Effects of soy isoflavones on the oxidative resistance of postprandial low-density lipoprotein
by Sharon Jean Tate

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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APPROVAL

Of a thesis submitted by

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TABLE OF CONTENTS

TABLE OF CONTENTS - CONTINUED

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF FIGURES – CONTINUED

CHAPTER 1

INTRODUCTION

A considerable amount of research has been done to investigate the potential benefits of soy on cardiovascular disease (CVD) (1). The research has shown that most people would benefit from the incorporation of soy into their diets. Based on this research, the Food and Drug Administration (FDA) approved a health claim in 1999 stating, "Diets low in saturated fat and cholesterol that include 25 grams of soy protein may reduce the risk of heart disease" (2).

Setchell was the first to suggest that the phytoestrogens contained in soy could contribute to soy's beneficial effects on CVD. Soy protein containing foods are a rich source of phytoestrogens. Phytoestrogens can be divided into three categories: isoflavones, coumestans, and lignans. Isoflavones are found in particularly high concentrations in soybeans, primarily in the form of the β-glycoside conjugates, genistin and daidzin. This conjugated form of the isoflavones readily undergoes hydrolysis via β-glucosidase in the small intestine, releasing the bioactive aglycones, genistein and daidzein (3). The aglycones are the biologically active form of the isoflavones. The daidzein can be metabolized by intestinal bacteria to the isoflavone equol. However, not all subjects that consume soy can metabolize daidzein to equol. Equol appears to be a more potent antioxidant than genistein or daidzein (4).

The oxidation of low-density lipoprotein (LDL), a process that alters the metabolic properties of the LDL and allows for the development of atherosclerosis, has been a focus of intense research. Currently, interest is focused on a possible link between the inhibition of low-density lipoprotein oxidation and protection against CVD. LDL oxidation *ex vivo* has emerged as the method of choice for obtaining a relative measure of the degree of oxidative stress occurring *in vivo* (5). Antioxidants, such as the isoflavones (genistein and daidzein) present in soy, may act to inhibit LDL oxidation by converting lipid peroxyl or alkoxyl radicals to hydroperoxides or hydroxides, thus blocking oxidation (6). Since it is the oxidative modification of the LDL that facilitates its uptake by macrophages, the ability of soy isoflavones to inhibit this modification would reduce foam cells as well as the fatty streaks they form, resulting in a reduction in atherosclerotic plaques and eventually CVD (7). Studies have been able to demonstrate a relationship between soy consumption and a reduction in the susceptibility of LDL to oxidative stress (8, 9, 10).

Past research has mainly focused on quantifying LDL after an overnight fast because it is thought to be more reproducible in research studies. However, evidence suggests that postprandial LDL is more susceptible to oxidation (11). Most individuals exist predominantly in the postprandial state due to a regular consumption of meals throughout the day. Antioxidants, such as soy isoflavones, in food may serve to balance pro-oxidants (cholesterol, polyunsaturated fatty acids), explaining a key relationship between the

composition of a meal and CVD. Thus, there exists a need to investigate the effect of meal composition, specifically with or without soy isoflavones, on the oxidative resistance of postprandial LDL.

Goal

The goal of this study is to examine the effects of a meal containing 50 g soy protein (100 mg aglycone isoflavones) versus 50 g whey protein (0 mg isoflavones) on the oxidative resistance of postprandial LDL. Specifically, this study will determine if a difference exists between soy and whey protein in relation to their protection against oxidative damage postprandially.

Hypothesis

Soy protein (100 mg aglycone isoflavones) consumption significantly enhances the oxidative resistance of postprandial LDL as compared to whey protein consumption.

CHAPTER 2

REVIEW OF LITERATURE

The ability of soy protein to reduce the risk of heart disease through its hypolipidemic effects on total cholesterol, LDL cholesterol and triglyceride concentrations has been demonstrated in numerous studies and is further documented in a meta-analysis and FDA health claim (2). In addition to the hypolipidemic effects of soy protein, soy isoflavones present in the soy protein posses antioxidant properties that may serve to protect LDL cholesterol from oxidative stress. A large body of evidence supports the belief that oxidative modification of LDL contributes to atherosclerosis and that certain antioxidants can serve to protect the LDL. There exists a need to investigate the effects of soy protein with isoflavones on LDL oxidation in the postprandial state when LDL is most susceptible to oxidation. This review will progress through an explanation of the postprandial state, the theory of LDL modification, dietary influences on the postprandial state, and research investigating soy and LDL oxidation.

The Postprandial State

Over twenty years ago Zilversmit (12) hypothesized that atherosclerosis was a postprandial phenomenon due in part to an increase in triglycerides (TG), compounds consisting of three fatty acids esterified to a glycerol molecule,

following a meal. In response to a meal, the liver secretes very low-density lipoproteins (VLDL) whereas the intestine secretes chylomicrons (in the case of high fat meals). Both lipoproteins are rich in TG but also contain free cholesterol, esterified cholesterol and one molecule of apolipoprotein (apo) B. Intestinal and hepatic TG-rich lipoproteins can be distinguished by their apo B, intestinally derived lipoproteins yield apo B-48 and hepatically derived lipoproteins yield apo B-100. The chylomicrons are hydrolyzed by lipoprotein lipase (LPL) on the surface of endothelial cells and the majority of the TG are broken down into free fatty acids and monoglycerides. The resulting fatty acids (from the TG) are subsequently incorporated into the nascent VLDL which contain apo E and C in addition to the apo B-100. The remaining chylomicron remnants then pick up cholesterol ester (CE) from high-density lipoprotein (HDL), which are subsequently taken up by the liver in a process referred to as "reverse cholesterol transport". The chylomicron remnants are hydrolyzed by hepatic lipase and their CE is incorporated into nascent VLDL. The postprandial chylomicron uptake by the liver stimulates VLDL secretion and a cascade toward production of LDL, the major cholesterol carrying lipoprotein. The hepatically-derived nascent VLDL is hydrolyzed by LPL, resulting in VLDL remnants. Due to an increase in the concentration of these lipoproteins following a high fat meal, the smaller VLDL remnants are often unable to interact as efficiently as the larger VLDL remnants with the LDL receptor and are less likely to be taken up into the liver and degraded. The smaller VLDL remnants remaining in circulation

eventually lose the apo E and C along with some of the TG following hydrolysis with hepatic lipase to become LDL. The resultant LDL depleted of apo E, are slowly taken up by the liver via an interaction of a receptor-binding domain on apo B-100 (the sole protein of LDL) with the LDL receptor (13). As a result, the LDL spend more time in circulation than do the chylomicron or VLDL remnants.

The increase in chylomicron and VLDL levels postprandially (14), results in enhanced competition between chylomicrons and VLDL for common removal mechanisms (LPL, hepatic lipase). This competition contributes to the prolonged time of remnant particles spent in circulation which allows for increased exchange of TG and cholesterol for CE with the HDL and LDL, resulting in smaller, denser HDL (HDL3) and LDL (pattern B) particles (15). High plasma TG levels indicate that higher concentrations of TG are present in lipoproteins as well. Any TG present in the VLDL not delivered to muscle adipocytes or the liver, eventually becomes part of the LDL. The postprandial LDL particles are enriched with TG and cholesterol. The increase in the fatty acids, especially the polyunsaturated fatty-acids (PUFA), present in the LDL makes the LDL more susceptible to oxidative modification (16). Only oxidatively modified LDL can be taken up by macrophage LDL scavenger receptors, eventually leading to atherogenesis. Additionally, the increased cholesterol present provides more cholesterol for later incorporation into macrophages (17).

More recently, Lechleitner (18) has demonstrated that LDL isolated during the postprandial state were more susceptible to oxidation than LDL isolated from

the postabsorptive (i.e. after an overnight fast of 9-12 hours) state. The LDL were isolated from seventeen healthy donors in both the postabsorptive and postprandial states and incubated with the macrophage-like cell line P388. Postprandial LDL accumulated significantly more cholesteryl ester ($p<0.003$) and total cholesterol ($p<0.003$) than did the postabsorptive LDL (18). Oxidation studies were performed using the thiobarbituric acid-relating substances (TBARS) technique to assess lipid peroxidation via evaluation of by-products such as malondialdehyde. Samples were heated with thiobarbituric acid under acidic conditions, causing the breakdown of lipid peroxides which were then quantified spectrophotometrically (19). An increased TBARS concentration was observed in the postprandial LDL, indicating an increased susceptibility to oxidation, as compared to the postabsorptive LDL ($P<0.018$) (18).

Zilversmit (12) proposed that lipoprotein metabolism was altered following the ingestion of a meal. Lechleitner (18) expanded this theory by demonstrating that the change in lipoprotein metabolism seen following a meal was linked to atherosclerosis, specifically due to the increase in the susceptibility of the LDL to oxidation.

Theory of LDL Modification

In a recent review, Chisolm and Steinberg (20) discussed their original proposal on the oxidative modification hypothesis of atherosclerosis first made in the 1980s. Chisolm had observed, back in the early 1980s, that LDL could injure

cells and that the injury was dependent on the oxidative modification of the LDL. Steinberg, right around the same time as Chisolm, recognized that native LDL could not induce foam cell formation and demonstrated that it was the oxidatively modified form of the LDL that was recognized by the receptors on the macrophage. Since that time, research investigating the relationship between LDL oxidation and atherosclerosis has grown exponentially from a handful of studies in the 1980s to greater than 300 peer-reviewed publications in 2000 (21).

Low-density lipoprotein oxidation occurs when the unsaturated lipids present in LDL are subject to peroxidative damage (i.e. lipid peroxidation). The damage results when a free radical extracts a hydrogen atom from the carbon chain, leaving a carbon-centered radical. The carbon-centered radical is stabilized by a molecular rearrangement that yields a conjugated diene. The conjugated diene reacts with oxygen to produce a hydroperoxyl radical which propagates the reaction by extracting hydrogens just as the initiating free radical did (see Figure 1). This process continues until stopped by formation of a non-radical product or the presence of a hydrogen donor (e.g.vitamin E, glutathione) (22).

The oxidized lipoproteins facilitate the development of atherosclerosis. The oxidized LDL binds to scavenger receptors on macrophages, not to the LDL receptors (found on liver cells, adipocytes, smooth muscle cells, and fibroblasts), resulting in an increase in circulating LDL concentration (22). The increase in the concentration of circulating oxidized LDL provides more opportunity for the

uptake of the oxidized LDL by macrophages. The macrophages are able to adhere to the arterial walls and penetrate the wall between the endothelial cells. The macrophages accumulate cholesterol from the LDL and form foam cells which grow into a fatty streak as additional macrophages and T-cells are attracted to the area. The fatty streak may progress to a fibrous plaque and then into a complex lesion as the increasing volume of the plaque slowly begins to restrict blood flow (23). It is thought that only oxidized LDL can be taken up by the macrophages within the endothelial cells lining the arterial walls (as demonstrated by Steinberg); therefore, prevention of the oxidation of LDL should in turn reduce the rate of lesion formation (24).

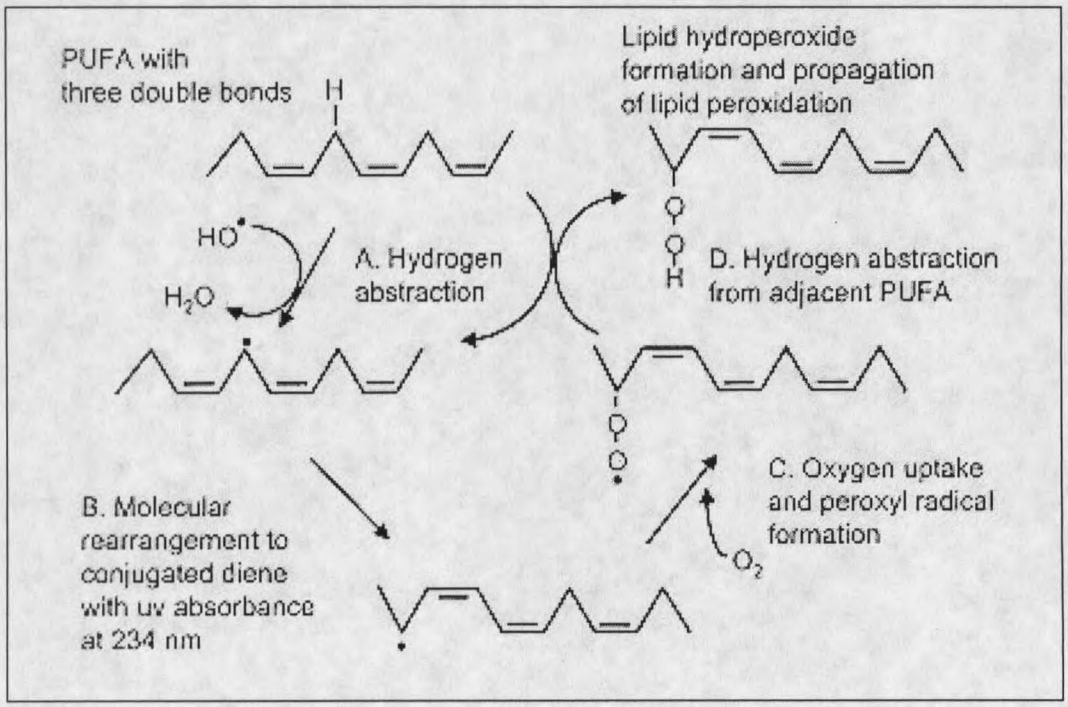

Figure 1. Lipid Peroxidation

Although research has expanded our understanding of the role of oxidized LDL, one fact remains, oxidized LDL does not define a well characterized molecular species. Native LDL is a heterogeneous and complex particle comprised of a variety of oxidation products. Oxidized forms of cholesterol, phospholipids, CE, TG and protein could all exist at varying degrees. LDL oxidation is a relative measure of the LDL particles that have acquired new functions as a result of oxidation; methods of analysis, therefore, require strict adherence to assure reliable results for comparison (20).

One of the most widely used methods of analysis has been Easterbauer's "conjugated diene" method with copper as a pro-oxidant (25). The rearrangement of double bonds in the unsaturated fatty acids that results in the formation of conjugated dienes provides an absorbance change at 234 nm. This absorbance change allows for monitoring of LDL oxidation as it passes through its three phases: lag phase, propagation phase and decomposition phase (5). The lag phase occurs during the time in which antioxidants are able to protect the LDL against oxidation. The propagation phase follows the lag phase occurring as the antioxidants are consumed, resulting in the rapid conversion to lipid hydroperoxides also referred to as conjugated dienes. Finally, the decomposition phase is the last phase characterized by breakdown of the lipid peroxides formed during the previous phase. The division of LDL oxidation into phases is used for

conceptual purposes only since the physiological process is actually a continuum and is not divided into distinct stages (26).

Dietary Influences on the Postprandial State

Although the exact mechanisms of atherosclerosis are unknown, one hypothesis suggests that pro-oxidants present in food cause lipid peroxidation of the PUFA (present in LDL) due to the high concentration of double bonds. Lipid peroxidation is a radical–initiated chain reaction that facilitates the oxidative modification of other LDL resulting in increased levels of oxidatively modified postprandial LDL (27).

In a study with ten Type 2 diabetic individuals who were fed two test meals differing in carbohydrate (CHO) content (A: high CHO, 80g; B: low CHO, 47g), Ceriello et al. (14) demonstrated that meal induced oxidative stress occurs. The susceptibility of the LDL to oxidation was significantly increased in terms of duration of lag phase ($p<0.01$), time of diene peak ($p<0.01$) and rate of diene formation ($p<0.01$) at two hours after the meal versus baseline for both meals A and B. The high CHO meal was found to produce a significantly higher degree of hyperglycemia ($p<0.05$) and shorter lag phase ($p<0.05$) than the low CHO meal, providing evidence that meal composition can affect meal-induced oxidative stress.

