
COMPARISON OF TWO FEEDING REGIMENS ON ENDOTHELIAL FUNCTION
VARIABILITY MEASURED BY PERIPHERAL ARTERIAL TONOMETRY

by
Yibin Liu

Submitted in partial fulfillment of the requirements
for the degree
Master of Science
in the School of Health Physical Education and Recreation
Indiana University

May, 2010

© 2010

(Yibin Liu)

ALL RIGHTS RESERVED

ABSTRACT

Consumption of a high-fat meal has been shown to elicit endothelial dysfunction that contributes to cardiovascular events. The US diet pattern of frequent meals and snacks extends the postprandial state. The focus of this research was to optimize a feeding model to study the acute effects of two high-fat meals on endothelial-dependent vasodilation (reactive hyperemia index) and a biomarker of oxidative stress. The study was conducted to compare changes in endothelial function induced by two different feeding regimens (standard feeding regimen and body surface area-based feeding regimen) and to study the peripheral vascular function in the extended postprandial state. A 2×4 within subject design was used to investigate variability in reactive hyperemia index and oxidative stress. Ten male subjects (age 19-30 years, BMI 18.5-24.9 kg/m², Body surface area 1.5-2.2 m²) were recruited and assigned to two feeding regimens, in random order, on different days, with a 1-week washout period. Each feeding regimen contained two meals that were consumed as “breakfast” (0h) and “lunch” (4h) on the test day. Each meal in feeding regimen 1 provided 850 kilocalories; whereas, each meal in feeding regimen 2 provided 460 kilocalorie/m² subject body surface area. Reactive hyperemia index was measured, followed by blood draw at 0, 2, 4, 6 hours. Blood was analyzed for plasma malondialdehyde as a measure of oxidative stress. Blood glucose and triglyceride were measured to monitor the postprandial response at 0, 1, 2, 3, 4, 5, 6 hours. Power was too low to detect a significant difference in regimen \times time interaction and main effect of feeding regimen for reactive

hyperemia index and oxidative stress ($p > 0.05$, $1 - \beta < 0.6$). However, the feeding regimen based on body surface area might be a more effective model to use than the standard meal as shown by the greater effect size (η_p^2 and ω^2). More subjects are needed to confirm this finding. Reactive hyperemia index increased from the fasting state to the postprandial state ($p < 0.05$). Oxidative stress was elevated 2 hours after “breakfast” ($p < 0.05$) and decreased by 4 hours after “breakfast” ($p < 0.05$).

ACKNOWLEDGEMENT

It is a pleasure to thank all my committee members, Prof. Alyce D. Fly, Prof. David M. Koceja, and Prof. David L. Daleke, who made this thesis possible. First, I owe my deepest gratitude to my advisor, Prof. Fly, whose encouragement and support from the initial to the final stage enabled me to gain knowledge and clear understanding of the project. I am heartily thankful to Prof. Koceja, who has made available his support in the data analysis and statistical interpretation. I am also grateful that Prof. Daleke guided me through the assay development of thiobarbituric acid reactive substances, personally trained my laboratory skills, and allowed me to work in his laboratory.

A special thank to Stephanie Dickinson, a senior consultant in the Center for Statistical and Mathematical Computing, who helped me frame the initial method for data analysis. Thanks to Senta K. Baker in the Office of Human Subjects for her help with the review process of Institutional Review Board. Last but not least, I offer my regards and blessings to my lab mates, John Fyolek, Yi-Chun Lin, and all of those who supported me in any respect during the completion of the project.

TABLE OF CONTENTS

Chapter		Page
1.	INTRODUCTION.....	2
	Statement of the Problem.....	3
	Purpose of the Study.....	4
	Need for the study.....	4
	Delimitations.....	6
	Limitations.....	8
	Assumptions.....	8
	Hypotheses.....	9
	Secondary Hypotheses	9
	Definition of Terms.....	10
2.	REVIEW OF THE LITERATURE.....	15
	Endothelial-dependent Vasodilation.....	15
	Measurements of Endothelial-dependent Vasodilation.....	19
	Postprandial Oxidative Stress and Reactive Oxygen Species.....	21
	Postprandial Hyperglycemia and Hypertriglyceridemia.....	24
	Measurement of Postprandial Oxidative Stress.....	32
	Influence of Single High-fat Meal Consumption on Endothelial Function.....	35
	Influence of Two High-fat Meal Consumption on Endothelial Function.....	41
	Influence of Feeding Meal by Body Surface Area on Endothelial Function.....	42
	Summary.....	44
3.	PROCEDURES.....	45
	Design of the Study.....	45
	Selection of Subjects.....	48
	Arrangements for Conducting the Study.....	50
	Test Procedures.....	50
	Treatment of Data.....	60

4.	ANALYSIS OF DATA AND DISCUSSION OF RESULT.....	65
	Demographic Data.....	65
	Within Subject Analysis of Variance.....	72
	Power and Subject Estimates.....	85
	Effect Size Estimates.....	89
5.	DISUSSION, CONCLUSIONS, IMPLEMENTATIONS AND RECOMMENDATIONS	90
	Discussion	90
	Conclusions.....	99
	Implementations and Recommendations.....	100
	REFERENCE.....	102
	APPENDIXES	
	A. INSTITUTIONAL REVIEW BOARD APPROVAL AND DOCUMENTATIONS.....	118
	B. THIOBARBITURIC ACID REACTIVE SUBSTANCE ASSAY PROTOCOL.....	126

Chapter 1

INTRODUCTION

The endothelium is a single layer of cells lining the walls of the blood vessels that has physical contact with the blood as the blood circulates in the body (Bonetti, Lerman, & Lerman, 2003; Lerman & Burnett Jr, 1992). A function of the endothelium is to mediate relaxation of the muscle layer of the vessels through the production of nitric oxide (NO). Relaxation is an important function of the vascular smooth muscle. Dysfunction of the endothelium is a key contributor to all stages of atherosclerosis and associated with the risk of cardiovascular events. Consumption of a single high-fat meal leads to endothelial dysfunction; perhaps because the high-fat meal induces oxidative stress due to metabolism of cellular fatty acids as well as exogenous dietary

fatty acids, which increase production of modified proteins, fatty acids and DNA. The usual US meal pattern is repeated meals and snacks every few hours, where the postprandial state is extended to a significant part of a day; endothelial function under this prolonged oxidative state is poorly studied. In prior research, the postprandial state has been elicited by ingestion of either a standard high-fat meal, or a meal based on individual body surface area, or a meal based on individual body weight; however, none of these studies proposed a rationale for prescribing a certain feeding method over another or compared the postprandial effects of different feeding methods. In addition, flow-mediated dilation has been widely used as the major indicator of postprandial endothelial function changes although it is operator-dependent and generates variations in results. To avoid the controversy, this study will apply an automated system, peripheral arterial tonometry (tested by EndoPAT 2000), as a substitute for flow-mediated dilation to assess the endothelial-dependent vasodilation. The present study was a pilot investigation (n=10) and served as a tentative design for future research. Power analysis was conducted to determine the effect size of the feeding regimens and to estimate the sample size for future research.

Statement of the Problem

The focus of the research was to optimize a feeding model to study the acute effects of two high-fat meals on endothelial-dependent vasodilation (reactive hyperemia index) and a biomarker of oxidative stress (plasma malondialdehyde). Blood triglyceride and glucose were measured to describe the model of the extended postprandial state.

Purpose of the Study

The study was proposed to:

1. compare the changes in endothelial-dependent vasodilation induced by two different feeding regimens (one consisting of feeding subjects two standard meals, separated by four hours or one consisting of feeding subjects two variable sized meals, where the amount of the meal was proportional to body surface area).
2. study the peripheral vascular function in the extended postprandial state.

Need for the Study

Endothelial dysfunction has been characterized as a key contributor for cardiovascular disease and it is referred to as the hallmark of atherosclerosis (Bonetti, Lerman, et al., 2003; Deanfield, Halcox, & Rabelink, 2007; Lerman & Zeiher, 2005). In fact, a recent multivariate analysis of studies involving close to 2500 patients with follow-ups between 1 and 92 months, found that endothelial dysfunction was strongly and independently associated with cardiovascular events (Lerman & Zeiher, 2005). The lack of direct correlation between the presence of endothelial dysfunction and other traditional risk factors further supported the hypothesis that endothelial dysfunction may be an integrated, independent and sensitive marker for cardiovascular disease (Bonetti, Lerman, et al., 2003).

Though endothelial dysfunction has commonly been measured using the technique of flow-mediated dilation of the brachial artery, this technique requires a highly trained technician to read the ultrasound films, and hours of laborious analysis. A newer technique using signals of peripheral arterial tonometry obtained from two

finger cuffs has been developed that removes much of the operator-dependent analysis, bias, and non-endothelial mediated artifacts that may complicate the measures. The technique uses a system called the EndoPAT 2000 (Itamar Medical, Caesarea, Israel). EndoPAT 2000 is an emerging non-invasive instrument used to examine the endothelial-dependent vasodilation. In clinical research, Endo-PAT2000 has been shown to be valid and reliable but less operator-dependent in comparison to flow mediated dilation (FMD) (Bonetti, Pumper, Higano, Holmes, & Lerman, 2002; Deanfield, et al., 2007; Kuvin, et al., 2003).

Prior research in the area of postprandial oxidative stress has focused on the change of endothelial function and oxidative stress that occurs 2-3 hours after a single meal (Barringer, Hatcher, & Sasser, 2008; Nappo, et al., 2002; Vogel, Corretti, & Plotnick, 1997). However, the common US eating behaviors, including frequent snacks and repeated meals every few hours, expose the human body to prolonged oxidative stress, which may further impair the relaxation function of the vessels (Tushuizen, et al., 2006). Therefore, it is important to know how our vascular function responds to the oxidative stress generated by this particular meal pattern. In addition, prior research has been conducted by varying feeding methodologies: feeding a constant quantity of the high-fat meal, feeding a variable sized meal based on individual body weight, or feeding a variable sized meal based on individual body surface area. It is not clear whether one feeding method is superior to the other in terms of reducing the unexplained variance and optimizing the postprandial outcome markers.

High-fat meal ingestion has been shown to produce excessive reactive oxygen

species that oxidize proteins, lipids and DNA. Much of the research has focused on the lipid peroxidation; because lipids that are found in cell membranes are easily damaged by exogenous oxidative stress and damage to these lipids interferes with subsequent function of the cells. When lipids deteriorate, a number of byproducts are formed, including isoprostanes, and two aldehydes, malondialdehyde, and 4- hydroxynonenal. These byproducts serve as biomarkers for examining oxidative stress status. Recently, a protocol using two high-fat meals was adopted for several studies. A study by Tushuizen et al. (2006) indicated that flow-mediated dilation of the brachial artery (a measure of endothelial function) was significantly impaired after two consecutive high-fat meals compared to the baseline; plasma oxidized low-density lipoprotein (LDL) and plasma malondialdehyde were significantly increased. But other research has questioned the validity of oxidized LDL or damaged LDL assay, because it was shown that these measurements were not consistently related to oxidative stress (Sjogren, et al., 2005), which may be due to problems with the methodology to measure oxidized LDL.

Delimitations

The study was delimited to the following:

1. Subjects were male (ages 19-30 y, BMI 18.5-24.9 kg/m², Body surface area 1.5-2.2 m²).
2. Subjects were recruited based on their BMI and body surface area. Subjects were also screened and excluded for history of: coronary artery disease, cerebrovascular disease, heart failure, diabetes mellitus, hepatic and renal disease,

uncontrolled hypertension, dyslipidemia, untreated or clinically evident thyroid disease (Barringer, et al., 2008) and tobacco use (Shimabukuro, et al., 2007). Subjects were also excluded for the use of regular medication, including aspirin, anti-platelet drugs, anti-inflammatory drugs, lipid-lowering drugs and blood pressure medication (Hall, Sanders, Sanders, & Chowienczyk, 2008; Nappo, et al., 2002).

3. Subjects were introduced to the testing procedures before tests begin.

4. Subjects were required to exclude caffeine, alcohol, multiple vitamin supplements and any other supplements 24 hours prior to and on each test day (Berry, et al., 2008; Hall, et al., 2008).

5. Subjects were required to refrain from physical activity 24 hours prior to and on each test day (Berry, et al., 2008; Hall, et al., 2008).

6. Subjects were required to undergo an overnight fasting period of at least 12 hours before each test day; water drinking was permitted.

7. The study was carried out in a temperature-controlled lab 079, HPER building at Indiana University.

8. The study included assessments of endothelial-dependent vasodilation, oxidative stress status, blood glucose and triglyceride.

9. Endothelial-dependent vasodilation was assessed by the reactive hyperemia index, generated by peripheral arterial tonometry tested by EndoPAT 2000.

10. A measure of postprandial oxidative stress (lipid peroxidation): plasma malondialdehyde, tested by thiobarbituric acid reactive substances (TBARS) assay.

11. Blood glucose and triglyceride were obtained by finger stick.

12. The study included two test days and lasted for 6-7 hours/day.

Limitations

Results of the study were interpreted while considering the following limitations:

1. Subjects in this study did not represent both genders or all ages of the US population so that the results were limited to apparently healthy, normal weighted young male subjects, without history of heart diseases, chronic illnesses, or tobacco use.

2. Dietary pattern and physical activity were lightly controlled.

3. EndoPAT 2000 could not calculate the shear stress and blood flow velocity, therefore, the calculated reactive hyperemia index was a downstream outcome indicator of peripheral endothelial function.

Assumptions

The basic assumptions underlying this study were:

1. Subjects followed instructions to undergo a fasting period for 12 hours before testing.

2. Subjects complied with the investigator's request of no additional food other than the provided meals during the test.

3. The impairment of endothelial-dependent vasodilation was reflected by decreased reactive hyperemia index (Barringer, et al., 2008).

4. The measurement of reactive hyperemia index was valid for assessing endothelial-dependent vasodilation.

5. The measure of plasma malondialdehyde (tested by TBARS assay) was a valid

indicator of oxidative stress.

6. Blood glucose and triglyceride levels obtained by finger stick were valid substitutes for the results by needle blood draw and analysis.

Hypotheses

This study was designed to test the following hypotheses:

1. H_A: The feeding regimens affect the reactive hyperemia index differently over time.

1a. H_A: The reactive hyperemia index is significantly different over time after feeding standard meals (feeding regimen 1).

1b. H_A: The reactive hyperemia index is significantly different over time after feeding meals by body surface area (feeding regimen 2).

1c. H_A: The reactive hyperemia index is significantly different between feeding regimen 1 and feeding regimen 2 at hour 2 post ingestion.

1d. H_A: The reactive hyperemia index is significantly different between feeding regimen 1 and feeding regimen 2 at hour 4 post ingestion.

1e. H_A: The reactive hyperemia index is significantly different between feeding regimen 1 and feeding regimen 2 at hour 6 post ingestion.

2. H_A: The reactive hyperemia index changes over time.

Secondary Hypotheses

1. H_A: The feeding regimens affect the plasma malondialdehyde level differently over time.

1a. H_A: The plasma malondialdehyde level is significantly different over time after

feeding standard meals (feeding regimen 1).

1b. H_A: The plasma malondialdehyde level is significantly different over time after feeding meals by body surface area (feeding regimen 2).

1c. H_A: The plasma malondialdehyde level is significantly different between feeding regimen 1 and feeding regimen 2 at hour 2 post ingestion.

1d. H_A: The plasma malondialdehyde level is significantly different between feeding regimen 1 and feeding regimen 2 at hour 4 post ingestion.

1e. H_A: The plasma malondialdehyde level is significantly different between feeding regimen 1 and feeding regimen 2 at hour 6 post ingestion.

2. H_A: The plasma malondialdehyde changes over time.

Definition of Terms

Butylated hydroxytoluene (BHT). A lipophilic antioxidant food additive that is used in cosmetics, pharmaceuticals and etc.

Caveolae. It is the omega-shaped invaginations on the membrane of endothelial cells; it is responsible for signal transduction by shuttling molecules in and out of the cell (Bruns & Palade, 1968; Palade, 1953).

Endothelial-dependent vasodilation. The vessel relaxation process that is mediated by endothelium-derived vasodilators, such as nitric oxide (NO).

Endothelium-derived nitric oxide (NO). An important endothelium-derived vasodilator, which inhibits the development of atherosclerosis, including synthesis of pro-inflammatory cytokines, the expression of leukocyte adhesion molecules, the activation and aggregation of platelets, and the proliferation of vascular smooth muscle

cell (Rush, Denniss, & Graham, 2005). NO is synthesized enzymatically from the amino acid L-arginine and NADPH in a reaction catalyzed by endothelial NO synthase.

Endothelin. A potent endothelium-derived vasoconstrictive peptide comprised of 21 amino acid residues. Endothelin and NO acts on the same nonselective cation channel but endothelin elicits constriction of vascular smooth muscle and promotes the growth of endothelial cells and smooth muscle cells. The circulating level of endothelin was shown to be elevated in cardiovascular disease, including chronic heart failure, ischemic heart disease, hypertension, atherosclerosis and etc. (Masaki, 2004).

Endo-PAT 2000 (Itamar Medical, Caesarea, Israel). A device that measures tonometry of the peripheral arterial, more specifically, measures changes in fingertip blood volume in response to reactive hyperemia (Dhindsa, et al., 2008). Two probes are place on the index fingers of both hands to record the fingertip blood volume changes (Figure 1-1). The blood cuff is put on the upper arm and inflated for 5 minutes to completely occlude the brachial artery to induce ischemia (Figure 1-1).

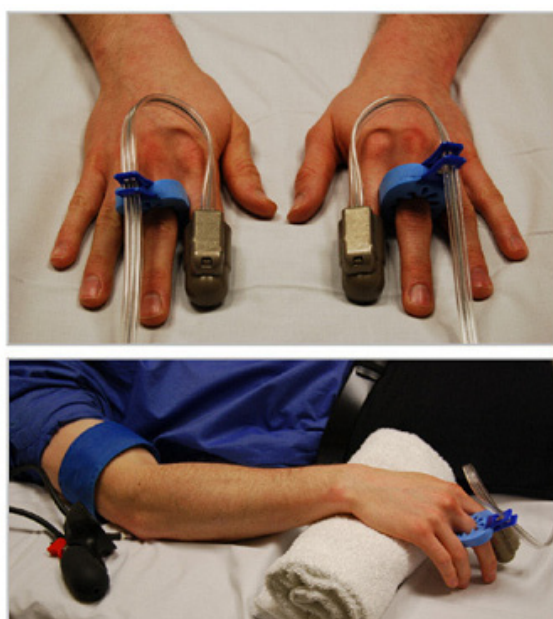


Figure 1-1. EndoPAT 2000 overview (Hamburg & Benjamin, 2009)

After 5 minutes, the blood cuff is deflated and EndoPAT 2000 software calculates the reactive hyperemia index. This index is used to evaluate the vascular function, in particular, endothelial-dependent vasodilation. A higher reactive hyperemia index represents better endothelial responses; a reactive hyperemia index of less than or equal to 1.6 indicates endothelial dysfunction whereas greater than 1.6 indicates normal endothelial function, characterized by a distinct increase in the post-occlusion signal amplitude in comparison to the baseline (Figure 1-2).

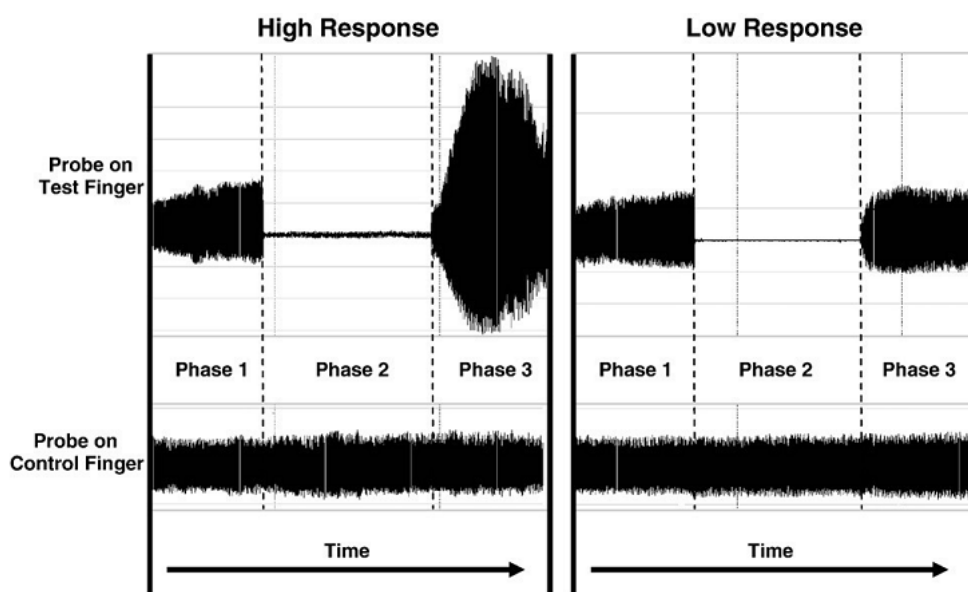


Figure 1-2. High and low endothelial responses tested by EndoPAT 2000 (Hamburg & Benjamin, 2009)

Lipid peroxidation. A chain reaction that leads to structural and functional damage of polyunsaturated fatty acids in cell membranes (Spiteller, 2006); during the reaction, lipid peroxides (radicals) are formed but unstable and can degrade to secondary oxidation products such as malondialdehyde, 4-hydroxynonenal and various

isoprostanes.

L-arginine. The substrate for NO synthesis, which undergoes a five-electron oxidation to generate L-citrulline and NO (Voetsch, Jin, & Loscalzo, 2004).

Reactive hyperemia. Complex hemodynamic responses of the vasculature that occur to accelerate the delivery of oxygen to tissues as well as the removal of metabolic byproducts after a period of ischemia (Dhindsa, et al., 2008).

Reactive hyperemia index (RHI). The ratio of the average pulse wave amplitude during the 1 minute period following the release of blood pressure cuff to the average pulse wave amplitude during a 210-second baseline period. It is a measure of micro-vascular function. The coefficient variation of the reactive hyperemia index was shown to be 12% (Dhindsa, et al., 2008) in comparison to 14.7% for flow-mediated dilation (Donald, et al., 2008).

Sensitivity. “The ability of a test to detect disease when it is truly present, e.g., it is the probability of a positive test result given that the patient has the disease” (Vasan, 2006).

Specificity. “The ability of a test to exclude the disease in patients who do not have the disease, e.g., it is the probability of a negative test result given that the patient does not have the disease” (Vasan, 2006).

Superoxide dismutase. In humans, there are three forms of the superoxide dismutase enzyme: cytosolic Cu-Zn superoxide dismutase, mitochondrial Mn superoxide dismutase, and extracellular Cu-Zn superoxide dismutase (Faraci & Didion, 2004). These enzymes convert superoxide radicals to hydrogen peroxides and therefore

prevent superoxide-induced cellular damage, e.g., inactivation of mitochondrial proteins containing Fe-S centers and subsequent hydroxyl radical formation (Faraci & Didion, 2004). In addition, superoxide dismutase preserves NO bioavailability by inactivating the reaction between NO and superoxide, which reduces peroxynitrite radical production and prevent endothelial NO synthase from uncoupling (Faraci & Didion, 2004).

Thiobarbituric acid reactive substances assay (TBARS). Under high temperature and acidic condition, malondialdehyde and thiobarbituric acid react to generate a malondialdehyde-thiobarbituric acid adduct, which can be measured colorimetrically at 530-540 nm. The result may be overestimated due to artifacts, reactions between thiobarbituric acid and other compounds, such as sugar, amino acid, albumin and bilirubin; because products of these reactions overlap the colorimetric absorbance range of malondialdehyde-thiobarbituric acid adduct (Grotto, et al., 2009).

Urinary nitric oxide metabolites (NO_x). NO metabolites (nitrite and nitrate) are indirect biomarkers of NO production (Feelisch, et al., 2002).

Venoarteriolar reflex. The vasoconstriction of the adjacent capillaries, in this way, blood flow and capillary pressures in the limb are declined to prevent the venous blood pooling and formation of edema under dependent position (Gabrielsen & Norsk, 2007).

Chapter 2

REVIEW OF THE LITERATURE

Endothelial-dependent Vasodilation

Cardiovascular disease has been the leading cause of death in the US every year since 1900, except 1918 (Lloyd-Jones, et al., 2009). The American Heart Association and the Centers for Disease Control and Prevention reported that approximately 80,000,000 American adults (approximately 1 in 3) suffered from at least one type of cardiovascular disease; 41,900,000 of these people were estimated to be under 61 years of age (Lloyd-Jones, et al., 2009). Mortality data showed that cardiovascular diseases accounted for 1 of every 2.8 deaths in the United States (Lloyd-Jones, et al., 2009). Endothelium dysfunction is associated with several recognized cardiovascular risk factors, including smoking, diabetes mellitus, systemic inflammation, obesity and hyperlipidemia (Bonetti, Lerman, et al., 2003; Hamburg, et al., 2008; Urbich & Dimmeler, 2005; Vita & Keaney, 2002). Increasing evidence shows that endothelial dysfunction is clinically relevant. Patients with endothelial vasomotor dysfunction in the coronary or peripheral circulation have increased risk for cardiovascular events including myocardial infarction, stroke, and etc. (Widlansky, Gokce, Keaney, & Vita, 2003).

The endothelium plays a key role in cardiovascular health, as it regulates vessel dilation and constriction, blood fluidity, growth of vascular smooth muscle cells, local inflammation and thrombosis by elaborating a number of endothelium-originated paracrine factors such as NO and endothelin (Shenouda & Vita, 2007; Tam, et al., 2005;

Widlansky, et al., 2003). Endothelium-derived NO is a potent vasodilator and acts to inhibit platelet activity, vascular smooth muscle cell growth, and adhesion of leukocytes to the endothelial surface (Widlansky, et al., 2003). Nitric oxide is produced in endothelial cells from L-arginine catalyzed by endothelial NO synthase located in caveolae (invaginations in cell membranes) under the presence of cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), and oxygen as shown in Figure 2-1 (Davignon & Ganz, 2004). Shear stress increases intracellular Ca^{++} which displaces the inhibitor caveolin from calmodulin (CaM); then endothelial NO synthase is activated for NO production (Davignon & Ganz, 2004).

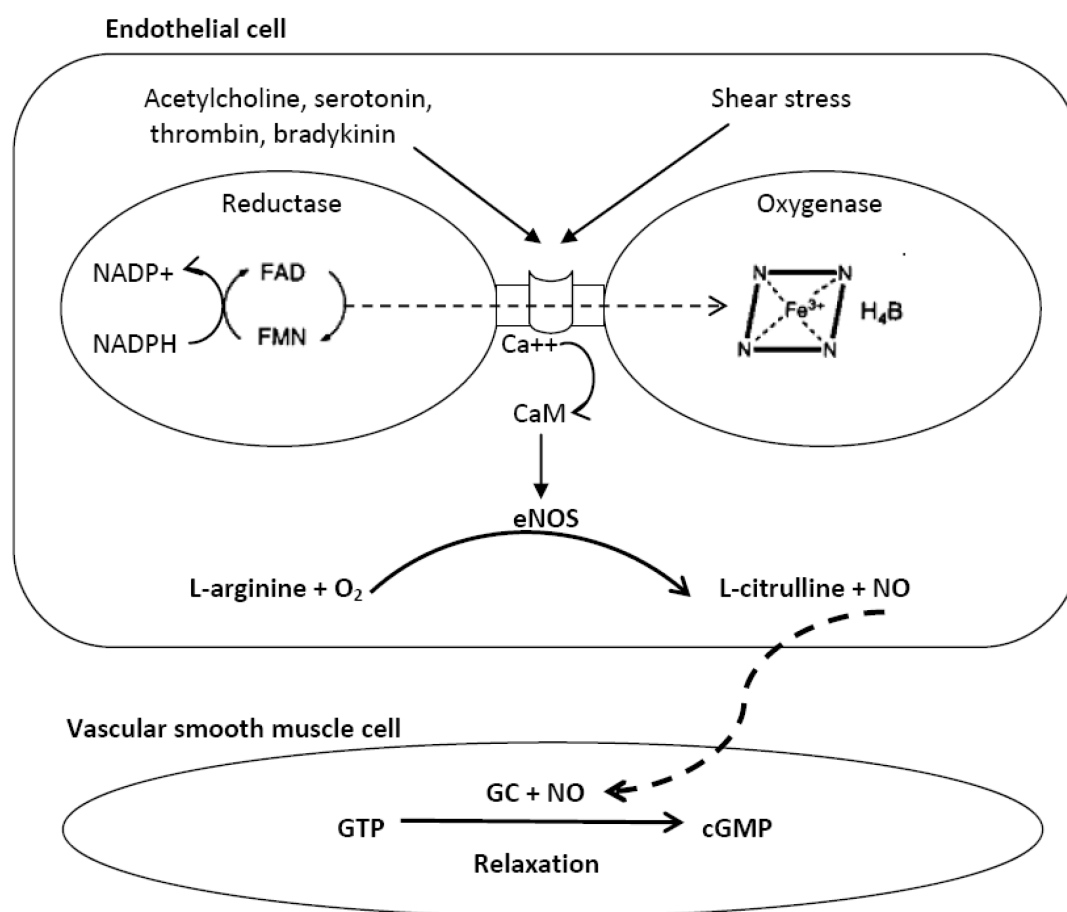


Figure 2-1. Endothelium-derived NO production

Reduced NO bioavailability has been identified as an important indicator of endothelial dysfunction and is referred to as the hallmark sign of early atherosclerosis (Malek, Alper, & Izumo, 1999; Vita & Keaney, 2002). Endothelial dysfunction facilitates inflammation, thrombosis, vasoconstriction, and atherosclerotic plaque formation and thus contributes to all stages of atherosclerosis (Huang & Vita, 2006; Shenouda & Vita, 2007).

Endothelial function is influenced by age, gender, and physical activity. A study conducted by Taddei et al. (2001) showed that forearm blood flow response to acetylcholine was negatively correlated with age ($r=-0.65$, $p<0.0001$) among normotensive subjects; forearm blood flow response was more impaired in subjects ages 30-45 years in comparison to younger ages less than 30 years and the response further declined in subjects ages 45-60 years. In addition, Celermajer et al. (1994) proposed that the age-related endothelial dysfunction occurred earlier in men than women. For young women, flow-mediated dilation was shown to change correspondingly with serum estrogen during menstrual cycle (Hashimoto, et al., 1995); in addition, flow-mediated dilation was higher in the follicular phase than in luteal and menstrual phases (Hashimoto, et al., 1995; Kawano, et al., 2001). For postmenopausal women, estrogen treatment was shown to increase flow-mediated dilation (Gerhard, et al., 1998). Kalantaridou et al. (2006) observed similar results among women (age <40 y) with premature ovarian failure and low estrogen level. Exercise training was shown to elevate NO availability thus likely increasing exercise capacity and cardiovascular

protection, both in healthy individuals and patients with cardiovascular risk factors and established diseases (Kingwell, 2000).

