprietary product, hereafter referred to as the 'supplement', containing (per capsule) grape seed and skin extract (165 mg), green tea extract (50 mg, decaffeinated, 90% phenols), quercetin dehydrate (25 mg), resveratrol (25 mg, Polygonum Cuspidatum—50%) and a 5 mg combination of gingko biloba, bilberry extract, bromelain and fungal protease (Melaleuca Inc, Idaho Falls, ID, USA). The placebo consisted of cornstarch packaged in identical capsules to that of the supplement. At the end of each arm, bottles of supplement and placebo were collected to determine compliance via pill count.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	
Blood pressure (mm Hg) ⩾ 130 systolic or ⩾ 85 diastolic	
Fasting glucose (mmol/l)	⩾ 5.55
Waist circumference (cm)	⩾ 102 men or ⩾ 88 women
Triglyceride levels (mmol/l)	⩾ 1.69
HDL (mmol/l)	⩽ 1.03 men or ⩽ 1.29 women
Exclusion criteria	
Alcohol consumption > 12 drinks weekly	
BMI over 40 kg/m^2	
Diabetes	
Liver disease	
Renal insufficiency	
History of prior cardiovascular event	
Chronic disease that might interfere with participation	
Unwillingness to stop current dietary supplement intake	
Use of calcium/magnesium antacids	

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein.

Overview of study design and blood pressure measurement

Eligible subjects were recruited between March 2010 and June 2011 and were entered into this double-blinded, placebo-controlled, crossover study. The primary outcome measure was blood pressure, and secondary outcome measures were ACE activity and urinary nitrate/nitrite concentrations as potential regulators of blood pressure. The study was conducted at the University of Utah Nutrition Clinic on a final sample of 18 subjects who had resting blood pressure of ⩾ 130 mm Hg systolic or ⩾ 85 mm Hg diastolic, and who completed all study protocols. A prospective power calculation using a difference of 6 mm Hg in mean arterial pressure between placebo and supplement, a s.d. of 9 mm Hg and a $P < 0.05$ determined that $n = 20$ would be required to produce a power of $\beta = 0.80$. This analysis provided us a recruitment target goal when we began the study. The study was halted after a significant difference in blood pressure was achieved. After a 1-week 'wash-in phase' where subjects discontinued any existing supplements, they were assigned (random draw) to a 28-day supplement or a 28-day placebo arm. Identically sealed bottles containing either placebo or the supplement were produced by Melaleuca, Inc. and given identical labels that contained a number identifier, along with the

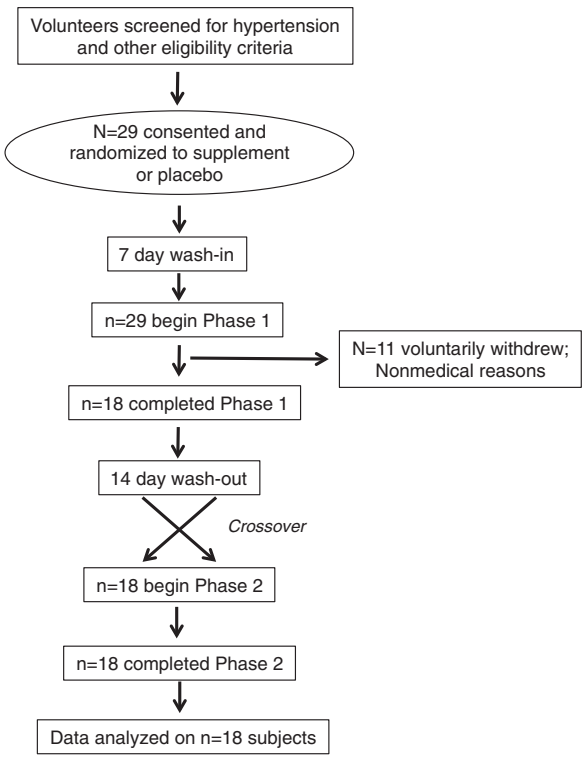

Figure 1. Overview of participant flow throughout the human study.