Nielson et al. (28) examined the postprandial effects of meals containing various fats on measures of atherosclerotic risk. Six meals of various fat

composition (low-fat, sunflower oil, rapeseed oil, olive oil, palm oil, butter) were fed on six different occasions to 18 healthy male subjects. The low-fat meal provided 7% of energy from fat, 12% from protein and 81% from CHO. The fat-rich meals contained 41% of energy from fat (enriched with one of the five test fats), 10% from protein and 49% from CHO. LDL and VLDL were isolated from those samples collected in the fasted state and five and a half hours after the first meal. No differences were observed in the resistance of the LDL to copper-mediated oxidation between the fasted and postprandial states following consumption of any of the test meals. A significant difference was observed between the fasting and postprandial VLDL; the propagation rates for VLDL were higher with the sunflower oil and rapeseed oil when compared with the other four diets ($p<0.001$). A longer lag time was also observed for the VLDL after the palm oil meal when compared with the sunflower oil meal ($P=0.0018$). Results here provide evidence that meal fat composition can affect the susceptibility of VLDL, but not LDL, to oxidation.

A pilot study by Ursini et al. (27) investigated the effects of a high fat (55% fat, 11% protein and 34% carbohydrate) meal. The plasma lipid peroxide levels of nine male volunteers aged 30-60 years were evaluated from plasma collected at baseline and two hours following ingestion of the high fat meal. At the two-hour time point, plasma peroxide concentrations increased an average of 153% in the subjects. Lipid peroxides were elevated following a high fat meal, providing evidence that diet can be a source of lipid peroxides. Assuming that

the lipid peroxides are eventually incorporated into LDL, diet can be considered a direct source of lipid peroxides in lipoproteins which, in turn, may serve to indirectly stimulate atherosclerosis via propagation of lipid peroxidation.

Based on epidemiological studies indicating a reduced risk of CVD with moderate wine intake, Natella et al. (17) investigated the capacity of red wine to counteract the oxidative stress induced by a meal. Red wine, containing antioxidant polyphenols, was compared to an isocaloric ethanol solution, without polyphenols, to discriminate the effect of ethanol from that of the polyphenols. Six healthy men were fed the same test meal (14% of energy form protein, 24% from fat, 38% from CHO, and 24% from alcohol) on two different occasions, with either red wine (3.2 g/l polyphenols) or an isocaloric ethanol solution (no polyphenols). The resistance of the fasting and postprandial LDL to oxidative modification was assessed by the formation of conjugated dienes. The findings suggested that a meal consumed with wine, did not elicit any difference in the oxidative resistance of the postprandial LDL as compared to fasted LDL. However, the postprandial LDL appeared more resistant to oxidative modification following the wine-meal than the ethanol-meal. It is important to note that no statistical information was provided for the LDL oxidation data, a distinct limitation to this study. This study suggests that antioxidants, in this case the polyphenols present in wine, may serve a protective role in the susceptibility of postprandial LDL to lipid peroxidation.

The studies reviewed here present evidence relating meal composition to the susceptibility of postprandial LDL to oxidation. Preliminary data on fats, CHO, and wine polyphenols supports the roles of both pro- and antioxidants in postprandial lipid oxidaton.

Soy and LDL Oxidation

Lifestyle and diet changes are the preferred approach to the reduction of CVD risk factors. Pharmacological and medical interventions are to occur secondarily. In 1999 the Food and Drug Administration (FDA) issued a health claim stating that soy protein intake could reduce heart disease (2). This health claim was pre-empted by, and likely a result of, a meta-analysis of 38 clinical trials conducted by Anderson et al. (1). The analysis revealed that a soy protein intake of 47 grams per day on average (with 37% of the studies using 31g per day or less) can significantly decrease serum cholesterol (a decrease of 23.2 mg/dl; 95% confidence interval, 13.5 to 32.9 mg/dl), LDL cholesterol (a decrease of 21.7 mg/dl; 95% confidence interval, 11.2 to 31.7 mg/dl) and triglyceride concentrations (a decrease of 13.3 mg/dl; 95% confidence interval, 0.3 to 25.7mg/dl). Anderson estimated that 25 to 50 grams of soy protein daily would decrease serum cholesterol concentrations by 8.9 or 17.4 mg/dl, with severely hypercholesterolemic (>250 mg/dl) individuals exhibiting even greater decreases.

A balance between antioxidants and pro-oxidants in food may be a key factor in the relationship between the postprandial state and CVD. The body is

able to defend itself against free radicals (pro-oxidants) with antioxidants that can inhibit LDL oxidation by scavenging free radicals, terminating lipid peroxidation or quenching reactive oxygen species (29).

Experiments conducted to investigate the antioxidant activity of soy isoflavones in *in vitro* models of LDL oxidation have yielded mixed results. Kanazawa et al. (30) analyzed the isolated lipoproteins from thirty-six subjects. The subjects included ten patients with cerebral thrombosis who were administered soycreme (61.9% water, 10.6% protein, 8.2% lipid, 16.8% CHO, 3.6% linoleic acid, 2.5% ash) orally for six months, 15 patients with cerebral thrombosis, and eleven healthy subjects who did not receive soycreme. The lipoproteins were incubated with copper dichloride solution and the lipid constituents in the lipoproteins were measured by thin-layer chromatography (TLC). The TLC spots reflecting lipid peroxidation were significantly higher in the cerebral thrombosis patients without soycreme than in the cerebral thrombosis patients who were administered the soycreme ($p<0.01$), although no difference was noted between the two patient groups before copper oxidation. Soycreme suppressed the appearance of the TLC spots indicating a reduction in lipid peroxidation and possibly atherosclerosis.

Kerry et al. (31) specifically investigated the antioxidant activity of the soy isoflavone, genistein, on copper mediated oxidation of LDL *in vitro*. In the first part of the study, LDL from a single volunteer was oxidized using copper in the presence of 0.2 – 5 μmol/l genistein or 1.3% ethanol (control). LDL oxidation

was inhibited by the addition of 1, 2.5 and 5 μmol/l genistein as shown by an increase in lag time ($p<0.001$). In the second part of the study designed to investigate the incorporation of genistein into LDL, plasma from the same volunteer was pre-incubated with 25 - 100 μmol/l genistein or ethanol control for 24 hours at 37°C. The LDL was isolated from the plasma and oxidized using copper sulphate. The oxidation of the control LDL and pre-incubated LDL was not significantly different. The lag times for the control LDL and LDL isolated from plasma incubated with 25, 50 and 100 μmol/l genistein were 60.5 ± 9.2 minutes, 60.9 ± 11.3 minutes, 63.0 ± 6.3 minutes and 58.6 ± 8.9 minutes, respectively. Through the use of high performance liquid chromatography (HPLC), it was also determined that only 3-4% of the genistein present in the plasma was incorporated into the LDL. The PUFA present in the phospholipids of the LDL are most susceptible to peroxidative damage. It is thought that genistein requires incorporation into the lipophilic phase of the membrane to terminate the propagation of lipid peroxidation via donation of a hydrogen atom to lipid radicals. The inability of the genistein to incorporate into the LDL has led to speculation that genistein may not be an effective physiological antioxidant however, it is difficult to come to any conclusions based on one study with a single subject.

Meng et al (32) designed *in vitro* experiments to explore possible mechanisms for the incorporation of isoflavones into LDL. Fatty acid esters of genistein and daidzein were synthesized *in vitro* to increase lipid solubility.

Daidzein and genistein are structurally similar to the endogenous human steroid estradiol, which is known to form fatty acid esters *in vivo* to enable incorporation into LDL (33). Unesterified daidzein and genistein were not found to significantly influence the oxidative resistance of the LDL, while the esterified daidzein and genistein increased the lag times by 46% and 202%, respectively. This study demonstrated that esterification allows for the incorporation of isoflavones into the LDL particle *in vitro* (32). It is important to note that although esterified endrogenous estrogens are known to occur in *vivo*, it is not clear whether isoflavones can form similar substances or if these substances could become incorporated into the LDL (33). Further studies are needed to determine if this mechanism functions *in vivo.*

Hwang et al. (4) found that *in vitro* oxidation of LDL could be inhibited by soy isoflavones. Genistein, daidzein or equol, at concentrations ranging from 0.5 to 10 μM, were added to cuvettes containing 200 μg/ml of LDL protein isolated from one adult volunteer; copper was added to the cuvettes and formation of conjugated dienes was monitored at 234 nm for up to 16 hours. The results demonstrated a prolongation of lag time that was significant ($p<0.05$) at concentrations greater than 0.5 μM for equol, 1 μM for genistein, and 2.5 μM for daidzein. Equol, a daidzein metabolite formed *in vivo* by gut microflora, was the most potent antioxidant tested. There has been speculation that interindividual variability in gut miroflora may influence the conversion of daidzein to equol, leading to varying results among subjects. The use of samples from one

individual in the current study serves as a limitation. The findings of Hwang et al. (2000), along with the other *in vitro* studies cited, provide insight into the role of soy isoflavones as antioxidants yet demonstrate a need for further investigation.

Anderson et al. (8) investigated the effects of antioxidant rich foods or supplements to determine if a diet containing isoflavone-rich soy protein could minimize the oxidization of lipoproteins. Sixty male rats were randomly assigned to groups of ten and fed one of six test diets [control, green tea, ß-carotene, low isoflavones (0.08mg genistein/g protein, 0.11mg total isoflavones/g protein), high genistein (1.45mg genistein/g of protein, 2.39 mg isoflavones/g protein), high genistein and vitamin E] for three weeks. The lag time, a measure of the resistance of LDL to oxidation, increased by 49% ($p=0.01$) in the high-genistein group and 43% ($p=0.0019$) in the low-genistein group compared to the control group. Decreases in conjugated diene (decreased by 28%; $p=0.01$), lipid peroxide (decreased by 31%; $p=0.0059$) and TBARS production (35% lower; $p=0.019$) were noted in the high-genistein group compared to the control group. The measures of oxidative stress studied support the role of the chronic consumption of genistein, a soy isoflavone, in reducing LDL oxidation, however is should be noted that this study is in rats.

Tikkanen et al. (34) investigated the hypothesis that soy isoflavones could be incorporated into LDL and thus possibly provide protection against oxidation. Six healthy volunteers (three men and three women) consumed three soy bars containing 21.3 g soy protein, 36 mg genistein, and 21 mg daidzein for two

weeks. Following two weeks of soy consumption, isolated LDL subjected to copper-mediated oxidation yielded a significant mean extension of the lag phase (15.3% longer) when compared to baseline values ($p<0.02$). Isoflavone (genistein and daidzein) concentrations were analyzed using isotope dilution-gas chromatography-mass spectrometry in the selected ion monitoring mode. Although large increases in plasma isoflavone levels occurred following two weeks of soy consumption, the incorporation of isoflavones into the LDL was determined to be less than 1% of the total plasma isoflavones. Caution is needed when interpreting the results, since only the soy isoflavones genistein and daidzein were analyzed, not equol. The results indicated that following chronic soy consumption LDL was resistant to oxidation for a longer period of time. However, none of the findings were able to elucidate the underlying mechanism by which soy isoflavones impacted the resistance of LDL to oxidation. Further investigation is needed into other soy isoflavones (i.e. equol) that may be present in the LDL as well as exploration into the possibility that LDL may not require the incorporation of isoflavones in order to elicit their antioxidant effects.

Jenkins et al. (9) conducted a randomized crossover study to investigate the effects of soy protein foods on LDL oxidation. The 31 subjects (19 men and 12 postmenopausal women) consumed a test diet (33 g/d soy protein) containing 86 milligrams of isoflavones per 2000 kcal per day for a month and a control diet with no soy protein or isoflavones for a month. Results demonstrated a reduction in oxidized LDL, assessed as conjugated dienes, following the test diet versus

the control diet (-10.8% ± 2.9%; $p<0.001$). The antioxidant effects appeared to be additive in those nine subjects taking vitamin E supplements (no statistics given), providing evidence that soy isoflavones may function via a mechanism entirely different from that of vitamin E (a hydrogen donor).

In a separate study, Jenkins et al. (10) investigated the effects of a soy-based breakfast cereal on blood lipids and oxidized LDL in 25 hyperlipidemic subjects (15 men, 10 postmenopausal women). The subjects were provided with soy (36 g/d soy protein and 168 mg/d isoflavones) and control (8 g/d wheat protein) breakfast cereals, each for three weeks in a randomized crossover study with a two-week washout period between treatments. Blood samples were collected after an overnight fast at weeks two and three of each treatment. Oxidized LDL was significantly reduced in the soy diet compared with the control diet (9.2% ± 4.3%, $p<0.05$) at week three. However, there were no significant differences seen in the serum lipids. This study indicates that soy intake is capable of reducing oxidized LDL without effecting the concentration of the LDL overall.

In a randomized crossover study, 19 premenopausal women and 5 men consumed a textured soy protein (15 g/d) diet high (56 mg/d) in isoflavones for 17 days and low (1.9 mg/d) in isoflavones for 17 days. Treatment periods were separated by a 25 day washout period. Plasma concentrations of F_2 – isoprostanes, a biomarker of *in vivo* lipid peroxidation, as well as resistance of LDL to copper-induced oxidation were evaluated. Findings revealed a longer lag

time for the copper-induced LDL oxidation (9% longer; p=0.017) as well as a lower concentration of 8-epi-prostaglandin $F_{2\alpha}$ (19.5% lower; p=0.028) when subjects consumed the high isoflavone diet. The results indicate that soy isoflavones appear to protect lipoproteins against oxidative damage. This study is unique in that it utilizes both an *in vivo* method, isoprostanes, and an *ex vivo* method, copper-induced LDL oxidation, to assess the effects of soy on oxidation (35).

The effects of supplementation with isoflavone tablets on LDL oxidation in premenopausal women was investigated by Samman et al. (36). Fourteen volunteers with regular menstrual cycles consumed 86 milligrams of isoflavones a day in tablet form for two menstrual cycles. The women then served as their own controls and consumed a placebo supplement for two menstrual cycles. Fasted blood samples were collected on two consecutive days at baseline and the end of each treatment period. There were no significant treatment effects found on the oxidisability of LDL (lag time: 32.9 $\pm$ 3.1 vs. 30.4 $\pm$ 2.9 min).

Abbey et al. (37) found similar results when 21 postmenopausal women consumed 80 mg of isoflavones daily for five to ten weeks in a placebo controlled crossover trial. No difference was found between the placebo and isoflavone treatments in relation to the oxidisability of the LDL isolated from the plasma of the 21 women. The values for placebo and isoflavone treatments were 53.5 $\pm$ 5.7 and 53.2 $\pm$ 5.2 minutes for lag time and 14.4 $\pm$ 2.3 and 14.1 $\pm$ 1.9 nmol diene/mg protein/minute for oxidation rate, respectively. The results from both

this study and the previous Samman study, provide evidence against the use of soy isoflavones alone. It would appear that both the consumption of soy protein and the isoflavones are necessary to elicit an effect.

The use of isoflavones in conjunction with soy protein appears most useful in modifying parameters of oxidative stress. Furthermore, there appears no consensus on the appropriate dose of protein or isoflavones. Based on the above human *ex vivo* studies with both soy protein and isoflavones present, a range of 21.3 – 36 g of protein accompanied by 56 – 168 mg aglycone isoflavone would be recommended to enhance the oxidative resistance of the LDL. However, it should also be noted that based on the Anderson et al. (1) meta-analysis, 47 g/d of soy protein on average can significantly decrease serum lipid levels (cholesterol, LDL, TG) in humans. The studies reviewed here represent long-term feeding studies. No data is yet available on the acute effects of soy consumption in relation to oxidative stress or any other parameter, supporting the need to investigate the effects of soy on LDL oxidation in the fed state.