Endothelial function is also influenced by antioxidant level. Studies by Pleiner et al. (2008) and Cangemi et al. (2007) showed that ascorbic acid infusion was inversely correlated with the changes in flow-mediated dilation and oxidative stress. Fisher et al. (2006; 2003) found that 1-week consumption of flavanol-rich cocoa (821 mg/day) improved the endothelial response (reactive hyperemia index, tested by EndoPAT 2000) to the ischemia among healthy middle aged and older subjects; a similar result was observed by Schroeter et al. (2006) and Heiss et al. (2005) using flow-mediated dilation. Dixon et al. (1994) showed the protective effect of β -carotene against oxidative damage; they found that β -carotene-deficient diet (58 μ g β -carotene/day with sufficient vitamin A, C and E) depressed erythrocyte superoxide dismutase activity ($p=0.0001$) and elevated plasma malondialdehyde ($p=0.0001$) compared to the β -carotene-rich diet (>1500 μ g/day). In addition, supplementation with multiple antioxidant vitamins was found to be protective against atherosclerosis. Plantinga (2007) showed that an 8-week supplementation of vitamin C (1 g) and E (400 IU) had beneficial effects on flow-mediated dilation and arterial stiffness in hypertensive patients. Neri (2005) showed that supplementation with vitamin E (300 mg/day, although the article mentioned 300 g/day) and C (250 mg/day) reduced the plasma level of malondialdehyde, 4-hydroxynonenal, oxidized LDL, and soluble vascular cell adhesion molecule-1 (sVCAM-1), while significantly increasing plasma nitrite and nitrate from radial arterial blood (tested by a kit from Cayman Chemical, Ann Arbor,

MI). Title et al. (2000) showed that a supplementation with vitamin C (2 g) and vitamin E (800 IU) prevented the hyperglycemia-induced (75 g oral glucose loading) impairment of flow-mediated dilation.

Measurements of Endothelial-dependent Vasodilation

In clinical studies, endothelial function were studied by infusing acetylcholine intravenously and measuring the change in vessel diameter by quantitative coronary angiography (Cox, et al., 1989; Ludmer, et al., 1986). In the intact endothelium, infused acetylcholine induced vasodilation by stimulating endothelium-derived NO production; whereas infused acetylcholine led to vasoconstriction in the dysfunctional endothelium. The valid contribution of NO to the endothelial responses has been examined by N(G)-mono-methyl-L-arginine, an endothelial NO synthase inhibitor (Goodhart & Anderson, 1998). The disadvantage of this technique is that it is extremely invasive and hard to test repeatedly.

Several noninvasive techniques have also been developed, including the flow-mediated dilation technique which has been widely used in clinical research. Flow-mediated dilation utilizes an ultrasound-based test to assess the brachial artery diameter change induced by reactive hyperemia, therefore evaluating conduit artery vascular function in the peripheral circulation (Corretti, et al., 2002). Flow-mediated dilation requires high levels of ultrasound expertise in conduct and analysis, minimization of environmental and physiological influences, and may be burdened by considerably large intra- and inter-operator variability. Several researchers have questioned whether this methodology will ever be applied and implemented in routine

clinical practice (Bhagat, Hingorani, & Vallance, 1997; Corretti, et al., 2002; Tschakovsky & Pyke, 2005).

A new technique using peripheral arterial tonometry has been developed that removes much of the operator analysis, bias, and non-endothelial mediated artifacts that may complicate the measures. The technique employs a system called the EndoPAT 2000 (Itamar Medical, Caesarea, Israel). EndoPAT 2000 is another non-invasive method to examine the endothelial-dependent vasodilation. In clinical research, Endo-PAT2000 has been shown to be valid and reliable but less operator-dependent comparing to flow-mediated dilation (Bonetti, et al., 2002; Deanfield, et al., 2007; Kuvin, et al., 2003). A study (n=94) by Bonetti et al. (2004) showed that the sensitivity and specificity of the EndoPAT system were 80% and 85% respectively. The EndoPAT system measures the fingertip blood volume changes that are continuously recorded by bio-sensors in the finger probes (Kuvina, et al., 2003). By inflating the blood pressure cuff to supra-systolic pressure between 200-300 mmHg (70 mmHg above systolic pressure), the brachial artery and other small resistance vessels are occluded which causes an ischemia in the upper arm (Kuvina, et al., 2003). This local ischemia provokes the reactive hyperemia response and induces endothelium dependent vasodilation after deflating the blood cuff (Bonetti, Barsness, et al., 2003). The magnitude of the reactive hyperemia response indicates the function of endothelium (Bonetti, Barsness, et al., 2003). The EndoPAT system automatically analyzes these blood volume changes and generates a reactive hyperemia index. This reactive hyperemia index was shown to be significantly associated with values obtained

by flow-mediated dilation (Dhindsa, et al., 2008). The reactive hyperemia index (RHI) is calculated according to the following formula (Bonetti, et al., 2004; Kuvin, et al., 2003):

$$\text{RHI} = \frac{\text{Average amplitude of the PAT signal during 1-min starting 90 s after deflation}}{\text{Average amplitude of the PAT signal during 210-s preocclusion baseline}}$$

The EndoPAT system has several unique features: the finger probe imparts a uniform pressure field to the distal 2/3 of the fingers including the tips so that the vasoconstriction due to venoarteriolar reflex (distal venous blood pooling) is avoided; the EndoPAT system corrects the index to the control arm thus it decreases the potential systemic changes during ischemia (Kuvin, et al., 2003). These systemic changes may result from variation in temperature, noise, light and water drinking.

There are several other techniques for assessing peripheral vascular health, for example, radial artery tonometry, pulse wave analysis, and pulse contour analysis by digital photoplethysmography. They have been shown to correlate with flow-mediated dilation testing, yet still need further validation because the relative contribution of structural changes in the vessel wall to endothelial dependent biology remains uncertain (Deanfield, et al., 2007).

Postprandial Oxidative Stress and Reactive Oxygen Species

Oxidative stress was defined by Blomhoff (2005) as “an accumulation of non-enzymatic oxidative damage to molecules that threatens the normal functions of a cell or an organism” (Blomhoff, 2005). Dietary oxidative overload indicates an imbalance between oxidants and antioxidants (Sies, Stahl, & Sevanian, 2005); thus oxidative stress can be a combination of overproduced oxidants and reduced

antioxidant capacity, in which the antioxidants are not sufficient to protect the cell membranes and cells from oxidant attack. Oxidants refer to the reactive oxygen species that have been correlated with a large number of human diseases: cancer, diabetes mellitus, coronary heart disease, Parkinson's disease, Alzheimer's disease, hepatitis, inflammatory bowel diseases, and rheumatoid arthritis (Blomhoff, 2005).

Reactive oxygen species are produced continuously by cells during the metabolism of carbohydrate, protein, and fat (Urso & Clarkson, 2003). They can be formed by either breakage of covalent bonds, addition of electrons to a molecule or removal of hydrogen by other radicals; these highly reactive species act as electrophiles or oxidant agents. They are divided into two categories: radicals (superoxide radical $O_2^{\cdot-}$, hydroxyl radical OH^{\cdot} , nitric oxide radical NO^{\cdot} , peroxy radical ROO^{\cdot} , and alkoxy radical RO^{\cdot}) and non-radicals (hydrogen peroxide H_2O_2 , organic peroxides, aldehydes, hyperchlorous acid $HOCl$, and ozone O_3). These species cause oxidative damage to lipid (Blomhoff, 2005), DNA (Barzilai & Yamamoto, 2004), and protein (Davies, 1987; Grune, Reinheckel, & Davies, 1997) as shown in Figure 2-2. Radicals, such as superoxides, peroxy radicals, and peroxy nitrite radicals, are highly relevant in the lipid peroxidation-induced damages.

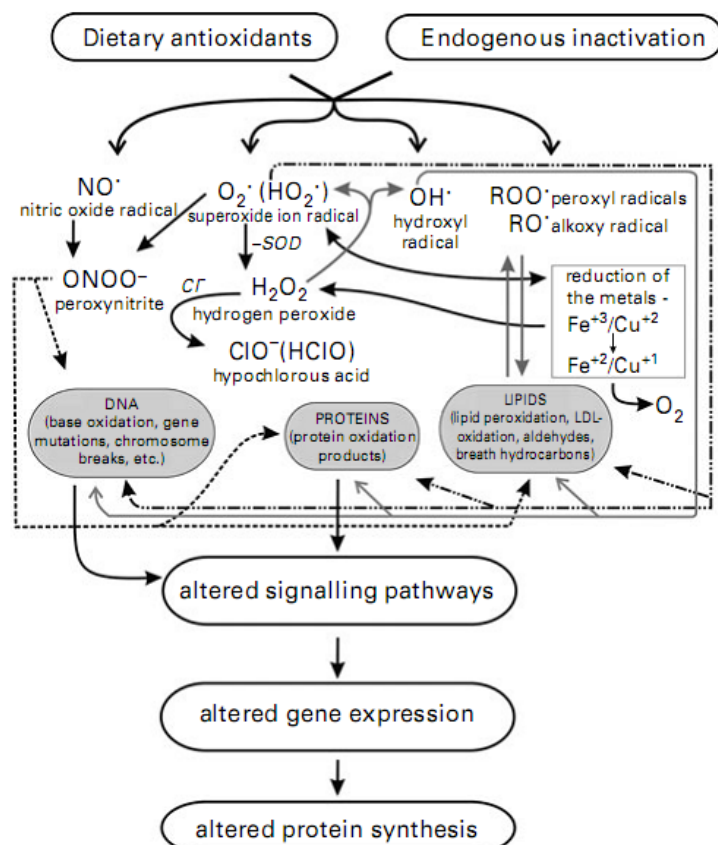


Figure 2-2. Different forms of reactive oxygen species (Knasmüller, et al., 2008)

Radicals are highly reactive because they contain unpaired electrons and thus have the ability to donate or receive other atoms to obtain stability (Blomhoff, 2005). DNA can be modified by hydroxyl radical through interaction with the sugar phosphate chain and oxidation of bases, which produce thymine glycol, 5-hydroxy-uracil and 8-hydroxy-desoxyguanosine (Barzilai & Yamamoto, 2004). Protein can be modified by some radicals through interactions with nucleophilic amino acids (Davies, 1987; Grune, et al., 1997). For example, superoxide and hydrogen peroxide directly oxidize the thiol group of cysteine (Levine & Stadtman, 2001); hydroxyl radical can hydroxylate lysine and proline. Peptide bonds can also be destroyed by radicals thus causing drastic alterations of protein structure and cellular function. For example, peroxynitrite radical

reacts with the Fe–S centers of proteins which affects electron transport functions of mitochondria (Kushnareva, Murphy, & Andreyev, 2002).

Non-radicals are also deleterious to the cells, for example, hydrogen peroxide can penetrate the cell membrane and damage cells at low concentrations by degrading heme proteins and oxidizing DNA, enzymes, thiol groups and keto acids. Hydrogen peroxide is also the source of more deleterious species such as hyperchlorous acid and hydroxyl radicals (Fridovich, 1986; Halliwell & Gutteridge, 1999).

Normally, reactive oxygen species are eliminated by antioxidant defenses through cellular enzymatic and non-enzymatic defenses. The enzymatic defense includes the reaction of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase converts the superoxide radical into hydrogen peroxide, which is then metabolized into water and oxygen by catalase and glutathione peroxidase (Andreazza, et al., 2008). The non-enzymatic defense refers to reactions with small molecules such as glutathione, ascorbic acid, tocopherol, carotenoids, polyphenols, and uric acid (Prior, Wu, & Schaich, 2005).

Postprandial Hyperglycemia and Hypertriglyceridemia

One form of the oxidative stress, postprandial oxidative stress, is characterized by increased oxidative damage after consumption of a high-fat and/or high-carbohydrate meal (Bowen & Borthakur, 2004; Ursini & Sevanian, 2002). Postprandial hyperlipidemia increases the risk of coronary artery disease (Verschuren, et al., 1995); both hyperglycemia and hypertriglyceridemia have been associated with atherosclerosis, type 2 diabetes, and obesity (Bae, et al., 2001; WeiChuan, YiHeng,

ChihChan, TingHsing, & JyhHong, 2004). Prior research suggested that hyperglycemia (Ceriello, et al., 2002; Marfella, Quagliaro, Nappo, Ceriello, & Giugliano, 2001; Marfella, et al., 1995) and hypertriglyceridemia (R. Anderson, et al., 2001; Bae, et al., 2001; Ceriello, et al., 2004) produced an overload of reactive oxygen species that mediated the endothelial dysfunction and inflammatory diseases, especially atherosclerosis (Ceriello, et al., 2005; Shimabukuro, et al., 2007). It is hypothesized that high glucose and fat intake increase acetyl CoA derived from glycolysis and fatty acid β -oxidation. Acetyl CoA accelerates the citric acid cycle and thus increases NADH production which enters mitochondrial electron transport chain (oxidative phosphorylation) for ATP production; however, excessive NADH elevate the mitochondrial proton gradient and thus single electrons are transferred to oxygen, which lead to the formation of reactive oxygen species, especially superoxide as shown in Figure 2-3 (Ceriello, et al., 2004). Several *vivo* studies supported this hypothesis and showed the cumulative and independent effect of glucose and triglyceride in mediating postprandial oxidative stress (Ceriello, et al., 2004; Ceriello, et al., 2002; Esposito, et al., 2002; Nappo, et al., 2002; Tripathy, et al., 2003).

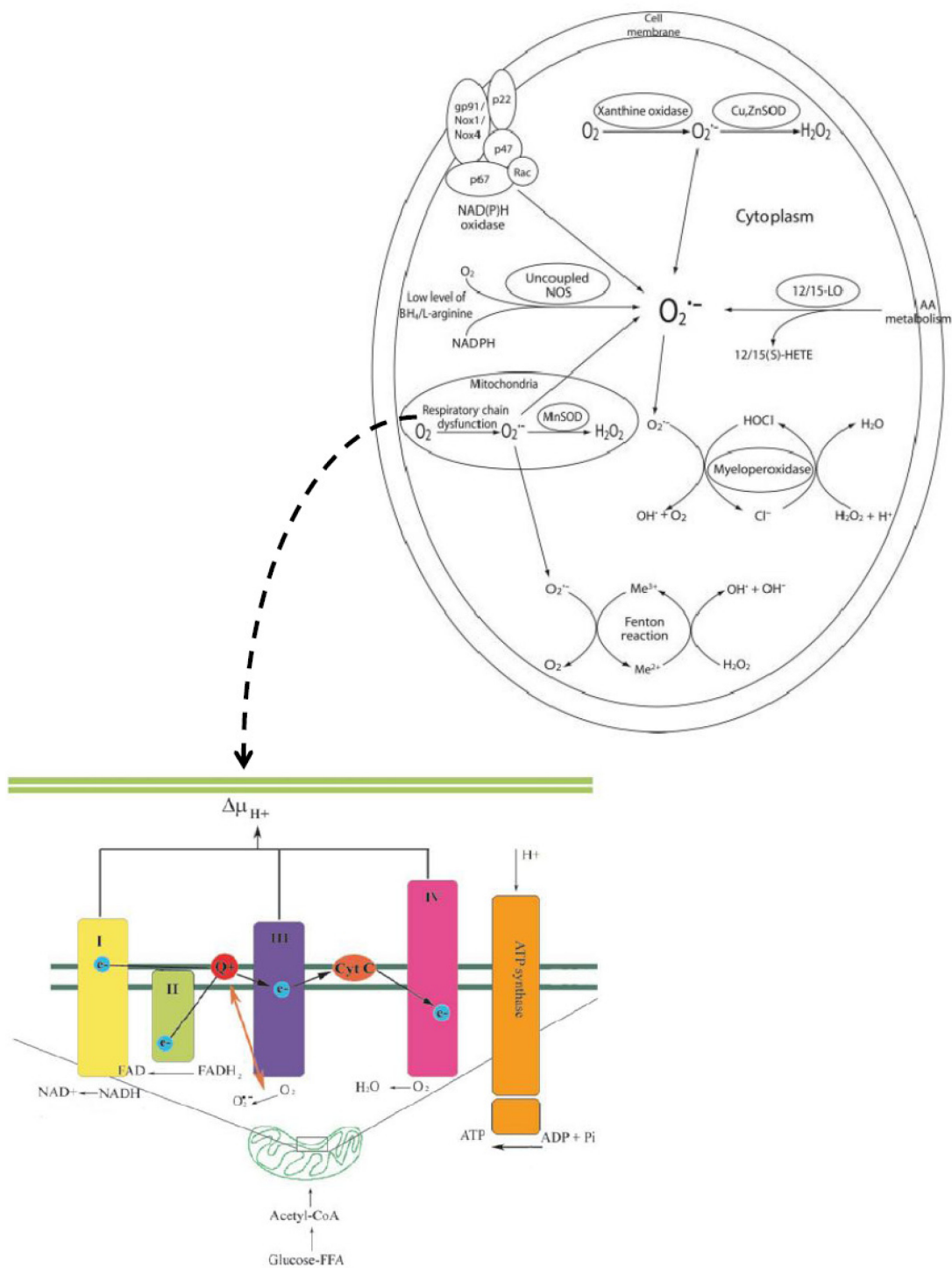


Figure 2-3. Production of mitochondrial superoxide. Adapted from Ceriello et al. (2004) and Madamanchi et al. (2005).

Superoxide is one of the major oxygen-derived radicals. It is mainly formed by uncoupled mitochondrial electron transport chain (non-enzymatically) and can also be produced by activated membrane-bound NAD(P)H oxidase in vascular smooth muscles, and xanthine oxidase in plasma and endothelial cell as shown in Figure 2-3 (Madamanchi, et al., 2005). Superoxide reacts with endothelium-derived NO at a rate three times faster than with superoxide dismutase, and therefore favors the production of peroxynitrite radical that reduces the NO availability and impairs the endothelial-dependent vasodilation.

Furthermore, the increased peroxynitrite can oxidize and deplete BH₄, a cofactor for NO production, and thus result in the uncoupling of endothelial NO synthase, which directs the reaction towards superoxide production (Davignon & Ganz, 2004). The endothelial NO synthase is composed of two globular protein domains (reductase and oxygenase/heme domains) that are connected via a flexible protein strand as shown in Figure 2-1 (Davignon & Ganz, 2004). In the reductase domain, electrons are generated when NADPH reduces FAD that in turn reduces FMN. In the presence of intracellular Ca⁺⁺, calmodulin (CaM, Figure 2-1) is activated to accelerate the transfer of electrons across the protein strand to the oxygenase/heme domain. Electrons bind ferric heme and are transferred to L-arginine. Under the presence of cofactor BH₄, as the electrons flow, oxygen is incorporated to produce L-citrulline and NO (Davignon & Ganz, 2004). However, when the cofactor BH₄ is oxidized by peroxynitrite, the electron from the ferric heme reduces oxygen instead to form superoxide (Davignon & Ganz, 2004; Madamanchi, et al., 2005). Endothelial NO synthase is then switched from a coupled

state to an uncoupled state that further decreases the NO production.

Superoxide as well as other hyperglycemia- and hypertriglyceridemia-induced reactive oxygen species oxidize polyunsaturated fatty acid (PUFA); damage to these PUFAs interferes with subsequent function of the cells (Spiteller, 2006). Essential PUFAs include omega-6 (n-6) and omega-3 (n-3) fatty acids, both of which are present in cereals, walnuts, algae, fish, shellfish, and plant oils (Margioris, 2009; Russo, 2009). Among the plant sources, n-6 fatty acids are more abundant in soybean, corn, sunflower, safflower oils, whereas n-3 fatty acids are present mainly in linseed and canola oils (Russo, 2009). The parent fatty acid of the n-6 PUFAs is linoleic acid (18:2n-6), which can be elongated and desaturated to form long-chain n-6 PUFA such as arachidonic acid (20:4n-6) that is found in the phospholipids of cell membrane as shown in Figure 2-4 (Margioris, 2009).

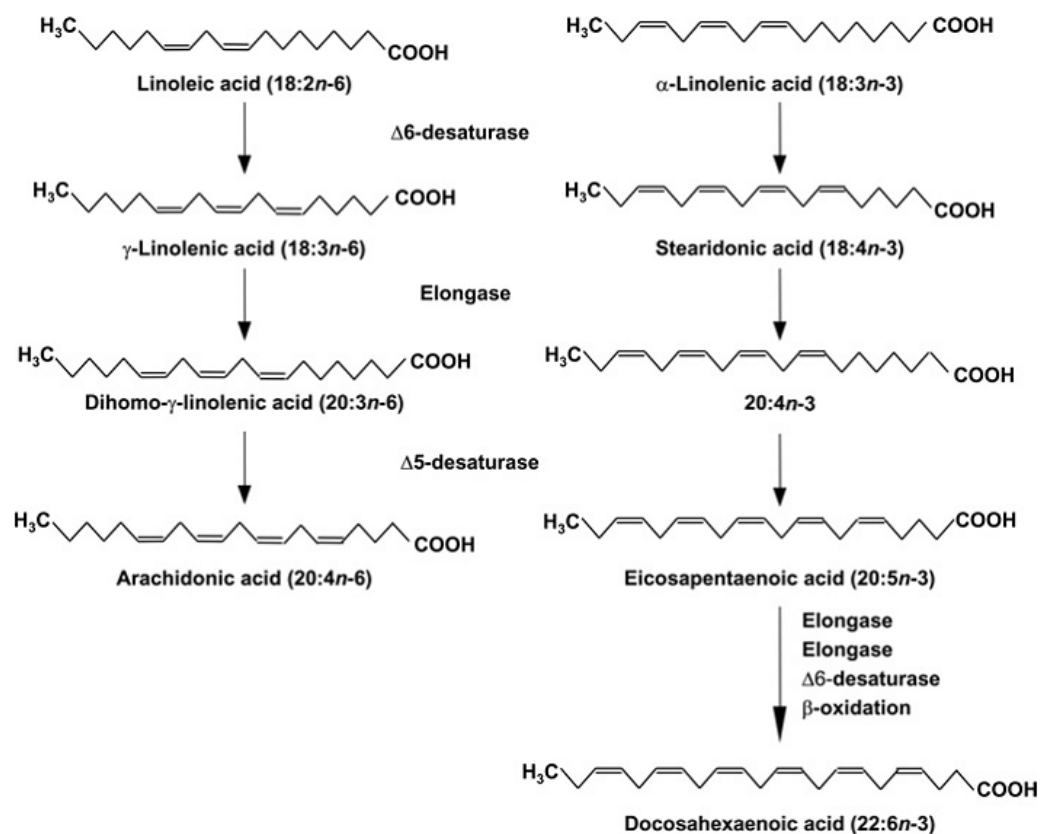


Figure 2-4. The n-6 and n-3 PUFAs (Calder, 2005)

Membrane 20:4n-6 is involved in the lipid peroxidation reaction, which is a radical-mediated chain of reactions directed towards membrane n-6 PUFA. During this process, radicals oxidize 20:4n-6 to form lipid peroxides, which are unstable and can decompose to produce reactive aldehydes, such as malondialdehyde and 4-hydroxynonenal (Figure 2-5).

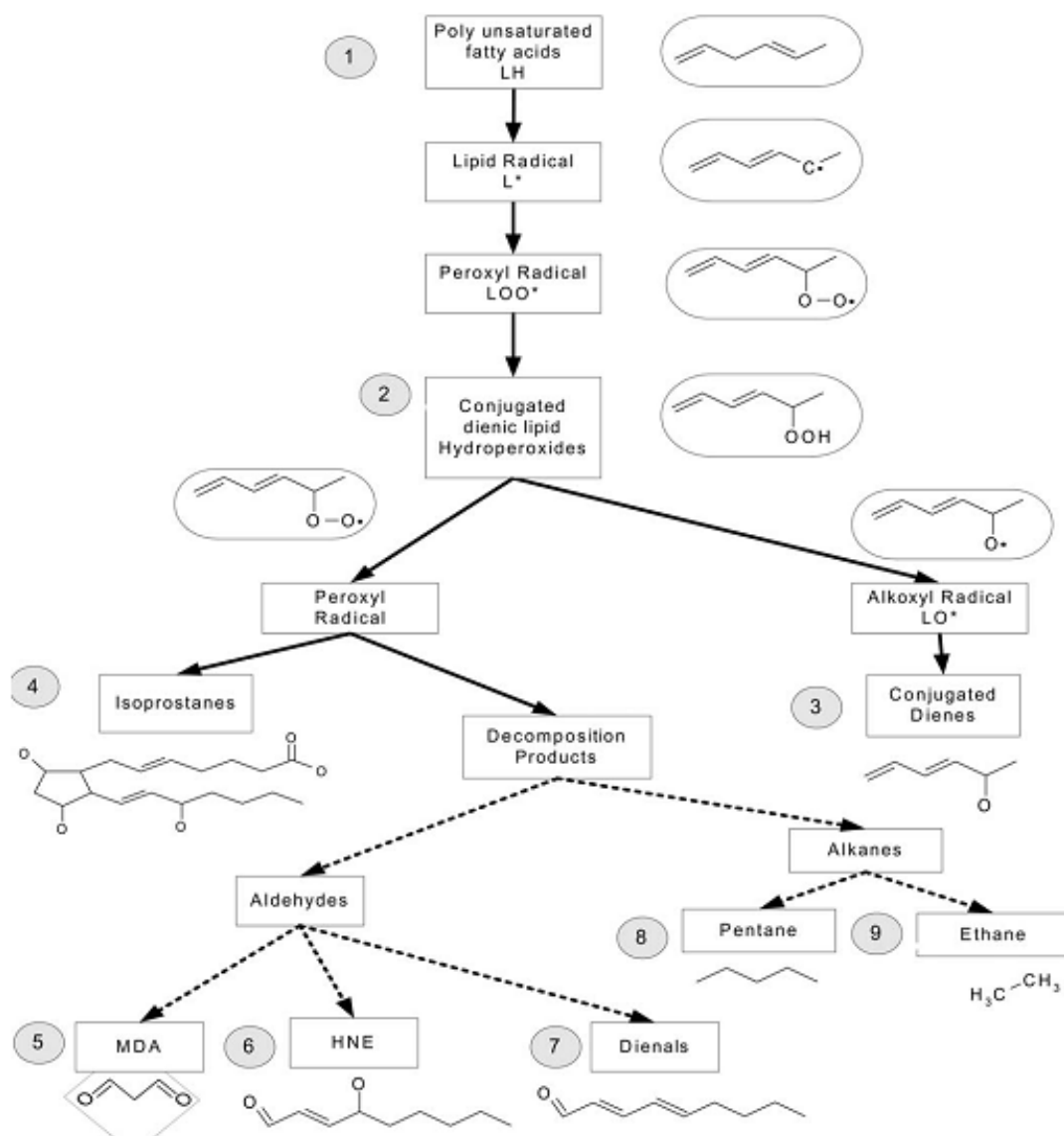


Figure 2-5. Lipid peroxidation process (Dotan, Lichtenberg, & Pinchuk, 2004)

Malondialdehyde, 4-hydroxynonenal, and isoprostanes are cytotoxic aldehydes and can diffuse through membranes and attack targets intracellularly or extracellularly (Esterbauer, Schaur, & Zollner, 1991). Therefore, lipid peroxidation is an amplified oxidative modification of the initial radical attack. In contrast, n-3 PUFAs (Figure 2-4) were shown to suppress oxidative stress by partially displacing 20:4n-6, thus reducing substrate for known mediators of inflammation such as 4-series leukotrienes and

2-series thromboxanes (Calder, 2005). N-3 PUFAs were also shown to increase the production of prostaglandins E₃ that has anti-inflammatory property, and thromboxanes A₃ with low inflammatory, pro-aggregatory and vasoconstrictive properties (Russo, 2009). In addition, n-3 PUFAs were shown to induce NO production and suppress inflammatory indices such as C-reactive protein, soluble intercellular adhesion molecule-1 (sICAM-1), sVCAM-1, E-selectin and etc. (Paschos, et al., 2004; Zhao, et al., 2007).

Increased reactive oxygen species also activate the endothelial cells and induce an inflammatory response through pro-inflammatory cytokine production, such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), interleukin-18 (IL-18) and etc., which activate the production of adhesion molecules, such as sICAM-1 and sVCAM-1, etc. (Esposito, Nappo, Giugliano, Palo, et al., 2003; Plotnick, Corretti, & Vogel, 1997).

Cytokines are small, non-structural proteins that are expressed by nearly every cell under stressful events such as ultraviolet light, heat-shock, etc. (Dinarello, 2000).

Cytokines can be categorized into: pro-inflammatory family, such as TNF- α , IL-1, IL-12, IL-18, and anti-inflammatory family, such as IL-4, IL-10, IL-13 (Tedgui & Mallat, 2006). TNF- α is correlated with the progression of atherosclerosis and serves as a marker for predicting the severity of peripheral arterial disease (Tedgui & Mallat, 2006). IL-1 and IL-18 activate NF- κ B pathway that regulates inflammation through controlling the expression of pro-inflammatory cytokines, adhesion molecules, growth factors, and inducible NO synthase (Tedgui & Mallat, 2006). Adhesion molecules, such as sVCAM-1 and sICAM-1, are immunoglobulins induced by pro-inflammatory

cytokines. They are expressed on activated endothelial cells, macrophages, and vascular smooth muscles; their release from endothelial cell membranes may reflect endothelial activation (Jenny, et al., 2006; Nahrendorf, et al., 2006). Adhesion molecules participate in atherosclerosis lesion formation (Jenny, et al., 2006; Nahrendorf, et al., 2006) and mediate leukocyte attachment to the endothelial cells and their transmigration in the arterial intima.

Measurement of Postprandial Oxidative Stress

Malondialdehyde is one of the most widely accepted and reliable markers for oxidative damage; it is detectable in several biological fluids (plasma, serum, and urine) and tissues. Lipid peroxidation is also accompanied by the formation of conjugated dienes (Figure 2-5), which have also been used as an indicator of oxidative stress, yet the measurement is confounded by the continued *ex vivo* formation of conjugated dienes. In addition, the absorbance (230-235 nm) range used to quantify conjugated diene confounded by interfering substances with similar absorbance range. Biomarkers for measuring DNA damage (single cell gel electrophoresis assay, 5-hydroxymethyl-2'-deoxyuridine measurement) and protein modification (catalase, glutathione peroxidase) have been used in prior research; but these markers many times could be formed by pathways other than reactions with reactive oxygen species, therefore are not specific indicators for oxidative stress (Grotto, et al., 2009). Isoprostane is one of the widely used lipid peroxidation biomarker; it has been quantified by several methods, including gas chromatography-mass spectrometry (GC-MS), and liquid chromatography tandem mass spectrometry (Berdeaux, Scruel,

Cracowski, & Durand, 2006), however, these methods are expensive, time consuming, and not easy to apply to large numbers of samples. Although ELISA was developed as an alternative to GC-MS, results from ELISA showed inconsistencies that may be due to interfering substances in plasma samples (Samuelsson, et al., 1978). Inconsistencies between ELISA and GC-MS were also observed in prior research (Proudfoot, et al., 1999). Before performing the ELISA, biological samples may be purified by affinity column or solid phase extraction cartridge C-18. If plasma samples are analyzed, there is an additional shortcoming: the generation of artifactual isoprostanes by autoxidation of plasma 20:4n-6 if the sample was not handled properly (Roberts & Morrow, 2000).

Malondialdehyde is a three-carbon, low-molecular weight aldehyde (Figure 2-6) and it is a secondary breakdown product from lipid peroxide during lipid peroxidation (Armstrong & Browne, 1994; Dahle, Hill, & Holman, 1962; Yagi, 1998).

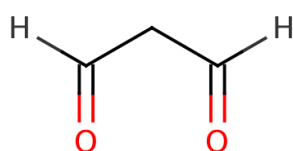


Figure 2-6. The structure of malondialdehyde

Malondialdehyde has been recognized as an important indicator of lipid peroxidation due to its mutagenic and genotoxic characteristics that are involved in cancer, heart disease and many other diseases. Cirak et al. (2003) suggested that serum and tissue malondialdehyde levels were higher in malignant brain tumors than healthy controls. Scott et al (2003) showed that plasma malondialdehyde was elevated in hemodialysis

patients with cardiovascular complications. Kesavulu et al. (2001) found that malondialdehyde was higher in type 2 diabetics than in healthy controls; in addition, malondialdehyde was even higher among diabetic patients with heart disease than those diabetics without heart disease.