Table 2. Initial subject characteristics[a]

Age, years	44 (3)
Sex	*n* = 15 Males *n* = 3 Females
Plasma total cholesterol, mmol/l	5.53 (0.23)
LDL, mmol/l	3.49 (0.18)
HDL, mmol/l	1.19 (0.13)
Triglycerides, mmol/l	2.39 (0.34)
Glucose, mmol/l	5.55 (0.17)
Body weight, kg	104.5 (12.9)
BMI, m/kg^2	33 (1.5)
Systolic BP, mm Hg	146 (4)
Diastolic BP, mm Hg	86 (3)
Mean arterial pressure, mm Hg	106 (3)

Abbreviations: BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein. [a]Data presented as mean (standard error).

name and contact information of the study Principle Investigator. Subjects and research assistants were blinded to the nature of the bottles of supplement/placebo. Only the Principle Investigator, who did not have a direct patient contact, was aware of the interventions. Subjects were instructed to take two capsules with the meal of their choice. Research assistants randomized subjects to begin either placebo or supplement arm first by blindly selecting a bottle of capsules out of a box. After a 2-week washout period, subjects crossed over to the other arm. The 2-week washout period was selected on the basis of our previous human studies using quercetin, which have indicated no trace of quercetin metabolites in plasma beyond 1 week after supplementation.[22,25] Compliance was monitored by pill count; each bottle was sealed with 58 capsules, two more than required for full compliance. During the course of the study subjects were counseled to maintain their regular diet and physical activity but not take any additional supplements. For each study arm, baseline and end-point blood pressure was measured using an ambulatory blood pressure cuff (Meditech ABPM-05, Budapest, Hungary) fitted on the left arm that recorded heart rate and systolic and diastolic blood pressure every 30 min from ~0800 to 2200 h. This technique has clinical advantages over traditional acute clinic-based blood pressure measurement because it is free from the 'white coat effect' and similar confounding variables, and is a better predictor of cardiovascular events.[26]

Dietary records

To verify diet consistency, subjects logged a 3-day food record during the second week of both the placebo and the supplement phase. Data were entered and analyzed using the Food Processor dietary analysis program (ESHA Research, Salem, OR, USA).

Blood lipids and glucose

A fasting venous sample of blood (12 ml) was drawn into two serum separator tubes. The blood was allowed to sit for 1 h before being centrifuged at 1000 RCF for 15 min at 4 °C. Serum was placed into aliquots and stored at − 80 °C. Fasting total cholesterol, low-density lipoprotein, high-density lipoprotein, very low-density lipoprotein, triglycerides and glucose in whole blood were determined using a Cholestech (Orlando, FL, USA) blood lipid analyzer as previously described.[22]

Serum cytokines

Interleukin-8 (IL-8), C-reactive protein (CRP), monocyte chemoattractant protein-1 and interferon gamma-induced protein 10 (IP-10) were measured in fasting serum using multiplex kits from Quansys Biosciences (Logan, UT, USA).

Serum ACE activity

ACE activity was measured using the Buhlmann Assay Kit (Basel, Switzerland) as previously described.[25] This method is based on the ability of ACE to cleave the synthetic substrate *N*-[3-(2–furly)acryloyl]-L-phenylalanine-L-glycyl-L-glycine) into an amino acid derivative and a di-peptide. The kinetics of this cleavage is measured by reading the decrease in absorbance at 340 nM at time 0 and 10 min after the addition of the synthetic substrate to the serum sample.

Nitrates and nitrites measurement

NO_2^- and NO_3^-, surrogates for NO bioavailability, were quantified in urine collected from fasting human subjects and in HAECs grown in culture using a colorimetric kit (Cayman Chemical, Ann Arbor, MI, USA) as previously described.[25] Total NO_2^- and NO_3^- concentrations were normalized to protein concentration.