CHAPTER 3

METHODS

Subjects

Ten healthy male volunteers, 19-33 years of age, were chosen from the greater Bozeman area. Subjects were recruited through newspaper and bulletin board advertisements. Males were selected based on a study by Cohn et al. (38) which indicated that males tend to have greater postprandial triglyceridemia than females. Exclusion criteria included: 1) regular use of a vitamin or mineral supplement (> 3 times per week); 2) use of any medications except for intermittent use of aspirin, acetaminophen or ibuprofen; 3) a regular consumption of soy protein ($\geq$ 2 servings of 6.25g/day); 4) known existence of chronic diseases (e.g. thyroid disease, diabetes, liver or renal disease); 5) obesity (BMI >33 kg/m^2); 6) cigarette smoking within the last 3 years (39); 7) known food allergies (soy, milk, peanuts); 8) lacto-ovo or strict vegetarian status (40); 9) regular alcohol consumption (>1 drink/d for women and >2 drinks/d for men; 1 drink = 12 oz beer, 6 oz wine or 1.5 oz distilled alcohol); and 10) the regular use of an herbal supplement with antioxidant capacity (> 3 times per week).

Screening

Individuals expressing interest in the study were asked to complete a medical history questionnaire (see Appendix A), which was then reviewed by the principal investigator for assessment of their eligibility. If the individual met the inclusion (male, 20 to 40 years of age) and exclusion criteria for the study, they were asked to sign a Human Subjects Consent Form (see Appendix B), previously approved by the Montana State University Human Subjects Committee.

Experimental Design

Subjects were asked to report to the Nutrition Research Lab (NRL) on two separate occasions for participation in a double-blind feeding study with a crossover design. Subjects were asked to report in a fasted state (no food or beverage, except water, for 10 hours) and to have refrained from any strenuous physical activity (41) or alcohol consumption (17) for at least 24 hours prior to the study date. Subjects were instructed to bring completed weighed (requires that subjects weigh all food and beverage) diet records (see Appendix C) for the three days just prior to each study date. Subjects were also required to complete activity logs (see Appendix D) for the day just prior to each study date. During the study period, the participants remained in Herrick Hall, the building in which the NRL is located; a television and videos were available for them to watch.

On each of the feeding study days, subjects were given a dose meal and two background meals (see Figure 2). The subjects were then given 20 minutes to consume the dose meal, assigned in a random order, containing 50 g of soy protein powder with aglycone isoflavones (100 mg) or control powder (50 g whey protein and 0 mg isoflavones). The participants also received a background diet, which they had 20 minutes to consume at each feeding point.

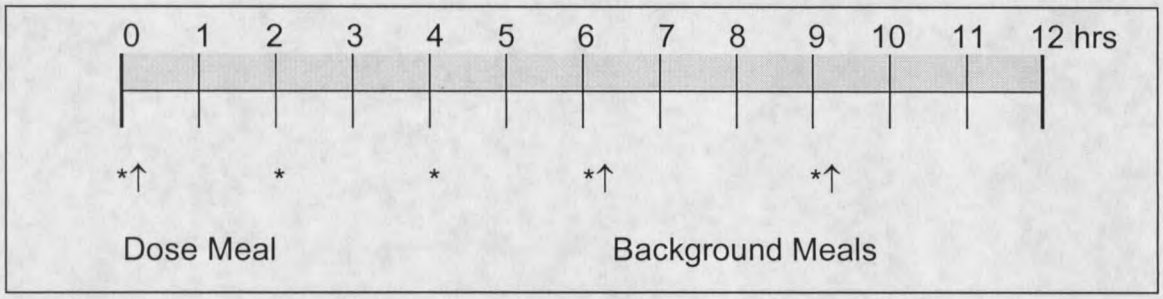

Figure 2. Timeline (* = point of analysis)

On each of the feeding study days, participants entered the NRL at 0630 hours and stayed until 1930 hours. After the measurement of body weight and height, a small forearm Insyte Autoguard shielded IV catheter (Becton Dickinson Infusion Systems Inc, Sandy, UT) with a LifeShield latex-free macrobore extension set (Abbott Laboratories, North Chicago, IL) was placed by a Registered Nurse (RN) for blood sampling purposes.

Feeding Study Diet

The dose meal contained 50 g of soy protein powder with aglycone isoflavones (100 mg) or control powder (50 g whey protein and 0 mg isoflavones). The protein powder was mixed into a shake using a banana (100g), 12-16 oz of water, and 3-4 ice cubes. Protein Technologies Inc. (St.Louis, MO) supplied the soy and control proteins (see Appendix E).

The background diet, consumed at hour six and hour nine, contained 1077 kcal for both meals. The background diet at hour six consisted of two slices of white bread, 28 g of Kraft Singles Pasteurized Process Swiss Cheese, mustard, one medium apple (gala), 15 Pringles Original Potato Chips, and 250 ml of water. The background diet provided at hour nine was comprised of two slices of white bread, 14 g Kraft Colby Jack Cheese, mustard, one medium apple (gala), 15 Tostitos Bite Size Tortilla Chips, 250 ml of unsweetened apple juice, and 22 honey-flavored Teddy Grahams (42). The nutrient composition (Nutritionist Pro) for the dose and background meals was 1429 kcal, 72.3 g protein (51.0 g protein from the dose meal and 21.3 g protein from the background meal), 205.8 g carbohydrate (35.4 g carbohydrate from the dose meal and 170.4 g carbohydrate from the background meal), 38.4 g fat (2.5 g fat from the dose meal and 35.9 g fat from the background meal), 15.0 g dietary fiber, and 31.5 mg vitamin C (see Appendix F). The diet (both dose and background meals) contained 19.8% protein, 56.5% carbohydrate and 23.7% fat. This diet did not meet the daily

nutrient requirements for most nutrients but the subjects were allowed to eat a normal dinner following the completion of the study period.

Blood Sampling

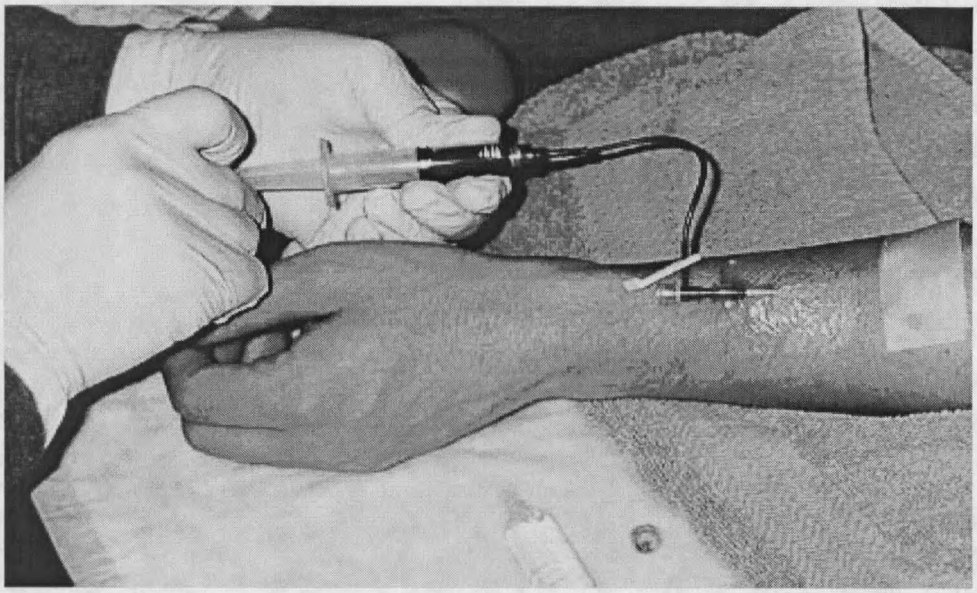

Figure 3. IV Catheter Used for Obtaining Blood Samples Hourly

A Tegaderm transparent dressing (3M, St. Paul, MN) was used to secure and protect the forearm IV catheter. At each sampling time point, the catheter was wiped with an alcohol swab and 3 ml were drawn back into a 3 ml plastic syringe (Becton Dickinson, Franklin Lakes, NJ) and wasted. The RN then drew back 6 ml of blood into a plastic 10 ml syringe (Terumo Medical Corporation, Elkton, MD), attached a 21 gauge short bevel needle (Becton Dickinson, Rutherford, NJ) to the syringe and injected the blood into the first ethylenediaminetetraacetic acid (EDTA) Vacuette vacutainer (Greiner Bio-one,

Monroe, NC) (see Figure 3). The same procedure was repeated for a second EDTA vacutainer. The catheter was then flushed with 2-3 ml of saline and the area was washed with an alcohol swab.

Two 6 ml vacutainers containing EDTA were used to collect blood samples at baseline and then hourly, following the consumption of the dose meal for a total of 156 ml of blood. The RN remained present for the duration of the study.

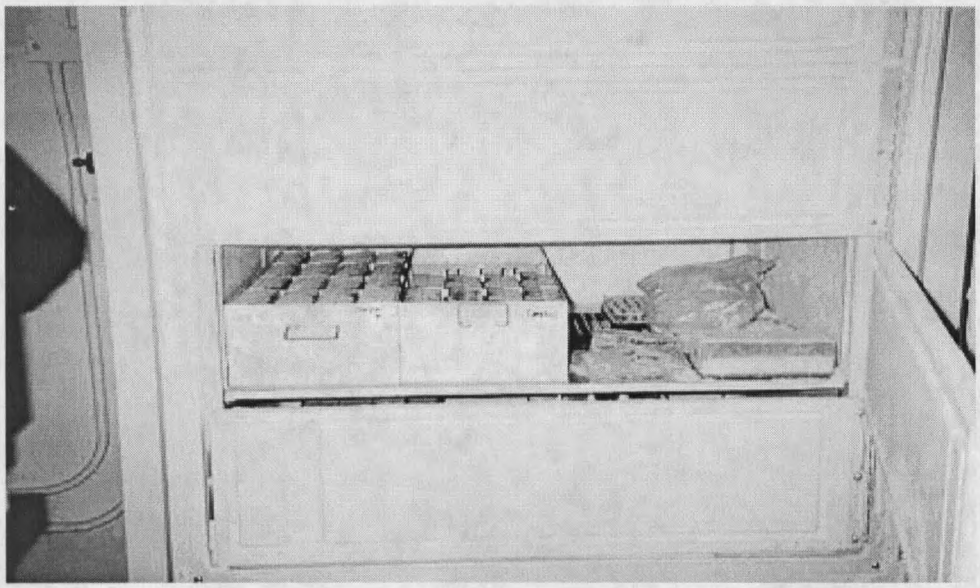

Figure 4. Storage of Samples in Revco Ultima II Freezer

Blood samples were centrifuged in a 21000 Marathon centrifuge (Fisher Scientific, Pittsburgh, PA) for 15 minutes at 3000 rpm and 16°C. Plasma supernatant was aspirated off, 700μl aliquots were then placed into labeled plastic microcentrifuge tubes. The microcentrifuge tubes were then stored in a

Revco Ultima II freezer (Legaci Refrigeration Systems, Ashville, NC) at -80°C until the time of analysis (see Figure 4). Each microcentrifuge tube was labeled with the subject's identification number, the sampling time point, the vacutainer number and date.

Diet Records

Participants were given detailed instructions (see Appendix G) on how to weigh and record all food and beverage consumed. Participants were provided with a Cuisinart SA-110A dietary scale (Cuisinart, East Windsor, NJ) to facilitate the process. The collected weighed diet records were analyzed using the Nutritionist Pro (First DataBank, San Bruno, CA) software package to assess their dietary status and compliance to study protocol. Dietary components of the study protocol included no regular consumption of soy protein ($\geq$ 2 servings of 6.25 g/day), no strict vegetarian status, no regular consumption of alcohol (>2 drinks/d) and no regular use of antioxidant supplements (>3 times per week).

Physical Activity Logs

Subjects were asked to complete an activity log designed to estimate energy expenditure for the day prior to each study date (see Appendix D). In the activity log, a day was divided into 96 periods of 15 minutes each (43). The procedure for recording the data was explained to the subjects and a list of activities along with their corresponding ratings, on a scale from one to nine, was

provided. The collected physical activity logs were analyzed using the approximate median energy cost for each category to assess compliance to study protocol (to refrain from any strenuous physical activity for at least 24 hours prior to each study date).

LDL Oxidation

The LDL was isolated by sequential density-gradient untracentrifugation in an Optima TLX Ultracentrifuge (Beckman Instruments, Palo Alto, CA) (see Appendix H). First, 0.5 ml of plasma was placed into a 1 ml polyallomer centrifuge tube (#347287, Beckman Instruments, Palo Alto, CA) and 0.5 ml of 0.9% sodium chloride solution was added. Next, up to ten centrifuge tubes were placed into the TLA-120.2 fixed angle rotor (#362046,Beckman Instruments, Palo Alto, CA) and secured in the centrifuge. The centrifuge operated for 2.5 hours at 100,000 rpm (436,000 x g; k = 12) and 16°C. The rotor was removed upon completion of the run. The centrifuge tubes were removed from the rotor and sliced at the 0.5 ml mark using a Beckman CentriTube Slicer (Beckman Instruments, Palo Alto, CA) (see Figure 5). The top fraction, containing VLDL, was discarded and the bottom layer, LDL and HDL, was transferred to a new 1ml polyallomer centrifuge tube. A volume of 0.5 ml of 16.7% sodium chloride was added to the HDL and LDL in each new centrifuge tube. The tubes were again placed in the rotor and spun for 2.5 hours at 100,000 rpm and 16°C. Upon completion of the run, the centrifuge tubes containing the samples were removed

and sliced in the same manner as before, retaining the top layer, the LDL, for analysis (44).

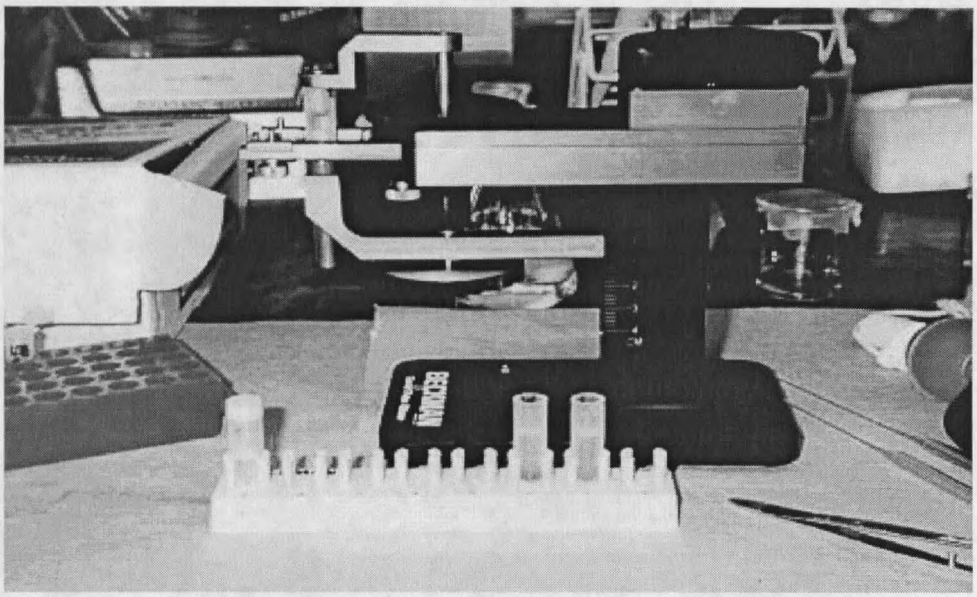

Figure 5. CentriTube Slicer

The isolated LDL was then desalted using pre-packed columns (#732-2010, Econo-Pac 10DG, Bio-Rad, Richmond, CA) filled with Bio-Gel P6 desalting gel (see Figure 6). The columns were preconditioned with 20 ml phosphate-buffered saline (PBS) immediately prior to addition of the sample. After preconditioning, 350 μl of isolated LDL was added to the column. Once the LDL was loaded onto the column, 2650 μl of PBS was added to the column; the first 3 ml (approximate void volume of the column) of effluent were collected and discarded. A 525 μl volume of PBS was then added and used to elute the

desalted LDL. The desalted LDL was collected and analyzed for protein concentration (45, 46).

