Postprandial effects of soy isoflavones on low-density lipoprotein oxidative resistance with a high carbohydrate meal

by Bobbi Jo Miller

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Health and Human Development

Montana State University

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Abstract:

Cardiovascular disease (CVD) encompasses a wide array of health problems including atherosclerosis which results in 50% of all cardiac deaths. Currently the American Heart Association developed the Step I guidelines for reducing the risk of CVD limits total fat to ≤30% of total energy, saturated fat to <10% of total energy, and cholesterol to < 300 mg/day. Reducing dietary fat generally decreases plasma cholesterol however carbohydrate (CHO) content typically rises accompanied by increasing plasma triacylglycerol (TC). Elevated TG may possibly be a risk factor for CVD, referred to as "CHO-induced hypertriacylglycerolemia" (HPTG). The disease process of atherogenesis has been hypothesized by Zilversmit as a postprandial phenomenon based on the formation of chylomicron remnants, low-density lipoproteins (LDL), and the uptake of these cholesterol and TG rich molecules by arterial cells. Oxidation of LDL and phagocytic immune system cells have been implicated in the mechanism involving fatty streaks and occlusion of the arterial lumen. The isoflavones diadzein and genistein in soy-protein have been associated with oxidative resistance of LDL due to their antioxidant activity. The purpose of this study was to determine if the oxidative resistance of postprandial LDL is enhanced with the consumption of a meal containing 39.0 g of soy protein (80 mg aglycone isoflavones) vs 39.9 g milk protein (0 mg aglycone isoflavones) in combination with a high carbohydrate meal. Fifteen healthy male subjects participated in a double-blind, crossover feeding study in the Nutrition Research Lab (NRL) at Montana State University. Subject's height, weight, and baseline blood draw were completed before consuming the challenge meal consisting of 2 high carbohydrate muffins and a soy or milk protein shake (899 calories, 22% fat, 58.6% CHO, 19.4%). Blood samples were collected by venipuncture postprandially at hours 2, 4, and 6.

Isolated LDL was subjected to ex vivo copper-induced oxidation. Initial absorbance, lag time, and propagation rate were calculated for each time point. Results indicated no significant difference ($p>0.05$) between the protein treatments or their interaction on LDL oxidation parameters. Additional research is needed to ascertain the function of soy in prevention of CVD, specifically in the postprandial state.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Cardiovascular disease (CVD) encompasses a wide array of health problems including atherosclerosis which results in 50% of all cardiac deaths. Currently the American Heart Association developed the Step I guidelines for reducing the risk of CVD limits total fat to ≤30% of total energy, saturated fat to <10% of total energy, and cholesterol to < 300 mg/day. Reducing dietary fat generally decreases plasma cholesterol however carbohydrate (CHO) content typically rises accompanied by increasing plasma triacylglycerol (TG). Elevated TG may possibly be a risk factor for CVD, referred to as "CHO-induced hypertriacylglycerolemia" (HPTG). The disease process of atherogenesis has been hypothesized by Zilversmit as a postprandial phenomenon based on the formation of chylomicron remnants, low-density lipoproteins (LDL), and the uptake of these cholesterol and TG rich molecules by arterial cells. Oxidation of LDL and phagocytic immune system cells have been implicated in the mechanism involving fatty streaks and occlusion of the arterial lumen. The isoflavones diadzein and genistein in soy-protein have been associated with oxidative resistance of LDL due to their antioxidant activity. The purpose of this study was to determine if the oxidative resistance of postprandial LDL is enhanced with the consumption of a meal containing 39.0 g of soy protein (80 mg aglycone isoflavones) vs 39.9 g milk protein (0 mg aglycone isoflavones) in combination with a high carbohydrate meal. Fifteen healthy male subjects participated in a double-blind, crossover feeding study in the Nutrition Research Lab (NRL) at Montana State University. Subject's height, weight, and baseline blood draw were completed before consuming the challenge meal consisting of 2 high carbohydrate muffins and a soy or milk protein shake (899 calories, 22% fat, 58.6% CHO, 19.4%). Blood samples were collected by venipuncture postprandially at hours 2, 4, and 6. Isolated LDL was subjected to *ex vivo* copper-induced oxidation. Initial absorbance, lag time, and propagation rate were calculated for each time point. Results indicated no significant difference ($p>0.05$) between the protein treatments or their interaction on LDL oxidation parameters. Additional research is needed to ascertain the function of soy in prevention of CVD, specifically in the postprandial state.

CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) is currently the leading cause of death (1) encompassing a wide array of health problems including atherosclerosis, hypertension, stroke, and hyperlipidemia (2). Atherosclerosis is a degenerative disease of the vascular endothelium that is associated with vessel damage and impeded blood flow (3). It is the most deadly CVD resulting in 50% of all cardiac deaths (2). Due to the morbidity and mortality rates, CVD has become a major health problem (2). More than 58 million Americans have at least one form of CVD, with associated costs exceeding $274 billion yearly (2), therefore, research is directed towards lowering associated risk factors that could potentially lead to the formation of CVD. Epidemiological studies have repeatedly shown high degrees of correlation between the intake of dietary fats and cholesterol with the prevalence of CVD (4).

Health promotion efforts now emphasize the importance of reducing risk factors such as high dietary fat and cholesterol intake. Currently the American Heart Association's Step I guidelines for reducing the risk of CVD limits total fat to ≤30% of total energy, saturated fat to <10% of total energy, and cholesterol to <300 mg/day. As individuals reduce their dietary fat, dietary carbohydrate (CHO) content typically rises to replace lost calories. The desired reduction in total cholesterol is frequently reached, but is typically accompanied by an elevation in triacylglycerol (TG) level (5). For the purpose of research, a high CHO diet has been termed "low-fat, high-carbohydrate" if ≤30% of the

total energy is derived from fat and ≥55% of energy is derived from CHO (5). Concern now stems from a possible increase in CVD occurrence when TG levels are elevated as a result of lowered dietary fat and increased dietary CHO, a phenomenon referred to as CHO-induced hypertriacylglycerolemia (HPTG) (5). If this phenomenon shares a physiological foundation with endogenous HPTG, commonly observed in high-fat diets, a similar atherogenic risk may ensue following high CHO diets.

Triglyceride-rich lipoproteins are derived through processes involving the intestine (chylomicrons) and the liver (very low-density lipoproteins [VLDL]). TheseTG -rich lipoproteins have been determined to come from four distinct sources; *de novo* lipogenesis, lipolysis, chylomicron remnant formation, and TG droplets stored in the liver (6). The chylomicron and VLDL are metabolized in the blood stream by the action of lipoprotein lipase on the surface of endothelial cells in the muscle and adipose tissue (7, 8). Multiple interactions lead to the hydrolysis of TG-rich molecules and to the formation of low-density lipoproteins (LDL) (7, 8). Low-density lipoprotein becomes the major cholesterol carrying lipoprotein and resides much longer in the blood than do chylomicron or VLDL remnants (days for LDL vs. minutes to hours for VLDL) (7). This increased exposure time in the blood allows free radicals a greater chance to manipulate the native LDL into an oxidized form (3). The associated risk of CVD has been related to the persistence in the circulation of the remnant particles that carry the dietary TG in the plasma (7, 9).

Atherogenesis begins with phagocytic immune system cells, primarily monocytes and T lymphocytes, responding to endothelial cell injury caused by high levels of oxidized

LDL (3). The oxidized LDL penetrates the endothelium into the arterial intima causing adherence of monocytes and platelets (3). The perpetuation of atherogenesis occurs by two routes. First, monocytes, activated by the presence of oxidized LDL, release chemoattractants which draw additional monocytes to the endothelium (3). Phagocytosis of oxidized LDL leads to the transformation of monocytes to macrophages, inhibiting motility and trapping the macrophages in the endothelial space (3). At this point, the lipid-engorged macrophages become foam cells. A second, proposed mechanism is through the release of growth factors stimulating the proliferation of smooth muscle cells in the arterial media. Oxidized LDL rapidly accumulates within the smooth muscle cells leading to the accumulation of foam cells, which contributes to fatty streaks and occlusion of the arterial lumen compromising blood flow (3).

Zilversmit (4) hypothesized that atherosclerosis was a postprandial phenomenon based on the formation of chylomicron remnants, LDL, and the uptake of these cholesterol and TG rich molecules by arterial cells after a meal (4, 10). Fasting blood cholesterol concentrations are used clinically to determine if an individual is at elevated risk for the development of CVD, however most individuals exist in a postprandial state 16-18 hours per day (6). Disease risk may be better predicted by a postprandial test than a fasting test due to the amount of time an individual spends in a postprandial state (6). The measurement of blood TG has also been shown to be elevated in the postprandial state in patients with documented CVD (11). A high CHO diet increases postprandial TG, which can further contribute to increased atherogenic risk (6).

The cholesterol lowering effects of soy protein as compared to animal protein on lowering CVD risk has been recognized in animal studies for more than 80 years (12). In 1995, Anderson et al. (12) conducted a meta-analysis to determine the effect of soy protein intake on serum lipids. Beneficial effects were associated with the substitution of soy protein for animal protein foods in diets already low in saturated fat (<10% of total energy) and dietary cholesterol (<200 mg/d) (13). Significant reductions in serum concentrations of total cholesterol (23.2 mg/dL), LDL cholesterol (21.7 mg/dL), and TG (13.3 mg/dL) without significantly influencing high-density lipoprotein (HDL) cholesterol (2.4 mg/dL increase) were observed (12, 14). These lipid lowering effects have generally not been seen in humans fed isoflavones separated from the soy protein (13), suggesting there may be additional factors within the intact soy protein that contributes to the hypocholesterolemic effects.

Isoflavones are one of the three main categories of phytoestrogens (15), a plant derived estrogen analog (16). Soy products are rich in isoflavones, daidzin and genistin, that possess antioxidant activity. These isoflavones are both hydrolyzed from the β-glycoside form to the highly bioavailable aglycone form, diadzein and genistein, by bacterial glycosidases in the large intestine (15). Several studies suggest that soy isoflavone consumption may protect LDL cholesterol from oxidative damage due to its antioxidant properties (12, 16, 17, 18).

Although there have been advances in research regarding the consumption of soy products for beneficial effects related to CVD, much is still unknown. Whether isoflavones are effective in reducing atherogenesis needs to be studied further.

Understanding the mechanism behind LDL oxidation and the beneficial components of soy products, researchers can begin to examine how soy can protect against a variety of diets. For example, soy may offer protection against HPTG observed after ingesting a high CHO diet. This will enable researchers to gain further knowledge of the possible anti-atherosclerotic effects of soy.

Purpose

The purpose of this study was to determine if the oxidative resistance of postprandial LDL was enhanced with the consumption of a shake containing 39.0 g soy protein (85 mg aglycone isoflavones) vs. 39.9 g milk protein (0 mg aglycone isoflavones) in combination with a high carbohydrate meal.

Hypothesis

Postprandial oxidative stress, as measured by copper-induced LDL oxidation following the consumption of a high carbohydrate meal, will be reduced following the 39.0g of soy (85mg aglycone isoflavones) as compared to the same meal with 39.9g of milk protein (0mg aglycone isoflavone) due to the isoflavone content of the soy protein.

CHAPTER 2

REVIEW OF LITERATURE

The randomized clinical trial has the potential to provide a compelling rationale for accepting or rejecting a treatment. One drawback of clinical trials of diet in CVD is their high cost and impracticality for testing many nutrients and foods. Also, clinical trials may not last long enough to detect such effects that take more than a few years to make them seen. An alternative to using CVD as an outcome in a dietary trial is to select surrogate end points that are in the causal path between a food or nutrient and CVD. Plasma lipoproteins such as total and LDL cholesterol are an important surrogate end point because of their strong link to the pathogenesis of atherosclerosis and their strong predictive association with CVD which has been supported strongly by a consistent body of evidence from clinical trials and epidemiological studies (19). National health organizations advocate dietary changes that decrease intake of saturated and trans-unsaturated fat and cholesterol to prevent CVD. The rationale is to reduce LDL concentration; however, diets affect not only LDL but also HDL and TG, which are also independent lipid risk factors.

Postprandial Metabolism

In 1910, the presence of cholesterol in lesions of diseased arteries was described by Windaus (4). Since that time, epidemiological studies have repeatedly confirmed a high degree of correlation between the intake of cholesterol and other lipids with the

occurrence of CVD (4). Over 20 years ago, Zilversmit hypothesized that atherosclerosis was a postprandial phenomenon (10). This hypothesis was based on the formation of chylomicron remnants, LDL, and the normal process of lipid absorption by arterial cells after a meal (4, 10).

Many studies of lipoprotein metabolism have been carried out in the fasted state, because this was thought to be more reproducible. However, most individuals exist in the postprandial state 16-18 hours per day and subside in a fasted state for approximately 6 hours per day, typically during sleep (6). Therefore, the assessment of disease risk could be improved by a postprandial test of lipoproteins, rather than a fasted test. Indeed, studies have shown blood TG levels are elevated in the postprandial state in patients with documented CVD (11).

The metabolism of chylomicrons and VLDL molecules are similar in many respects. Absorption, synthesis, and secretion by the enterocyte and transportation of dietary lipids are functions of the intestine dependent on chylomicron formation. The chylomicron transports dietary fatty acids in the form of TG through the lymph to the liver. In addition to apolipoprotein (apo) B-48, the chylomicron acquires apo E and apo C from HDL in circulation (20). Once in the blood, chylomicrons interact with lipoprotein lipase on the surface of mainly muscle and adipose endothelial cells, catalyzing the hydrolysis of TG molecules into monoglycerides and fatty acids (21). The hydrolytic products are then transported across the endothelium where they are oxidized by the muscles or reesterified for storage in the adipose tissue. Some of the free fatty acids from the chylomicron escape the uptake and become bound to albumin and are transported to the liver for

reesterification or inclusion into native VLDL. The apo C of the chylomicron is then transferred back to the HDL as cholesterol esters are gained (21).

The remaining chylomicron particle is termed a "chylomicron remnant". The remnant interacts with the hepatic lipase to hydrolyze any remaining TG and expose the apo E, which is essential for recognition and clearance of the chylomicron remnant by the liver (10). Postprandial uptake of chylomicron remnants and TG stimulates the hepatocytes to produce native VLDL.

Once the apoB-100 containing VLDL particle is in circulation, apo E and C are transferred to the VLDL particle from HDL. Lipoprotein lipase hydrolyzes large VLDL-TG similar to chylomicrons, but smaller VLDL are hydrolyzed more slowly because of limited interaction with lipoprotein lipase due to their size and the increased concentration of lipoproteins postprandially. The large VLDL remnants are then taken up rapidly by the liver's LDL receptors and degraded, while the small VLDL interact with hepatic lipase, eventually losing their apo E and C protein, the majority of their TG, and ultimately become LDL particles. The apo E receptor is essential for binding with the liver and ultimate clearance of the particles from circulation. The number of LDL particles in the blood significantly surpasses that of their precursors due to the lack of apo E receptor-binding domain. They are eventually taken up by the liver via interactions with a receptor-binding domain on the apo B-100 with the LDL receptor (7).

The rapid increase in chylomicrons and VLDL-TG in the postprandial phase saturates the liver's capacity by increasing competition for the common removal mechanism (apo E receptor) by the hepatocytes, leading to a longer duration that the TG-rich lipoprotein

remnants remain in circulation (4, 10). This increased circulation time enhances the possibility that the lipoprotein particles may become oxidized and enter into the endothelial space (10, 22). This progression further increases the risk for atherosclerosis and CVD.

Oxidative Hypothesis of Atherosclerosis

In the 1970's, two groups of researchers simultaneously proposed the oxidative modification hypothesis of atherogenesis. Chisolm et al. (23) witnessed that the injury of endothelial cells *in vitro* was dependent on oxidatively modified LDL, while Steinberg et al. (23), determined that foam cell formation could not be induced by native LDL. Both Chisolm and Steinberg later demonstrated that modification of LDL was possible *in vivo* and that the modified LDL was recognized by savenger receptors on macrophages due to oxidative modification. Oxidized LDL causes endothelial injury initiating an immune system response drawing phagocytic monocytes and T lymphocytes to the arterial site (3). Macrophages rapidly up-take oxidized LDL due to the abundance of binding sites for LDL modified by oxidation (7, 24). These engorged macrophages become trapped in the endothelial spaces and become lipid-laden foam cells (25). As the foam cells increase in size, their lipid contents form fatty streaks in the intima of the arterial wall, enlarging and occluding the arterial lumen and impeding blood flow (3). In more recent years oxidative stress has become the term used to define the imbalance between free-radical producing pro-oxidants (e.g. copper, iron) and antioxidant defenses (e.g. soy isoflavones, vitamin E)

that contributes to LDL oxidation and the pathogenesis of vascular complications, such as athrosclerosis.

The field of research regarding the oxidation of LDL has rapidly developed over the last 20 years beginning with only a few published articles, manifesting into hundreds. The widely accepted process of oxidative stress leading to the pathogenesis of atherosclerosis involves a cascade of cellular events in the endothelium of the arterial wall contributing to plaque formation (26, 27). The formation of atherosclerotic lesions in the arterial intima is believed to be caused by a number of factors including, endothelial injury brought about by hyperlipidemia or toxic agents (28). The reactions causing oxidation of LDL vary; the presence of metal ions *in vitro* (e.g. copper and iron), superoxide radicals and heme-containing compounds *in vivo*, have all been suspected of modifying LDL (3).

The initial stage of LDL oxidation involves the free radical peroxidation of predominately polyunsaturated fatty acids (PUFA) and to a lesser extent monounsaturated fatty acids (MUFA) in LDL, due to the double bonds possessed by these fatty acids (29). The process occurs such that hydrogen is removed by a free radical and a molecular rearrangement occurs, forming a conjugated diene (29). Oxygen is then taken up and a peroxy radical is formed initiating the removal of hydrogen from another fatty acid. It is important to remember that these processes are occurring simultaneously and are at different phases *in vivo*. In a controlled environment *in vitro*, this phase is known as the propagation stage of the oxidation process and represents a chain reaction

leading to the formation of lipid hydroperoxides (30). Likewise, the decomposition stage refers to the conversion of lipid hydroperoxides into reactive aldehydes and ketones and is the final phase of LDL oxidation *in vitro* (30).

A study conducted by Lechleitner et al. (31), examined the hypothesis that modified LDL but not native LDL are capable of leading to foam cell formation in the course of postprandial lipemia. Macrophages were incubated with fasted and postprandial native LDL from 17 healthy volunteers. After the LDL was isolated, postprandial LDL was found to be more susceptible to *in vitro* oxidation than fasting LDL. A significantly higher cellular cholesterol ester accumulation (postprandial: 477 ± 286%; fasted: 212 ± 173% respectively; $p<0.003$) was induced by postprandial LDL than fasting LDL (31). The increase in cellular cholesterol ester synthesis is evidence that oxidized postprandial LDL but not native LDL leads to foam cell formation in macrophages.

Regnstrom et al. (32) investigated the relationship between the ability of LDL to resist oxidation *in vitro* and the severity of CVD. Low-density lipoprotein was isolated from 35 young male survivors of myocardial infarction. The LDL was subjected to copper induced oxidation and the lag time was measured. Oxidative modification of LDL cholesterol correlated independently with the severity level of CVD ($r = -0.45$, $p<0.02$). The lag time was also related to the TG content of the LDL fraction ($r = -0.55$; $p<.002$). The finding that the susceptibility of LDL to oxidation is positively associated with the severity of atherosclerosis indicates that lipid oxidation promotes premature CVD and that individuals with LDL rich in TG are at particularly high risk. This experiment is

beneficial in directing further research toward the cause of elevated TG levels and possible mechanisms to increase oxidative resistance of LDL.

The expanded research surrounding the oxidative modification hypothesis of atherogenesis proposed over 20 years ago has provided a strong body of evidence to suggest that oxidized LDL has an important pathological role in the development of atherosclerosis. Further research must now be conducted to determine if oxidative resistance of LDL can aid in the prevention of CVD. One potential mechanism may be antioxidants, such as soy isoflavones, which may lend protection to LDL, thus increasing resistance and reducing the risk of atherosclerosis.

Benefits of Soy in CVD

In 1999, the Food and Drug Administration authorized the use of a health claim for soy protein based on data reviewed from 38 clinical studies in a meta-analysis, conducted by Anderson et al. (13). The study concluded that 25 g of soy protein included in a diet low in total fat (<30% of total daily calories), saturated fat (<10% of total daily calories), and cholesterol (<200 mg of total daily calories) may reduce the risk of CVD by significantly lowering blood cholesterol levels (33). The claim indicated that four daily soy servings of 6.25 g/serving can reduce levels of LDL by as much as 10% (33). The scientific community generally agrees that a 1% drop in total cholesterol can equal a 2% drop in heart disease risk (33). Dietary interventions that can lower cholesterol are important tools in the fight against CVD.

The combined LDL-lowering and antioxidant effects of soy protein and their isoflavones may contributeto the lower rates of CVD among the Asian population, who consume 30 to 50 times (34) more soy protein in their diet as compared to the intakes of Americans (14). Whole soy foods are a good source of fiber, B vitamins, calcium, and omega-3 essential fatty acids, as well as offering a complete protein profile and soy isoflavones. Soy contains many other potentially active components, including saponins, plant sterols, and PUFAs, all of which may contribute to plasma cholesterol reduction (16). The amino acid content in soy protein is different from animal and most other vegetable proteins, and appears to alter the synthesis and metabolism of cholesterol in the liver (33).

The isoflavones in soy are phytoestrogens, a plant derived estrogen analog (16). Isoflavones in soy have several features in common with β-estradiol including an aromatic ring with a hydroxyl group substitution and a second hydroxyl group in the same plane, which enable isoflavones to bind to estrogen receptors (35) (see Figure 1). The isoflavones in soy may have effects that are similar to those of estradiol, which increases LDL receptor expression and decreases hepatic lipase activity, thus having a favorable effect on plasma LDL concentrations (36).

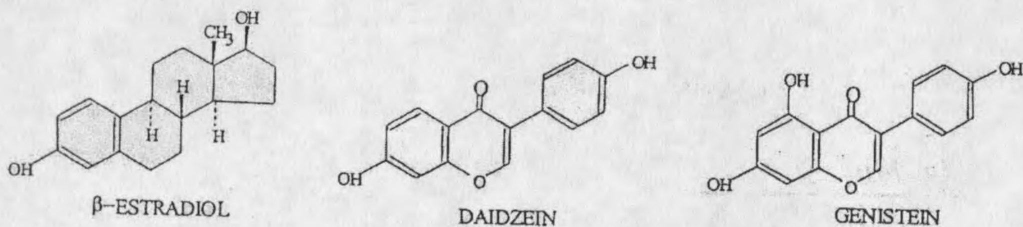

Figure 1. Structural Comparison of Estrogen to Isofalvones (35).

Isolates of soy protein (ISP) are commonly used products in soy research and are beneficial because of the control they provide. The content of isoflavones and the ratio of individual isoflavones can be manipulated enabling researchers to prepare isocaloric meals thatcan be produced and easily compared to placebos (37). These benefits are also due to the negligible content of CHO, fiber, and fat in ISP.

The major isoflavones, daidzein and genistein, exist in four chemical forms: aglycone, glycoside, acetylglucoside and malonylglucoside. After ingestion of the isoflavones, daidzin and genistin are hydrolyzed from the β-glycoside form to the bioavailable aglycone form, daidzein and genistein, by bacterial glycosidases in the large intestine (15, 38, 39). The metabolism of daidzein *in vivo* yields an isoflavone, equol. The isoflavone equol, is a more potent antioxidant *in vitro* than either daidzein or genistein and has shown the greatest antioxidant activity (15). Some individuals however, may differ in their ability to metabolize this isoflavone. Only 30% of the population are capable of complete metabolism of daidzin to equol (40, 41), therefore 70% of individuals may not reap the antioxidant health benefit associated with the consumption of soy (42).