Urinary F2-α-isoprostane

F2-α-isoprostane, an indicator of oxidative stress, was measured in fasting urine samples as previously described.[22]

Cell culture and treatment with polyphenols

HAECs (Lonza, Carlsbad, CA, USA) were cultured as previously described[27] in media containing 2% FBS and endothelial growth supplements EGM-2 (Life Technologies, Carlsbad, CA, USA) in a humidified atmosphere (5% CO2 / 95% O_2, 37 °C). Cells were passaged at 80% confluence, and all experiments were conducted on cells between passages 3 and 6. Reported data are the average of 4–6 independent experiments. Prior to polyphenol treatment, HAECs were incubated overnight in serum-free medium. Next, HAECs were treated with metabolites of polyphenols for 24 h in media containing 2% FBS and 5 mM glucose. After 24 h, HAECs were treated with or without insulin (100 nM) for 10 min. Following insulin treatment, cells were lysed and prepared for detection of eNOS and measurement of nitrate/nitrite concentrations. HAECs were grown in glucose concentration (5 mM) that mimicked the average fasting blood glucose levels of subjects in the clinical trial. Insulin was used to activate eNOS in our cell-based model as it is known to stimulate eNOS activation and NO production in human endothelial cells, rodents and humans.[28,29]

HAECs were treated with metabolites of polyphenols found in the supplement used in the human trial. Metabolites, rather than the parent polyphenols, were selected because human studies have demonstrated that polyphenols are rapidly metabolized after ingestion and very little parent compound remains in circulation,[30] thus indicating that metabolites may be more biologically relevant than the originating compounds. As multiple metabolites are produced for each compound *in vivo*, we attempted to use metabolites for our *in vitro* study that were reported to be prevalent in circulation. Accordingly, metabolites of proanthocyanins, epicatechin and epigallocatechin (major components of grape seed and green tea extract), as well as quercetin and resveratrol, were employed. We acknowledge the use of a single metabolite of each ingredient as a limitation of these *in vitro* studies. However, we opted for this approach because of the difficulty inherent in employing multiple metabolites for each individual ingredient in combination in cell culture media.

Proanthocyanins are found in grape seed extracts, whereas catechin and epicatechin are abundant in green tea extract. Proanthocyanins are poorly absorbed and converted by colonic microflora to the phenolic acid 3-hydroxyphenylpropionic acid,[31] which has been detected in human plasma.[32] Similarly, catechin and epicatechin can undergo similar conversion.[31,33–36] On the basis of these data, we used 3-hydroxyphenylpropionic acid (purchased from Sigma Chemical, St Louis, MO, USA) in our *in vitro* studies to represent a common metabolite for compounds found in both grape seed and green tea extract. Piceatannol is an analog of resveratrol found in grapes and is a product of reservatrol metabolism by cytochrome P450.[37–39] Therefore, we used piceatannol (purchased from A. G. Scientific Inc., San Diego, CA, USA) as a resveratrol metabolite. Finally, for quercetin, quercetin-3-*O*-glucoronide (purchased from TRC Chemicals, Toronto, ON, Canada) was used as it represents a major metabolite previously reported in human plasma.[33,40–42] All three metabolites were combined in cell culture media for in vitro experiments. Concentrations were selected by performing a preliminary dose–response experiment using 1–50 μM of the various metabolites, individually. The lowest concentrations that produced significant insulin-stimulated eNOS phosphorylation were selected. These levels were 1 μM for 3-hydroxyphenylpropionic acid, 5 μM for Piceatannol and 2 μM for quercetin-3-*O*-glucoronide. Regarding these concentrations, the amount of quercetin used in our *in vitro* experiments was similar to the human plasma levels (1.42 μM) we have measured in our supplement trials,[22] albeit following a larger quercetin dose of 730 mg. Other studies have reported ~0.3–0.4 μM quercetin in plasma following ingestion of 50–200 mg quercetin.[23,41,43] For 3-hydroxyphenylpropionic acid and piceatannol there is a paucity of data on their plasma levels following ingestion of food or supplements. This lack of data prompted our rationale for employing a dose–response approach to first identify the lowest effective dose able to augment NO production.