Malondialdehyde molecules are unstable, polar and water-soluble, therefore they are difficult to extract. Also they contains no electrophore, chromophore or fluorophore that would aid in detection (Grotto, et al., 2009). TBARS assay is a well-established method for screening and monitoring malondialdehyde in plasma, serum, tissues and urine (Armstrong & Browne, 1994; Grotto, et al., 2009; Yagi, 1998). The assay results can be read colorimetrically (532-535 nm) or fluorimetrically. Normal human plasma contains 1.86-3.94 $\mu\text{mol/L}$ TBARS (Richard, et al., 1992; Yagi, 1998). Tinahones et al. (2008) found that postprandial plasma TBARS value was significantly higher at 3 hours post ingestion of a high-fat meal ($1.31 \pm 0.72 \mu\text{mol/L}$) compared to baseline ($1.02 \pm 0.7 \mu\text{mol/L}$) in severely obese subjects ($n=29$, 9 males and 20 females, ages 44.9 ± 8.6 y, BMI $54.5 \pm 6.4 \text{ kg/m}^2$). Yesilova et al. (2005) found that fasting plasma TBARS value was significantly higher in patients with nonalcoholic fatty liver disease ($0.5 \pm 0.2 \mu\text{mol/L}$, $n=51$, male, ages 20-65 y, BMI $28.12 \pm 4.06 \text{ kg/m}^2$) compared to 30 healthy controls ($0.36 \pm 0.09 \mu\text{mol/L}$, $n=30$, male, ages 20-60 y, BMI $27.54 \pm 3.27 \text{ kg/m}^2$). Oteiza (1997) found that fasting plasma TBARS value was significantly higher in patients with sporadic amyotrophic lateral sclerosis ($1.30 \pm 0.1 \mu\text{mol/L}$, $n=13$, 6 males and 7 females, ages 22-66 y) compared to health controls ($1.03 \pm 0.06 \mu\text{mol/L}$, $n=11$, 8 males and 3 females, ages 32-66 y). Chalasani et al. (2004) found that fasting

plasma TBARS value was significantly increased in patients with nonalcoholic steatohepatitis ($3.4 \pm 1.3 \mu\text{mol/L}$, $n=21$, 11 males and 10 females, ages 41 ± 13 y, BMI $33 \pm 4 \text{ kg/m}^2$) compared to healthy controls ($1.8 \pm 0.9 \mu\text{mol/L}$, $n=19$, 9 males and 10 females, ages 43 ± 14 y, BMI $31 \pm 4 \text{ kg/m}^2$). There is a controversy cited in the literature regarding the lack of sensitivity and specificity of TBARS (Armstrong & Browne, 1994), because thiobarbituric acid reacts with other compounds in the sample, such as sucrose, amino acids and albumin, thus interfering by overlapping with the absorbance of malondialdehyde (Grotto, et al., 2009) resulting in an overestimate of lipid peroxidation.

Influence of Single High-fat Meal Consumption on Endothelial Function

The endothelial function can be impaired by long term consumption of saturated fat-rich meal. Barringer et al. (2008) found that 4-week of high-fat meals led to decreased reactive hyperemia index 3 hours post ingestion in 23 healthy subjects (-12.37% , $p=0.005$), but no decline when supplemented with 250 mg flavonoid daily (-3.16% , $p=0.663$). This was a double-blind, crossover treatment design; 23 healthy subjects were involved (mean age 43.4 ± 10.4 , 78% female, BMI not listed). Each subject was assigned in random sequence, to receive a high-fat meal with additional flavonoid supplement (for 1 week) or a high-fat meal with placebo (for 1 week). Between the two study periods, there was a 2-week washout period to minimize the carryover effect. After the washout period, subjects received the other treatment. The high-fat meal provided 1010 kilocalories (49.9% from fat), including 56 g total fat (15 g saturated fat and 225 mg cholesterol). The meal consisted of one cheese omelet (Jimmy

Dean[®] Three Cheese Omelet[™]), one croissant (Jimmy Dean[®] Croissant with Egg and Cheese), 4.75 oz of French fries (Ore-Ida[®] Extra Crispy Fast Food Fries[™]), and one bottle of water.

Similar endothelial impairment was observed by Cuevas et al. (2000) and Plotnick et al. (2003). Cuevas et al. (2000) showed that flow-mediated dilation was impaired ($p=0.014$) in 11 healthy subjects (male, ages 20-28 y, BMI 20-25 kg/m²) by 4-week of a high-saturated fat diet compared to a control diet with antioxidant enriched; however, after 4-week supplementation of red wine (240 mL/day), flow-mediated dilation was raised to a level comparable to the control diet. The high-fat diet provided an average of 2565 kilocalories daily (39.9% from fat), including 112.7±12.9 g total fat (35.8±9.6 g saturated fat, 35.3±4.2 g monounsaturated fat, 32±4.5 g polyunsaturated fat, 0.12±0.04 g very long-chain omega-3 fatty acids, and 610±120 mg cholesterol). The control diet also provided 2565 kilocalories (27.3% from fat) but contained 77±2.6 g total fat (22.7±2.3 g saturated fat, 37.9±2.5 g monounsaturated fat, 9.6±1.4 g polyunsaturated fat, 0.38±0.13 g very long-chain omega-3 fatty acids, and 290±90 mg cholesterol) with 3-fold higher fruit and vegetable intake (675 g) than the high-fat diet. Plotnick et al. (2003) found that flow-mediated brachial artery reactivity was significantly reduced in 38 healthy subjects (14 males and 24 females, ages 36.4±10.1 y, BMI not listed) after 4 weeks of high-fat meals ($p<0.05$), but was not impaired when the high-fat meal was supplemented with the fruit/vegetable juice concentrate. In this double-blind design, subjects received 3 test meals (high-fat meal with placebo, high-fat meal with 4 capsules of Juice Plus, and high-fat meal with 4 capsules of Juice Plus and 4 capsules of

Vineyard) in a random sequence; Juice Plus was a powdered fruit vegetable juice concentrate and Vineyard was a supplement of antioxidants with herbal extracts. The high-fat meal provided 897 kilocalories (50.2% from fat) and contained 50 g total fat (14 g saturated fat and 225 mg cholesterol). It consisted of one Egg McMuffin[®], Sausage McMuffin[®], two hash brown patties (McDonald's Corporation), and one non-caffeinated beverage.

Besides long term consumption, an acute consumption of saturated fat-rich meal also led to endothelial dysfunction. A study by Padilla et al. (2006) showed that brachial artery flow-mediated dilation was significantly reduced between 2 and 6 hours post ingestion in 8 healthy subjects (5 males and 3 females, ages 25.5±0.8 y, BMI not listed). The high-fat meal consisted of one Egg McMuffin[®], one Sausage McMuffin[®], two hash brown patties (McDonald's Corporation), and water. The nutrient composition was reported from company literature as providing 940 kilocalories and containing 48 g fat (16.5 g saturated fat, 4.5 g trans fat and 280 mg cholesterol), 91 g carbohydrates and 33 g proteins. This result agreed with early studies by Vogel (1997) and Plotnick (1997) using similar high-fat meals (McDonald's Corporation) providing 900 kilocalories, 50 g fat, 14 g saturated fat and 255 mg cholesterol.

It has been shown that the endothelial cells were activated after acute consumption of a saturated fat-enriched meal. Nappo et al. (2002) proposed that the postprandial hyperlipidemia and hyperglycemia significantly increased the endothelial activation by elevating TNF- α , IL-6, sICAM-1 and sVCAM-1 at 0, 2, and 4 hours in both diabetic patients (10 males and 10 females, ages 46±5 y, BMI 27.5±1.3 kg/m²) and healthy

controls (10 males and 10 females, ages 44 ± 5 y, BMI 26.8 ± 1.2 kg/m²). The increase of cytokines and adhesion molecules sustained longer following the high-fat meal than the high-carbohydrate meal. The high-fat meal provided 760 kilocalories (59.2% from fat) and contained 58 g carbohydrate, 2.8 g fiber, and 50 g fat (20.4 g saturated fat and 246 mg cholesterol); it consisted of two sausages (80 g), six bread slices (90 g), one small egg (40 g), butter (15 g) and olive oil (5 g). The isocaloric (20.6% of total calories from fat) high-carbohydrate meal contained 144 g carbohydrate, 4.5 g fiber, and 17 g fat (2.2 g saturated fat and no cholesterol); it consisted of one cheese-free pizza (300 g) with tomatoes (60 g). A study by Esposito et al. (2003) found that postprandial plasma triglyceride concentration was correlated with the elevated platelet aggregation 4 hours after high-fat meal ingestion ($r=-0.23$, $p<0.05$) in 25 healthy subjects (13 males and 12 females, ages 23-40 y, BMI 23.7 ± 1.9). The high-fat meal provided 760 kilocalories (59.2% from fat) and contained 50 g fat (20.4 g saturated fat and 246 mg cholesterol), 58 g carbohydrate and 2.8 g fiber; it consisted of two sausages, six bread slices, one small egg, butter and olive oil).

Similar to the effect of saturated fat, a high dose of monounsaturated fat (18:1n-9) was shown to reduce endothelial-dependent vasodilation and increase oxidative stress. Berry et al. (2008) found that the brachial artery flow-mediated dilation was decreased ($p<0.001$) while the plasma 15-F_{2t}-isoprostane (+10.4 ng/L; $p=0.005$) and the plasma triglyceride were significantly increased 3 hours after the 18:1n-9 enriched meal in 17 healthy subjects (male, ages 18-40 y, BMI 24.3 ± 3.0 kg/m²). The 18:1n-9 enriched meal provided 850 kilocalories and contained 15 g protein, 89 g carbohydrate and 43.3 g fat

(42.5 g 18:1n-9, 4 g 18:2n-6, and 0.8 g stearic acid from sunflower oil); it consisted of two muffins and one chocolate flavored sweetened milkshake.

A mix of monounsaturated fat and saturated fat was also shown to increase oxidative stress. Raff et al. (2008) found that 5-week consumption of a high-fat meal increased urinary 15-F_{2t}-isoprostane concentration (+1.47 ng/mL, $p < 0.001$) in the experimental group (18 male subjects, ages 20-35 y, BMI 19.2-26.7 kg/m²) compared to the control group (20 male subjects, ages 19-33 y, BMI 18.8-27 kg/m²). The meal provided 1642 kilocalorie (60% from fat) and contained 115 g fat, including 14:0 (11.2 g), 16:0 (32.2 g), 18:0 (6.44 g), 18:1n-9 (14.03 g), and 18:2n-6 (6 g); it consisted of two bread rolls, one piece of cake, a cup of chocolate milk, and butter.

Epidemiological data suggested that essential fatty acids, n-6 and n-3 PUFAs, had protective effect on cardiovascular health (Hu, et al., 1997; Mozaffarian, et al., 2005). Nestel et al. (1997) found that arterial compliance was enhanced after 4-week supplementation of 18:3n-3 (providing 9% of total energy) in 15 obese subjects (BMI 25-36 kg/m², ages < 65 y), although oxidative stress was significantly increased that was indicated by elevated TBARS in oxidized LDL. Tholstrup et al. (2004) found that 3 weeks of fish oil supplemented high-fat meal did not increase the plasma and urinary 15-F_{2t}-isoprostane concentration compared to high-oleic acid meal in 16 healthy subjects (male, ages 35-75 y, BMI 21.9-32.5 kg/m²). The high-oleic acid meal provided 1523.8 kilocalories (40% from fat) and contained 80 g fat, including 16:0 (7.4 g), 18:0 (11.9 g), 18:1n-9 (34.1 g) and 18:2n-6 (22.9 g). The isocaloric fish oil meal replaced 5% of the oleic acid with PUFA (20:5n-3, 22:5n-3 and 22:6n-3); it consisted of one bun, one

piece of cake, one small package of spread, and a ready-made dinner. Romieu et al. (2008) found that fish oil (2 capsules/day, each containing 83.2% n-3 PUFA) or soy oil (2 capsules/day, each containing 52.7% 18:2n-6, 22.5% 18:1n-9 and 16.3% saturated fat) led to increased Cu/Zn superoxide dismutase activity and increased plasma glutathione level in 52 subjects (42 males, ages 60-96 y, BMI 16.6-37.2).

Dietary n-6 PUFAs were considered to induce oxidative stress and inflammatory effects, because dietary 18:2n-6 was the precursor of membrane 20:4n-6 that was involved in lipid peroxidation (Griffin, 2008). Nevertheless, a meta-analysis by Mensink et al. (2003) suggested that 18:2n-6 had the most potent effect on lowering plasma LDL, triglycerides, and the ratio of total to HDL cholesterol. The mechanism was proposed as 18:2n-6 reduced LDL production and enhanced hepatic receptor-dependent LDL clearance (Spady & Dietschy, 1988; Woollett, Spady, & Dietschy, 1992). Prior human studies found that high 18:2n-6 diet, providing 16-29% of total energy, was shown to significantly lower plasma LDL by 16%-22% compared to high saturated fat diet, providing 19%-30% of total energy (J. Anderson, Grande, & Keys, 1976; Baudet, Dachet, Lasserre, Esteva, & Jacotot, 1984; Becker, Illingworth, Alaupovic, Connor, & Sundberg, 1983; Hegsted, McGandy, Myers, & Stare, 1965). Sanders et al. (2006) found that a n-6:n-3 ratio of 3:1 reduced both fasting and postprandial plasma triglyceride among 258 subjects (ages 45-70 y, BMI 20-35 kg/m²). Long term dietary treatments (6% energy from polyunsaturated fat, 11-13% from saturated fat, 11-15% from monounsaturated fat) with different n-6 to n-3 ratio (5:1, 3:1, and 10:1) was applied in this study and neither treatment significantly alter hemostatic

risk factors, such as fibrinogen, factor VIIc coagulant activity, activated factor VII, factor XII and postprandial activated factor XII.

Influence of Two High-fat Meal Consumption on Endothelial Function

Oxidized LDL and malondialdehyde (as measured by the TBARS assay with an additional alkaline hydrolysis step) were used to indicate oxidative stress by Tushuizen et al. (2006); however, the specificity and sensitivity of the testing methods and the biomarkers themselves were highly controversial. Tushuizen et al. (2006) showed that two consecutive high-fat meals, rich in saturated fat, significantly impaired flow-mediated dilation at hour 6 in 17 healthy subjects (male, ages 25.4 ± 3 y, BMI 23.6 ± 2 kg/m²). The plasma malondialdehyde ($p=0.01$) and the oxLDL/LDL cholesterol ratio ($p=0.001$) were increased at hour 6. Each high-fat meal provided 900 kilocalories (50% from fat) and contained 55 g carbohydrate, 30 g protein, and 50 g fat (30 g saturated fat). The first high-fat meal (at 8:30 am) consisted of one EggMcMuffin[®] (McDonald's), one croissant with butter and marmalade, 200 mL milk with 20 mL cream. The second high-fat meal (at 12:30 pm) consisted of one Quarterpounder[®] (McDonald's), one croissant with butter, and 200 mL milk.

N-3 polyunsaturated fat was shown to have a better protective effect on arterial stiffness in comparison to n-9 monounsaturated fat. Hall et al. (2008) found that an oleic acid (46% 18:1n-9) enriched meal impaired arterial compliance by increasing digital volume pulse-stiffness index, while an eicosapentaenoic acid (EPA)-enriched meal (39% 18:1n-9, 6% EPA and DHA) did not. In fact, the EPA (20:5n-3) enriched meal decreased stiffness index 3 hours post ingestion. The plasma NOx (nitrate and

nitrite) decreased significantly at 6 hours, indicating decreased NO production; the plasma 15-F_{2t}-isoprostane increased by 48% at 6 hours; however these changes may result from the ingestion of the second meal rich in saturated fat. In this single-blind crossover design, 17 healthy subject (male, ages 18-35 y, BMI 20-32 kg/m²) randomly received an EPA-enriched meal or a high-oleic acid meal followed by a second high-fat meal, in random sequence, on two separate study periods with 1-week washout period. The EPA-enriched meal provided 844.8 kilocalorie (54.7% from fat) and contained 51.3 g fat (4.7 g EPA, 0.7 g DHA, 36.6 g 18:1n-9, 1.1 g 16:0, 4.3 g 18:2n-6), 86.7 g carbohydrate, and 15.2 g protein; it consisted of two muffins and one milkshake. The isocaloric 18:1n-9 enriched meal differed only in the fatty acid composition: 43.6 g 18:1n-9, 1.3 g 16:0, and 5.1 g 18:2n-6. The second high-fat meal provided 658.6 kilocalorie and contained 43.7 g fat (25.6 g saturated fat), 46 g carbohydrate and 21 g protein.

Influence of Feeding Meal by Body Surface Area on Endothelial Function

Body surface area is the calculated surface of a human body. One recommended formula is the Mosteller formula (Mosteller, 1987)

$$BSA(m^2) = \sqrt{\frac{weight (kg) \times height (cm)}{3600}}$$

For example, for a person with 60 kg body weight and 168 cm height, his/her body surface area is 1.67 m².

Some investigators have fed the high-fat meal based on individual body surface area. Giannattasio et al. (2005) fed subjects 680 kilocalories/m². Results showed that the radial artery diameter and blood flow were markedly impaired (p<0.05) at 6 hours

in 16 subjects (ages 46.6 ± 2.5 y) with untreated mild hypertriglyceridemia and dyslipidemia, but were not significantly altered in 7 normotriglyceridemic subjects (ages 35.1 ± 2.4 y). Subjects were provided with the same oral high-fat load (83% fat, 5% proteins, 12% carbohydrates); food sources were not mentioned. Shimabukuro (2007) found that an acute high-fat meal (30 g fat/m^2) decreased the forearm blood flow ($p < 0.0001$) 2 and 4 hours post ingestion in 12 healthy subjects (6 males and 6 females, ages 30-43 y, BMI $23.3 \pm 0.9 \text{ kg/m}^2$), while neither an acute high-carbohydrate nor a standard meal had significant impact. In this crossover design, subjects received a high-fat meal, a high-carbohydrate meal or a standard meal in random order on different days separated with 1-week washout period. The high-fat meal contained 342 kilocalories/100 g meal (35% was from fat) and provided 1239-1332 kilocalories. The high-carbohydrate meal provided 300 kilocalories (100% from carbohydrate). The standardized meal provided 478 kilocalories (32.7% fat, 50.4% carbohydrate, 16.7% protein). A study by Muntwyler et al. (2001) did not find the high-fat meal ($700 \text{ kilocalorie/m}^2$, 83% from fat, 14% from carbohydrate, 3% from protein) have any significant impact on forearm blood flow at hour 4 although plasma triglyceride level was significantly elevated in 12 healthy subjects (male, ages 29.5 ± 1.5 y, BMI $23.2 \pm 1.2 \text{ kg/m}^2$). Here, the high-fat meal consisted of 180 mL dairy cream, 2 g lean milk powder, 20 mg sucrose (possibly a typographic error) and 10 g chocolate powder. The limitation is that this study did not specify the fatty acid composition, thus it is hard to compare with other postprandial studies; in addition, the test meal contained chocolate powder which might serve as an antioxidant source.

Meal feeding based on body weight has also been studied. Westphal et al. (2006) showed how protein-supplemented high-fat meal affected flow-mediated dilation in 16 healthy students (8 males and 8 females, ages 19-23 y, BMI 18.5-25 kg/m²). Results showed that the average flow-mediated dilation was reduced by 58% after a high-fat meal, however, not significantly altered when additional caseinate protein or soy protein was supplemented. This was a crossover design; each subject received three test meals in random sequence with 1-week washout period. One meal consisted of whipping cream and was given based on body weight (3 mL/kg body weight). For example for a subject with 60 kg body weight, the high-fat meal provided 577.8 kilocalories (92.5% from fat) and contained 59.4 g fat (32.8 g saturated fat and 16.3 g monounsaturated fat). The protein-rich meals provided an additional 50 g sodium caseinate or 50 g soy protein.

Summary

The literature reviewed provides a general background of high-fat meal induced postprandial oxidative stress and endothelial function change. Prior research primarily focused on the impact of single high-fat meal, yet overlooked the repeated eating and snacking pattern that prolonged the postprandial oxidative stress. In addition, literature regarding different feeding regimens (standard meal, variable sized meal based on body surface area and based on body weight) does not provide substantial evidence to indicate which approach is better at lowering the unexplained variance. Therefore, study is needed to compare the effects of different feeding regimens on oxidative stress and endothelial function. To support the optimization of the feeding model, saturated

fat-rich diet is used as the source of oxidative stress to elicit potent postprandial effects since saturated fat has been extensively studied for its deleterious effect on LDL, triglyceride, oxidative stress and endothelial function. Another additional emphasis needed is the application of EndoPAT system, which is an emerging substitution for flow-mediated dilation for endothelial function assessment in the research area.

Chapter 3

PROCEDURES

The focus of the research was to compare the effect of two feeding regimens on endothelial function assessed by reactive hyperemia index. The conduct of the study included the following organizational steps: (a) design of the study; (b) selection of subjects; (c) arrangements for conducting the study; (d) test procedures; (e) treatment of data.

Design of the Study

A two factor (2×4) Within Subject Design was selected, as shown in Table 3-1. The independent variables were feeding regimen (A), with two levels: feeding regimen 1 (two standard meals: two muffins and one milk drink per person as “breakfast” at 0h and as “lunch” at 4h) and feeding regimen 2 (two variable sized meals based on individual body surface area: muffins and milk drinks for 460 kilocalorie/m² each as “breakfast” at 0h and as “lunch” at 4h), and time (B) with four levels: 0, 2, 4, 6 hour. The dependent variables measured were reactive hyperemia index and plasma malondialdehyde (TBARS). Each subject completed two test days separated by a

1-week washout period; each test day started at 8:00 am and lasted 6-7 hours.

Table 3-1. ANOVA table of Within Subject Design¹ (Keppel, 2004d)

Source	df	SS	Bracket term
A	a - 1	[A] - [T]	$[A] = \frac{\sum A_j^2}{bn}$
B	b - 1	[B] - [T]	$[B] = \frac{\sum B_k^2}{an}$
A × B	(a - 1)(b - 1)	[AB] - [A] - [B] + [T]	$[AB] = \frac{\sum AB_{jk}^2}{n}$
S	n - 1	[S] - [T]	$[S] = \frac{\sum S_i^2}{ab}$
A × S	(a - 1)(n - 1)	[AS] - [A] - [S] + [T]	$[AS] = \frac{\sum SA_{ij}^2}{b}$
B × S	(b - 1)(n - 1)	[BS] - [B] - [S] + [T]	$[BS] = \frac{\sum SB_{ik}^2}{a}$
A × B × S	(a - 1)(b - 1) × (n - 1)	[Y] - [AB] - [AS] - [BS] + [A] + [B] + [S] - [T]	$[Y] = \sum Y^2$
Total	abn - 1	[Y] - [T]	$[T] = \frac{T^2}{abn}$

¹ A, feeding regimen; B, time; S, subject; Y, values of reactive hyperemia index; T, the grand sum, sum of all reactive hyperemia index scores.

Prior to the test day, subjects' height and weight were recorded. Individual body surface area was calculated by the following formula (Mosteller, 1987):

$$BSA(m^2) = \sqrt{\frac{weight (kg) \times height (cm)}{3600}}$$

Twenty-four hours prior to each test day, subjects were asked to exclude caffeine, alcohol, tobacco, multiple vitamin supplements and any other supplements; subjects were also asked to refrain from working out or other physical activity. Subjects were also required to undergo a fasting period of at least 12 hours. On the test day, subjects were asked if they had followed each of the directions (Table 3-2).

Table 3-2. Test day questionnaire

Did you consume any of the following substances in the past 24 hours?

- Caffeine containing drinks
- Alcohol
- Tobacco
- Multiple vitamin supplements
- Other supplements

2. Did you exercise during the past 24 hours?

3. Did you eat anything during the past 12 hours?

Subjects were assigned to the two feeding regimens in random order. Each feeding regimen contained two meals that were consumed at time point 0 and 4 hours of the test day (Figure 3-1). Each meal in feeding regimen 1 provided 850 kilocalories; whereas, each meal in feeding regimen 2 provided 460 kilocalorie/m² body surface area.

Subjects were given 15 minutes to finish each meal. Reactive hyperemia index was measured after the meal, and followed by blood draw at 0, 2, 4, 6 hours (Figure 3-1); 10 mL of blood was drawn each time (a total amount of 40 mL blood was drawn on each test day). Blood was frozen at -80°C and later analyzed for plasma malondialdehyde concentration. Blood glucose and triglyceride concentration were measured by finger stick at 0, 1, 2, 3, 4, 5, 6 hours to monitor the postprandial state.

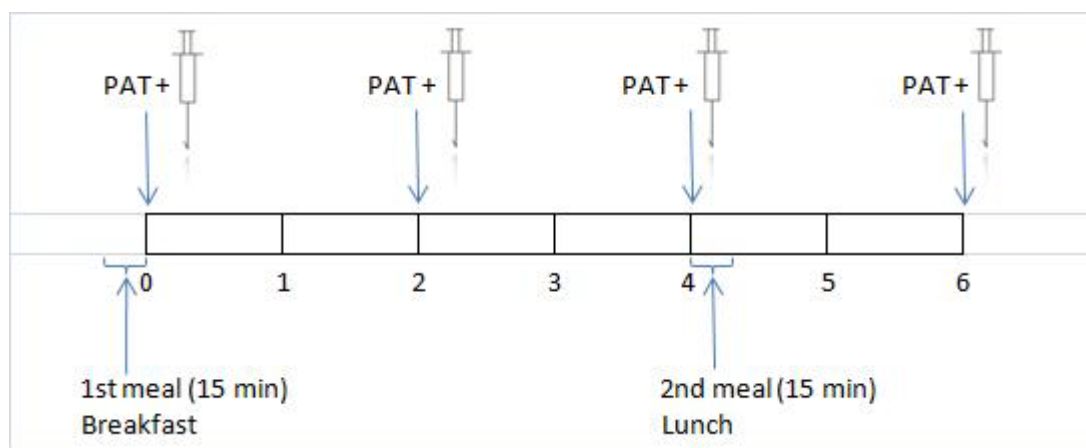


Figure 3-1. Timeline of the test procedure each day

Selection of Subjects

Subjects were selected using the following criteria:

- Male
- age 21-26 years
- healthy BMI (18.5-24.9 kg/m²)
- body surface area ranging 1.5-2.2 m² (Table 3-3).

Table 3-3. Reference for body surface area

Height (inches)	Weight (lb)	BMI (kg/m ²)	Body surface area (m ²)
65	149.5	24.9	1.76
65	111.0	18.5	1.52
70	173.5	24.9	1.97
70	129.0	18.5	1.70
75	199.0	24.9	2.19
75	148.0	18.5	1.89

Subjects were excluded using the following criteria:

- history of coronary artery disease, cerebrovascular disease, heart failure, diabetes mellitus, hepatic and renal disease, uncontrolled hypertension, dyslipidemia, and evident thyroid disease (Barringer, et al., 2008)
- tobacco use (Shimabukuro, et al., 2007)
- use of regular medication, including aspirin, antiplatelet drugs, anti-inflammatory drugs, lipid-lowering drugs and blood pressure medication; similar criteria was used before by Hall et al. (2008) and Nappo et al. (2002).

Health history was obtained through a questionnaire (Table 3-4).

Table 3-4. Screening questionnaire

1. What is the month and year of your birth?
 2. How often do you smoke?
 3. How often do you exercise?
 4. Do you have a history of _____?
 - Heart disease
 - Stroke
 - Diabetes mellitus
 - Uncontrolled hypertension
 - Thyroid disease
 - Dyslipidemia
 - elevation of plasma total cholesterol, LDL, or triglycerides
 - low level of HDL
 - Liver diseases
 - Kidney diseases
 5. Are you currently taking any of the following medications regularly?
 - Aspirin
 - Anti-platelet drugs
 - Anti-inflammatory drugs
 - Lipid-lowering drugs
 - Blood pressure medication
 6. Do you take supplements, such as vitamin and minerals etc.? If yes, how often?
 7. Do you have an allergy or intolerance to milk or wheat?
-

These criteria were selected to improve the external validity and allow the results to be generalized to a population of healthy male ages 21-26 years. A male population was selected because endothelial responses have been shown to vary between genders, therefore should be separately investigated during development of a model. The restrictions on age, BMI, cardiovascular disease history and smoking habit were imposed to reduce factors known to alter vascular function, and thus add power to the analysis of the experimental data by reducing the unexplained variance.

Arrangements for Conducting the Study

The study was approved by the Indiana University Institutional Review Board (#090800057). IRB approval and study forms are found in Appendix A. The study was conducted in a temperature-controlled laboratory. Subjects were informed of the test procedures as well as potential risks and signed a consent form. Subjects were compensated with \$25.00 cash, results of their fasting blood glucose and triglyceride concentration, and a handout on dietary guidance.

Test Procedures

Feeding regimens

The meal consisted of muffins and a milk drink. Nutrition Data System for Research software (Minneapolis, MN) was used to analyze the composition of the meal. Each muffin was formulated to provide 321 kilocalories and contained 21.1 g fat. Each milk drink provided 208 kilocalories and contained 8.5 g fat. The nutrient composition is shown in Table 3-5 and 3-6.

Table 3-5. Muffin nutrients composition¹

Nutrient	Quantity	Calories (%)
Energy, kcal	321.0	
Total fat, g	21.1	58.1
Saturated fatty acids, g	5.3	14.5
Monounsaturated fatty acids, g	9.4	25.7
Polyunsaturated fatty acids, g	5.5	15.2
Trans fatty acids, g	3.6	
Omega-3 fatty acids, g	0.3	
Cholesterol, mg	0.0	
Total carbohydrate, g	30.1	37.3
Total protein, g	3.3	4.3
Vitamins		
Vitamin A (Retinol), µg	12.0	
Vitamin D (calciferol), µg	0.2	
Vitamin E (α-tocopherol), mg	0.8	
Vitamin C (ascorbic acid), mg	0.0	
Vitamin K, µg	9.1	
Thiamin (vitamin B ₁), mg	0.1	
Riboflavin (vitamin B ₂), mg	0.1	
Niacin (vitamin B ₃), mg	1.7	
Vitamin B ₆ , mg	0.0	
Vitamin B ₁₂ , µg	0.1	
Folate, µg	21.0	

¹ per one muffin.

Table 3-6. Milk drink nutrients composition¹

Nutrient	Quantity	Calories (%)
Energy, kcal	208.0	
Total fat, g	8.5	36.2
Saturated fatty acids, g	2.5	10.5
Monounsaturated fatty acids, g	3.6	15.3
Polyunsaturated fatty acids, g	2.1	8.9
Trans fatty acids, g	1.4	
Omega-3 fatty acids, g	0.1	
Cholesterol, mg	5.0	
Total carbohydrate, g	24.8	47.0
Total protein, g	8.4	17.1
Vitamins		
Vitamin A (Retinol), µg	139.0	
Vitamin D (calciferol), µg	2.3	
Vitamin E (α-tocopherol), mg	0.3	
Vitamin C (ascorbic acid), mg	4.1	
Vitamin K, µg	3.6	
Thiamin (vitamin B ₁), mg	0.1	
Riboflavin (vitamin B ₂), mg	0.4	
Niacin (vitamin B ₃), mg	1.9	
Vitamin B ₆ , mg	0.2	
Vitamin B ₁₂ , µg	1.2	
Folate, µg	12.0	

¹ per 249 g milk drink.

Each meal in feeding regimen 1 consisted of two muffins and one milk drink per subject and contained 850 total kilocalories, 55 g fat (4 g loss in the muffin tins), 15 g protein, and 85 g carbohydrate. Each meal in feeding regimen 2 consisted of muffins and milk drink in a quantity that supplied 460 kilocalories/m² individual body surface area. For a subject with a body surface area of 1.85 m² (the average between 1.5-2.2 m² range), he consumed the same calories (850 kilocalories) in each meal in both feeding regimens. For a subject with a body surface area of 2.2 m², the meal quantity of feeding regimen 2 was determined by the following calculations:

- $460 \text{ kilocalories} \times 2.2 \text{ m}^2 = 1012 \text{ kilocalories}$

The subject consumed 1012 kilocalories in each meal in feeding regimen 2

- $$\begin{cases} 321a + 208b = 1012 \\ a = 2b \end{cases}$$

a, the quantity of muffin; b, the quantity of the milk drink

The above equation was solved and the muffin : milk drink ratio was 2.4 : 1.2, therefore 2.4 muffins and 1.2 milk drink were served.

