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THE INFLUENCE OF MENTAL STRESS AND A HIGH POLYPHENOL HERB AND SPICE BLEND ON POSTPRANDIAL LIPEMIA, OXIDATIVE STRESS AND VASCULAR DYSFUNCTION

A Dissertation in

Biobehavioral Health

by

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ABSTRACT

Postprandial elevations in triglycerides and glucose may be a novel and powerful predictor of increased CVD risk[1]. Specifically, exaggerated increases in postprandial triglycerides and glucose are believed to promote atherogenesis via increases in inflammation, oxidative stress, endothelial dysfunction, and the production of small dense LDL cholesterol [2-4]. For this reason, dietary interventions which reduce post meal spikes in triglycerides and glucose or the subsequent rise in oxidative stress and endothelial dysfunction may be particularly important for managing CVD risk.

First, this dissertation explores the effects of acute stress and a high antioxidant herb and spice blend on postprandial oxidative stress, inflammation and vascular function. Methods: Archived samples from a randomized, crossover clinical trial [5] were used to test the effects of mental stress (Trier Social Stress Test) and rest on postprandial oxidative stress, inflammation and endothelial function following the consumption of a high fat meal with or without 14.5 grams of polyphenol-rich spices (black pepper, cinnamon, cloves, garlic, ginger, oregano, paprika, rosemary, and turmeric). Twenty participants completed 4 visits in this 2x2 factorial design. At each visit, fasted baseline blood samples were collected, participants consumed the test meal and then underwent either the mental stress protocol or rest condition. Additional blood samples were collected at 105, 140, 180, and 210 minutes post meal (corresponding to 10, 45, 90 and 120 minutes post stress). Endpoints included plasma levels of malondialdehyde (MDA), oxidized low density lipoproteins (oxLDL), the inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α). Vascular function was assessed via peripheral arterial tonometry at 240 minutes post meal. Results: Compared to the fasting baseline, the meal increased oxidative stress regardless of the
presence or absence of spices (p = 0.003 for MDA and p = 0.02 for oxLDL). Similarly, the meal resulted in increased plasma IL-6 (p < 0.001). A stress by time interaction was also present for plasma IL-6 (p = 0.05), such that when stressed, the average change was elevated at time 2 compared to time 3 (1.05 ± 0.25 vs 0.44 ± 0.16, Tukey p = 0.03). However, the direct comparison of IL-6 at time 2 following stress versus rest revealed no difference. There was no effect of spices on oxidative stress, inflammation or vascular function.

Second, this dissertation describes a clinical study which examines the effects of an polyphenol-rich spiced meal on postprandial plasma triglyceride concentrations and rates of fecal fat excretion and gut motility. Building on previous studies in which we observed significant reductions in postprandial triglycerides following a meal rich in spices, the purpose of this study is to identify mechanisms by which this effect occurred.

Methods: Twelve healthy but overweight men participated in a randomized 2 period cross over study. At each of 2 visits, a fasting blood sample was followed by the consumption of a high fat meal (~1100 kcals, 57g fat) containing blue dye, 2.5g non absorbable fat and with or without the 14.5 g polyphenol-rich herb and spice blend. Within 15 minutes of finishing the meal, participants swallowed a single-use SmartPill wireless motility capsule. Blood was sampled every 60 minutes after the meal for 8 hrs for the analysis of plasma triglycerides and glucose. Fecal deposits were collected until the dyed sample was captured. The sucrose polybehenate test of fecal fat absorption was used to evaluate fat excretion in the dyed samples. Endothelial function (measured via flow-mediated dilation, FMD) was assessed at fasting and 210 minutes post meal.

Results: Serum triglycerides, glucose and insulin were increased postprandially, as expected. There was no effect of spices on these three endpoints (ps > 0.05). Gastric emptying time was lower after the spice meal compared to control (658 ± 98.2 vs. 884.5
± 98.2 minutes; p = 0.046), but small bowel and colonic transit time were unaffected by treatment. There was no effect of either meal on brachial artery diameter or FMD (ps > 0.05). The spiced meal increased blood flow compared to the fasting scan (ps = 0.06 and 0.05). Both blood flow volume at the start of the FMD scan (average increase of 57.78 ± 25.34 mL/min, post hoc p = 0.037) and following shear stress were increased (average increase of 188.867 ± 66.6 mL/minute post hoc p = 0.012). These findings suggest that spices may influence sympathetic activity.

Finally, this dissertation reports the test-retest reliability of pulse amplitude tonometry (PAT). PAT is an alternative to the noninvasive standard procedure of flow-mediated dilation (FMD) for assessing endothelial function. PAT uses automated methods to measures the change in pulse wave amplitude before and during reactive hyperemia (Itamar Medical Ltd, Caesarea, Israel). However, little is known about its ability to reliably assess vascular function in healthy adults across time periods of more than a few hours. Methods: Twenty healthy adults completed two PAT tests separated by a mean of 19.5 days. Standardized conditions included time of day, location, time since and contents of last meal, body position and menstrual phase for premenopausal females. Results: PAT-derived measures of endothelial function (reactive hyperemia index, RHI) and arterial stiffness (augmentation index, AI) showed strong repeatability (intra-class correlations = 0.74 and 0.83, respectively). To guide future research, we also analyzed sample size requirements for a range of effect sizes. A crossover design powered at 0.90 requires 28 participants to detect a 15% change in RHI. Our study is the first to show that PAT measurements are repeatable in adults over an interval greater than 1 week and thus appropriate for gauging change over longer term intervention trials.
In conclusion, this dissertation provides evidence that common culinary spices have little impact on metabolic, oxidative and inflammatory processes during the postprandial period. Evidence is also provided for the reliability of an inexpensive and operator-independent method of measuring endothelial dysfunction. The results of this dissertation may be useful in designing interventions to prevent and treat obesity, metabolic syndrome, and CVD.
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Chapter 1

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, and accounts for approximately 30% of all U.S. deaths annually [6]. Elevations in fasting triglyceride and glucose levels have long been associated with cardiovascular disease (CVD) and diabetes [4, 7]. However, only a small fraction of the day is spent in the fasted state. The postprandial or fed state is characterized by changes in plasma measures of metabolic substrates (e.g. glucose and triglycerides) and hormones (insulin and glucagon). The degree of change is dependent on the meal type (e.g. caloric content, macronutrient profile) and amount of time since the last meal [8]. The health of the individual also influences the rate and degree of macronutrient absorption, principally by affecting the timing and magnitude of insulin and glucagon secretions [9]. In those who are healthy, the consumption of a meal containing both carbohydrates and fat results in glucose and triglyceride increases that tend to peak at 1 and 2-4 hrs respectively ([10]).

However, exaggerated and/or delayed increases in postprandial triglycerides and glucose, a condition known as metabolic dysfunction, is believed to promote atherogenesis via increases in inflammation, oxidative stress, endothelial dysfunction, and the production of small dense LDL cholesterol [2-4]. As such, the oral glucose tolerance test (OGTT) has been integrated into standard medical practice to identify postprandial metabolic dysfunction. The test utilizes a measure of glycemia at 2 hrs post ingestion of a 75g glucose drink to diagnose metabolic abnormalities. Severity can range
from impaired glucose tolerance (blood glucose of 140-199mg/dL at 2hrs post drink) to diabetes diagnosis (≥ 200mg/dL) [11]. The relationship between glycemia at 2hrs post OGTT and CVD mortality is graded, with risk elevating long before the cutpoint of diabetes diagnosis [12]. Specifically, every 1.49mmol/L increase in glucose 2hrs post OGTT is associated with a 60% increased risk of CVD event in 13 years of follow-up[13]. Even in those with fasting blood glucose in the normal range, glucose 2hrs post OGTT is predictive of CVD mortality[14].

Those with dysfunctional glucose metabolism also present with abnormalities in triglyceride processing. Impaired lipid metabolism, commonly comorbid to insulin resistance, is a less understood facet of metabolic dysfunction [15]. Expert panels suggest that triglyceride concentrations of ≥2.5mmol/L at 4 hrs following a mixed meal are considered elevated [16]. These postprandial elevations in triglycerides and glucose are novel and powerful predictor of increased CVD risk [1]; in meta-analysis of 68 long-term prospective studies of over 300,000 initially disease free individuals, those with non-fasted triglyceride concentrations in the top third were 37% more likely to have coronary heart disease at follow-up than those with concentrations in the bottom tertile [17]. Notably, the relationship between triglyceride concentrations (fasted or non-fasted) is not independent of other lipid parameters [17]. Postprandial triglyceride concentrations also correlate with familial history of CVD [18] and atherosclerotic plaques in the carotid artery [19].

Mechanistically, exaggerated increases in postprandial triglycerides and glucose are believed to promote atherogenesis at least partially via elevated oxidative stress and endothelial dysfunction [2-4]. Thus, dietary interventions which reduce post meal spikes in triglycerides and glucose or the subsequent rise in oxidative stress and endothelial dysfunction may be particularly important for managing CVD risk.
We have previously studied the \textit{in vivo} effects of a large acute dose of high polyphenol spices (black pepper, cinnamon, cloves, garlic, ginger, oregano, paprika, rosemary and turmeric) on postprandial metabolism and antioxidant activity. The spice meal provides a polyphenolic dose similar to a 5 oz glass of red wine, 40 g dark chocolate, or an 8 oz glass of acai juice (554 gallic acid equivalents). In addition to being well-tolerated, the dose significantly improved some measures of plasma antioxidant status (FRAP and H-ORAC) \cite{20}. Li \textit{et al.} recently reported that a similar spice blend reduced lipid oxidation during the cooking of hamburger and levels of lipid oxidation byproducts \textit{in vivo} after consumption compared to an unspiced burger \cite{21}. In a second study, the same group showed that in those with diabetes, consumption of the spiced burger prevented postprandial endothelial dysfunction relative to the unspiced burger \cite{22}. Thus, Chapter 3 of this dissertation explores whether a high polyphenol herb and spice blend can attenuate oxidative and inflammatory processes provoked by the feeding of a high fat meal and acute psychological stress.

Our previous work demonstrates that the same large dose of spices discussed earlier also reduces the postprandial triglyceride response. Specifically, a spiced meal (same spice dose as described above) was associated with a \textasciitilde30\% lower plasma triglyceride response than the unspiced meal \cite{20}, a finding we recently replicated\cite{5}. The mechanism for this effect is unknown. However, others have also shown that high polyphenol foods, including spices, may inhibit fat absorption in the small intestine \cite{5, 23} and delay gastric emptying \cite{24, 25}. Chapter 4 of this dissertation reports the findings from a clinical trial that examines the effect of the same 14.5g spice dose on postprandial triglycerides, fat absorption and gastric emptying rate. This study also assesses postprandial endothelial dysfunction using the gold standard non-invasive
flow-mediated dilation technique (FMD), an improvement over previous work which uses a newer and less prognostically method.

Finally, although vascular endothelial dysfunction is known to robustly predict cardiovascular outcomes [26], its measurement has yet to be included in standard clinical risk assessment. The gold standard method, FMD, uses ultrasound to measure changes in brachial artery diameter in response to reactive hyperemia [27]. Many laboratories (including our own) have shown robust test-retest reliability for FMD when conditions are rigorously standardized [28-30]. However, the technique requires specialized training and high resolution sonography equipment. Thus, it is expensive and highly operator dependent. Many consider FMD testing to be impractical for either large-scale clinical trials or clinical use [31, 32]. Pulse amplitude tonometry (PAT), a recently FDA approved method which is relatively inexpensive and operator independent, is increasingly being used as an alternative measure of endothelium dependent dilation in response to reactive hyperemia [33]. However, the test-retest reliability of this method has not been established. The fifth chapter in this dissertation reports the reliability of the PAT method for assessing vascular function in a sample of 20 healthy but overweight adults.
Chapter 2

Review of Literature: Postprandial Oxidative Stress

Mechanisms

Oxidative stress or the imbalance in the production of reactive species and the antioxidant system in favor of the former [34], is common to the etiology of most chronic diseases. Free radicals, which include both oxygen and nitrogen derived species containing an unpaired electron, are highly reactive with and may damage surrounding molecules. Within the biological system, this results in injury and malfunction of bioactive molecules. Accordingly, it is associated with cancer, cardiovascular disease and insulin resistance/diabetes [35-38].

Oxidative stress is often stimulated by the consumption of food. Nutrients can serve as targets for oxidative damage and nutrients consumed in the oxidative state increase markers of oxidative injury [21, 39, 40]. However, many researchers have reported that in the postprandial period, the oxidative stress timeline parallels that of glucose and triglycerides [41] and there is ample in vitro evidence to suggest that glucose elevations may actually induce oxidation. In some human cell lines hyperglycemia increases the superoxide formation that accompanies normal mitochondrial ATP production [42]. The expression of subunits which activate NADPH oxidase (NOX), another significant source of intracellular superoxide, are also upregulated in response to both long term and acute elevated glucose levels [43, 44]. Accordingly, pharmacologic agents that minimize glucose fluctuations throughout the day also decrease oxidative stress and inflammation [45].
Mechanisms through which elevated free fatty acids may induce oxidative stress have also been reported: the mononuclear cells of those with hypertriglyceridemia produce more superoxide radicals than normotriglyceridemic subjects [46] and a correlation between all subject’s triglyceride levels and superoxide production was significant [47]. Further, supplementation with antioxidants blunts postprandial oxidative stress associated with triglyceride elevations despite having no effect on the triglyceride concentrations [48].

Several studies have considered the role of varying macronutrient content on the induction of postprandial oxidative damage. Despite molecular mechanisms linking both postprandial glucose and triglycerides to oxidation, many human studies suggest that high fat meal challenges are a more likely instigator than high glucose challenges [10, 49, 50]. In one of the cleaner designs, Fisher-Wellman et al. compared calorically identical meals of fat, carbohydrate, protein or a mix of 33% of each [51]. The fat meal alone produced significant increases in oxidative stress (both radical formation and lipid oxidation by-products) in the 6 hrs following the meal. Additionally, these studies reveal that a fatty acid profile dominated by saturated fat (SF) is likely to induce oxidative DNA damage [52] while lipid oxidation byproducts are likely to be elevated by glucose solutions versus fructose [53].

In a correlational analysis, Aaro et al. showed that postprandial glucose but not triglycerides was associated with increases in a urinary marker of ox (8-Ohdg) in those who had established CVD[54]. Others have shown that increases in MDA, LDL-C susceptibility to oxidation and decreases in TRAP are elevated in accordance with postprandial hyperglycemia [55]. But other oxidative stress markers do appear to increase in connection with postprandial triglycerides even in the absence of glucose [49, 56]. Further, two labs have shown that the degree of oxidative stress induction
following a high fat meal is correlated with the extent of very low density lipoprotein cholesterol (VLDL-C) triglyceride enrichment [48, 57]. Finally, Ceriello and colleagues compared 3 meals containing primarily fat (75g fat, 8g glucose, 700kcals/m² of body surface area), glucose (75g, 300kcals) or both. All three meals, but especially the third mixed meal with the highest energy content, produced the most dramatic increases in byproducts of oxidative damage [10].

In sum, it is likely that excess energy substrate from carbohydrates and fat have the ability to induce OS, and each can also overload the electron transport chain with overproduced acetyl CoA, resulting in elevated oxidative stress [58]. Thus any meal that contains excessive calories is likely to induce some oxidative increase.

Assessment

The magnitude and even the presence of oxidative increase observed following a meal challenge is dependent on the type of marker measured. Oxidative stress is a broad term that encompasses a complicated balance, and as such it can be measured in a variety of ways. For example, actual pro-oxidants such as the free radicals and reactive species superoxide (O2), peroxynitrite (ONOO), hydrogen peroxide (H2O2) and nitric oxide (NOx) can be measured. However, because they quickly react with other molecules, measuring oxidized molecules (e.g. Oxidized LDL-C or oxLDL-C) is often preferred. The byproducts of these radical-molecule reactions may also be evaluated (such as isoprostanes, malondialdehyde).

Notably, oxidative damage may be the result of overproduction of reactive species, or an inability of an antioxidant system to cope with oxidative demands. Therefore, depletion of the antioxidant system is another opportunity for assessment of
oxidative stress. In addition to all of these markers which reflect the current balance, the upregulation or downregulation of these systems via genetic alteration can also be assessed [34]. Thus, the measure of oxidative stress is remarkably complex with various measures often disagreeing on the state of redox balance. For example, Gradek and colleagues demonstrated that a fatty acid profile dominated by polyunsaturated fatty acids (PUFA) was associated with larger increases in MDA than SFA [59]. However, others have shown that SFAs produce more oxidative DNA damage than meal challenges rich in PUFAs [52].