Our experiments evaluated both basal and stimulated eNOS activation and nitric oxide production in order to better mimic the *in vivo* condition in which eNOS activation fluctuates between these two states. To stimulate eNOS we used insulin (100 nM, 10 min), whereas control cells were treated with saline. Vehicle control was dimethyl sulfoxide.

Western blotting

Western blots to detect eNOS and p-eNOS^{Ser1177} (Cell Signaling Technology, Beverly, MA, USA) were performed as previously described.[44]

Statistical analysis

Blood pressure, ACE activity, lipids, inflammatory activity and nitrates were analyzed using paired *t*-tests (SPSS, Armonk, NY, USA) to compare the difference achieved between placebo and supplement arms. Cell culture data were analyzed using analysis of variance (eNOS and NO_x data) and Tukey's *post-hoc* test when main effects were detected. Variance was

similar between groups in both human and cell studies. Data are reported as mean ± standard error and significance was accepted at $P < 0.05$.

RESULTS

Blood pressure and heart rate

Baseline blood pressure levels in placebo and supplement arms were similar to each other. A treatment effect was present as there was a significant decrease in diastolic pressure during the 4-week supplement arm from 88.8 ± 2.1 to 84.2 ± 1.9 mm Hg ($P = 0.024$, mean difference between placebo and supplement = 4.4, 95% CI, 0.6–8.1, Figure 2a). However, systolic pressure remained unchanged after supplement treatment (baseline 145.1 ± 3.3 vs end point 141.3 ± 2.2 mm Hg). Mean arterial pressure showed a trend ($P = 0.052$) toward reduction from 107.7 ± 2.3 to 102.8 ± 2.1 mm Hg after the supplement (mean difference between placebo vs supplement = 4.0, 95% CI, − 0.03 to 8.0). There was no order effect (that is, randomized to placebo or supplement first) on blood pressure reduction. During the placebo arm there was no change in systolic pressure (baseline 145.6 ± 3.5 vs end point 142.8 ± 3.0 mm Hg), diastolic pressure (baseline 85.8 ± 2.5 vs end point 85.4 ± 2.1 mm Hg) or mean arterial pressure (baseline 106.2 ± 2.9 vs end point 105.2 ± 2.6 mm Hg), Figure 2a). Placebo and supplement treatments did not alter the heart rate (Table 3).

Urinary nitrate and nitrite concentrations, serum ACE activity and F2-α-isoprostane.

Reduction in mean arterial pressure during the supplement arm was accompanied by a significant increase in total urinary nitrate and nitrite concentrations ($P = 0.022$, mean difference = 540 ± 214 μM, 95% CI, 87–993, Figure 2b). ACE activity, a known regulator of blood pressure, was unchanged between supplement and placebo phase (Table 3). F2-α-isoprostane concentration was also similar between the supplement and placebo periods (Table 3).