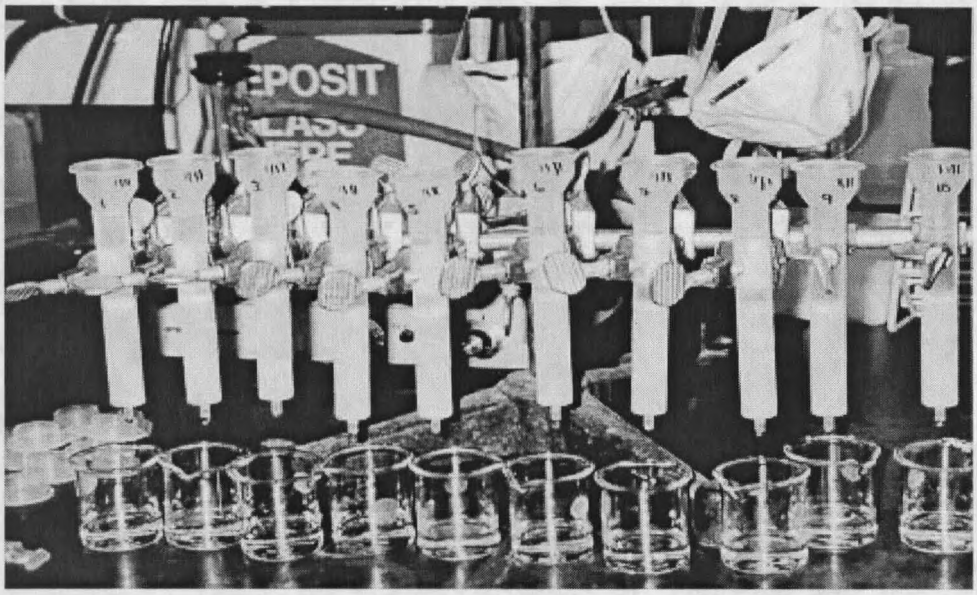

Figure 6. Econo-Pac 10DG Desalting Columns

The protein concentration of the LDL eluate was determined using a bicinchoninic acid (BCA) protein assay kit (#23225,Pierce, Rockford, IL). The BCA kit allowed for the colormetric detection and quantitation of total protein present in the samples (47, 48). A bovine serum albumin (BSA) standard of known concentration was diluted to encompass a working range of 25 to 2000 μg/ml. Twenty-five microliters of each standard of known concentration and each sample (isolated, desalted LDL) of unknown concentration were added to individual wells on a 96-well microplate (269620, Nalge Nunc International, Rochester, NY); a 200 μl volume of working reagent was then added to each well

(see Figure 7). All standards and samples were analyzed in duplicate. The microplate was then placed on a 6115 Rotator-Incubator (Eberbach, Ann Arbor, MI), shaken for 30 seconds, covered and incubated at 37°C for 30 minutes. Following incubation, the microplate was placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT) and absorbance was measured at 562 nanometers (nm). The BCA is a chelating agent, which combines with the cuprous cation (Cu^{1+}) product of the reduction of the cupric cation (Cu^{+2}) to Cu^{+1} caused by protein in alkaline medium. The colored product of the reaction absorbs strongly at 562 nm. The absorbance is proportional to the number of cations bound to the protein and, therefore, to the amount of protein present. A standard curve, corrected for a PBS blank, was then plotted. The sample protein concentrations, based on sample absorbance corrected for PBS blank absorbance, were determined from the standard curve using the KCjunior (Bio-Tek Instruments, Winooski, VT) software package. Using the results of the protein assay, the isolated and desalted LDL samples were then diluted with PBS to achieve a final concentration of 0.10 mg protein/ml for each sample (49).

The susceptibility of the LDL to oxidation was determined by measuring the formation of conjugated dienes following copper induced oxidation of the LDL. Low-density lipoprotein *in vivo* oxidation is mimicked *ex vivo* by adding a pro-oxidant, in this case copper, to the isolated LDL (desalted and adjusted for protein concentration). The double bonds in the polyunsaturated fatty acids of the

isolated LDL are converted to fatty acid hydroperoxides with conjugated double bonds also referred to as conjugated dienes. The conjugated dienes absorb light at 234 nm. Monitoring the increase in absorbance at this wavelengh reflects the formation of conjugated dienes and therefore the susceptibility of the LDL to oxidation (25, 45, 50, 51). A 100 μl volume of samples (isolated, desalted and adjusted for protein concentration) and blank (PBS) were incubated in separate wells of a 96-well microplate (#3635, Costar, Corning Incorporated, Corning, NY) with 10 μl of 500 mM cupric chloride solution. The plate was placed in the μQuant Universal Microplate Spectrophotometer and the absorbance read every ten minutes for eight hours at 234 nm and 37°C. Samples were analyzed in duplicate.

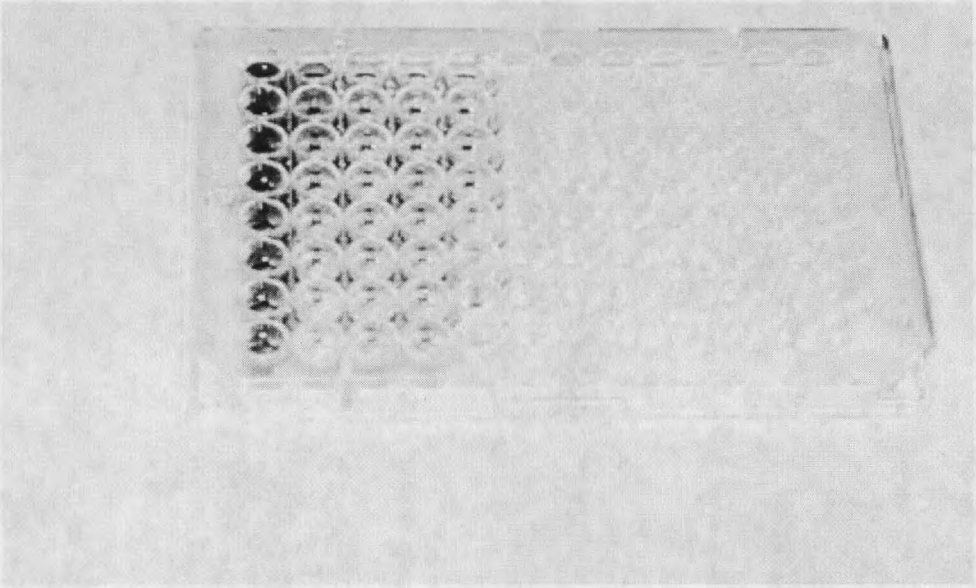

Figure 7. A 96-Well Microplate

To facilitate data interpretation, Excel (Microsoft Corporation, Redmond, WA) was used to construct separate graphs (see Figure 8) for each time point analyzed in which the absorbance readings (taken every ten minutes) were plotted against time for each individual well. The lag time for each sample (i.e. individual well) was calculated using a previously written program in Excel. The point of intersection of a best-fit line drawn tangent to the lag phase and a best-fit line drawn tangent to the propagation phase was defined as the lag time or duration of the lag phase. The mean of the lag times for duplicate sample wells was used for statistical analysis.

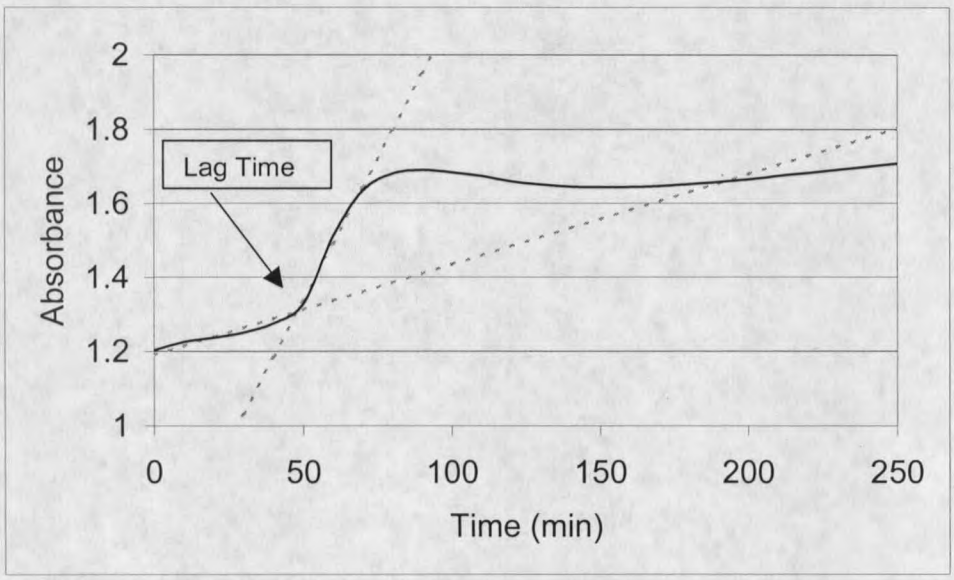

Figure 8. Determination of Lag Time for LDL Oxidation

Statistical Analysis

Based on studies conducted investigating the effects of soy on LDL oxidation (11, 35, 37, 38), effect sizes of 0.81 – 2.37 were calculated. With an expected effect size of at least 0.95 – 1.00 ($\alpha = 0.05$; $\beta = 0.80$), 10 subjects were determined necessary to detect a significant difference in LDL oxidation parameters.

Analysis was performed only on those samples colleted at baseline, two, four, six and nine hours. Analysis was limited to these five time points to allow all samples of an individual subject to be analyzed simultaneously as the centrifuge rotor is only able to accommodate ten samples at one time. Simultaneous analysis minimizes the effect of day-to-day method variation on the results. The use of five time points per subject for each protein allowed for the analysis of one subject's complete data in a day (two protein types with five time points each, for a total of ten samples). The data from these time points supply adequate information to determine if there is an acute effect on the oxidative resistance of LDL and, therefore, a need for further investigation in this area.

Statistix 7 (Analytical Software, Tallahassee, FL) and Statistica (Statsoft, Tulsa, OK) software packages were selected to analyze the data collected. A two-way analysis of variance (ANOVA), protein * time, with repeated measures was used to assess treatment differences (soy vs. whey protein) in LDL oxidation parameters at hours 0, 2, 4, 6, and 9. The α-level was set at 0.05.

An ANOVA model was selected because it allows for studying the relationship between a dependent variable and one or more independent variables. The ANOVA model also does not require any assumptions about the nature of the statistical relation between the dependent and independent variables, as is the case with regression analysis. A repeated measures design was selected because this study utilizes the same subjects for each of the treatments (soy, whey) under study. A principal advantage to the repeated measures design is that it provides good precision for comparing treatments because sources of variability between subjects are excluded from the experimental error. Only variation within subjects enters the experimental error, as the subjects serve as their own control (52).

A Tukey comparison of means was selected to detect the existence of any trends in the data. A Tukey's comparison of means test is conceptually similar to a t-test in that it identifies which pairs of means differ from each other. However, unlike t-tests, a Tukey's test protects against increasing Type I error (i.e. alpha is no longer 0.05). In this instance, differences in LDL oxidation between the various time points were of particular interest. If the differences between the extreme-most means were not significant, testing for differences between other pairs of means would be unnecessary.

A t-test is basically just a special case of a simple ANOVA utilized to assess differences between two sample means. In this case, a group of subjects was tested twice on the same independent variable (protein). A dependent t-test

was therefore appropriate to determine if a change occurred in the mean weight, BMI, energy output, or energy input between the two levels (soy, whey) of that same independent variable (protein) (53).

Box plots were used to display the distributional characteristics of the data. To interpret a box plot it is important to note that the box contains 50% of the sample values, the whiskers extend above and below the box to include the highest and lowest values in the data set (excluding outliers), and that the outliers are points which fall outside of the box by more than 1.5 times the middle 50% of the sample values (54).

All data was normalized by subtracting the lag time for the zero hour time point of that study day from the lag time for each of the other time points (2, 4, 6, and 9 hours). A positive number reflects a lag time greater than at baseline and a negative number reflects a shorter lag time.

CHAPTER 4

RESULTS

The individual characteristics for each subject are summarized in Table 1, while the descriptive statistics for subjects are included in Table 2.

Table 1. Subject Characteristics

Subject*	Protein Type	Age* (yrs)	Height* (in)	Weigh (lbs)	BMI ($kg/m^{2)}$	Energy (kcal/kg)	Energy (kcal)	Intake (kcal)
1	363	33	72.38	170.0	22.9	44.9	3469	2900
	658			167.9	22.6	61.8	4718	3497
2	363	32	68.50	209.0	31.4	42.5	4039	1433
	658			209.5	31.5	45.5	4335	1456
3	658	23	73.50	210.0	27.4	38.9	3715	1612
	363			214.0	27.9	33.4	3246	3175
4	363	20	70.13	226.8	32.5	44.6	4601	1475
	658			226.0	32.4	46.9	4815	2727
5	363	27	65.50	163.5	26.8	38.7	2872	1823
	658			163.0	26.8	38.7	2865	1823
6	658	19	64.75	144.8	24.3	34.0	2235	1979
	363			147.5	24.8	63.7	4269	1787
7	363	20	73.75	201.6	26.1	33.8	3092	1782
	658			205.5	26.6	39.9	3727	2475
8	363	20	70.25	163.0	23.3	37.4	2771	2819
	658			164.0	23.4	49.5	3686	2898
9	363	29	70.50	135.0	19.1	39.5	2426	2357
	658			132.0	18.7	45.0	2698	2244
10	658	20	70.88	179.0	25.1	66.8	5441	3089
	363			179.5	25.2	52.9	4618	2322

* = information does not change between study days
Protein Type: 363 = Soy, 658 = Whey

Table 2. Descriptive Statistics

Variable	Mean	Minimum	Maximum	Std. Dev.
Age	24	19.00	33.00	5.31
Height	70.01	64.75	73.75	2.96
Weight[1]	181.0	135.00	226.75	30.52
Weight[2]	180.2	132.00	226.00	31.23
BMI [1]	26.0	19.10	32.50	3.98
BMI [2]	25.9	18.70	32.40	4.07
Energy Expenditure (kcal/kg)[1]	43.1	33.36	63.72	9.28
Energy Expenditure (kcal/kg)[2]	46.7	33.97	66.84	10.44
Total Energy Expenditure(kcal)[1]	3511	2425.90	4601.40	750.81
Total Energy Expenditure (kcal)[2]	3852	2235.20	5440.80	1046.94
Intake (kcal)[1]	2187	1433	3175	620.31
Intake (kcal)[2]	2380	1456	3497	673.40

[1] = whey protein , [2] = soy protein

There were no significant differences found in weight, BMI, energy expenditure or energy intake for the subjects between study treatment days (see Table 3).

Table 3. Comparison of Soy vs. Whey Protein Study Days

Variable	t-value	df	p
Weight	0.059749	18	0.95
BMI	0.066667	18	0.95
Energy output (kcal/kg)	-0.806541	18	0.43
Total Energy (kcal)	-0.780280	18	0.45
Energy input (kcal)	-0.665569	18	0.51

Results of the 2-way ANOVA with repeated measures on both factors (protein type, time points) are presented in Table 4. No significant differences between the main effects (protein type, time points) or the interaction between the factors (protein * time) on LDL oxidation parameters (lag time, initial absorbance) were observed (see Appendix I).

Table 4. Analysis of Variance for LDL Oxidation (Lag Time)

Source	DF	SS	MS	F	p
Subject (A)	9	345.328	38.3698	2.78	0.0716
Protein (B)	1	3.53440	3.53440	.26	0.6247
A*B	9	124.044	13.7826		
Time (C)	4	90.8650	22.7163	1.77	0.1565
A*C	36	462.267	12.8407		
B*C	4	49.5966	12.3992	1.70	0.1721
A*B*C	36	263.015	7.30598		
Total	99	1338.65			

Results of the ANOVA confirm that no significant differences exist between the factors mean effects on LDL oxidation. To determine whether individual pairs of groups differ from each other a Tukey comparison of means was performed for comparison of means of LDL oxidation by time and by protein * time. The tests showed no significant pairwise differences among the means for LDL oxidation by time. In the comparison of means of LDL oxidation by protein * time, only two groups were found in which the means were significantly different from one another at the 0.05 level, soy protein at time point five and whey protein at time point three.