The scientific research investigating the effects of soy on CVD have examined the results of ISP, purified isoflavones, and whole soy foods on plasma lipids, all of which provide differing results (37). Simons et al. (42) conducted a randomized 16 week cross-over study with 20 postmenopausal women to evaluate the effects of a diet containing purified soy isoflavones as compared to a placebo. The soy isoflavone extract was provided daily in a tablet containing 80 mg of isoflavones. An isocaloric fat-restricted diet (30% energy from fat, <10% energy from saturated fat, cholesterol intake <300

mg/day) was consumed for 21 days prior to treatment. Fasted blood sample analysis showed no significant differences in plasma lipid or lipoprotein levels for total, LDL, and HDL cholesterol although isoflavone levels were significantly higher during treatment. The link between consumption of purified soy isoflavone and cholesterol-lowering effects was not observed. These results strongly suggest there is an interaction between the soy isoflavones or an additional component such as soy protein which must also be present for the cholesterol-lowing effects.

In contrast to the previous study, Crouse et al. (34) investigated the use of ISP in reducing total and LDL cholesterol in 156 moderately hypercholesterolemic (LDL cholesterol levels of 140-200 mg/dL) men and women. They compared 25 g/d of casein protein to 25 g/d of ISP containing 3, 27, 37, and 62 mg of isoflavones. The ISP containing 62 mg of isoflavones revealed the greatest reduction in plasma concentration of total and LDL cholesterol of 4% (243 ± 26 mg/dL vs. follow-up: 233 ± 20 mg/dL; p=0.04) and 6% (baseline: 166 ± 25 mg/dL vs. follow-up: 156 ± 17 mg/dL; p=0.01) respectively. For further analysis, subjects were divided into high and low LDL cholesterol groups based on their baseline values. The high LDL cholesterol group who received isolated soy protein containing 62 mg of isoflavones had a reduced total cholesterol of 9% (baseline: 261 ± 23 mg/dL vs. follow-up: 237 ± 21 mg/dL; p<0.001) and LDL cholesterol of 10% (baseline: 185 ± 21 mg/dL vs. follow-up: 163 ± 18 mg/dL; p=0.001). The 37 mg of isoflavones also lowered both the total (baseline: 260 ± 16 mg/dL vs. follow-up: 240 ± 25 mg/dL; p=0.007) and LDL (baseline: 182 ± 16 mg/dL vs. follow-up: 165 ± 22 mg/dL; p=0.02) cholesterol by 8% in the high LDL cholesterol

group. The plasma concentration of TG and HDL cholesterol was not adversely influenced for all isoflavone levels. This study exemplifies the potent interaction isoflavones possessin conjunction with the isolated soy protein to reduce total and LDL cholesterol, with the greatest effect on subjects with above average LDL levels and greater amounts of isoflavone provided.

Soy and Oxidation

The hypocholesterolemic effects of soy have been studied for years, more recently, the antioxidant potential of soy and its isoflavones has been examined. Products containing soy may protect against atherosclerosis not only by increasing antioxidants to plasma and lipoproteins, but also minimizing the postprandial increase in lipid hydroperoxides. It has been purposed thatthe Cu^{2+} metal ion acts as pro-oxidants and bind to the apo B on LDL, triggering lipid peroxidation (43). Therefore, it is conceivable that soy isoflavones could act as an antioxidant defense and become bound to apo B or in some way cause a steric hindrance blocking the Cu^{2+} from binding to the apo B site, thus inhibiting lipid peroxidation (43).

Preliminary works have studied vitamin E, soy protein isoflavones, plant phenols using green tea, and β-carotene *in vitro* and revealed the administration of antioxidants, significantly reduces LDL oxidation in humans (14). The antioxidant effect of genistein against LDL oxidation *in vitro* was investigated by Kerry et al. (44). The LDL was isolated from a single healthy volunteer, incubated with 0, 25, 50, or 100 μmol/L of genistein, and subjected to both copper-mediated and radical-mediated LDL oxidation.

Genistein inhibited LDL oxidation in a concentration-dependent manner by increasing the lag time (control: 54.1 ± 5.1 min vs. 5 μmol/l genistein: 107.1 ± 1.8 min; $p<0.001$) and decreased the propagation rate (control: 14.4 ± 1.9 nmol/mg/min vs. 5 μmol/l: 7.4 ± 1.1 nmol/mg/min; $p<0.001$). Approximately 3-4% of genistein present in the plasma was incorporated into LDL. This study demonstrates that isoflavones can inhibit LDL oxidation *in vitro*.

Tikkanen et al. (18) examined the effect of soy as a possible strategy for preventing oxidation through incorporation of isoflavones into LDL particles. Six healthy young subjects consumed one soy bar containing 12 mg of genistein and 7 mg of daidzein and 7.1 g of protein 3 times daily for 2 weeks. Fasting blood was drawn at baseline, at the end of the treatment period, and 12 days after discontinuation of soy. The proportion of genistein and daidzein in purified LDL was less than 1% of total plasma content of these substances, yet a significant mean prolongation of the lag phase by more than 20 minutes (baseline:147 ± 9 min vs. day 14: 173 ± 19 min, $p<0.02$) was observed after 2 weeks of soy treatment (18).

In support of Tikkanen, Meng et al. (43) also reported that dietary intake of soybean isoflavones resulted in increased oxidative resistance of isolated LDL *in vitro*. Following reports that human estrogen could be incorporated into lipoproteins *in vitro*, they hypothesized that isoflavones could become esterified by a similar mechanism and lead to oxidative resistance. Diadzein and genistein were esterified into several different isoflavones fatty acid esters to increase their lipid solubility. The incorporation of these isoflavones into LDL and the ability to protect isoflavone-containing LDL from copper-

mediated oxidation was analyzed. Relatively small amount (0.33 molecules of isoflavone per LDL particle, or less) of the unesterified isoflavones were incorporated into LDL particles. The esterified esters of daidzein and genistein were incorporated more effectively (2.19 molecules of isoflavone per LDL particle) and prolonged the lag times by 46% ($p<0.05$) and 202% ($p<0.01$), respectively. Both of these studies illustrate the antioxidant capabilities of soy isoflavones and their ability to aid in the protection of LDL from oxidation.

Jenkins et al. (16) assessed the effects of soy isoflavones on LDL oxidation using 31 hyperlipidemic subjects in a 2 month randomized crossover study. All subjects had an elevated serum LDL cholesterol of >158 mg/dL and a triglyceride level <154 mg/dL at recruitment. Subjects consumed a test meal providing 33 g/d of soy protein (86 mg isoflavones/ 2000 kcal/d) and a lacto-ovovegetarian control diet with low-fat milk products. A fasted blood sample at baseline showed no significant differences in pretreatment values for blood lipids between the test and control diets. However, fasted blood samples obtained following week 2 and 4 of treatment showed lower mean test values for oxidized LDL assessed by conjugated dienes formation (test: 56 ± 3 μmol/L vs. control: 63 ± 3, $p<0.001$). The ratio of conjugated dienes to LDL cholesterol was also reduced (15.0 ± 1.0 test vs. 15.7 ± 0.9 control, $p=0.032$) between the two dietary treatments. These findings demonstrate soy isoflavone consumption may protect LDL cholesterol from oxidative damage *in vitro* and reduce the concentration of oxidized LDL cholesterol as expressed by conjugated diene formation (16). This study also demonstrated the consumption of high isoflavones foods appears to be associated with

reduced levels of circulating oxidized LDL. The effect of dietary antioxidants in reducing the risk of atherosclerosis has potential value in CVD risk reduction, possibly without adverse side effects.

Further research by Jenkins et al. (17) assessed the effects of a soy-based breakfast cereal on serum lipids and oxidized LDL in a randomized crossover design with two three-week ad libitum diets. Twenty-five hyperlipidemic subjects with elevated serum LDL cholesterol concentrations of >74 mg/dL and triglyceride levels <72 mg/dL were placed on a NCEP Step 2 diet (<30% energy as total fat, <7% saturated fat, and <200 mg/d dietary cholesterol) 1 month prior to the study to control the subject's background diet. Following the run-in period, subjects consumed 36 g/d soy protein (168 mg/100 g isoflavones) daily for 3-weeks. Low-density lipoprotein was reduced compared with the control both as total dienes in LDL and as the ratio of conjugated dienes to cholesterol in the LDL fraction by 9.2 ± 4.3%, p=0.042 and 8.7 ± 4.2%, p=0.050. This study further demonstrates that daily consumption of soy can reduce the concentration of oxidized LDL cholesterol. These results may be attributed to the increased isoflavone intake in conjunction with soy protein (17).

In addition to the work conducted by Tikkanen and Jenkins, Weisman et al. (38) also examined the effects of soy protein on *in vivo* biomarkers of lipid peroxidation and oxidative resistance. Twenty-four healthy subjects participated in a randomized crossover design and consumed a high isoflavone (HI) burger (21.2 mg daidzein, 34.8 mg genistein) and a low isoflavone (LI) burger (0.9 mg daidzein, 1.0 mg genistein) once a day for 17 days along with an ad libitum dietseparated by a 25 day washout period.

Fasted blood samples revealed the HI soy dietsignificantly lowered F_2-isoprotstane, a biomarker of *in vivo* lipid peroxidation by 19.5% (HI: 326 ± 32 ng/L, LI: 405 ± 50 ng/L; p=0.03). The lag time of LDL oxidation was also extended by 9% (HI: 48 ± 2.4 min, LI: 44 ± 1.9 min; p=0.02), suggesting the consumption of soy isoflavones protect against lipid peroxidation *in vivo* as well at *in vitro* as seen in the Tikkanen (18) study.

Although the mechanism related to the ability of soy isoflavones to decrease oxidation is not completely understood it is understood that concentration is positively correlated with effect (16, 17, 18). The mechanism has been hypothesized to be the result of synergy between the soy protein and the natural isoflavones (34). Patel et al. (45) have proposed a scheme by which genistein inhibits lipid peroxidation (see Figure 2) and is regenerated by ascorbate, similar to vitamin E. Hypothesis such as this demonstrate a need for further studies to attain a greater understanding of how soy isoflavones increase the oxidative resistance of LDL.

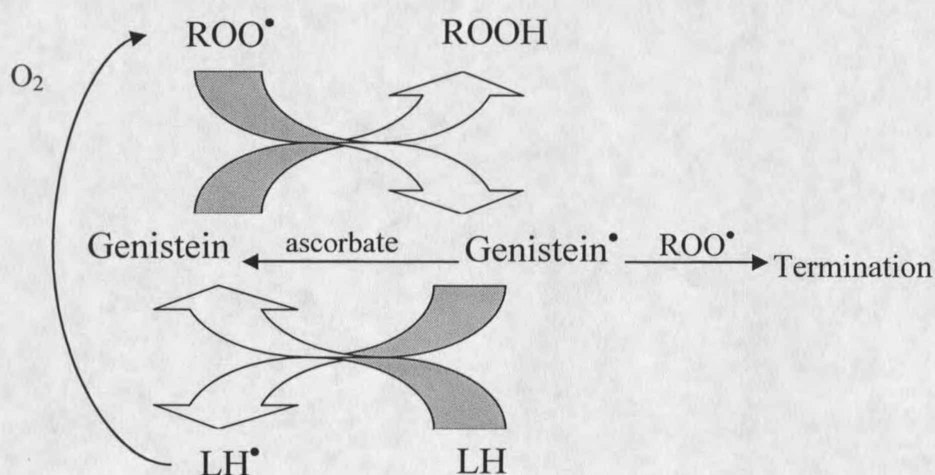

Figure 2. Antioxidant Effects of Isoflavones (45).

High Carbohydrate Diet

Recommendations to reduce fat intake in the diet is based upon well established evidence that variations in dietary saturated fat content represent the most powerful way of influencing LDL cholesterol concentration (46, 11). As fat intake is reduced in the diet, individuals tend to replace the missing calories with dietary CHO. The concentration of dietary CHO consumed can range greatly, but the high CHO diet, for the purpose of research, has been termed "low-fat, high-carbohydrate" if ≤30% of the total energy is derived from fat and ≥55% of energy is derived from CHO (5).

Given the importance of LDL cholesterol concentration as a risk factor for CVD, more specifically atherosclerosis, it seems appropriate to limit fat intake. Research has shown the desired reduction in plasma LDL cholesterol and total cholesterol is typically reached with a low-fat, high CHO diet (47, 48). Ironically, these benefits are accompanied by unfavorable effects as well. A rise in plasma TG concentrations above 200 mg/dL (5) is one unfavorable effect and has been termed HPTG. Decreasing fat without increasing CHO such as, replacing dietary fat with protein does not appear to elevate serum TG levels (49). This suggests that it is the addition of CHO, not the removal of fat that is associated with HPTG in persons consuming high CHO diets. Another unfavorable effect is a decrease in HDL cholesterol, proportionately more than the decrease in LDL (19, 21, 50). These unfavorable affects have both been shown to be independent risk factors for the development of and mortality from CVD (19, 47).

Ginsberg et al. (51) conducted The Delta Study, an important clinical trial that determined the effects of reducing dietary saturated fatty acids (SFA) in specific

subgroups of the population. The subjects consisted of 103 healthy, normolipidemic individuals (55% women and 45% men) who ranged in age from 22 to 67 years. Twenty percent of the men and 30% of the women were black, and of the total women, 32% were postmenopausal. Each subject was randomized to one of six diet sequences, participating in all three diets, in the double-blind crossover design. The three diets consisted of the average American diet (AAD: 34% fat, 48% CHO, 15% protein), the NCEP Step 1 diet (Step 1: 29% fat, 55% CHO, 15% protein), and the low-SFA diet (low-SFA: 25% fat, 59% CHO, 15% protein). The diet period lasted 8 weeks and fasted blood samples were obtained once each week during weeks 5, 6, 7, and 8 to ensure that there was adequate time to achieve steady-state levels of lipids, lipoproteins, and thrombogenic factors. The results showed a decrease in total cholesterol of ~5% from the AAD diet to the Step 1 diet and a ~4% decrease from the Step 1 to the low-SFA diet (AAD: 202.1 ± 2.8 mg/dL, Step 1: 191 ± 2.7 mg/dL, low-SFA: 183.4 ± 2.7 mg/dL; $p<0.01$). The LDL cholesterol also decreased ~7% from the AAD to the Step 1 diet and decreased an additional 4% on the low-SFA diet (AAD: 131.4 ± 2.7 mg/dL, Step 1: 122.2 ± 2.6 mg/dL, low-SFA: 116.9 ± 2.6 mg/dL; $p<0.01$). The TG concentrations increased ~9% between the AAD and Step 1, but did not change significantly from the Step 1 to the Low-Sat diet (AAD: 85.1 ± 3.4 mg/dL, Step 1: 92.4 ± 3.7 mg/dL, low-SFA: 93.0 ± 3.7 mg/dL; $p=0.054$). These results substantiate the findings that replacing as little as 5% of energy from saturated fat with CHO can lead to a significant increase in TG levels. This study also demonstrated the total and LDL cholesterol lowering effects of the Step 1 and low-SFA diets, and the increase in TG levels can be observed in healthy subgroups of the population, where

pervious works generally investigated the response of middle-aged white males in varying degrees of health.

The debate surrounding the use of high CHO diets has raised concerns regarding the atherogenicity of TG-rich lipoproteins due to the high correlation with the presence of postprandial TG-rich chylomicron and VLDL remnants (46). Understanding the mechanism as to how high CHO diets elevate plasma TG concentrations may aid in designing dietary guidelines that will promote the maintenance of good health. Current research is trying to determine whether CHO-induced HPTG results from an overproduction of TG, a decrease in clearance of TG containing lipoprotein from the blood, or a combination of the two.

Theories regarding an overproduction mechanism have been proposed by several researchers. Reaven et al. (52), hypothesized that insulin resistance of adipose tissue could result in failure to decrease lipolysis when insulin is elevated. Insulin concentration could lead to a reduction in fatty acid oxidation by suppressing hormone sensitive lipase and by increasing the activity of lipoprotein lipase thereby decreasing the rate of fatty acid entry in the mitochondria (47, 48). Fatty acids in the liver may then be driven towards esterification, therefore new TG is formed and exported into the plasma as VLDL-TG (48).

Although it has yet to be fully accepted whether two weeks of dietary modifications are sufficient to achieve a new steady physiologic state in enzyme concentrations and activities, substrate oxidation, and plasma TG concentrations, studies such as Mittendorfer and Sidossis (47), examined an acute feeding trials lasting 14 days. The

study examined the effect of a HC diet on rate of VLDL-TG secretion using *in vivo* labeled VLDL-TG tracers. Six healthy volunteers consumed both an isoenergetic HC (10% fat, 75% CHO, 15% protein) and HF (55% fat, 30% CHO, 15% protein) for 14 days on two separate occasions. Analysis of the isotope infusion followed a 15 hour fast after the last meal was consumed. Total TG (HF: 144 ± 18, HC: 134 ± 14 mg/dL) and VLDL-TG concentrations were higher ($p<0.01$) after the HC diet. The rate of VLDL-TG appearance was significantly higher ($p<0.04$) after the HC diet (0.76 ± 0.12 μmol/kg/min) than the HF diet (0.45 ± 0.15 μmol/kg/min), but there was no difference in VLDL-TG clearance between the 2 diets. This research confirmed the rise in fasting plasma TG concentrations is accompanied by an increase in VLDL-TG levels following a HC diet and was able to demonstrate it was the result of increased VLDL-TG secretion in conjunction without an increase in the clearance level.

The over production of VLDL-TG by CHO induced HPTG can occur through two methods. High plasma TG concentrations may be caused by an increase in carrying capacity of TG by each VLDL particle, while the number of particles remains the same. Mancini et al. (53) found the average ratio of VLDL-TG to protein increased 230% when subjects consumed a diet containing 20% fat and 80% CHO. The alternative is an increase in the number of VLDL particles produced, while the carrying capacity of TG remains constant (5). Abbott et al. (54) and Stacpoole et al. (55) discovered there is a large variability among subjects with respect to changes in VLDL particle number. The magnitude of change is related to many factors including the amount and type of CHO,

duration of feeding, route of administration, lipoprotein phenotype and the degree of glucose tolerance an individual possesses.

Abbott et al. (54) investigated a potential mechanism behind the reduction in LDL when SFA is replaced by CHO and to determine whether solid food, isocaloric, HC diets cause an increase in either plasma VLDL concentrations or hepatic VLDL production. Fourteen Pima Indians (7 nondiabetic (ND)and 7 NIDDM) were provided a HF diet (42% fat, 43% CHO, 15% protein) for 5 weeks and a low-fat, HC diet (21% fat, 65% CHO, 15% protein) for 5 -7 weeks, allowing for TG levels to stabilize. Metabolic changes were similar in ND and NIDDM. Analysis of fasted plasma samples showed a decrease in LDL on the HC diet (131 ± 8 mg/dL vs. 110 ± 7 mg/dL, $p<0.0001$) for all subjects. Both fasting and 24-hour TG concentrations remained unchanged as well as the mean production rates for VLDL, apo B and VLDL-TG. However, the mean VLDL apo B pool increased (303 ± 20 mg/dL vs. 371 ± 38 mg/dL, $p = 0.01$), illustrating the conversion pathway transporting VLDL apo B to intermediate-density lipoproteins (IDL) apo B decreased (10.7 ± 1.1 mg/kg ffm per day vs. 8.4 ± 0.9 mg/kg ffm per day, $p<0.001$). This explains the decrease in LDL apo B (70 ± 5 mg/dL vs. 61 ± 5 mg/dL, $p<0.001$) when subjects were consuming the HC diet. This experiment demonstrates the impact a high CHO diet can have on the conversion process in the lipoprotein pathway. The decrease in LDL and increase in VLDL suggests that the mechanism for converting VLDL to IDL and LDL was impaired by the high CHO diet.

Another hypothesis exists stating it is not only the synthesis of new TG that leads to HPTG, but the VLDL-TG clearance is decreased (6). Three clearance mechanisms have

been proposed. Reaven et al. (52) suggests lipoprotein lipase, the primary enzyme responsible for the removal of fatty acids from TG-rich lipoprotein particles as they circulate throughout the plasma, either becomes saturated or is down-regulated in amount or activity after a high CHO diet (6). The second mechanism involves the receptor mediated uptake of the TG-rich lipoproteins by the liver (6). The combined contribution of these mechanisms is still unknown. It is speculated that the down regulation of lipoprotein lipase could increase chylomicron TG postprandially, and down-regulation of hepatic receptors could lead to reduced VLDL-TG clearance, thus leading to a build up of TG-rich lipoproteins in circulation (6).

Mancini et al. (53) tested the hypothesis that the resistance of adipose and muscle tissue to insulin leads to a block in the peripheral utilization resulting in decreased clearance of TG. The half life of TG before HC feeding averaged 6.6 mg/dl/min, but after 5 days of HC feeding the half life increased significantly ($p<0.01$) to 14 mg/dl/min, which showed the peripheral uptake of TG, was decreased by HC diets in normal subjects (27). Research now needs to determine the cause behind the decreased clearance and the impact HC diets have on insulin resistance.

To determine how rapidly the effects of both HF and HC diets have on parameters of insulin resistance, HPTG, and gastrointestinal adaptations, Robertson et al. (48) recruited 12 healthy men who consumed both a HF meal (62% of energy from fat and 31% of energy from CHO) and a HC meal (16% of energy from fat and 75% of energy from CHO) in a crossover study. Fasting blood samples 12 hours after the evening meal were followed by either an oral fat tolerance test (OFTT) or an oral glucose tolerance test

(OGTT). Fasting plasma TG concentrations after consuming the meals were significantly higher ($p<0.0001$) in the HC diet (19.82 ± 1.80 mg/dL) compared to the HF diet (16.22 ± 1.80 mg/dL). The increase in TG levels rose within 20-30 minutes and remained elevated 12 hours after the HC meal (48). This study illustrates the effects of a HC meal, which had the strongest effect on subsequent TG metabolism. The HC diet had a rapid response and persisted for a long period of time. Additional studies have agreed with these findings and have also concluded that there can be great variability among subjects and diets (5).

The physical form of the diet (liquid or solid), and the presence of fiber in the diet are also factors that influence the time it takes for a HC diet to induce HPTG (5). Purified diets, regardless of CHO type, induce HPTG more readily than whole food diets (5). Hypotriacylglycerolemic effects of whole food are influenced by fiber content and are an important factor in moderating CHO-induced HPTG because a high fiber diet shows a decreased TG response (5).

Clinical trials have provided a consistent body of evidence that advocate dietary changes that decrease the intake of saturated fat and cholesterol to prevent CVD. These modifications to the diet have produced the desirable decreases in LDL, possibly diminishing the opportunity for oxidation leading to atherosclerosis. Research has now begun to examine the resulting effects of an increase in CHO in the diet the relationship to HPTG and CVD. The information gained from this type of research can aid diabetic patients and individuals who have a predisposition for HPTG and are at a high risk of developing CVD. Through the understanding of these dietary influences on lipoproteins,

researchers can begin to cultivate alternative methods of decreasing the risk of atherosclerosis through the use of antioxidants, such as soy isoflavones. It is evident that the need for additional research exists and would be valuable to the 58 million American suffering with at least one form of CVD.

CHAPTER 3

METHODS

Subjects

Fifteen healthy males between the ages of 18 and 44 years of age were recruited through the use of flyers posted on the MSU campus, the Bozeman Chronicle, and word of mouth. Power calculations ($\alpha = 0.50$; $\beta = 0.80$) from a project completed spring 2002 in the NRL investigating the effects of soy protein on postprandial oxidative stress indicated that future studies should use no less than 15 subjects for similar acute studies (56). Each subject gave written, informed consent and was not monetarily compensated. Male individuals were specifically selected for this study due to the increased risk of CVD in males and to control for influences of the menstrual cycle. Subjects were excluded based on the following criteria: 1) a known chronic disease or metabolic disorder (e.g. diabetes mellitus, abnormal hepatic or renal function); 2) use of prescription medications (e.g. insulin) or have been on antibiotic therapy within the last 3 months (57); 3) regular use (>3 times per week) of a vitamin or mineral supplement in the previous 3 months; 4) regular use of vitamin or herbal supplements (>3 times/week) with antioxidant capacity; 5) regular consumption of soy products (> 2 servings of 6.25 g/day); 6) a strict vegetaian status (58); 7) a known food allergy to dairy, soy, grains, or nuts; 8) obesity (BMI > 30 kg/m^2); 9) regular consumption of alcohol (>2 drinks/day; 1 drink = 12 oz beer, 6 oz wine, or 1.5 oz distilled alcohol); and 10) any smoking or tobacco use within 3 years (59).