Given the complexities of the pro- and antioxidant balance, the ability to observe increases in oxidative stress following consumption of excessive metabolic substrate may also be dependent on sample health. For example, in a cross sectional investigation of oxidative stress during the OGTT, Serin and colleagues reported increases in oxLDL and MDA at 2hrs post glucose ingestion in participants with impaired and diabetic glucose tolerance but not in those with normal glucose tolerance[60]. Conversely, an increase in the antioxidant marker PON1 was observed in those who were healthy but not in the impaired or diabetic glucose tolerant individuals. Others have found no effect of meal challenges on total antioxidant capacity and peroxide production in healthy subjects [61]. Finally, Graner and colleagues have reported that the magnitude of the oxLDL response to a HFM is related to severity of CAD [62]. Anderson et al. suggest that healthy levels of HDL-C may help to buffer postprandial oxidative insult [57]. High levels of congregated denines in postprandial HDL-C also demonstrate the possible role of HDL-C in reverse transport of oxidized lipids [40]. Thus, oxidative stress following meal consumption may be difficult to observe in healthy individuals (or those with normal HDL-C levels) given that their antioxidant systems are well poised to buffer the prooxidant effects of metabolic substrates. Additional support for this comes
from a study of healthy Koreans who consumed a high fat high carbohydrate meal at baseline and following 4 days of excessive caloric consumption. The single meal at baseline induced increases in radical production but after 4 days of excessive intake, insulin resistance increases and cellular antioxidant systems were elevated [63]. Some studies have shown that women generally react to meal challenges with less of an increase in radical production and oxidized molecules than men [64].

**Endothelial dysfunction: a functional assessment**

The vascular endothelium is the single-celled layer lining the lumen of all blood vessels. It is responsible for modulating permeability, preventing platelet adhesion and releasing the endothelium derived relaxation factor or nitric oxide (NO) [65, 66]. Atherosclerotic plaques form at sites of endothelial injury [67]. Thus the integrity of vascular endothelial cells is crucial to overall vascular health and the prevention of cardiovascular disease. NO dependent vasodilation, measured via flow mediated dilation, is a reliable predictor of vascular health and independently predicts cardiovascular events [68, 69]. Endothelial function reliably decreases in the postprandial period, a phenomenon mediated, at least in part, by hyperglycemia induced oxidative stress [53]. VLDL-C triglyceride enrichment is also associated with increases in reactive species which react rapidly with NO resulting in blunted flow-mediated vasodilatation [57].

In healthy vasculature, elevated intracellular levels of Ca^{2+} or mechanical stimulation via sheer stress act to stimulate the enzyme endothelium nitric oxide synthase (eNOS) to produce NO [65]. eNOS NO synthesis requires L-arginine, tetrahydrobiopterin (BH_4), and oxygen (O_2). eNOS binding to BH_4 and L-arginine is said
to occur in a somewhat cooperative process through which BH₄ first binds and then alters the configuration of the iron in the eNOS enzyme to increase its binding affinity for L-arginine [70]. This is crucial because L-arginine is often a limited resource being competitively sought by both the enzymes eNOS and arginase. After binding all three substrates, eNOS catalyzes a two-step reaction which utilizes the bound O₂ to hydroxylated and then oxidized L-arginine to produce NO and citrulline.[70] This NO can then diffuse through the basal membrane of the cell to stimulate vasodilation in the smooth muscle fibers of the vessel wall.

Oxidative stress impedes basal endothelial function by reducing levels of NO both via direct degradation and by altering the eNOS enzyme so that it can no longer produce sufficient NO (termed eNOS uncoupling) [70]. The main endogenous sources of free radicals include NADPH oxidases (the NOX family) and the mitochondria, both of which can produce the superoxide radical [65]. Intracellular antioxidant systems work to prevent noxious levels of superoxide but imbalances do occur leading to endothelial dysfunction. Type 2 diabetes mellitus and the metabolic syndrome are comorbid with vascular dysfunction and are associated with increased mitochondrial and NOX production of the superoxide radical [71, 72].

Once elevated levels of superoxide are present, intracellular NO is at risk of being quickly transformed into peroxynitrite (ONOO⁻). As seen in Figure 1-2, peroxynitrites can then act to oxidize BH₄ preventing it from binding eNOS and reducing the ability of L-arginine to bind as well. Under these conditions, eNOS continues to work. However, in the absence of BH₄ and L-arginine only O₂ is present in the reaction and ends up being reduced into additional superoxide. Thus free radicals both consume NO and prevent its production in a cyclical process [65].
Figure 2-1. The uncoupling of eNOS by reactive species.

Other more indirect mechanisms have been discovered between free radicals and endothelial dysfunction. For example, elevated levels of hydrogen peroxide (which can be produced by some of the NOX family) increases arginase activity in humans resulting in increased competition for the eNOS substrate L-arginine [70, 73]. Indeed, arginase activity is correlated with the production of reactive oxygen species that mediate endothelial dysfunction in diabetic rats [74]. ADMA or asymmetric dimethylarginine, is a redox sensitive compound which inhibits eNOS activity and therefore reduces NO production. Studies with endothelial cell lines have shown that NOX over production of superoxide increases ADMA both by inhibiting its degradation pathway and increasing the expression of its synthesis pathway [75]. This results in lower NO production and reduced capacity for vasodilation. Given this relationship, it is not surprising that ADMA level is associated with vasodilation capacity in human studies,
predicts cardiovascular mortality and interventions which reduce reactive species also reduce ADMA [76].

**Dietary interventions**

Given the overall predictability of the postprandial increase in OS, meal challenges are a particularly well-suited model for studying the *in vivo* antioxidant effects of foods. Studies testing the impact of spices, dietary patterns and other acute nutritional interventions are reviewed below.

**Spices and herbs**

The spices relevant to this dissertation are black pepper, cinnamon, cloves, garlic, ginger, oregano, paprika, rosemary, and turmeric. We have previously demonstrated that a 14g blend of these spices improves some measures of antioxidant capacity *in vitro* and is well tolerated in a meal employing ecologically valid recipes (coconut chicken curry, cheese muffins and dessert cookie). Additionally, this blend reduced postprandial insulin and triglycerides by 21 and 30% respectively when compared to an unspiced meal [20]. A follow-up study confirmed the postprandial triglyceride lowering effect during rest but not stress conditions with a larger sample (n = 20 vs previous n = 6) and with an additional 0.5g cinnamon in the spice dose [5].

However, two other labs have recently reported no effect of spice blends on post meal triglycerides. Li *et al.* found no effect of an 11.25g dose of spices (the same spices listed above minus turmeric) on postprandial triglycerides in adults with diabetes up to 6 hrs after consuming a hamburger (440kcals, 25gfat) [22]. Similarly, Nakayama *et al.*
reported no effect of a 10g dose of garlic, clove, red pepper, ginger, cumin, coriander and 9g of sautéed onion on triglycerides 1.5 hrs postprandial a curry meal (480kcals, 10gfat) [77]. Li et al. also reported a reduction in urinary MDA following the spiced burger, but MDA was reduced in the burger prior to consumption [21]. Nakayama et al. measured postprandial oxidative stress as MDA modified LDL-C and found no difference between the control and spiced curries. Both studies however reported that the spiced meals prevented postprandial decreases in endothelial function; Li et al. using pulse amplitude tonometry at 2hrs and Nakayama et al. using FMD at 1hr postmeal. Which individual spices are most responsible for the observed health benefits of these blends are unknown, although spices are known to work synergistically when influencing metabolism [78]. The amount of research considering the health benefits of individual spices varies widely, and is reviewed below. An overview of these studies can be viewed in Table 2-1.

Table 2-1. Overview of studies considering the influence of herb and spice blends on postprandial oxidative stress and endothelial function

<table>
<thead>
<tr>
<th>Author year</th>
<th>Subject(s) (n)</th>
<th>Meal description/preparation</th>
<th>Spice dose</th>
<th>PP OS effects</th>
<th>PP endothelial function effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li et al., 2010</td>
<td>Healthy (11)</td>
<td>Burger patty cooked with or w/o S</td>
<td>11.3g cloves, cinnamon, oregano, rosemary, ginger, black pepper, paprika, garlic powder</td>
<td>Plasma MDA: sig increased following S vs un-S; Urine MDA: 49% reduction following S vs un-S</td>
<td>NA</td>
</tr>
<tr>
<td>Li et al., 2013</td>
<td>Diabetic (18)</td>
<td>Burger patty cooked with or w/o S</td>
<td>Same as 2010 protocol</td>
<td>Urine MDA: reduced 31% following S vs un-S patty</td>
<td>PAT: higher following S vs un-S burger</td>
</tr>
<tr>
<td>Skulas-Ray et al., 2010</td>
<td>Healthy (6)</td>
<td>Coconut milk curry chicken meal cooked with or w/o S</td>
<td>14.0g of black pepper, cinnamon, cloves, garlic, ginger, oregano, paprika, rosemary, turmeric</td>
<td>FRAP: 2 fold increase following S vs un-S; H-ORAC: increased following S vs un-S</td>
<td>NA</td>
</tr>
<tr>
<td>Nakayama et al., 2014</td>
<td>Healthy (14)</td>
<td>Tomato base curry cooked with or w/o S</td>
<td>14.5g of clove, coriander, cumin, garlic, ginger, red pepper, turmeric +</td>
<td>MDA modified LDL: no effect of S; 8-isoprostanes: no effect of S</td>
<td>FMD: S increased pp FMD by1.5% compared to PP</td>
</tr>
</tbody>
</table>
### Cinnamon

A large body of work has evaluated the influence of cinnamon extracts on chronic disease risk indicators. In a review of seven human clinical trials, Deng concluded that there is strong evidence to support the hypoglycemic lowering effects of cinnamon with the exception of some specific population groups [79]. This effect may be partially due to blunting of the postprandial glucose response which has been observed in both animal and human models [80]. Mechanistically, cinnamon is known to inhibit amylases necessary for carbohydrate absorption [81] and promote GLUT4 translocation for cellular uptake of circulating glucose [82]. *In vitro*, cinnamon extracts also promote insulin secretion which could further influence postmeal glucose [83].

An effect of cinnamon on gastric emptying rate has also been described [84], although the human literature on this point is mixed [24, 67, 85]. A review of animal studies also suggests that cinnamon may improve the lipid profile in those with impaired glucose metabolism [86]. Human studies support this; 120 mg/d to 6 g/d of cinnamon consistently reduces fasting triglycerides, LDL-C and fasting blood glucose concentrations while increasing HDL-C within 4-18 weeks [87]. These metabolic effects
are thought to be responsible for at least one positive finding on body composition in humans [88].

The impact of cinnamon on oxidative balance is understudied. One protocol detected no difference in fasting MDA following 6 weeks of supplementation with 3g/day [89]. One human study considered oxidative stress prandially after a matched meal with and without cinnamon. In this protocol, Markey et al. fed 3g of cinnamon or placebo with a breakfast (42g fat 42g carbohydrates) and reported no effect on lipid hydroperoxides 3 hours later [85].

**Garlic**

Garlic supplementation has long demonstrated an ability to reduce blood pressure in those with hypertension [90]. Specifically, 600–900 mg/day of garlic powder reduces systolic blood pressure by 8.4 ± 2.8 mm Hg and diastolic pressure by 7.3 ± 1.5 mm Hg in those with hypertension. Garlic bioactives increase intracellular antioxidants and protect BH₄ from oxidant damage, both of which protect the vasodilator NO [91, 92], promoting endothelial health. Accordingly, the one clinical trial which evaluated vascular health following a high fat meal with or without garlic, showed that garlic prevented meal induced arterial stiffness [93]. Meta-analysis also supports the claim that garlic improves the lipid profile by reducing total and LDL-C cholesterol and reducing fasting blood glucose [94].

Very little work has described the effects of garlic singly on acute metabolism. In diabetes induced rats, garlic extract reduced glycemic response during OGTT acutely and after 3 weeks of supplementation [95]. Reductions in the activity of maltase and sucrose and increased expression of GLUT4 and insulin production genes by the extract
were also reported. In adults with diabetes, garlic + drug therapy (metformin) lowered fasting and postprandial glucose better than either garlic or drug therapy alone [96]. Two clinical trials have evaluated the effect of garlic on postprandial lipemia; one found a 35% reduction in post meal triglycerides in garlic vs. placebo [97], the other found no difference [98]. *In vitro*, garlic acts synergistically with polyphenols to protect LDL-C from oxidation, however human clinical trials on this point have not been published [99].

**Turmeric**

The anti-inflammatory and antioxidant characteristics of turmeric (or its constituent curcumin) have made it a popular topic of nutraceutical literature [100, 101]. However, since some components of the spice are poorly absorbed, its influence is thought to be somewhat limited to the gut [102]. A small number of clinical trials also suggests that turmeric has clinically significant effects on digestion and the health of the gastrointestinal tract in those with gut disorders [100]. Some effects on metabolism have also been reported. In patients with metabolic syndrome, 1g/d of turmeric + piperine from black pepper for 8 weeks reduced LDL-C, total cholesterol and fasting triglycerides and increased HDL-C [103]. The effects of turmeric on the lipid profile have been confirmed in one additional clinical trial [104]. The co-ingestion of 6g of turmeric and 75g of glucose solution used in the oral glucose tolerance test is reported to increase insulin secretion for up to 2 hrs [105].

*In vitro*, turmeric exhibits potent antioxidant activity [106, 107]. Findings from *in vivo* models also suggest that turmeric may be a potent *in vivo* antioxidant. For example, turmeric and curcumin alone improved basal oxidative balance in rats with induced diabetes [108]. However, turmeric’s ability to enhance circulating antioxidant potential
appears to be significantly less than that of garlic [101]. In healthy adults consuming 0.8g of curcumin per day reduced fasting triglycerides and and blood markers of vascular function (adhesion molecules) while increasing host antioxidant scavenging potential [109]. In those with type 2 diabetes, 1.5g/d of curcuminoids for 8wks improved fasted inflammatory markers, lipid oxidation byproducts and a blood marker of vascular health [110].

**Ginger**

Four trials in adults with diabetes report that 1 to 3g/day of ginger powder for 4-12 weeks, improves fasting measures of glycemic control, lipid parameters [111] insulin sensitivity [112, 113] and lipid oxidation [114]. However in a group with coronary artery disease (CAD), 4g/day for 12 weeks had no impact on blood clotting time, lipid profile or fasting glucose [115]. Notably, a single 10g dose of ginger in this same sample of CAD patients acutely reduced platelet aggregation. No difference was observed between fasting markers of endothelial health (adhesion molecules) when a sample of dialysis patients consumed 1g/day ginger vs. control for 10 weeks [116].

Postprandially, ginger is known to speed gastric emptying in those with disorders of delayed digestion [117, 118]. Additionally, an acute thermogenic effect of dried ginger has been reported along with increased satiety and reduced hunger [119]. Extract from 25g fresh grated ginger root has demonstrated ability to reduced postprandial glucose up to 2hrs postmeal in healthy adults [78]. No human work has evaluated postprandial oxidative stress in humans. In rats, ginger enriched diets reduce basal oxidative stress [120]. However, one human protocol found no difference in fasting oxidative parameters in female athletes following ginger supplementation for 6 weeks [89].
**Other: clove, rosemary, oregano, paprika, black pepper**

**Clove** is a potent antioxidant *in vitro* [121], and one study of zebra fish suggests that it has hypolipidemic properties *in vivo* as well [122]. **Rosemary** bioactives are potent *in vitro* antioxidants; they upregulate endogenous antioxidant potential [123], and when administered in concert, act synergistically with dietary polyphenols to protect LDL-C from oxidation [99]. **Oregano** oil has potent antimicrobial and anti-inflammatory characteristics [124] and in mice demonstrates an ability to prevent diabetes onset and scavenge radicals preventing oxidative damage [125]. **Paprika**, also referred to as red pepper or chili, contains a the well-known thermogenic compound capsaicin [126] and was associated with reduced postmeal insulin after daily and acute consumption [127]. Once thought to only enhance the bioavailability of other bioactives via piperine, **black pepper** is now known to possess antioxidant potential of its own [106]. In rats fed a high fat high carbohydrate diet, dietary piperine (the main bioactive in black pepper) enrichment normalized several measures of cardiovascular risk including oxidative parameters [128].

**Dietary pattern interventions**

Dietary patterns rich in antioxidants have demonstrated some ability to influence basal levels of OS. For example, in a randomized parallel arm design, 8wks of high polyphenol diet resulted in reduced urinary 8-isoprostane levels compared to baseline levels [129]. The studies reviewed tested the effects dietary patterns emphasizing fatty acid profile (Mediterranean diet [130], vegan diet [56]), glycemic index [131, 132], foods containing high levels of phenolics (cocoa [133], strawberry [134]) and even antioxidant
tablets [48, 135] for periods of 3ds to 6wks on oxidative stress following a standardized meal stimulus.

Dietary patterns with favorable fatty acid profiles (e.g. reductions in SFA) resulted in favorable alterations in the postprandial OS; 21ds of vegan diet reduced postprandial radical production and oxidative damage to lipids and proteins [56] while 4wks of a Mediterranean diet (38% MUFA) vs SFA diet (30% SFA) increased gene expression of cellular antioxidants [130]. These findings could be the result of the fatty acid profile’s influence on triglyceride and HDL-C levels and/or phytonutrients. Acute studies, which are too short to effectively change HDL-C, suggest that the phytonutrients are responsible. For example, despite similar postprandial triglyceride responses, when ice cream and avocado were fed in a calorically equivalent dose, the ice cream meal (and a meal with equivalent sugar only and one with equivalent fat only) increased postprandial oxidative stress but the avocado did not [136]. Others have shown that almonds produce less protein oxidation than meals which are relatively poor in phytonutrients but lack any significant fatty acid profile (e.g. rice, potatoes, bread) [137].