In this case, box plots (see Figures 9 and 10) are useful for comparison of distributions and means for both protein types at all five time points. A general difference between the protein types is the greater variability observed in lag time of the whey protein as compared to the soy protein. Differences between the proteins at certain time points could also be noted as baseline lag times were normalized by subtracting the lag time for the zero hour time point of that study day from the lag time for each of the other time points. A positive number reflects a lag time greater than at baseline and a negative number reflects a shorter lag time. At the two-hour time point soy protein appeared to be better than whey protein at lengthening the lag time, however both protein types produced mean lag times greater than the zero-hour lag time. At the four-hour time point, soy protein was again better at lengthening the lag time and at this time point soy protein was the only protein with a mean lag time greater than the zero-hour lag time. At the six-hour time point, both protein types have mean lag times longer than that at zero-time, however the lag time for whey protein was longer than that for soy protein although the variability of the whey lag time data was greater than that for the soy data. At the nine-hour time point, the whey protein lag time data again had greater variability and a mean lag time greater than that of the soy protein, the difference at this time point being that the mean lag time for the soy protein was less than at zero-time.

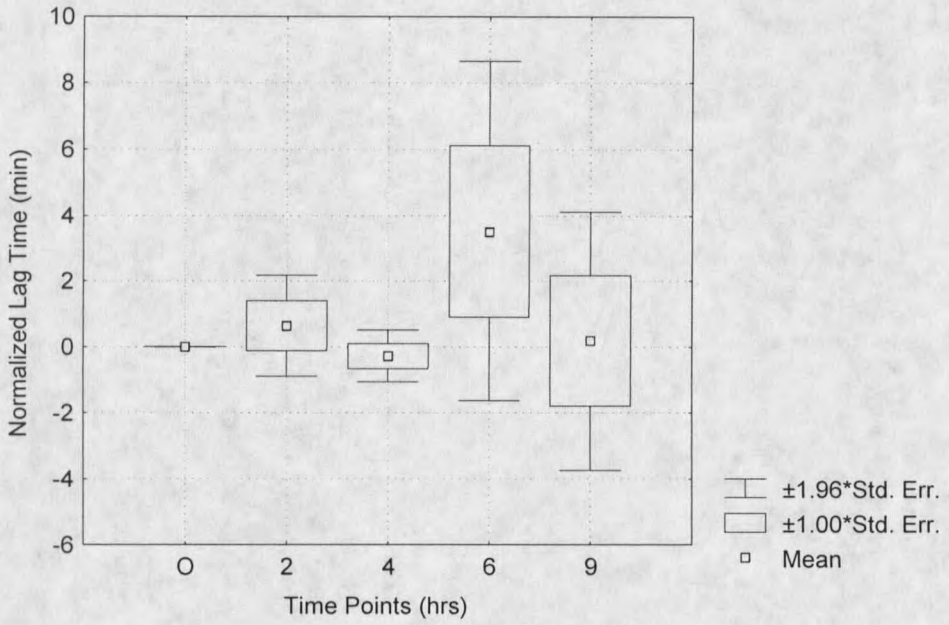

Figure 9. Whey Protein Box Plot

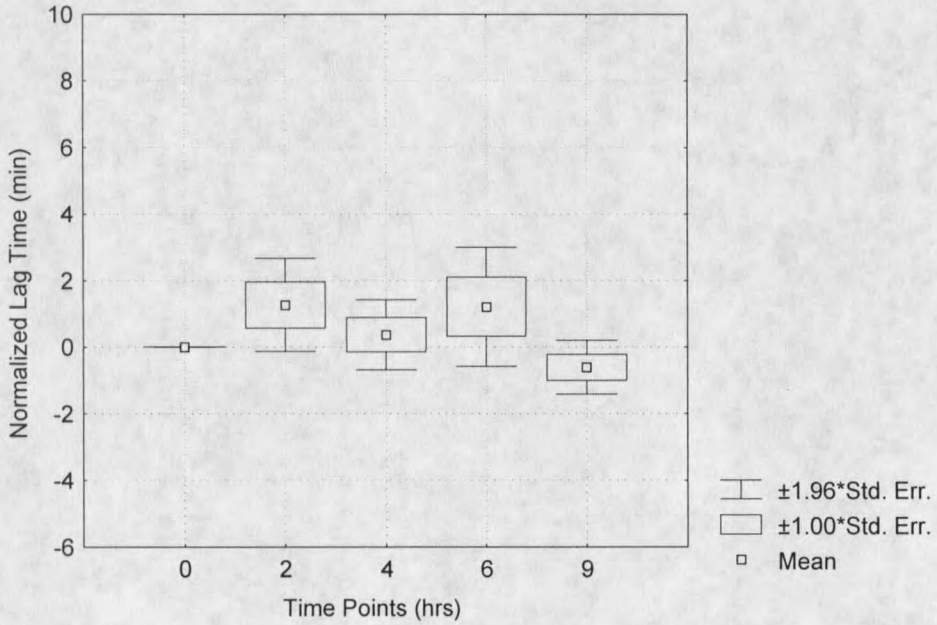

Figure 10. Soy Protein Box Plot

The mean data for a group of subjects can at times disguise important individual differences. Although the statistical analysis found insignificant ($p < 0.05$) differences between treatments, valuable information may still be provided. Examining the individual patterns of each of the subjects, may provide this useful information.

The following graphs (see Figures 11-21) serve as visual aids for comparison of mean lag times for each protein type for the group of subjects (see Figure 11) as well as each subject individually (see Figures 12-21).

The pattern of postprandial LDL oxidation varied considerably between subjects, such that four patterns were distinguishable. The first and most obvious pattern (A) occurs when both protein types do not affect a noticeable change in the lag time (selected as +/- 3 minutes based on visual inspection) from baseline. Pattern A was observed in 60% of the subjects (see Figure 13 - 15, 17, 19 and 21). A second pattern (B), defined by an increase in lag time for both protein types at one or more time point, emerged in 20% of the subjects (see Figures 12 and 18). Pattern C, indicated by an increase in lag time at one or more of the time points following the consumption of whey protein, was observed in 10% of the subjects (see Figure 16). Pattern D, a similar pattern to C apart from the fact that it occurs in subjects following the consumption of soy protein, was also observed in 10% of the subjects (see Figure 20).