A sample calculation of different body surface area meal is shown in Table 3-7.

Table 3-7. Sample reference for feeding meal based on body surface area

Body surface area (m ²)	Calories based on BSA (kilocalories)	Ratio of muffin to milk drink
1.52	699	1.64 : 0.82
1.70	782	1.84 : 0.92
1.76	811	1.90 : 0.95
1.89	867	2.04 : 1.02
1.97	907	2.14 : 1.07
2.19	1006	2.36 : 1.18

The muffin recipe was modified from previous studies in the literature (Berry, et al., 2008; Hall, et al., 2008). The dry ingredients (Table 3-8) were weighed using an electronic balance (Model #XS2002S Mettler-Toledo Inc., Columbus, OH). All dry ingredients were stirred into a large bowl using a large spoon and a spatula and were sifted twice using a sifter. Vanilla extract was measured with a 25 mL graduated cylinder. Water was measured with a 100 mL graduated cylinder. Skim milk was measured by a 250 mL graduated cylinder. The vegetable shortening was weighed in a pan, melted, and added to other ingredients. Dry and wet ingredients were stirred in a mixing bowl for 2 minutes. Batter was weighed into 40 individual foil muffin cups (78 g/cup) in muffin tins (75 mL). Muffin tins were baked in two preheated fan-assisted ovens at 400°F for 18 minutes. The recipe yielded 40 muffins. Muffins were cooled, covered with plastic wrap, put into large freezer storage plastic bags and frozen until needed for the study.

Table 3-8. Ingredients for muffins¹

Ingredients	Quantity	Percent Weight (%)
Cornstarch ² , g	187	6.0
Pastry flour ³ , g	550	17.6
Granulated sugar ⁴ , g	550	17.6
Baking powder ⁵ , g	38.5	1.2
Dried pasteurized egg white ⁶ , g	77	2.5
Skim milk ⁷ , mL	737	24.2
Vegetable shortening ⁸ , g	920	29.5
Pure vanilla extract ⁹ , mL	19	0.6
Water, mL	58	1.8
Total, g	3120	100

¹ Each muffin is made with 78 g batter.

² Cornstarch, Kroger Co., Cincinnati, OH.

³ Pastry Flour #76353, Bloomingfoods, Bloomington, IN.

⁴ Value Granulated sugar, Kroger Co., Cincinnati, OH.

⁵ Baking Powder, Kroger Co., Cincinnati, OH.

⁶ Just Whites, Deb-El Foods Co., Papetti Plaza, Elizabeth, NJ.

⁷ Skim Milk, Kroger Co., Cincinnati, OH.

⁸ Vegetable Shortening, Kroger Co., Cincinnati, OH; The ingredients of the vegetable shortening include: partially hydrogenated soybean and cottonseed oil with mono- and diglycerides.

⁹ Rodelle Gourmet Vanilla Extract, Custom Blending Inc., Fort Collins, CO.

The milk drink was prepared using skim milk, chocolate instant drink powder and vegetable shortening (Table 3-9). Chocolate instant drink powder was weighed in a large bowl. Skim milk was measured with a 250 mL graduated cylinder, and was added to the powder and mixed with an electric hand mixer (Kitchenaid[®], St. Joseph, MI). Melted vegetable shortening was weighed and stirred into the drink.

Table 3-9. Ingredients for one milk drink

Ingredients	Quantity	Percent Weight (%)
Skim milk ¹ , mL	220	90.8
Nesquik ^{®2} , g	15	6.0
Vegetable shortening ³ , g	8	3.2
Total, g	249	100

¹ Skim Milk, Kroger Co., Cincinnati, OH.

² Nestle USA, Inc., Glendale, CA.

³ Vegetable shortening, Kroger Co., Cincinnati, OH. The ingredients of the vegetable shortening include: partially hydrogenated soybean and cottonseed oil with mono- and diglycerides.

The endothelial function was measured using the EndoPAT 2000 (Itamar Medical, Caesarea, Israel). The instrument was turned on 20 minutes prior to use and left on for the day. The temperature of the room was maintained between 21-24°C. Lights were dimmed and the room was quiet. The subject rested on an exam table in the supine position for 20 minutes prior to measurements.

Subject's baseline blood pressure (systolic/diastolic) was measured from the dominant arm (non-tested arm) using an automated oscillometric blood pressure monitor (Omron M7, HEM 780, Omron Healthcare Corporation, Kyoto, Japan). The blood cuff was moved to the tested arm (non-dominant arm) and placed above the elbow. Test procedures were explained to the subject and subject's gender, age, and blood pressure were entered into the software, following the instructional prompts provided by the EndoPAT 2000 system. A deflated probe was inserted into a groove of each arm-support. The subject's index fingers were inserted into the probes. Probes were inflated using the EndoPAT2000 software, then index fingers were removed from

the grooves. A foam anchor was placed on the root of each middle finger. To secure the tubing, the free end of the tubing was taped and formed a “U turn”, which would not touch the back of the hand (Figure 3-2).

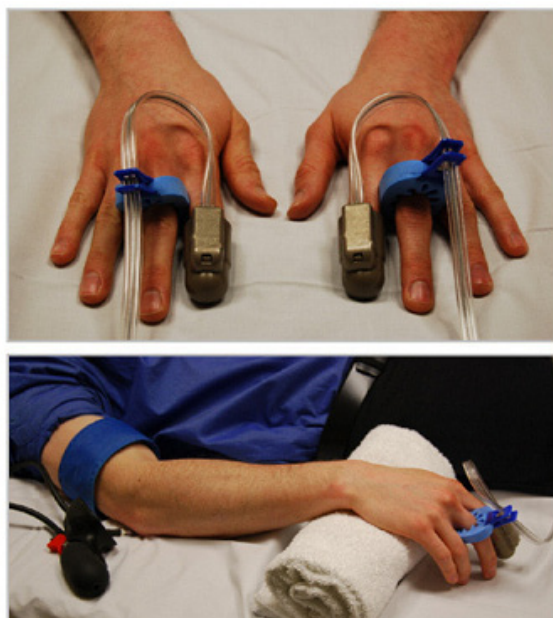


Figure 3-2. EndoPAT 2000 overview (Hamburg & Benjamin, 2009)

Signal quality was monitored for 1 minute to inspect for leaks and abnormal connections. The baseline signals of blood volume change were recorded for 5-10 minutes. Before occlusion, the timer was adjusted to 5 minutes, the gain of the occluded arm was adjusted to 20,000 g, and the time base was adjusted to 00:00:15 sec. The blood pressure cuff was inflated to 70 mmHg above baseline systolic blood pressure and held for exactly 5 minutes, then released rapidly. The time base was changed back to 1 minute. The post occlusion signal was continuously recorded for 10 minutes. After completing the signal recording, the probes were detached and discarded.

The baseline signals and the post occlusion signals were automatically analyzed

by the EndoPAT 2000 software and expressed as reactive hyperemia index (Hamburg & Benjamin, 2009; Hamburg, et al., 2008):

$$\text{RHI} = \frac{\text{Average amplitude of the PAT signal during 1-min starting 90 s after deflation}}{\text{Average amplitude of the PAT signal during 3.5-min preocclusion baseline}}$$

Blood samples for glucose and triglyceride analysis were collected at 0-premeal, 1, 2, 3, 4-premeal, 5, 6 hours during testing by 1.8 mm, 21 gauge clinical safety lancets (Stat-Let[®], North Miami, FL) and capillary tube from finger tips. Blood was analyzed in a CardioChek[®] PA analyzer (Polymer Technology Systems Inc., Indianapolis, IN) that had been fitted with a memory chip, turned on, and activated by insertion of a color coded test panel (Metabolic Syndrome Panel, Polymer Technology Systems Inc., Indianapolis, IN). Capillary tube blood was immediately applied to the test panel and results were read and recorded. Although not relevant to this study, HDL concentration was also obtained as a component of the metabolic test panel.

Blood samples for thiobarbituric acid reactive substances assay was drawn from subjects at each of the 4 time points (0-premeal, 2, 4-premeal, 6 hour). At each collection time, a 21 gauge needle was used to draw 10 mL of blood from the median cubital vein or median cephalic vein into a 15-mL EDTA-treated Vacutainer tube. Plasma was separated in a centrifuge using a swinging bucket rotor (JS4.3, J2-HC Beckman, Fullerton, CA) set at 910 x g (2000 rpm) for 10 minutes at 4°C. After centrifugation, the top layer of plasma was transferred into four 1.5-mL microfuge tubes (about 300 µL /tube) using a transfer pipet (#73990-016, VWR Scientific, Batavia, IL). All plasma samples were then supplemented with 0.005% BHT (3 µL/300 µL

plasma) and 2 mM indomethacin (1.5 μ L/300 μ L plasma). Microfuge tubes were placed into microfuge tube boxes and frozen at -78°C until analysis.

Thiobarbituric Acid reactive substances (TBARS) was assayed as a measure of malondialdehyde (MDA), utilizing the reaction between MDA and TBA to form an MDA-TBA adduct under high temperature and acidic conditions measured colorimetrically at 530-540 nm. Four separate assays were conducted to measure TBARS in plasma from 10 subjects. All samples from 3 subjects were measured with each assay; thus plasma from a subject's 4 time points were measured in the same assay.

Reagents were constituted according to Buege and Aust (1978) and Park et al. (2005) as shown in appendix B. Standards were prepared using 99% 1,1,3,3-Tetramethoxypropane and ultrapure water (RO/Elix-treated, Millipore, Billerica, MA) to create MDA concentrations of 0, 0.625, 1.25, 2.5, 5.0, 10.0, 25.0, 50.0 μM . Standards or treatment plasma (250 μL) were transferred by pipette (P1000 pipetman, Gilson Inc., Middleton, WI) using 1000 μL capacity sterile tips, into 1.5 mL polypropylene screw cap centrifuge tubes. BHT solution (0.202 M, 10 μL) and SDS solution (10% w/v, 50 μL) were added to each tube and tubes were swirled briefly to mix contents. Color reagent (500 μL) was added forcefully to each tube. Caps were placed on tubes and tubes were placed in foam stabilizers and boiled for 30 minutes. After boiling, vials were removed and incubated in an ice bath for 10 minutes to stop the reaction. Tubes were subjected to centrifugation in a fixed angle rotor microcentrifuge at 6000~7000 x g for 10 minutes at 4°C . Cold samples from each tube (200 μL) were transferred to wells in a microtiter plate and absorbance was measured at

532 nm and 600 nm using a plate reader (Biotek Powerwave, Winooski, VT) within 30 minutes of removal from centrifuge. Each treatment sample and standard was read in triplicate.

Average absorption of each standard and sample at both wavelengths were calculated separately from the readings. To adjust for the light scattered, OD₆₀₀ was subtracted from OD₅₃₂. Mean $\Delta(\text{OD}_{532} - \text{OD}_{600})$ was plotted for each standard concentration to form the standard curve. The concentration of MDA in the test samples were determined from the MDA standard curve: $\text{MDA } (\mu\text{M of sample}) = [(\text{OD}_{532} - \text{OD}_{600}) \text{ of treatment sample} - (\text{y intercept})] / \text{slope}$.

Treatment of Data

SPSS version 18.0 (Chicago, IL) was used for data analysis.

Subject characteristics

Descriptive statistic was used to generate the mean, standard deviation and range for subjects' age, body mass index, body surface area, blood pressure, fasting heart rate, fasting blood glucose, triglyceride and HDL. Within Subject (2×7) ANOVA was used to analyze blood glucose, triglyceride, and HDL data. Within Subject (2×4) ANOVA was used to analyze the heart rate data. When the interaction term was significant, simple main effects were analyzed.

Testing of statistical assumptions

Normality was examined by Shapiro-Wilk and Kolmogorov-Smirnov tests. The

distribution was evaluated by residuals histogram, skewness and kurtosis and a box plot was used to identify outliers (Keppel, 2004c; Tabachnick & Fidell, 2007a). When the deviation from the normality assumption was significant or skewness and kurtosis were beyond the range of -1 to 1, data were transformed (Tabachnick & Fidell, 2007a).

Assumption of sphericity was examined by Mauchly's test to determine if the correlations between group means were consistent (not significantly different) for each independent variable (Keppel, 2004b).

Tests of hypotheses

1. H_A : The feeding regimens affect the reactive hyperemia index differently over time.

For hypothesis 1, a two factor (2×4) Within Subject Analysis of Variance (ANOVA) was used to determine if there was a significant feeding regimen \times time interaction and feeding regimen main effect for reactive hyperemia index (Keppel, 2004d). Planned comparisons were used to determine the difference between two feeding regimens. When the interaction was significant, hypotheses 1a, 1b, 1c, 1d and 1e for simple main effect were tested.

1a. H_A : The reactive hyperemia index is significantly different over time after feeding standard high-fat meals (feeding regimen 1).

1b. H_A : The reactive hyperemia index is significantly different over time after feeding high-fat meals based on body surface area (feeding regimen 2).

Reactive hyperemia index from each feeding regimen was analyzed separately, for

the simple main effect, using one-way ANOVA to determine if there was a significant difference between 4 time points (Keppel, 2004d).

1c. H_A : The reactive hyperemia index is significantly different between feeding regimen 1 and feeding regimen 2 at hour 2 post ingestion.

1d. H_A : The reactive hyperemia index is significantly different between feeding regimen 1 and feeding regimen 2 at hour 4 post ingestion.

1e. H_A : The reactive hyperemia index is significantly different between feeding regimen 1 and feeding regimen 2 at hour 6 post ingestion.

Reactive hyperemia index at each time point except for 0 hour was analyzed separately, for simple main effect, using one-way ANOVA to determine if there was a significant difference between two feeding regimens (Keppel, 2004d).

2. H_A : The reactive hyperemia index changes over time.

For hypothesis 2, the main effect of time in the 2×4 ANOVA was used to examine how reactive hyperemia index differed between four time points. Planned comparisons were used to determine the difference between fasting state and postprandial state.

Tests of secondary hypotheses

1. H_A : The feeding regimens affect the plasma malondialdehyde level differently over time.

For hypotheses 1 and 6, a two factor (2×4) Within Subject Analysis of Variance (ANOVA) was used to determine if there was a significant feeding regimen \times time interaction and feeding regimen main effect for plasma malondialdehyde (Keppel,

2004d). If interaction was significant, then hypotheses 1a, 1b, 1c, 1d and 1e were analyzed.

1a. H_A : The plasma malondialdehyde level is significantly different over time when feeding standard meals (feeding regimen 1).

1b. H_A : The plasma malondialdehyde level is significantly different over time when feeding meals by body surface area (feeding regimen 2).

Plasma malondialdehyde from each feeding regimen was analyzed separately, for simple main effect, using one-way ANOVA to determine if there were any significant differences between the 4 time points (Keppel, 2004d).

1c. H_A : The plasma malondialdehyde level is significantly different between feeding regimen 1 and feeding regimen 2 at hour 2 post ingestion.

1d. H_A : The plasma malondialdehyde level is significantly different between feeding regimen 1 and feeding regimen 2 at hour 4 post ingestion.

1e. H_A : The plasma malondialdehyde level is significantly different between feeding regimen 1 and feeding regimen 2 at hour 6 post ingestion.

Plasma malondialdehyde at each time point except for 0 hour was analyzed separately, for simple main effect, using one-way ANOVA to determine if there was a significant difference between two feeding regimens (Keppel, 2004d).

2. H_A : The plasma malondialdehyde changes over time.

For hypothesis 2, main effect of time in the 2×4 ANOVA was used to examine how plasma malondialdehyde differed between four time points. Planned comparisons were used to examine the difference between fasting and postprandial state. Post-hoc

was also used for exploratory purposes.

Power analysis and effect size

Variation (sum of squares) of the tested effect and error were obtained for dependent variables from the ANOVA and used to calculate power. SPSS was used to estimate the observed power and effect size (partial eta squared, η_p^2). Effect size estimates the proportion of variance in a dependent variable that is associated with an independent variable and it assesses how levels of the independent variable predict the total variance of the dependent variable (Tabachnick & Fidell, 2007b). Partial eta squared (η_p^2) shows the proportion of variance in the dependent variable, $SS_{\text{effect}} + SS_{\text{error}}$, attribute to the effect, SS_{effect} (Tabachnick & Fidell, 2007b). The effect size is small when η_p^2 is 0.01, medium when η_p^2 is 0.09, and large when η_p^2 is 0.25 (Tabachnick & Fidell, 2007b). Effect size (ω^2) in the population was also estimated for each effect by the following formula: $\omega^2 = \frac{(a-1)(F-1)}{(a-1)(F-1)+an}$, where “a” was the level of a independent variable, “F” was the F ratio of a tested effect and n was the subject number in each level of the independent variable (Keppel, 2004a). The effect size is small when ω^2 is 0.01, medium when ω^2 is 0.06, and large when ω^2 is 0.15 (Keppel, 2004a). G*Power 3.0 software (Parkville, Australia) was used to generate graphs of power by subject number which provided estimates of sample size necessary for adequate study power ($1-\beta=0.6$) for dependent variables to strengthen the ability of the model to detect significant differences in future studies (Erdfelder, Faul, & Buchner, 1996).

Chapter 4

ANALYSIS OF DATA AND DISCUSSION OF RESULTS

The focus of the study was to optimize a feeding model to study the acute effects of two consecutive high-fat meals on endothelial-dependent vasodilation (reactive hyperemia index) and a biomarker of oxidative stress (plasma malondialdehyde). Blood triglyceride and glucose were monitored to describe the model of the extended postprandial state. The analysis of the data is presented in this chapter according to the following topics: (a) demographic data; (b) Within Subject Analysis of Variance; (c) power and subject estimates; (d) effect size estimates.

Demographic Data

The baseline characteristics of subjects in this study are shown in Table 4-1. All ten subjects were healthy males. The mean age of subjects was 24 years (range of 20-30 years), body mass index 22.4 kg/m² (range of 20.4-25 kg/m²), and body surface area 1.91 m² (range of 1.617-2.109 m²). Fasting blood pressures were within normal ranges for adults that diastolic blood pressure was less than 80 mm Hg and systolic blood pressure was less than 120 mm Hg (Chobanian, et al., 2003). Mean fasting blood glucose was within the normal range: less than 100 mg/dL (American Diabetes Association, 2010). Fasting triglycerides was less than 150 mg/dL which was in the normal range (American Heart Association, 2010). Mean fasting HDL was below the desirable level (60 mg/dL, heart disease protection level), however was above 40

mg/dL below which was associated with increased risks of heart disease in men

(American Heart Association, 2010).

Table 4-1. Anthropometric and clinical characteristics of subjects prior to study

Characteristics	Mean ¹
Age, y	24.4 ± 3.4 (20-30)
Body mass index, kg/m ²	22.4 ± 1.5 (20.4-25)
Body surface area, m ²	1.9 ± 0.1 (1.617-2.109)
Systolic blood pressure, mm Hg	119.5 ± 7.7 (109-130)
Diastolic blood pressure, mm Hg	71.7 ± 5.5 (63-81)
Fasting heart rate, bpm	58.1 ± 8.9 (44-74)
Fasting glucose, mg/dL	93.9 ± 7.9 (75-105)
Fasting HDL, mg/dL	43.4 ± 12.3 (30-79)
Fasting triglycerides, mg/dL	69.9 ± 24.8 (49-143)

¹ Values are means ± SD, n=10 (male).

The average of blood glucose concentrations at each time point (0-6h) from feeding regimen 1 (standard meal) and feeding regimen 2 (body surface area-based meal) are shown in Table 4-2 and Figure 4-1. In both feeding regimens, blood glucose concentration changed in a similar pattern. Blood glucose level started low (93.9 mg/dL) in the fasting state and was elevated one hour (112.9 mg/dL) into the postprandial state, reached the peak (113.1 mg/dL) 2 hours after the second high-fat meal ($p < 0.05$) at time point 6h.

Table 4-2. Mean blood glucose concentration¹

Time post meal (hours)	Blood glucose Regimen 1 (standard) mg/dL	Blood glucose Regimen 2 (body surface area) mg/dL	Mean ² mg/dL
0	93.5 ± 3.2	94.3 ± 1.7	93.9 ^a ± 2.2
1	113.5 ± 6.8	112.3 ± 6.4	112.9 ^c ± 5.2
2	99.6 ± 4.5	97.0 ± 2.9	98.3 ^{ab} ± 3.1
3	95.4 ± 2.3	100.7 ± 2.8	98.1 ^b ± 1.8
4	95.4 ± 2.8	98.3 ± 3.2	96.9 ^a ± 1.8
5	113.2 ± 4.8	104.8 ± 2.6	109.0 ^c ± 3.2
6	117.3 ± 3.9	108.9 ± 3.5	113.1 ^c ± 3.2

¹ Values are mean ± SEM, n=10 (male).

² Data are analyzed by 2 × 7 Within Subject ANOVA. Means that do not share the same superscript are significantly different (Fisher's Least Significant Difference Test, p<0.05).

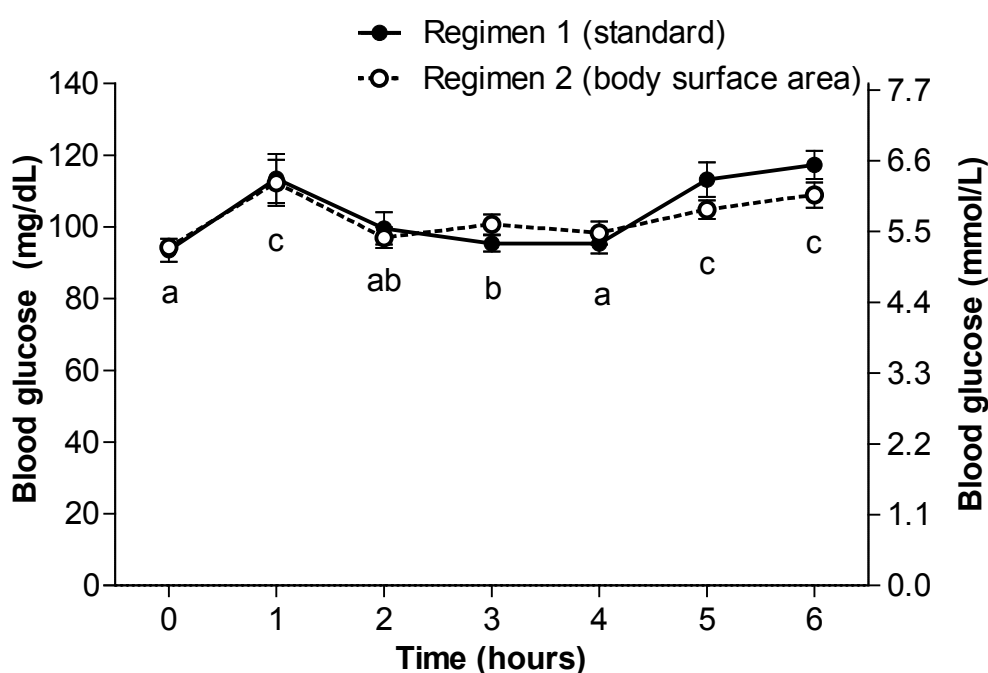


Figure 4-1. Mean glucose concentration (± SEM) over time. Data are analyzed by 2 × 7 Within Subject ANOVA. Means that do not share the same letter are significantly different (Fisher's Least Significant Difference Test, p<0.05).

The average of blood triglyceride concentration at each time point (0-6h) from both feeding regimens is shown in Table 4-3 and Figure 4-2. In both feeding regimens, blood triglyceride concentration was low in the fasting state (69.9 mg/dL) and was increased 3 hours after the first high-fat meal (121.5 mg/dL, $p<0.05$). Blood triglyceride concentration reached peak level 1 hour after the second high-fat meal (173.0 mg/dL, $p<0.05$) and decreased 2 hours after the second high-fat meal (122.2 mg/dL, $p<0.05$).

Table 4-3. Mean blood triglyceride concentration¹

Time post meal (hours)	Blood triglyceride Regimen 1(standard) mg/dL	Blood triglyceride Regimen 2 (body surface area) mg/dL	Mean ² mg/dL
0	59.8 ± 4.5	79.9 ± 9.3	69.9 ^a ± 5.4
1	75.3 ± 8.4	85.4 ± 7.8	80.4 ^a ± 5.2
2	72.6 ± 6.6	91.6 ± 7.1	82.1 ^a ± 4.5
3	113.0 ± 14.9	130.0 ± 13.4	121.5 ^b ± 11.5
4	109.5 ± 12.0	115.0 ± 16.7	112.3 ^b ± 9.8
5	155.0 ± 16.9	190.9 ± 24.0	173.0 ^c ± 18.7
6	105.4 ± 10.0	138.9 ± 21.5	122.2 ^b ± 12.5

¹ Values are mean ± SEM, n=10 (male).

² Data are analyzed by 2 × 7 Within Subject ANOVA. Means that do not share the same superscript are significantly different (Fisher's Least Significant Difference Test, $p<0.05$).

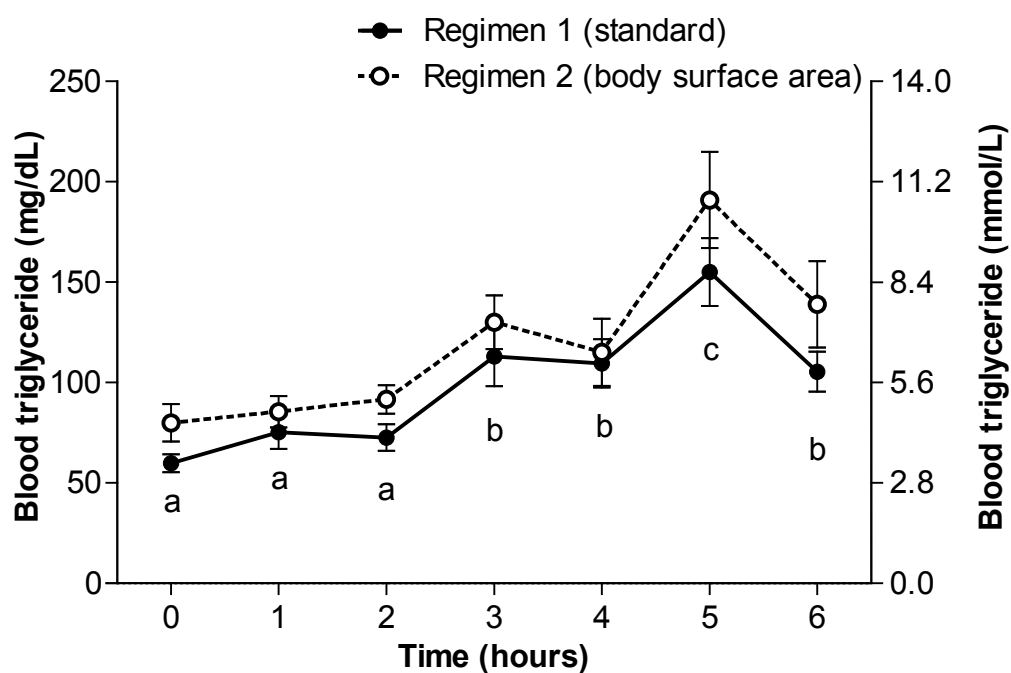


Figure 4-2. Mean triglyceride concentration (\pm SEM) over time. Data are analyzed by 2×7 Within Subject ANOVA. Means that do not share the same letter are significantly different (Fisher's Least Significant Difference Test, $p < 0.05$).

As shown in Table 4-4 and Figure 4-3, mean HDL level were similar over time (0-6h) in two feeding regimens ($p > 0.05$).

Table 4-4. Mean HDL concentration¹

Time post meal (hours)	HDL		Mean ² mg/dL
	Regimen 1 (standard) mg/dL	Regimen 2 (body surface area) mg/dL	
0	44.0 \pm 4.5	42.8 \pm 3.4	43.4 ^a \pm 3.8
1	45.0 \pm 3.6	45.0 \pm 4.3	45.0 ^a \pm 3.8
2	45.4 \pm 4.0	43.1 \pm 3.5	44.3 ^a \pm 3.7
3	44.2 \pm 3.9	43.0 \pm 3.7	43.6 ^a \pm 3.7
4	46.1 \pm 4.5	44.6 \pm 4.4	45.4 ^a \pm 4.3
5	46.4 \pm 4.4	46.3 \pm 4.9	46.4 ^a \pm 4.6
6	46.6 \pm 4.1	44.6 \pm 4.9	45.6 ^a \pm 4.4

¹ Values are mean \pm SEM, n=10 (male).

² Data are analyzed by 2×7 Within Subject ANOVA. Means that do not share the same superscript are significantly different (Fisher's Least Significant Difference Test, $p < 0.05$).

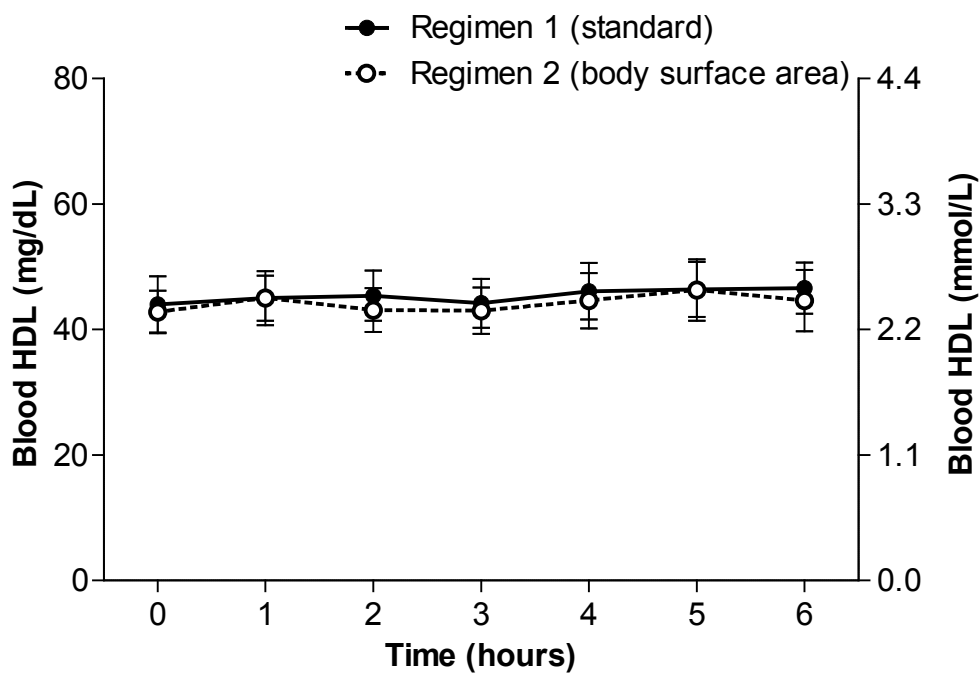


Figure 4-3. Mean HDL concentration (\pm SEM) over time. Data are analyzed by 2×7 Within Subject ANOVA. Means that do not share the same letter are significantly different (Fisher's Least Significant Difference Test, $p < 0.05$).

Mean heart rate slightly increased 2 hours after the first high-fat meal ($p < 0.05$), decreased to baseline 4 hours after the first high-fat meal ($p < 0.05$), and then slightly increased to reach a peak at 6h (2 hours after the second high-fat meal, $p < 0.05$), as shown in Table 4-5 and Figure 4-4.