In both studies of varying glycemic index diets, low glycemic index diets resulted in less postprandial oxidative stress than high glycemic diet controls. In one study, the increases in ORAC seen postprandial after the low glycemic period were confounded by differences at the fasting blood draw [132]. However, Kwak et al. reported reduced MDA and improvements in NO even when controlling for baseline [131]. Stable glucose levels likely stimulate less radical production. Finally, the four studies which evaluated the effects antioxidant supplements or foods containing high amounts of antioxidants (cocoa and strawberry) [48, 133-135] all demonstrated an ability to reduce postprandial oxidative stress likely via traditional antioxidant pathways.
**Other acute interventions**

Acute ingestion of foods or compounds hypothesized to blunt the postprandial oxidative stress induced by a meal challenge were reviewed. Of the 15 studies reviewed, 13 reported improvement in postprandial oxidative balance by the intervention tested: red wine [138], orange juice [139, 140], spices [20, 21], Asiatic plantain [141], strawberry [134], tomato [142], walnuts [143], L-arginine [144] or vitamin E [46] supplementation and oils (extra virgin olive oil [145, 146], antioxidant supplemented oil [146]). Blackhurst et al. failed to see any effect of wine on a variety of assessments of postprandial chylomicron oxidation [147]. One study of vitamin E failed to observe a postprandial increase in their oxidative stress measure (MDA) following either the control or treatment meals [148].

Several of the successful interventions (wine, spices, tomato, olive oil) contained polyphenolic compounds. Indeed, a study of *in vivo* phenolic kinetics after ingestion of 144g of raisins showed total plasma phenolics peaked at 1hr, the same time at which serum oxidation resistance peaked [149]. However, some have argued that polyphenols are poorly absorbed and are therefore unlikely to be potent bioactors in quantities typically consumed in foods [150]. Despite this, many of the polyphenol-rich interventions produced statistically significant *in vivo* changes in oxidative balance. Interestingly, one lab reported that although plasma phenolics from orange juice were not detectable in circulation during the 3hr following orange juice consumption, the orange juice did effectively increase both ORAC and the lag time for lipoprotein oxidation [140].

In a recent report, Carnevale et al. demonstrated that extra virgin olive oil could blunt postprandial NOX2 (the catalytic subunit of NADPH oxidase, a major contributor to
cellular ROS) and prevent postprandial decreases in vitamin E when compared to corn oil [145]. This suggests that vitamin E may be an important mechanism through which the oil interventions influence postprandial OS. The γ-tocopherol component of vitamin E in particular, surfaced as the correlate of decreased postprandial oxidative stress after walnut consumption [143] and supplementation with γ-tocopherol effectively blunts postprandial increases in glucose mediated OS [151]. Importantly, in vitro work suggests that high antioxidant extracts, including those derived from rosemary and garlic, work synergistically to produce antioxidant protection that is greater than the sum of its parts [99]. Thus, polyphenols and vitamins may work together to produce effects that are not explainable by plasma polyphenol content alone.
Chapter 3

A meal challenge acutely alters some measures of inflammation, lipid oxidation and vascular function regardless of the presence of high polyphenol spices

Abstract

**Background:** Culinary spices have demonstrated the ability blunt oxidation and inflammatory responses *in vitro*, but it is unknown whether this effect would exist in a human model of induced inflammation. Acute psychological stress has been shown to induce inflammation *in vivo*. **Objective:** The objective of this study was to examine whether adding 14.5 g of a high polyphenol herb and spice blend to a 945 kcal meal could ameliorate the oxidative stress, inflammation and vascular dysfunction stimulated by a high fat meal in a setting of acute psychological stress. **Methods:** Healthy overweight (BMI = 30.4 ± 0.9) men (n = 14) and women (n = 6) completed 4 testing sessions for a 2 X 2 factorial design examining the effects of spice, acute stress, and their interaction. Testing sessions were separated by at least one week. Blood was sampled at 5 time points per session for oxidative stress markers (oxLDL and MDA) and plasma immune markers (TNF-α and IL-6). Endothelial function (EndoPAT reactive hyperemia index or RHI, Framingham reactive hyperemia index or FRHI and augmentation index or AI) was assessed once at baseline and once following the meal + condition sequence (twice per visit). **Results:** Compared to the fasting baseline, the meal increased oxidative stress regardless of the presence or absence of spices (p = 0.003 for MDA and p = 0.02 for oxLDL). Similarly, the meal resulted in increased plasma IL-6 (p <0.001). A stress by time interaction was also present for plasma IL-6 (p = 0.05), such that when stressed, the average change was elevated at time 2 compared to time 3.
(1.05 ± 0.25 vs 0.44 ± 0.16, Tukey p = 0.03). However, the direct comparison of IL-6 at time 2 following stress versus rest revealed no difference. There was no effect of spices on oxidative stress, inflammation or vascular function. **Conclusions:** A high fat/high carbohydrate meal induces increased oxidative stress and inflammation. A blend of common culinary spices does not influence either of these parameters. Researchers should be aware that food intake confounds the expected physiological response to stress.
Introduction

Oxidative stress and inflammation are key players in the etiology of chronic disease [152]. In addition to direct oxidative modification of lipids and proteins, amplified oxidative stress promotes inflammation and vascular dysfunction [153]. Thus, interest in dietary antioxidants may prevent oxidative stress and inflammation in vivo has increased. Spices are potent sources of polyphenols [154] and have among the highest in vitro ORAC values on a per gram basis [155]. As such, spices are a promising low calorie vehicle for dramatically increasing dietary antioxidant intake without increasing overall energy intake. Polyphenol rich foods (such as spices, cocoa and red wine), have been shown to blunt postprandial increases in oxidative stress [21, 22, 133, 156, 157]. Many high polyphenol spices have also demonstrated benefits on glucose, lipid, and inflammatory homeostasis [87, 158-160].

We have previously studied the in vivo effects of a large acute dose of high polyphenol spices (black pepper, cinnamon, cloves, garlic, ginger, oregano, paprika, rosemary and turmeric) providing a polyphenolic dose similar to a 5 oz glass of red wine, 40 g dark chocolate, or 8 oz glass of acai juice (554 gallic acid equivalents) [20]. In addition to being well-tolerated, the dose significantly improved some measures of plasma antioxidant status (FRAP and H-ORAC) and the triglyceride response to a high fat meal [5, 20]. Others have demonstrated that a similar spice blend added to hamburger prior to cooking reduced lipid oxidation byproducts both in the meat and in vivo post consumption [21, 22]. Further, two protocols have reported that spiced meals blunt endothelial function deteriorations observed following consumption of their unspiced counterparts [22, 77]. These human clinical trials suggest that high polyphenol spices may blunt in vivo oxidation and its result, inflammation.
High fat, high carbohydrate meals are pervasive in Western culture and the metabolic response to such meals (e.g. plasma glucose and triglycerides) is now recognized as an indicator of cardiovascular risk [161]. The excessive energy substrate provided by these meals provokes increased oxidative stress, inflammation and vascular endothelial dysfunction [2, 3]. Likewise, the experience of psychological stress influences disease risk partially through the promotion of inflammation [162]. The Trier Social Stress Test (TSST) is well studied laboratory model of moderate acute psychological stress which reliably provokes inflammatory responses [163]. Laboratory mental stress challenges are also known to induce vascular dysfunction [164]. Dietary interventions capable of attenuating the oxidative and inflammatory responses provoked by a high fat meal and/or psychological stress could be of great benefit to public health.

This study was designed to assess whether a high polyphenol herb and spice blend was able to attenuate oxidative and inflammatory processes stimulated by the feeding of a high fat meal and acute psychological stress. Endpoints include plasma levels of malondialdehyde (MDA), oxidized low density lipoproteins (oxLDL), the inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α), and vascular function (assessed via PAT-pulse amplitude tonometry). In an effort to determine the source of lipid oxidation changes in blood samples (e.g. formation during meal preparation versus in vivo processes), we also considered the magnitude of lipid oxidation in the test and control meal.

Hypothesis 1: We hypothesize that the high fat/high carbohydrate meal will induce increases in inflammation, oxidative stress and endothelial dysfunction.

Hypothesis 2: We hypothesize that the acute mental stressor will also stimulate increases in inflammation.
Hypothesis 3: We hypothesize that when participants consume the high fat/high carbohydrate meal with the herb and spice blend, the inflammation, oxidative stress and endothelial function induced by the meal and stress will be less pronounced than after control.

Methods

The 2 x 2 factorial design employed to test our hypotheses has been described previously in detail [5]. In brief, we conducted a randomized, controlled, 4-period crossover study with at least one week separating testing sessions. Participants were randomized to the following conditions, presented in counterbalanced order: 1) spice meal + rest, 2) spice meal + stress, 3) control meal + rest, and 4) control meal + stress.

Participants

Twenty healthy men and women completed this study (N = 6 females). Interested individuals (n=118) were screened by phone after answering advertisements from community bulletin boards and email lists. Thirty-one met initial criteria were scheduled for clinic screening (2 cancelled their visits). Blood was sampled for a complete blood count and chemistry panel; weight and height were measured. Blood pressure was measured according to JNC 7 guidelines [165]. Inclusion was limited to those who were aged 30-65 y, free from any serious illness (including any inflammatory conditions, liver or kidney dysfunction, a history of heart disease), had body mass index (BMI) of 25-40 kg/m², resting blood pressure < 160/100 mm Hg (if blood pressure was > or = 140/90, approval for study participation was requested from the participant’s
physician), fasting glucose <126 mg/dL, and willingness to discontinue all dietary supplements during the study. Additionally, potential participants were excluded if they used tobacco products, were training for athletic competition (>2 h aerobic activity a week), or used medications relating to birth control, hormone replacement therapy, lipid lowering, blood pressure, and psychosis or depression, with the exception of selective serotonin reuptake inhibitors.

Twenty four individuals were approved but only 22 started the study. Two participants were withdrawn during the study due to exceeding limits for blood pressure (n = 1) and emotional response to the stress task (n =1). Data are reported for 20 healthy participants.

Procedures

In the 48 hrs prior to each of 4 testing session, participants consumed one meal per day (provided) which matched their treatment assignment for the laboratory session (e.g. a meal containing spices was consumed vs. a meal without spices + placebo capsules). Participants were asked to avoid foods containing antioxidants in the 24 hours prior to visits, and a list of such foods was provided (supplemental materials [5]). Participants fasted overnight and consumed a standardized, low antioxidant breakfast (white bagel and nonfat spread were provided) at 8:00am on the morning of each visit.

A timeline of study procedures can be viewed in Figure 3-1. Upon arrival, the first vascular function assessment was made via EndoPAT at approximately 11 am followed by the placement of an intravenous catheter. After a baseline blood sample, participants were given 30 minutes in which to consume the high fat meal meal (with or without spices) followed by a stress or rest condition. The second blood sample was collected
approximately 75 minutes after the start of test meal (105 minutes after the fasted baseline blood draw). Additional blood samples were collected at 140, 180 and 210 minutes (Figure 3-1). The second vascular function test was administered at 240 minutes post meal.
Figure 3-1. Study schematic and visit timeline
Vascular function testing

Endothelial function was assessed twice at each visit (baseline and 4hrs post meal) in a quiet, dimly lit room at 71 - 75 °F (21 - 24 °C). The EndoPAT2000 (Itamar Medical, Ltd.) was used to measure fingertip pulse wave amplitude (PWA) pre and post ischemia via two flexible probes placed on the index finger of the right (ischemic) and left (control) hands [166]. Ischemia was induced by a blood pressure cuff inflated on the forearm by an automated rapid cuff inflator set to 250 mm Hg (Hokanson, Bellevue, WA) for five minutes. Measurements were made during baseline (5 min), occlusion (5 min), and reactive hyperemia (5 min). The Reactive Hyperemia Index (RHI) was calculated as the ratio of the average PWA during hyperemia (60 sec to 120 sec of the post-occlusion period) compared with the average PWA during baseline in the occluded hand over the same values in the control hand multiplied by a baseline correction factor. The Framingham RHI (F-RHI) is an alternative calculation derived from the same data that others have identified as being most strongly associated with traditional CVD risk indicators [167]. The EndoPAT device also generates the augmentation index (AI), a measure of vascular stiffness (pulse wave reflection) that is calculated from the shape of the pulse waves. AI was adjusted to a heart rate of 75 beats per minute.

Test meal

The 945 kcal lunch (43g fat, 98g carbohydrate, 43g protein) meal consisted of a coconut chicken curry and white rice dish, a corn muffin, and pastry dessert. The fatty acid profile of the meal can be viewed in detail in Chapter 4. In brief, the meal contained 128mg of cholesterol, 24.6g saturated fatty acids (SFAs), 9.2g monounsaturated fatty
acids (MUFAs) and 5g polyunsaturated fatty acids (PUFAs). The control meal was prepared without spices, but served with placebo capsules. The herb and spice blend (14.5 g/meal) was incorporated into the various meal items as follows: chicken curry and rice (turmeric, garlic, black pepper, cinnamon, cloves, ginger, oregano, paprika), muffin (rosemary, oregano, garlic, black pepper, paprika, turmeric), and a dessert pastry (cinnamon, cloves, ginger). The exact amounts of spices present in each menu item and other details about the meal have been previously published [5] and can be viewed in Figure 3-2. The spice dose is estimated to provide 404 mg GAE of phenolics, 8690μmol TE hydrophilic oxygen reducing absorbance capacity (ORAC) and 5157μmol TE lipophilic ORAC. The spices were obtained from the Characterized Samples Program at the McCormick Science Institute (Hunt Valley, MD) and weighed with a balance accurate to 0.01 g (Mettler Toledo, Columbus, OH).

Table 3-1. Spice blend constituents, antioxidant potential and distribution in test meal

<table>
<thead>
<tr>
<th>Spice</th>
<th>Dessert biscuit, g</th>
<th>Coconut chicken, g</th>
<th>Cheese bread, g</th>
<th>Total dose, g</th>
<th>H-ORAC contribution,(^1)μmol TE</th>
<th>L-ORAC contribution,(^1)μmol TE</th>
<th>Phenolic contribution,(^1)mg GAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamon</td>
<td>0.88</td>
<td>0.23</td>
<td></td>
<td>1.11</td>
<td>1590</td>
<td>37</td>
<td>50</td>
</tr>
<tr>
<td>Cloves</td>
<td>0.30</td>
<td>0.31</td>
<td></td>
<td>0.61</td>
<td>680</td>
<td>1091</td>
<td>101</td>
</tr>
<tr>
<td>Garlic powder</td>
<td>0.91</td>
<td>0.90</td>
<td></td>
<td>1.81</td>
<td>118</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Ginger</td>
<td>0.38</td>
<td>0.75</td>
<td></td>
<td>1.51</td>
<td>138</td>
<td>451</td>
<td>10</td>
</tr>
<tr>
<td>Oregano (Mediterranean)</td>
<td>1.13</td>
<td>1.13</td>
<td></td>
<td>2.26</td>
<td>3745</td>
<td>510</td>
<td>86</td>
</tr>
<tr>
<td>Paprika</td>
<td>1.43</td>
<td>1.42</td>
<td></td>
<td>2.85</td>
<td>392</td>
<td>233</td>
<td>47</td>
</tr>
<tr>
<td>Rosemary</td>
<td>0.61</td>
<td>0.61</td>
<td></td>
<td>0.61</td>
<td>684</td>
<td>324</td>
<td>30</td>
</tr>
<tr>
<td>Turmeric</td>
<td>2.09</td>
<td>0.70</td>
<td></td>
<td>2.79</td>
<td>1249</td>
<td>2296</td>
<td>77</td>
</tr>
<tr>
<td>Black pepper</td>
<td>0.45</td>
<td>0.45</td>
<td></td>
<td>0.91</td>
<td>93</td>
<td>212</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>14.5</td>
<td></td>
<td></td>
<td></td>
<td>8690</td>
<td>5157</td>
<td>404</td>
</tr>
</tbody>
</table>

\(^1\) ORAC values (in vitro) are from the USDA 2010 Report [155]. Abbreviations: H-ORAC, hydrophilic ORAC; L-ORAC, lipophilic ORAC. Contribution of ORAC and phenolic compounds was calculated by weight using the values reported in the table.
**Stress testing**

The Trier Social Stress Test (TSST) is a standardized and scripted procedure for producing reliable increases in cortisol, blood pressure and heart rate [168]. In brief, subjects prepare (10 min) and deliver (5 min) a speech and complete difficult arithmetic problems (8 min), while in the presence of “expert panelists” who provide no encouragement and offer negative verbal feedback. Subjects listened to gentle music during a 20 min rest period which preceded the stressors and a 10 min recovery which followed them. To prevent habituation, the topic for the speech task and the numerical base of the mental arithmetic was changed for the second stress visit. Blood pressure and heart rate were monitored repeatedly throughout each task using a standard blood pressure (BP) cuff (Dinamap Pro 100 oscillometric monitor, GE Medical Systems).