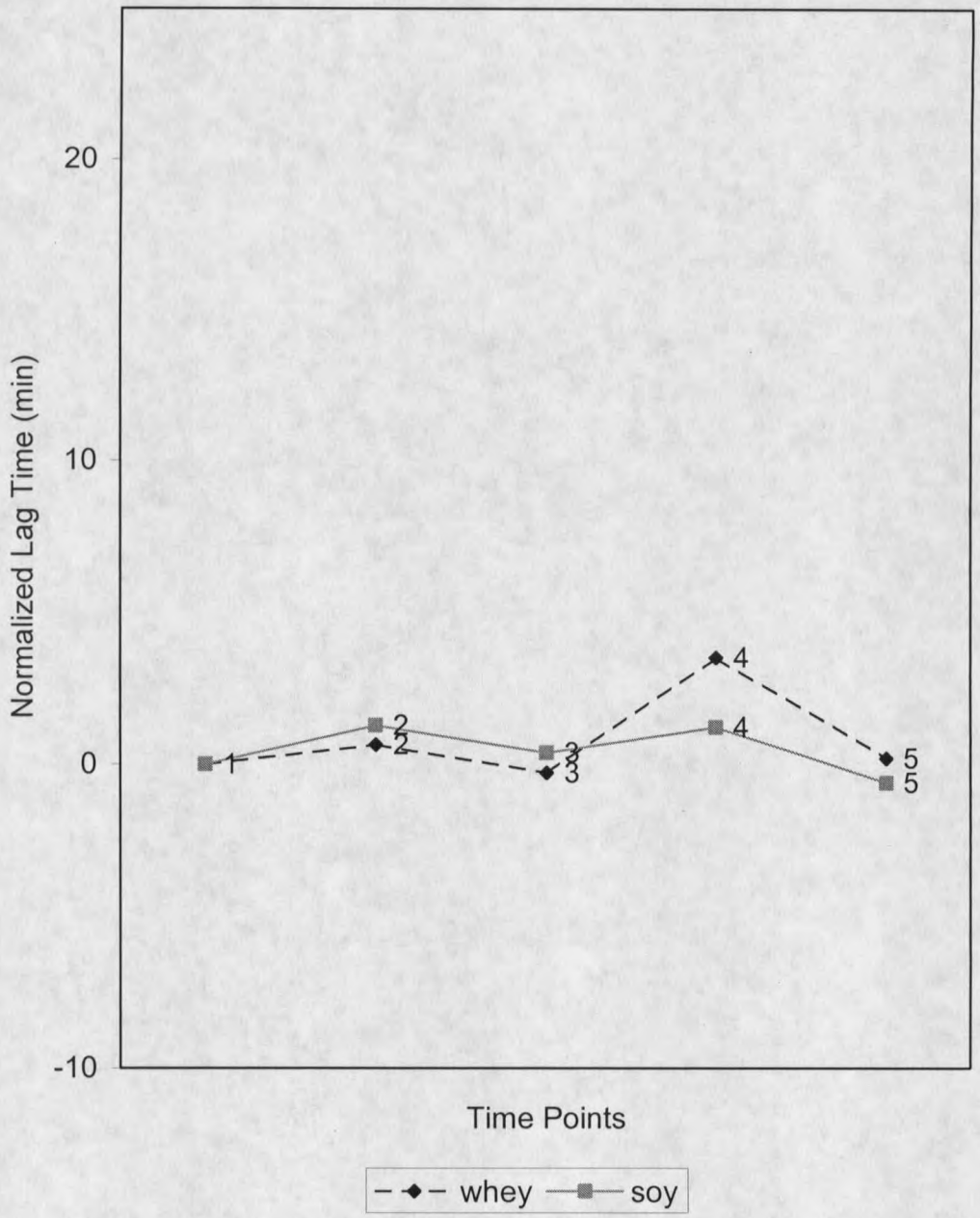

Figure 11. Average Lag Times (normalized) for All Subjects

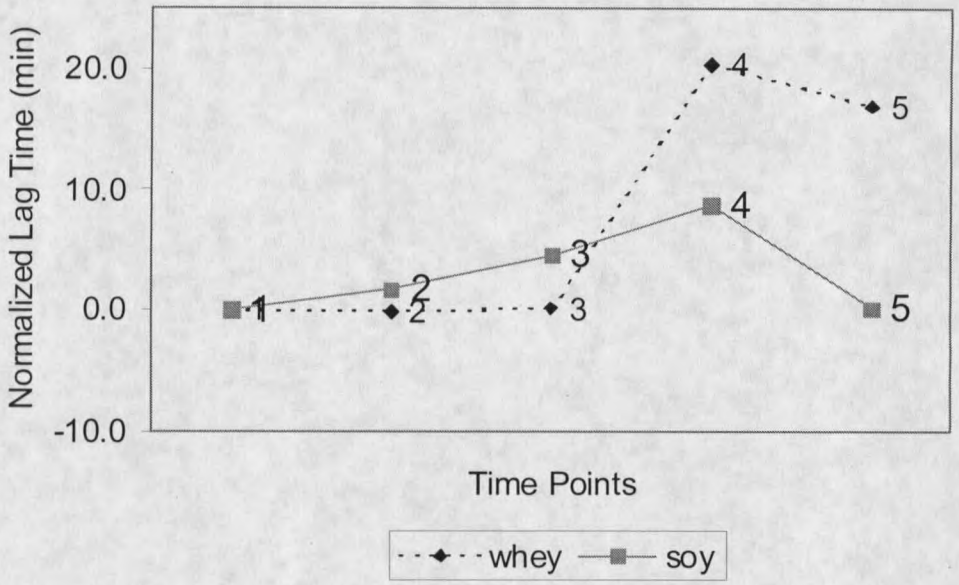

Figure 12. Individual Lag Times (normalized) for Subject 1

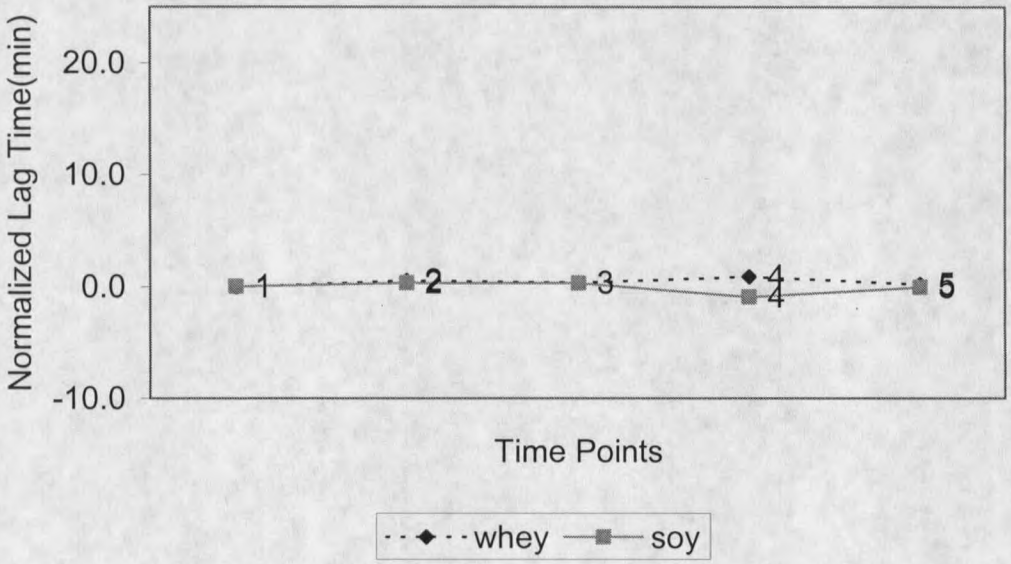

Figure 13. Individual Lag Times (normalized) for Subject 2

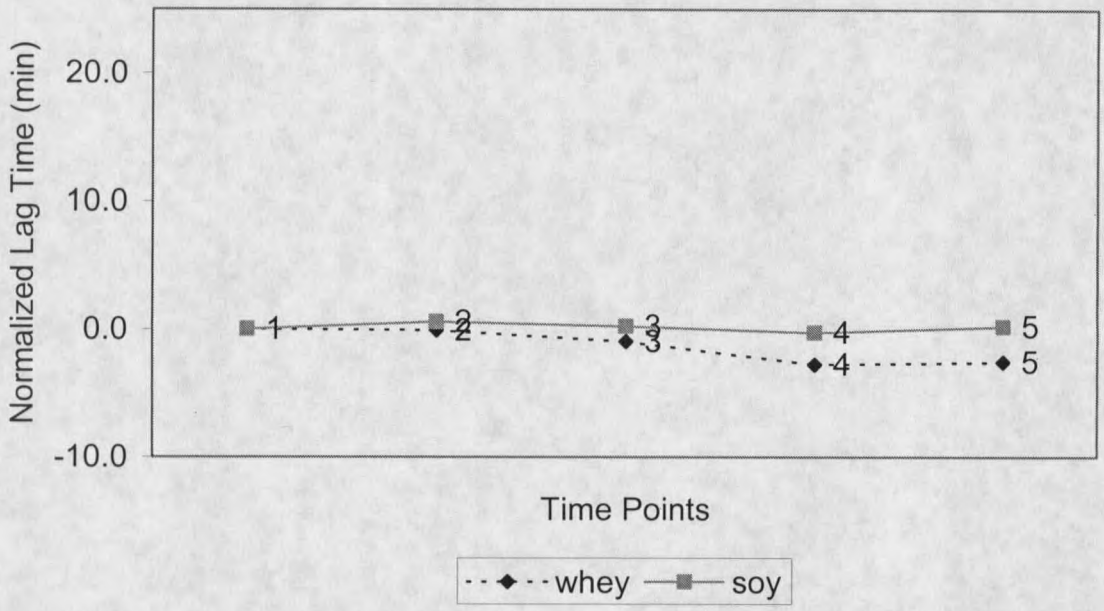

Figure 14. Individual Lag Times (normalized) for Subject 3

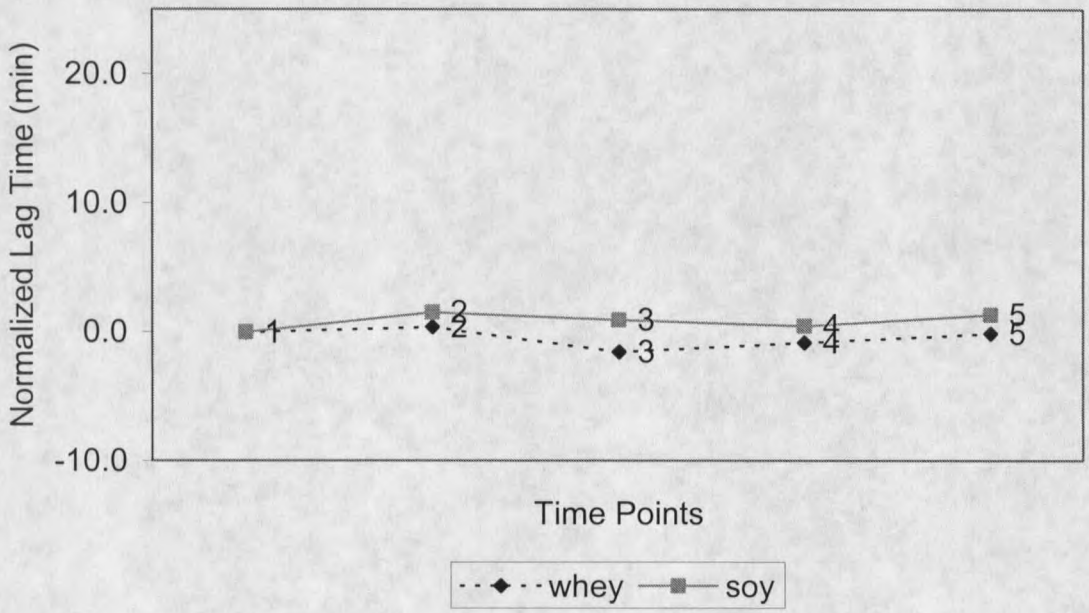

Figure 15. Individual Lag Times (normalized) for Subject 4

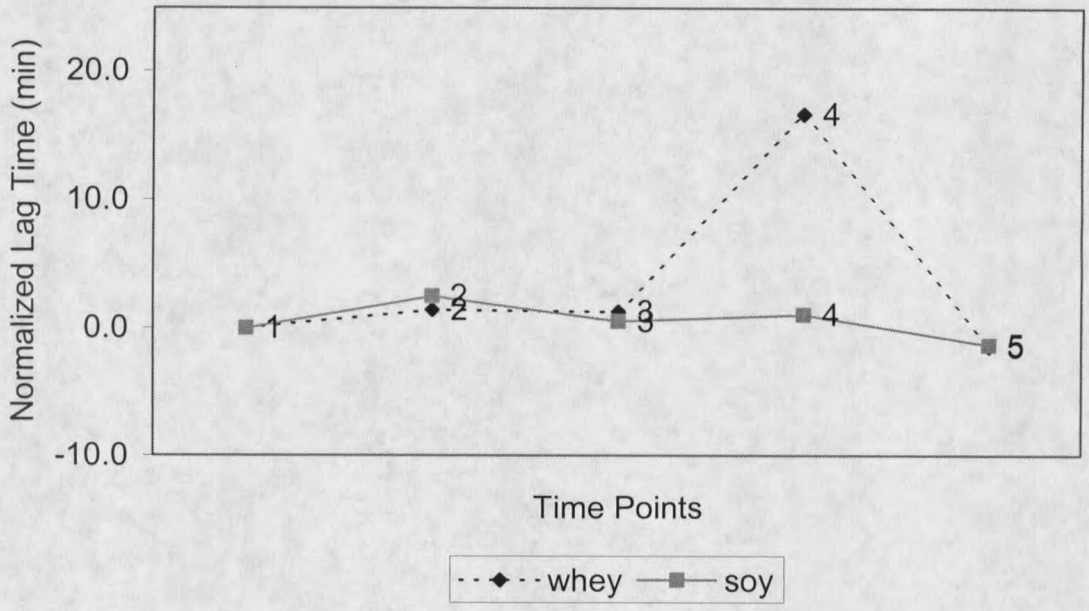

Figure 16. Individual Lag Times (normalized) for Subject 5

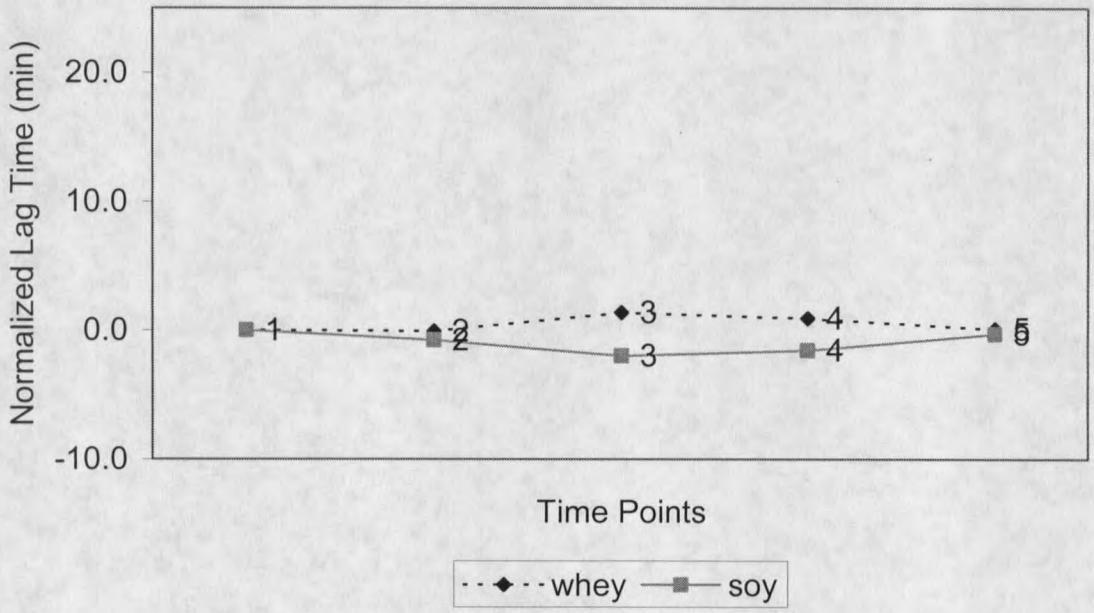

Figure 17. Individual Lag Times (normalized) for Subject 6

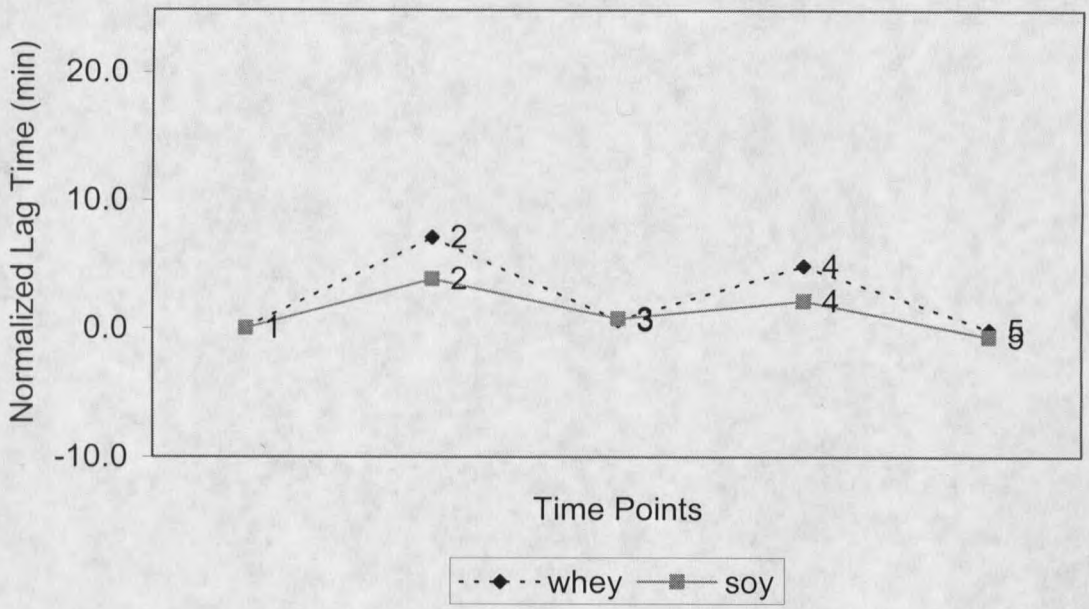

Figure 18. Individual Lag Times (normalized) for Subject 7

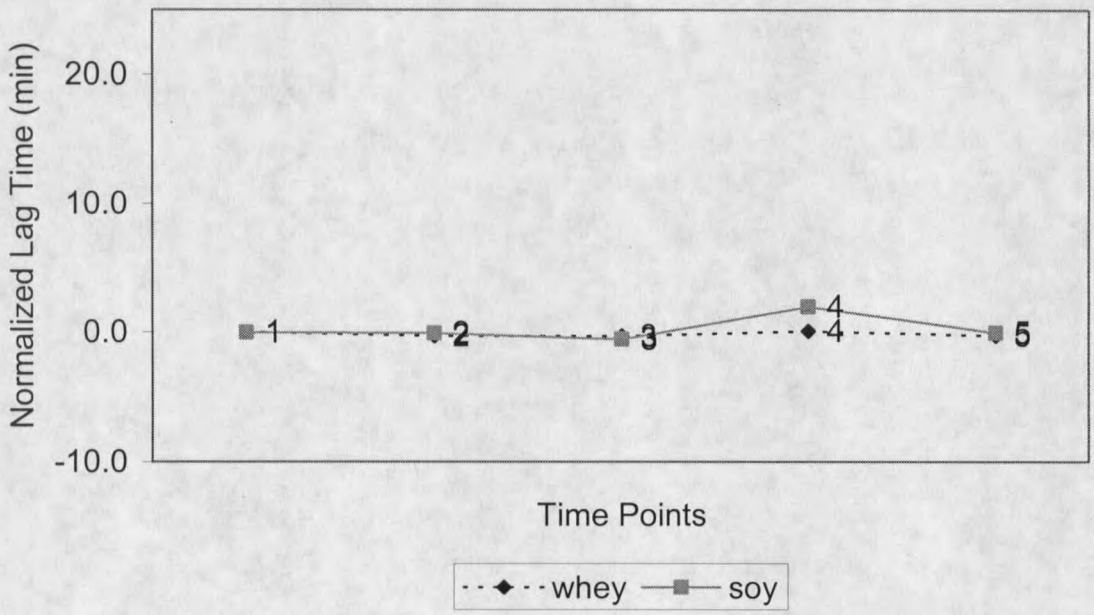

Figure 19. Individual Lag Times (normalized) for Subject 8

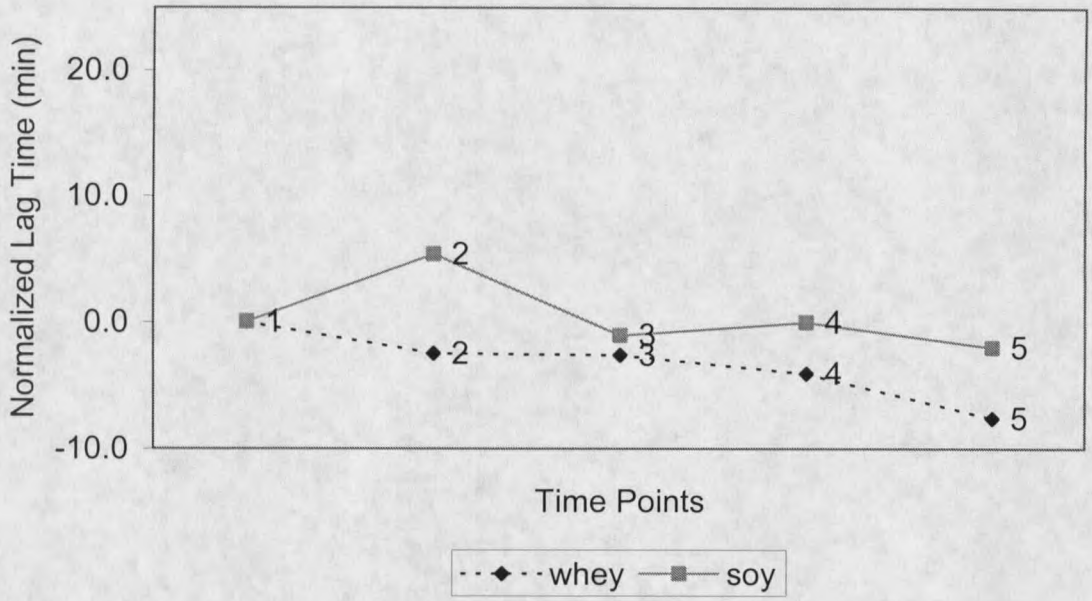

Figure 20. Individual Lag Times (normalized) for Subject 9

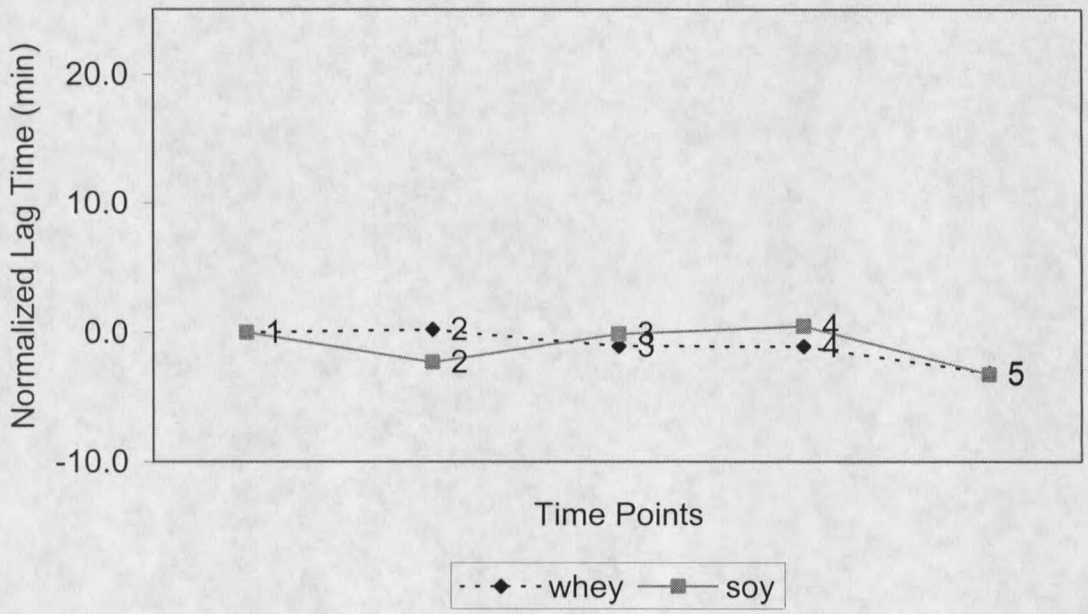

Figure 21. Individual Lag Times (normalized) for Subject 10

CHAPTER 5

DISCUSSION

The aim of this study was to compare the acute effects of two dietary treatments, soy protein containing 100 mg isoflavones and whey protein without isoflavones, on the oxidative resistance of the postprandial LDL of ten healthy men. We examined the effects of these two different dietary treatments on lag time, a marker of the resistance of LDL to oxidation. No significant difference was found between the soy and whey proteins in regards to their protection against the oxidative damage incurred following a meal.

The reason for the failure of soy protein to decrease oxidative damage, by comparison with control, in this study is not clear but could relate to a number of factors. These factors likely include inadequate number of subjects, inherent variability between subjects, lack of an atherogenic challenge meal, and a need for more than one method of analysis.