Table 4-5. Heart rate (bpm) before and after standard high-fat meals or high-fat meals based on body surface area in healthy men¹

	Regimen 1 (standard meal)	Regimen 2 (body surface area meal)	Mean ²
Time post 1 st meal			
0 h	58.20 ± 2.81	57.90 ± 2.94	58.05 ^a ± 2.65
2 h	61.70 ± 2.91	61.80 ± 2.79	61.75 ^b ± 2.61
4 h	58.90 ± 1.95	56.80 ± 2.08	57.85 ^a ± 1.95
6 h	62.5 ± 2.09	62.00 ± 2.02	62.25 ^b ± 1.81
<i>P-values³ (1-β)</i>			
<i>Feeding regimen</i>		0.68 (0.07)	
<i>Time</i>		0.002 (0.94)	
<i>Feeding regimen × time</i>		0.56 (0.18)	

¹ Values are means ± SEM, n=10.

² Means do not share the same superscript are significantly different (Fisher's Least Significant Different Test, p<0.05).

³ P value and power, calculated using Within Subject ANOVA, p<0.05. Adequate power should be at least 0.6.

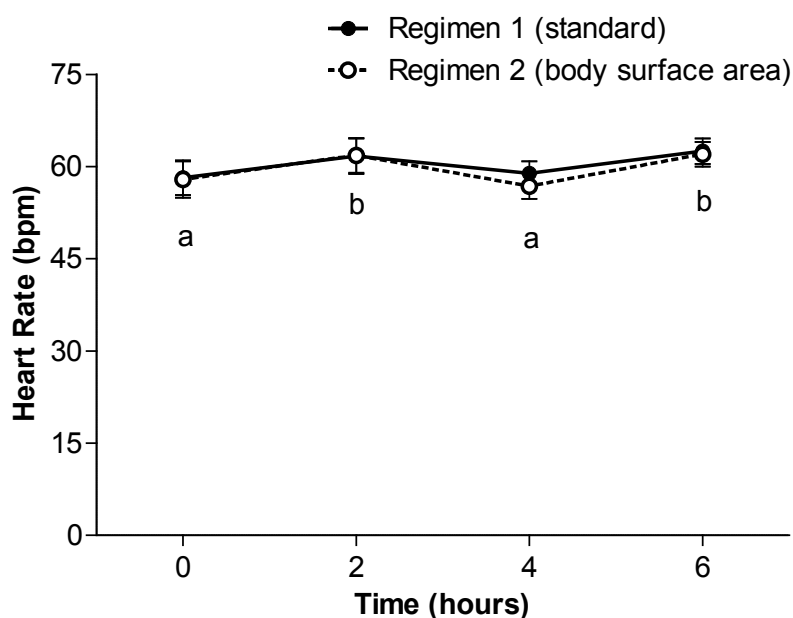


Figure 4-4. Mean heart rate (±SEM) over time. Data are analyzed by Within Subject ANOVA. Values are means ± SEM, n=10. Feeding regimen effect, p=0.68; time effect, p=0.002; feeding regimen × time, p=0.56. Means that do not share the same letter are significant difference between time points across feeding regimens (Fisher's Least Significant Difference Test, p<0.05).

Within Subject Analysis of Variance

Reactive hyperemia index

Residuals of reactive hyperemia index data were not normally distributed ($p < 0.05$) as shown in Table 4-6, therefore the assumption of Analysis of Variance was violated. A histogram of the residuals (Figure 4-5) showed a positive skew; two outliers were identified from subject 9 and 10 using a box plot of the residuals (Figure 4-6). The skewness (1.03) and kurtosis (1.27) were beyond the range from -1 to +1 (Table 4-6).

Table 4-6. Normality assumption test for reactive hyperemia index

	Test Values
Residuals ¹	0 ± 0.06
95% Confidence interval	(-0.12,0.12)
Skewness ²	1.03
Kurtosis ²	1.27
Shapiro-Wilk normality test ³	$p=0.001$
Kolmogorov-Smirnov normality test ³	$p=0.01$

¹ Values are mean \pm SEM, n=80.

² Skewness and Kurtosis show the asymmetry and peakness of the residuals and should be zero for normal distributed data.

³ Data are not normally distributed when $p < 0.05$

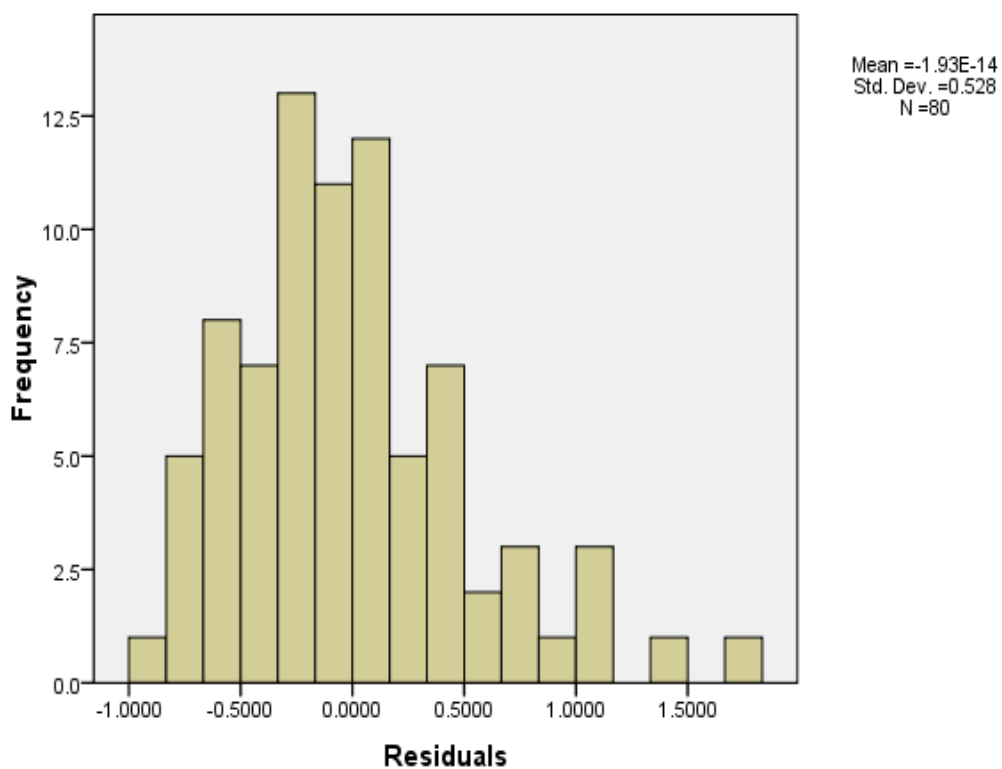


Figure 4-5. Distribution of residuals for reactive hyperemia index

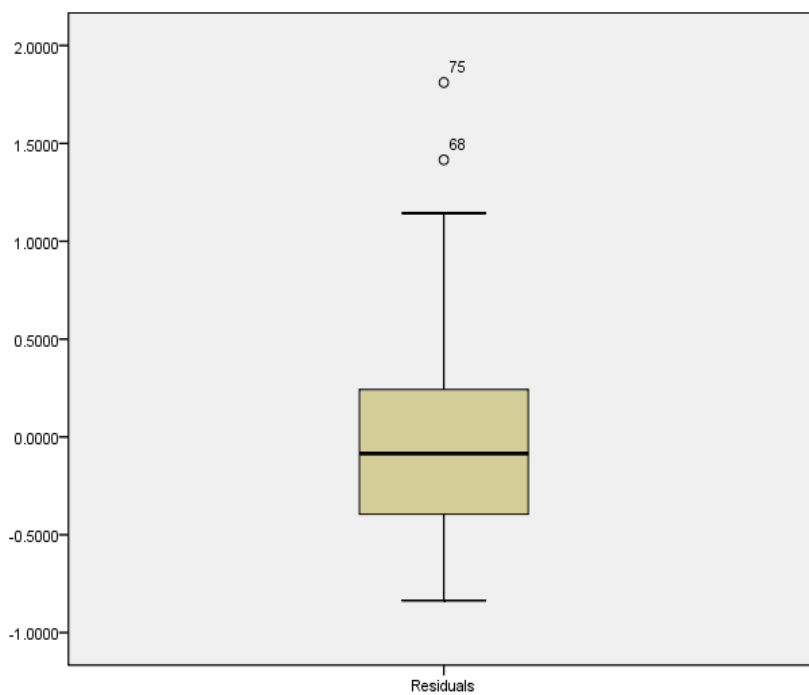


Figure 4-6. Box plot of reactive hyperemia index residuals

As shown in Table 4-7, Mauchly's W was not significant either for time ($p=0.54$) or feeding regimen \times time interaction ($p=0.11$), therefore the assumption of sphericity was maintained. Mauchly's test did not generate a result for feeding regimen since there were only two levels of feeding regimen.

Table 4-7. Sphericity assumption test for reactive hyperemia index

Source of variance	Mauchly's W ¹	df	Significance	Epsilon ²
Feeding regimen	1	0	.	1
Time	0.59	5	0.54	0.80
Feeding regimen*Time	0.31	5	0.11	0.56

¹ Mauchly's test is used to test the null hypothesis that the error covariance matrix of the orthonormalized dependent variables is proportional to an identity matrix.

² Epsilon index may be used to adjust the degrees of freedom for the averaged tests of significance.

Data was transformed by reflection (inverse) to normalize data. Statistical analysis of reflected reactive hyperemia index gave the same results as unreflected data, thus results are presented for unreflected reactive hyperemia index.

The ANOVA table for reactive hyperemia index is shown in Table 4-8 and results are summarized in Figure 4-7 and Table 4-9. As shown in Table 4-8, there was not enough power to detect a significant time \times feeding regimen interaction ($p=0.42$, $\omega^2=0$, $1-\beta=0.24$), therefore a conclusion cannot be drawn as to whether the two feeding regimens impact reactive hyperemia index differently over time. Power was also too low to detect a significant difference between feeding regimen 1 and 2 ($p=0.46$, $\omega^2=0$, $1-\beta=0.12$). However, there were significant differences between time points ($p=0.01$, $\omega^2=0.12$, $1-\beta=0.84$). Analysis of the planned comparison showed that reactive

hyperemia index at 0h (fasting state) was significantly lower in comparison to 2h, 4h and 6h respectively, and was significantly lower than the pooled postprandial reactive hyperemia index from 2h-6h (Table 4-9 and 4-10). Reactive hyperemia index at 0h from regimen 2 (body surface area meal) was lower than 6h (2 hours after the second meal, $p < 0.05$), whereas reactive hyperemia index at 0h from regimen 1 (standard meal) was not different ($p > 0.05$), as shown in Table 4-9 and 4-10.

Table 4-8. Within Subject ANOVA for reactive hyperemia index^a

Source of variation	Sum of squares	df	Mean squares	F	Significance	ω^2 ^b	η_p^2 ^c	$1-\beta$ ^d
Feeding regimen	0.2	1	0.2	0.6	0.46	0.00	0.06	0.12
Error (feeding regimen)	2.9	9	0.3					
Time	2.4	3	0.8	4.6	0.01	0.12	0.34	0.84
Error (time)	4.7	27	0.2					
Feeding regimen×Time	1.0	3	0.3	1.0	0.42	0.00	0.10	0.24
Error (Regimen×Time)	8.7	27	0.3					

^a Values are mean \pm SEM, n=10 (male).

^b Omega squared is an estimate of the dependent variance accounted for by the independent variable in the population for a fixed effects model.

^c Partial eta squared is the proportion of the effect plus error variance that is attributable to the effect.

^d Power calculated by SPSS. Adequate power should be at least 0.6.

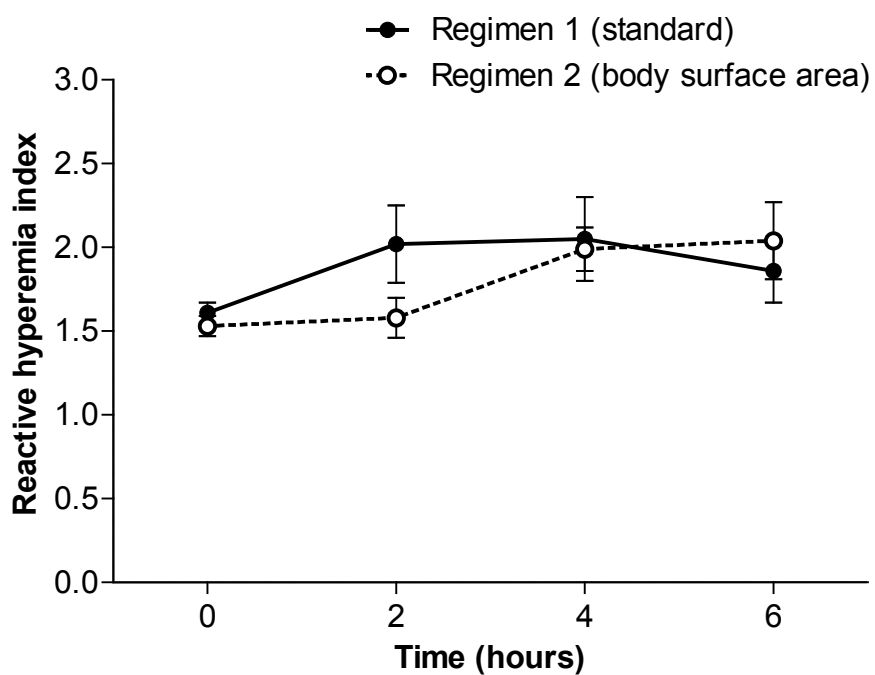


Figure 4-7. Mean reactive hyperemia index (\pm SEM) over time. Data are analyzed by Within Subject ANOVA. Values are means \pm SEM, $n=10$. Feeding regimen effect, $p=0.46$; time effect, $p=0.01$; feeding regimen \times time, $p=0.42$.

Table 4-9. Mean reactive hyperemia index before and after standard high-fat meals or high-fat meals based on body surface area in healthy men¹

	Regimen 1 (standard meal)	Regimen 2 (body surface area meal) ²	Mean
Time post 1st meal ^{3,4}			
0 h	1.61 ± 0.06	1.53 [‡] ± 0.06	1.57 ^{a*} ± 0.05
2 h	2.02 ± 0.23	1.58 ± 0.12	1.80 ^b ± 0.13
4 h	2.05 ± 0.25	1.99 ± 0.13	2.02 ^b ± 0.13
6 h	1.86 ± 0.19	2.04 [‡] ± 0.23	1.95 ^b ± 1.51
<i>P-values⁵(1-β)</i>			
<i>Feeding regimen</i>		0.46 (0.12)	
<i>Time</i>		0.01 (0.84)	
<i>Feeding regimen × time</i>		0.42 (0.24)	
Changes post meal ⁶			
"Breakfast" Δ 2 h - 0 h	0.41 ± 0.24	-0.19 ± 0.3	0.23 ^a ± 0.12
"Lunch" Δ 6 h - 4 h	0.05 ± 0.12	0.05 ± 0.25	-0.07 ^b ± 0.13
<i>P-values⁵(1-β)</i>			
<i>Feeding regimen</i>		0.85 (0.05)	
<i>Time</i>		0.03 (0.66)	
<i>Feeding regimen × time</i>		0.17 (0.27)	

¹ Values are means ± SEM, n=10.

² ‡ indicates a significant difference between 0h and 6h after body surface area meal (planned comparison, p<0.05).

³ Means that are significantly different from the baseline (0h) do not share the same letter (planned comparison, p<0.05). * indicates a significant difference between fasting state at 0h and a pooled postprandial state 2-6 h (planned comparison, p<0.05).

⁴ 0h, baseline at fasting state; 2h, 2 hours after the first high-fat meal; 4h, 4 hours after the first meal and right before the second meal; 6h, 6 hours after the first meal and 2 hours after the second meal.

⁵ P value and power, calculated using Within Subject ANOVA, p<0.05. Adequate power should be at least 0.6.

⁶ Means that do not share the same superscript are significantly different, p<0.05.

Table 4-10. Planned comparisons for reactive hyperemia index

Contrast	Regimen 1 (standard meal)				Regimen 2 (body surface area meal)				ΣC^{2a}	ϕ^{2b}	Mean squares	F ^c
	0h	2h	4h	6h	0h	2h	4h	6h				
1	1	-1	0	0	1	-1	0	0	4	0.21	0.53	3.12*
2	1	-1	0	0	0	0	0	0	2	0.17	0.85	2.83
3	0	0	0	0	1	-1	0	0	2	0.00	0.01	0.04
4	1	0	-1	0	1	0	-1	0	4	0.81	2.03	11.94*
5	1	0	-1	0	0	0	0	0	2	0.19	0.95	3.17
6	0	0	0	0	1	0	-1	0	2	0.21	1.05	3.50
7	1	0	0	-1	1	0	0	-1	4	0.58	1.45	8.53*
8	1	0	0	-1	0	0	0	0	2	0.06	0.30	1.00
9	0	0	0	0	1	0	0	-1	2	0.26	1.30	4.33*
10	0	1	0	-1	0	1	0	-1	4	0.09	0.23	1.35
11	0	1	0	-1	0	0	0	0	2	0.03	0.15	0.50
12	0	0	0	0	0	1	0	-1	2	0.21	1.05	3.50
13	0	1	1	0	0	-1	-1	0	4	0.25	0.63	1.97
14	3	-1	-1	-1	3	-1	-1	-1	24	4.50	1.88	11.06*
15	3	-1	-1	-1	0	0	0	0	12	1.21	1.01	3.37
16	0	0	0	0	3	-1	-1	-1	12	1.04	0.87	2.89

^a The sum of the contrast squared.

^b The sum of the cross products of mean and contrast.

^c F ratio, * indicates significant difference, $p < 0.05$.

The ANOVA table for changes in reactive hyperemia index is shown in Table 4-11.

There was not enough power to detect a significant time \times feeding regimen interaction ($p=0.17$, $\omega^2=0.03$, $1-\beta=0.27$). The power was also too low to detect a significant main effect of feeding regimen ($p=0.85$, $\omega^2=0$, $1-\beta=0.05$). There were significant differences between time points ($p=0.03$, $\omega^2=0.13$, $1-\beta=0.66$) which indicated reactive hyperemia index changed differently 2 hours after the first meal in comparison to the second meal.

As shown in Table 4-9 and Figure 4-8, reactive hyperemia index increased from 0 hour

to 2 hour whereas it decreased from 4 hour to 6 hour ($p < 0.05$).

Table 4-11. Within Subject ANOVA for changes in reactive hyperemia index^a

Source of variation	Sum of squares	df	Mean squares	F	Significance	ω^2 ^b	η_p^2 ^c	$1-\beta$ ^d
Feeding regimen	0.1	1	0.1	0.0	0.85	0.00	0.00	0.05
Error (feeding regimen)	11.2	9	1.3					
Time	0.9	1	0.9	7.1	0.03	0.13	0.44	0.66
Error (time)	1.1	9	0.1					
Feeding regimen×Time	0.9	1	0.9	2.2	0.17	0.03	0.20	0.27
Error (Regimen×Time)	3.6	9	0.4					

^a Values are mean \pm SEM, n=10.

^b Omega squared is an estimate of the dependent variance accounted for by the independent variable in the population for a fixed effects model.

^c Partial eta squared is the proportion of the effect plus error variance that is attributable to the effect.

^d Power is calculated by SPSS. Adequate power should be at least 0.6.

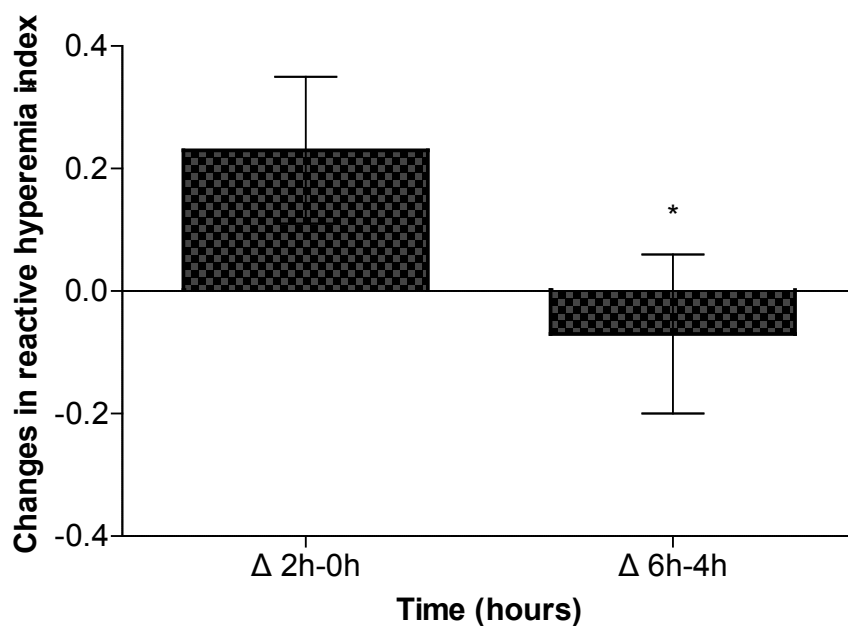


Figure 4-8. Changes in reactive hyperemia index (\pm SEM) 2 hours post each meal. Data are analyzed by Within Subject ANOVA. Values are means and standard error, n=10.

Feeding regimen effect, $p=0.85$; time effect, $p=0.03$; feeding regimen \times time, $p=0.17$.

*Different from changes in reactive hyperemia index at 2h-0h time interval, $p < 0.05$.

Thiobarbituric acid reactive substances

Residuals of thiobarbituric acid reactive substances data were not normally distributed ($p < 0.05$) as shown in Table 4-12, therefore the assumption of ANOVA was violated. The histogram of residuals (Figure 4-9) had a slightly negative skew (skewness=-0.09) but was close to zero, whereas kurtosis(-1.34) was beyond the range of -1 to +1 that indicated a slightly flat normal curve. No outliers were observed in the box plot (Figure 4-10). Although there was a violation of the normality assumption, considering the value of the skewness, kurtosis, the shape of the residual plot and the small subject number, Within Subject ANOVA was still conducted.

Table 4-12. Normality assumption test for thiobarbituric acid reactive substances data

	Test Values
Residuals ¹	0 ± 0.04
95% Confidence interval	(-0.07,0.07)
Skewness ²	-0.09
Kurtosis ²	-1.34
Shapiro-Wilk normality test ³	$p=0.001$
Kolmogorov-Smirnov normality test ³	$p=0.002$

¹ Values are mean \pm SEM, n=80.

² Skewness and Kurtosis show the asymmetry and peakness of the residuals and should be zero for normal distributed data.

³ Data are not normally distributed when $p < 0.05$.

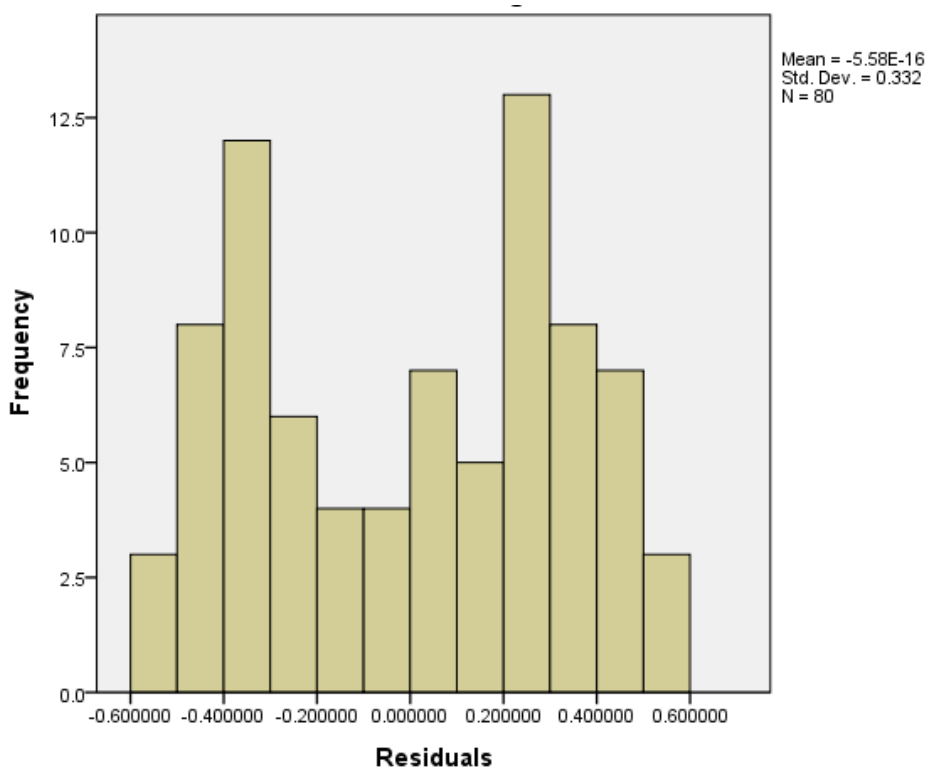


Figure 4-9. Distribution of thiobarbituric acid reactive substances residuals

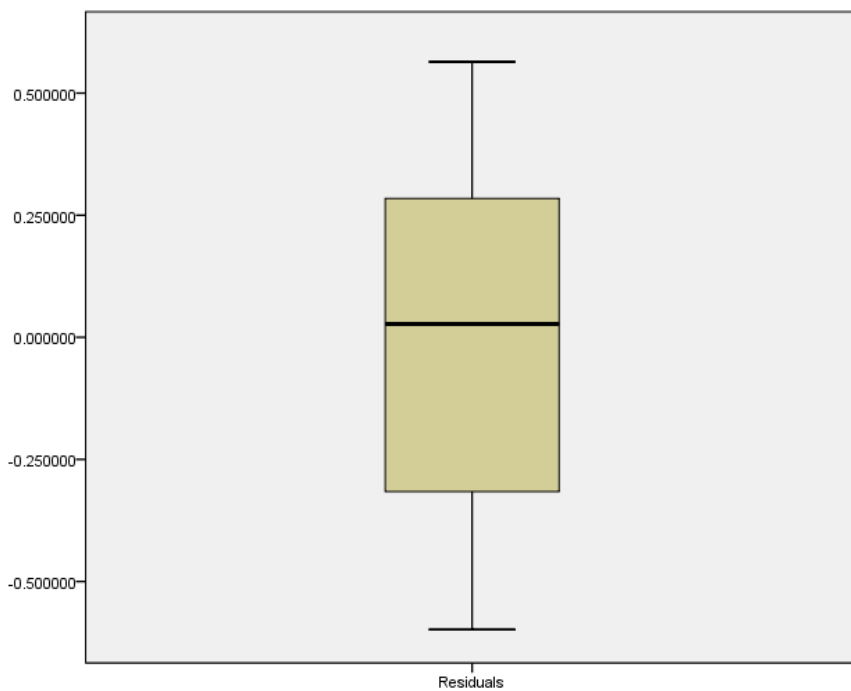


Figure 4-10. Box plot of thiobarbituric acid reactive substances residuals

The assumption of sphericity was satisfied as shown in Table 4-13. Mauchly's W was not significant for time ($p=0.65$) or the feeding regimen \times time interaction ($p=0.91$). Mauchly's test did not give a result for feeding regimen since there were only two levels of feeding regimen.

Table 4-13. Sphericity assumption test for thiobarbituric acid reactive substances

Source of variance	Mauchly's W^1	df	Significance	Epsilon ²
Feeding regimen	1	0	.	1
Time	0.65	5	0.65	0.8
Feeding regimen \times Time	0.82	5	0.91	0.9

¹ Mauchly's test is used to test the null hypothesis that the error covariance matrix of the orthonormalized dependent variables is proportional to an identity matrix.

² Epsilon index may be used to adjust the degrees of freedom for the averaged tests of significance.

ANOVA table for thiobarbituric acid reactive substances was shown in Table 4-14 and results were summarized in Table 4-15 and Figure 4-11. As shown in Table 4-14 and 4-15, there was not enough power to detect a significant time \times feeding regimen interaction ($p=0.65$, $\omega^2=0$, $1-\beta=0.15$). Power was too low to detect a significant difference between feeding regimen 1 and 2 ($p=0.17$, $\omega^2=0.01$, $1-\beta=0.27$). There were significant differences between four time points ($p=0.04$, $\omega^2=0.07$, $1-\beta=0.66$). Thiobarbituric acid reactive substances increased from 0h to 2h (planned comparison, $p<0.05$, Table 4-16); there was also a significant decrease in thiobarbituric acid reactive substances from 2h to 4h (Fisher's Least Significant Different Test, $p<0.05$), as shown in Table 4-15.

Table 4-14. Within Subject ANOVA for thiobarbituric acid reactive substances^a

Source of variation	Sum of squares	df	Mean squares	F	Significance	ω^2 ^b	η_p^2 ^c	$1-\beta$ ^d
Feeding regimen	0.04	1	0.04	2.21	0.17	0.01	0.20	0.27
Error (feeding regimen)	0.17	9	0.02					
Time	0.09	3	0.03	3.11	0.04	0.07	0.26	0.66
Error (time)	0.27	27	0.01					
Feeding regimen \times Time	0.02	3	0.07	0.55	0.65	0.00	0.06	0.15
Error (Regimen \times Time)	0.33	27	0.01					

^a Values are mean \pm SEM, n=10.

^b Omega squared is an estimate of the dependent variance accounted for by the independent variable in the population for a fixed effects model.

^c Partial eta squared is the proportion of the effect plus error variance that is attributable to the effect.

^d Power is calculated by SPSS.

Table 4-15. Thiobarbituric acid reactive substances ($\mu\text{mol/L}$) before and after standard high-fat meals or high-fat meals based on body surface area in healthy men¹

	Regimen 1 (standard meal) ²	Regimen 2 (body surface area meal)	Mean ³
Time post 1 st meal			
0 h	0.62 [‡] \pm 0.11	0.62 \pm 0.10	0.62 ^a \pm 0.10
2 h	0.72 [‡] \pm 0.11	0.67 \pm 0.12	0.70 ^b \pm 0.11
4 h	0.64 \pm 0.11	0.57 \pm 0.11	0.61 ^a \pm 0.11
6 h	0.67 \pm 0.12	0.60 \pm 0.10	0.64 ^{ab} \pm 0.11

P-values⁴ (1- β)

Feeding regimen

0.17 (0.27)

Time

0.04 (0.66)

Feeding regimen \times time

0.65 (0.15)

¹ Values are means \pm SEM, n=10.

² ‡ indicates a significant increase from 0h to 2h after standard meal (planned comparison, p<0.05).

³ Means do not share the same superscript are significantly different (planned comparison and Fisher's Least Significant Different Test, p<0.05).

⁴ P value and power, calculated using Within Subject ANOVA, p<0.05. Adequate power should be above 0.6.

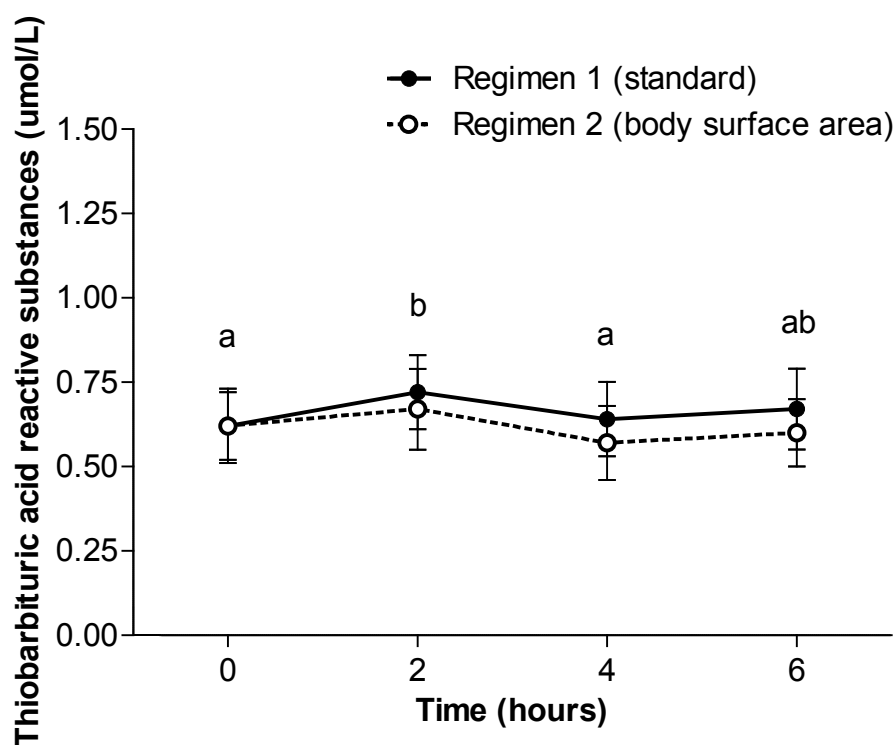


Figure 4-11. Mean thiobarbituric acid reactive substances (\pm SEM) over time. Data are analyzed by Within Subject ANOVA. Values are means \pm SEM, n=10. Feeding regimen effect, $p=0.27$; time effect, $p=0.66$; feeding regimen \times time, $p=0.15$. Means that do not share the same letter are significant difference between time points across feeding regimens (planned comparison and Fisher's Least Significant Difference Test, $p<0.05$).