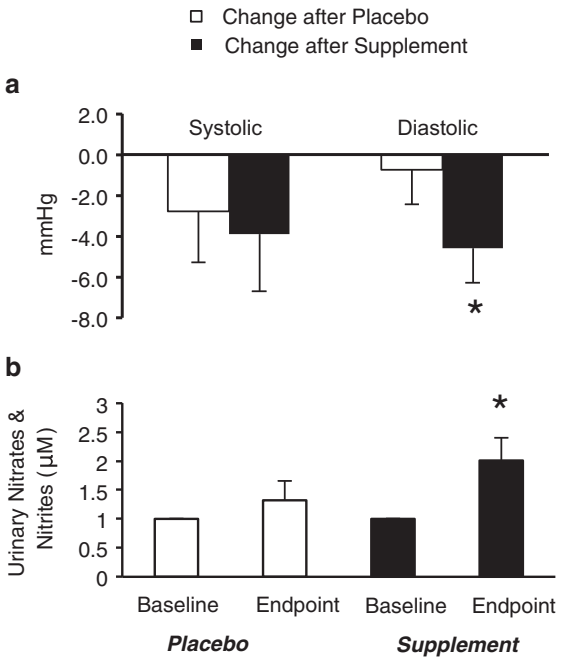

Figure 2. (**a**) Diastolic (diastolic blood pressure) but not systolic (systolic blood pressure) pressure (mm Hg) was reduced ($P < 0.024$) from baseline after 28 days of supplement treatment compared with placebo ($n = 18$/group). (**b**) Urinary nitrate/nitrite concentration (μM) was lower ($P < 0.022$) after 28 days of supplementation compared with placebo ($n = 17$/group). Values are normalized to baseline for each treatment phase and presented as fold change from baseline. *$P < 0.05$.

Markers of inflammation, blood lipids, body weight

There was no change in serum concentrations of CRP, IL-8, monocyte chemoattractant protein-1 or IP-10 between baseline and end point in either the placebo or the supplement arm (Table 3). Fasting total cholesterol, low-density lipoprotein, high-density lipoprotein, triglycerides and glucose also remained unchanged during the course of the study (Table 3). Body weight and body mass index remained unchanged during the study (Table 3).

Diet analysis and supplement compliance

Analysis of 3- day food records indicated that there were no significant differences in the intake of carbohydrates, proteins and fats between the placebo and supplement arms (Table 4). Micronutrient intake, including sodium, potassium and calcium, was similar between supplement and placebo arms (Table 4). Pill count at the end of each study arm indicated that 86 ± 5% of allocated supplements were consumed during the supplement phase and 93 ± 4% during the placebo phase.

Nitric oxide and eNOS in HAECs

We coupled our human study with *in vitro* experiments to shed light on potential mechanisms regulating the observed increase in the urinary marker of NO bioavailability. HAECs were treated with metabolites of the polyphenols used in the clinical supplement trial. Under basal (no insulin stimulation) conditions NO production and eNOS phosphorylation was similar in polyphenol-treated vs untreated HAECs (Figures 3a and b). Insulin stimulation did not alter NO production in untreated HAECs, but did produce a robust increase of NO ($P < 0.001$), eNOS phosphorylation ($P = 0.005$) and the p-eNOS to total eNOS ratio ($P = 0.05$) in polyphenol-treated HAECs (Figures 3a and b).

DISCUSSION

The major finding of this study is that diastolic blood pressure was reduced in hypertensive subjects in the supplement arm. There was also a trend ($P = 0.052$) toward reduction of mean arterial pressure. These results were achieved in the absence of adverse events. It is unclear why only diastolic pressure was reduced in this study, but differential blood pressure findings have previously been reported. For example, a study examining grape-derived polyphenols reported a reduction of only systolic pressure, but not diastolic pressure.[45] In the present study, reduction of diastolic blood pressure was accompanied by a significant increase in urinary nitrate and nitrite concentrations. This observation suggests that the mechanism responsible for blood pressure reduction could be related to an increase in NO bioavailability. Additional evidence for this was obtained in HAECs, where incubation with polyphenol metabolites markedly increased insulin-stimulated NO production and eNOS phosphorylation. As there were no changes in body weight, body mass index or micronutrient intake, we conclude that the attenuated blood pressure was due to the supplement employed. Although we did not see changes in micronutrients related to blood pressure control (sodium, magnesium, potassium and calcium), our dietary analysis did not evaluate intake of dietary nitrate, which could affect blood pressure levels. For example, beetroot juice is a dietary source of nitrate and is reported to lower blood pressure.[46,47] Therefore, it is possible that acute changes in nitrate consumption could also have influenced blood pressure.