**Biochemical analysis**

Whole blood was transferred into serum separator tubes, allowed to clot, and centrifuged. Serum high-sensitivity C-reactive protein was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%). Plasma concentrations of IL-1β, IL-6, TNF-α were measured via high sensitivity ELISA kits from R&D Systems (Minneapolis, MN) in duplicate (assay CV < 11% for all). Serum hs-CRP was measured by latex-enhanced immunonephelometry (Quest Diagnostics, Pittsburgh, PA; assay CV < 8%). Malondialdehyde was assessed by fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm via the Thiobarbituric Acid Reactive Substances Assay Kit (Caymen Chemicals, Ann Arbor, MI, mean CV 2.4%). Oxidized
low density lipoprotein cholesterol was assessed via Sandwich ELISA based on the mouse monoclonal antibody 4E6 (Mercodia, Uppsala, Sweden, average CV 3.6%).

**Statistical Analysis**

The mixed models procedure (PROC MIXED) was used to test the fixed effects of treatment (spice or placebo), condition (stress or rest testing session), time and their interaction on each outcome variable. Outcomes were first modeled as levels/concentrations to evaluate any differences at baseline (effects of preloading with spices or placebo). These models revealed no differences and the use of change scores improved model fit significantly. Thus, the results reported herein are based on models of change scores (value - baseline). Bonferroni adjustment was applied to an α of 0.05 to determine the significance level for comparisons of the change score being significantly different from 0. SAS produced Tukey p-values for all other post hoc analyses. Non-transformed least-squares means and standard errors are reported. Model selection (including best covariance structure) was based on optimizing fit statistics, or lowest Bayesian Information Criterion.

**Results**

A description of this sample has been previously published. In brief, the sample was overweight (average BMI of 30.4 ± 0.9 kg/m²), middle aged (average age of 43.2 ± 2.3 years), and normotensive (average blood pressure of 117.8 ± 2.2mmHg systolic and 81.8 ± 1.3mmHg diastolic). The meal produced expected increases in triglycerides,
glucose and insulin and the stress task induced expected increases in blood pressure
and heart rate (published previously here [5]).

Oxidative stress markers

As expected, there was a significant positive change in both MDA and oxLDL
immediately following the meal (mean change of $0.36 \pm 0.11$ nmol/ml, $p = 0.003$ and
$3.54 \pm 1.38$ U/L, $p = 0.01$ from baseline to time 2, respectively; Figure 3-2). Elevations
above baseline were not observed at any later time. Congruently, a main effect of time
was present for both MDA ($p = 0.007$) and oxLDL ($p = 0.02$). Peaks were observed at
time 2 (75 minutes after the meal): change in MDA was higher at time 2 than all later
time points (Tukey $ps < 0.03$) while change in oxLDL was higher at time 2 compared with
time 4 (Tukey $p = 0.019$). No effects of spices or the stressor were present for either
MDA or oxLDL.
Figure 3-2. The oxidative stress markers malondialdehyde by spice, stress and time (A) and by significant main effect of time only (B) and oxidized low density lipoprotein cholesterol by spice, stress and time (C) and by significant main effect of time only (D).
Inflammatory markers

As expected, there was a significant positive change in plasma IL-6 at time 2 (75 minutes post meal) compared to baseline (average change of 0.82 ± 0.18 units, p < 0.001; Figure 3-3). IL-6 remained elevated above baseline at all post meal time points (ps < 0.001). A stress by time interaction was also present for plasma IL-6 (p = 0.05), such that when stressed a peaking effect occurred; IL-6 was higher at time 2 than time 3 for the stress condition only (average change of 1.05 ± 0.25 vs 0.44 ± 0.16, Tukey p = 0.03). However, when comparing stress versus rest, no significant difference was found. There was no effect of the spices on plasma IL-6.

Plasma TNF-α values exhibited a main effect of stress (0.04) and both a treatment by time (p = 0.03) and stress by time (0.02) interaction. During post hoc testing, no comparison for either interaction was significant. Therefore, we interpreted the main effect of stress: plasma TNF-α values were reduced to a greater degree following stress (mean change of -0.12 ±0.03) versus following rest (mean change of -0.02 ± 0.03; Figure 3-3). In fact, following the rest condition there was no significant change from baseline at any time point. However following stress, plasma TNF-α was significantly lower than baseline at time 3, 4 and 5 (ps < 0.002).
Figure 3-3. Change in plasma cytokines IL-6 and TNF-α by time, stress and spice (A, C) and by significant stress effects only (B, D).

**Vascular function**

Neither measure of reactive hyperemia (RHI or F-RHI) was altered following the meal (Table 3-2). Additionally, neither the presence of spices nor the experience of stress significantly predicted change in either score. In contrast, augmentation index decreased (which reflects an improvement in arterial stiffness) following the meal. This change was similar regardless of the presence of spices or stress.
Table 3-2. Average pre and post intervention EndoPAT vascular function scores

<table>
<thead>
<tr>
<th>Vascular parameter</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Reactive Hyperemia Index (RHI)</td>
<td>1.85</td>
<td>± 0.05</td>
</tr>
<tr>
<td>Framingham RHI</td>
<td>0.34</td>
<td>± 0.03</td>
</tr>
<tr>
<td>Augmentation Index(^1)</td>
<td>-14.11</td>
<td>± 1.11</td>
</tr>
</tbody>
</table>

\(^1\) Adjusted for a heart rate of 75bpm

*postmeal significantly different than premeal, p <0.05

Figure 3-4. Change in measures of PAT measures vascular endothelial function from baseline to 4 hrs post meal by treatment (spice or placebo) and condition (stress and rest).
In this study of otherwise healthy overweight/obese adults, we confirmed hypothesis 1 and partially confirmed hypothesis 2: the high fat meal induced acute elevations in lipid oxidation and plasma IL-6. Stress caused a peak in the postprandial plasma IL-6 response, but failed to provoke a difference compared to the rest condition. The meal did not directly influence plasma TNF-α, but reductions were observed following the psychological stress condition. Endothelial function was unaffected by the meal or stress, except for an unexpected overall improvement in arterial stiffness at the post intervention time point. There was no evidence to support hypothesis 3: the presence of spices in the high fat meal did not influence any of these endpoints.
As expected, the high fat meal induced an increase in lipid oxidation byproducts and the oxidation of LDL-C. This increase was only observed at the blood draw most immediately following the meal (105 minutes post meal) but not at subsequent time points (140-210 min post meal). Despite our earlier findings that a similar herb and spice blend augmented some measure of antioxidant capacity in vivo [20], we found no effect of the spices on plasma MDA or the oxidation of LDL-C in this larger study. Notably, two rigorously conducted studies have shown that when a similar spice blend is added to hamburger meat prior to cooking, levels of the lipid oxidation byproduct MDA are reduced postprandially in blood and urine compared to an unspiced burger [21, 22]. MDA production due to lipid oxidation is a well-known result of cooking [169] and indeed the spiced burger was reported to have 71% lower levels of MDA compared to the unspiced burger after cooking [21]. Thus the difference in oxidative byproducts in blood and urine observed in these studies may reflect differential consumption of MDA rather than altered in vivo processes.

Whether our spiced and unspiced meals were similar with respect to lipid oxidation markers is yet to be determined. However, we hypothesize that the lipid oxidation content of our test meal and control meal were similar, that is, the spices did not influence lipid oxidation dramatically during cooking. This is likely given that over half of the fat in our test meal was oxidation-resistant SFAs (primarily from coconut milk in the curry) [170]. Additionally, our menu items containing the most oxidation-susceptible PUFAs were baked; baking has been shown to produce minimal lipid oxidation in alveolar cereal products (such as our muffin and biscuit) [171]. Given our null findings for both MDA and oxLDL-C, we propose that the herb and spice blend is likely to prevent oxidation of susceptible fats during cooking (as in the burger studies), but limited ability to provide in vivo protection to lipids in humans.
Only a small number of studies have considered the influence of acute laboratory mental stress on indices of oxidative stress. One series of studies showed that mental stress reduces reactive oxygen species via suppression of superoxide release from neutrophils [172, 173], but others have not been able to confirm any change in ROS or total antioxidant capacity following acute laboratory stress [174, 175]. This is the first study of which we are aware to test the effects of metal stress on the oxidation of lipids specifically. Our final models showed a null effect of stress on both measures of lipid oxidation in this young and healthy population. Measurable changes in oxidative stress following acute stress may be more likely in aging adults with generally less resilient oxidative balance.

Unexpectedly, we did not observe clear elevations in inflammatory markers following the stress condition. The stressor did induced a peak in plasma IL-6 at 10 minutes post stress compared to 45 minutes post stress. This peaking was not observed following rest, but the direct comparison of IL-6 following stress and rest was not significant. We, like others [3], observed an increase in plasma IL-6 due to the high fat meal. It is plausible that the meal-induced increases in IL-6 prevented detection of stressor effects. Indeed, many studies reporting an inflammatory response to stress have instructed participants to refrain from eating at least 1 hour prior to testing [176-179]. Plasma TNF-α was reduced following stress but not rest. There was no effect of the herb and spice blend on these cytokine responses.

Finally, measures of vascular endothelial function were unchanged and an overall improvement in arterial stiffness was observed following the meal. No effects of spice vs control or stress vs rest were observed for any vascular parameter. This was unexpected as high fat/high carbohydrate meals have been repeatedly shown to cause endothelial dysfunction [180, 181] (although not always and perhaps dependent on
fitness level, sex and age [182-184]). Similar spice blends have twice been reported to prevent this dysfunction [22, 77]. Notably, participants consumed a very low fat but high carbohydrate breakfast 3hrs prior to the baseline PAT scan. Since high carbohydrate meals, especially simple carbohydrates with high glycemic index are known to induce vascular dysfunction [185], endothelial function may have still been deteriorated 3hrs post breakfast during the baseline scan. This may have confounded vascular measures.

Alternatively, the lack of change in endothelial function observed may be the result of limited oxidized fats in the meal. Others have shown that a high fat meal rich in oxidized lipids impairs endothelial function while a high fat meal rich in stable fat (non-oxidized) does not [186]. Finally, one similar protocol in which participants were fed a high or low fat meal followed by repeated psychological stress or rest, found that endothelial function 4hrs post meal unexpectedly improved following stress versus rest [187]. This suggests a complex interaction between food intake and stress.

Although the measure of endothelial function used in the current protocol has been linked to hard CVD outcomes, is reliable [188, 189] and is alterable by acute nutritional intervention [95], it does not correlate well with the gold standard method [167]. It has been suggested that the two methods measure separate physiological phenomenon, a possible confound for our results. Our use of the TBARS assay to evaluate levels of the lipid oxidation byproduct MDA limits our ability to compare this study to other similar studies of spices which utilized more robust HPLC methods [21]. However, the null effect of spices we observed on MDA was confirmed with oxLDL data.

In conclusion, a high fat/high carbohydrate meal induces increases in lipid oxidation and inflammation in vivo. This specific curry meal, although high in saturated fat, did not impair endothelial function relative to a non-fasted baseline scan. Stress did not influence any of these parameters except a postprandial TNF-α response. Spices did
not exert any anti-inflammatory, antioxidant or vaso-protective effects \textit{in vivo}.

Researchers should be aware that food intake and acute stress may dynamically interact to influence inflammatory and oxidative stress indices.
Chapter 4

The effect of culinary spices on lipid metabolism, fat excretion and gastric emptying rate; possible mechanisms for triglyceride lowering

Abstract

A blend of common culinary spices has demonstrated an ability to blunt the triglyceride response to a high fat/high carbohydrate meal by 30% in two previous samples. The spice blend inhibits lipase activity in vitro, suggesting a possible mechanism. Ginger and cinnamon, components of the blend, have individually been found to influence gastric emptying rate. This protocol was designed to examine the effects of the herb and spice blend on postprandial plasma triglyceride concentrations, rates of fecal fat excretion and gut motility. Methods: Twelve healthy but overweight men participated in a randomized 2 period cross over study. At each of 2 visits, a fasting blood sample was followed by the consumption of a high fat meal (~1100 kcals, 57g fat) containing blue dye, 2.5g non absorbable fat and with or without the 14.5g polyphenol-rich herb and spice blend. Within 15 minutes of finishing the meal, participants swallowed a single-use SmartPill wireless motility capsule. Blood was sampled every 60 minutes after the meal for 8 hrs for the analysis of plasma triglycerides and glucose. Fecal deposits were collected until the dyed sample was captured. The sucrose polybehenate test of fecal fat absorption was used to evaluate fat excretion in the dyed samples. Endothelial function (measured via flow-mediated dilation, FMD) was assessed at fasting and 210 minutes post meal. Results: Serum triglycerides, glucose and insulin were increased postprandially, as expected. There was no effect of spices on these three endpoints (ps > 0.05). Gastric emptying time was lower after the spice meal compared to control (658 ± 98.2 vs. 884.5 ± 98.2 minutes; p = 0.046), but small bowel
and colonic transit time were unaffected by treatment. There was no effect of either meal on brachial artery diameter or FMD (ps > 0.05). The spiced meal increased blood flow volume compared to the fasting scan (ps = 0.06 and 0.05). Both blood flow volume at the start of the FMD scan (average increase of 57.78 ± 25.34mL/min, post hoc p = 0.037) and following shear stress were increased (average increase of 188.867 ± 66.6 mL/minute post hoc p = 0.012). These findings suggest that spices may influence sympathetic activity.
Introduction

Overweight and obesity are reaching pandemic levels and are primary risk factors for the development of diabetes and heart disease [190, 191]. There is tremendous public and scientific interest in nutritional strategies that promote health and reduce disease risk through weight management. In tightly controlled clinical feeding studies, remarkable improvements in disease risk factors including inflammation, blood pressure, and the lipid profile can be accomplished through weight reduction [192, 193]. However, ingestive behavior is driven largely by contextual and sociocultural habits making adherence to complex dietary interventions difficult to maintain outside of a controlled setting [194]. In contrast, nutritional supplements or beneficial food ingredients (such as spices) can be incorporated into an established diet.

In two previous studies, we have shown that a large dose of common culinary spices (14.5g of black pepper, cinnamon, cloves, garlic, ginger, oregano, paprika, rosemary and turmeric) incorporated into a high fat meal results in a 30% reduction in circulating triglycerides when compared to an unspiced meal up to 4 hrs postprandially [5, 20]. The blend also increased some measures of in vivo antioxidant capacity and reduced the postmeal insulin response [20]. However, others have failed to observe a triglyceride effect when studying similar blends [22, 77] and two component spices (cinnamon [24] and garlic [98]). Notably, in those with very low HDL-C concentrations, Rotzsch et al. observed a 35% reduction in postprandial triglycerides after 900mg of garlic powder daily for 6 weeks [97]. Further, daily cinnamon consumption consistently reduces fasting triglyceride concentrations in within 4-6 weeks [87, 195]. However, the mechanism through which our previously studied herb and spice blend influences post meal triglycerides is yet to be confirmed.
Most dietary fat requires enzymatic hydrolysis via lipases before absorption is possible [196]. Lipase inhibition is the mechanism through which the popular pharmaceutical weight loss therapy tetrahydrolipstatin reduces fat absorption [196] and is a possible route for spice induced triglyceride reductions. *In vitro* experiments demonstrate that several spices, but especially cinnamon, clove, turmeric and rosemary inhibit pancreatic lipase and phospholipase A<sub>2</sub> activity [5]. In rats, consumption of a diet enriched with rosemary extract for 8wks reduced plasma triglycerides without influencing food intake; a result of gastric lipase inhibition [197]. Other high polyphenol dietary components such as cocoa and tea have demonstrated an ability to inhibit lipases *in vitro* [198, 199] and mice, resulting in lower triglyceride concentrations [126]. We hypothesize that a high polyphenol herb and spice blend may reduce postprandial triglyceride concentrations by inhibiting lipases *in vivo*, resulting in less absorption and therefore more excretion of dietary fat.

Altered gastrointestinal transit is another avenue through which spices may influence lipid metabolism. Slower gastric emptying is associated with greater satiety [200] and inversely related to cholesterol absorption in mice [201]. Regulated by the postprandial glucagon-like peptide 1 (GLP-1) and insulin cascade, gastric emptying rate is the target of experimental GLP-1 mimetics for hyperglycemia [202]. Cinnamon, in a dose of just 3g, has demonstrated an ability to increase GLP-1 postprandially [203]; however the influence of cinnamon on gastric emptying rate is inconsistent [24, 85]. Turmeric has demonstrated an ability to slow gastric emptying in a rat model [204], possibly due to increased GLP-1 secretion [205]. A bioactive compound isolated from garlic slowed gastric emptying dose dependently in mice [206]. Ginger and ginger extract however, have been shown to increase gastric emptying rate in those with abnormal digestion [117, 118]. It is unknown if the triglyceride lowering effect observed
following the herb and spice blend previously is due to any effects on gastric emptying.