Table 4-16. Planned comparisons for thiobarbituric acid reactive substances

Contrast	Regimen 1 (standard meal)				Regimen 2 (body surface area meal)				ΣC^{2a}	ϕ^{2b}	Mean squares	F ^c
	0h	2h	4h	6h	0h	2h	4h	6h				
1	1	-1	0	0	1	-1	0	0	4	0.02	0.05	5.00*
2	1	-1	0	0	0	0	0	0	2	0.01	0.05	5.00*
3	0	0	0	0	1	-1	0	0	2	0.00	0.01	1.00
4	1	0	-1	0	1	0	-1	0	4	0.00	0.00	0.00
5	1	0	-1	0	0	0	0	0	2	0.00	0.00	0.00
6	0	0	0	0	1	0	-1	0	2	0.00	0.01	1.25
7	1	0	0	-1	1	0	0	-1	4	0.00	0.00	0.00
8	1	0	0	-1	0	0	0	0	2	0.00	0.01	1.25
9	0	0	0	0	1	0	0	-1	2	0.00	0.00	0.00
10	0	1	0	-1	0	1	0	-1	4	0.01	0.03	2.50
11	0	1	0	-1	0	0	0	0	2	0.00	0.01	1.25
12	0	0	0	0	0	1	0	-1	2	0.00	0.03	2.50
13	0	1	1	0	0	-1	-1	0	4	0.01	0.03	1.25
14	3	-1	-1	-1	3	-1	-1	-1	24	0.00	0.00	0.00
15	3	-1	-1	-1	0	0	0	0	12	0.00	0.00	0.00
16	0	0	0	0	3	-1	-1	-1	12	0.00	0.00	0.00

^a The sum of the contrast squared.

^b The sum of the cross products of mean and contrast.

^c F ratio, * indicates significant difference, $p < 0.05$.

Power and Subject Estimates

Graphs of power \times subject estimate were generated using G*Power software (Parkville, Australia) for each main effect and interaction term in the ANOVA of each dependent variable. Graphs of power \times subject estimates are presented by feeding regimen main effect, time main effect and feeding regimen \times time interaction.

To achieve an adequate power of 0.6 for feeding regimen main effect, 54 subjects might be needed for reactive hyperemia index and 17 subjects might be needed for

thiobarbituric acid reactive substances to detect significant statistical differences

(Figure 4-12 and 4-13).

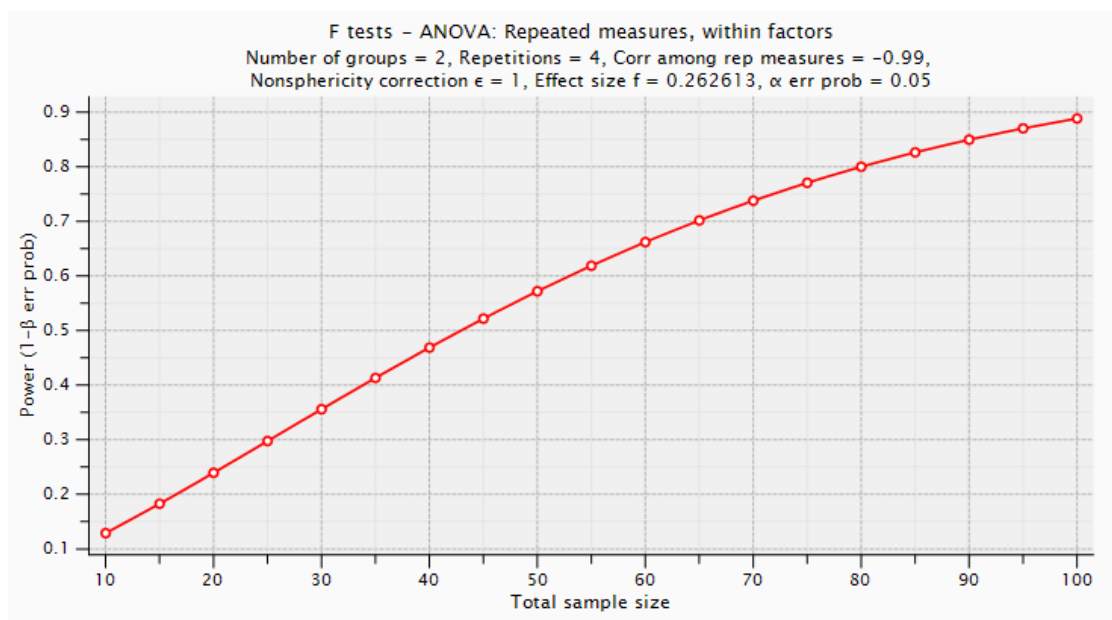


Figure 4-12. Power \times subject estimates of feeding regimen main effect for reactive hyperemia index

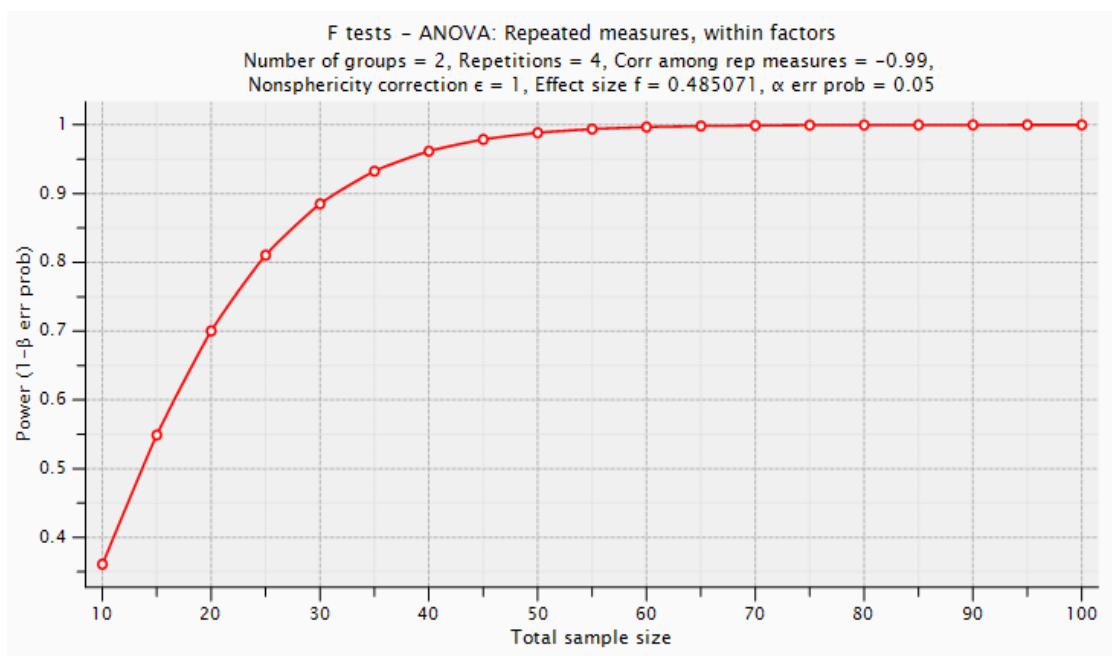


Figure 4-13. Power \times subject estimates of feeding regimen main effect for thiobarbituric acid reactive substances

Adequate power was achieved for time main effect for reactive hyperemia index

($1-\beta=0.84$) and thiobarbituric acid reactive substances ($1-\beta=0.66$) analysis (Figure 4-14 and 4-15).

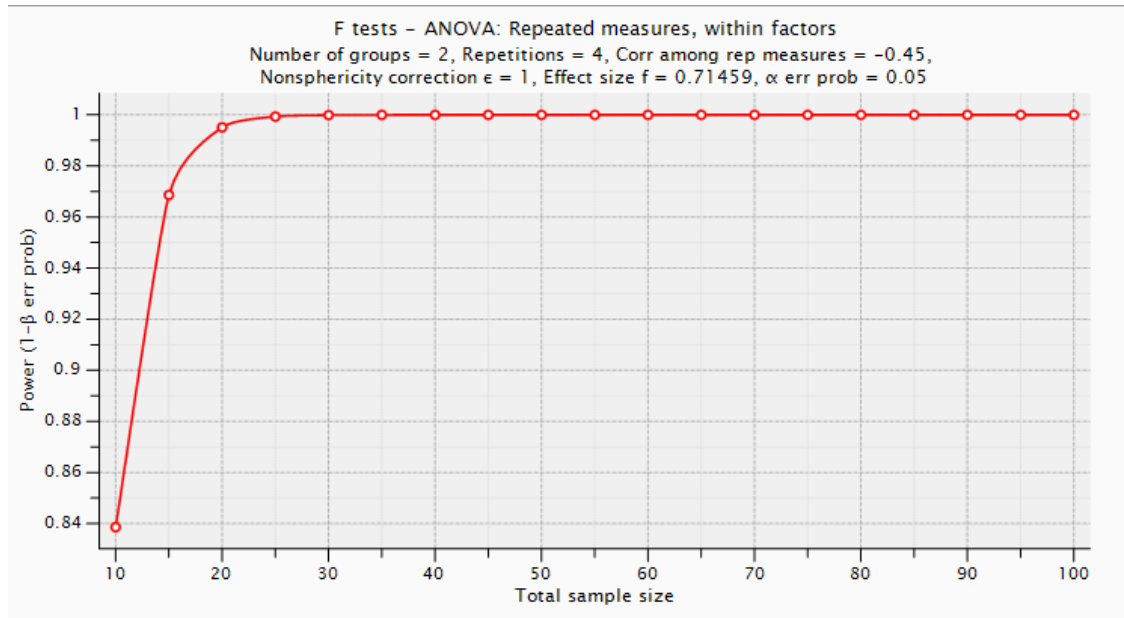


Figure 4-14. Power \times subject estimates of time main effect for reactive hyperemia index

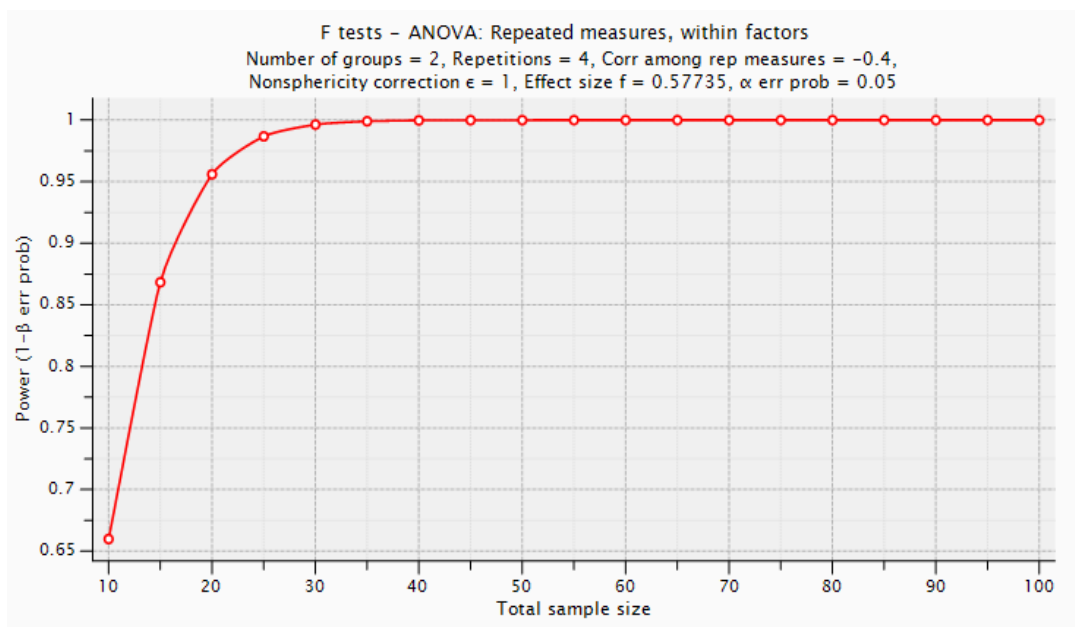


Figure 4-15. Power \times subject estimates of time main effect for thiobarbituric acid reactive substances

To reach an adequate power of 0.6 for regimen \times time interaction, 25 subjects

might be needed for reactive hyperemia index and 43 subjects might be needed for thiobarbituric acid reactive substances to detect statistical significance (Table 4-16 and 4-17).

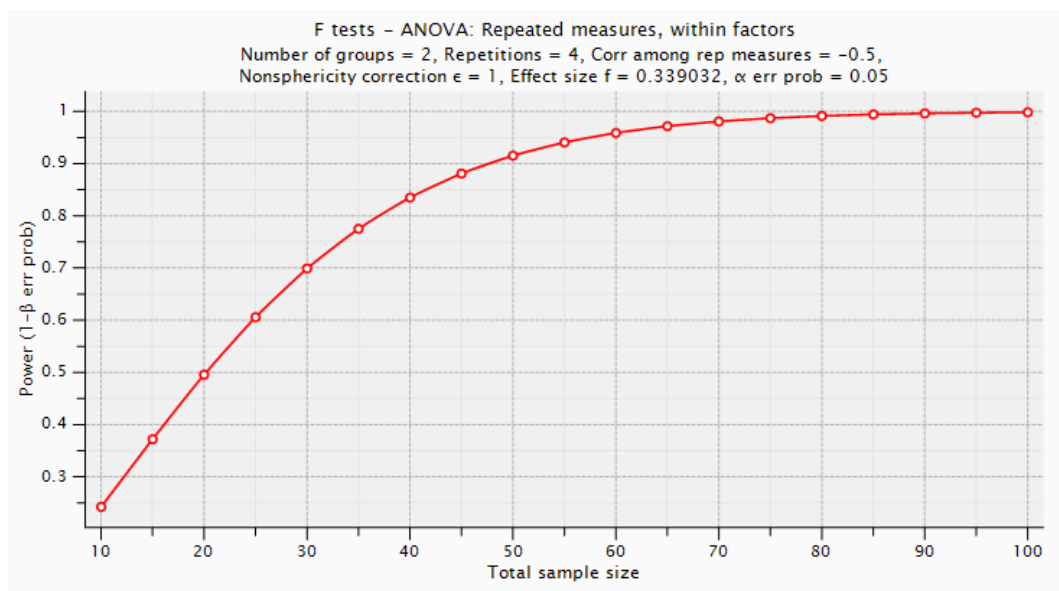


Figure 4-16. Power × subject estimates of feeding regimen × time interaction for reactive hyperemia index

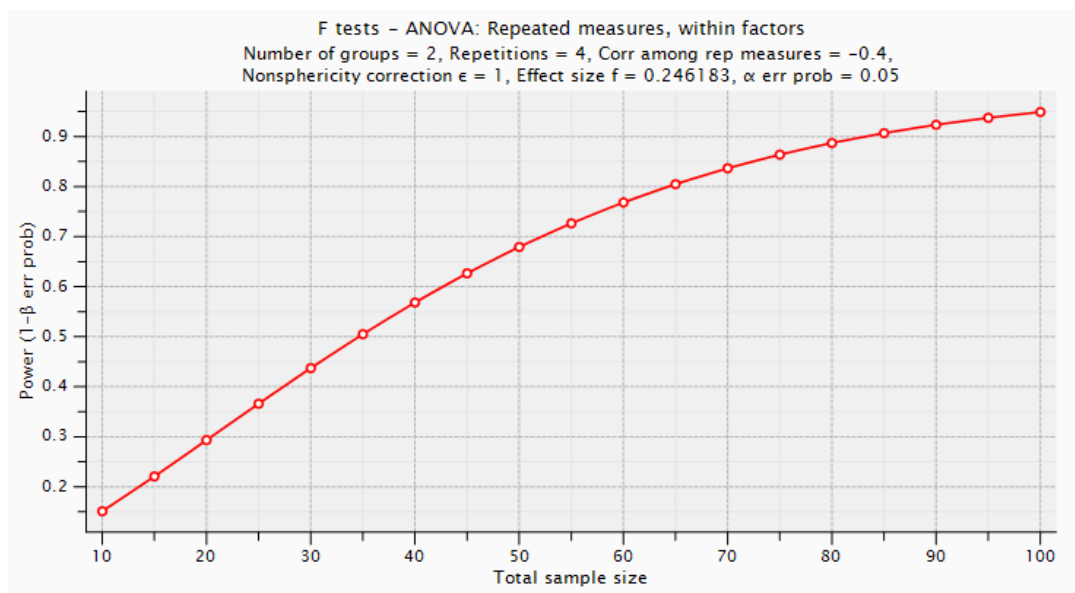


Figure 4-17. Power × subject estimates of feeding regimen × time interaction for thiobarbituric acid reactive substances

In summary, more subjects are needed to achieve adequate power and statistical

significance.

Effect Size Estimates

Effect sizes, analyzed in two Within Subject ANOVAs, are shown for each feeding regimen in Table 4-18. Regimen 2 shows a greater effect on reactive hyperemia index than regimen 1. The effect of both feeding regimens on thiobarbituric acid reactive substances measured at these time points was small. Both regimens have large effect on heart rate, but regimen 2 had a much greater effect compared to regimen 1.

Table 4-18. Effect size estimates, η^{p2} and ω^{2b} , for reactive hyperemia index, thiobarbituric acid reactive substances and heart rate

Time effect	η_p^{2a}	ω^{2b}
Regimen 1 (standard meal)		
Reactive hypremia index	0.12	0.00
Thiobarbituric acid reactive substances	0.21	0.03
Heart Rate	0.29	0.16
Regimen 2 (body surface area meal)		
Reactive hypremia index	0.32	0.07
Thiobarbituric acid reactive substances	0.13	0.01
Heart Rate	0.39	0.27

^a Partial eta squared is the proportion of the effect plus error variance that is attributable to the effect. Small effect ($\eta_p^2=0.01$); medium effect ($\eta_p^2=0.09$); large effect ($\eta_p^2=0.25$).

^b Omega squared is an estimate of the dependent variance accounted for by the independent variable in the population for a fixed effects model. Small effect ($\omega^2=0.01$); medium effect ($\omega^2=0.06$); large effect ($\omega^2=0.15$).

Chapter 5

DISCUSSION, CONCLUSIONS, IMPLEMENTATIONS AND

RECOMMENDATIONS

Discussion

There was not enough power to determine whether dietary regimens had different effects on postprandial vascular function, as shown by the insignificant main effect of feeding regimen ($p > 0.05$, $\omega^2 = 0$, $1 - \beta = 0.12$). Power was also too low to detect a significant time \times feeding regimen interaction ($p > 0.05$, $\omega^2 = 0$, $1 - \beta = 0.24$). Thus, no conclusions can be drawn as to whether the two feeding regimens affect the reactive hyperemia index differently over time. Additional subjects are needed to complete exploration of this model. Reactive hyperemia index has been investigated in other studies with the subject number ranging from 20 (Haller, et al., 2007) to 1957 (Hamburg, et al., 2008). The sensitivity and specificity of the EndoPAT were reported as 80% and 85% respectively using 94 subjects by Bonetti et al (2004), however, power and effect size in these studies were not reported.

This study found significant differences between the four time points ($p = 0.01$, $\omega^2 = 0.12$, $1 - \beta = 0.84$). Peripheral vascular function, as measured by reactive hyperemia index, before “breakfast” (0h) was significantly lower than that of 2h, 4h, and 6h (or 2 hours past “lunch”), respectively, and was significantly lower than the postprandial reactive hyperemia index pooled over 2h-6h (Table 4-9 and 4-10). This result occurred possibly because the meals caused an increase in hyperemic pulse wave amplitude or a decrease in resting pulse wave amplitude (numerator and denominator in reactive

hyperemia index).

The decrease in resting pulse wave amplitude is supported by several papers in the literature. Jakulj et al. (2007) found a high-fat meal increased total peripheral resistance (mean arterial pressure divided by cardiac output) which indicated increased vasoconstriction (Simon, et al., 1982). The increased vasoconstriction may decrease the resting pulse wave amplitude, just as shown by Nohria (2006) that a vasoconstriction drug (phenylephrine, nitric oxide inhibitor) decreased the resting pulse wave amplitude but not the final reactive hyperemia index. That the final index remained constant was possibly because the hyperemic pulse wave amplitude decreased at the same time due to suppression of nitric oxide production. In the case of the meal though, with no external nitric oxide inhibitor, the production of nitric oxide during hyperemia would continue to elevate the hyperemic pulse wave amplitude and thus elevate the index. The total peripheral resistance may increase while the brachial artery diameter increases, because changes in the brachial artery diameter is only a local response in the arm and cannot be used to represent the systemic changes in the body. Simon et al. (1982) found that a vasodilator (nitroglycerin) increased the brachial artery diameter but did not change the total peripheral resistance ($p < 0.05$).

The increase in hyperemic pulse wave amplitude may be explained by a heightened hyperemic nitric oxide production mediated by the increased postprandial digital blood flow due to the activation of sympathetic nerve system (Nohria, et al., 2006). Fingertips are rich in arteriovenous anastomoses and the amount of blood flow in the digits are reported to be largely affected by peripheral blood flow (Nohria, et al.,

2006). Raitakari et al. (2000) found an increased in resting brachial artery diameter and forearm blood flow ($p < 0.001$, $p < 0.001$) 3 hours and 6 hours after feeding 12 healthy subjects (7 males and 5 females, aged 33 ± 7 years) a high-fat meal, whereas the flow mediated dilation did not change over time ($p > 0.05$). Forearm blood flow was used as a measure of microvascular function, calculated from brachial artery diameter and mean blood velocity and tested by venous occlusion strain-gauge plethysmography. The meal contained 1030 kcal and consisted of a sausage, two muffins and two hash browns cooked in tallow fat, providing 48% saturated, 40% monounsaturated, 7.4% polyunsaturated, and 4.6% trans fatty acids. A similar result was found by Gokce et al. (2001), in which the high-fat meal induced a 36% increase in forearm blood flow ($p = 0.03$) at 2, 4, and 6 hours from a baseline.

Although these meals were rich in fat, the increased forearm blood flow might have been a carbohydrate-induced response. Young et al. (2010) found that a mixed-meal (527 ± 12 kilocalories) induced an increase in plasma glucose, insulin concentration, muscle sympathetic nerve activity, and forearm blood flow in healthy subjects. In addition, Berne et al. (1989) and Scott et al. (2002) found a strongly positive correlation between plasma insulin concentration and the muscle sympathetic nerve activity. These two factors facilitate the glucose uptake and the blood flow redistribution into the skeletal muscle (Scherrer, Randin, Vollenweider, Vollenweider, & Nicod, 1994). Forst et al. (2005) found that the microvascular response to acetylcholine was increased within the first postprandial hour ($p < 0.05$), indicating an increased nitric oxide-mediated vasodilation. Scherrer et al. (1994) found that a nitric

oxide inhibitor (N^{G} -monomethyl-L-arginine, L-NMMA) but not nitric oxide-independent vasoconstrictor (norepinephrine) blunted the increase in forearm blood flow ($p < 0.05$). Insulin-independent mechanisms should also be considered. Fujimura et al. (1997) found that meal consumption activated tension receptors in the stomach wall through vagal afferent fibres during stomach distension, thus increased muscle sympathetic nerve activity and subsequently increased superior mesenteric artery flow (O'Donovan, Feinle, Tonkin, Horowitz, & Jones, 2002; Rossi, et al., 1998).

In summary, two mechanisms are proposed to explain the increased reactive hyperemia index. First, the high fat in the meals may trigger the increase in total peripheral resistance which decreases resting pulse wave amplitude and thus increases the final index. Secondly, the carbohydrate component of the meal may increase the peripheral blood flow due to the heightened muscle sympathetic nerve activity mediated by insulin concentration and/or stomach distension. This may increase the nitric oxide production during hyperemic and thus increase the hyperemic pulse wave amplitude and subsequently lead to an elevated reactive hyperemia index.

High-fat meals induced increased reactive hyperemia index in our study, whereas they have been shown to decrease flow mediated dilation in other studies, as shown in Table 5-1. The increase in postprandial reactive hyperemia index may also be a transient effect due to short-term high-fat meal ingestion, because a study by Barringer et al. (2008) showed that 4 weeks of 1010 kcal high-fat meals led to a decreased reactive hyperemia index 3 hours post ingestion (-12.37%, $p = 0.005$). Thus long-term consumption of high-fat meals may assert more potent effects as endothelial

dysfunction.

In addition, the change in reactive hyperemia index 2 hours after “breakfast” was larger than the changes 2 hours after “lunch” ($p < 0.05$), in other words, the postprandial increase in reactive hyperemia index was significantly diminished after the second high-fat meal which may indicate impaired endothelial function (Table 4-9, Figure 4-8). A similar result was observed by Tushuizen et al. (2006), suggesting that flow mediated dilation of the brachial artery was significantly impaired after two consecutive high-fat meals compared to the baseline (Table 5-1).

Table 5-1. Summary of other research studies with healthy subjects that examine endothelial function in response to a high-fat meal

Author(year)	Subject	High-fat meal composition	Comments
Cuevas et al. (2000)	11 males 20-28 y BMI 20-25 kg/m ²	2565 kilocalories daily (39.9% from fat, 112.7±12.9 g fat with 35.8±9.6 g saturated fat)	Flow-mediated dilation decreased after 4-week of high-fat meals (p=0.014)
Plotnick et al. (2003)	38(14 males) 36.4±10.1 y	897 kilocalories (50.2% from fat, 50 g fat with 14 g saturated fat; consisted of 1 Egg McMuffin®, Sausage McMuffin®, 2 hash brown patties, and 1 non-caffeinated beverage.	Flow-mediated dilation decreased after 4-week of high-fat meals (p<0.05).
Berry et al. (2008)	17 males 18-40 y BMI 24.3±3 kg/m ²	850 kilocalories, 43.3 g fat with 42.5 g 18:1n-9, 4 g 18:2n-6, and 0.8 g stearic acid; consisted of 2 muffins and 1 chocolate flavored milkshake.	Brachial artery flow mediated dilation decreased (p<0.001) while the plasma 15-F ₂ -isoprostane increased 3 hours post meal (p<0.05).
Tushuizen et al. (2006)	17 males 25.4±3 y BMI 23.6±2 kg/m ²	900 kilocalories (50% from fat, 50 g fat with 30 g saturated fat). The first meal (at 8:30 am) consisted of 1 EggMcMuffin®, 1 croissant with butter and marmalade, 200 mL milk with 20 mL cream. The second meal (at 12:30 pm) consisted of 1 Quarterpounder® (McDonald's), 1 croissant with butter, and 200 mL milk.	After two consecutive high-fat meal flow mediated dilation was impaired significantly at 6 hour; plasma malondialdehyde (p=0.01) and the oxLDL/LDL cholesterol ratio (p=0.001) were increased.
Ayer et al. (2009)	11 obese and 11 healthy subjects (14 males, 32.1±6.3 y BMI 18-25 kg/m ²)	1000 kilocalories (60 g fat with a ratio of saturated fat 1.0 : monounsaturated fat 0.85 : polyunsaturated fat 0.39); consisted of a slice of carrot cake and a milkshake.	Flow mediated dilation, reactive hyperemia index, hyperemic forearm blood flow and brachial pulse wave velocity did not differ between healthy and obese subjects or 3 hours after the meal. Heart rate increased 3 hours post ingestion (p<0.001).

Our study also found that the heart rate, an index of cardiovascular reactivity, was increased 2 hours after “breakfast”, then decreased to baseline, and subsequently increased 2 hours after “lunch” ($p < 0.05$). This finding agreed with Jakulj et al. (2007) that meal consumption induced an increase in heart rate, blood pressure, stroke volume, total peripheral resistance, and cardiac output ($p < 0.05$), indicating an increased postprandial cardiovascular reactivity to stress. Jakulj (2007) also found that high-fat meal consumption may be associated with increased cardiovascular reactivity to behavioral stress tasks (mental arithmetic, public speech task, arm ischemia, and cold pressor) as the high-fat meal increased the total peripheral resistance ($p < 0.05$) compared with a low-fat meal. A possible mechanism was put forth based on results from prior studies that found increased cardiovascular reactivity led to peripheral vascular resistance which contributed to microvascular hypertension and microvessel damage in obese individuals (Mitchell, et al., 2005; Serne, de Jongh, Eringa, IJzerman, & Stehouwer, 2007) and formation of plaques on vessels walls eventually resulted in the reduction of vessel diameter and elasticity (Stamler, Wentworth, & Neaton, 1986). These changes may not apply to healthy populations.

The marker of oxidative stress (thiobarbituric acid reactive substances) used in this study was detected in the range of $0.40 \mu\text{mol/L}$ to $0.85 \mu\text{mol/L}$. A similar range was observed in a study by Yesilova et al. (2005), in which fasting plasma thiobarbituric acid reactive substances were significantly higher in patients with nonalcoholic fatty liver disease ($0.5 \pm 0.2 \mu\text{mol/L}$, $n=51$, male, ages 20-65 y, BMI $28.12 \pm 4.06 \text{ kg/m}^2$) compared to healthy controls ($0.36 \pm 0.09 \mu\text{mol/L}$, $n=30$, male, ages 20-60 y, BMI

27.54 ± 3.27 kg/m²). Many other studies have reported higher values of thiobarbituric acid reactive substances. For example, Richard et al. (1992) and Yagi (1998) found that normal human plasma contains 1.86-3.94 µmol/L thiobarbituric acid reactive substances; Oteiza (1997) found that fasting plasma thiobarbituric acid reactive substances were significantly higher in patients with sporadic amyotrophic lateral sclerosis (1.30 ± 0.1 µmol/L, n=13, 6 males and 7 females, ages 22-66 y) compared to healthy controls (1.03 ± 0.06 µmol/L, n=11, 8 males and 3 females, ages 32-66 y). Chalasani et al. (2004) found that fasting plasma thiobarbituric acid reactive substances were significantly increased in patients with nonalcoholic steatohepatitis (3.4 ± 1.3 µmol/L, n=21, 11 males and 10 females, ages 41 ± 13 y, BMI 33 ± 4 kg/m²) compared to healthy controls (1.8 ± 0.9 µmol/L, n=19, 9 males and 10 females, ages 43 ± 14 y, BMI 31 ± 4 kg/m²).

As with reactive hyperemia index, this study did not have enough power to determine whether dietary regimens had different effects on postprandial oxidative stress, as shown by the insignificant main effect of feeding regimen ($p=0.17$, $\omega^2=0.01$, $1-\beta=0.27$). Power was also too low to detect a significant time × feeding regimen interaction ($p>0.05$, $\omega^2=0$, $1-\beta=0.24$). Thus, no conclusions can be drawn as to whether the two feeding regimens impact the reactive hyperemia index differently over time. Similarly, the power was also not high enough to detect a significant time × feeding regimen interaction ($p=0.65$, $\omega^2=0$, $1-\beta=0.15$). Thus it is not known whether feeding standard meals or those based on body size affect oxidative stress differently over time.