The subjects enrolled in our study met at least three out of the five criteria accepted for metabolic syndrome,[5] a condition that is associated with elevations in inflammatory cytokines and chemokines.[48] It is unclear whether polyphenols can reduce inflammatory markers, such as CRP, a widely used marker of inflammation that is linked to greater CVD risk. For example,

Table 3. Body mass, blood and urinary parameters[a]

	Placebo		*Supplement*		
	Baseline	*End point*	*Baseline*	*End point*	*Difference (95% CI)*
Heart rate, beats/min	82.0 (2)	84.0 (3)	85.0 (3)	82.0 (3)	4.1 (−2.1 to 10.3)
ACE[b], U/L	70.8 (8.6)	69.8 (9.5)	63.4 (7.5)	54.7 (6.3)	−3.4 (−22.1 to 15.3)
Urinary isoprostanes	712 (121)	697 (104)	681 (80)	713 (98)	−32 (−197 to 260)
pg/mg Creatinine					
IL-8, pg/ml	9.30 (1.75)	6.74 (1.28)	6.68 (1.07)	8.80 (2.50)	−4.36 (−10.70 to 1.97)
IP-10, pg/ml	70.0 (14.1)	53.8 (5.6)	46.6 (4.6)	48.0 (8.5)	−17.4 (−53.6 to 18.8)
MCP-1, pg/ml	264 (35)	249 (22)	251 (23)	259 (22)	−22.7 (77.1 to 31.6)
CRP, μg/ml	14.6 (1.5)	14.8 (0.8)	15.4 (0.9)	14.3 (1.0)	1.4 (−1.2 to 4.1)
Body weight, kg	104.4 (5.8)	104.9 (5.9)	105.6 (6.1)	106.0 (6.2)	0.35 (−1.5 to 0.8)
BMI, kg/m^2	32.9 (1.5)	33.0 (1.5)	33.8 (1.6)	33.7 (1.6)	0.14 (−0.5 to 0.2)
Total Cholesterol (mmol/l)	5.51 (0.23)	5.74 (0.30)	5.60 (0.34)	5.69 (0.28)	0.14 (−0.61 to 0.90)
LDL (mmol/l)	3.49 (0.18)	3.71 (0.21)	3.90 (0.28)	4.03 (0.24)	0.48 (−1.13 to 2.09)
HDL (mmol/l)	1.18 (0.12)	1.27 (0.21)	1.18 (0.12)	1.17 (0.13)	0.10 (−0.13 to 0.33)
Triglycerides (mmol/l)	5.47 (0.77)	5.84 (0.85)	6.36 (0.72)	6.30 (0.64)	0.41 (−0.20 to 1.02)
Glucose (mmol/l)	5.46 (0.09)	5.57 (0.18)	5.57 (0.14)	5.58 (0.14)	0.01 (−0.61 to 0.62)

Abbreviations: ACE, angiotensin-converting enzyme; BMI, body mass index; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein, MCP-1, monocyte chemoattractant protein-1. [a]Data presented as mean (standard error). [b]One unit of ACE activity is defined as the amount of ACE required to release one μmol of Hippuric acid per minute, per unit of serum at 37 °C. $n = 18$ for heart rate, body weight, BMI. $n = 14$–16 for all others parameters.

Table 4. Analysis of 3-day food records[a]

Nutrients	*Placebo*	*Supplement*
Energy, kJ	12 483 (1030)	13 039 (1662)
Energy, kcal	2982 (246)	3115 (397)
Protein, g	111 (10)	114 (11)
Carbohydrates, g	367 (28)	358 (48)
Fiber, g	24 (4)	27 (4)
Fat, g	122 (12)	139 (20)
Vitamin A, IU	7033 (1337)	7497 (2212)
Vitamin E, mg	3.7 (0.5)	4.6 (1.6)
Vitamin K, μg	28.6 (4.4)	33.9 (9)
Calcium, mg	1086 (124)	1401 (189)
Magnesium, mg	195 (24)	210 (26)
Potassium, mg	2059 (229)	2066 (285)
Sodium, mg	5253 (540)	6183 (643)