Figure 4-1 Possible mechanisms through which high polyphenolic herbs and spices blunt postprandial triglyceride increases

The present protocol was designed to examine the underlying mechanism through which polyphenol-rich spices affect lipid concentrations (as seen in Figure 4-1) by assessing gut motility and fecal fat excretion. We hypothesize that as compared to an un-spiced high fat/high carbohydrate meal, the inclusion of spices in the same meal will result in the following postprandial changes:
1. less dramatic elevations in plasma triglycerides up to 8 hrs postprandial
2. greater fat excretion in the feces
3. slower gastric emptying rate and
4. improved endothelial function

We also report on the use of lauric/behenate fecal fat testing [207] and SmartPill wireless motility capsule (WMC; Given Imaging, Yoqneam, Israel), both novel applications for assessing the effects of dietary bioactives postprandially.

**Methods**

A randomized, placebo controlled, two period crossover study design was used to evaluate study hypotheses. Participants completed 2 visits each (with at least 1 week in between) during which they received a high fat meal + placebo capsules and a high fat meal + spices in random order. Randomization was performed using the online tool randomization.com and was blocked using the two body mass index (BMI) categories of overweight (BMI of 25-30 kg/m², N=6) and obese (BMI of 30-35 kg/m²). All participants provided informed consent and all procedures were approved by the Institutional Review Board at The Pennsylvania State University.

**Participants**

Twelve adult men aged 28-65 years and with BMI of 25-35 kg/m² completed the study. Community board flyers, newspaper advertisements and email announcements were used to recruit interested individuals in University Park and State College, Pennsylvania. Phone and clinic screening procedures ensured that participants met
study criteria (Figure 4-2). Inclusion criteria were as follows: overweight and obese men (BMI of 25-35 kg/m²) but at weighing least 110 pounds, English speaking and aged 28-65 years of age. Exclusions were made if individuals had a medical history of chronic disease (heart, inflammatory or hepatic disease, diabetes) or were using lipid-lowering, blood pressure lowering or insulin sensitizing medications, steroids or daily use of non-steroid anti-inflammatory medications or fish oil. Vitamins and supplements were discontinued for study duration. Further exclusions were made for those who were athletes (more than 1 hour of moderate to vigorous exercise 4 times per week) or had lost >10% of total body weight in the last 6 months. Given the use of the Smartpill technology (Given Imaging, Yoqneam, Israel), we excluded those with an inability/unwillingness to swallow large pills, a history of gastrointestinal disorders including gastric bezoars, hernia, strictures, fistulas or physiological/mechanical GI obstruction, Crohn’s disease or diverticulitis.
Procedures

In the 48 hrs prior to each visit, participants were asked to avoid foods containing antioxidants and a list of such foods was provided (a complete list has previously published in supplemental materials [25]). Participants also abstained from alcohol and exercise in the 24 hrs prior to and were provided with a standardized dinner to be consumed the night before each visit (687 kcal, 34g fat, 71g carbohydrate, 18g protein).
Participants were provided with and required to drink 32oz of water after 6pm the night before and 16oz of water on the morning of each visit.

After a 12hr overnight fast, participants reported to the Pennsylvania State University on-campus Clinical Research Center. Upon arrival, the first vascular function assessment was made via flow mediated dilation followed by the placement of an intravenous catheter and collection of a baseline blood sample. Then participants were given 15 minutes in which to consume the high fat meal (with spices or placebo capsules) and blue dye solution. A Smartpill (Given Imaging, Yoqneam, Israel) was consumed immediately following the meal. To control for the effects of stress on glucose and insulin parameters seen in the previous study [5], participants completed a stress questionnaire about stressors in the last 24 hours (see Appendix B). Hourly blood samples were obtained for 8hrs and a second vascular function test was administered at 3.5hrs post meal.

A complementary dinner was provided at the end of the study visit and participants were discharged with instructions to collect whole bowel movements until the sample with the blue dye was obtained. Samples were kept in a cooler on ice until being returned to study personnel. A monitor to telemetrically collect data from the Smartpill was also worn until pill exit via bowel movement.

**Flow Mediated Dilation**

Measurements of brachial artery diameter and blood flow velocity were made by a single, well-trained sonographer (P.W.) using a Logiq e ultrasound imaging system (GE Healthcare, Tyrone, PA) with a 10-MHz linear-array transducer. Longitudinal, 2-dimensional images of the brachial artery were captured at 5 frames per second above
the elbow of the right arm. The sonographer identified and imaged a relatively straight segment of artery during the following procedure: baseline (1 minute), arterial occlusion (5 minutes) via inflation of a cuff on the forearm (distal to the segment being imaged) at 250 mm Hg, and reactive hyperemia (2 minutes). Measurements were taken to promote similar placement for subsequent testing. Images were scored using edge-detection software (Brachial Analyzer, MIA, Iowa City, IA), with manual review by a technician blinded to treatment. Peak diameter was taken as the largest average diameter in the 2-minute deflation sequence (typically observed 40–70 seconds postdeflation). FMD was measured as the percentage change in arterial diameter from baseline to after hyperemia.

Average flow velocity (m/s) across the cardiac cycle, maximal flow velocity, and velocity time integral across the cardiac cycle (m) were measured by using duplex pulsed Doppler during the resting baseline and immediately after cuff release. Flow (ml/min) was calculated using the following equation: velocity time integral × cross-sectional area of the vessel × heart rate.

**Test Meal**

The 1098 kcal test meal (58g fat, 98g carbohydrate, 43g protein) meal consisted of a coconut chicken curry and white rice dish, a corn muffin, and pastry dessert. Fatty acid profile of the meal can be viewed in Table 4-1 and Figure 4-3. The control meal was prepared without spices, but served with placebo capsules. The herb and spice blend (14.5 g/meal) was incorporated into the various meal items as has been previously described (Chapter 3). The spice dose is estimated to provide 404mg GAE of phenolics, 8690μmol TE hydrophilic oxygen reducing absorbance capacity (ORAC) and 5157μmol
TE lipophilic ORAC based on data from the USDA database [5]. The spices were obtained from the Characterized Samples Program at the McCormick Science Institute (Hunt Valley, MD) and weighed with a balance accurate to 0.01 g (Mettler Toledo, Columbus, OH). The fatty acid profile of the test meal was assayed using GC mass spectrometry.

Table 4-1. Calculated\(^1\) test meal fatty acid composition

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Previous Meal (g)(^2)</th>
<th>Current Meal (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA 4:00 butyric</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>SFA 6:00 caproic</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>SFA 8:00 caprylic</td>
<td>1.37</td>
<td>2.49</td>
</tr>
<tr>
<td>SFA 10:00 capric</td>
<td>1.17</td>
<td>2.03</td>
</tr>
<tr>
<td>SFA 12:00 lauric</td>
<td>8.50</td>
<td>16.40</td>
</tr>
<tr>
<td>SFA 14:00 myristic</td>
<td>3.91</td>
<td>7.41</td>
</tr>
<tr>
<td>SFA 16:00 palmitic</td>
<td>6.41</td>
<td>7.96</td>
</tr>
<tr>
<td>SFA 17:00 margaric</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>SFA 18:00 stearic</td>
<td>3.01</td>
<td>3.51</td>
</tr>
<tr>
<td>SFA 20:00 arachidic</td>
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<td>0.02</td>
</tr>
<tr>
<td>SFA 22:00 behenic</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>MUFA 14:01 myristoleic</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>MUFA 16:01 palmitoleic</td>
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<td>0.32</td>
</tr>
<tr>
<td>MUFA 18:01 oleic</td>
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<td>10.08</td>
</tr>
<tr>
<td>MUFA 20:01 gadoleic</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>MUFA 22:01 erucic</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PUFAs 18:02 linoleic</td>
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<td>4.98</td>
</tr>
<tr>
<td>PUFAs 18:03 linolenic</td>
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<td>0.44</td>
</tr>
<tr>
<td>PUFAs 18:04 parinaric</td>
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<td>0.00</td>
</tr>
<tr>
<td>PUFAs 20:04 arachidonic</td>
<td>0.08</td>
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<tr>
<td>PUFAs 20:05 eicosapentaenoic</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>PUFAs 22:05 docosapentaenoic</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>PUFAs 22:06 docosahexaenoic</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>TRANS 16:01 trans-hexadecenoic</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>TRANS 18:01 trans-octadecenoic</td>
<td>1.32</td>
<td>1.32</td>
</tr>
<tr>
<td>TRANS 18:02 trans-octadecadienoic</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>CLA cis-9 trans-11</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>CLA</td>
<td>cis-12</td>
<td>trans-10</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
<td>----------</td>
</tr>
<tr>
<td><strong>Total fat</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Calculated using Nutrition Data System for Research
2 Meal used in McCrea & West et al. [5]

Figure 4-3. Assayed test meal fatty acid profile

The meal was served with 30mL of water and 13 large drops of blue food dye (McCormick and Company, Inc, Hunt Valley, MD). The blue dye and, when appropriate, the placebo capsules were consumed in the middle of the meal, half way through food consumption (as judged subjectively by participant). To allow for calculation of fecal fat excretion, a non-absorbable fat substitute, sucrose polybehenate (2.5g per meal, Cincinnati, OH) was melted simultaneously and mixed until homogenous with coconut oil before addition to the curry (for more details on food preparation, see Appendix A). Water was limited to the 30mL provided with the blue dye and an additional 16oz from
arrival until 6hrs post meal after which participants were allowed to drink water ad libitum.

**Gut transit assessment**

Participants consumed the biocompatible single-use SmartPill wireless motility capsule (WMC; Given Imaging, Yoqneam, Israel) immediately after consuming the meal. The capsule measures pressure, pH and temperature as it transits the gastrointestinal tract, transmitting data to a hip monitor. Participants were instructed to wear the monitor at all times (except when bathing or sleeping, during which it had to remain within 3 feet) and keep a diary of bowel movements, waking, sleeping, eating and exercise until the pill passed. An automated software package (MotiliGI, Given Imaging, Yoqneam, Israel) provided measures of transit using temperature, pressure and pH data.

**Biochemical Analysis**

Whole blood was transferred into serum separator tubes, allowed to clot, and centrifuged. Total cholesterol and triglycerides were determined by enzymatic procedures (Quest Diagnostics, Pittsburgh, PA; CV < 2% for both). HDL-C was estimated according to the modified heparin-manganese procedure (CV < 2%). Insulin was measured by radioimmunoassay using $^{125}$I-labeled human insulin and a human insulin antiserum (Quest Diagnostics). Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose & Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH).
Approximately 1 g of feces was removed from the blue sample (or subjectively “bluest,” in the case of multiple fecal deposits with coloring) for lauric acid:behenic acid analysis by gas chromatography techniques and previously used by Dorsey et al. [208] and conducted at the Mouse Metabolic Phenotyping Center (University of Cincinnati, Cincinnati, OH). Fatty acid compositions of the test meal and feces were analyzed by gas chromatography using the method of Metcalfe et al.[209]. The percent of absorbed fat was calculated using the following equation: absorbed fat = 100* [(Fd/Bd) - (Ff/Bf)]/(Fd/Bd), where Fd = mass of lauric fatty acid (LFA) in test meal, Bd = mass of behenic acid in meal, Ff = mass of lauric acid in fecal sample and Bf = mass of behenic acid in fecal sample. The same was calculated for myristic fatty acid (MFA).

**Statistical Analysis**

Based on our pilot data, a sample size of 10 was necessary to observe a difference of 21 units in the postprandial change in triglycerides between treatment and control (power = 0.90, α = 0.05). The mixed models procedure (PROC MIXED) was used to test the fixed effects of treatment (spice or placebo), time, visit, BMI category and their interaction on each outcome variable. Stress variables were considered as covariates for glucose, insulin and TG data. Severity of the most recent stressor was retained in the insulin model and both total severity of stressors in the last 24 hrs and severity of most recent stressor were retained in the glucose model. Stress parameters had no effect on the TG model. One participants glucose, insulin and TG data was set to missing for hours 7 and 8 post meal due consumption of food after hour 6. Differences in baseline values were tested via paired-t-tests. Outcomes are modeled as change scores (value - baseline) for all blood variables and FMD. Gut transit variables were assessed as levels.
Bonferroni adjustment was applied to an \( \alpha \) of 0.05 to determine the significance level for all post hoc comparisons. Change in triglyceride required a log transformation to produce normalized residuals. Non-transformed least-squares means and standard errors are reported. Model selection (including best covariance structure) was based on optimizing fit statistics, or lowest Bayesian Information Criterion.

Results

Sample characteristics can be viewed in Table 4-2. On average, participants had healthy lipid profiles and fasting glucose concentrations but were overweight. On average, participants reported experiencing 8.8 ± 1.6 stressors in the past 24 hours with an average stress severity total of 25.2 ± 3.6. An average of 8.3 ± 1.3 hours had elapsed since the most recent stressor and the average stress rating of the most recent stressor was 3.0 ± 0.03 out of 7.

Table 4-2. Sample characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>40.38 ± 3.17</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>29.80 ± 0.84</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>181.20 ± 9.88</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>116.45 ± 8.53</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>41.29 ± 3.26</td>
</tr>
<tr>
<td>Total to HDL-C ratio</td>
<td>5.15 ± 0.66</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>117.00 ± 17.79</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>84.04 ± 1.46</td>
</tr>
<tr>
<td>Insulin (IU/L)</td>
<td>5.44 ± 0.85</td>
</tr>
</tbody>
</table>
Blood results

A main effect of time was present for triglycerides \( (p = 0.0004) \), however no effect of spices was observed \((0.166; \text{Figure 4-4})\). A main effect of BMI category was also present \((p=0.01)\) such that average triglyceride levels in obese subjects were higher (average change of 78.6mg/dL) than those of overweight subjects (average of 60.9mg/dL). Eight hrs postmeal, triglyceride concentrations were still elevated above baseline (change of 40.0 ± 8.1 mg/dL was significantly different from 0, \( p <0.0001 \)).

The glucose model revealed a treatment by BMI category (OW vs OB) interaction \((p = 0.0126)\) such that in OW subjects only, average change in glucose was 5.81mg/dL less following the spice meal vs the control meal \((p = 0.0184; \text{Figure 4-5})\). In the OB group, no treatment effect on glucose was observed. For insulin, an interaction between BMI category (OW vs OB) and time was present \((p = 0.038)\). Post hoc analysis revealed a trend for OB subjects to exhibit greater change in insulin at the first two postprandial time points as compared to OW subjects \((1 \text{ and } 2 \text{ hour post meal } p_s = 0.04, 0.01; \text{ Bonferroni correction required } p < 0.00625 \text{ as significance}; \text{Figure 4-6})\). Expectedly, a main effect of time was present for both glucose and insulin \((ps < 0.0001)\).
Figure 4-4. Change in triglyceride concentrations following the meal with spice and placebo.

Figure 4-5. Average change in glucose concentrations following the meal in OB and OW subjects.
Gut transit results can be viewed in Figure 4-7. Gastric emptying was effected by both treatment (p = 0.046) and visit (p = 0.015). Longer gastric emptying time was seen after the control meal vs spice meal (884.5 ± 98.2 vs 658 ± 98.2 minutes, respectively) and at visit 1 vs 2 (920.9 ± 98.2 vs 621.8± 98.2 minutes, respectively).

Small bowel transit was unaffected by spices or any other factor. Average small bowel transit was 209.67 ± 19.89 minutes. The average colonic transit time was 1236.90 ± 209.71 minutes. There was no effect of spices (p = 0.42) or any other factor on colonic transit time. Finally, a treat by visit interaction was present for whole gut transit time (p=0.05), however post hoc analysis revealed no significant differences. Average whole gut transit time was 2219.23 ± 233.59 minutes.
Fecal fat excretion results

Average absorption of lauric and myristic fatty acids after spice and control meals can be viewed in Table 4-3. There was no effect of spices on fat absorption (p = 0.71 for LFA and p = 0.47 for MFA). There was a treat by visit by BMI category effect for MFA absorption that was driven by a trend for OW subjects to absorb more MFA than OB subjects when both were presented with the control meal at visit 2. However, it did not reach significance (p = 0.039, Bonferroni correction requires p < 0.008).

Table 4-3. Absorption of lauric and myristic fatty acids from spiced and control meals

<table>
<thead>
<tr>
<th></th>
<th>Lauric fatty acid absorption</th>
<th>Myristic fatty acid absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spice</td>
<td>658.17</td>
<td>220.84</td>
</tr>
<tr>
<td>Placebo</td>
<td>884.51</td>
<td>203.04</td>
</tr>
</tbody>
</table>

Figure 4-7. Gut transit measures displayed in minutes.
<table>
<thead>
<tr>
<th></th>
<th>% from test meal</th>
<th>% from test meal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SE</td>
<td>mean ± SE</td>
</tr>
<tr>
<td>Spice</td>
<td>99.20 ± 0.20</td>
<td>94.19 ± 1.82</td>
</tr>
<tr>
<td>Control</td>
<td>99.29 ± 0.20</td>
<td>92.33 ± 1.82</td>
</tr>
</tbody>
</table>

**Endothelial function results**

Basal artery diameter, peak artery diameter and flow mediated dilation (percent change in artery diameter) were unaffected by time (pre to post meal) and treatment (spice vs. placebo). Averages and p-values can be seen in Table 4-4.