There were significant differences between the four time points ($p=0.04$, $\omega^2=0.07$,

1- β =0.66). Thiobarbituric acid reactive substances increased from 0 hour to 2 hour ($p<0.05$) and decreased from 2 hour to 4 hour after “breakfast” ($p<0.05$, Table 4-14). These results are similar to findings by Tinahones et al. (2008), where postprandial plasma thiobarbituric acid reactive substances were significantly higher at 3 hours post ingestion of a high-fat meal ($1.31 \pm 0.72 \mu\text{mol/L}$) compared to baseline ($1.02 \pm 0.7 \mu\text{mol/L}$) in severely obese subjects ($n=29$, 9 males and 20 females, ages 44.9 ± 8.6 y, BMI $54.5 \pm 6.4 \text{ kg/m}^2$). The increase in postprandial thiobarbituric acid reactive substances indicated heightened oxidative stress 2 hours after a high-fat meal as described in prior research (Armstrong & Browne, 1994; Dahle, et al., 1962; Yagi, 1998). There was not an increase in thiobarbituric acid reactive substances after the second high-fat meal at 6h. This indicated no elevated oxidative stress after the second high-fat meal. This result is contrast to findings by Tushuizen et al. (2006) in which thiobarbituric acid reactive substances were increased after two consecutive 900 kcal high-fat meal compared to the fasting state ($p=0.01$). The discrepancy may be due to increased antioxidant defense, such as by superoxide dismutase, an enzyme that converts the superoxide radical into hydrogen peroxide and is subsequently metabolized into water and oxygen by catalase and glutathione peroxidase (Andreazza, et al., 2008). Prior (2005) suggested a non-enzymatic defense, such as ascorbic acid, tocopherol, carotenoids, polyphenols, and uric acid to reduce oxidative load. This discrepancy may also result from the inconsistency of the semi-quantitative thiobarbituric reactive substances assay (Armstrong & Browne, 1994).

The power analyses indicated that at least 50 subjects are needed to study reactive

hyperemia index and 43 subjects to study thiobarbituric acid reactive substances to improve the statistical power and the ability to detect significance. Although the main effect of feeding regimen for each dependent variable was not significantly different, there was an increase in reactive hyperemia index from 0h to 6h in the body surface area-based meal regimen (planned comparison, $p < 0.05$, Table 4-8), whereas not in the standard meal. In addition, the body surface area-based regimen showed a greater effect on reactive hyperemia index ($\eta_p^2 = 0.32$, $\omega^2 = 0.07$) and heart rate ($\eta_p^2 = 0.39$, $\omega^2 = 0.27$) than regimen 1 ($\eta_p^2 = 0.12$, $\omega^2 = 0$; $\eta_p^2 = 0.29$, $\omega^2 = 0.16$), as shown in Table 4-17. The body surface area-based meal may be a more effective model to study the vascular reactivity in comparison to the standard meal; however, more subjects are needed to confirm this finding.

The effect of both feeding regimens on thiobarbituric acid reactive substances measured at these time points was small ($\omega^2 < 0.06$). It is possible the effect is small because the peak of the oxidative stress may have been missed using these testing time points, therefore, other time points, such as 3h and 8h, may be considered. The small effect size and the large estimated subject number ($n = 43$) also indicated that thiobarbituric acid reactive substances may not be a sensitive marker of oxidative stress in this model. Other biomarkers, such as F₂-isoprostane and hydrogen peroxide, may be more useful to reflect postprandial oxidative stress.

Conclusions

1. Postprandial vascular reactivity (reactive hyperemia index) increased from fasting state to postprandial state ($p < 0.05$), possibly due to increased total peripheral

resistance induced by the high fat content or increased peripheral blood flow induced by the elevated muscle sympathetic nerve activity, insulin response and/or stomach distension after “breakfast” and “lunch”. Endothelial function was impaired as reflected in smaller increase in reactive hyperemia index after the second high-fat meal “lunch” than after the first high-fat meal “breakfast”(p<0.05).

2. Oxidative stress (thiobarbituric acid reactive substances) was elevated 2 hours after “breakfast” (p<0.05) and decreased by 4 hours after “breakfast” (p<0.05).

3. The feeding regimen based on body surface area may be a more effective model to use than the standard meal as shown by the greater effect size (η_p^2 and ω^2). More subjects are needed to confirm this finding.

Implementations and Recommendations

The study used automated peripheral arterial tonometry (via EndoPAT 2000), as a substitute for flow-mediated dilation to assess the endothelial-dependent vasodilation, to avoid the operator-dependent bias in the ultrasound measurement and correct for uncontrollable physiological and environmental variables.

Consumption of two high-fat meals in healthy young adult males leads to endothelial dysfunction, perhaps because the high-fat meal induces oxidative stress through metabolism of cellular fatty acids as well as exogenous dietary fatty acids which increase production of modified proteins, fatty acids and DNA. The protocol studied here focused on understanding the repeated eating and snacking/meal pattern that extends the postprandial oxidative stress to a significant part of a day. The same

model could be applied to study a different population such as overweight or patients with diabetes mellitus to understand how vascular function is affected by chronic diseases and health complications.

To obtain a more comprehensive view of the oxidative status in response to the high-fat meal, supplementary oxidative stress markers may be considered, for example F₂-isoprostanes, oxidized LDL, hydrogen peroxide, and superoxide dismutase enzyme. Markers to measure the body's ability to quench oxidative stress, such as total antioxidant capacity, may also be useful in understanding the effects of a prolonged postprandial state.

Blood pressure and total peripheral resistance should be measured along with endothelial function and blood biomarkers to demonstrate the effects of the high-fat meal on cardiovascular reactivity. Plasma insulin concentration and peripheral blood flow should be measured to prove the mechanism of how high-fat meals affect the endothelial function. Based on these data, future studies with more subjects may provide adequate power to detect differences in healthy young adult males. The feeding regimen based on body surface area may be a more effective model to use due to the larger effect size.

REFERENCE

- American Diabetes Association (2010). High blood glucose, from <http://www.diabetes.org/diabetes-basics/prevention/checkup-america/highbg.html>
- American Heart Association (2010). What Your Cholesterol Levels Mean, 2010, from <http://www.americanheart.org/presenter.jhtml?identifier=183#HDL>
- Anderson, J., Grande, F., & Keys, A. (1976). Independence of the effects of cholesterol and degree of saturation of the fat in the diet on serum cholesterol in man. *The American journal of clinical nutrition*, 29(11), 1184-1189.
- Anderson, R., Evans, M., Ellis, G., Graham, J., Morris, K., Jackson, S., et al. (2001). The relationships between post-prandial lipaemia, endothelial function and oxidative stress in healthy individuals and patients with type 2 diabetes. *Atherosclerosis*, 154(2), 475-483.
- Andreazza, A., Kauer-Sant'anna, M., Frey, B., Bond, D., Kapczynski, F., Young, L., et al. (2008). Oxidative stress markers in bipolar disorder: a meta-analysis. *Journal of Affective Disorders*, 111(2-3), 135-144.
- Armstrong, D., & Browne, R. (1994). The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory. *Advances in Experimental Medicine and Biology*, 366, 43-58.
- Bae, J., Bassenge, E., Kim, K., Kim, Y., Kim, K., Lee, H., et al. (2001). Postprandial hypertriglyceridemia impairs endothelial function by enhanced oxidant stress. *Atherosclerosis*, 155(2), 517-523.
- Barringer, T. A., Hatcher, L., & Sasser, H. C. (2008). Potential Benefits on Impairment of Endothelial Function after a High-fat Meal of 4 weeks of Flavonoid Supplementation. *Evidence-based Complementary and Alternative Medicine: eCAM*.
- Barzilai, A., & Yamamoto, K. (2004). DNA damage responses to oxidative stress. *DNA Repair*, 3(8-9), 1109-1115.
- Baudet, M., Datchet, C., Lasserre, M., Esteva, O., & Jacotot, B. (1984). Modification in the composition and metabolic properties of human low density and high density lipoproteins by different dietary fats. *Journal of lipid research*, 25(5), 456-468.
- Becker, N., Illingworth, D., Alaupovic, P., Connor, W., & Sundberg, E. (1983). Effects of saturated, monounsaturated, and omega-6 polyunsaturated fatty acids on

plasma lipids, lipoproteins, and apoproteins in humans. *The American journal of clinical nutrition*, 37(3), 355-360.

Berdeaux, O., Scruel, O., Cracowski, J., & Durand, T. (2006). F2-Isoprostanes: review of analytical methods. *Current Pharmaceutical Analysis*, 2, 69-78.

Berne, C., Fagius, J., & Niklasson, F. (1989). Sympathetic response to oral carbohydrate administration. Evidence from microelectrode nerve recordings. *Journal of Clinical Investigation*, 84(5), 1403-1409.

Berry, S., Tucker, S., Banerji, R., Jiang, B., Chowienczyk, P., Charles, S., et al. (2008). Impaired postprandial endothelial function depends on the type of fat consumed by healthy men. *Journal of Nutrition*, 138(10), 1910-1914.

Bhagat, K., Hingorani, A., & Vallance, P. (1997). Flow associated or flow mediated dilatation? More than just semantics. *Heart (British Cardiac Society)*, 78(1), 7-8.

Blomhoff, R. (2005). Dietary antioxidants and cardiovascular disease. *Current Opinion in Lipidology*, 16(1), 47-54.

Bonetti, P. O., Barsness, G. W., Keelan, P. C., Schnell, T. I., Pumper, G. M., Kuvin, J. T., et al. (2003). Enhanced external counterpulsation improves endothelial function in patients with symptomatic coronary artery disease. *Journal of the American College of Cardiology*, 41(10), 1761-1768.

Bonetti, P. O., Lerman, L. O., & Lerman, A. (2003). Endothelial dysfunction: a marker of atherosclerotic risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 23(2), 168-175.

Bonetti, P. O., Pumper, G. M., Higano, S. T., Holmes, D. R., & Lerman, A. (2002). Reactive hyperemia peripheral arterial tonometry, a novel non-invasive index of peripheral vascular function, is attenuated in patients with coronary endothelial dysfunction. *Circulation*, 106, 579.

Bonetti, P. O., Pumper, G. M., Higano, S. T., Holmes, D. R. J., & Kuvin, J. T., Lerman, A. (2004). Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. *Journal of the American College of Cardiology*, 44(11), 2137-2141.

Bowen, P., & Borthakur, G. (2004). Postprandial lipid oxidation and cardiovascular disease risk. *Current Atherosclerosis Reports*, 6(6), 477-484.

Bruns, R., & Palade, G. (1968). Studies on blood capillaries I. General organization of blood capillaries in muscle. *Journal of Cell Biology*, 37(2), 244-276.

Buege, J., & Aust, S. (1978). Microsomal lipid peroxidation. *Methods in enzymology*, 52, 302-310.

Calder, P. (2005). Polyunsaturated fatty acids and inflammation. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, 75(3), 197-202.

Cangemi, R., Angelico, F., Loffredo, L., Del Ben, M., Pignatelli, P., Martini, A., et al. (2007). Oxidative stress-mediated arterial dysfunction in patients with metabolic syndrome: effect of ascorbic acid. *Free Radical Biology and Medicine*, 43(5), 853-859.

Celermajer, D., Sorensen, K., Spiegelhalter, D., Georgakopoulos, D., Robinson, J., & Deanfield, J. (1994). Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *Journal of the American College of Cardiology*, 24(2), 471-476.

Ceriello, A., Assaloni, R., Da Ros, R., Maier, A., Piconi, L., Quagliaro, L., et al. (2005). Effect of atorvastatin and irbesartan, alone and in combination, on postprandial endothelial dysfunction, oxidative stress, and inflammation in type 2 diabetic patients. *Circulation*, 111(19), 2518-2524.

Ceriello, A., Quagliaro, L., Piconi, L., Assaloni, R., Da Ros, R., Maier, A., et al. (2004). Effect of postprandial hypertriglyceridemia and hyperglycemia on circulating adhesion molecules and oxidative stress generation and the possible role of simvastatin treatment. *Diabetes*, 53(3), 701-710.

Ceriello, A., Taboga, C., Tonutti, L., Quagliaro, L., Piconi, L., Bais, B., et al. (2002). Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: effects of short-and long-term simvastatin treatment. *Circulation*, 106(10), 1211-1218.

Chalasani, N., Deeg, M., & Crabb, D. (2004). Systemic levels of lipid peroxidation and its metabolic and dietary correlates in patients with nonalcoholic steatohepatitis. *The American journal of gastroenterology*, 99(8), 1497-1502.

Chobanian, A., Bakris, G., Black, H., Cushman, W., Green, L., Izzo Jr, J., et al. (2003). Seventh report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. *Hypertension*, 42(6), 1206.

Cirak, B., Inci, S., Palaoglu, S., & Bertan, V. (2003). Lipid peroxidation in cerebral tumors. *Clinica Chimica Acta*, 327(1-2), 103-107.

Corretti, M., Anderson, T., Benjamin, E., Celermajer, D., Charbonneau, F., Creager, M., et al. (2002). Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery a report of the International Brachial Artery Reactivity Task Force. *Journal of the American College*

of Cardiology, 39(2), 257-265.

Cox, D., Vita, J., Treasure, C., Fish, R., Alexander, R., Ganz, P., et al. (1989). Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. *Circulation*, 80(3), 458-465.

Cuevas, A., Guasch, V., Castillo, O., Iribarra, V., Mizon, C., San Martin, A., et al. (2000). A high-fat diet induces and red wine counteracts endothelial dysfunction in human volunteers. *Lipids*, 35(2), 143-148.

Dahle, L. K., Hill, E. G., & Holman, R. T. (1962). The thiobarbituric acid reaction and the autoxidations of polyunsaturated fatty acid methyl esters. *Archives of Biochemistry and Biophysics*, 98, 253-261.

Davies, K. (1987). Protein damage and degradation by oxygen radicals. I. general aspects. *Journal of Biological Chemistry*, 262(20), 9895-9901.

Davignon, J., & Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. *Circulation*, 109(23), 27-32.

Deanfield, J. E., Halcox, J. P., & Rabelink, T. J. (2007). Endothelial function and dysfunction: testing and clinical relevance. *Circulation*, 115(10), 1285-1295.

Dhindsa, M., Sommerlad, S., DeVan, A., Barnes, J., Sugawara, J., Ley, O., et al. (2008). Interrelationships among noninvasive measures of postischemic macro- and microvascular reactivity. *Journal of Applied Physiology*, 105(2), 427-432.

Dinarello, C. (2000). Proinflammatory cytokines. *Chest*, 118(2), 503-508.

Dixon, Z., Burri, B., Clifford, A., Frankel, E., Schneeman, B., Parks, E., et al. (1994). Effects of a carotene-deficient diet on measures of oxidative susceptibility and superoxide dismutase activity in adult women. *Free Radical Biology & Medicine*, 17(6), 537-544.

Donald, A., Halcox, J., Charakida, M., Storry, C., Wallace, S., Cole, T., et al. (2008). Methodological approaches to optimize reproducibility and power in clinical studies of flow-mediated dilation. *Journal of the American College of Cardiology*, 51(20), 1959-1964.

Dotan, Y., Lichtenberg, D., & Pinchuk, I. (2004). Lipid peroxidation cannot be used as a universal criterion of oxidative stress. *Progress in Lipid Research*, 43(3), 200-227.

Erdfelder, E., Faul, F., & Buchner, A. (1996). GPOWER: A general power analysis program. *Behavior Research Methods, Instruments & Computers*, 28(1), 1-11.

Esposito, K., Nappo, F., Giugliano, F., Giugliano, G., Marfella, R., & Giugliano, D. (2003). Effect of dietary antioxidants on postprandial endothelial dysfunction induced by a high-fat meal in healthy subjects. *American Journal of Clinical Nutrition*, 77(1), 139-143.

Esposito, K., Nappo, F., Giugliano, F., Palo, C., Ciotola, M., Barbieri, M., et al. (2003). Meal modulation of circulating interleukin 18 and adiponectin concentrations in healthy subjects and in patients with type 2 diabetes mellitus. *American Journal of Clinical Nutrition*, 78(6), 1135-1140.

Esposito, K., Nappo, F., Marfella, R., Giugliano, G., Giugliano, F., Ciotola, M., et al. (2002). Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. *Circulation*, 106(16), 2067-2072.

Esterbauer, H., Schaur, R., & Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology & Medicine*, 11(1), 81-128.

Faraci, F., & Didion, S. (2004). Vascular protection: superoxide dismutase isoforms in the vessel wall. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 24(8), 1367-1373.

Feelisch, M., Rassaf, T., Mnaimneh, S., Singh, N., Bryan, N., Jourd'Heuil, D., et al. (2002). Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *The FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 16(13), 1775-1785.

Fisher, N., & Hollenberg, N. (2006). Aging and vascular responses to flavanol-rich cocoa. *Journal of Hypertension*, 24(8), 1575-1580.

Fisher, N., Hughes, M., Gerhard-Herman, M., & Hollenberg, N. (2003). Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. *Journal of Hypertension*, 21(12), 2281-2286.

Forst, T., Forst, S., Strunk, K., Löig, M., Welter, K., Kazda, C., et al. (2005). Impact of insulin on microvascular blood flow and endothelial cell function in the postprandial state in patients with Type 1 diabetes. *Journal of diabetes and its complications*, 19(3), 128-132.

Fridovich, I. (1986). Biological effects of the superoxide radical. *Archives of Biochemistry and Biophysics*, 247(1), 1-11.

Fujimura, J., Camilleri, M., Low, P., Novak, V., Novak, P., & Opfer-Gehrking, T. (1997). Effect of perturbations and a meal on superior mesenteric artery flow in patients with orthostatic hypotension. *Journal of the autonomic nervous system*, 67(1-2), 15-23.

Gabrielsen, A., & Norsk, P. (2007). Effect of spaceflight on the subcutaneous venoarteriolar reflex in the human lower leg. *Journal of Applied Physiology* 103(3), 959-962.

Gerhard, M., Walsh, B., Tawakol, A., Haley, E., Creager, S., Seely, E., et al. (1998). Estradiol therapy combined with progesterone and endothelium-dependent vasodilation in postmenopausal women. *Circulation*, 98(12), 1158-1163.

Giannattasio, C., Zoppo, A., Gentile, G., Failla, M., Capra, A., Maggi, F., et al. (2005). Acute effect of high-fat meal on endothelial function in moderately dyslipidemic subjects. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 25(2), 406-410.

Gokce, N., Duffy, S., Hunter, L., Keaney, J., & Vita, J. (2001). Acute hypertriglyceridemia is associated with peripheral vasodilation and increased basal flow in healthy young adults. *The American Journal of Cardiology*, 88(2), 153-159.

Goodhart, D., & Anderson, T. (1998). Role of nitric oxide in coronary arterial vasomotion and the influence of coronary atherosclerosis and its risks. *American Journal of Cardiology*, 82(9), 1034-1039.

Griffin, B. (2008). How relevant is the ratio of dietary n-6 to n-3 polyunsaturated fatty acids to cardiovascular disease risk? Evidence from the OPTILIP study. *Current Opinion in Lipidology*, 19(1), 57-62.

Grotto, D., Maria, L., Valentini, J., Paniz, C., Schmitt, G., Garcia, S., et al. (2009). Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. *Química Nova*, 32, 169-174.

Grune, T., Reinheckel, T., & Davies, K. (1997). Degradation of oxidized proteins in mammalian cells. *The FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 11(7), 526-534.

Hall, W., Sanders, K., Sanders, T., & Chowienczyk, P. (2008). A high-fat meal enriched with eicosapentaenoic acid reduces postprandial arterial stiffness measured by digital volume pulse analysis in healthy men. *Journal of Nutrition*, 138(2), 287-291.

Haller, M. J., Stein, J., Shuster, J., Theriaque, D., Silverstein, J., Schatz, D. A., et al. (2007). Peripheral artery tonometry demonstrates altered endothelial function in children with type 1 diabetes. *Pediatric Diabetes*, 8(4), 193-198.

Halliwell, B., & Gutteridge, J. (1999). Free radicals, other reactive species and disease. *Free Radicals in Biology and Medicine*, 617-683.

Hamburg, N., & Benjamin, E. (2009). Assessment of endothelial function using digital pulse amplitude tonometry. *Trends in Cardiovascular Medicine*, 19(1), 6-11.

- Hamburg, N., Keyes, M., Larson, M., Vasan, R., Schnabel, R., Pryde, M., et al. (2008). Cross-sectional relations of digital vascular function to cardiovascular risk factors in the Framingham Heart Study. *Circulation*, *117*(19), 2467-2474.
- Hashimoto, M., Akishita, M., Eto, M., Ishikawa, M., Kozaki, K., Toba, K., et al. (1995). Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation*, *92*(12), 3431-3435.
- Hegsted, D., McGandy, R., Myers, M., & Stare, F. (1965). Quantitative effects of dietary fat on serum cholesterol in man. *The American journal of clinical nutrition*, *17*(5), 281-295.
- Heiss, C., Kleinbongard, P., Dejam, A., S, P., Schroeter, H., Sies, H., et al. (2005). Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. *Journal of the American College of Cardiology*, *46*(7), 1276-1283.
- Hu, F., Stampfer, M., Manson, J., Rimm, E., Colditz, G., Rosner, B., et al. (1997). Dietary fat intake and the risk of coronary heart disease in women. *The New England Journal of Medicine*, *337*(21), 1491-1499.
- Huang, A., & Vita, J. (2006). Effects of systemic inflammation on endothelium-dependent vasodilation. *Trends in Cardiovascular Medicine*, *16*(1), 15-20.
- Jakulj, F., Zernicke, K., Bacon, S., van Wielingen, L., Key, B., West, S., et al. (2007). A high-fat meal increases cardiovascular reactivity to psychological stress in healthy young adults. *Journal of Nutrition*, *137*(4), 935-939.
- Jenny, N., Arnold, A., Kuller, L., Sharrett, A., Fried, L., Psaty, B., et al. (2006). Soluble intracellular adhesion molecule-1 is associated with cardiovascular disease risk and mortality in older adults. *Journal of Thrombosis and Haemostasis*, *4*(1), 107-113.
- Kalantaridou, S., Naka, K., Bechlioulis, A., Makrigrannakis, A., Michalis, L., & Chrousos, G. (2006). Premature ovarian failure, endothelial dysfunction and estrogen-progestogen replacement. *Trends in Endocrinology & Metabolism*, *17*(3), 101-109.
- Kawano, H., Motoyama, T., Ohgushi, M., Kugiyama, K., Ogawa, H., & Yasue, H. (2001). Menstrual cyclic variation of myocardial ischemia in premenopausal women with variant angina. *Annals of Internal Medicine*, *135*(11), 977-981.
- Keppel, G. (2004a). Effect size, power, and sample size. In J. Heffler & L. Jewell (Eds.), *Design and analysis: A researcher's handbook* (4th ed., pp. 159-180). Upper Saddle River, NJ: Pearson Education.
- Keppel, G. (2004b). Further within-subject topics. In J. Heffler & L. Jewell (Eds.),

Design and analysis: A researcher's handbook (4th ed., pp. 369-399). Upper Saddle River, NJ: Pearson Education.

Keppel, G. (2004c). The linear model and its assumptions. In J. Heffler & L. Jewell (Eds.), *Design and analysis: A researcher's handbook* (4th ed., pp. 132-158). Upper Saddle River, NJ: Pearson Education.

Keppel, G. (2004d). The two-factor within-subject design. In J. Heffler & L. Jewell (Eds.), *Design and analysis: A researcher's handbook* (4th ed., pp. 401-431). Upper Saddle River, NJ: Pearson Education.

Kesavulu, M., Rao, B., Giri, R., Vijaya, J., Subramanyam, G., & Apparao, C. (2001). Lipid peroxidation and antioxidant enzyme status in type 2 diabetics with coronary heart disease. *Diabetes Research and Clinical Practice*, 53(1), 33-39.

Kingwell, B. (2000). Nitric oxide-mediated metabolic regulation during exercise: effects of training in health and cardiovascular disease. *The FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 14(12), 1685-1696.

Knasmüller, S., Nersesyan, A., Mišik, M., Gerner, C., Mikulits, W., Ehrlich, V., et al. (2008). Use of conventional and-omics based methods for health claims of dietary antioxidants: a critical overview. *British Journal of Nutrition*, 99(E-S1), ES3-52.

Kushnareva, Y., Murphy, A., & Andreyev, A. (2002). Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD (P)⁺ oxidation-reduction state. *Biochemical Journal*, 368(Pt 2), 545-553.

Kuvin, J. T., Patel, A. R., Sliney, K. A., Pandian, N. G., Sheffy, J., Schnall, R. P., et al. (2003). Assessment of peripheral vascular endothelial function with finger arterial pulse wave amplitude. *American Heart Journal*, 146(1), 168-174.

Lerman, A., & Burnett Jr, J. (1992). Intact and altered endothelium in regulation of vasomotion. *Circulation*, 86(6 Suppl), 12-19.

Lerman, A., & Zeiher, A. (2005). Endothelial function: cardiac events. *Circulation*, 111(3), 363-368.

Levine, R., & Stadtman, E. (2001). Oxidative modification of proteins during aging. *Experimental Gerontology*, 36(9), 1495-1502.

Lloyd-Jones, D., Adams, R., Carnethon, M., De Simone, G., Ferguson, T., Flegal, K., et al. (2009). Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*, 119(3), 480-486.

- Ludmer, P., Selwyn, A., Shook, T., Wayne, R., Mudge, G., Alexander, R., et al. (1986). Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *The New England journal of medicine*, 315(17), 1046-1051.
- Madamanchi, N. R., Vendrov, A., & Runge, M. S. (2005). Oxidative stress and vascular disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 25(1), 29-38.
- Malek, A., Alper, S., & Izumo, S. (1999). Hemodynamic shear stress and its role in atherosclerosis. *The Journal of the American Medical Association*, 282(21), 2035-2042.
- Marfella, R., Quagliari, L., Nappo, F., Ceriello, A., & Giugliano, D. (2001). Acute hyperglycemia induces an oxidative stress in healthy subjects. *Journal of Clinical Investigation*, 108(4), 635-636.
- Marfella, R., Verrazzo, G., Acampora, R., La Marca, C., Giunta, R., Lucarelli, C., et al. (1995). Glutathione reverses systemic hemodynamic changes induced by acute hyperglycemia in healthy subjects. *American Journal of Physiology- Endocrinology And Metabolism*, 268(6), 1167-1173.
- Margioris, A. (2009). Fatty acids and postprandial inflammation. *Current Opinion in Clinical Nutrition and Metabolic Care*, 12(2), 129-137.
- Masaki, T. (2004). Historical review: endothelin. *Trends in Pharmacological Sciences*, 25(4), 219-224.
- Mensink, R., Zock, P., Kester, A., & Katan, M. (2003). Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *The American journal of clinical nutrition*, 77(5), 1146-1155.
- Mitchell, G., Vita, J., Larson, M., Parise, H., Keyes, M., Warner, E., et al. (2005). Cross-sectional relations of peripheral microvascular function, cardiovascular disease risk factors, and aortic stiffness: the Framingham Heart Study. *Circulation*, 112(24), 3722-3728.
- Mosteller, R. (1987). Simplified calculation of body-surface area. *The New England Journal of Medicine*, 317(17), 1098.
- Mozaffarian, D., Ascherio, A., Hu, F., Stampfer, M., Willett, W., Siscovick, D., et al. (2005). Interplay between different polyunsaturated fatty acids and risk of coronary heart disease in men. *Circulation*, 111(2), 157.
- Muntwyler, J., Sutsch, G., Kim, J., Schmid, H., Follath, F., Kiowski, W., et al. (2001). Post-prandial lipaemia and endothelial function among healthy men. *Swiss Medical Weekly*, 131(15/16), 214-218.

- Nahrendorf, M., Jaffer, F., Kelly, K., Sosnovik, D., Aikawa, E., Libby, P., et al. (2006). Noninvasive vascular cell adhesion molecule-1 imaging identifies inflammatory activation of cells in atherosclerosis. *Circulation*, *114*(14), 1504-1511.
- Nappo, F., Esposito, K., Cioffi, M., Giugliano, G., Molinari, A., Paolisso, G., et al. (2002). Postprandial endothelial activation in healthy subjects and in type 2 diabetic patients: role of fat and carbohydrate meals. *Journal of the American College of Cardiology*, *39*(7), 1145-1150.
- Neri, S., Signorelli, S., Torrisi, B., Pulvirenti, D., Mauceri, B., Abate, G., et al. (2005). Effects of antioxidant supplementation on postprandial oxidative stress and endothelial dysfunction: a single-blind, 15-day clinical trial in patients with untreated type 2 diabetes, subjects with impaired glucose tolerance, and healthy controls. *Clinical Therapeutics*, *27*(11), 1764-1773.
- Nestel, P., Pomeroy, S., Sasahara, T., Yamashita, T., Liang, Y., Dart, A., et al. (1997). Arterial compliance in obese subjects is improved with dietary plant n-3 fatty acid from flaxseed oil despite increased LDL oxidizability. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *17*(6), 1163-1170.
- Nohria, A., Gerhard-Herman, M., Creager, M., Hurley, S., Mitra, D., & Ganz, P. (2006). Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. *Journal of Applied Physiology*, *101*(2), 545-548.
- O'Donovan, D., Feinle, C., Tonkin, A., Horowitz, M., & Jones, K. (2002). Postprandial hypotension in response to duodenal glucose delivery in healthy older subjects. *The Journal of Physiology*, *540*(Pt 2), 673-679.
- Oteiza, P., Uchitel, O., Carrasquedo, F., Dubrovski, A., Roma, J., & Fraga, C. (1997). Evaluation of antioxidants, protein, and lipid oxidation products in blood from sporadic amyotrophic lateral sclerosis patients. *Neurochemical research*, *22*(4), 535-539.
- Padilla, J., Harris, R., Fly, A., Rink, L., & Wallace, J. (2006). The effect of acute exercise on endothelial function following a high-fat meal. *European Journal of Applied Physiology*, *98*(3), 256-262.
- Palade, G. (1953). Fine structure of blood capillaries. *Journal of Applied Physiology*, *24*(1), 1424-1436.
- Park, J., Ferrell, R., Park, J., Hagberg, J., Phares, D., Jones, J., et al. (2005). NADPH oxidase p22phox gene variants are associated with systemic oxidative stress biomarker responses to exercise training. *Journal of Applied Physiology* *99*(5), 1905-1911.
- Paschos, G., Rallidis, L., Liakos, G., Panagiotakos, D., Anastasiadis, G., Votteas,

V., et al. (2004). Background diet influences the anti-inflammatory effect of alpha-linolenic acid in dyslipidaemic subjects. *The British Journal of Nutrition*, 92(4), 649-655.

Plantinga, Y., Ghiadoni, L., Magagna, A., Giannarelli, C., Franzoni, F., Taddei, S., et al. (2007). Supplementation with vitamins C and E improves arterial stiffness and endothelial function in essential hypertensive patients. *American Journal of Hypertension*, 20(4), 392-397.

Pleiner, J., Schaller, G., Mittermayer, F., Marsik, C., MacAllister, R., Kapiotis, S., et al. (2008). Intra-arterial vitamin C prevents endothelial dysfunction caused by ischemia-reperfusion. *Atherosclerosis*, 197(1), 383-391.

Plotnick, G., Corretti, M., & Vogel, R. (1997). Effect of antioxidant vitamins on the transient impairment of endothelium-dependent vasoactivity following a single high-fat meal. *Journal of the American Medical Association*, 278, 1682-1686.

Plotnick, G., Corretti, M., Vogel, R., Hesslink, R., & Wise, J. (2003). Effect of supplemental phytonutrients on impairment of the flow-mediated brachial artery vasoactivity after a single high-fat meal. *Journal of the American College of Cardiology*, 41(10), 1744-1749.