[a]Data presented as mean (standard error).

resveratrol supplementation does not alter CRP in healthy subjects,[49] but reduces CRP in smokers.[50] On the other hand, a meta-analysis of human trials using grape seed extract found no change in CRP, despite widely reported reductions in blood pressure.[11] In our present study, the observed benefits to blood pressure were not accompanied by reduction in inflammatory markers. Taken together, there is a lack of conclusive evidence supporting the ability of polyphenols to reduce plasma markers of inflammation in humans.

Clinical trails evaluating combinations of compounds used in this supplement are very rare, but the individual polyphenols and extracts used in this supplement have been previously evaluated. For example, acute[25] (10 h, 1000 mg) and long-term[22,23,51] (>4 weeks, 150–750 mg/day) quercetin supplementation reduces blood pressure in subjects with hypertension. Freeze-dried grape extract (46 g/day, ~232 mg of total phenols and 31 mg anthocyanins) reduces systolic blood pressure (−6 mm Hg), but not diastolic blood pressure.[45] Purified grape seed extract supplements
(150 and 300 mg/day) lower systolic and diastolic blood pressure by 11 and 6 mm Hg, respectively.[10] The blood pressure effects of resveratrol in humans are mixed. Some studies demonstrate that

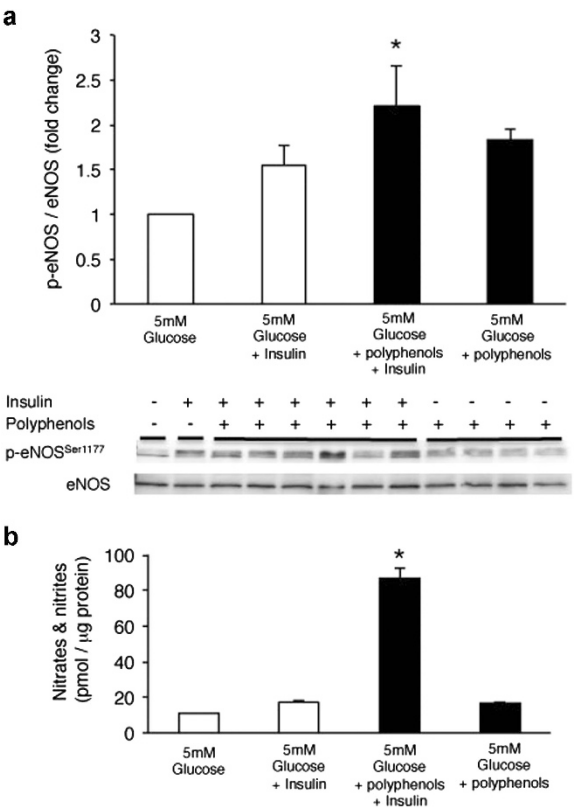

Figure 3. Human aortic endothelial cells were incubated for 24 h with polyphenol metabolites, and then insulin was used to activate eNOS to stimulate NO production. (**a**) Cells that received the mix of polyphenol metabolites prior to insulin stimulation exhibited increased eNOS phosphorylation ($P = 0.005$) and a greater ratio of p-eNOS to total eNOS ($P = 0.05$) vs vehicle-treated insulin-stimulated cells. (**b**) Accumulation of nitrates and nitrites was also increased ($P < 0.001$) in human aortic endothelial cells incubated with polyphenol metabolites compared with vehicle-treated cells. $N = 4$–6 for all experiments. *$P < 0.05$.

chronic (30+ days) supplementation of resveratrol in doses ranging from 150 to 250 mg/d (vs 60 mg/d employed in the present study) reduces only systolic blood pressure in subjects with metabolic syndrome or diabetes.[52,53] However, others report no change.[54,55] Similarly, green tea extract has variable effects on blood pressure, ranging from almost no change (using 379–400 mg catechins/day)[56,57] to nearly 4 mm Hg reduction (using 456-583 mg catechins/day). It is notable that the blend of polyphenols tested in our experiment produced similar reductions in diastolic blood pressure as some of the aforementioned studies, but used smaller amounts of quercetin, resveratrol and green tea extract to do so. On the other hand, there may be no differences in the magnitude of the antihypertensive effect resulting from a combination supplement versus the use of the individual ingredients given at a higher dose.