Blood flow volume at the start of the FMD scan (basal blood flow in Table 4-4) was predicted by a treat by time trend (p = 0.06), such that basal flow had a tendency to increase postprandially only when spices were present in the meal (average change of 57.78 ± 25.34mL/min, p = 0.037). Blood flow following shear stress (peak deflation blood flow in Table 4-4) also demonstrated a treat by time interaction (p = 0.05). Again, post hoc tests revealed that flow volume was increased after the meal compared to pre-meal only when spices were present (average change of 188.867 ± 66.6 mL/min, p = 0.012). The interaction is depicted in Figure 4-8. Percent change in blood flow volume was not influenced postprandially or by treatment.
Table 4-4. Treatment and prandial effects on vascular parameters

<table>
<thead>
<tr>
<th></th>
<th>Spice</th>
<th>Placebo</th>
<th></th>
<th>p</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
<td>Postprandial</td>
<td>Fasting</td>
<td>Postprandial</td>
<td>prandial effect</td>
</tr>
<tr>
<td></td>
<td>mean ± SE</td>
<td>mean ± SE</td>
<td>mean ± SE</td>
<td>mean ± SE</td>
<td></td>
</tr>
<tr>
<td>Basal artery diameter, mm</td>
<td>4.60 ± 0.12</td>
<td>4.71 ± 0.18</td>
<td>4.64 ± 0.12</td>
<td>4.66 ± 0.18</td>
<td>0.72</td>
</tr>
<tr>
<td>Peak deflation diameter, mm</td>
<td>4.89 ± 0.11</td>
<td>5.06 ± 0.19</td>
<td>4.98 ± 0.12</td>
<td>5.04 ± 0.19</td>
<td>0.43</td>
</tr>
<tr>
<td>Flow-mediated dilation, %</td>
<td>6.36 ± 0.85</td>
<td>8.24 ± 0.85</td>
<td>7.65 ± 0.89</td>
<td>7.80 ± 0.85</td>
<td>0.15</td>
</tr>
<tr>
<td>Basal blood flow volume, mL/min</td>
<td>164.98 ± 29.52</td>
<td>222.77 ± 31.43</td>
<td>187.52 ± 31.23</td>
<td>182.72 ± 32.92</td>
<td>0.23*</td>
</tr>
<tr>
<td>Peak deflation blood flow volume, mL/min</td>
<td>949.56 ± 98.43</td>
<td>1138.4 ± 85.04</td>
<td>971.57 ± 104.48</td>
<td>955.25 ± 89.19</td>
<td>0.11*</td>
</tr>
<tr>
<td>Change in blood flow, %</td>
<td>594.12 ± 89.08</td>
<td>493.59 ± 85.03</td>
<td>473.49 ± 92.09</td>
<td>476.84 ± 88.12</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Data are mean + standard error. Flow-mediated dilation and percent change in blood flow are calculated as: (peak – baseline)/baseline x 100. *indicates an interaction, see text for details.
Discussion

In this protocol, we tested the mechanism through which the addition of a herb and spice blend to a high fat/high carbohydrate meal attenuates postprandial triglyceride concentrations. However, we did not replicate our previous findings and found no support for any of our 4 hypotheses; in this sample of 12 healthy but overweight and
obese men, adding a 14.5g dose of spices to a high fat/high carbohydrate meal had no effect of postprandial triglyceride, glucose or insulin concentrations. In obese subjects only, there was a trend for spices to cause a spike in insulin postprandially 2 hrs. Further, the spiced meal traveled through the stomach more quickly than the control meal, with no differences on small bowel or colonic transit time. There was no difference between lauric and myristic fatty acid absorption when participants consumed the spiced or control meals. Endothelial function was not influenced by either meal. Interestingly, blood flow volume tended to increase following spices.

We have twice previously reported a 30% reduction in postprandial triglyceride concentrations using the same spice dose in the same population [5, 20]. However in the current analysis, we found no difference between spice and control up to 8 hrs post-meal. Our recipes were nearly identical to those previously used except some additions for the fat absorption assay: 17g coconut oil, 2.5g sucrose polybehenate and blue dye solution. Triglyceride concentrations 4hrs postprandially were higher in the current study than those seen 4hrs after the control meal in our latest publication (change of 101.35 vs. 87.17mg/dL, respectively), which reflects the addition of more fat. Sucrose polyesters are known to interfere with the absorption of some lipophilic pharmaceuticals [210] and vitamins [211], possibly via sequestration in the oily phase of the indigestible fat [212]. A large portion of the antioxidant capacity of the herb and spice blend is lipophilic (Chapter 3, Table 3-1). The sucrose behenate may have blunted absorption of lipophilic bioactives in the spices preventing their triglyceride lowering effect. Notably, others have failed to observe any effect of similar blends on post-meal metabolism [22, 77].

Gastric emptying time was delayed (> 4 hrs) after both spiced and control meals; likely a result of the large bolus of fat [213]. Surprisingly, the spiced meal was associated with shorter gut transit than the control meal with placebo. This was contrary to our
hypothesis and may be due to the 1.5g dose of ginger in the spiced meal. Just 1.2g of ginger powder has demonstrated an ability to reduce gastric emptying rate by ¼, perhaps due to increased antral contractions but with no effect of GLP-1 [117]. There was no difference between fat absorption after the spiced and control meals. Given the parallel triglyceride response, the similarity in fat absorption rates was not surprising. Notably, we used the medium MFA and LFA to indicate fat absorption. Medium chain fatty acids can be absorbed directly into the portal vein without the use of lipases [214]. Therefore, they are likely not the optimal indicator of attenuated absorption by a treatment that is proposed to inhibit lipases. Future analysis on samples from this protocol will consider fat absorption using long chain fatty acid indicators.

The high fat/high carbohydrate meal administered in this protocol did not induce endothelial dysfunction. This is consistent with our previous work using a different measure of endothelial function (Chapter 3). However, high fat meals generally [180, 181, 215], though not always [182-184], induce endothelial dysfunction. While diets rich in SFA are known to impair endothelial function [215], several studies suggest that single meals rich in SFA may not be as detrimental to postprandial endothelial function as meals containing other types of fat [216, 217]. This may be the result of SFAs’ relative resistance to oxidation [59].

Interestingly, we observed a trend for blood flow volume to be increased following the spiced meal but not the control meal. This may be the result of increased heart rate, one variable of the flow equation. In one previous study, we reported that the herb and spice blend augmented heart rate during stress but not at rest [5]. Further analysis will consider any effect of the herb and spice blend on heart rate in this sample. Elevated heart rate via increased sympathetic activity is the primary pathway for food induced augmentation of energy expenditure or thermogenesis [218-220]. Capsaicin
from hot peppers (paprika) and constituents of ginger, black pepper and garlic elevate sympathetic activity by stimulating catecholamine release from the adrenal medulla [221]. Taken together with our previous findings, this suggests that the herb and spice blend may enhance energy expenditure.

In conclusion, we find no evidence that a blend of commonly used culinary spices incorporated into a high fat/high carbohydrate meal inhibits postprandial fat absorption or endothelial dysfunction. Spices did shorten gastric emptying time and increase blood flow. Limitations of this protocol include the lack of availability of data for sample size calculations for two main outcomes (fecal fat excretion and WMC Smartpill assessment of gut transit). Additionally, lack of control over diet, medications and water intake after the test meal may have prevented the observance of treatment effects on some gut transit parameters.
Chapter 5

Test Test-retest Reliability of Pulse Amplitude Tonometry Measures of Vascular Endothelial Function: Implications for Clinical Trial Design

Abstract

Endothelial dysfunction is an important outcome for assessing vascular health in intervention studies. However, reliability of the standard noninvasive method (flow mediated dilation) is a significant challenge for clinical applications and multicentre trials. We evaluated repeatability of pulse amplitude tonometry (PAT) to measure change in pulse wave amplitude during reactive hyperemia (Itamar Medical Ltd, Caesarea, Israel). Twenty healthy adults completed 2 PAT tests (mean interval = 19.5 days) under standardized conditions. PAT-derived measures of endothelial function (reactive hyperemia index, RHI) and arterial stiffness (augmentation index, AI) showed strong repeatability (intraclass correlations = 0.74 & 0.83, respectively). To guide future research, we also analyzed sample size requirements for a range of effect sizes. A crossover design powered at 0.90 requires 28 participants to detect a 15% change in RHI. Our study is the first to show that PAT measurements are repeatable in adults over an interval greater than one week.
Introduction

It is now well established that vascular endothelial dysfunction is positively associated with traditional CVD risk factors [167], and independently predicts cardiovascular events over intervals of 1 to 6 years [33, 68, 222-225]. Vascular dysfunction is evident in high risk populations as early as adolescence,[226] and intervention studies have shown that it is responsive to dietary [227][228], behavioral [229], pharmacological [230] and biomechanical interventions [231, 232]. The gold standard, non-invasive measurement of endothelial function (flow mediated dilation, FMD) uses ultrasound to measure changes in brachial artery diameter in response to reactive hyperemia [27]. Many laboratories (including our own) have shown robust test-retest reliability for FMD when conditions are rigorously standardized [28-30]. However, the technique requires specialized training and high resolution sonography equipment. Thus, it is expensive and highly operator dependent. Many consider FMD testing to be impractical for either large-scale clinical trials or clinical use [31, 32].

Pulse amplitude tonometry (PAT), a recently FDA approved method which is relatively inexpensive and operator independent, is increasingly being used as an alternative measure of endothelium dependent dilation in response to reactive hyperemia [33]. The PAT device records digital pulse wave amplitude (PWA) using fingertip plethysmography (EndoPAT, Itamar Medical Ltd, Caesarea, Israel) [233]. PWA is measured continuously during three phases: a quiet baseline period, 5 min forearm occlusion, and reactive hyperemia following cuff release. Unlike FMD, PAT testing is not dependent on a highly skilled technician and post-test analysis is largely automated. Most importantly, one longitudinal study has shown that PAT measures of endothelial
function predict CVD events over a 6 year follow up period [33]. These significant advantages may make PAT testing suitable for clinical practice if prognostic significance and reliability can be verified. In the text below, we define and discuss each of the PAT-derived measures of vascular function before considering their reliability under controlled laboratory conditions.

**Reactive Hyperemia Scores**

The reactive hyperemia index (RHI) measures nitric-oxide dependent changes in vascular tone [234]. The continuous monitoring of blood volume reaching the finger tips allows the hyperemic response to be quantified as a ratio, comparing PWA from pre to post occlusion. As seen in Figure 5-1, RHI is calculated as follows: the ratio of the occluded arm's mean PWA 90 to 150 seconds post occlusion (A) to the mean PWA from baseline readings of the same arm (B). The result is further divided by the same ratio from the control arm (C/D), which allows the device to account for systemic vascular changes during testing. The final ratio is then multiplied by a proprietary baseline correction factor (Itamar Medical Ltd, Caesarea, Israel). An alternative reactive hyperemia score, proposed by Framingham Heart Study researchers (fRHI), uses the natural logarithmic transformation of the RHI ratio, does not include the baseline correction factor and utilizes only the readings from 90-120 seconds for post occlusion [167]. RHI and fRHI are highly correlated (r’s = 0.92 to 0.97 in this sample), however their relationships to CVD risk factors may differ in important ways [167], and researchers should be encouraged to present both measures in future studies.
Figure 5-1. The RHI score as derived from EndoPAT peripheral arterial tonometry

**Augmentation Index Scores**

As a pressure wave moves through the arterial tree, it encounters impedance resulting in a reflected wave that moves back toward the heart and may augment peak systolic pressure [235]. Arterial stiffness increases pulse wave velocity, causing early reflection of this waveform [236]. The EndoPAT derived augmentation index (AI) provides a measure of arterial stiffness by considering the timing and magnitude of this wave reflection in the digital pulse [237]. Calculated from baseline resting pulse waves, AI represents the relative contribution of augmented pressure due to wave reflection to the pressure wave form. Proprietary software automatically identifies inflection points distinguishing the systolic peak (P₁) and the reflected peak (P₂) for the calculation of this
ratio and converts it into a percentage \(\left(\frac{P_1 - P_2}{P_1} \times 100\right)\) [236]. Because AI is inversely related to heart rate, values are sometimes mathematically adjusted to represent arterial stiffness at a standard heart rate of 75 beats per minute (AI@75). PAT arterial stiffness measures are associated with abnormal ventricular-vascular coupling [236] and correlate well with AI measures from other devices [239].

**Reliability of PAT scores**

Although these tests have been widely adopted by investigators, relatively few studies have examined test-retest reliability of PAT derived indices of vascular health [226, 232, 240]. Importantly for clinical researchers, no previous studies have used these data to provide sample size calculations for a wide variety of study designs and hypothesized treatment effect sizes. For example, one of the most widely cited papers on repeatability of RHI utilizes a Bland-Altman plot to demonstrate test-retest consistency [232]. While these plots provide a clear visual representation of reliability of paired measurements, they allow neither sample size calculations nor direct comparison of reliability metrics across studies. Precision of the sample size estimate is particularly important for crossover studies, in part because the standard deviation of change from one test to the next (SD\(_{\text{within}}\)) is not often provided in published manuscripts. Therefore, the current study was designed to provide reliability data on PAT measures of endothelial function and arterial stiffness, including newly proposed metrics derived from the PAT signal. Our goal is to inform the design of future clinical trials by providing sample size calculations for a wide range of experimental conditions.
Methods

Participants

Characteristics of the sample are shown in Table 5-1. The sample was comprised of 20 disease-free but overweight, normotensive participants (14 men & 6 women). Participants were recruited for a study of antioxidant effects on vascular function, and only the fasting vascular tests are reported here. Age ranged from 31 to 63 years with a mean of 41.2 ± 2.4. The average BMI was 30.5 ± 0.86. Exclusion criteria included: tobacco use, fasting blood glucose > 126mg/dL, blood pressure > 160/100mmHg, clinically significant arrhythmia, history of heart attack, stroke, renal or hepatic disease. Use of the following also resulted in exclusion: anti-inflammatory medication, statins, anti-hypertensive medications, hormones, daily aspirin, non-SSRI psychotropic medication & dietary supplements/vitamins. Participants were excluded if they were unable to tolerate wheat, gluten or certain spices due to dietary intervention demands that were required as part of the larger clinical trial.
Table 5-1. EndoPAT scores and metabolic parameters measured in a healthy sample across days

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Visit 1</th>
<th>Visit 2</th>
<th>Average CV (%)</th>
<th>Mean Variability</th>
<th>Pearson’s r</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHI</td>
<td>1.81 ± 0.12</td>
<td>1.82 ± 0.12</td>
<td>12.22 ± 2.19</td>
<td>0.31 ± 0.06</td>
<td>0.68</td>
<td>0.74</td>
</tr>
<tr>
<td>fRHI</td>
<td>0.29 ± 0.09</td>
<td>0.34 ± 0.1</td>
<td>...</td>
<td>0.23 ± 0.04</td>
<td>0.77</td>
<td>0.77</td>
</tr>
<tr>
<td>AI</td>
<td>-4.23 ± 3.18</td>
<td>-2.53 ± 4.17</td>
<td>...</td>
<td>5.40 ± 1.85</td>
<td>0.89</td>
<td>0.83</td>
</tr>
<tr>
<td>AI@75</td>
<td>-11.16 ± 3.29</td>
<td>-8.81 ± 4.25</td>
<td>...</td>
<td>5.60 ± 2.04</td>
<td>0.88</td>
<td>0.81</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>63.9 ± 1.5</td>
<td>64.9 ± 1.7</td>
<td>3.6 ± 0.7</td>
<td>3.3 ± 0.6</td>
<td>0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>121.9 ± 2.3</td>
<td>121.3 ± 2.0</td>
<td>4.3 ± 0.8</td>
<td>7.4 ± 1.3</td>
<td>0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>80.2 ± 1.5</td>
<td>81 ± 1.2</td>
<td>3.2 ± 0.8</td>
<td>3.6 ± 0.8</td>
<td>0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td>C-Reactive Protein (nmol/L)</td>
<td>16.0 ± 3.5</td>
<td>13.7 ± 2.3</td>
<td>22.2 ± 5.5</td>
<td>5.7 ± 1.9</td>
<td>0.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>57.9 ± 7.9</td>
<td>64.1 ± 14.0</td>
<td>29.9 ± 5.8</td>
<td>32.6 ± 10.4</td>
<td>0.70</td>
<td>...</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.01 ± 0.14</td>
<td>4.99 ± 0.19</td>
<td>6.9 ± 1.3</td>
<td>0.51 ± 0.09</td>
<td>0.19&lt;sup&gt;a, ns&lt;/sup&gt;</td>
<td>...</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.54 ± 0.15</td>
<td>4.62 ± 0.15</td>
<td>5.5 ± 1.0</td>
<td>0.36 ± 0.06</td>
<td>0.78</td>
<td>...</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.11 ± 0.05</td>
<td>1.10 ± 0.06</td>
<td>4.7 ± 1.0</td>
<td>0.07 ± 0.02</td>
<td>0.92</td>
<td>...</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.68 ± 0.13</td>
<td>2.67 ± 0.13</td>
<td>9.9 ± 1.5</td>
<td>0.37 ± 0.06</td>
<td>0.71</td>
<td>...</td>
</tr>
<tr>
<td>Total / HDL cholesterol (mmol/L)</td>
<td>0.11 ± 0.005</td>
<td>0.11 ± 0.005</td>
<td>5.5 ± 1.1</td>
<td>0.09 ± 0.002</td>
<td>0.90</td>
<td>...</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.65 ± 0.11</td>
<td>1.86 ± 0.17</td>
<td>12.6 ± 2.2</td>
<td>0.34 ± 0.08</td>
<td>0.89</td>
<td>...</td>
</tr>
</tbody>
</table>

Mean ± SEM; CV = (100 x SD)/mean; ICC = Intra-class correlation, calculated as $S_b^2 - S_w^2 / S_b^2 + S_w^2$; RHI: Reactive Hyperemia Index, AI: Augmentation Index, AI@75: Augmentation Index at a heart rate of 75 beats per minute, fRHI: Framingham Reactive Hyperemia Index.