Prior, R., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53(10), 4290-4302.

Proudfoot, J., Barden, A., Mori, T., Burke, V., Croft, K., Beilin, L., et al. (1999). Measurement of urinary F2-isoprostanes as markers of in vivo lipid peroxidation- a comparison of enzyme immunoassay with gas chromatography/mass spectrometry. *Analytical Biochemistry*, 272(2), 209-215.

Raff, M., Tholstrup, T., Basu, S., Nonboe, P., Sørensen, M., & Straarup, E. (2008). A diet rich in conjugated linoleic acid and butter increases lipid peroxidation but does not affect atherosclerotic, inflammatory, or diabetic risk markers in healthy young men. *Journal of Nutrition*, 138(3), 509-514.

Raitakari, O., Lai, N., Griffiths, K., McCredie, R., Sullivan, D., & Celermajer, D. (2000). Enhanced peripheral vasodilation in humans after a fatty meal. *Journal of the American College of Cardiology*, 36(2), 417-422.

Richard, M., Portal, B., Meo, J., Coudray, C., Hadjian, A., & Favier, A. (1992). Malondialdehyde kit evaluated for determining plasma and lipoprotein fractions that react with thiobarbituric acid. *Clinical Chemistry*, 38(5), 704-709.

Roberts, L., & Morrow, J. (2000). Measurement of F2-isoprostanes as an index of oxidative stress in vivo. *Free Radical Biology and Medicine*, 28(4), 505-513.

Romieu, I., Garcia-Esteban, R., Sunyer, J., Rios, C., Alcaraz-Zubeldia, M., Velasco, S., et al. (2008). The effect of supplementation with omega-3 polyunsaturated fatty acids on markers of oxidative stress in elderly exposed to PM (2.5). *Environmental Health Perspectives*, 116(9), 1237-1242.

Rossi, P., Andriessse, G., Oey, P., Wieneke, G., Roelofs, J., & Akkermans, L. (1998). Stomach distension increases efferent muscle sympathetic nerve activity and blood pressure in healthy humans. *Journal of the neurological sciences*, 161(2), 148-155.

Rush, J., Denniss, S., & Graham, D. (2005). Vascular nitric oxide and oxidative stress: determinants of endothelial adaptations to cardiovascular disease and to physical activity. *Applied Physiology, Nutrition, and Metabolism*, 30(4), 442-474.

Russo, G. (2009). Dietary n-6 and n-3 polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention. *Biochemical Pharmacology*, 77(6), 937-946.

Samuelsson, B., Goldyne, M., Granstrom, E., Hamberg, M., Hammarstrom, S., & Malmsten, C. (1978). Prostaglandins and thromboxanes. *Annual Review of Biochemistry*, 47(1), 997-1029.

Sanders, T., Lewis, F., Slaughter, S., Griffin, B., Griffin, M., Davies, I., et al. (2006). Effect of varying the ratio of n-6 to n-3 fatty acids by increasing the dietary intake of alpha-linolenic acid, eicosapentaenoic and docosahexaenoic acid, or both on fibrinogen and clotting factors VII and XII in persons aged 45-70 y: the OPTILIP study. *American journal of clinical nutrition*, 84(3), 513-522.

Scherrer, U., Randin, D., Vollenweider, P., Vollenweider, L., & Nicod, B. (1994). Nitric oxide release accounts for insulin's vascular effects in humans. *Journal of Clinical Investigation*, 94(6), 2511-2515.

Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C., Hollenberg, N., et al. (2006). (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proceedings of the National Academy of Sciences*, 103(4), 1024-1029.

Scott, B., Deman, A., Peeters, P., Van den Branden, C., Stolar, J., Van Camp, G., et al. (2003). Cardiac troponin T and malondialdehyde modified plasma lipids in haemodialysis patients. *Nephrology, Dialysis, Transplantation*, 18(4), 737-742.

Scott, E., Greenwood, J., Vacca, G., Stoker, J., Gilbey, S., & Mary, D. (2002). Carbohydrate ingestion, with transient endogenous insulinaemia, produces both sympathetic activation and vasodilatation in normal humans. *Clinical science (London, England: 1979)*, 102(5), 523-529.

Serne, E., de Jongh, R., Eringa, E., IJzerman, R., & Stehouwer, C. (2007). Microvascular dysfunction: a potential pathophysiological role in the metabolic syndrome. *Hypertension*, *50*(1), 204-211.

Shenouda, S. M., & Vita, J. A. (2007). Effects of flavonoid-containing beverages and EGCG on endothelial function. *Journal of the American College of Nutrition*, *26*(4), 366S-372S.

Shimabukuro, M., Chinen, I., Higa, N., Takasu, N., Yamakawa, K., & Ueda, S. (2007). Effects of dietary composition on postprandial endothelial function and adiponectin concentrations in healthy humans: a crossover controlled study. *American Journal of Clinical Nutrition*, *86*(4), 923-928.

Sies, H., Stahl, W., & Sevanian, A. (2005). Nutritional, dietary and postprandial oxidative stress. *Journal of Nutrition*, *135*(5), 969-972.

Simon, A., Levenson, J., Levy, B., Bouthier, J., Peronneau, P., & Safar, M. (1982). Effect of nitroglycerin on peripheral large arteries in hypertension. *British Journal of Clinical Pharmacology*, *14*(2), 241-246.

Sjogren, P., Basu, S., Rosell, M., Silveira, A., de Faire, U., Vessby, B., et al. (2005). Measures of oxidized low-density lipoprotein and oxidative stress are not related and not elevated in otherwise healthy men with the metabolic syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *25*(12), 2580-2586.

Spady, D., & Dietschy, J. (1988). Interaction of dietary cholesterol and triglycerides in the regulation of hepatic low density lipoprotein transport in the hamster. *The Journal of clinical investigation*, *81*(2), 300-309.

Spiteller, G. (2006). Peroxyl radicals: inductors of neurodegenerative and other inflammatory diseases. Their origin and how they transform cholesterol, phospholipids, plasmalogens, polyunsaturated fatty acids, sugars, and proteins into deleterious products. *Free Radical Biology & Medicine*, *41*(3), 362-387.

Stamler, J., Wentworth, D., & Neaton, J. (1986). Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). *JAMA: the journal of the American Medical Association*, *256*(20), 2823-2828.

Tabachnick, B., & Fidell, L. (2007a). Cleaning up your act: screening data prior to analysis. In S. Hartman (Ed.), *Using multivariate statistics* (5 ed., pp. 61-114). Boston, NC: Pearson Education Inc.

Tabachnick, B., & Fidell, L. (2007b). Review of univariate and bivariate statistics. In S. Hartman (Ed.), *Using multivariate statistics* (5 ed., pp. 33-58). Boston, NC:

Pearson Education Inc.

Taddei, S., Viridis, A., Ghiadoni, L., Salvetti, G., Bernini, G., Magagna, A., et al. (2001). Age-related reduction of NO availability and oxidative stress in humans. *Hypertension*, 38(2), 274-279.

Tam, L., Li, E., Leung, V., Griffith, J., Benzie, I., Lim, P., et al. (2005). Effects of vitamins C and E on oxidative stress markers and endothelial function in patients with systemic lupus erythematosus: a double blind, placebo controlled pilot study. *Journal of Rheumatology*, 32(2), 275-282.

Tedgui, A., & Mallat, Z. (2006). Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiological Reviews*, 86(2), 515-581.

Tholstrup, T., Hellgren, L., Petersen, M., Basu, S., Straarup, E., Schnohr, P., et al. (2004). A solid dietary fat containing fish oil redistributes lipoprotein subclasses without increasing oxidative stress in men. *Journal of Nutrition*, 134(5), 1051-1057.

Tinahones, F. J., Murri-Pierri, M., Garrido-Sánchez, L., García-Almeida, J. M., García-Serrano, S., García-Arnés, J., et al. (2008). Oxidative Stress in Severely Obese Persons Is Greater in Those With Insulin Resistance. *Obesity*, 17(2), 240-246.

Title, L., Cummings, P., Giddens, K., & Nassar, B. (2000). Oral glucose loading acutely attenuates endothelium-dependent vasodilation in healthy adults without diabetes: an effect prevented by vitamins C and E. *Journal of the American College of Cardiology*, 36(7), 2185-2191.

Tripathy, D., Mohanty, P., Dhindsa, S., Syed, T., Ghanim, H., Aljada, A., et al. (2003). Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. *Diabetes*, 52(12), 2882-2887.

Tschakovsky, M., & Pyke, K. (2005). Counterpoint: Flow-mediated dilation does not reflect nitric oxide-mediated endothelial function. *Journal of Applied Physiology*, 99(3), 1235-1237.

Tushuizen, M., Nieuwland, R., Scheffer, P., Sturk, A., Heine, R., & Diamant, M. (2006). Two consecutive high-fat meals affect endothelial-dependent vasodilation, oxidative stress and cellular microparticles in healthy men. *Journal of Thrombosis and Haemostasis*, 4(5), 1003-1010.

Urbich, C., & Dimmeler, S. (2005). Risk factors for coronary artery disease, circulating endothelial progenitor cells, and the role of HMG-CoA reductase inhibitors. *Kidney International*, 67(5), 1672-1676.

Ursini, F., & Sevanian, A. (2002). Postprandial oxidative stress. *Biological Chemistry*, 383(3-4), 599-605.

- Urso, M., & Clarkson, P. (2003). Oxidative stress, exercise, and antioxidant supplementation. *Toxicology*, *189*(1-2), 41-54.
- Vasan, R. (2006). Biomarkers of cardiovascular disease: molecular basis and practical considerations. *Circulation*, *113*(19), 2335-2362.
- Verschuren, W., Jacobs, D., Bloemberg, B., Kromhout, D., Menotti, A., Aravanis, C., et al. (1995). Serum total cholesterol and long-term coronary heart disease mortality in different cultures. Twenty-five-year follow-up of the seven countries study. *Journal of the American Medical Association*, *274*(2), 131-136.
- Vita, J. A., & Keaney, J. F. (2002). Endothelial function: a barometer for cardiovascular risk? *Circulation*, *106*(6), 640-642.
- Voetsch, B., Jin, R., & Loscalzo, J. (2004). Nitric oxide insufficiency and atherothrombosis. *Histochemistry and Cell Biology*, *122*(4), 353-367.
- Vogel, R., Corretti, M., & Plotnick, G. (1997). Effect of a single high-fat meal on endothelial function in healthy subjects. *American Journal of Cardiology*, *79*(3), 350-354.
- WeiChuan, T., YiHeng, L., ChihChan, L., TingHsing, C., & JyhHong, C. (2004). Effects of oxidative stress on endothelial function after a high-fat meal. *Clinical Science*, *106*(3), 315-319.
- Westphal, S., Taneva, E., Kätner, S., Martens-Lobenhoffer, J., Bode-Böger, S., Kropf, S., et al. (2006). Endothelial dysfunction induced by postprandial lipemia is neutralized by addition of proteins to the fatty meal. *Atherosclerosis*, *185*(2), 313-319.
- Widlansky, M. E., Gokce, N., Keaney, J. F., & Vita, J. A. (2003). The clinical implications of endothelial dysfunction. *Journal of the American College of Cardiology*, *42*(7), 1149-1160.
- Woollett, L., Spady, D., & Dietschy, J. (1992). Saturated and unsaturated fatty acids independently regulate low density lipoprotein receptor activity and production rate. *Journal of lipid research*, *33*(1), 77-88.
- Yagi, K. (1998). Simple assay for the level of total lipid peroxides in serum or plasma. *Methods in Molecular Biology*, *108*, 101-106.
- Yesilova, Z., Yaman, H., Oktenli, C., Ozcan, A., Uygun, A., Cakir, E., et al. (2005). Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic Fatty liver disease. *The American journal of gastroenterology*, *100*(4), 850-855.
- Young, C., Deo, S., Kim, A., Horiuchi, M., Mikus, C., Uptergrove, G., et al.

(2010). Influence of endurance training on central sympathetic outflow to skeletal muscle in response to a mixed meal. *Journal of Applied Physiology*, 882-890.

Zhao, G., Etherton, T., Martin, K., Gillies, P., West, S., & Kris-Etherton, P. (2007). Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. *American Journal of Clinical Nutrition*, 85(2), 385-391.

APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL AND DOCUMENTATIONS



INDIANA UNIVERSITY
OFFICE OF RESEARCH ADMINISTRATION

To: Alyce D. Fly
Applied Health Science

From: IUB Human Subjects Office
Office of Research Administration – Indiana University

Date: November 20, 2009

RE: EXPEDITED APPROVAL – AMENDMENT
Protocol Title: Comparison of Two Feeding Regimens on Endothelial Function Variability Measured
by Peripheral Arterial Tonometry
Protocol #: 0908000572
Sponsor: N/A

An amendment to your above-referenced protocol was approved by the IRB on NOV 19, 2009. The protocol continues to meet the requirements for expedited review pursuant to §46.110, Category(ies) 2&4. The changes described in the amendment can now be implemented, unless any departmental or other approvals are required.

If you submitted a revised informed consent document(s), study information sheet(s), advertisement(s), etc., a copy of the approved stamped document(s) are enclosed and must now be used.

You should retain a copy of this letter and any associated approved study documents for your records. All documentation related to this protocol must be maintained in your files for audit purposes for at least three years after closure of the research; however, please note that research studies subject to HIPAA may have different requirements regarding file storage after closure. Please refer to the project title and number in future correspondence with our office. Additional information is available on our website at http://researchadmin.iu.edu/HumanSubjects/TUB/hs_home.html. Please contact our office if you have questions or need further assistance.

Thank you.

/ Enclosures

INDIANA UNIVERSITY BLOOMINGTON INSTITUTIONAL REVIEW BOARD (IRB) REVIEW
STUDY AMENDMENT

NOTE: Modifications included in this amendment *may not* be implemented until approval from the IRB is granted.

IRB STUDY NUMBER: 0908000572

AMENDMENT NUMBER: 1

SECTION I: INVESTIGATOR INFORMATION

Principal Investigator: Alyce D. Fly Department: Applied Health Science
(Last, First, Middle Initial)

Building/Room No.: HPER 005 Phone: 812-855-7975 E-Mail: afly@indiana.edu

Faculty Sponsor: _____ Department: _____
(Last, First, Middle Initial)

Building/Room No.: _____ Phone: _____ E-Mail: _____

Project Title: **Comparison of Two Feeding Regimens on Endothelial Function Variability Measured by Peripheral Arterial Tonometry**

Sponsor/Funding Agency: _____

SECTION II: AMENDMENT DESCRIPTION

This form must be typed and submitted to: iub_hsc@indiana.edu with any applicable revised documents.

1. Please provide a complete description of the proposed change(s) included in this amendment:
- 1) The age range is changed from 21-26 y to 19-30 y;
 - 2) The BMI range is changed from 18.5-24.9 to 18.5-25 kg/m²;
 - 3) Additional recruitment method is added: recruitment flyer (Appendix B) is sent out using email list from HPER, Chinese Student Association, and International center.
2. Please state the justification/rationale for this amendment:
The principle investigator had difficulty recruiting subjects following the previous exclusion/inclusion criteria. The age, BMI range need to be expanded to recruit sufficient subjects. Additional recruitment methods are also needed.
3. Is the study sponsored by a granting agency or other external entity?
- No.
- Yes. Check the appropriate line below and provide with this amendment, as applicable:
- a copy of the agency's or external entity's amendment.
- a copy of your notice to the agency or external entity of this change, if you initiated the amendment.
- a copy of the approved amendment will be sent to the agency or external entity.
- none of the above apply.
4. Do the proposed change(s) described in this amendment alter the balance of risks and benefits presented to the subjects?
- No.
- Yes. Please describe how the assessment is altered:
5. Do the proposed change(s) described in this amendment affect any of the following documents?
- Summary Safeguard Statement Authorization
- Surveys, questionnaires, etc. Protocol
- Recruitment materials (advertisements, flyers, scripts, etc.)
- Other, Please describe: Consent form

NOTE: Any document selected above (i.e. any document that was revised due to the amendment) must be included with the submission of the amendment. Please be sure to highlight or otherwise show revisions to the above documents.

6. Do the proposed change(s) described in this amendment require changes to an informed consent and/or assent document?
- No. Informed consent, written documentation of informed consent, and/or assent has been waived for this study.
 - No. Skip to item 7 below.
 - Yes. Check the appropriate line below.
 - The new informed consent and/or assent document(s) are in addition to the current one(s).
 - The new informed consent and/or assent document(s) replace the current one(s).
- If there are multiple consent and/or documents for this study, please indicate which consent and/or assent document(s) are to be replaced.

NOTE: Please be sure to highlight or otherwise show revisions to the informed consent document(s).

7. Amendment includes:
- | | |
|---|--|
| <input checked="" type="checkbox"/> Informed Consent and/or Assent, dated: 11/17/09 | <input type="checkbox"/> Surveys, questionnaires, dated: |
| <input checked="" type="checkbox"/> Summary Safeguard Statement, dated: 11/17/09 | <input checked="" type="checkbox"/> Recruitment materials, dated: 11/17/09 |
| <input type="checkbox"/> Authorization, dated: | <input type="checkbox"/> Protocol, dated: |
| <input type="checkbox"/> Other, please describe: | |

NOTE: Only include documents that were checked in items 5 and 6 above (as being changed because of the amendment).

NOTE: Listing document dates are optional and only necessary if required by the investigator or sponsor.

Please indicate the type of amendment you are submitting. See Guidelines for Determining an Amendment Type for additional information (<http://www.iupui.edu/~resgrad/spon/amend-guide.htm>). **Please note, however, that the IRB makes the final determination with regard to whether or not the amendment is acceptable for expedited review or if it requires review at a convened IRB meeting.**

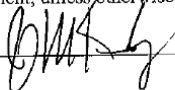
- Minor Amendment.** Change(s) do not significantly affect the safety of subjects and is acceptable for expedited review per 45 CFR 46.110(b)(2)/21 CFR 56.110(b)(2).
- Major Amendment.** Changes potentially involve increased risks or discomforts or decrease potential benefit. The amendment requires review at a convened IRB meeting.

Signature of Principal Investigator: Alyce D. Fly Date: 10/27/2009
Note: An e-mail originating from the Principal Investigator's e-mail address, which contains the submission of the amendment serves in place of the Principal Investigator's actual signature.

Signature of Faculty Sponsor: _____ Date: _____
Note: An e-mail originating from the Faculty Sponsor's e-mail address acknowledging continued oversight responsibilities for the research study, including all changes represented in the amendment serves in place of the Faculty Sponsor's actual signature.

SECTION III: IRB APPROVAL

This amendment, including documentation noted in item 7 above, has been reviewed and approved as meeting the criteria for IRB approval as outlined in 45 CFR 46.111(a) by the Indiana University HSC. I agree with the investigator's assessment above regarding whether the amendment is a minor or major amendment, unless otherwise noted.

Authorized IRB Signature:  IRB Approval Date: 11/19/09

**INDIANA UNIVERSITY BLOOMINGTON
INFORMED CONSENT STATEMENT****Comparison of Two Feeding Regimens on Endothelial Function Variability Measured by Peripheral Arterial Tonometry**

You are invited to participate in a research study of the effects of two snacks on vascular health. We ask that you read this form and ask any questions you may have before agreeing to be in the study. The study is being conducted by Alyce D. Fly, Ph.D. Nutrition Science, Department of Applied Health Science, School of HPER, Indiana University Bloomington.

STUDY PURPOSE

The purpose of this study is to determine an optimal feeding regimen to use to study vascular health after eating.

NUMBER OF PEOPLE TAKING PART IN THE STUDY:

If you agree to participate, you will be one of [10] subjects who will be participating in this research.

PROCEDURES FOR THE STUDY:

If you agree to be in the study, you will do the following things:

- Complete 3 sessions including, screening and two testing sessions.
- The study will be conducted in HPER, Indiana University-Bloomington, rooms 079 (testing) and 007 (snacks).
- For the screening session (about 10 minutes):
 - You will answer some questions about your personal history and have your height and weight measured. This information will be used to determine your suitability to participate in the study. Study subjects will also be male, between the ages of 19-30 years of age, without any history of heart disease, stroke, diabetes, uncontrolled hypertension, liver and kidney diseases, thyroid disease, and dyslipidemia; nonsmoking and do not use any tobacco products, do not use any medications regularly, including, aspirin, anti-platelet drugs, anti-inflammatory drugs, lipid-lowering drugs, and blood pressure medication, and are not allergic or intolerant of milk and/or wheat foods. If you do not meet these criteria you will not be able to participate in the testing sessions.
- For each testing session (6 - 7 hours/session, on two different days, one week apart):
 - Your blood pressure and weight will be measured and you will answer some questions to see if you meet the study day criteria that include excluding caffeine-containing drinks, alcohol, tobacco and vitamin or mineral supplements, refraining from exercise for the past 24 hours, and undergoing a 12-h fasting period.
 - You will be assigned to eat two snacks, one at 8 am, and the second at 12 noon.
 - Your vascular function will be measured using a noninvasive instrument (EndoPAT 2000) 4 times. This machine uses a blood pressure cuff to activate your vessels and measures volume changes in your fingertips with two finger cups.
 - Your blood will be drawn from a vein in your arm 4 different times, 10 mL (2 teaspoons) will be taken at each time for a total of 40 mL (or 8 teaspoons). Laboratory analyses will be performed on the blood samples.
 - A tiny blood sample (40 µL) will also be taken by finger stick 7 times (total of 280 µL or 6/100ths of a teaspoon) to collect blood for other laboratory measures like triglycerides and glucose.
 - All of the blood samples will be taken by a graduate student that has been trained in blood collection techniques by a professor of physiology, a medical doctor and a nurse in the IU School of Medicine.
 - You may read a book, study, or watch movies any time you are not eating the snack or undergoing testing.

RISKS OF TAKING PART IN THE STUDY:

While on the study, the risks of the blood draw and finger stick may include irritation and bruising of the skin, pain, multiple puncture sites, discomfort, lightheadedness, fainting, or rarely, an infection. The risks of the assessment of vascular function may include skin irritation. There is also a possible risk to lose confidentiality of your information.

Precautions will be taken to avoid the potential risks listed above. The puncture site of blood draw and finger sticks will be cleaned with an alcohol wipe and sterile technique will be used to collect blood. A sterile bandage will be applied to the puncture site and you will be observed for 5 minutes following blood collection. Your personal information will be protected by the investigators by securing all the information in a locked cabinet and keeping the electronic files protected with a secret password.

BENEFITS OF TAKING PART IN THE STUDY:

The benefits to participation that are reasonable to expect are test results of your fasting blood glucose and triglyceride after completing the study, and a handout on healthy eating guidelines.

ALTERNATIVES TO TAKING PART IN THE STUDY:

Instead of being in the study, you have the option not to participate.

CONFIDENTIALITY

Efforts will be made to keep your personal information confidential. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. Your identity will be held in confidence in reports in which the study may be published.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the study investigator and his/her research associates, the IUB Institutional Review Board or its designees, and (as allowed by law) state or federal agencies, specifically the Office for Human Research Protections (OHRP), who may need to access your research records.

COSTS

Taking part in this study may lead to added costs to you. You will be responsible for these study-specific costs: transportation to the test location at School of HPER, Indiana University Bloomington.

PAYMENT

You will receive a \$25 payment for taking part in this study, that is, \$12.50 for completing each testing session. Even though you come in for the screening session and agree to take part in the study, it does not ensure that you will be selected to be a subject in the study. You may be excluded if the investigators recruit other suitable subjects before you.

CONTACTS FOR QUESTIONS OR PROBLEMS

For questions about the study or a research-related injury, contact the researcher Alyce D. Fly at 812-855-7975 or afly@indiana.edu.

For questions about your rights as a research participant or to discuss problems, complaints or concerns about a research study, or to obtain information, or offer input, contact the IUB Human Subjects office, 530 E Kirkwood Ave, Carmichael Center, 203, Bloomington IN 47408, 812-855-3067 or by email at iub_hsc@indiana.edu

VOLUNTARY NATURE OF STUDY

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. Your decision whether or not to participate in this study will not affect your current or future relations with the investigator(s).

SUBJECT'S CONSENT

In consideration of all of the above, I give my consent to participate in this research study. I will be given a copy of this informed consent document to keep for my records. I agree to take part in this study.

Subject's Printed Name: _____

Subject's Signature: _____ Date: _____
(must be dated by the subject)

Printed Name of Person Obtaining Consent: _____

Signature of Person Obtaining Consent: _____ Date: _____

Form date: November 19, 2009

IRB Approval Date: NOV 19, 2009

Expiration Date AUG 21, 2010

IUB Institutional Review Board (IRB)

APPENDIX A – Statement for Recruiting Subjects

Hello ____,

Would you like to eat snacks to help us study vascular health? We are conducting a study on the effects of snacking on vascular health. Your benefits of the study include test results of blood triglycerides and blood glucose. We will also give you a handout about dietary guidance and \$25.

Are you interested in hearing more and considering participating as a volunteer?

(if yes, proceed below)

The study has 3 sessions including, a quick screening session and two longer testing sessions:

The screening session will take about 10 minutes. At the screening session you will answer some questions about your personal history and we will measure your height and weight. This information will be used to determine your suitability to participate in the study. You may be eligible if you are male, between the ages of 19-30 years of age, without any history of heart disease, stroke, diabetes, uncontrolled hypertension, liver and kidney diseases, thyroid disease, and dyslipidemia. You also need to be a nonsmoker and do not use any tobacco products. You can't be using any medications regularly, including, aspirin, anti-platelet drugs, anti-inflammatory drugs, lipid-lowering drugs, and blood pressure medication. Lastly you can't be allergic or intolerant of milk or wheat-containing foods, because our snack contains these ingredients. Even though you come in for the screening session and agree to take part in the study, it does not ensure that you will be selected to be a subject in this study. You may be excluded if the investigators recruit other suitable subjects before you. Payment is only made if you are selected to participate in the study and participate in the testing sessions.

Each of the two testing sessions will take 6 to 7 hours each. The testing sessions are on different days about a week apart. On the testing day you are assigned to eat a snack in our building, in HP 007. You eat the same snack twice, once at 8:00 am and a second one at 12:00 noon. After each snack you will be tested in room HP 079, of the HPER building, at Indiana University Bloomington.

The tests include a weight measurement, blood pressure measurement, vascular function measurement, and blood marker measurements. Small amounts of blood, about two teaspoons each time, will be collected 4 times and tiny amounts of blood, a few 100ths of a teaspoon will be collected through a series of 7 finger sticks. But most of the time you can chill out, read a book or watch movies in our laboratory.

If you are interested, would you like to set up an appointment for the screening session?

(If yes, set up a time at the subjects convenience).

(If no, thank them anyway)

End with,

If you have further questions, please contact Yibin Liu (yibliu@indiana.edu) or Professor Fly (afly@indiana.edu), 812-855-7975, School of HPER, Indiana University Bloomington.

IRB APPROVED

NOV 19, 2009

IUB Institutional Review Board (IRB)

APPENDIX B
THIOBARBITURIC ACID REACTIVE SUBSTANCE ASSAY PROTOCOL

 PROTOCOL

1. Make up reagents.

Materials	Health Hazard	Molecular weight (g/mol)	Density	Preparation
≥99% TCA	3	163.39		15 g
TBA	0	144.15		0.375 g
12 N HCl	0	36.46		Mix 2.1 mL with 97.9 mL water
BHT	2	220.35		0.446 g in 10 mL ethanol
≥98% SDS	2	288.38		0.5 g in 5 mL water
≥99.5% Ethanol	2	46.07	0.789 g/mL	256 μL ethanol in 11.9 mL water (2.1% ethanol/water)
99% 1,1,3,3-Tetramethoxypropane	1	164.2	0.997 g/mL	1 mg in 12.18 mL 2.1% ethanol/water (0.00821% w/v)
Ultrapure water				300 mL

Solution	Stock solution	Volume/test tube	Final Concentration
TCA ¹	15% w/v in 0.25 N HCl		0.567 M
TBA ¹	0.375% w/v in 0.25 N HCl	500 μL	0.016 M
0.25 N HCl ¹	100 mL		0.156 M
BHT	10 mL (0.202 M)	10 μL	0.002 M
SDS	5 mL (10% w/v in water)	50 μL	0.021 M
Sample OR stock standard		250 μL	
Total		810 μL	

¹Color reagent = TCA (15 g) + TBA (0.375 g) + 0.25 N HCl (100 mL).

	Health Hazzard	MW (g/mol)	Concentration	Density	Preparation	Solution
99% Ethanol	2	46.07		0.789 g/mL	10 mL	
BHT	2	220.35	0.045 M		0.05 g in 5 mL ethanol	1.00%
BHT	2	220.35	0.225 mM		10 μ L 1% BHT + 1.99 mL ethanol	0.005%

	MW (g/mol)	Concentration	Density	Mass (mg)
Indomethacin 1 (Concentrated)	357.78	10 μ M x 200folds= 2 mM	2 mM x 357.78 g/mol = 0.72 mg/mL in ethanol	6.1

2. Labeling and utensils.

9 disposable glass tubes (Stock, A-H), flask (Ultrapure Water), 32 1.5-mL microcentrifuge tubes (S1-S8, 1-24), pipette man (1000 μ L, 200 μ L, 10 μ L), water bath, floating rack, ice bath.

3. Prepare standards.

- To obtain the Standard Stock Solution (125 μ M): dilute the 1,1,3,3 – tetramethoxypropane (250 μ L, 0.00821% w/v in 2.1% ethanol/water) with ultrapure water (750 μ L) in a clean glass tube.
- Take 8 clean glass tubes A-H.
- Add the standard stock solution (125 μ M) and water to each glass tube as follows.

Glass tube	Standard stock solution w/ water (μL)	Water (μL)	MDA concentration (μM)
A	0	1,000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

- For standard curve: take 1.5-mL micro centrifuge tubes S1-S8. Add reagents as follows.

	Standard A-H	BHT (0.005% in ethanol)	IDM (2 mM)	BHT (0.202 M)	SDS (10% w/v)	Color reagent
S1	250 μL	2.5 μL	1.25 μL	10 μL	50 μL	500 μL
S2	250 μL	2.5 μL	1.25 μL	10 μL	50 μL	500 μL
S3	250 μL	2.5 μL	1.25 μL	10 μL	50 μL	500 μL
S4	250 μL	2.5 μL	1.25 μL	10 μL	50 μL	500 μL
S5	250 μL	2.5 μL	1.25 μL	10 μL	50 μL	500 μL
S6	250 μL	2.5 μL	1.25 μL	10 μL	50 μL	500 μL
S7	250 μL	2.5 μL	1.25 μL	10 μL	50 μL	500 μL
S8	250 μL	2.5 μL	1.25 μL	10 μL	50 μL	500 μL

- Prepare plasma samples (samples had been supplemented with 0.005% BHT/EtOH and 2 mM indomethacin). Take 24 1.5-mL centrifuge tubes labeled as 1-24. Add reagents as follows.

	Plasma sample	BHT (0.202 M)	SDS (10% w/v)	Color reagent
1	250 μL	10 μL	50 μL	500 μL
.	250 μL	10 μL	50 μL	500 μL
.	250 μL	10 μL	50 μL	500 μL
.	250 μL	10 μL	50 μL	500 μL
24	250 μL	10 μL	50 μL	500

-
5. Incubate centrifuge tubes in boiling water for 30 min (Park, et al., 2005). Then put centrifuge tubes on an ice bath to stop the reaction.
 6. Centrifuge tubes at 14,000 rpm (6000~7000 x g) for 10 min.
 7. Transfer 200 μ L supernatant to 96-well microplate. Transfer each standard in duplicate. Transfer each sample in triplicate.
 8. Read absorbance at 532 nm and 600 nm. The malondialdehyde concentration can be calculated from [532 nm] - [600 nm] or using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Buege & Aust, 1978).