We evaluated ACE activity and urinary nitrite/nitrate concentrations to gain insight into the mechanism responsible for reduction of blood pressure. No change was observed in ACE activity; however, urinary nitrite/nitrate concentrations were increased during the supplement arm, which could indicate enhanced nitric oxide bioavailability. Our subsequent studies using HAECs support this notion as cells treated with ingredients contained in the polyphenol supplement had greater insulin-stimulated NO production and eNOS phosphorylation compared with untreated cells. Our results are in general agreement with previous work using endothelial cells,[58] rodents[59] and humans.[45] A limitation inherent to our *in vitro* studies is that many metabolites are produced after ingestion of the polyphenols found in this supplement. Therefore, it is possible that the metabolites selected for the *in vitro* studies may have lesser or greater impact upon NO bioavailability compared with the *in vivo* situation. As such, we note that our *in vitro* experiments may convey proof of principle that metabolites of polyphenols used in this supplement could generally impact NO bioavailability but are not sufficient to identify a specific mechanism that reflects the *in vivo* state.

Public interest in the use of supplements, including polyphenols, to prevent CVD is high. For example, in the United States the 2007–2010 National Health and Nutrition Examination Survey reported that 49% of all adults use dietary supplements as a tool to 'improve or maintain' health.[60] Furthermore, 7.5% of adults reported the use of botanical supplements, which include polyphenol-containing compounds and extracts such as those evaluated in this study, to promote overall health.[60] This reality underscores the need for research to evaluate the efficacy and mechanisms regulating the biological effects of polyphenols in humans. To this end, we found that the blend of isolated polyphenols and botanical extracts used in the present study reduced diastolic blood pressure in subjects with metabolic syndrome and hypertension. Data from our human and cell-based experiments support the theory that this supplement reduced diastolic blood pressure through potentiation of eNOS phosphorylation and NO bioavailability, a novel mechanism of action compared with most traditional antihypertensive medications. Although our study attempted a careful investigation of this polyphenol supplement to reduce blood pressure, there are limitations that must be acknowledged. Volunteers were counseled to maintain their normal daily activity to minimize this effect on weight control and blood pressure, but physical activity records were not collected to verify this. Also, the final sample size analyzed was smaller than originally planned because of the greater than expected dropout rate. Therefore, the study was slightly underpowered, as a retrospective power analysis using the actual results of the study indicated that the observed power was $\beta = 0.70$.

The fact that diastolic but not systolic pressure was reduced has clinical relevance as diastolic pressure is a better predictor of CVD risk in people less than 50 years of age.[61–63] It is possible that this type of polyphenol blend could be useful in those under age 50 who prefer to initially avoid a conventional pharmaceutical and try a naturopathic approach to reduce diastolic blood pressure. Given the general prevalence of supplement use to reduce CVD risk coupled with results of small studies such as ours, we believe pursuit of larger-scale clinical trials is warranted to evaluate the efficacy of polyphenols used alone, or even in coordination with traditional pharmaceutical treatments, to achieve optimal blood pressure control.

CONFLICT OF INTEREST

TJ received research funding from Melaleuca, Inc. for this study. ABR is an employee of Melaleuca, Inc. Other authors do not declare any conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge Maureen Murtaugh, PhD, RD, for providing statistical consultation. This study was funded by a grant from Melaleuca, Inc., and by the University of Utah College of Health Research Incentive grant.

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