<sup>a</sup>Spearman rank order correlation. All correlations were statistically significant (P < 0.05), except for glucose.
Procedures

Subjects underwent two EndoPAT tests with a mean interval of 19.5 ± 6.2 days, but not less than 7 days. Tests occurred at approximately 11 am, after a standardized low fat, low antioxidant breakfast (low fat cream cheese and a white bagel) had been consumed between 7 and 8 am. Subjects consumed the breakfast after completing a 12 hour overnight fast. Premenopausal females (n = 2) were tested during the first 7 days of their menstrual cycle. The protocol from which these fasting data were taken required that participants limit their consumption of high antioxidant foods 48 hrs prior to testing (a list was provided). Food and beverage records were reviewed by study personnel to assure compliance. The study protocol was reviewed and approved by The Pennsylvania State University Institutional Review Board and all participants gave written informed consent.

PAT tests were performed in a supine position, in a dimly lit and temperature controlled room (70-75 F). After application of the occlusion cuff to the right forearm and finger tip probes to the index fingers of each hand, the study began with a 10 min quiet rest period. Measures of PWA and heart rate were captured continuously by pneumatic finger probes throughout testing, as outlined above. Using PWA recordings, proprietary EndoPAT software calculated the RHI, fRHI, AI and Al@75. Seated blood pressure measurements were obtained using a Dinamap oscillometric device (GE Healthcare, Fairfield, CT) before each PAT test and blood samples were taken immediately following PAT testing.
Statistical Analysis

Analyses were conducted with SAS v. 9.2 (Cary, NC). Reported variability measures include: (i) Pearson’s correlations for measures across visits, (ii) mean variability expressed as the absolute value of the difference between visit 1 & 2 scores, and (iii) the intra-class correlation (ICC) calculated using a conventional formula: $S_b^2 - S_w^2 / S_b^2 + S_w^2$, where “$S_b^2$” represents between subjects variance and “$S_w^2$” represents within subjects variance [241]. As in previous studies,[240, 242, 243] the ICC was computed using raw, non-normalized data for each individual and a mean ICC is reported for each variable. The coefficient of variation (CV) was also appropriate for RHI given its non-negative values [244] and was therefore calculated using the following equation: $CV = (100 \times SD) / \text{mean}$. Variables were tested for normality and transformed when appropriate. Of the 4 PAT scores, all but fRHI required transformation. For normally distributed variables, the Pearson correlation was used. When distributions remained non-normal post transformation, the Spearman rank order correlation was used on the original data.

Sample size calculations were computed using two-tailed tests, $\alpha = 0.05$ (CI = 95%), $\beta = 0.80$ or 0.90. Consistent with previous reports [245, 246], sample size calculations for the crossover design were performed using $SD = \sqrt{2} \times SD_{\text{within subject}}$, where $SD_{\text{within subject}} = \sqrt{\text{MSE}}$ as provided by a one way ANOVA with subject as a main effect. All values are reported as mean ± SEM unless otherwise indicated.
Results

Reliability of EndoPAT measures

Table 5-1 lists blood chemistry, blood pressure and heart rate measures of participants and reliability calculations for EndoPAT measurements taken on two separate days using carefully standardized procedures. No statistically significant differences between days were observed for any of these variables. Similarly all measures correlated well across visits with the exception of glucose (p = 0.43). Variability in glucose was not correlated with change in any PAT score (data not shown). Values for RHI and fRHI were similar to those from a recent sample with similar BMI, age, and lipid profiles [247]. All four EndoPAT scores were repeatable across days. RHI scores were correlated across the two testing days (r = 0.68, p < 0.001, Figure 5-2).
Figure 5-2. PAT RHI scores and Framingham RHI scores plotted by visit. (A) Plot of paired measurements of log RHI illustrating a significant correlation ($r = 0.68$, $p < 0.001$). (B) Plot of paired measurements of fRHI also demonstrate a significant correlation ($r = 0.77$, $p < 0.0001$).

Reliability estimates for RHI (ICC = 0.74, and CV = 12.2 ± 2.2) were within acceptable ranges. FRHI appeared to be slightly more reliable (ICC = 0.77) and, as seen in Figure 2, the correlation between visit 1 and visit 2 scores for fRHI was stronger ($r = 0.77$) than that of RHI ($r = 0.68$). Of the measures of arterial stiffness, both AI (ICC = 0.83) and AI@75 (ICC = 0.81) were found to be highly reliable. Correlations for AI and AI@75 collected across the two testing days were high (0.89 & 0.88, respectively).
Table 5-2 compares our reliability statistics to the small number of published studies that have assessed test-retest reliability of EndoPAT measures. The studies that we selected met two criteria: 1) they were specifically designed to test reliability, and 2) they presented adequate data to allow comparison to the present study. Previous studies reported ICCs of 0.78 and 0.73 for repeated RHI measurements across days in healthy participants (Table 5-2). The ICC from our sample was 0.74, indicating robust reliability, in keeping with previous studies. We found that our estimate of test-retest reliability for fRHI (0.77) was also similar to that found in a previous study (0.88, Table 2).

Table 5-2. Comparison studies reporting test-retest analysis for the Reactive Hyperemia Index (RHI) and Framingham Reactive Hyperemia Index in healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>Current study(^a)</th>
<th>Selmat \textit{et al.}, 2009(^b)</th>
<th>Tomfohr \textit{et al.}, 2008(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>20</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Age</td>
<td>41.2 ± 2.4</td>
<td>17.3(^c)</td>
<td>26.8</td>
</tr>
<tr>
<td>RHI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit 1 Mean ± SEM</td>
<td>1.81 ± 0.12</td>
<td>1.91 ± 0.10</td>
<td>---</td>
</tr>
<tr>
<td>Visit 2 Mean ± SEM</td>
<td>1.82 ± 0.12</td>
<td>1.78 ± 0.09</td>
<td>---</td>
</tr>
<tr>
<td>Mean CV</td>
<td>12.2 ± 2.2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Mean variability (</td>
<td>t_1-t_2</td>
<td>)</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>(r) value (t_1) vs (t_2)</td>
<td>0.68</td>
<td>---</td>
<td>0.76</td>
</tr>
<tr>
<td>Intra-class correlation</td>
<td>0.74</td>
<td>0.78</td>
<td>0.73</td>
</tr>
<tr>
<td>fRHI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit 1 Mean ± SEM</td>
<td>0.29 ± 0.09</td>
<td>0.57 ± 0.08</td>
<td>---</td>
</tr>
<tr>
<td>Visit 2 Mean ± SEM</td>
<td>0.34 ± 0.10</td>
<td>0.49 ± 0.07</td>
<td>---</td>
</tr>
<tr>
<td>Mean CV</td>
<td>1.59 ± 0.27</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Mean variability (</td>
<td>t_1-t_2</td>
<td>)</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>(r) value (t_1) vs (t_2)</td>
<td>0.77</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Intra-class correlation</td>
<td>0.77</td>
<td>0.88</td>
<td>---</td>
</tr>
</tbody>
</table>

Comparison literature standard deviations converted to SEM, SEM = SD/√n;
\(^a\) 3 hrs post standardized breakfast preceded by 12 hour fast; \(^b\) post 12 hour fast; \(^c\) age expressed as a median.
--- indicates that information was not reported in manuscript.
Sample size for experimental design

Table 5-3 contains sample size calculations for both crossover and parallel design studies, using varying magnitudes of treatment effects for RHI, fRHI and AI@75, with $\alpha = 0.05$ (confidence interval fixed at 95%), and $\beta = 0.80$ or 0.90. For example, a crossover design powered at 0.90 would require 28 participants to detect an absolute change in RHI of 0.25 units (corresponding to ~15% increase in RHI from pre to post-treatment). In a parallel arm design, 96 participants would be required to detect the same effect size. For AI@75, a treatment effect of 9 units would require 24 participants to detect at 0.90 power in a crossover design and 95 in a parallel arm design.

Discussion

In this sample of 20 overweight (but otherwise healthy) adults, we showed robust reliability of measures collected during PAT testing conducted in the same individuals under controlled conditions. Despite the long list of factors that are known to cause short-term fluctuations in vascular endothelial function, interclass correlations ranged from 0.74-0.83, indicating strong repeatability in this relatively small sample of healthy adults. We provide preliminary evidence that PAT measures may be more repeatable measures than FMD [28, 245, 246]. Few published studies have directly addressed test-retest reliability of these measures, and there is little consistency in the metrics reported (Table 2). Existing studies of adults have assessed repeatability within a single day [243], or across intervals up to 7 days [232, 240, 243, 248]. The current findings illustrate the robust repeatability of these variables across a mean follow-up interval of nearly 3 weeks. This is especially useful for intervention researchers, who
Table 5-3. Sample size required to detect treatment effects for the RHI, fRHI and AI at EndoPAT endpoints in both parallel arm and crossover designs.

<table>
<thead>
<tr>
<th>Magnitude of treatment effect</th>
<th>RHI Parallel Arm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Crossover&lt;sup&gt;b&lt;/sup&gt;</th>
<th>fRHI Parallel Arm&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Crossover&lt;sup&gt;d&lt;/sup&gt;</th>
<th>AI@75 Parallel Arm&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Crossover&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power</td>
<td>0.80 0.90 0.80 0.90 0.80 0.90</td>
<td>0.80 0.90 0.80 0.90 0.80 0.90</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
</tr>
<tr>
<td>0.05</td>
<td>1765 2363 485 648</td>
<td>0.04 1732 2318 406 543</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
</tr>
<tr>
<td>0.1</td>
<td>442 592 123 164</td>
<td>0.08 434 581 103 138</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
</tr>
<tr>
<td>0.15</td>
<td>197 264 56 74</td>
<td>0.12 194 259 47 63</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
</tr>
<tr>
<td>0.2</td>
<td>112 149 33 43</td>
<td>0.16 110 146 28 36</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
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<tr>
<td>0.25</td>
<td>72 96 22 28</td>
<td>0.20 71 94 19 24</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
</tr>
<tr>
<td>0.3</td>
<td>50 67 16 20</td>
<td>0.24 50 66 14 18</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
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<tr>
<td>0.35</td>
<td>37 50 12 16</td>
<td>0.28 37 49 11 14</td>
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<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
</tr>
<tr>
<td>0.4</td>
<td>29 38 10 13</td>
<td>0.32 29 38 9 11</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
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<td>23 31 9 11</td>
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</tr>
<tr>
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<td>19 25 7 9</td>
<td>0.40 19 25 7 8</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
</tr>
<tr>
<td>0.55</td>
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<td>0.44 16 21 6 7</td>
<td>1.8 1.8 1.8 1.8 1.8 1.8</td>
<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
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<td>14 18 6 7</td>
<td>0.48 14 18 6 7</td>
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<td>3.6 3.6 3.6 3.6 3.6 3.6</td>
<td>5.4 5.4 5.4 5.4 5.4 5.4</td>
<td>7.2 7.2 7.2 7.2 7.2 7.2</td>
</tr>
</tbody>
</table>

AI: Augmentation Index; AI@75: Augmentation Index at heart rate of 75 beats/min; fRHI: Framingham Reactive Hyperemia Index; RHI: Reactive Hyperemia Index; Calculated using: <sup>a</sup>mean ± SD = 1.82 ± 0.53; <sup>b</sup>mean = 1.82, SD = √2 x SD<sub>within</sub> = 0.392; <sup>c</sup>mean ± SD = 0.313±0.42; <sup>d</sup>mean = 0.313, SD = √2 x SD<sub>within</sub> = 0.287; <sup>e</sup>mean ±SD = -9.99 ±16.79; <sup>f</sup>mean = -9.99, SD = √2 x SD<sub>within</sub> = 10.52. All α = .05, all confidence intervals fixed at 95%.
attempt to alter and measure changes in endothelial function within the same individual over intervals longer than a few hours or days.

Given the relatively recent introduction of RHI and fRHI as measures of endothelial function, few studies have examined their clinical utility or prognostic significance. After an exhaustive analysis in which many different components of the PAT signal were evaluated, Hamburg and colleagues determined that the fRHI ratio (referred to as the “PAT ratio”) had the strongest relationship to known CVD risk factors such as smoking, BMI, diabetes mellitus and ratio of total-to-high density lipoprotein cholesterol [167]. More recent work suggests that RHI is similarly useful; the natural log of RHI joined age and prior coronary bypass as significant independent predictors of late cardiovascular adverse events in 270 outpatients followed over 6 years [33]. As this field has developed, investigators have proposed new scoring equations for calculating indices of vascular health from the PAT signal. It is critically important that future publications provide complete information about how the vascular measurements were derived from the PAT signal.

PAT measures of augmentation index (AI and AI@75), a commonly reported indicator of vascular stiffness [236, 249], also displayed robust repeatability in this sample. While little work has considered the relationship between this PAT device’s arterial stiffness measures and CVD risk, previous literature has found that AI measured by a different device (SphymoCor) correlates well with CVD risk [250, 251]. One study of men referred for coronary angiography found that when compared to the lowest quartile of SphygmoCor AI scores, individuals whose AI score fell within the fourth quartile were 4 times more likely to have coronary artery disease [252]. To our knowledge, our study provides the first published data analysis of the repeatability of augmentation index from
the EndoPAT device. Power and sample size calculations for these measures should assist future research evaluating new interventions for reducing arterial stiffness.

The present study reports a wide range of test-retest statistics that are particularly useful for the design of crossover studies. The within-subject standard deviation, presented in Table 3, is required for accurate sample size calculations for crossover studies. However, this variable is not typically reported in the existing literature. Given the potential for day to day fluctuations in measures of endothelial function, future studies should directly assess statistical power before reporting intervention data. Effect sizes were chosen to reflect the magnitude of change reported in previous intervention studies using the EndoPAT device. For example, one study of mandibular advancement splint use for obstructive sleep apnea resulted in a significant change in RHI of 0.33 units [253]. A separate intervention utilizing a standard 35-hour course of enhanced external counterpulsation in patients with symptomatic coronary artery disease reported a 0.26 unit change in RHI [232].

**Study strengths and limitations**

Many variables known to acutely influence endothelial function were controlled for in this protocol. These variables include testing during the follicular menstrual cycle phase [254] (for premenopausal women), standardized time of day [255] and room temperature [256], limiting postprandial effects [216], limiting recent exercise [257] and disallowing certain medication use [258, 259]. The testing procedure also calls for the standardization of posture, probe placement and a timed resting period to eliminate sympathetic stimulation prior to testing [260]. As per findings from previous literature,
alcohol [261], caffeine [262, 263] and antioxidant intake [264] were also limited in the 48 hrs prior to testing. Future studies should consider including these controls for reliable measurement of endothelial function via PAT. However recent work suggests that even in the absence of stringent controls, RHI scores are a valuable tool for discriminating between patients with and without coronary artery disease [265].

Limitations of this study include the small sample size, the small number of women (n = 6), the healthy nature of the participants and the limited number of retests. It should be noted that sample size calculations for FMD studies are commonly based on a classic paper which reported reliability over four testing occasions, with a sample size double that of our own [266]. Finally, many CVD risk interventions require treatment periods of longer than 3 weeks. Future investigations should consider the stability of these EndoPAT measures over longer periods of time.