Screening

Individuals who were interested in participating in this study were asked to sign a Human Subjects Consent Form (see Appendix A), which was previously approved by the Montana State University Human Subjects Committee, and to complete a medical history questionnaire (see Appendix B), which was reviewed by the principal investigator for assessment of their eligibility. Individuals who met the inclusion criteria (male, 18–45 years of age) and the exclusion criteria for this study were asked to participate.

As an additional component of the screening, each subject was asked to come to the Nutrition Research Lab (NRL) after a 10 hour fast (no food or beverage with the exception of water) and provide a blood sample for the purpose of obtaining a lipid profile. The blood samples were obtained by venipuncture and collected in two 6 ml Vacuette vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) (Greiner Bio-one, Monroe, NC). From this sample, fasted total cholesterol, HDL, LDL, and TG levels were reviewed. If any lipid profiles fell outside the norms (total cholesterol >200 mg/dl, HDL < 35 mg/dl, LDL > 100 mg/dl, and triglycerides > 200 mg/dl), the individual was excluded from participating in the study (60). Those individuals who passed the initial blood draw by meeting all criteria were asked to participate in the study.

Experimental Design

Subjects who passed the initial screening were required to report to the NRL to participate in the double blind crossover study. The subjects were required to bring their weighed food records (see Appendix C) for the preceding three days as well as a

completed physical activity log (see Appendix D) for the day prior to testing. The subject's height and weight were recorded at this time and they were required to remain in Herrick Hall, the building in which NRL is located, for the duration of the six-hour study. A television and videos were provided for entertainment and a computer and telephone were available for the subjects to use during this time.

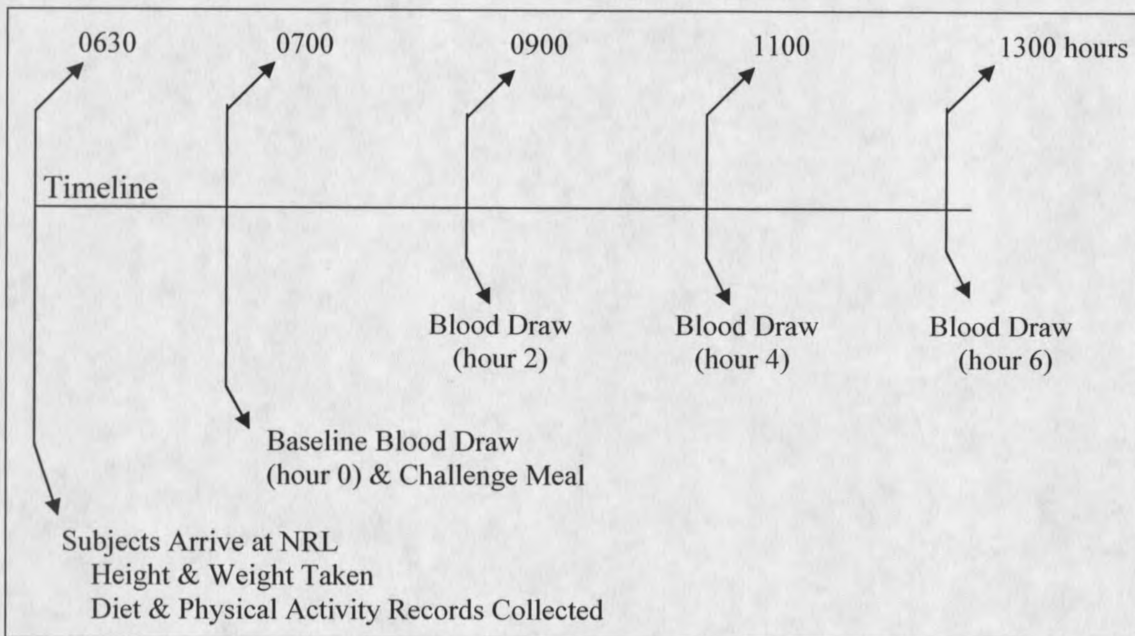

Figure 3. Timeline of each treatment day. After the baseline blood draw was completed, the clock was started. Either a soy protein or a milk protein (control) shake was served and blood draws were obtained every 2 hours thereafter.

At 0700 hours, the baseline blood draw was collected and subjects were randomly assigned a challenge meal, which included either a soy protein or a milk protein (control) and two high carbohydrate muffins. The subjects then consumed the challenge meal and blood draws followed at hours 2, 4, and 6 (see Figure 3).

Challenge Meal

The challenge meal provided to each subject at 0700 consisted of two high-carbohydrate muffins, formulated and produced in the Food Science Laboratory at Montana State University (see Appendix E), and either a treatment shake made with soy protein powder or a placebo shake made with milk protein powder. The protein powder was provided by the Solae Company (see Appendix F) and arrived blinded in 44 g servings packets. Two packets (88 g) of the protein powder was mixed into shake form using 100 g of banana, 18 oz of water, and 3-4 ice cubes to aid palatability. The treatment shake contained 39.0 g soy protein and 85 mg of aglycone isoflavones, while the placebo shake containing 39.9 g of milk protein and 0 mg of aglycone isoflavones.

The nutrient composition of the challenge meal was 19.4% protein, 58.6% carbohydrate, and 22% fat (see Figure 4). The percentage of fat was further broken down into a 4:1:1 ratio of saturated fat (13.1 g), monounsaturated fat (3.2 g), and polyunsaturated fat (3.2 g). The nutrient composition of the meal was calculated using Nutritionist Pro (First DataBank, San Bruno, CA) (see Table 1). Subjects were encouraged to consume water ad lib during the course of the treatment days. The meal was designed to approximate 1/3 of the recommended daily intake for this population of male individuals. Subjects were able to resume their normal eating habits after the final blood draw at approximately 1300 hours.

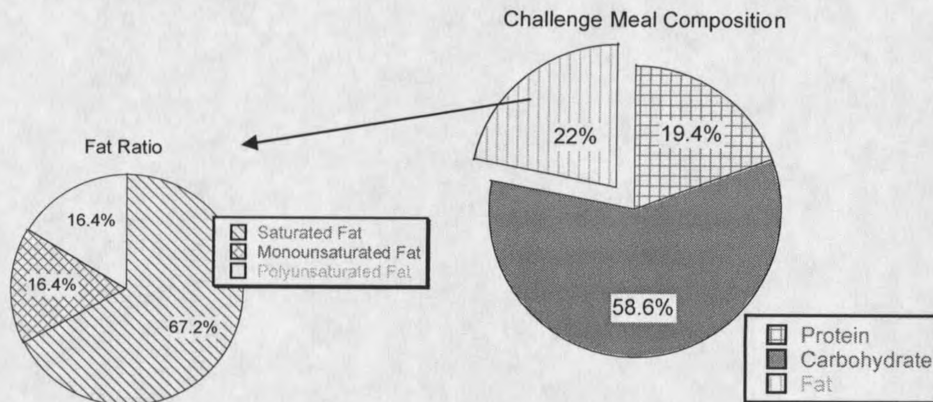

Figure 4. Challenge Meal Nutrient Composition

Table 1. Challenge Meal Nutrient Composition

Nutrient	Challenge Meal	Shake	Muffin
Serving Size	357.1g	188.0g	169.1g
Kilocalories	899 kcal	412 kcal	487.4 kcal
Protein	44.4g	41.0g	3.4g
Carbohydrate	134.3g	59.4g	74.9g
Fat, Total	22.4g	2.0g	20.4g
Saturated	13.1g	0.2g	12.9g
Monounsaturated	3.2 - 3.35g	.15-.1g	3.2g
Polyunsaturated	3.2 – 3.8g	.05-.6g	3.1g
Cholesterol	38.3g	5.0mg	33.3g
Dietary Fiber, Total	3.5g	2.4g	1.1g

Blood Sampling

Blood samples were collected by a phlebotomist through venipuncture. Samples were collected in two 6 ml vacutainers tubes containing EDTA at baseline, hours 2, 4 and 6, for a total of 48 ml of blood per subject. Blood samples were immediately centrifuged using a 21000 Marathon centrifuge (Fisher Scientific, Pittsburgh, PA) for 15 minutes at

3000 rpm (5529 G) and 16°C to recover the plasma. Plasma supernatant was aspirated off and aliquots were placed into a labeled plastic microcentrifuge tube. Each microcentrifuge tube was labeled with the date, sampling time point and initials of the subject. The plasma was then stored in a Revco Ultima II freezer (Legaci Refrigeration Systems, Ashville, NC) at -80°C until analysis.

Diet Records

Subjects were issued a Cuisinart SA-110A dietary scale (Cuisinart, East Windsor, NJ) to aid in accurate diet records. Subjects were also given explicit instructions (see Appendix C) on how to weigh all food and beverages consumed as well as how to properly record this information. Each subjects weighed diet records were analyzed with the Nutritionist Pro (First Data Bank, San Bruno, CA) software package to assess their dietary habits for the three days prior to the study. Subjects were required to comply with the following dietary components for this study: 1) no vitamins or mineral supplements on a regular basis; 2) no herbal supplements containing antioxidants; 3) no regular consumption of alcohol; and 4) no regular consumption of soy or products containing soy.

Physical Activity Logs

To estimate energy expenditure for the 24 hour period prior to each blood draw, subjects were asked to complete a Bouchard physical activity record (see Appendix D).

The collected physical activity records determined if the subjects refrained from strenuous physical activity during the twenty-four hour period prior to the blood draw.

The log consists of 24 hours broken out into 15 minute increments. A list of activity codes, which coincide with a specific physical activity level and range on a scale of one to nine was provided for the subject's selection. The procedure for recording data was explained to each subject.

LDL Oxidation

The LDL was isolated by sequential density-gradient ultracentrifugation in an Optima TLX Ultracentrifuge (Beckman Instruments, Palo Alto, CA). A plasma aliquot of 500 μl was pippetted into a 1 ml polyallomer Beckman centrifuge tube with 500 μl of 0.9% sodium chloride solution. The tubes were centrifuged for 2.5 hours at 100,000 rpm (184,313 G) and 16°C. The centrifuge tubes were then sliced at the 0.5 ml mark using a Beckman CentriTube Slicer (Beckman Instruments, Palo Alto, CA). The top layer of VLDL was discarded allowing for extraction of the bottom layer of LDL and HDL. The LDL and HDL were then transferred to new centrifuge tubes containing 500 μl of 0.16% sodium chloride solution and spun again for 2.5 hours at 100,000 rpm (184,313 G) and 16°C. After complete centrifugation, the tubes were sliced again in the same manner as before and the top layer of LDL was saved in microcentrifuge tubes for analysis.

The LDL samples were individually desalted using columns containing Bio-Gel P6 desalting gel, preconditioned with 20 ml phosphate-buffered saline (PBS). After preconditioning, 350 μl of the LDL sample was pippetted into each column and followed

with 2650µl of PBS. The first 3 ml of effluent were collected and discarded (approximate void volume of the column). To elute the desalted LDL, 525 µl of PBS was added to each column and the LDL was collected for protein concentration analysis.

A bicinchoninic acid (BCA) protein assay kit (#23225, Pierce, Rockford, IL) was used to determine the protein concentration of the LDL sample. Twenty-five microliters of eight known standards and each sample of isolated, desalted LDL were added to individual wells on a 96-well microplate in duplicate (269620, Nalge Nunc International. Rochester, NY). The standard of bovine serum albumin (BSA) provided a working range of 25-2000 µg/ml. A working reagent totaling 200 µl was then added to each well. The microplate was then placed in a 6115 Rotator-Incubator (Eberbach, Ann Arbor, MI) and shaken for 30 seconds then covered and incubated at 37°C for 30 minutes.

After incubation, the microplate was allowed to cool to room temperature for 5 minutes before being placed in a µQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Total protein present in the sample was quantified through colormetric detection. Absorbance was measured at 562 nanometers (nm) and a standard curve was determined using the KCjunior (Bio-tech Instruments, Winooski, VT) software package (see Figure 5). Based on the absorbance, the sample protein concentrations corrected for the wells containing a PBS blank. The results from this protein assay provided the correct dilution required, of the isolated desalted LDL samples with PBS, to achieve a final concentration of 0.10 mg protein/ml for each sample.

A 100 µl sample of LDL (isolated, desalted and adjusted for protein concentration), and the blank (PBS) were added in duplicate to a 96-well microplate (#3635, Costar,

Corning Incorporated, Corning, NY). To replicate LDL oxidation *in vivo*, 10 μl of 500 nm cupric chorine solution was added to each well as a pro-oxidant to oxidize *ex vivo*. The absorbance at 234 nm and 37°C was read every 10 minutes for 8 consecutive hours, by a μQuant Universal Microplate Spectrophotometer.

Excel (Microsoft Corporation, Redmond, WA) was used to create separate graphs in which the absorbance at each time point was plotted against each well of the microplate. The lag time, for each sample was determined by the intersection of a best-fit line constructed tangent to the lag phase and a best-fit line constructed tangent to the propagation phase (see Figure 5). The lag time, expressed in minutes, represents the period of time in which the LDL is protected against oxidation by the isoflavones present in the protein treatment. The propagation rate follows the lag time and is observed as an increasing slope and illustrates the formation of conjugated dienes from the hydrolysis of lipid peroxides. The mean of the lag times, propagation rate, and initial absorbance (the measure of each subject's oxidation level prior to the treatment) for duplicate sample wells was used for the statistical analysis.

The rotor size limited the analysis to one subject per day, which included one sample from each time point for both treatments. A control plasma sample was also run along with each subject's plasma throughout all analysis days. Samples with a coefficient of variance (CV) over 10% were rerun and the average interassay and intraassay was 3% and 2% respectively.

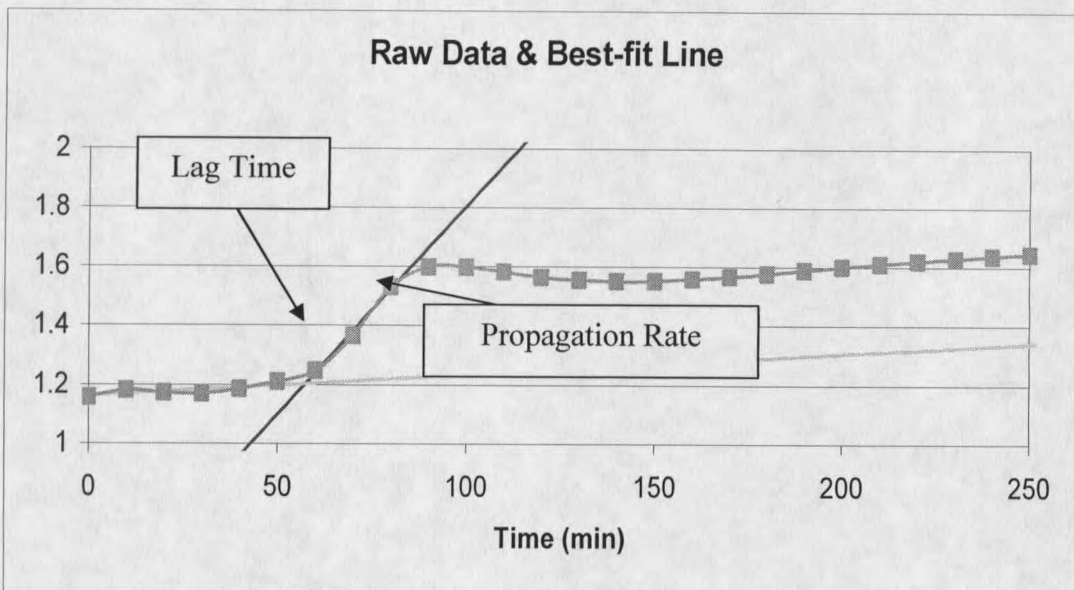

Figure 5. Standard Curve of LDL Oxidation Based on Absorbance

Statistical Analysis

All lag time data was normalized by subtracting the baseline lag time from the lag time of the other three time points (hour 2, 4, and 6). A positive number reflects an increase in lag time from baseline at that specific time point and treatment. A negative number reflects a shortened lag time with respect to the time point and treatment as well. These results were input into Excel (Microsoft Corporation, Redmond, WA) and used to graph each subject's individual lag times. These graphs were used to determine any visual patterns.

A two-way analysis of variance (ANOVA) with repeated measures was used to assess treatment differences for LDL oxidation parameters (lag time, propagation rate, and initial absorbance) for all time points (hours 0, 2, 4, and 6) using Minitab (Minitab Inc., State College, PA). The purpose of the ANOVA is to determine whether there are any

significant mean differences between the treatments (soy and milk) and the time points. In addition to evaluating the main effect of each independent variable individually, the two-way ANOVA allows for evaluation of other mean differences that may result from a unique combination of the two variables referred to as an interaction. The α-level for the statistical analysis was set at 0.05, indicating the probability is less than 5% that the obtained mean difference between treatments is simply due to chance or sampling error.

A repeated measures design was selected because this study utilizes the same set of subjects for each of the two treatment days (soy and milk). A principal advantage to the repeated measures design is that it provides adequate precision for comparing treatments because sources of variability between subjects are excluded from the experimental error. Only variation within subjects enters the experimental error (61).

A dependent t-test was used to assess differences between two treatment means. The independent variable (protein), was tested in duplicate for each of the 15 subjects. In this case, the t-test was used to determine if a change occurred in the subject's characteristics; mean weight, BMI, energy expenditure, or energy intake between the two treatment days.

CHAPTER 4

RESULTS

The characteristics of individual subjects and averaged descriptive statistics for all subjects are presented in Table 2 and 3, respectively.

Table 2. Individual Subject Characteristics

Subject*	Protein Type	Age* (yrs)	Height* (cm)	Weight (kg)	BMI (kg/(m)^2)	Expenditure (Kcal/kg)	Expenditure (Kcal)	Intake (kcal/kg)	Intake (kcal)
1	Soy	49	170.2	77.4	26.7	38.3	2968	16.2	1253.6
	Milk			77.3	26.7	37.8	2925	21.5	1658.2
2	Soy	30	177.8	67.5	21.4	48.7	3289	35.1	2367.9
	Milk			57.7	18.3	55.5	3201	42.4	2445.5
3	Soy	23	176.6	63.4	20.3	46.6	2957	35.8	2268.7
	Milk			64.1	20.6	41.5	2660	52.5	3367.8
4	Soy	22	174.3	68.6	22.6	41.0	2814	25.3	1734.4
	Milk			67.7	22.3	47.5	3214	41.4	2804.3
5	Soy	29	183.9	92.3	27.3	40.0	3688	19.7	1819.9
	Milk			92.7	27.4	42.1	3900	32.9	3046.9
6	Soy	33	187.4	82.7	23.5	45.4	3753	35.4	2930.3
	Milk			82.8	23.6	46.8	3874	33.5	2771.8
7	Soy	24	174.0	67.2	22.2	41.9	2816	47.5	3193.0
	Milk			64.7	21.4	37.9	2454	40.7	2634.8
8	Soy	44	169.2	70.0	24.5	36.3	2538	45.4	3175.4
	Milk			71.4	24.9	38.2	2727	31.0	2212.0
9	Soy	27	174.6	76.9	25.2	39.9	3067	45.4	3491.4
	Milk			76.8	25.2	51.0	3913	39.8	3055.7
10	Soy	25	181.6	67.3	20.4	44.1	2968	45.5	3062.6
	Milk			67.3	20.4	37.1	2498	42.1	2833.0
11	Soy	22	181.8	75.0	22.7	43.3	3248	30.1	2256.1
	Milk			74.1	22.4	46.6	3452	27.3	2023.2
12	Soy	22	175.7	72.7	23.5	38.6	2804	38.5	2798.4
	Milk			73.2	23.7	37.2	2724	24.3	1779.1
13	Soy	18	182.9	67.3	20.1	35.4	2380	35.0	2353.7
	Milk			69.1	20.7	36.0	2486	25.3	1745.9
14	Soy	26	183.2	83.0	24.7	46.2	3832	40.9	3393.1
	Milk			82.0	24.4	50.8	4169	38.1	3125.9
15	Soy	22	190.1	101.5	28.1	45.1	4575	27.2	2758.8
	Milk			100.2	27.7	43.6	4370	17.9	1796.7

* information did not change between study days

Table 3. Mean Subject Characteristics

Variable	Mean	Minimum	Maximum	Std. Dev.
Age (yrs)	28	18	49	8.6
Height (cm)	178.9	169.2	190.1	6.1
Weight (kg)[1]	75.5	63.4	101.5	10.6
Weight (kg)[2]	74.7	57.7	100.2	11.2
BMI (kg/m^2)[1]	23.6	20.1	28.1	2.5
BMI (kg/m^2)[2]	23.3	18.3	27.7	2.8
Energy Expenditure (kcal/kg)[1]	42.1	35.4	48.7	4.0
Energy Expenditure (kcal/kg)[2]	43.3	36.0	55.5	6.1
Total Energy Expenditure (kcal)[1]	3180	2380	4575	572.4
Total Energy Expenditure (kcal)[2]	3238	2454	4370	664.4
Intake (kcal/kg)[1]	34.9	16.2	47.5	9.6
Intake (kcal/kg)[2]	34.0	17.9	52.5	9.5
Intake (kcal)[1]	2591	1254	3491	655.0
Intake (kcal)[2]	2487	1658	3368	576.3

[1] soy protein , [2] milk protein

A t-test analysis of subject's characteristics revealed no significant differences ($p>0.05$) in weight, BMI, energy expenditure or energy intake between study treatment days as seen in Table 4.

Table 4. Comparison of Soy vs. Milk Protein Study Days

Variable	t-value	df	p
Weight	1.11	14	0.29
BMI	1.04	14	0.32
Energy Expenditure (kcal/kg)	-1.01	14	0.33
Total Energy Expenditure (kcal)	-0.67	14	0.52
Energy Intake (kcal/kg)	0.76	14	0.12
Energy Intake (kcal)	0.60	14	0.34

Table 5. Average Lag Time, Propagation Rate, and Initial Absorbance (all subjects)

	Protein	T0	T2	T4	T6
Lag Time (minutes)	Soy	69.81 ± 6.54[1]	67.74 ± 5.85	65.50 ± 5.18	66.68 ± 6.54
	Milk	69.29 ± 7.04	68.32 ± 7.55	71.68 ± 9.08	69.07 ± 5.45
	p values	Protein	0.41		
		Time Points	0.85		
		Interaction	0.07		
Propagation Rate (absorbance)	Soy	0.006 ± 0.001	0.007 ± 0.001	0.006 ± 0.001	0.006 ± 0.001
	Milk	0.007 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001
	p values	Protein	0.57		
		Time Points	0.31		
		Interaction	0.80		
Initial (absorbance)	Soy	1.13 ± 0.02	1.14 ± 0.03	1.12 ± 0.03	1.13 ± 0.03
	Milk	1.13 ± 0.03	1.13 ± 0.03	1.12 ± 0.03	1.11 ± 0.02
	p values	Protein	0.56		
		Time Points	0.31		
		Interaction	0.80		

[1] mean ± SD, significance at $p<0.05$

Three data points were greater than two standard deviations away from the mean and defined as outliers (61). The standard deviation is used to describe the spread of a group of scores and is the average amount that the scores differ from the mean. These three data points (T4 and T6 for subject 8 with soy consumption; T4 for subject 14 with milk consumption) were treated as missing data and were excluded from the statistical analysis in order to prevent misinterpretation of the results. The cause of these outliers could not

be determined but may be do to such effects as subject's physiological response or experimental error.

Results of the 2-way ANOVA with repeated measures on both factors (protein treatment, time points) for initial absorbance, lag time, and propagation rate are shown in Table 5. No significant difference was observed ($p>0.05$) between the main effects (protein treatment and time points) or in any interaction between the two (protein * time) on LDL oxidation parameters (initial absorbance, lag time, and propagation rate) (see Appendix H). Table 5 illustrates the small variation between subject's responses in regards to the LDL oxidation. For example, the shortest mean lag time was 65.35 ± 5.15 minutes, while longest lag time was 69.82 ± 5.53 minutes.