Power is the probability of rejecting the null hypothesis when the null hypothesis is false. Power ($\alpha = 0.05;\beta = 0.80$) calculations based on each individual subject's best response to the soy protein indicate that 15 – 25 subjects would be needed to detect a significant difference in LDL oxidation. The use of ten subjects in the current study is slightly less than this calculation possibly contributing to insignificant results, however it is recommended that future studies use no less than 15 subjects.

In the present study, variability between individuals was observed in both the magnitude and pattern of the postprandial response as was evident by the existence of four distinct patterns (A – D). The extent of the response to treatments was greater in certain subjects and virtually nonexistent in others.

Considerations for individual differences include the rate of output of TG from the intestine, the rate of secretion of secretion of TG from the liver, the activity of LPL and hepatic lipase, and the rate of uptake of TG containing lipoproteins by receptors. Cohn et al. (38) monitored lipoprotein changes in 22 subjects (9 males, 13 females) for 12 hours following a high fat meal (1 g fat/kg body weight). Water, but no food, was allowed during the study period. Overall, TG peaked four hours after the meal and remained elevated for nine hours. Variability between subjects was noted. Upon closer inspection of individual data, it was discovered that multiple postprandial TG peaks often existed but were disguised when the mean data was presented. Of the subjects tested, five had one TG peak, 11 had two TG peaks, and six had three TG peaks. The results show that after a high fat meal the plasma TG concentration may have multiple peaks providing insight into postprandial TG metabolism. It was beyond the scope of the study to determine which of the factors, mentioned above, may be responsible for the one, two or three peak patterns observed. This study does provide evidence that variations in the postprandial lipoprotein metabolism of subjects exists. The classification of subjects as mono or multiple postprandial TG peak producers may be a potentially important tool. Interpretation of LDL

oxidation data for plasma obtained following a meal could be affected by the number of TG peaks. Therefore, screening subjects to determine the number of TG peaks produced may simplify data interpretation.

The bioavailability of the isoflavones must be considered as well. Mitchell et al. (55) noted that inter-individual variations existed in the plasma concentrations of genistein and daidzein and proposed that this was possibly related to gut bacteria. Setchell et al. (56) investigated the bioavailability of soy isoflavones in 19 healthy premenopausal women following consumption of a standardized 50 mg dose of isoflavones. The subjects were assigned to one of four goups. Each group received a specific isoflavone: daidzein (n=6), genistein (n=6), daidzin (n=4) or genistin (n=3). Blood samples were obtained at baseline, 2, 4, 6, 8, 12 and 24 hours following the isoflavone dose. The concentrations of the isoflavones in the plasma were measured by gas chromatography-mass spectrometry. Peak plasma concentrations of the isoflavones occurred 4 - 7 hours after ingestion of the aglycones (daidzein and genistein) and 8 – 11 hours after ingestion for the β-glycosides (daidzin and genistin). The β-glycosides absorption was slowed by the hydrolysis of the glycosidic moiety. In the current study, both glycosides and aglycones were provided with the majority of isoflavones provided as aglycones.

It should not be assumed that all subjects metabolize daidzein to equol. Studies have reported that only 33% of subjects were able to metabolize daidzein to equol (32). In the same study described above, Setchell et al. (56)

measured plasma equol in the subjects fed daidzein (aglycone) or daidzin (glycoside). Equol was found in the plasma of only three of the nine subjects, none of which had been in the daidzein group. Those individuals with the physiological ability to convert daidzin to daidzein (if not provided as daidzein in the diet), then daidzein to equol are referred to as "converters". The equol appeared in the plasma of the "converters" 6 – 8 hours following the consumption of the isoflavone dose.

In the current study, isoflavones were provided primarily in the aglycone form along with soy protein isolate. Studies of the acute effects of soy consumption on LDL oxidation have not been done previously. Therefore, it is worth consideration that adequate time may not have been provided for the isoflavones to peak in the blood, fulfill their antiatherogenic roles and affect the lag time of copper-induced LDL oxidation. Also, if the data to date is correct, it is highly likely that only 33% of the subjects were "converters" and therefore plausible that the subjects responsive to the soy were the equol "converters". Ideally subject screening should include testing for "converters" as equol is the most potent antioxidant of the isoflavones.

Interindividual differences in pre-existing plasma lipid peroxide and antioxidant levels may have influenced LDL oxidisability and contribute to the variations seen. Specially designed diets may be required for the days prior to the study to ensure that dietary antioxidants and pro-oxidants are the same for all subjects. Cohen et al. (57) used a pretest meal (2 slices of white bread, 40 g

peanut butter, 20 g jam, 250 ml orange juice and 500 ml whole milk) the evening before a study day to eliminate such variables between individuals.

The feeding study diet, particularly the dose meal, for the current study may also require some adjustment as some dietary "challenge" may be required to induce postprandial lipemia. The lack of heightened postprandial lipemia may not allow for an environment necessary to assess the effects of soy on the oxidative resistance of postprandial LDL, similar to the situation in which lipid lowering effects are more evident in hyperlipidemic individuals. It is also important to remember that Zilversmit (12) hypothesized that atherosclerosis was a postprandial phenomenon due in part to an increase in TG following a meal.

Cohen et al. (57) examined the dose response of serum TG concentrations to the fat content of a meal. Twelve male subjects consumed fat meal consisting of dairy cream in amounts containing 40, 80, and 120 g of fat. The subjects were provided with a standard pretest meal the evening before a test day, fasted for ten hours, and returned for one of the fat meals. Blood was sampled at baseline and again at two-hour intervals for eight hours following consumption of the fat meal. The test procedure was repeated at seven-day intervals for each of the remaining fat meals. Mean fasting serum TG was similar on each test day ($p>0.25$), however serum postprandial TG concentrations increased in proportion to the dose given (no statistical information provided). The results of this study indicate that the changes in serum TG concentration following a fat meal vary linearly with the fat content of the meal. If it is in fact

the dietary TG that led to the postprandial response, this study provides evidence that the postprandial TG response, at least in part, would be linked to the fat content of the meal.

Diet derived lipid peroxides may act as initiators for LDL oxidative modification. A meal containing fat, also likely to contain lipid peroxides, may serve as a source of oxidized lipids. The dietary lipid peroxides are secreted into chylomicrons and intestinal VLDL and presumably transferred to the LDL by the action of plasma lipid transfer proteins (17). Findings by Ursini et al. (27) indicated that lipid peroxide levels are elevated in the plasma following a high fat meal, providing evidence that dietary fat can be a source of lipid peroxides. Nielson et al. (28) provided further evidence related to the effects of dietary oxidized lipids. They observed a greater increase in the susceptibility of the VLDL after a PUFA meal, containing fatty acids with double bonds more prone to oxidation, than after a saturated fat meal that cannot become oxidized due to its lack of double bonds. These studies provide information into the effects of feeding a high fat meal, these effects may be necessary to observe the actions of soy isoflavones in the heightened state of postprandial lipemia.

Both the fatty acid composition and antioxidant content of the diet are believed to affect the oxidative resistance of lipoprotein particles. Antioxidants are thought to reduce the pro-oxidant effect of these dietary lipid peroxides. Plotnick et al. (57) investigated the short-term effects of antioxidants on high fat (50g fat; 50% kcal from fat) meals. They found that a single high fat meal

decreased endothelial function (measured by flow-mediated vasodilation), an important early process in the development of atherosclerosis, as compared with baseline ($p<0.001$). The same high fat meal fed following pretreatment with vitamins C (1 g) and E (800 IU) did not show a change in vasodilation from baseline. The high fat meal was found to differ significantly from both the high fat meal with vitamins and low fat (0 g fat) meal ($p<0.001$) in relation to vasodilation. However, it is important to note that the low-fat meal with vitamins did not differ from the low fat meal without vitamins (no statistical data provided) in relation to vasodilation. Considering the dose meal provided in the current study was a low fat meal (whey protein) on one day and a low fat meal with antioxidants (soy protein with isoflavones) on the other day they would be similar to the above example despite the difference in the measured outcome. This provides evidence as to the lack of an observed effect for the soy protein with isoflavones.

A high fat challenge, containing predominantly PUFA, may be needed in order to discern any difference between treatments, soy protein containing 100 mg isoflavones and whey protein without isoflavones, on the oxidative resistance of the postprandial LDL. Diets that do not contain an atherogenic fat challenge may not elicit the postprandial lipemia necessary to observe the antioxidant effects of the soy protein and isoflavones. The dose diet for the current study provided 51 g of protein (55.4% of total kcal), 35 g of CHO (38.5% of total kcal), and 2.5 g (6.1% of total kcal) fat; it was not until the six hour time point that 11 g protein (9.0% of total kcal), 63 g CHO (52.2% of total kcal), and 21 g (38.6% of

total kcal) fat were provided to the subjects. The hour six meal was consumed immediately following the collection of the hour six blood sample, the effects of the meal were therefore observed only at the hour nine time point. Oxidative damage was evident in the shortened lag time occurring at hour 9 following the first background meal provided at hour six for both soy and whey protein.

Postprandial lipemia is due in part to an increase in serum TG following a meal. The TG increase may occur as a result of dietary fat or possibly as a consequence of metabolic changes occurring following CHO consumption. Although, typical dietary fat contains 95% TG, the ingestion of a meal changes fat metabolism such that it suppresses the entry of endogenous TG into the plasma and increases the disposal of TG from plasma (58). A study by van Wijk et al.(59) evaluated the effects of nutrient intake on plasma TG concentrations. The capillary TG was self-measured by 58 subjects at six fixed time points over three days. Food intake was recorded in a diary. The area under the curve for changes in TG concentration was associated with absolute intake of CHO, energy, and protein, however, the strongest correlation was with CHO intake ($p<0.005$, $r = 0.38$).

Mittendorfer and Sidossis (60) investigated the effects of high CHO diets on plasma TG concentrations. Six subjects (five men, one woman) were studied after two weeks on a high CHO diet (75% CHO, 10% fat, 15% protein) and again after two weeks on an isocaloric high fat diet (30% CHO, 55% fat, 15% protein). After completion of 14 days on one of the study diets (high CHO or high fat),

catheters were inserted into the femoral artery and a hepatic vein for tracer ([U-^{13}C] fatty acid) infusion. The rate of appearance of the VLDL-TG was significantly higher ($p<0.04$) after the high CHO diet than after the high fat diet. No difference was found between the high CHO and high fat diets in VLDL-TG clearance (1.5 ± 0.5 compared with 1.7 ± 0.5 ml/kg/min). High CHO diets appear to increase plasma TG concentrations via increased hepatic secretion of the VLDL-TG significantly more than a high fat diet.

Ten Type 2 diabetic individuals were fed two test meals differing in CHO content (meal A: high CHO, 80g; meal B: low CHO, 47g) to determine the impact of blood glucose on LDL modification (14). The high CHO meal was found to produce a significantly higher degree of hyperglycemia ($p<0.05$) and shorter lag phase ($p<0.05$) than the low CHO meal, providing evidence that meal composition can affect meal-induced oxidative stress, furthermore a high CHO meal can produce more oxidative stress than a high fat meal. This study was conducted in diabetics and considerations for insulin resistance must be made. Insulin inhibits hepatic VLDL-TG secretion in normal situations, however in cases of insulin resistance where resistant adipose tissue continues lipolysis in the presence of insulin, the rate of VLDL secretion by the liver increases (62).

It is difficult to discern a single dietary component that results in the increase in the plasma TG concentration following a meal. Postprandial lipid metabolism is a complex coordinated physiological event that likely has more

than one stimulus and is even more likely to vary based on individual differences (e.g. multiple TG peaks, insulin resistance).

The dose meal for the current study may also require some additional considerations in order to optimize the bioavailability of the isoflavones. Greater equol production has been observed in subjects consuming diets higher in CHO (62). Sixty subjects (30 men and 30 women) participating in an equol excreter study consumed 34 g soy protein (22 mg genistein and 8 mg daidzein) along with their usual diets for four days. On day four, subjects provided 24-hour urine samples for urinary isoflavonoid analysis via gas chromatography-mass spectrometry. Equol excreters were defined as subjects with urinary excretion rates of > 2000 nmol equol/d, while non-excreters excreted < 250 nmol equol/d. The prevalence of equol excreters in the study group was 35% (43% of the men, 27% of the women). Analysis of the subjects' recorded food intakes for the four study days, revealed that equol excreters consumed a higher percentage of their energy from CHO and consumed greater amounts of plant protein and fiber (soluble and insoluble). This study suggests that the CHO content of the diet may be an important variable in determining equol production. Dietary fiber may promote the growth of the colonic bacteria necessary for conversion of daidzein to equol. This may be an extremely important consideration as equol is currently considered the most powerful antioxidant isoflavone. It should be noted that this is based on a single study where the recorded food intakes of the subjects were used to determine diet composition.

The use of one method of analysis may have been a limitation to the current study. The oxidation process is characterized by several occurrences. A number of methods have been developed to measure various compounds, such as conjugated dienes, that are formed throughout the oxidation process. Each method of analysis has its limitations, making no one method applicable to all circumstances. It is therefore reasonable to suggest the use of more than one method for evaluation of oxidation status.

Isoprostanes are products of lipid peroxidation *in vivo*. The isoprostanes are prostaglandin-like compounds formed *in vivo* from nonenzymatic free radical catalyzed oxidation of PUFA (63). Cell membrane bound arachidonic acid, a PUFA, undergoes attack by free radicals yielding isoprostanes. Therefore, isoprostane measurement is representative of the *in vivo* rates of lipid peroxidation and is a specific index of oxidative stress (64, 65). A disadvantage to isoprostane testing is their relatively low concentration, while an advantage is their specificity (63).

One of the more popular methods to assess degree of oxidation is TBARS. Malondialdehyde (MDA), a byproduct of lipid peroxidation, reacts with thiobarbituric acid to produce a fluorescent product ($[TBA]_2$-MDA) with an absorption maximum near 532 nm. The TBARS test for detection of MDA is rapid and inexpensive, but has inherent shortcomings in that aldehydes other than MDA can form the fluorescent product and MDA can be generated during sample preparation if no antioxidants are present (66).

The total radical trapping parameter (TRAP) assay was one of the most widely used methods for measuring total antioxidant capacity over the past ten years. The TRAP assay uses peroxyl radical generated by addition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) to the plasma. Oxidation is monitored by measuring the oxygen consumed during the reaction. The length of the lag phase is compared to that of an internal standard, Trolox, and then quantitatively related to the antioxidant capacity of the plasma, however the meaning of this data remains in question. A major problem with the TRAP assay lies in determination of the endpoint, as an oxygen electrode will not maintain stability over the period of time required (67).

As was stated by Prior and Cai (67), "A battery of measurements are necessary to adequately assess oxidative stress in biological systems". Although LDL oxidation may be an ample tool for the evaluation of the oxidative resistance of postprandial LDL, the addition of at least one other test method would be beneficial as no single measurement of antioxidant status appears sufficient. Soy is a unique challenge as the mechanism by which it functions as an antioxidant remains unknown, knowledge as to how an antioxidant functions can help in identifying adequate assessment techniques.

This study was designed to prepare the basis for further postprandial soy feeding studies. In that regard it has succeeded in providing valuable information. However, future studies should address the concerns discussed above in regards to composition of an atherogenic challenge consumed in

conjunction with the dose meal, variability between subjects, and methods of analysis.

CHAPTER 6

SUMMARY

The specific ability of soy to reduce total cholesterol, LDL cholesterol and TG concentrations has been documented in a meta-analysis by Anderson et al. (1995). The combination of the lipid lowering effects of soy and the antioxidant properties of soy's isoflavones may contribute to a beneficial effect on LDL oxidation. Past research has been able to demonstrate a relationship between soy consumption and a reduction in LDL oxidation (8, 9, 10). Evidence suggests that postprandial LDL is more susceptible to oxidation indicating a relationship between the composition of a meal and CVD (11). The effect of meal composition, specifically with or without soy isoflavones, on the oxidative resistance of postprandial LDL requires further consideration.