Sample characteristics are also an important consideration because intervention studies are typically targeted toward individuals at high risk of CVD, diabetes, or other chronic disease. While these participants were overweight and had less than ideal HDL and triglyceride levels, future research should consider the reliability of PAT measures in other populations, particularly in subjects at higher risk of CVD. For example, we previously studied day to day variation in FMD in a sample of adults with Type 2 diabetes. We found that higher day-to-day variability in glucose and insulin was associated with greater variability in endothelial function [245]. This pattern was not observed in the present study (data not shown), perhaps because of the narrow range of fasting glucose values in these participants.
Conclusion

As one of the precursors to atherosclerosis, endothelial dysfunction is a novel target for CVD risk reduction and therefore is of great interest to interventionists. This study is the first to show that PAT measures of endothelial function are highly repeatable across intervals greater than 1 week in healthy adults. In addition, the present study provides some guidance for sample size and power calculations for future intervention design. Specifically, it appears that well controlled crossover designs of relatively small size (n = 15-30) can detect treatment effects for RHI and fRHI that are plausible given the current literature.
Chapter 6

Summary and Concluding Remarks

This dissertation examined the influence of a high polyphenol herb and spice blend on the metabolic, oxidative, vascular and inflammatory response of overweight but healthy individuals to consumption of a high fat/high carbohydrate meal and psychological stress. Further, it explored the mechanism for the twice reported observation of reduced plasma triglyceride concentrations following consumption of a meal containing the herb and spice blend versus one without. Two methods novel to postprandial nutrition intervention trials were employed: the lauric to behenic acid test [207] of fat absorption and the wireless motility capsule (SmartPill, Given Imaging, Yoqneam, Israel). Finally, it examined the test-retest reliability of a novel pulse amplitude tonometry test of endothelial function. All three studies were conducted in samples of healthy but overweight individuals. Such individuals are representative of the U.S. population (2/3rds of which is overweight/obese) and likely to have metabolic abnormalities, including moderate hypertriglyceridemia and insulin resistance [190].

First, we confirm that a high fat/high carbohydrate meal induces lipid oxidation and inflammatory response (Chapter 3). We were only able to observe the effects of stress on one cytokine measure (TNF-α) given that meals’ influence on these endpoints was so pronounced. We did not observe any influence of the herb and spice blend on these outcomes, suggesting that the antioxidant potential of spices may be limited to ex vivo processes or its activity is unable to conserve lipids in vivo. This confirms work by others that suggests that the health benefits of polyphenols may be limited to the gut [267-269]. Researchers attempting to evaluate the in vivo antioxidant abilities of foods using postprandial methods should be aware that ex vivo processes (e.g. storage, cooking) also influence oxidation [270] and the background oxidation in the meal challenge should be assessed and reported.
To our knowledge, only a few other studies have examined the interaction of food intake and stress [187, 271]. Our work adds to this body, highlighting that food intake and acute stress may dynamically interact to influence inflammatory and oxidative stress indices.

In two separate protocols we were unable to observe any effect of our high fat/high carbohydrate meal on endothelial dysfunction using both the gold standard FMD technique (Chapter 4) and a pulse amplitude tonometry technique (Chapter 3). This occurred despite increases in lipid oxidation and inflammation known to promote endothelial dysfunction (Chapter 3). While diets rich in SFA are known to impair endothelial function [215], several studies suggest that single meals rich in SFA may not be as detrimental to postprandial endothelial function as meals containing other types of fat [216, 217]. This may be the result of SFAs' relative resistance to oxidation [59]. Researchers should be aware that meals containing large amounts of SFA may confound observations of postprandial endothelial dysfunction. Future studies which are aimed at determining the in vivo antioxidant potential of food ingredients should use a large bolus of oxidizable (e.g. polyunsaturated) but well preserved fat (free of lipid oxidation prior to consumption due to exposure to light, heat (including cooking) and oxygen).

We did observe an influence of the culinary herb and spice blend on triglyceride concentrations 8hrs post high fat/high carbohydrate meal ingestion. This is inconsistent with our previous work using identical spices which reduced postprandial triglycerides by ~30% [5, 20]. This may be the result of lipophilic bioactive sequestration by a non-absorbable fat substitute (sucrose polybehenate) [210, 212]. This suggests that non-absorbable fat additives in processed foods may prevent the beneficial actions of food-based bioactives. In the future, researcher should be aware that sucrose polybehenate required for the lauric/behenic acid test of fat absorption may interfere with the ability of food based phenolics to act in biological systems. Our spiced meal was associated with shortened gut transit time and acutely increased blood flow compared to a control meal with placebo. Spices have previously been shown to alter gastric emptying rate when given supplemental to a meal [117, 118]. This finding may be clinically
relevant for those with delayed gastric emptying. Our work adds to this literature by demonstrating that a palatable and ecologically valid spiced meal influences blood flow and gut transit. We demonstrated that a novel method for assessing endothelial function is reliable (Chapter 5; [272]). This relatively new method is similar to the gold standard flow mediated dilation in that it evaluates reactive hyperemia following ischemia induced by a blood pressure cuff inflated on the arm for 5 minutes. However, instead of ultrasound images for calculation hyperemia, pulse amplitude tonometry of fingertip blood flow is used. This simple and operator independent method is attractive for large scale clinical trials. We have previously demonstrated its reliability in adults with diabetes [189]. This dissertation adds to the body of work by demonstrating good test re-test reliability in healthy adults.

The crossover design and heterogeneous samples used in these studies is a considerable strength for the determination of biological mechanisms which may explain links between food ingredients and alterations in disease risk factors. However, more intensive sampling of endothelial function (e.g. the addition of assessments at postprandial OS peaks) would be recommended for future research. Additionally, studies which are aimed at considering the gastointesitnal transit of a specific meal should tightly control pre-intervention meals (specifically fiber content) and water intake.

In the future, researchers considering postprandial oxidative measures and endothelial function should be aware that meals containing large amounts of oxidation resistant fat (e.g. SFAs) may confound results. Researchers should also note that conclusions about postprandial in vivo antioxidant effects of food based interventions can best be made when considering the amount of oxidized products in the meal when it is consumed.
Appendix A

Spice Mechanism Study Recipes (Chapter 4)

SPICY CORN MUFFINS

NOTE: Muffins may be made in batches and frozen. Remove muffins the day before test meal. Heat in microwave on defrost for 25 seconds before delivery.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>1 serv</th>
<th>3 serv</th>
<th>6 serv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jiffy Corn Muffin Mix, g</td>
<td>58</td>
<td>174</td>
<td>348</td>
</tr>
<tr>
<td>salt</td>
<td>0.3</td>
<td>0.9</td>
<td>1.8</td>
</tr>
<tr>
<td>oregano</td>
<td>1.13</td>
<td>3.39</td>
<td>6.78</td>
</tr>
<tr>
<td>rosemary</td>
<td>0.61</td>
<td>1.83</td>
<td>3.66</td>
</tr>
<tr>
<td>black pepper</td>
<td>0.45</td>
<td>1.35</td>
<td>2.70</td>
</tr>
<tr>
<td>Garlic powder</td>
<td>0.90</td>
<td>2.70</td>
<td>5.40</td>
</tr>
<tr>
<td>Paprika</td>
<td>1.42</td>
<td>4.26</td>
<td>8.52</td>
</tr>
<tr>
<td>turmeric</td>
<td>0.70</td>
<td>2.10</td>
<td>4.20</td>
</tr>
<tr>
<td>skim milk, g</td>
<td>47</td>
<td>141</td>
<td>282</td>
</tr>
<tr>
<td>Mozzarella cheese, part skim</td>
<td>27.9</td>
<td>83.7</td>
<td>167.4</td>
</tr>
<tr>
<td>Beaten egg</td>
<td>14</td>
<td>42</td>
<td>84</td>
</tr>
<tr>
<td>Total raw weights</td>
<td>152.4</td>
<td>457.2</td>
<td>914.5</td>
</tr>
</tbody>
</table>

1. Preheat oven to 350°F. Spray 12-well muffin tin with Pam. Weigh the empty bowl.
2. Sift together muffin mix and all spices until well blended. This means sift the mixture TWICE and then further mix in the bowl with a whisk-- spice needs to be very evenly distributed to ensure uniformity of each muffin’s spice content.
3. Mix in cheese, milk and egg.
4. Weigh the bowl & batter, subtract weight of empty bowl to be sure all ingredients have been added. Weight of mixture should be very close to amounts listed above.
5. Weigh 75.0 g of batter into each of 2-6-12 wells of muffin tin. Bake for 25 minutes or until golden and springs back to the touch. Place in Baggies and label SPICE muffins with date made.

CONTROL CORN MUFFINS

Follow above recipe OMITTING ALL SPICES (highlighted), but including salt. Batter for each muffin: 73.5g. Label CONTROL muffins.
SPICE STUDY RECIPES (to be made on test day)

**CHICKEN CURRY – (allow 10-15 minutes to cook)**
- *96.6g boneless, skinless chicken breast, raw*
- *91.2g coconut milk*
- 0.3g salt
- 1.43g Paprika
- 1.13g Oregano
- 0.31g Cloves
- 0.46g Black Pepper
- 2.09g Turmeric
- 0.91g Garlic Powder
- 0.23g Cinnamon
- 0.76g Ginger
- *120g canned chicken broth*
- 6.4g cornstarch
- 3 Tbsp. cold water
- *102.7g cooked white rice*
- 17g coconut oil
- 2.5g sucrose polybehenate (non-absorbable fat)

◊ Place chicken in glass Pyrex bowl in which you have combined the coconut milk, salt, paprika, oregano, cloves, pepper, turmeric, garlic powder, cinnamon and ginger. Mix all together and marinate chicken in refrigerator for 30 minutes).

◊ Mix cold water and cornstarch and set aside.

◊ Allow 10-15 minutes to cook chicken. Lightly spray wok with cooking spray. When wok is hot, add chicken mixture. Sauté until chicken is white and mixture bubbling.

◊ While chicken cooks, measure coconut oil and SPB into glass bowl. Heat in microwave until it is completely clear, not cloudy (about 1 to 1 ½ minutes). Swirl the mix gently to ensure that it is evenly dispersed.

◊ Add chicken broth, coconut oil/SPB mixture to chicken. Combine.

◊ When mixture comes to a simmer, add cornstarch mixture and combine to thicken. Serve over white rice in large white bowl.

**- CONTROL - COCONUT CHICKEN**

Follow all instructions above, except marinate chicken in coconut milk and salt only. OMIT ALL SPICES.

This entrée will be served in a large bowl covered with a fitted foil-wrapped, microwave cover.
SPICE STUDY RECIPES

CINNAMON BISCUITS (can make ahead)
39.5g Wegmans Buttermilk Biscuits from 7.5 oz refrigerated can (9-16-09)
2.5g Olivio soft margarine (changed from Fleischmann's 9/14/09)
5g brown sugar
0.88g Cinnamon (increased by .5g from .83 – 6/18/09)
0.75g Ginger
0.30g Cloves
NOTE: Biscuits may be made in batches and frozen. Remove biscuit the day before test meal.
Heat in microwave on defrost for 25 seconds before delivery.

Preheat oven to 350°F. Measure spices and brown sugar together and mix well. Place measured dough on sheet of no-stick foil and cover with another sheet of no-stick foil. With rolling pin, make 8-inch circle. Remove top foil and place bottom foil with dough on scale; tare. Spread 2.5 g soft margarine over surface of dough. Sprinkle with brown sugar, cinnamon, ginger and cloves. Fold dough over mixture to seal so it won’t leak and push foiled “bundle” into muffin well. Bake for 15 minutes. Do not over bake! Cool on wire rack and remove foil. Place in labeled snack baggies and freeze in labeled gallon baggie. Be sure to label SPICE biscuits and the date. Remove biscuit to the refrigerator the day before meal is to be served.

– CONTROL -BISCUITS
Follow all instructions above, except brush biscuit with margarine and brown sugar only. OMIT ALL SPICES. Be sure to label CONTROL biscuits. Remove biscuit to the refrigerator the day before meal is to be served.
Appendix B Daily Stress Inventory

Below are listed events that may be viewed as stressful. Read each item carefully:

- If the event did not occur in the last 24 hours, place an “X” in the space next to that item.
- If the event did occur in the last 24 hours, indicate the amount of stress that it caused you by placing a number from 1 to 7 in the space next to that item (see numbers above).

<table>
<thead>
<tr>
<th>Question</th>
<th>Stress rating</th>
<th>When did this occur?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EXAMPLE:</strong> performed poorly at a task</td>
<td>4</td>
<td>Yesterday around 4pm</td>
</tr>
<tr>
<td>1. Performed poorly at a task</td>
<td></td>
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<td>2. Performed poorly due to others</td>
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<tr>
<td>3. Thought about unfinished work</td>
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<tr>
<td>4. Hurried to meet a deadline</td>
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<tr>
<td>5. Interrupted during task/activity</td>
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<td></td>
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<tr>
<td>6. Someone spoiled your completed task</td>
<td></td>
<td></td>
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<tr>
<td>7. Did something you are unskilled at</td>
<td></td>
<td></td>
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<tr>
<td>8. Unable to complete a task</td>
<td></td>
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<tr>
<td>9. Was unorganized</td>
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<tr>
<td>10. Criticized or verbally attacked</td>
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<tr>
<td>11. Ignored by others</td>
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<tr>
<td>12. Spoke or performed in public</td>
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<tr>
<td>13. Dealt with rude waiter/waitress/salesperson</td>
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<tr>
<td>14. Interrupted while talking</td>
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<tr>
<td>15. Was forced to socialize</td>
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<tr>
<td>16. Someone broke a promise/appointment</td>
<td></td>
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<td>17. Competed with someone</td>
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<tr>
<td>18.</td>
<td>Was stared at</td>
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<td>19.</td>
<td>Did not hear from someone you expected to hear from</td>
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<tr>
<td>20.</td>
<td>Experienced unwanted physical contact (crowded, pushed)</td>
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<tr>
<td>21.</td>
<td>Was misunderstood</td>
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<td>22.</td>
<td>Was embarrassed</td>
<td></td>
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<tr>
<td>23.</td>
<td>Had your sleep disturbed</td>
<td></td>
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<tr>
<td>24.</td>
<td>Forgot something</td>
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<tr>
<td>25.</td>
<td>Feared illness/pregnancy</td>
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<tr>
<td>26.</td>
<td>Experienced illness/physical discomfort</td>
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<tr>
<td>27.</td>
<td>Someone borrowed something without your permission</td>
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<tr>
<td>28.</td>
<td>Your property was damaged</td>
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<tr>
<td>29.</td>
<td>Had minor accident (broke something, tore clothing)</td>
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<tr>
<td>30.</td>
<td>Thought about the future</td>
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<tr>
<td>31.</td>
<td>Ran out of food/personal article</td>
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<tr>
<td>32.</td>
<td>Argued with spouse/boyfriend/girlfriend</td>
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<tr>
<td>33.</td>
<td>Argued with another person</td>
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<tr>
<td>34.</td>
<td>Waited longer than you wanted</td>
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<tr>
<td>35.</td>
<td>Interrupted while thinking</td>
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<tr>
<td>36.</td>
<td>Someone cut ahead of you in line</td>
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<tr>
<td>37.</td>
<td>Performed poorly at sport/game</td>
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<tr>
<td>38.</td>
<td>Did something you did not want to do</td>
<td></td>
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<tr>
<td>39.</td>
<td>Unable to complete all plans for today</td>
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<tr>
<td>40.</td>
<td>Had car trouble</td>
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<tr>
<td>41.</td>
<td>Had difficulty in traffic</td>
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<tr>
<td>42.</td>
<td>Money problems</td>
<td></td>
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<tr>
<td>43.</td>
<td>Store lacked a desired item</td>
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<tr>
<td>44.</td>
<td>Misplaced something</td>
<td></td>
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<tr>
<td>45.</td>
<td>Bad weather</td>
<td></td>
</tr>
<tr>
<td>46.</td>
<td>Unexpected expenses (fins, traffic ticket, etc.)</td>
<td></td>
</tr>
<tr>
<td>47.</td>
<td>Had confrontation with authority figure</td>
<td></td>
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<tr>
<td>48.</td>
<td>Heard some bad news</td>
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<tr>
<td>49. Concerned over personal appearance</td>
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<tr>
<td>50. Exposed to feared situation or object</td>
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<td></td>
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<tr>
<td>51. Exposed to upsetting TV show, movie, book</td>
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<tr>
<td>52. Pet peeve violated (someone fails to knock, etc)</td>
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<tr>
<td>53. Failed to understand something</td>
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<td></td>
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<tr>
<td>54. Worried about another’s problems</td>
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<tr>
<td>55. Experienced narrow escape from danger</td>
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<tr>
<td>56. Stopped unwanted personal habit (overeating, smoking, nail-biting)</td>
<td></td>
<td></td>
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<tr>
<td>57. Had problems with kid(s)</td>
<td></td>
<td></td>
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<tr>
<td>58. Was late for work/appointment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>59. Any stressors that we missed? (List below)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
References


Recent patents on foo


248. Reisner, Y. Reproducibility of endothelial function and arterial stiffness assessed using finger peripheral arterial tonometry. in European Society of Cardiology Congress. 2008. Munich, Germany.
264. Franzini, L., et al., *Food selection based on high total antioxidant capacity improves endothelial function in a low cardiovascular risk population*. Nutrition, Metabolism and Cardiovascular Diseases. **In Press, Corrected Proof**.


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2014-2015 Kligman Fellowship Award
2014-2015 Hintz Graduate Student Enrichment Award
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2014 American Psychosomatic Society Young Scholar Award
2010 Fund for Excellence in Graduate Recruitment, Pennsylvania State University
2010 Phi Kappa Phi National Fellowship Award
2010 Top Ten Graduating Scholar Award, Boise State University
2008-2010 Dean’s List with highest honors, Boise State University
2007-2010 Idaho Opportunity Scholarship
2007-2010 Ronald E. McNair Scholar
2006-2010 Scholarship America’s Access to Education Scholarship

SELECTED PUBLICATIONS