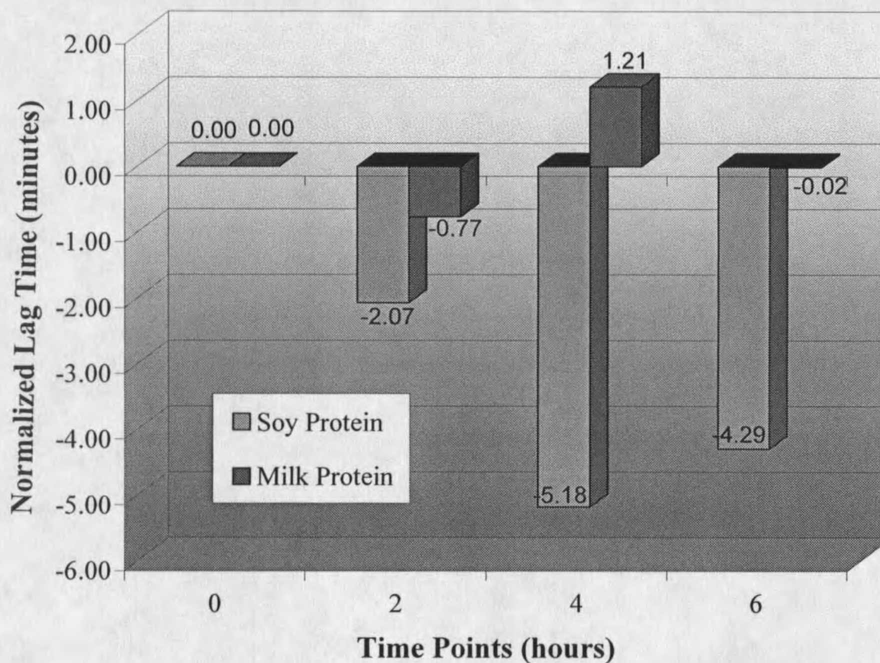

Figure 6. Normalized Lag Time for Protein Treatments

A visual comparison of the normalized lag time for both protein treatments related to the specific time points demonstrates the soy protein treatment did not have a significant positive effect on lag time (see Figure 6). A decrease of 5.18 minutes is observed in the mean values from baseline to T4 with soy consumption, which rises to 4.29 minutes at T6 but still remains below baseline levels. A slightly increased lag time (1.21 minutes), occurs at T4 returning to near baseline levels by T6 with the milk treatment.

Graphing each individual's normalized response to the soy and milk treatment (see Figures 7-21) generated three distinct patterns. Pattern A represents two individuals (13.3% of subjects) who consistently responded more positively to the soy protein treatment as compared to the milk (see Figure 7 and Figure 14) over all time points. Pattern B represents ten individuals (66.7%) who responded more positively to the milk protein treatment as compared to the soy (see Figures 8-13 and Figures 17-20) over all time points. Pattern C (see Figures 15, 16, and 21) represents three individuals (20%) whose response reversed during the course of treatment. For example, subject 9 had a positive response to soy protein from baseline through hour 2, but ended with a positive response to milk protein from hour 4 through hour 6. Although the statistical analysis of the lag time data was not significant ($p>0.05$), this visual analysis provides an excellent illustration of how individuals' physiological response can differ when provided the same treatment.

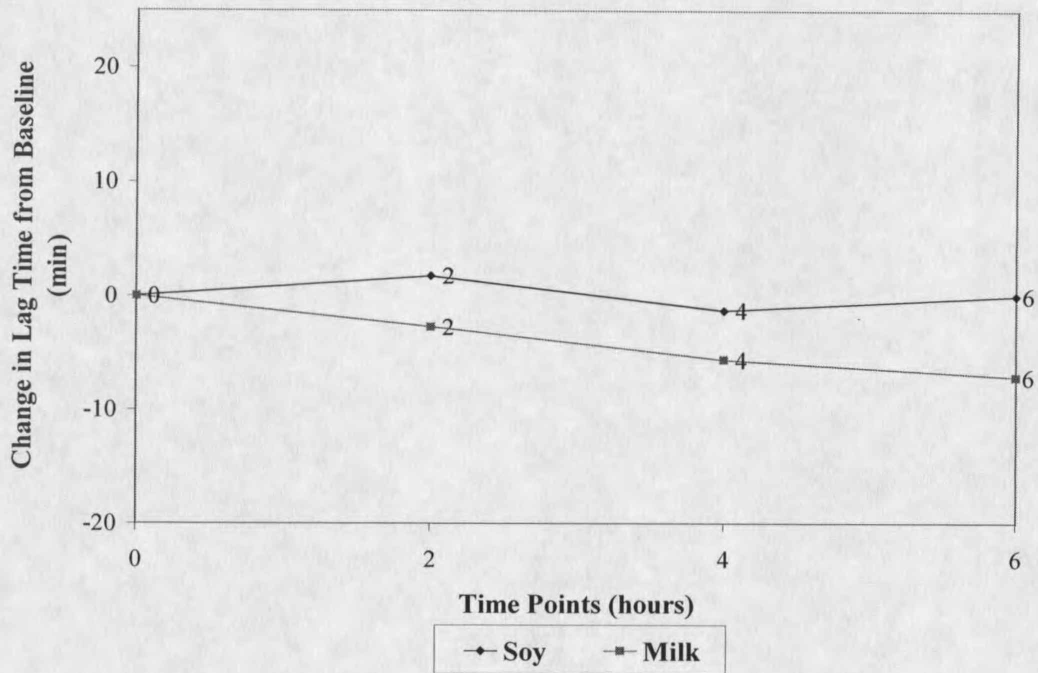

Figure 7. Individual Lag Times (normalized) for Subject 1

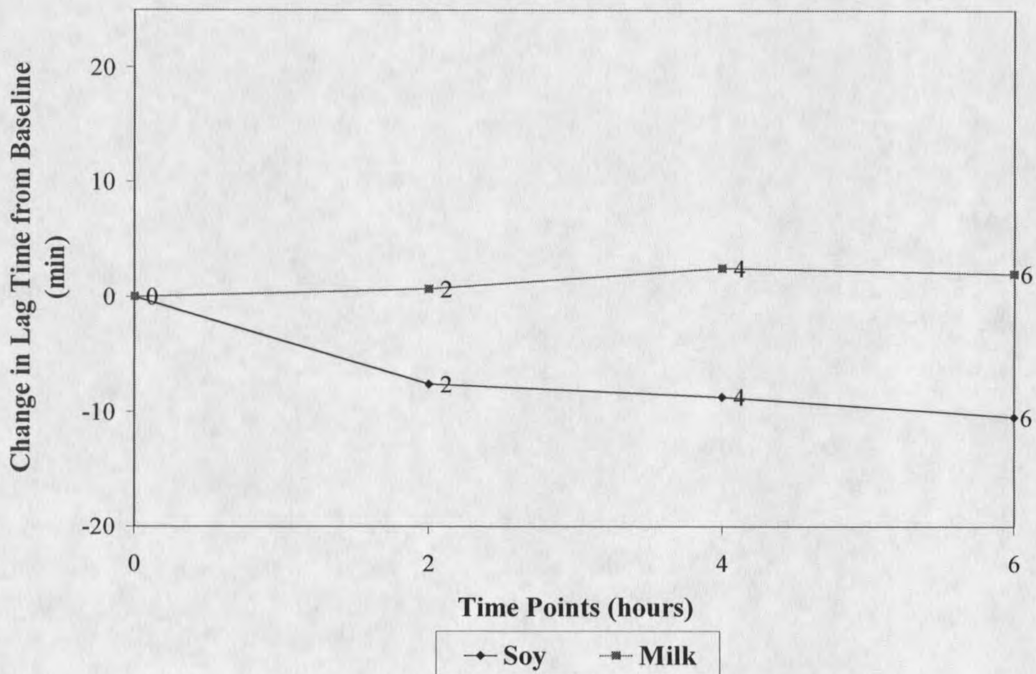

Figure 8. Individual Lag Times (normalized) for Subject 2

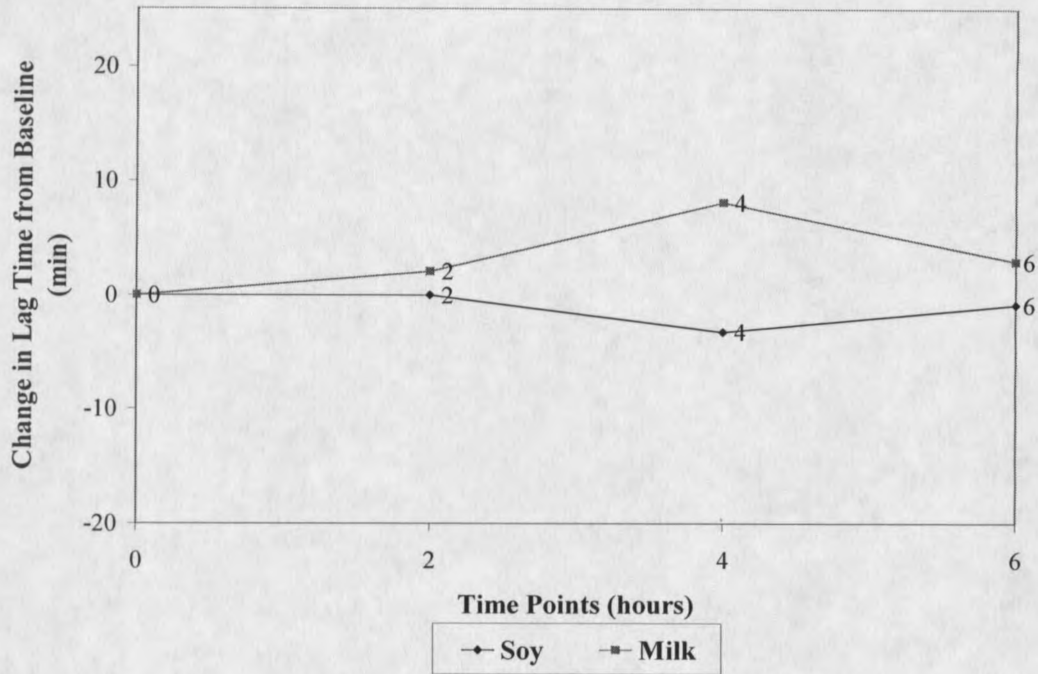

Figure 9. Individual Lag Times (normalized) for Subject 3

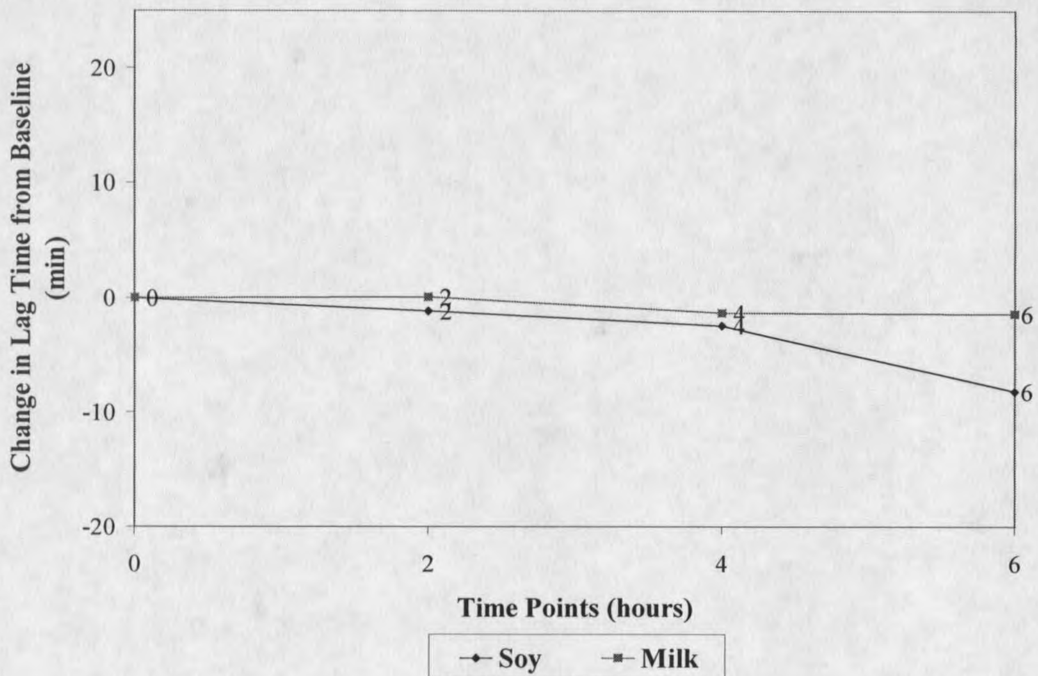

Figure 10. Individual Lag Times (normalized) for Subject 4

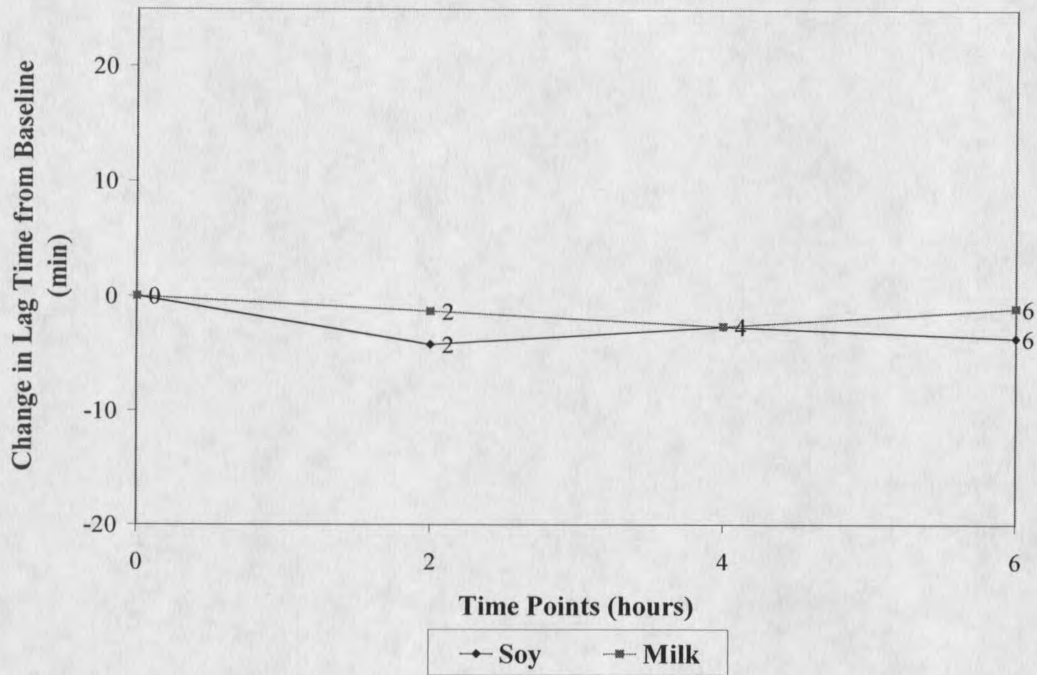

Figure 11. Individual Lag Times (normalized) for Subject 5

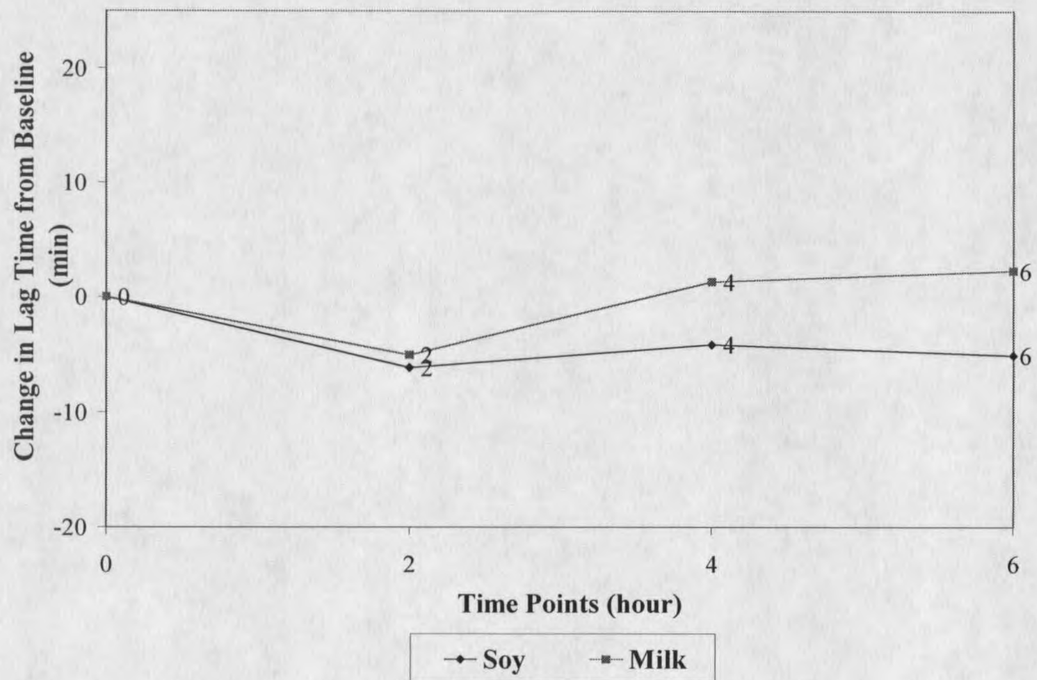

Figure 12. Individual Lag Times (normalized) for Subject 6

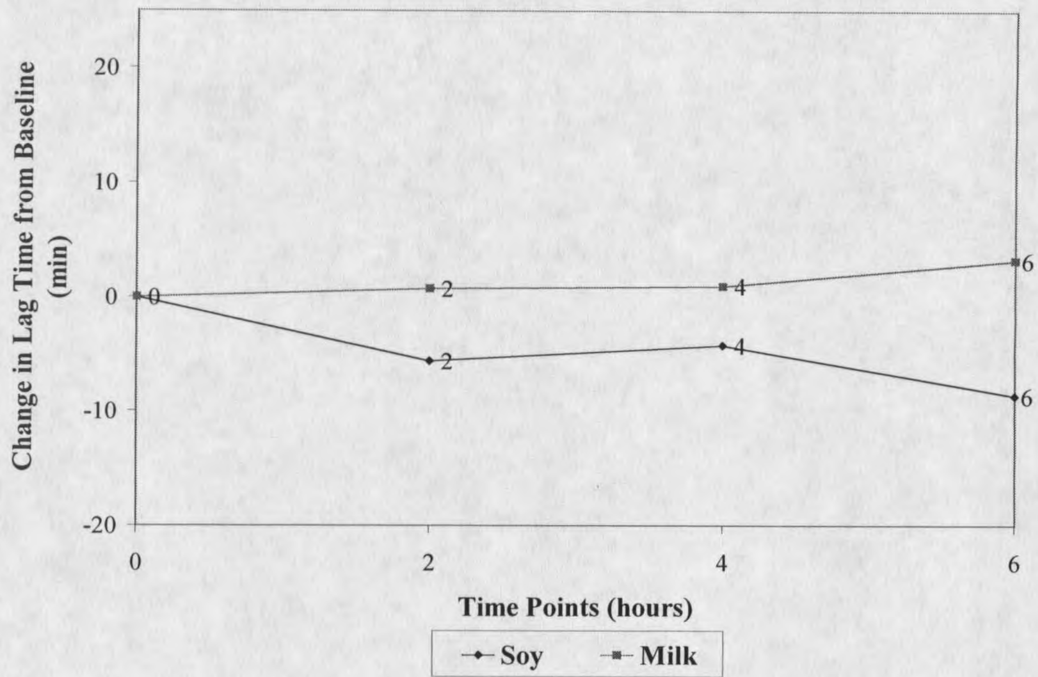

Figure 13. Individual Lag Times (normalized) for Subject 7

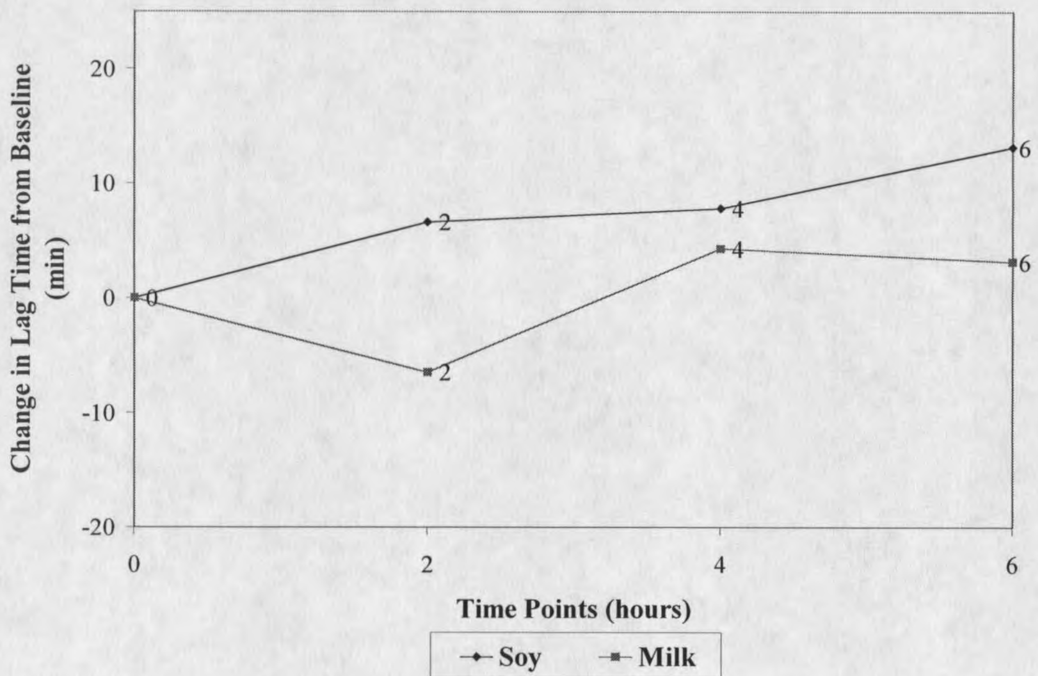

Figure 14. Individual Lag Times (normalized) for Subject 8

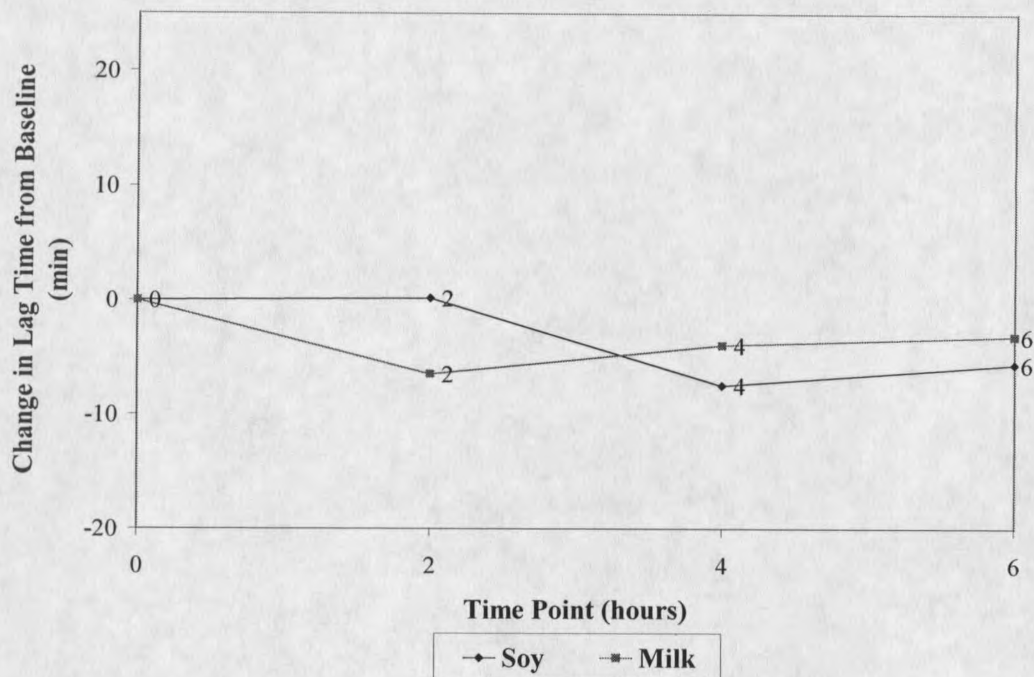

Figure 15. Individual Lag Times (normalized) for Subject 9

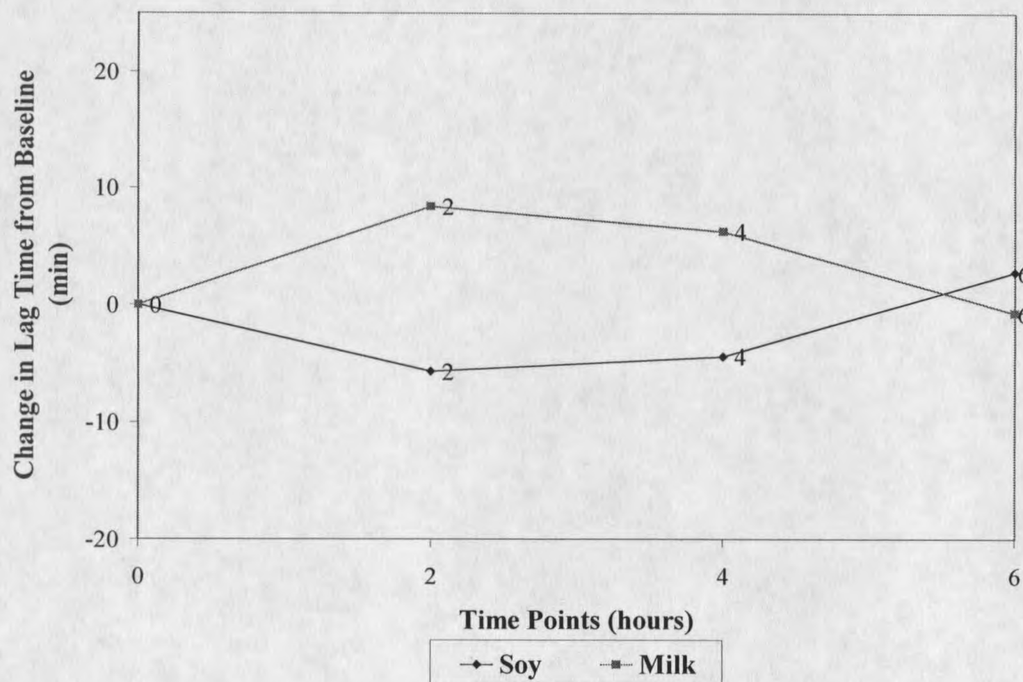

Figure 16. Individual Lag Times (normalized) for Subject 10

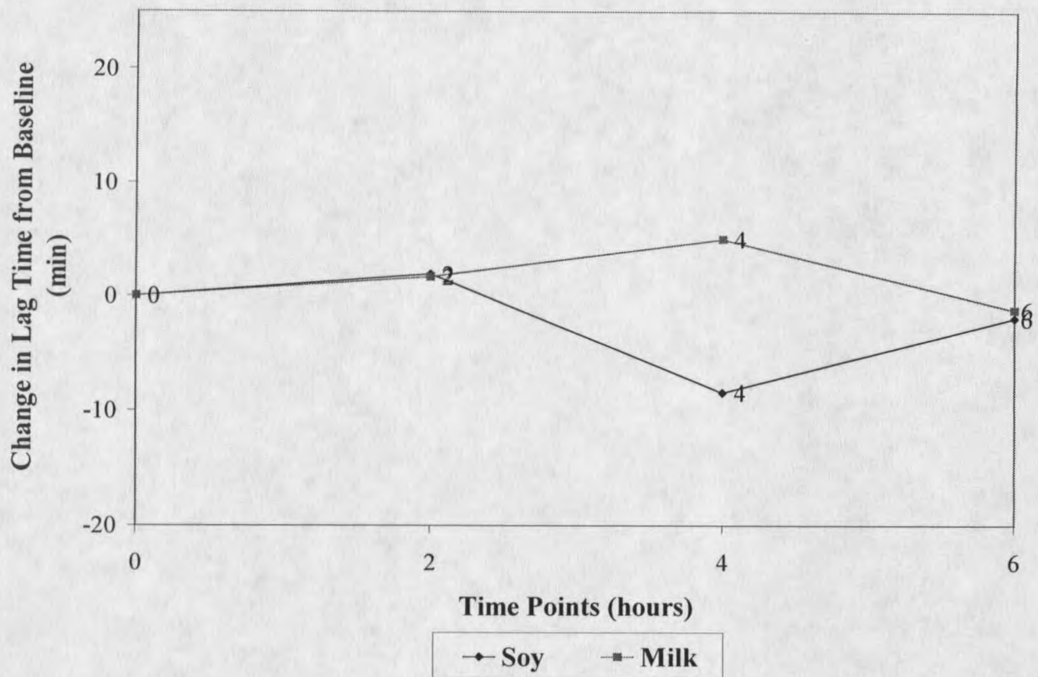

Figure 17. Individual Lag Times (normalized) for Subject 11

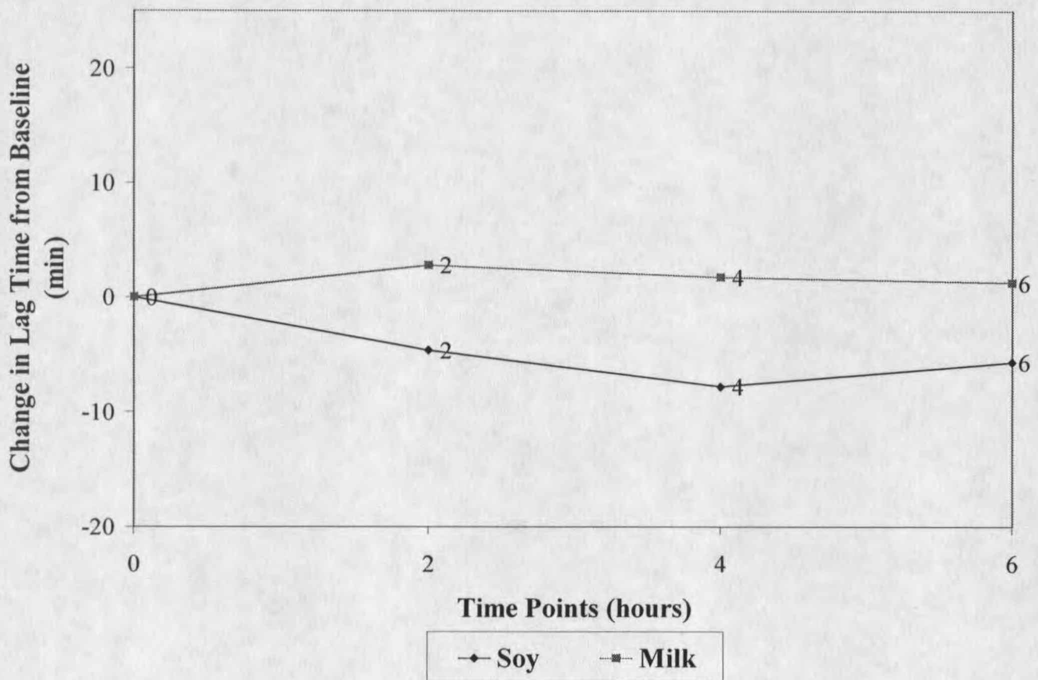

Figure 18. Individual Lag Times (normalized) for Subject 12

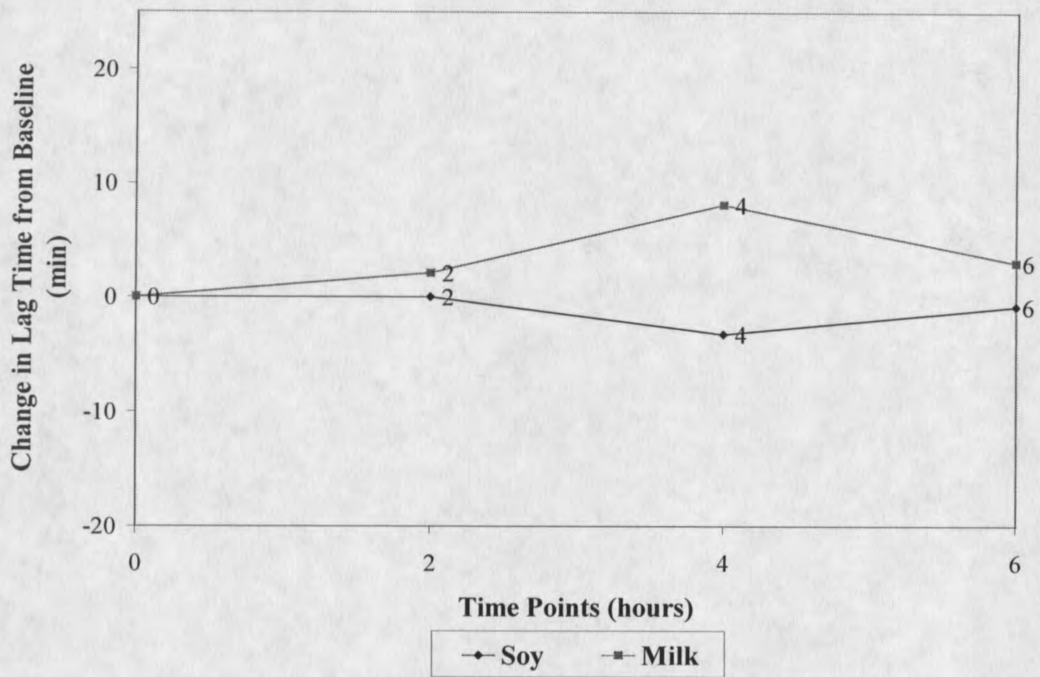

Figure 19. Individual Lag Times (normalized) for Subject 13

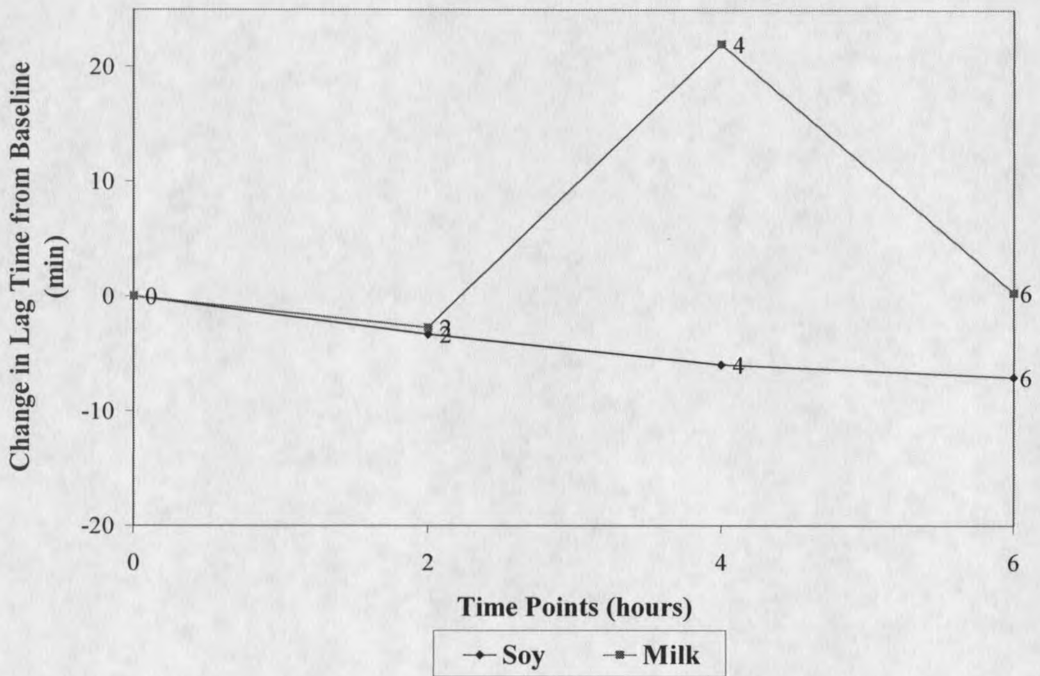

Figure 20. Individual Lag Times (normalized) for Subject 14

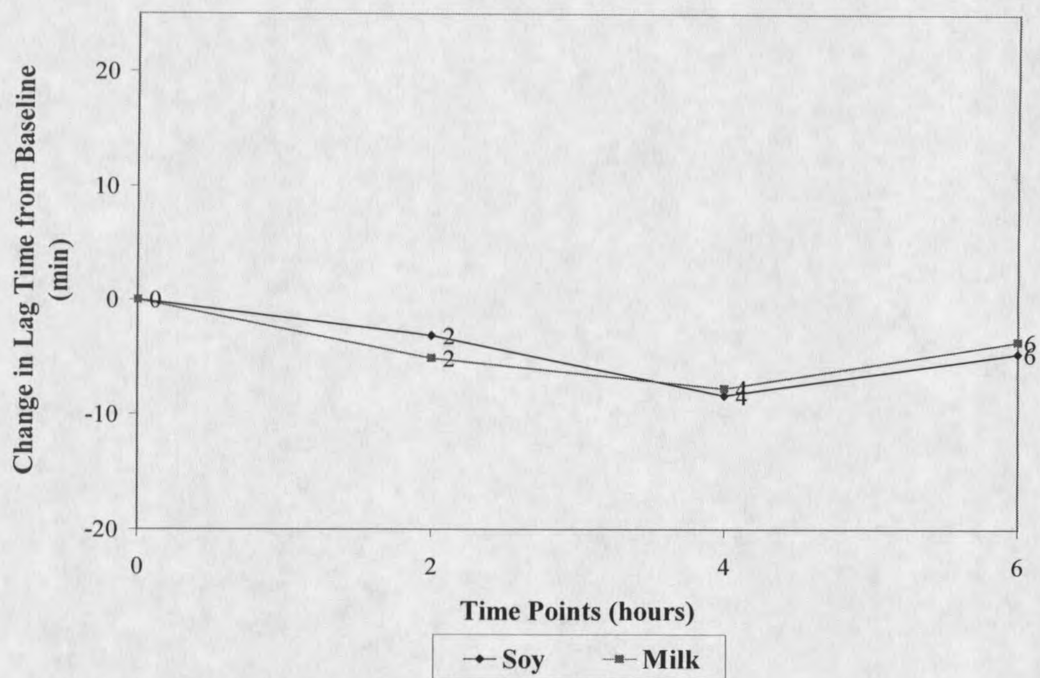

Figure 21. Individual Lag Times (normalized) for Subject 15

CHAPTER 5

DISCUSSION

The purpose of this study was to establish the acute effects of a high carbohydrate meal (899 kcal, 22% fat, 59% carbohydrate, 19% protein) including either a soy protein (85 mg aglycone isoflavones) or milk protein (0 mg aglycone isoflavones) shake on the oxidative resistance of 15 healthy men. We examined the initial absorbance, lag time, and propagation rate of these effects by copper induced LDL oxidation. Initial absorbance was measured to determine the baseline level of oxidation for each subject. This data was calculated by subtracting the first absorbance reading for all four time points from that of a blank well containing PBS. The lag time, expressed in minutes, represents the period of time in which the LDL is protected against oxidation by the isoflavones present in the protein treatment. A longer lag time can therefore be equated with more protection and is seen as a more favorable result. Following the lag phase in the graphical representation of LDL oxidation is the propagation rate (see Figure 5). This is observed as an increasing slope and illustrates the formation of conjugated dienes from the hydrolysis of lipid peroxides. A steep slope results from a rapid conversion to conjugated dienes and is less favorable than a gradual slope alternative.

No signifiant difference s in the interaction between the soy protein and milk protein treatments for initial absorbance, lag time, and propagation ratewere measured . There are several possible explanations why a statistical significance was not observed between the

interaction of the soy or milk protein treatment and the high carbohydrate meal. This could include 1) the use of healthy young male (normocholesterolemic) instead of individuals in a high risk group or hypercholesterolemic individuals; 2) the length of the study (acute vs. long-term); 3) individual variability (subject's background diet, antioxidant status, metabolism); 4) effects of equol producers; and 5) the need for additional lipid assessment.

Healthy vs. High Risk Individuals

The use of normocholesterolemic subjects in this experiment may have contributed to the inability to observe statistically significant findings. Researchers who have observed beneficial effects have used subjects with moderate to severe hypercholesterolemia (258 to ≥335 mg/dL) (62). In order to determine if isoflavones can protect individuals from LDL oxidation, using a population of individuals who are at high risk for CVD, may increase the chance of observing statistically significant results. Growing evidence has indicated that oxidative stress is involved in the pathogenesis of CVD in patients with type 2 diabetes mellitus and the role of hyperlipidemia in this group is still debated (49, 63).

Ceriello et al. (49) has demonstrated there is a higher susceptibility of LDL to oxidation and oxidative stress postprandially in type 1 and type 2 diabetic patients. This randomized crossover design included 10, type 2 diabetics. The subjects were provided 2 standard meals designed to produce different levels of postprandial hyperglycemia (400 ml Ensure Plus: 64% CHO, 16% fat, 20% protein and 500 ml Glucerna: 49% CHO, 29%

fat, 22% protein). Blood samples were taken at baseline, 60, and 120 minutes after the meal and plasma levels of glucose, insulin, cholesterol, TG nonesterified fatty acid (NEFS), malondialdehyde (MDA), and the total radical-trapping antioxidant parameter (TRAP) were measured. LDL susceptibility to oxidation in terms of the length of the lag time ($p<0.01$) and propagation rate ($p<0.01$) were significantly higher for both meals after 2 hours. The LDL was also found to be more susceptible to oxidation ($p<0.05$) with the high CHO meal which also produced a higher degree of hyperglycemia. This data reveals that meal related oxidative stress may be a pathogenic factor of atherosclerosis in diabetic patients (49). Therefore, susceptibility of LDL to oxidation and related studies of antioxidants may be more easily observed in diabetic patients due to the postprandial amplification of atherogenesis.

A study by Chiu et al. (64) is an excellent example of the increase in lipid peroxides and oxidative susceptibility of plasma lipoproteins in patients with CVD compared to healthy individuals. The subjects included 92 healthy controls (mean total cholesterol: 184 ± 35 mg/dL, TG: 112 ± 68 mg/dL) and 48 CVD patients (mean total cholesterol: 195 ± 34 mg/dL, TG: 151 ± 68 mg/dL). Following an overnight fast, levels of lipid peroxides were estimated as thiobarbituric acid-reactive substances (TBARS) and were found to be significantly greater in the plasma and VLDL of the CVD patients (plasma: 285 ± 41 nmol MDA/dL; $p<0.01$ and VLDL: 213 ± 0.43 nmol MDA/dL protein; $p<0.05$) than in the controls (plasma: 231 ± 38 nmol MDA/dL; $p<0.01$and VLDL: 174 ± 0.40 nmol MDA/dL; $p<0.05$). The oxidative susceptibility was evaluated with *in vitro* copper-mediated oxidation. The VLDL and LDL of CVD patients were oxidized at 1-2.5 μM

Cu^{2+}, significantly lower than the control subjects at 5-10 μM Cu^{2+}. The results of this study demonstrate that plasma VLDL and LDL of patients with CVD are more susceptible to *in vitro* oxidative modification than those of healthy individuals. The predisposition of CVD patients for oxidation at relatively lower oxidative stress than healthy individuals is an important factor influencing related research. Researchers examining the effects of antioxidants, such as soy protein and isoflavones, should take advantage of these findings which may allow significant results to be observed using high risk subjects as compared to healthy individuals.

In studies performed by Jenkins et al. (13, 16, 17) hyperlipidemic subjects possessing an elevated serum LDL cholesterol of >158 mg/dL and a TG level <154 mg/dL at recruitment were continually used to evaluate the effects of soy isoflavones on lipid levels and oxidation. The effect of soy-based breakfast cereal consumption on serum lipids and oxidized LDL was assessed in 25 hyperlipidemic subjects (17). The test diet provided 36 g of soy protein/d and 168 mg of isoflavones/d for 6 weeks. Total conjugated dienes were significantly reduced on the test diet compared to the control (9.2% ± 4.3%, p=0.42), and the ratio of conjugated dienes to cholesterol in the LDL fraction was also decreased (8.7% ± 4.2%, p=0.05). This study using hyperlipidemic individuals is another example of a high risk population which enabled researchers to observe beneficial effects in the reduction of oxidative stress with the consumption of soy isoflavones.

It is apparent that high risk subjects, such as those with diabetes mellitus (type 1 and type 2), patients with known CVD, and hyperlipidemic individuals demonstrate an increased susceptibility to oxidative modification of LDL. The subjects with

hyperlipidemia also responded favorable to soy treatment. Due to these findings it is reasonable to hypothesize that these individuals may also respond favorably to soy isoflavone consumption as a means to increase the oxidative resistance of LDL.

Length of the Study

The randomized clinical trial has the potential to provide a compelling rationale for accepting or rejecting a treatment, however one drawback of clinical trials of diet in CVD is their high cost which can lead to a shorter term study that may not last long enough to detect possible significant effects. Terms used to describe the length of studies such as acute and long term are used loosely because they do not define a specific length of time. For the purpose of this study, acute was defined as less than one day and long term, greater than one day. As the literature illustrates, the duration of time needed for a study to provide compelling evidence varies widely. Many studies provide treatment over several months while other studies obtain significant findings with studies lasting days to weeks.

As an example, Parks et al. (65) recruited 25 patients for a 3 month atherosclerosis treatment program to examine the effects of a diet containing 10% fat and 75% carbohydrate on the oxidative susceptibility of LDL. After treatment, a significant 24% increase (baseline: 120 ± 7 min vs. 3 mo: 149 ± 7 min, p=0.003) in the mean lag time and a reduction of 29% (baseline: 6.6 ± 0.3 nmol/min/μg LDL vs. 3 mo: 4.7 ± 0.3 nmol/min/μg LDL, p<0.0002) in the propagation rate was observed. A study of this

length supports the rationale that longer periods of treatment may be required to observe significant effects.

When the study length cannot be months, several days can provide significant results as well. Weisman et al. (38) fed 24 subjects a high and low soy protein meal, in a randomized crossover design, once a day for 17 days separated by a 25 day washout period. *In vivo* biomarkers of lipid peroxidation and oxidative resistance were examined and a decrease in lipid peroxidation and an increase in lag time was observed (19.5%, p=0.028 vs. 9%, p=0.017, respectively). Tikkanen et al. (18) was also able to observe an increase in the mean lag phases of LDL oxidation by 20 minutes ($p<0.02$) after feeding six healthy volunteer 3 soy bars containing 57 mg aglycone isoflavones total, daily for 2 weeks.

Lengthening the treatment period to months, as in Park et al. (65), may not be essential. However, these studies suggest a longer termed feeding study such as weeks, may be necessary to observe significant results when examining the effects of oxidative resistance in conjunction with a soy based diet. Continued studies using these assessment parameters should extend the length of study at least over several days to increase the confidence of the results.

Variability within Subjects

The individual variability between the subjects in this study was evident through three distinct patterns (A, B, and C). Individuals exhibiting pattern A (13.3%) consistently responded more positively over all time points to the soy protein treatment as compared

to the milk. The majority of subjects (66.7%) responded more positively at all time points to the milk protein treatment as compared to the soy as represented in pattern B. Pattern C included 20% of the subjects who initially responded positively to soy protein, but concluded the study with a positive response to milk protein. Several factors including plasma lipoprotein concentrations, hydration status, and the subject's background diet could be attributed to the variability between subjects.

Since plasma lipoprotein (specifically TG) concentration varies 10-20% from day to day (5), it is important to attempt to control factors that can affect the status of lipoproteins when conducting research studies. However, not all factors effecting lipoprotein concentrations can be controlled. For example, the rate of TG output from the intestine and secretion from the liver are factors that can not be controlled for. Additional factors include the activity of LPL, hepatic lipase, and the rate of uptake of lipoproteins containing TG by receptors. These mechanisms should be taken into consideration as possible influences causing inherent variability, as observed through the patterns A, B, and C, between subjects.

Cohn et al. (66) monitored plasma lipoprotein changes for 12 hours in 22 subjects after a HF meal (1g fat/kg body weight). The magnitude of postprandial triglyceridemia varied considerably between subjects. Plasma TG was measured hourly and results showed 5 subjects had 1 TG peak, 11 subjects had 2 TG peaks, and 6 had 3 TG peaks. These multiple postprandial TG peaks were hidden when the mean data was presented. Concluding evidence showed a significant rise in mean plasma TG concentration within 1 hour (100 mg/dL at baseline to 130 mg/dL at hour 1; $p<0.001$) which peaked 4 hours

after the meal (240 mg/dL; $p<0.001$) and remained elevated for 9 hours (150 mg/dL; $p<0.01$). It was beyond the scope of the study to determine which of the factors mentioned above may be responsible for variation between individuals. However, this study illustrates that variations in the postprandial lipoprotein metabolism of individuals exists. Pre-screening subjects as mono or multiple postprandial TG peak producers could provide researchers with more accurate results when examining postprandial LDL oxidation data.

In addition to pre-screening, there were two additional factors in our study that could dramatically affect plasma lipoprotein and were uncontrolled. These are the effects of a subject's background diet and hydration status (6). Variation in the subject's background diet could produce pre-existing differences in antioxidant levels and plasma peroxides which have the potential to influence LDL oxidizability (56). Control diets used for several days prior to the study may be required to ensure that dietary antioxidants, pro-oxidants, and macronutrient proportions are equally consumed by all subjects.

The control diet could therefore aid in the effects of the challenge meal. It is important to remember, Zilversmit hypothesized that atherosclerosis was a postprandial phenomenon due in part to an increase in TG following a meal (4). The inability of the HC meal to produce adequate oxidative stress may be influenced by a subject's chronic consumption of CHO or the type of CHO consumed. The lack of a heightened postprandial lipemia may not allow for an environment necessary to assess the effects of soy on the oxidative resistance of postprandial LDL. The time it takes for a HC diet to induce HPTG to develop depends on several factors including the type of CHO (5).

Simple CHO produces a more immediate metabolic response which may be more favorable when observing an acute study as compared to complex CHO consisting of a greater amount of fiber thus producing a slower metabolic response. The rate of delivery of dietary CHO into the circulation is a function of not only CHO type and background diet but processes including intestinal digestion, absorption, the synthesis of chylomicron particles in the enterocyte and their export into the lacteals, and the rate of lymphatic flow (56).

Controlling the proportion of macronutrients prior to a study can be beneficial. Robertson et al. (48) fed 12 healthy men isoenergetic evening meals of both a HF isoenergetic (62% of energy from fat & 31% of energy from CHO) and a HC (16% of energy from fat and 76% of energy from CHO) on 4 separate occasions. The following morning (12 hours postprandially) an oral fat tolerance test (OFTT) consisting of 40 g of fat or an oral glucose tolerance test (OGTT) consisting of 100 g of glucose was provided randomly between the study meals. The results illustrated the ratio of fat to CHO in the evening meal produced significant effects on plasma TG. Fasting plasma concentration of TG were significantly elevated (HF-to-low CHO: 34.7±6.9 mg/dL, HC-to-low fat: 42±3.9 mg/dL) 12 hours after each evening meal. This study clearly demonstrated the composition of the evening meal (fat or CHO) persists at least overnight and suggests that knowledge of recent dietary history or control of the diet prior to a study is essential to the effective design and results of metabolic studies such as ours. Since plasma lipoprotein can be elevated after a single meal fed the night before blood is drawn,

controlling a research subject's background diet before blood sampling is essential to control for these confounding (extraneous) variables (6).

A study by Simons et al. (37) evaluated the effects of purified soy isoflavones to investigate the effect on plasma lipids and lipoproteins in a double-blind placebo-controlled, randomized crossover study. Twenty subjects with a plasma cholesterol <309 mg/dL and TG <116 mg/dL were provided with a prescribed diet containing 30% energy from fat, <10% energy from saturated fat, and cholesterol intake <300 mg/day for 21 days prior to treatment. Immediately following, subjects were randomly assigned to receive 2 tablets per day for an 8 week phase of either the treatment (containing 40 mg of isoflavones per tablet) or a matching placebo. A washout period of 8 weeks was followed by the second 8 week phase. Fasting baseline blood samples were compared to fasting samples taken after each phase. There were no significant effects between the isoflavone treatment and the placebo although isoflavones levels were significantly higher during the intake of the treatment tablets. However, total, LDL, and HDL cholesterol levels were on average 6% to 8% lower after the prescribed diet than at the time of recruitment. This is a prime example of how the prestudy diet can influence the results of a study as well illustrating that purified soy isoflavones do not result in the same benefits as complete soy products. Administering this type of a control diet could alleviate many of the questions regarding a subject's background diet but considerable caution should be taken to administer a diet that does not influence the results of the study by inhibiting the subject's average intakes.

Effects of Equol Producers

Although most research has centered on the aglycone form of genistein and dadzein, metabolites such as equol, may contribute significantly to the biological effects of the isoflavone molecules (35). A study conducted by Sathyamoorthy and Wang (40) to differentiate the effects of dietary phytoestrogens daidzein and equol demonstrated that equol was 100-fold more potent than daidzein in stimulating an oestrogenic response.

Hwang et al. (38) assessed the copper-induced oxidation of LDL in response to isoflavones added *in vitro*. The activity of the three major phytoestrogens; genistein, daidzein, and equol, in terms of LDL oxidation susceptibility was measured. Fasted plasma samples from adult male volunteers showed genistein and daidzein levels ranged from 0.55-0.68 μM/L after consumption of a soy beverage for 2 weeks. These results provide evidence of phytoestrogens inhibitory effect by prolonging oxidation of LDL ($p<0.05$). This was determined by measurement of *in vitro* lag time. These researchers also noted that equol, a daidzein metabolite formed *in vivo* by gut flora was the most potent antioxidant examined.

The metabolism of daidzein to equol differs between individuals. The conversion of daidzein to equol by intestinal microbes occurs only in 30% of the population (40, 41) (see Figure 22). These individuals have been referred to as "responders" or "excreters" and the level of equol in their plasma or urine is 150-900 times higher than individuals termed "non-responders" or "non-excreters" (40, 67). It is not clear what the physiological impact of this might be, however "responders" may be expected to have a

longer lag time and decreased propagation rate due to the additional antioxidant protection they receive. The length of time needed to completely convert daidzin to equol in unknown at this time, but may require a longer period of time than our study allowed for. Therefore, the actual effect equol producers may have had on this study is possibly irrelevant.

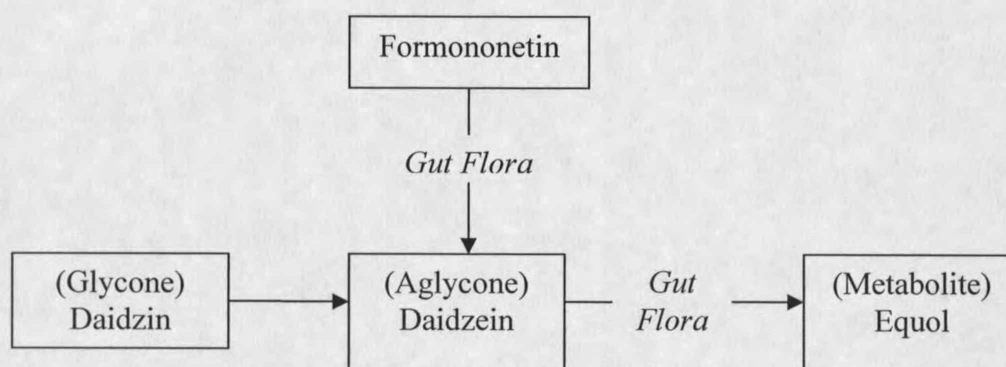

Figure 22. Metabolism of Isoflavones in the Gut Lumen.

This is an interesting hypothesis that could be examined further by placing subjects in either a responder group or non-responder group based on the level of equol found in their plasma or urine. Isoflavones were first quantified in human urine by gas chromatography and mass spectrometry and UV detection can now be done with plasma, urine, and fecal analysis (41). Current advances in methods for detection and quantification of isoflavones use a radioimmunoassay for formononetirin plsama and urine (68). This method allows a 10-100 fold increase in sensitivity allowing for the analysis of the enormous variation in urine between individuals. Using this method, a crossover study using a predetermined amount of soy isoflavones and a control could

determine if lag time is increased and propagation rate is decreased in responders as compared to non-responders.

Second, gut transit time may alter isoflavone bioavailability in interaction with isoflavone degradation (41). The longer the isoflavones are in the gut, the more opportunity for isoflavone degrading organisms to act, and the less isoflavones available for absorption. Hence, the regular consumption of high fiber foods may influence an acute study such as ours.

Another factor is gastrointestinal flora, which exerts a profound influence on bioavailability of equol. Individuals who recently used antibiotics were excluded from this study due to the negative impact antibiotics have on intestinal integrity. Antibiotics kill both beneficial and pathogenic bacterial populations in the GI tract, altering the balance of intestinal microflora which changes the ability of the gut to breakdown and absorb nutrients (2). Chronic disease and metabolic disorder were also exclusion criteria for this study due to influences on the digestion and absorption processes as well. Although these factors were controlled for, the extensive gastrointestinal process and the state of an individual's intestinal flora naturallyvary between subjects (67). The overall composition of the diet prior to experimental testing, may need to be taken into consideration in clinical studies investigating the potential efficacy of isoflavones, and the extent of intestinal bacterial metabolism to determine the bioavailability of dietary isoflavones and influence the potential for physiologic effects.

Adequate Lipid Assessment

Lipoproteins are an important surrogate endpoint and alternative to using CVD as an outcome in a dietary trial because they are in the causal path between a food or nutrient and CVD (19). Lipoproteins have a strong link to the pathogenesis of atherosclerosis and predictive association with CVD (19). Studies have shown that cholesterol of exogenous and endogenous origins are equally atherogenic. When studying the phenomenon of CHO induced lipemia, observing LDL cholesterol may not be adequate. This postprandial phenomenon caused by chylomicrons and eventually leading to oxidized LDL cholesterol should measure all lipid parameters including TC, VLDL, HDL, and TG levels in order to more fully understand the mechanism and results of feeding a high CHO diet (46, 69, 70).

The level of plasma TG is an important determinant of the evolution of postprandial plasma lipids. Plasma TG concentrations have been shown to be an independent risk factor for coronary disease, whether measured in the fasting or postprandial state (19). Elevated plasma TG measured at late time points after a high CHO meal can reveal a state of CHO intolerance linked to an elevated risk of CVD (57). Plasma TG concentrations should therefore be measured in our type of acute study to examine the level of meal induced lipemia. This would be possible to measure in our type of acute study since results show that TG concentrations generally begin to rise significantly with in one hour after a meal, peak at 4 hours, and remain significantly elevated for 9 hours after a meal (60). Males are more appropriate subjects for this type of analysis because

research has shown males tend to have greater postprandial triglyceridemia than females (60). These results also demonstrate that older subjects have a greater postprandial triglyceridemia than younger subjects (60). The increase in TG caused by CHO overfeeding leads to increased VLDL secretion, which is not always apparent at the level of plasma TG (20). Therefore, it would be beneficial to measure these lipoproteins (e.g. TC, VLDL, HDL and TG) also in order to examine more completely the metabolic process leading to lipemia. In addition to increasing the measurement of lipoproteins, the method of oxidation of LDL needs to be examined.

Our copper-induced oxidation of LDL cholesterol may not have been an appropriate marker of oxidative stress for this type of study due to the setting in which it is executed. *In vivo* antioxidants, such as soy isoflavones are suspended in the water-soluble environment of the plasma. This environment may offer a higher level of protection to surrounding the LDL against oxidation. Our method of oxidation essentially depleted the aqueous environment which may have altered the ability of the isoflavones in contributing to the oxidative resistance of LDL. It would be beneficial for future studies to examine alternative methods of oxidation in comparison to the one used in this study.

The examination of lipid peroxidation products, specifically isoprostanes is another method of assessment that could also prove used. Isoprostane measurement represents the *in vivo* rate of lipid peroxiation and is a specific indicator of oxidative stress. Isoprostanes are formed at the site of free radical attack in the cell membrane. They are eventually cleaved and are released and circulated in the plasma, eventually being excreted in the urine (71). This allows researchers to determine either plasmaor urine

isoprostane level. Accumulating research has supported this analysis as both reliable and non-invasive indices for examining lipid peroxidation *in vivo* (71). This method is expensive and requires an involved practice consisting of a 24 hour urine collection.

The analysis of plasma malondialdehyde (MDA) is another widely used index of lipid peroxidation and is measured by a technique known as TBARS. Malondialdehyde is a byproduct of lipid peroxidation that reacts with thiobarbituric acid. The product produces a fluorescence which can be read at 532 nm. This test is relatively quick and inexpensive (3, 49).

The most widely used method for measuring total antioxidant capacity of plasma or serum is the total radical-trapping antioxidant parameter (TRAP) assay. It has been defined as a global measure of the antioxidant capacity of plasma, taking into account all known and unknown antioxidant activity present in plasma, as well as their synergy (49). In fact, the overall antioxidant capacity of plasma is determined not only by the absolute concentration of the various antioxidant compounds but also by their interactions. In this method the production of peroxyl radicals obtained by thermal decomposition of 2,2'-Axobis(2-amidinopropane) dihydrochloride c(ABAP), leads to a linear decrease in R-phycoerythrin (R-PE) fluorescence emission over 1 hour. When plasma is added to the reaction mixture, a period of complete protection of R-PE is observed. The length of this lag time is considered to be directly related to total plasma antioxidant capacity. To quantify TRAP, the lag time produced by the plasma is compared with the lag time produced by a known standard, Trolox. The major drawback of the TRAP assay is using

an electrode to determining the endpoint oxidation, which may not be capable of maintaining accuracy of the period of time required (49, 56).

The growing importance of understanding the role of CHO intake on hyperlipidemia should lead to future experiments that focus on direct measures of lipolysis and hepatic fatty acid reesterification during CHO feeding. It is important to remember that each method of analysis has limitations, making no one method appropriate for all experiments. It is therefore reasonable to suggest the use of more than one method of evaluation of oxidation. The method, extent, and conditions in which LDL is oxidized, can also vary between laboratories and results in differing composition of LDL (33). Therefore, future studies in which LDL oxidation is utilized, should include a variety of methods and assessment tools in the procedures.

CHAPTER 6

SUMMARY

Cardiovascular disease is currently the leading cause of death in the U.S. and reducing the risk of CVD through dietary and other lifestyle practices had become a driving force behind current healthcare professionals. The major contributor to CVD is atherosclerosis, which was first determined to be a postprandial phenomenon by Zilversmit et al. (2). This important finding is essential to our understanding because today's society continuously consumes foods throughout the day, contributing to the existence of a postprandial state. Zilversmit also retained the perspective that lipids accumulated as the result not only of abnormally high concentrations of LDL in the plasma, but also as a consequence of the natural process of lipid absorption and transport. This process has the potential to be pathogenic in individuals who consume a diet rich in carbohydrates, therefore dietary practices are of significance in regard to research and prevention of the atherosclerotic process.

The ability of soy protein to reduce total cholesterol, LDL cholesterol, and TG concentrations was documented in 1995 when Anderson et al. (12) conducted a meta-analysis of 38 human studies to illustrate the combination of serum lipid lowing effects of soy protein as compared with animal protein. Further research by Jenkins et al. (13, 16, 17) has been able to demonstrate a reduction in LDL oxidation with soy consumption.

On October 26, 1999, the FDA authorized the use health claims supporting the role of soy protein in reducing the risk of CVD. This claim is based on scientific studies that show 25 grams of soy protein daily in the diet is needed for a significant cholesterol lowering effect. It also states that foods containing soy protein be included in a diet low in saturated fat and cholesterol in order to reduce the risk of CVD by decreasing blood cholesterol levels (33).

The purpose of this experiment was to determine if there was a significant difference in copper-induced oxidative resistance of postprandial LDL between 39 g soy protein (86 mg isoflavones) and 39.9 g milk protein (0 mg isoflavones) served in conjunction with a high CHO meal. A significant difference was not observed ($p>0.05$) between the protein treatment (soy vs. milk), the time points (baseline and hours 2, 4, 6), or any interaction between these two factors on the measurements of LDL oxidation, including initial absorbance, lag time, and propagation rate.

Postprandial lipemia and the atherogenic process are complex metabolic processes affected by an unknown number of factors and interactions. Incredible variation also exists between individuals, confounding the examination of a single dietary component, such as soy isoflavones. Therefore, the inability of this study to produce any significant results could possibly be due to any one, a combination of, but not limited to the factors discussed earlier. These included: 1) the use of healthy young males (normocholesterolemic) instead of individuals in a high risk group or hypercholesterolemic individuals; 2) the length of the study (acute vs. long-term); 3) individual variability (subject's background diet, antioxidant status, metabolism); 4)

effects of equol producers; and 5) the need of additional lipid assessment. Although, significant findings were not found, this study could provide useful information and a basis for future studies in which the postprandial effects following the use of soy and milk protein or the use of a high CHO meal are examined.

Studies such as ours which relate specific foods to specific disease states may provide the most useful information for nutrition policy decisions. However, it is important to remember that it is the whole food that humans ingest daily as part of the diet. Researchers must be able to incorporate these findings into the daily diet of the public in order to impact the deadly progression of CVD.

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APPENDICES

APPENDIX A

HUMAN SUBJECTS CONSENT FORM

SUBJECT CONSENT FORM
FOR
PARTICIPATION IN HUMAN RESEARCH
MONTANA STATE UNIVERSITY

PROJECT TITLE: Effects of soy isoflavone consumption following a high-fat or high-carbohydrate meal on the oxidative resistance of low-density lipoprotein in healthy, young men.

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PURPOSE AND RATIONALE OF THE STUDY:

A considerable amount of research has been done to investigate the potential benefits of soy on cardiovascular disease (CVD). The research has shown that most people would benefit from the incorporation of soy into their diets. Interventions lasting 1-2 months have been able to demonstrate a relationship between soy consumption and a reduction in the modification to the low-density lipoprotein (LDL), also known as the "bad cholesterol" that may promote the development of CVD. Past research has mainly focused on measuring the "bad cholesterol" during the fasted state, yet evidence suggests that LDL is more susceptible to modification following the consumption of food. The relationship between meal composition and measures of LDL modification following food consumption may provide useful insight into the prevention of CVD.

The purpose of this study is to determine if the effects of a meal containing soy protein and soy's natural antioxidants, isoflavone, versus milk protein (no isoflavones) can minimize measures of LDL modification when the soy meal is consumed along with a high-fat or high-carbohydrate (CHO) meal, both of which have been shown to increase the concentration of circulating lipids in the blood.

You are being asked to participate because you are a healthy, non-vegetarian, 20-40 year old individual living in the communities surrounding Montana State University who has shown interest in our study by responding to our recruiting flyer. You have been identified as a possible participant based on several criteria including: no regular use of a vitamin or mineral supplement in the last 3 months, no regular consumption of soy protein ($\geq$ 2 servings of 6.25g/day), no known existence of metabolic disorders (thyroid disease, diabetes, liver or renal disease), non-use of oral prescription medications (including antibiotics), non-obese (BMI<30kg/m^2), no cigarette smoking within the last 3 years, absence of certain food allergies (soy, milk, peanuts), no strict vegetarian status and no regular alcohol consumption (regular = >2 drinks/d for men; 1 drink = 12 oz beer, 6 oz wine or 1.5 oz distilled alcohol):

STUDY OUTLINE

If you agree to participate, you will be asked to complete the following:

You will be required to come into the Nutrition Research Lab (NRL) at Herrick Hall on the campus of Montana State University on three separate occasions. During the 1st visit, we will draw a fasted blood sample for a preliminary screening to determine your levels of total cholesterol, high-density lipoprotein and triglycerides. If your levels are within normal limits, you will be eligible to participate in the study. On the other two occasions, you will be asked to report to the NRL in a fasted state (no food or beverage, except water, for 10 hours) and to have refrained from any moderate to strenuous physical activity or alcohol consumption for at least 24 hours prior to the study date. You will be required to bring completed 3-day weighed (requires that subjects weigh all food and beverage) diet records for the days just prior to the study date. A dietetics student that has been trained on giving dietary analysis instructions will give you detailed instructions on how to properly complete the forms and tips on accurately weighing food. You will be provided with a dietary scale, at no cost to you, for use during the study to facilitate the process. You may perceive this to be a tedious process, however it is the most accurate means of collecting dietary intake information..

On each of the of the feeding study days, you will enter the NRL at 0630 h and stay until 1300 h. After the measurement of body weight and height, an individual trained in drawing blood from the forearm will take a blood sample. Following the collection of the fasted blood, you will be given 20 minutes to consume the test meal. The meal (shake and muffin) will provide approximately 850 calories, which is equivalent to one third of your daily calories. You will randomly be assigned to one of two groups; Group A, the high-fat meal or B, the high-CHO meal. Within your group, you will randomly be assigned to receive meal 1 or 2 first. On your subsequent visit to the NRL, you will receive the meal you have not yet had. The study is a double blind crossover, which means that neither you nor the research team knows which meal contains the soy or the milk protein. Utilizing this research design helps to minimize the placebo effect.

During the study period you will remain in Herrick Hall; a television and videos will be available for you to watch. Blood will be collected from your forearm at 2, 4 and 6 hours following the consumption of the test meal for a total of 72 mL of blood. You will not be allowed to eat or drink anything except water for the six hours following the test meal.

Group A
Test Meal 1
High fat muffin
Protein shake
40 g soy protein (80 mg isoflavones)
1 banana
12-16 oz water
20% protein, 40% fat, 40% CHO

Test Meal 2
High fat muffin
Protein shake
40 g milk protein (0 mg isoflavones)
1 banana
12-16 oz water
20% protein, 40% fat, 40% CHO

Group B
Test Meal 1
High CHO muffin
Protein shake
40 g soy protein (80 mg isoflavones)
1 banana
12-16 oz water
20% protein, 20% fat, 60% CHO

Test Meal 2
High CHO muffin
Protein shake
40 g milk protein (0 mg isoflavones)
1 banana
12-16 oz water
20% protein, 20% fat, 60% CHO

RISKS:

Approximately 1 tablespoon of blood will be removed by putting a needle in your vein on 4 occasions. This is the standard medical method used to obtain blood for tests. There is momentary pain at the time the needle is inserted into the vein, but other discomfort should be minimal. In about 10% of the cases there is a small amount of bleeding under the skin, which will produce a bruise. This risk of infection is less than 1 in 1,000.

Subjects may experience gastrointestinal distress as a result of protein shake consumption.

Soy protein does not pose any unusual risks of allergenic responses. Soy protein is less allergenic than cow's milk. There are, of course, certain people who may be allergic to soy. Allergy to soy is reported as approximately 0.5 percent incidence in the adult population.

BENEFITS:

All participants will be provided with a summary and explanation of their results from the study including the lipid panel profile and dietary intake analysis.

FUNDING:

This is currently not a funded project.

CONFIDENTIALITY:

The data obtained from the study will be regarded as privileged and confidential. Your privacy will be maintained in any future analysis and/or presentation of the data with the use of coded identification for each participant's data. All data will be stored in a locked file cabinet with access only by the principal investigator. Additionally, any data entered into the computer will be available with restricted password only.

FREEDOM OF CONSENT:

Participation in this study is completely voluntary. You may withdraw consent in person with the principal investigator, Dr. Christina Campbell, at any time. In the event of any physical injury occurring in connection with the study, Montana State University will not provide any special compensation or any medical treatment. We will advise and assist the participant in receiving medical treatment. Additionally, Montana State University will not be held responsible for injury or accidents that may occur when traveling to and from campus.

Please feel free to ask any questions or express your concerns regarding this study. The investigator will attempt to answer all of your questions. Contact Dr. Christina Campbell at 994-5002.

Please address any questions relating to the rights of human subjects to Mark Quinn, Chair, Human Subjects Committee, 994-5721.

AUTHORIZATION:

If over 18 years of age:
I have read the above and understand the discomforts, inconveniences and risk of this study. I, ________________________________ *(your name)*, agree to participate in the project. I understand that I may later refuse to participate, and that I may withdraw from the study at any time. I have received a copy of this consent form for my own records.

Signed: ________________________________

Witness: ________________________________

Investigator: ________________________________

Date: ____________________

APPENDIX B

MEDICAL HISTORY QUESTIONNAIRE

Medical History Questionnaire

Please answer the following questions honestly and to the best of your knowledge. All information provided here is completely confidential. Please ask for clarification if needed.

Subject Name: ____
(Last) *(First)* *(MI)*

Phone Number we can reach you at: ______________

Address: ____

Sex: M F Age:___yrs ___mo Date of Birth:__________

Weight: ______lbs Height: ___ft ___in

State of birth: ______________

Do you smoke cigarettes? Yes No
Are you an ex-smoker? Yes No
If so, how long ago did you quit? ______________

Race (circle):
1. American Indian or Alaska Native
2. African American
3. Caucasian
4. Asian
5. Hispanic
6. Other (specify): __________

Marital Status (circle one):
1. single
2. married
3. divorced/separated
4. widowed

Medical History (circle any, and give age at diagnosis):

	Age
1. Diabetes	___
2. Thyroid Disease	___
3. Cirrhosis	___
4. Hepatitis	___
5. Gall Stones	___
6. Kidney Stones	___

7. Nephritis ___
8. Cancer (specify) ___
9. High Blood Pressure ___
10. Angina ___
11. Allergies (specify) ___
12. Goiter ___
13. Cardiovascular Disease ___
14. Depression requiring medication ___
15. Insomnia requiring medication ___

Drug History:

1. Do you currently take any medications on a regular basis? ____
   If yes, please specify ______________________________
2. Have you take medication regularly in the past? _____
   If yes, please specify ______________________________
   How long ago was medication taken regularly? _____
3. Do you currently take vitamin supplements on a regular basis? ___
   If yes, please specify? ______________________________
   Have you in the past? ______________
   If so, how long ago? ______________
4. Do you currently take herbal supplements on a regular basis? ___
   If yes, please specify? ______________________________
   Have you in the past? ______________
   If so, how long ago? ______________

Diet History:

a. Are you currently on a diet to lose weight? Yes No
   i. If yes, please explain: ______________________________
   ____

b. Are you a vegetarian? Yes No

c. If yes, circle one of the following:
   i. Lacto-ovo (consume milk, milk products and eggs)
   ii. Ovo (consume eggs but no milk or milk products)

iii. Lacto (consume milk and milk products but no eggs)

iv. Vegan (consume no animal products)

d. Please specify any food allergies (soy, milk, peanut, etc) : ____________

e. How many alcoholic drinks (12 oz beer, 6 oz wine or 1.5 oz distilled alcohol) do you typically consume (circle one)?

i. 0-1 drinks/day

ii. 1-2 drinks/day

iii. >2 drinks/day

If yes,how many days/week do you consume > 2 drinks/day? ____

f. Do you consume soy on a regular basis? Yes No

g. If yes, how often? ____________________

h. Which soy products do you typically eat (please circle)? How often (servings/ day, week, month, year)?

i. Tofu (1/2 cup = 1 serving) ________________

ii. Soy milk (1 cup = 1 serving) ________________

iii. Soy nuts (1/4 cup = 1 serving) ________________

iv. Soy protein powder concentrate (1/4 cup = 1 serving) __________

v. Soy bar (i.e. Luna Bars, Genisoy Bars) ______________

vi. Soybeans (1/2 cup = 1 serving) ______________

vii. Soy burgers (i.e. Garden Burger, Boca Burger) ____________

viii. Tempeh (1/2 cup = 1 serving) ____________

Portion sizes from the 2000 Soyfoods Guide

Video Rental suggestions for days spent at the Nutrition Research Lab: __________
____

Please list dates (preferably weekend days) that would be best for you to spend 12 hours at the Nutrition Research Lab: ______________________________________
____

Please list any weekend for which you would be ***unable*** to spend 12 hours at the Nutrition Research Lab: ______________________________________
____

APPENDIX C

DIET RECORD INSTRUCTIONS AND FORM

Directions for
3-Day Weighed Diet Records

- Please weigh and record all foods and beverages consumed for the three days prior to reporting to the Nutrition Research Lab for a feeding study day (i.e. If you will be reporting to the Lab on a Sunday, please record for Thursday, Friday and Saturday).

- Keep your food record current. List foods immediately after they are weighed. Do not wait until the end of the day to record entries.

- Please print all entries.

- Be as specific as possible when describing the food or beverage:
  - include the method of preparation used (boiled, baked, broiled, fried, grilled, steamed, raw, etc); *example: chicken breast, skinless, broiled*
  - include a well detailed description of the food item (fresh, canned, packed in heavy or light syrup, packed in water or oil, skinless, boneless, cut of meat, brand name); *examples: peaches in heavy syrup, tuna in oil, broiled T-bone steak, microwave heated canned corn*
  - include label with the nutritional information for any unusual items or if unsure how to record

- Include the name of restaurant if eating out

- Report only the portion of the food that was actually eaten; *example: T-bone steak, grilled - 100g (do not include the weight of the bone)*

- Record amounts in either grams or ounces (wt)

- Remember to record condiments (ketchup, soy sauce, mustard, ranch dressing, etc) as well as any fats used in cooking (oils, butter, margarine, etc); it is acceptable to measure these (Tbsp, tsp, etc)

- And finally, try not to alter your normal diet during the period that you keep this record

- Please bring the scale and diet records to the Nutrition Research Lab with you on your scheduled blood draw day…THANK YOU!!!

3-Day Weighed Food Record Form

Name: ______________
Subject ID# ______________
Date Recorded: ______________
Day: M T W Th F Sa Su
Typical Day? ______________

Time	Description of Food Item Eaten & Cooking Method	Weight

APPENDIX D

PHYSICAL ACTIVITY LOG

Bouchard Three-Day Physical Activity Record

Activity component(s) assessed:
Leisure and occupational
Time frame of recall:
Three d (2 weekdays and 1 weekend day)
Original mode of administration:
Self-administered with instruction provided by interviewer
Primary source of information:
Dr. Claude Bouchard
Laval University
Physical Activity Sci. Lab.
P.E.P.S.
Ste-Foy, PQ G1K7P4
Canada
Primary reference:
BOUCHARD, C., A. TREMBLAY, C. LEBLANC, G. LORTIE, R. SAVARD, and G. THERIAULT. A method to assess energy expenditure in children and adults. *Am. J. Clin. Nutr.* 37:461–467, 1983.

RELIABILITY AND VALIDITY STUDIES

TABLE 4. Reliability studies of the Bouchard Three-Day Physical Activity Record.

Reference	Methods	Sample	Summary Results	
Bouchard et al. (1)	Relationships between first test and 6- to 10-d retest (intraclass correlations)	61 adults and children of French descent between the ages of 10 and 50 yr	Children	0.91*
			Adults	0.97*
			Total	0.96*

* $P < 0.05$.

TABLE 5. Validation studies of the Bouchard Three-Day Physical Activity Record.

Reference	Methods	Sample	Summary Results	
Bouchard et al. (1)	Relationships with a submaximal cycle test (PWC) and % body fat (BF) (interclass correlations)	150 adults with a mean age (±SD) of 42 ± 4 yr and 150 children with a mean age of 15 ± 3 yr	PWC	0.31*
			BF	−0.13*

* $P < 0.05$.
PWC = physical working capacity.

Activity Codes for the Bouchard Three Day Physical Activity Record

Category of activity	Example of activity for each category	Approximate energy expenditure (kcal/kg/15 min)
1	Lying down: - sleeping - resting in bed	0.26
2	Seated: - listening in class - eating - writing by hand or typing - reading - listening to the radio or T.V. - taking a bath	0.38
3	Standing; light activity: - washing oneself - shaving - combing hair - dusting - cooking	0.57
4	Getting dressed Taking a shower Driving a car Taking a walk (strolling)	0.70
5	Light manual work: - housework (washing windows, sweeping etc.) - tailor - baker - printer - brewer - cobbler - mechanic - electrician - painter - lab-work - carpentry - masonry - driving a farm tractor - cleaning trees - working in the chemical or electric industries - feeding animals on a farm - doing the bed Riding a moped Moderately quick walking (going to school, shopping)	0.83
6	Light sport or leisure activities: - light canoeing - volleyball - table tennis - baseball (except the pitcher) - golf - rowing - archery - ninepins - croquet - sailing - cycling (leisure)	1.20
7	Moderate manual work: - machine operating (building industry) - repairing a fence - loading bags or boxes - plantation work - forest work (machine sawing and log handling) - mine work - shoveling snow	1.40
8	Moderate sport or leisure activities: - baseball (pitcher) - badminton - canoeing - cycling (race bike) - dancing - tennis - jogging (slow running) - horseback riding - Alpine skiing - cross-country skiing (leisure) - swimming - gymnastics - brisk walking	1.0

Category of activity	Example of activity for each category	Approximate energy expenditure (kcal/kg/15 min)
9	Intense manual work: - felling a tree with an ax - sawing with a hand-saw - working with a pitchfork (on a farm) - cutting tree branches Intense sport or leisure activities: - running in a race - boxing - mountain-climbing - squash - cross-country skiing - ice hockey - basketball - football - racquetball	1.95

BOUCHARD THREE DAY PHYSICAL ACTIVITY RECORD

Day 1

Date: ______/______/______
day month year

Last name: ____________________

First name: ____________________

In each box, write the number which corresponds to the activity which you have carried out during this 15 minute period. Please consult the activity card that follows to establish the proper coding. If an activity is carried out over a long period (e.g. sleeping), you can draw a continuous line in the rectangular boxes which follow until such a time when there is a change in activity. To understand this better, we suggest that you take a look at the example that follows.

Minute / Hour	0-15	16-30	31-45	46-60
0				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				

APPENDIX E

DIET ANALYSIS OF CHALLENGE MEAL

Client Diet Record Nutrient Analysis

First:
Middle:
Last: Muffin and Shake
Company:

Identification Number: Muffin and Shake
Date of Birth:
Height:
Weight:

Total Days: 1
Avg. Daily Kcals: 899.386
Total Foods: 3
Diet Name: New Diet Record

Percentage of Kcals

Protein	19.4%
Carbohydrate	58.6%
Fat, total	22.0%
Alcohol	0.0%

Exchanges

Fat	0.00
Fruit	1.50

Nutrient	Value	Unit	Goal	%
Weight	357.109	g		
Kilocalories	899.386	kcal		
Protein	44.425	g		
Carbohydrate	134.311	g		
Fat, Total	22.386	g		
Alcohol	0.000	g		
Cholesterol	38.270	mg		
Saturated Fat	13.093	g		
Monounsaturated Fat	3.202	g		
Polyunsaturated Fat	3.200	g		
MFA 18:1, Oleic	2.649	g		
PFA 18:2, Linoleic	2.828	g		
PFA 18:3, Linolenic	0.120	g		
PFA 20:5, EPA	0.000	g		
PFA 22:6, DHA	0.002	g		
Sodium	600.927	mg		
Potassium	1169.557	mg		
Vitamin A (RE)	95.369	RE		
Vitamin A (IU)	1456.111	IU		
Beta-Carotene	36.023	µg		
Alpha-Carotene	9.800	µg		
Lutein (+ Zeaxanthin)	7.200	µg		
Beta-Cryptoxanthin	0.000	µg		
Lycopene	0.000	µg		
Vitamin C	10.079	mg		
Calcium	1542.249	mg		
Iron	5.918	mg		
Vitamin D (ug)	0.149	µg		
Vitamin D (IU)	205.954	IU		
Vitamin E (mg)	1.989	mg		
Vitamin E (IU)	2.964	IU		
Alpha-Tocopherol	1.522	mg		
Thiamin	0.059	mg		
Riboflavin	1.087	mg		
Niacin	0.758	mg		
Pyridoxine (Vitamin B6)	0.601	mg		
Folate	113.066	µg		
Cobalamin (Vitamin B12)	1.904	µg		
Biotin	6.561	µg		
Pantothenic Acid	0.396	mg		
Vitamin K	7.240	µg		
Phosphorus	1070.737	mg		
Iodine	0.000	µg		

Client Diet Record Nutrient Analysis

First:
Middle:
Last: Muffin and Shake
Company:

Identification Number: Muffin and Shake
Date of Birth:
Height:
Weight:

Nutrient	Value	Unit	Goal	%
Magnesium	161.886	mg		
Zinc	0.285	mg		
Copper	0.204	mg		
Manganese	0.298	mg		
Selenium	6.774	µg		
Fluoride	44.101	µg		
Chromium	0.003	mg		
Molybdenum	0.000	µg		
Dietary Fiber, Total	3.517	g		
Soluble Fiber	0.500	g		
Insoluble Fiber	1.202	g		
Crude Fiber	0.633	g		
Sugar, Total	74.976	g		
Glucose	6.032	g		
Galactose	0.000	g		
Fructose	4.032	g		
Sucrose	30.690	g		
Lactose	0.000	g		
Maltose	0.016	g		
Sugar Alcohol				
Other Carbohydrate				
Tryptophan	48.600	mg		
Threonine	170.760	mg		
Isoleucine	199.224	mg		
Leucine	324.023	mg		
Lysine	253.152	mg		
Methionine	109.707	mg		
Cystine	91.074	mg		
Phenylalanine	206.668	mg		
Tyrosine	141.170	mg		
Valine	234.358	mg		
Arginine	211.404	mg		
Histidine	149.080	mg		
Alanine	207.896	mg		
Aspartic Acid	416.376	mg		
Glutamic Acid	510.726	mg		
Glycine	140.059	mg		
Proline	160.674	mg		
Serine	254.387	mg		
Moisture	142.810	g		
Ash	2.301	g		
Caffeine	0.000	mg		

Bobbi's High Carbohydrate Muffin

Nutrient Analysis
Source: Custom

Yield: 16 (2.00 serving(s))
No. Ingredients: 23

Category: Basic Food

Nutrient Goal Template: DAILY VALUES/RDI - ADULT/CHILD

Percentage of Kcals

Protein	2.7%
Carbohydrate	60.3%
Fat, total	37 0%
Alcohol	0.0%

Exchanges

Nutrient	Value	Unit	Goal	%
Weight	169.109	g		
Kilocalories	487.386	kcal	2000.000	24 %
Protein	3.395	g	50.000	7 %
Carbohydrate	74.881	g	300.000	25 %
Fat, Total	20.406	g	65.000	31 %
Alcohol	0.000	g		
Cholesterol	33.270	mg	300.000	11 %
Saturated Fat	12.908	g	20.000	65 %
Monounsaturated Fat	3.161	g		
Polyunsaturated Fat	3.111	g		
MFA 18:1, Oleic	2.622	g		
PFA 18:2, Linoleic	2.772	g		
PFA 18:3, Linolenic	0.087	g		
PFA 20:5, EPA	0.000	g		
PFA 22:6, DHA	0.002	g		
Sodium	289.927	mg	2400.000	12 %
Potassium	163.557	mg	3500.000	5 %
Vitamin A (RE)	87.369	RE		
Vitamin A (IU)	375.111	IU	5000.000	8 %
Beta-Carotene	15.023	µg		
Alpha-Carotene	4.800	µg		
Lutein (+Zeaxanthin)	7.200	µg		
Beta-Cryptoxanthin	0.000	µg		
Lycopene	0.000	µg		
Vitamin C	0.979	mg	60.000	2 %
Calcium	136.249	mg	1000.000	14 %
Iron	1.288	mg	18.000	7 %
Vitamin D (ug)	0.149	µg	10.000	1 %
Vitamin D (IU)	5.954	IU	400.000	1 %
Vitamin E (mg)	1.719	mg	20.000	9 %
Vitamin E (IU)	2.561	IU	30.000	9 %
Alpha-Tocopherol	1.252	mg		
Thiamin	0.014	mg	1.500	1 %
Riboflavin	0.137	mg	1.700	8 %
Niacin	0.218	mg	20 000	1 %
Pyridoxine (Vitamin B6)	0.023	mg	2.000	1 %
Folate	4.066	µg	400.000	1 %
Cobalamin (Vitamin B12)	0.104	µg	6.000	2 %
Biotin	2.561	µg	300.000	1 %
Pantothenic Acid	0.136	mg	10.000	1 %
Vitamin K	6.740	µg	80.000	8 %
Phosphorus	50.737	mg	1000.000	5 %
Iodine	0.000	µg	150.000	0 %
Magnesium	12.886	mg	400.000	3 %
Zinc	0.125	mg	15.000	1 %

Bobbi's High Carbohydrate Muffin

Nutrient Analysis
Source: Custom

Yield: 16 (2.00 serving(s))
No. Ingredients: 23

Category: Basic Food

Nutrient	Value	Unit	Goal	%
Copper	0.100	mg	2.000	5 %
Manganese	0.146	mg	2.000	7 %
Selenium	5.674	µg	70.000	8 %
Fluoride	21.118	µg		
Chromium	0.003	mg	0.120	3 %
Molybdenum	0.000	µg	75.000	0 %
Dietary Fiber, Total	1.117	g	25.000	4 %
Soluble Fiber	0.000	g		
Insoluble Fiber	0.000	g		
Crude Fiber	0.166	g		
Sugar, Total	27.362	g		
Glucose	1.830	g		
Galactose	0.000	g		
Fructose	1.330	g		
Sucrose	24.190	g		
Lactose	0.000	g		
Maltose	0.016	g		
Sugar Alcohol				
Other Carbohydrate				
Tryptophan	36.600	mg		
Threonine	136.760	mg		
Isoleucine	166.224	mg		
Leucine	253.023	mg		
Lysine	205.152	mg		
Methionine	98.707	mg		
Cystine	74.074	mg		
Phenylalanine	168.668	mg		
Tyrosine	117.170	mg		
Valine	187.358	mg		
Arginine	164.404	mg		
Histidine	68.080	mg		
Alanine	168.896	mg		
Aspartic Acid	303.376	mg		
Glutamic Acid	399.726	mg		
Glycine	103.059	mg		
Proline	120.674	mg		
Serine	207.387	mg		
Moisture	68.550	g		
Ash	1.501	g		
Caffeine	0.000	mg		

Soy and Placebo Beverage 461 & 858

Nutrient Analysis — Yield: 1 (1.00 item(s)) — Category: Basic Food
Source: Custom

Nutrient Goal Template: DAILY VALUES/RDI - ADULT/CHILD

Percentage of Kcals

Protein	50.4%
Carbohydrate	45.4%
Fat, total	4.3%
Alcohol	0.0%

Exchanges

Nutrient	Value	Unit	Goal	%
Weight	44.000	g		
Kilocalories	160.000	kcal	2000.000	8 %
Protein	20.000	g	50.000	40 %
Carbohydrate	18.000	g	300.000	6 %
Fat, Total	0.750	g	65.000	1 %
Alcohol				
Cholesterol	2 500	mg	300.000	1 %
Saturated Fat			20.000	
Monounsaturated Fat				
Polyunsaturated Fat				
MFA 18:1, Oleic				
PFA 18:2, Linoleic				
PFA 18:3, Linolenic				
PFA 20:5, EPA				
PFA 22:6, DHA				
Sodium	155.000	mg	2400.000	6 %
Potassium	305.000	mg	3500.000	9 %
Vitamin A (RE)				
Vitamin A (IU)	500.000	IU	5000.000	10 %
Beta-Carotene				
Alpha-Carotene				
Lutein (+ Zeaxanthin)				
Beta-Cryptoxanthin				
Lycopene				
Vitamin C			60.000	
Calcium	700.000	mg	1000.000	70 %
Iron	2.160	mg	18.000	12 %
Vitamin D (ug)			10.000	
Vitamin D (IU)	100.000	IU	400.000	25 %
Vitamin E (mg)			20.000	
Vitamin E (IU)			30.000	
Alpha-Tocopherol				
Thiamin			1.500	
Riboflavin	0.425	mg	1.700	25 %
Niacin			20.000	
Pyridoxine (Vitamin B6)			2.000	
Folate	45.000	µg	400.000	11 %
Cobalamin (Vitamin B12)	0.900	µg	6.000	15 %
Biotin			300.000	
Pantothenic Acid			10.000	
Vitamin K			80.000	
Phosphorus	500.000	mg	1000.000	50 %
Iodine			150.000	
Magnesium	60.000	mg	400.000	15 %
Zinc			15.000	

Soy and Placebo Beverage 461 & 858

Nutrient Analysis
Source: Custom

Yield: 1 (1.00 item(s))

Category: Basic Food

Nutrient	Value	Unit	Goal	%
Copper			2.000	
Manganese			2.000	
Selenium			70.000	
Fluoride				
Chromium			0.120	
Molybdenum			75.000	
Dietary Fiber, Total			25.000	
Soluble Fiber				
Insoluble Fiber				
Crude Fiber				
Sugar, Total	16.000	g		
Glucose				
Galactose				
Fructose				
Sucrose				
Lactose				
Maltose				
Sugar Alcohol				
Other Carbohydrate				
Tryptophan				
Threonine				
Isoleucine				
Leucine				
Lysine				
Methionine				
Cystine				
Phenylalanine				
Tyrosine				
Valine				
Arginine				
Histidine				
Alanine				
Aspartic Acid				
Glutamic Acid				
Glycine				
Proline				
Serine				
Moisture				
Ash				
Caffeine				

APPENDIX F

THE SOLAE COMPANY INFORMATION

P.O. Box 88940
St. Louis, MO 63188
Phone: 314-982-1268
Fax: 314-982-3960
Email: brownjl@solae.com

The Solae Company

Dr. Christina Campbell
Montana State University
20 Herrick Hall
Bozeman MT 59717
406-994-5002

April 1, 2003

Dear Dr. Campbell:

The undersigned acknowledges that the Powdered Nutritional beverages, manufactured by Solae L.L.C, shipped to you with the designations Sample 858 and 461 contain the following ingredients:

Sample 858 – The Soy Vanilla Nutritional Beverage Powder (formulated to deliver 20 g soy protein with 2.0 mg isoflavones* /g of soy protein) *aglycone weight
Ingredients: Isolated soy protein, fructose, sucrose, maltodextrin, potassium citrate, artificial flavor and guar gum. **Vitamins & Minerals:** Calcium phosphate, magnesium phosphate, riboflavin, vitamin A palmitate, folic acid, vitamin D3 and vitamin B12.

Sample 461 – The Placebo Vanilla Nutritional Beverage Powder
Ingredients: Milk protein isolate, fructose, sucrose, maltodextrin, potassium citrate, artificial flavor and guar gum. **Vitamins & Minerals:** Calcium phosphate, magnesium phosphate, riboflavin, vitamin A palmitate, folic acid, vitamin D3 and vitamin B12.

The U.S. Food and Drug Administration's Regulations (21 CFR 101.100 (d)(2)) require that a copy of this agreement be kept on file for two (2) years beyond the final shipment or delivery of the above-referenced products. This document should be available for inspection, upon request, by any officer or employee of the FDA.

AGREED TO AND ACCEPTED BY:

A representative of Solae study number 506 titled: "The postprandial effect of soy isoflavone consumption following a high-fat or high-carbohydrate meal on oxidative resistance of low-density lipoprotein in healthy, young men".

BY: Christina Campbell

TITLE: Assistant Professor - MT St. Univ.

The Solae Company
P.O. BOX 88940
ST. LOUIS, MO 63188

AF1.2 CI VA 70CA 44
Lot # G265-0

Composition

Protein (%)	44.3
Calcium (mg/100g)	1630
Ash (%)	8.68
Fat, acid hydrolysis (%)	2.35
Moisture (%)	2.37
Phosphorus (mg/100g)	1290
Potassium (mg/100g)	1450
Sodium (mg/100g)	418

Microbiology

Aerobic Plate Count (/g)	<10
Coliform count (MPN/g)	<3
E. coli (MPN/g)	<3
Mold (/g)	<10
Salmonella (/375g)	Negative
Yeast (/g)	<10

Isoflavone Analysis

All Forms*	mg/g product as is	mg/g protein
Genistein-containing compounds	0.87	1.96
Daidzein-containing compounds	0.65	1.47
Glycitein-containing compounds	0.14	0.31
TOTAL ISOFLAVONES	1.66	3.74

Aglycone Components	mg/g product as is	mg/g protein
Genistein	0.51	1.15
Daidzein	0.38	0.86
Glycitein	0.08	0.19
Total Aglycone Components	0.97	2.20

*Aglycones, Glycosides, Glycoside esters

The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements made are intended only as a source of information. Also, we may suggest technical solutions for incorporating this ingredient into products, however, it is the user's responsibility to comply with appropriate government standards and requirements. No warranties, expressed or implied, are made. On the basis of this information, it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product. The information contained herein shall not be construed as permission for violation of patent rights.

The Solae Company

P.O. BOX 88940
ST. LOUIS, MO 63188

Summary of product data for Study 509 (Campbell/Montana State Univ)

Study title: The postprandial effect of soy isoflavone consumption following a high-fat or high-carbohydrate meal on oxidative resistance of low-density lipoprotein in healthy, young men

Products: Sample 858 – Soy Vanilla Nutritional Beverage Powder (formulated to deliver 20 g soy protein with 2.0 mg isoflavones, aglycone weight /g of soy protein)
Sample 461 – Milk Placebo Vanilla Nutritional Beverage Powder

Product	Sample#	Lot#	Protein g/44 g serv	Protein g/day	Isoflavones (aglc) mg/44 g serv	Isoflavones (aglc) mg/day
AF1.2 CI VA 70CA 44	858	G265-0	19.5	39.0	42.7	85.4
AFTMP CI VA 70CA 44	461	G266-0	19.9	39.9		

summary for Campbell G265-0 & G266-0.xls

The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements made are intended only as a source of information. Also, we may suggest technical solutions for incorporating this ingredient into products, however, it is the user's responsibility to comply with appropriate government standards and requirements. No warranties, expressed or implied, are made. On the basis of this information, it is suggested that you evaluate the product on a laboratory scale prior to use in a finished

APPENDIX G

LDL OXIDATION PROCEDURE

LDL Oxidation Procedure

Notes: Gloves should be worn throughout the procedure (when in contact with bodily fluids).

All solutions are prepared fresh daily unless otherwise stated.

I. Plasma Preparation

1. Collect blood samples in 6ml Vacuette Blood Collecting Tubes (1-2) with EDTA (purple top) by venipuncture
2. Place tubes into rotor, being sure that they are balanced, and check to ensure that the nut on top is tight and that the rotor is secure.
3. Close the lid on the Marathon 21000R centrifuge and set parameters at 2500 rpm, 16°C for 10minutes and start.
4. Aspirate off plasma with Pasteur pipet and place into µcentrifuge tubes, about 1 ml in each tube (2-3 tubes)
5. Place in -80°C Revco freezer for storage (be sure to record in log where samples are place, box & shelf) *or* continue on with isolation immediately

II. 3 days before:

Turn on incubator to achieve constant 37°C – KEEP TRACK OF TEMPS!

IV. Night before:

1. Set samples out (10 max) in freezer.for the time points and samples you will be working with tomorrow
2. Make $NaCl_2$ (2 solutions) and PBS spin these to mix and set at room temp – this only needs to be done once per week (Takes ~20-30 minutes to prepare samples)

PREPARE 0.9% NACL SOLUTION (WEIGH 900MG NACL AND 100MG EDTA IN A PIECE OF WEIGHING PAPER, TRANSFER TO A 125ML ERLENMEYER FLASK WITH THE AID OF 100ML OF DEIONIZED (DI) WATER, ADD A SMALL STIR BAR, STIR ON A MAGNETIC STIR PLATE TO DISSOLVE); NEED ½ TO 1 ML PER SAMPLE

a. Prepare the 16.7% NaCl solution (weigh 16.7g NaCl and 100mg EDTA on a piece of weighing paper, transfer to a 125ml erlenmeyer flask with the aid of 100ml of DI water, add a small stir bar, stir on a magnetic stir plate to dissolve, this solution may take a long time to dissolve); need ½ to 1 ml per sample [soln good for 1 week]. Will take about an hour to dissolve
b. Prepare PBS buffer (St. Lukes procedure): 22.8mg (.228 g) Sodium Phosphate Monobasic + 115mg (1.15 g) Sodium Phosphate Dibasic + 935mg (9.35 g) Sodium Chloride + 90ml DI water in a 100 ml volumetric flask, add stir bar and stir on magnetic stir plate to dissolve,

pH 7.4 (adjust with NaOH or HCl if necessary), add DI water to volume (*100 ml of PBS is required for each 1-2 samples) [soln good for 1 week]. Use 1000 ml beaker and MULTIPLY ALL BY 10 TO MAKE 1 L.

V. Day of:

1. Remove samples from -80°C freezer (if necessary) and allow to defrost. To defrost the samples takes about 15-20 minutes (Note: it helps to defrost samples quicker if you hold them in your hand!!)
2. Turn on centrifuge in comp lab
3. Label first set of Beckman tubes for VLDL and LDL isolation step
4. Spin to mix PBS and $NaCl_2$ mixtures – if needed
5. Turn on plate shaker , plate reader, and computer

VI. Isolation (ref. Lipoprotein Separations Using TL-100 Tabletop Ultracentrifuge, H.K. Naito) Takes approximately 6 hours

TO BECKMAN CENTRIFUGE TUBES (POLYALLOMER, 7/16 X 1 3/8 IN; REORDER # 347287) ADD 500ML OF THE 0.9% NACL SOLUTION AND 500ML OF PLASMA (EDTA); REPEAT THIS STEP INTO A SECOND CENTRIFUGE TUBE FOR EACH SAMPLE (TO DO IN DUPLICATE)

1. Centrifuge (Door-Enter/Display-Start if on correct program) at 100,000 rpm for 2.5 hours at 16°C in the Beckman Optima TLX Ultracentrifuge with a Beckman TLA 120.2 Rotor (S.N. 94U635; 120K RPM) [Door (to open),Enter/Display, Start (if on correct program)]
2. When step 2 has been completed, remove centrifuge tubes (view with a black background, the top layer should be cloudy/white) and slice with the Beckman Centritube Slicer at approximately the 500 µl mark (determine this by pipetting 500ul on 0.9% NaCl into an empty centrifuge tube and using that tube to set up the slicer), discard the top layer (VLDL layer) in a biohazards box and transfer the bottom layer (LDL and HDL layer) to a new centrifuge tube with a Pasteur pipet (mix the viscous bottom by carefully drawing it up into the pipet and expelling it, repeat 3-4 times), pipet in 500 µL of the 16.7% NaCl solution. Wash slicer with water.
3. Centrifuge again as in step 2 of Isolation
4. Label microcentrifuge tubes for Isolated LDL, Desalted LDL, and Adjusted LDL.
5. Wash centrifuge slicer with KimWipe and water
6. When last centrifuge has approximately 20 mintues take the caps off of Econo-Pac columns
7. When step 4 has been completed (view centrifuge tube with a white background, it should have a yellow/gold top layer), slice centrifuge tube and remove top 500µl (LDL layer), make a composite of the 2 centrifuge tube's top layers for each sample and place in a microcentrifuge tube and cover, discard the bottom

layer in a biohazards box; The LDL has now been isolated. Wash centrifuge slicer with water and KimWipe

8. MIX the isolated LDL thoroughly!

While samples are spinning (either during spin 1 or 2):

1. Prepare Working Reagent (WR) for the protein assay: using the Pierce BCA Protein Assay Kit (23225) mix 50 parts of BCA reagent A with 1 part of BCA reagent B keeping in mind that 200µl is required per well used (solution can be stored for 1 day at room temperature)
   a. Ex: 8 tubes of standard + blank (both in duplicate) = 18
      10 ml reagent A = $\frac{10000\ \mu l}{50}$ = 200 µl reagent B (Seal with parafilm)
2. Prepare standard solutions with bovine serum albumin (BSA) (in hands box) standard provided according to Pierce table 1 by pipeting into separate microcentrifuge tubes and mixing :

Label	Concentration (µg/ml)	Volume BSA	Volume PBS (µl)
Stock	2000	300µl stock	0
A	1500	300µl stock	100
B	1000	100µl stock	100
C	750	175µl A	175
D	500	100µl B	100
E	250	100µl D	100
F	125	100µl E	100
G	50	100µl F	400

* Remember to mix THOURGHLY!!!
* Empty glass contents into µcent tube (this is the stock)

VII. <u>Desalting</u> (ref.Econo-Pac 10DG Columns Manual #732-2010; Palomaki et al, Journal of Lipid Research v39, 1998, 1430-7)

1. Add ≤ 3ml of isolated LDL to the column (350-**450**µl), allow to enter column
2. Add PBS to achieve a total volume of 3ml (3000 µl):
   3ml – volume of isolated LDL = volume of PBS to be added to the column
   Ex: 3000 – 450 = 2550 µl of PBS to be added to the column
3. Use a 10ml graduated cylinder to measure the first 3ml of effluent and discard (solution should stop flowing at about this point)
4. Add [1.5 X volume of isolated LDL] to elute the higher molecular weight components, collect in test tube and cover, MIX
   Ex: 1.5 x 450 = 675 µl PBS

STORE COLUMN IN PBS, REPLACE CAP AND TIP, PUT SAMPLES IN FRIG

5. Turn on plate reader and allow to warm up (~30 minutes)

IX. <u>Protein Concentration</u> (ref. Pierce BCA Protein Assay Kit 23225, microwell plate protocol)

1. Pipet 25µl of each of the standard solutions from step 1 (stock-G), sample solutions(isolated and desalted LDL) and blank solutions (PBS) into separate wells of a 96 well **<u>Nunc</u>** microwell plate; Prepare in duplicate (2 wells for each!) Ex:

	1
A	PBS
B	"
C	Stock
D	"
E	A
F	"
G	B
H	"

2. Add 200µl of WR (from step 8b of Isolation)to each well
3. Shake plate for 30 seconds on a plate shaker
4. Cover plate with a plate sealer (Seal Plate NonSterile 100-THER-PLT, Excel scientific 760-249-6371)
5. Incubate for 30 minutes at 37°C in plate shaker/incubator
6. Prepare Cupric Chloride Dihydrate solution (weigh 85.2mg [.0852 g] on weighing paper and transfer into a 100ml volumetric flask with the aid of DI water, bring solution to 100ml volume with DI water and shake to dissolve (stock solution), dilute 1 ml of this stock solution plus 9 ml DI water and mix (this is the Cupric Chloride Dihydrate solution for the LDL oxidation, part V) .PREPARE DAILY
7. Allow plate to cool to room temperature
8. KC Junior: START – PROGRAM – KC JR - OK
9. Read absorbance at 562 nm on KC Junior plate reader (p. 6-10 of User's Guide)
10. Open existing protocol for protein assay by going to Protocol, then Open Protocol, then selecting BCAprotein
11. Once in the BCAprotein (ST-protein)protocol, open the Modify Protocol, go to Read Method and check to make sure that the primary wavelength is correct and that the plate geometry encompasses the wells that you need to read (if not go to Read and change the first and last locations or click to have it read the full plate)
12. In the Modify Protocol screen, go to template and enter in your Well ID's; the concentrations of the standard solutions can also be entered at this time (or after the read)
13. Click OK to exit the Modify Protocol Screen

14. Click Read Plate to initiate reading (you can enter a results ID at this time or at a later time), the dialog will prompt you to place the plate on the reader and press OK
15. When the read is complete, results are automatically displayed
16. Want a $R^2 = .99$ or .98
17. Print Std. Curve and Results
18. To save results, select Results/Save Results (enter the results ID at this time if it was not done earlier), click OK to save results

X. <u>LDL oxidation</u> (ref. Esterbauer et al, St.Lukes protocol)

1. Using the results of the protein assay, dilute (with PBS) the samples of isolated and desalted LDL to <u>achieve a concentration of 0.10 mg protein/ml (or 100 µg/ml)</u> from column report:

Calculation of Dilution (using 200µl of the isolated, desalted sample):

$$\mu l\ PBS = \frac{\text{sample concentration in } \mu g/ml \times 200 \mu l \text{ sample}}{100\ \mu g/ml} - 200\ \mu l \text{ sample}$$

- Change the # of µl sample in both places, if 200 µl is not the desired amount
- Change final concentration if 100 µg/ml is not desired
- Use the sample concentration from the protein assay
- Note: 1000 µg = 1 mg

Ex: D1 = 353.6; D2 = 242.1

$$\frac{(353.6 \times 200)}{(100)} - 200 = 507\ \mu l \text{ PBS for D1}$$

$$\frac{(242.1 \times 200)}{(100)} - 200 = 284\ \mu l \text{ PBS for D2}$$

2. Dilute as calculated above into microcentrifuge tubes and mix : 200 µl sample + _µl PBS to dilute. Label these new tubes with an A
3. Read on KC Junior plate reader using existing LDL oxidation (ST-LDLox) protocol at a wavelength of 234 nm and at a temperature of 37°C
   a. Open existing protocol for LDL oxidation by going to Protocol, then Open Protocol, then selecting LDL Ox.
   b. Once in the LDL Ox protocol, open the Modify Protocol, go to Read Method and check to make sure that the primary wavelength is correct and that the plate geometry encompasses the wells that you need to read (if not go to Read and change the first and last locations or click to have it read the full plate)
   c. In the Modify Protocol screen, go to template and enter in your Well ID's
   d. Click OK to exit the Modify Protocol Screen

   e. Click Read Plate to initiate reading (you can enter a results ID at this time or at a later time), the dialog will prompt you to place the plate on the reader and press OK
4. Pipet 100μl of each LDL sample solution (isolated, desalted and adjusted for protein concentration) into separate wells of a Costar 96 well UV flat bottom plate (370μl, 3635); prepare in duplicate. DO QUICKLY!
5. Pipet 10μl of the Cupric Chloride Dihydrate solution (step 8d of Isolation) into each well; seal with plate sealer. DO QUICKLY!
6. Run KC Junior
   a. When the read is complete, results are automatically displayed
   b. To save results, select Results/Save Results (enter the results ID at this time if it was not done earlier), click OK to save results
   c. The slope of the propagation phase can be determined by: selecting Open Results, selecting Kinetic Curve under the Kinetic View Options, clicking on the well of interest to enlarge, determining the points included in the propagation phase and clicking OK to close. Then, click the Calculations Options to open the Kinetic Calculations dialog, the first and last read points can then be changed to encompass the points of interest only, the slope will automatically recalculate based on the new range
   d. Protocol – save protocols
7. Write down temperature can leave @ read 3, keep checking and logging temp

VI. Data:

ENTER VALUES INTO EXCEL PROGRAM VIA KCJR→EXCEL COLUMNS DIRECTIONS (ATTACHED)

1. To Copy the column:
   a. Select cells and copy to clipboard
   b. Open in "lag & prop #2" template in Excel and paste (click on first cell and paste)
2. Record lag time and propagation rate; print data and graphs
   a. Lag phase and propagation phase duration
      i. Longer = better
   b. Maximal oxidation rate (measured within propagation phase)/maximum dienes

APPENDIX H

STATISTICAL DATA

Paired T-test Analysis for Subject's Characteristics

Student t Test for paired data
Group 1: Weight 858
Group 2: Weight 461

	Group 1	Group 2
Count	15	15
Mean	75.52	74.74
Variance	112.259	125.305
Std. Dev.	10.5952	11.194
Std. Err	2.73568	2.89028

Mean Difference 0.780001
Degrees of Freedom 14
t Value 1.1105
t Probability

Student t Test for paired data
Group 1: BMI 858
Group 2: BMI 461

	Group 1	Group 2
Count	15	15
Mean	23.5467	23.3133
Variance	6.43552	7.73124
Std. Dev.	2.53683	2.78051
Std. Err	0.655008	0.717925

Mean Difference 0.233333
Degrees of Freedom 14
t Value 1.0418
t Probability 0.3151

Paired T-test Analysis for Subject's Characteristics

Student t Test for paired data
Group 1: Energy 858
Group 2: Energy 461

	Group 1	Group 2
Count	15	15
Mean	42.0533	43.3067
Variance	15.8312	37.4492
Std. Dev.	3.97885	6.11958
Std. Err	1.02733	1.58007

Mean Difference -1.25333
Degrees of Freedom 14
t Value -1.0059
t Probability 0.3315

Student t Test for paired data
Group 1: Total Energy 858
Group 2: Total Energy 461

	Group 1	Group 2
Count	15	15
Mean	3179.8	3237.8
Variance	327600	441387
Std. Dev.	572.364	664.37
Std. Err	147.784	171.54

Mean Difference -58
Degrees of Freedom 14
t Value -0.66758
t Probability 0.5153

Paired T-test Analysis for Subject's Characteristics

Student t Test for paired data
Group 1: Intake 858
Group 2: Intake 461

	Group 1	Group 2
Count	15	15
Mean	34.8667	34.0467
Variance	92.6352	90.6484
Std. Dev.	9.62472	9.52094
Std. Err	2.48509	2.4583

Mean Difference 0.82
Degrees of Freedom 14
t Value 0.30871
t Probability 0.7621
Correlation 0.4226
Corr. Probability 0.1166

Student t Test for paired data
Group 1: Total Intake 858
Group 2: Total Intake 461

	Group 1	Group 2
Count	15	15
Mean	2590.49	2486.72
Variance	428988	332178
Std. Dev.	654.972	576.349
Std. Err	169.113	148.813

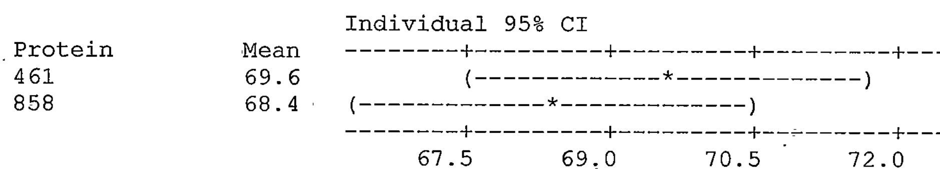
Mean Difference 103.767
Degrees of Freedom 14
t Value 0.53727
t Probability 0.5995
Correlation 0.26706
Corr. Probability 0.3359

Min tab Results for: Worksheet 5

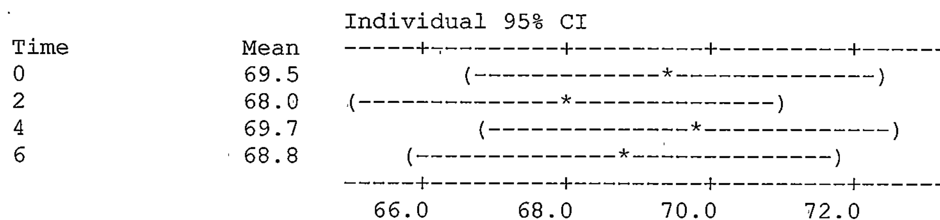
Two-way ANOVA: Adjusted for Missing Values

Lag Time Avera ges versus Protein, Time

```
Analysis of Variance for Lag Avg
Source        DF        SS         MS        F        P
Protein        1      39.9       39.9     0.60    0.439
Time           3      52.5       17.5     0.26    0.851
Interaction    3      99.9       33.3     0.50    0.681
Error        112    7405.1       66.1
Total        119    7597.3
```



```
                         Individual 95% CI
Protein         Mean     --------+---------+---------+---------+---
461             69.6              (-------------*-------------)
858             68.4     (-------------*-------------)
                         --------+---------+---------+---------+---
                              67.5      69.0      70.5      72.0
```



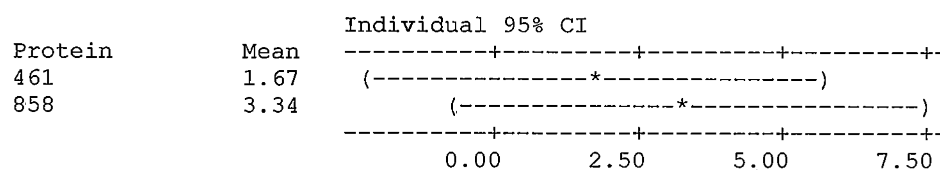
```
                         Individual 95% CI
Time            Mean     -----+---------+---------+---------+------
0               69.5              (-------------*--------------)
2               68.0     (--------------*--------------)
4               69.7               (--------------*-------------)
6               68.8         (--------------*--------------)
                         -----+---------+---------+---------+------
                           66.0      68.0      70.0      72.0
```

Two-way ANOVA: Adjusted for Missing Values

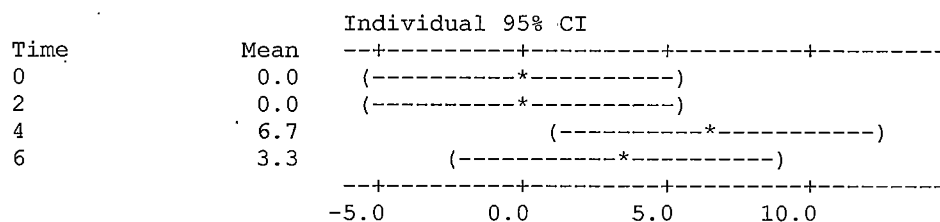
Propagation Rate Average versus Protein, Time

Analysis of Variance for Prop Avg

Source	DF	SS	MS	F	P
Protein	1	83	83	0.33	0.565
Time	3	916	305	1.22	0.305
Interaction	3	250	83	0.33	0.801
Error	112	27991	250		
Total	119	29241			



```
                        Individual 95% CI
Protein        Mean   ----------+---------+---------+---------+-
461            1.67    (---------------*---------------)
858            3.34          (---------------*----------------)
                      ----------+---------+---------+---------+-
                              0.00      2.50      5.00      7.50
```



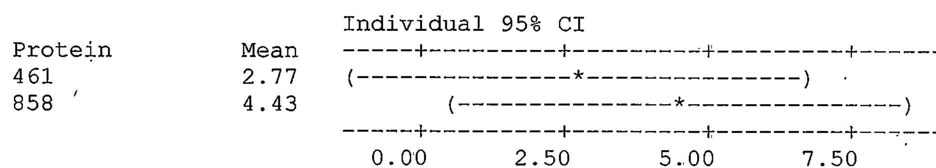
```
                        Individual 95% CI
Time           Mean   --+---------+---------+---------+---------
0               0.0    (----------*----------)
2               0.0    (----------*----------)
4               6.7                 (----------*-----------)
6               3.3          (-----------*----------)
                      --+---------+---------+---------+---------
                      -5.0       0.0       5.0      10.0
```

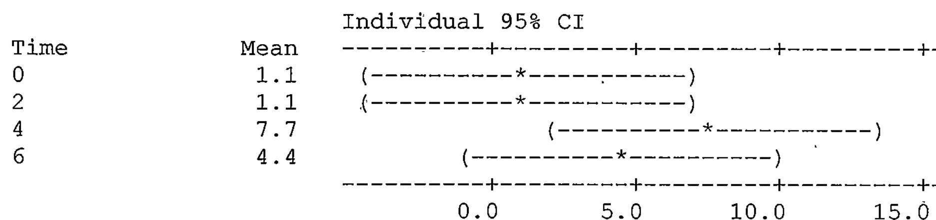
Two-way ANOVA: Adjusted for Missing Values

Initial Absorbance Average versus Protein, Time

Analysis of Variance for Initial

Source	DF	SS	MS	F	P
Protein	1	82	82	0.34	0.563
Time	3	894	298	1.22	0.306
Interaction	3	245	82	0.33	0.800
Error	112	27368	244		
Total	119	28589			





2-way ANOVA on Both Factors (Protein Treatment, Time Points)

Initial Absorbance, Lag Time, and Propagation Rate

Analysis of Variance for LDL Oxidation (Initial Absorbance)

Source	DF	SS	MS	F	p
Protein	1	82	82	0.34	0.56
Time Points	3	894	298	1.22	0.31
Interaction	3	245	82	0.33	0.80
Error	112	27368	244		

Analysis of Variance for LDL Oxidation (Lag Time)

Source	DF	SS	MS	F	p
Protein	1	39.9	39.9	0.60	0.41
Time Points	3	52.5	17.5	0.26	0.85
Interaction	3	99.9	33.3	0.50	0.68
Error	112	7405.1	66.1		

Analysis of Variance for LDL Oxidation (Propagation Rate)

Source	DF	SS	MS	F	p
Protein	1	83	83	0.33	0.57
Time Points	3	916	305	1.22	0.31
Interaction	3	250	83	0.33	0.80
Error	112	24991	250		

93 474MT TH 1477
10/03 30568 33 NLB

MONTANA STATE UNIVERSITY - BOZEMAN
3 1762 10388563 6