The purpose of this study was to examine the effects of a meal containing 50 g soy protein (100 mg aglycone isoflavones) versus 50 g whey protein (0 mg isoflavones) on the oxidative resistance of postprandial LDL. In doing so, no significant differences were observed between the main effects (protein type, time points) or the interaction between the factors (protein * time) on LDL oxidation (lag time, initial absorbance). However, this study does provide evidence that an atherogenic challenge meal consumed in conjunction with the dose meal may be required to observe the antioxidant effects of soy protein (100 mg aglycone isoflavones) postprandially.

Postprandial lipemia is a complex metabolic process affected by a variety of external and internal factors. Interindividual differences as well as meal composition impact lipid metabolism. Studying a single dietary component affects the entire dynamic system within the human body. Findings in the current study demonstrate a need for consideration of all dietary components; inclusion of an atherogenic challenge meal consumed in conjunction with the dose meal is warranted in future studies.

The current study has provided information on the postprandial effects of a whey protein meal versus a soy (with isoflavones) protein meal. This information should serve as a basis for future studies in which other macro- and micronutrients should be considered as components of the dose meal. The current study's findings suggest that any differences in LDL oxidation seen in future postprandial studies with soy (with isoflavones) and whey protein cannot be directly attributed to the protein content alone, but possibly due to an interaction between the protein or isoflavone content with other dietary components, including fat and CHO intake.

The current study should serve as an example for careful consideration of all dietary components and their effects on metabolism. Pre-screening (equol production, number of TG peaks produced, and insulin sensitivity) subjects for additional factors may decrease interindividual differences and facilitate data interpretation.

Nutritional science is the study of the nutrients in foods and the body's handling of them including ingestion, digestion, absorption, transport, metabolism, interaction, storage and excretion (68). Nutritional science is a young science, the first vitamin was identified in 1897 and the first protein structure was not fully described until 1945 (69). It is important to remember that it is the whole food that humans ingest daily as a part of their daily diet. Although, the study of a single component of the diet represents an approach to potentially identifying physiological roles of specific nutrients, it may be unrealistic. Dietary components interact with each other and in doing impact all of system of the human body. Much remains to be learned about foods, nutrients, and their effects on the human body.

REFERENCES CITED

1. Anderson JW, Johnstone BM, Cook-Newll ME. Meta-analysis of the effects of soy protein intake on serum lipids. N Engl J Med 1995;333:276-82.

2. Food and Drug Administration. FDA approves new health claim for soy protein and coronary heart disease. Available at: http://vm.cfsan.fda.gov/~lrd/tpsoypr2.html Accessed June 8, 2000.

3. Setchell KDR. Absorption and metabolism of soy isoflavones – from food dietary supplements and adult to infants. J Nutr 2000;130:654S-5S.

4. Hwang J, Sevanian A, Hodis H, Ursini, F. Synergistic inhibition of LDL oxidation by phytoestrogens and ascorbic acid. Free Radic Biol Med 2000;29;79-89.

5. Ahotupa M, Vasankari TJ. Baseline diene conjugation in LDL lipids: an indicator or circulating oxidized LDL. Free Radic Biol Med 1999;27:1141-50.

6. Leake DS. Flavonoids and the oxidation of low-density lipoproteins. Nutrition 2001;17:63-6.

7. Stocker R. Natural antioxidants and atherosclerosis. Asia Pacific J Clin Nutr 1993;2(supp 1):15-20.

8. Anderson JW, Diawadkar VA, Bridges SR. Selective effects of different antioxidants on oxidation of lipoproteins from rats. Proc Soc Ex Biol Med 1998;218:376-81.

9. Jenkins DJA, Kendall CWC, Garsetti K et al. Effects of soy protein foods on low-density lipoprotein oxidation and ex vivo sex hormone receptor activity-a controlled crossover trial. Metabolism 2000;49:537-43.

10. Jenkins DJA, Kendall CWC, Vidgen E et al. Effects of soy-based breakfast cereal on blood lipids and oxidized low-density lipoproteins. Metabolism 2000;49:1496-1500.

11. Diwadkar VA, Anderson JW, Bridges SR, Gowri MS, Oelgten PR. Postprandial low-density lipoproteins in type 2 diabetes are oxidized more extensively than fasting diabetes and control samples. Proc Soc Ex Biol Med 1999;222:178-84.

12. Zilversmit DB. Atherogenesis: a postprandial phenomenon. Circulation 1979;60:473-85.

13. Havel R. McCollum award lecture, 1993: triglyceride-rich lipoproteins and atherosclerosis-new perspectives. Am J Clin Nutr 1994;59:795-9.

14. Ceriello A, Bortolotti N, Motz E et al. Meal-induced oxidative stress and low-density lipoprotein oxidation in diabetes: the possible role of hyperglycemia. Metabolism 1999;48:1503-8.

15. Sethi S, Gibney MJ, Williams CM. Postprandial lipoprotein metabolism. Nutri Res Rev 1993;6:161-183.

16. Mero N, Syvanne M, Taskinen MJ. Postprandial lipid metabolism in diabetes. Atherosclerosis 1998;141:S53-5.

17. Natella F, Ghiselli A, Guidi A, Ursini F, Scaccini C. Red wine mitigates the postprandial increase of LDL susceptibility to oxidation. Free Radic Biol Med 2001;30:1036-44.

18. Lechleitner M, HoppichlerF, Foger B, Patsch JR. Low-density lipoproteins of the postprandial state induce cellular cholesteryl ester accumulation in macrophages. Arterioscler Thromb 1994;14:1799-807.

19. Hamilton CA. Low-density lipoprotein and oxidized low-density lipoprotein: their role in the development of atherosclerosis. Pharmacol Ther 1997;74:55-72.

20. Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: and overview. Free Radic Biol Med 2000;28:1815-26.

21. Hazen SL. Oxidation and atherosclerosis. Free Radic Biol Med 2000;28:1683-4.

22. Jones PJH, Kubow S. Lipids, sterols, and their metabolites. In: Modern Nutrition in Health and Disease. Baltimore, MR:Lippincott Williams & Wilkins, 1999:67-94.

23. Jialal I, Devaraj S. The role of oxidized low density lipoprotein in atherogenesis. J Nutr 1996;126:1053S-1057S.

24. Raines EW, Collins AR. Biology of atherosclerosis plaque formation: possible roles of growth factors in lesion development and the impact of soy. J Nutr 1995;125:625S-30S.

25. Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Red Res Comms 1989;6:67-75.

26. Keaney JF. Atherosclerosis: from lesion formation to plaque activation and endothelial dysfunction. Mol Aspects Med 2000;21:99-166.

27. Ursini F, Zamburlini A, Cazzolato G, Maiorino M, Bon GB, Sevanian A. Postprandial plasma lipid hydroperoxides: a possible link between diet and atherosclerosis. Free Radic Biol Med 1998;25:250-2.

28. Nielsen NS, Marckmann P, Hoy CE. Effect of meal fat quality on oxidation resistance of postprandial VLDL and LDL particles and plasma triacylglycerol level. Br J Nutr 2000;84:855-63.

29. Gaziano JM. Dietary antioxidants and cardiovascular disease. Vitamins and Hormones 2000;58:299-320.

30. Kanazawa T, Osanai T, Zhang XS et al. Protective effects of soy protein on the peroxidizability of lipoproteins in cerebrovascular diseases. J Nutr 1995;125:639S-46S.

31. Kerry N, Abbey M. The isoflavone genistein inhibits copper and peroxyl radical mediated low density lipoprotein oxidation in vitro. Atherosclerosis 1998;140:341-7.

32. Meng Q-H, Lewis P, Wahala K, Aldercreutz H, Tikkanen. Incorporation of esterified soybean isoflavones with antioxidant activity into low density lipoproteins. Biochimica et Biophysica Acta 1999;1438:369-76.

33. Tikkanen MJ, Aldercreutz H. Dietary soy-derived isoflavone phytoestrogens. Biochem Pharmacol 2000;60:1-5.

34. Tikkanen MJ, Wahala K, Ojala V, Aldercreutz H. Effect of soybean phytoestrogens intake on low density lipoprotein oxidation resistance. Proc Natl Acad Sci USA 1998;95:3106-3110.

35. Wiseman H, O'Reilly JD, Aldercreutz H et al. Isoflavone phytoestrogens consumed in soy decrease F_2-isoprostane concentrations and increase resistance of low-density lipoproteins to oxidation in humans. Am J Clin Nutr 2000;72:395-400.

36. Samman S, Wall PML, Chan GSM, Smith SJ, Petocz P. The effects of supplementation with isoflavones on plasma lipids and oxidisability of low density lipoprotein in premenppausal women. Atherosclerosis1999;147:277-83.

37. United Soybean Board. Abbey M, King R, Kerry N, Owen A, Record I, Nestel P. Technical Bulletin: Soy isoflavones: bioavailability, antioxidant activity, cancer and cardiovascular disease. Available at: http://www.asases.com/technical/hn30-1998.html Accessed August 18, 2000.

38. Cohn JS, McNamara JR, Cohn SD, Ordovas JM, Schaefer EJ. Postprandial plasma lipoprotein changes in human subjects of different ages. J Lipid Res 1988;29:469-79.

39. Moro E, Alessandrini P, Zambon C et al. Is glycation of low density lipoproteins in patients with type 2 diabetes mellitus a pre-oxidative condition? Diabetic Med 1999;16:663-9.

40. Barnard ND, Scialli AR, Bertron P Hurlock D, Edmonds K, Talev L. Effectiveness of a low-fat vegetarian diet in altering serum lipids in healthy promenopausal women. Am J Cardiol 2000;85:969-72.

41. Konig D, Wagner K-H, Elmadfa I, Berg A. Exercise and oxidative stress: significance of antioxidants with reference to inflammatory, muscular and systemic stress. Ex Immunol Rev 2001;7:108-33.

42. Xu X, Wang HJ, Murphy PA, Hendrich S. Neither background diet nor type of soy food affects short-term isoflavone bioavailability in women. J Nutr 2000;130:798-801.

43. Bouchard C, Tremblay A, Leblanc C, Lortie G, Savard R, Theriault G. A method to assess energy expenditure in children and adults. Am J Clin Nutr 1983;37:461-467.

44. Naito HK. Lipoprotein separations using the TL-100 tabletop ultracentrifuge. 1986

45. Puhl H, Waeg G, Esterbauer H. Methods to determine oxidation of low-density lipoproteins. Methods Enzymol 1994;233:425-41.

46. Palomaki A, Malminiemi K, Solakivi T, Malminiemi O. Ubiquinone supplementation during lovastatin treatment: effect on LDL oxidation ex vivo. J Lipid Res 1998;39:1430-7.

47. Smith PK, Krohn RI, Hermanson GT et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76-85.

48. Redinbaugh MG, Turley RB. Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. Anal Biochem 1986;153:267-71.

49. Gillotte KL, Horkko S, Witztum JL, Steinberg D. Oxidized phospholipids, linked to apolipoprotein B of oxidized LDL, are ligands for macrophage scavenger receptors. J Lipid Res 2000;41:824-33.

50. Schreier L, Pagliero F, Sanguinetti S, Wikinski R. Influence of the medium on the assessment of LDL resistance to oxidation: lag time in phosphate buffered saline is longer then in sodium chloride solution. Atherosclerosis 1997;129:127-8.

51. Ahotopa M, Marniemi J, Lehtimaki T. Baseline diene conjugation in LDL lipids as a direct measure of *in vivo* LDL oxidation. Clin Biochem 1998;31:257-61.

52. Neter J, Kutner MH, Nachtsheim CJ, Wasserman W. Applied linear statistical models. Boston, MA: McGraw-Hill, 1996.

53. Rao PV. Statistical research methods in the life sciences. Pacific Grove, CA: Brooks/Cole, 1998.

54. Glass GV, Hopkins KD. Statistical methods in education and psychology. Needhan Heights, MA: Simon and Schuster, 1996.

55. Mitchell JH, Collins AR. Effects of a soy milk supplement on plasma cholesterol levels and oxidative DNA damage in men - a pilot study. Eur J Nutr 1999;38:143-8.

56. Setchell KDR, Brown NM, Desai P et al. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. J Nutr 2001;131:1362S-75S.

57. Cohn JC, Noakes TD, Benade AJS. Serum triglyceride responses to fatty meals: effects of meal fat content. Am J Clin Nutr 1988;47:825-7.

58. Frayn KN. Dietary factors and postprandial lipaemia. Br J Nutr 1998;80:409-10.

59. van Wijk JPH, Cabezas MC, Halkes CJM, Erkelens DW. Effects of different nutrient intakes on daytime triacylglycerolemia in healthy, normolipemic, free living men. Am J Clin Nutr 2001;74:171-8.

60. Mittendorfer B, Sidossis LS. Mechanisms for the increase in plasma triacylglycerol concentrations after consumption of short-term, high-carbohydrate diets. Am J Clin Nutr 2001;73:892-9.

61. Zammit VA, Waterman IJ, Topping D, McKay G. Insulin stimulation of hepatic triacylglycerol secretion and the etiology of insulin resistance. J Nutr 2001;131:2074-7.

62. Slavin JL, Karr SC, Hutchins AM, Lampe JW. Influence of soybean processing, habitual diet, and soy dose on urinary isoflavonoid excretion. Am J Clin Nutr 1998;68(suppl):1492S-5S.

63. Salonen JT. Markers of oxidative damage and antioxidant protection: assessment of LDL oxidation. Free Rad Res 2000;33:S41-6.

64. Richelle M, Turini ME, Guidoux R, Tavazzi I, Metairon S, Fay LB. Urinary isoprostane excretion is not confounded by the lipid content of the diet. FEBS Letters 1999;459:259-62.

65. Pratico D, Lawson JA, Rokach J, Fitzgerald GA. The isoprostanes in biology and medicine. Trends in Endocrinology and Metabolism 2001;12:243-7.

66. Ordovas JM. Lipoprotein protocols. Totowa, NJ: Humana Press, 1998.

67. Prior RL, Cai G. In vivo total antioxidant capacity: comparison of different analytical methods. Free Radic Biol Med 1999;27:1173-81.

68. Whitney, EN, Cataldo CB, Rolfes SR. Understanding normal and clinical nutrition. Belmont, CA: Wadsworth, 1998.

69. Sizer FS, Whitney EN. Nutrition concepts and controversies. Belmont, CA: Wadsworth, 2000.

APPENDICES

APPENDIX A

MEDICAL HISORY 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 B

HUMAN SUBJECTS CONSENT FORM

SUBJECT CONSENT FORM
FOR
PARTICIPATION IN HUMAN RESEARCH
MONTANA STATE UNIVERSITY

PROJECT TITLE: Effects of soy isoflavones on the oxidative resistance of postprandial low-density lipoproteins.

PRINCIPLE INVESTIGATOR: Christina Gayer Campbell, PhD, RD
Assistant Professor, Nutrition
Department of Health and Human Development
Herrick Hall, Room 20, Montana State University
Bozeman, MT 59717-3540
406-994-5002, ccampbel@montana.edu

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 objective of this study is to examine the effects of a meal containing soy protein and soy's natural antioxidants, isoflavone, versus milk protein (no isoflavones) on measures of LDL modification.

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 selected to participate 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, 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 (>1 drink/d for women and >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 assigned to 1 of 2 groups. The only difference between the groups is the order in which you receive the soy protein or milk protein test meals. 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.

You will be required to come into the Nutrition Research Lab (NRL) at Herrick Hall on the campus of Montana State University on two separate occasions for participation in the feeding study. 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 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. You will be given detailed instructions on how to properly complete the forms and tips on accurately weighing food by a dietetics student that has been trained on giving dietary analysis instructions. 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 0700 h and stay until 1930 h. After the measurement of body weight and height, a Registered Nurse (RN) trained in such procedures will place a small indwelling catheter with a needle in a forearm vein. The indwelling catheter will be flushed before and after each blood draw with a saline solution to prevent the catheter from clotting. At 0720, you will be given 20 minutes to consume the test meal consisting of a protein shake (352 kcal). Test meal A will consist of 50 grams of soy protein with 100 mg of soy isoflavones. Test meal B will consist of 50 grams of milk protein with 0 mg of isoflavones. Both test meals will be in the form of a protein shake (protein powder [50 g protein; milk or soy], a banana, ice cubes and water). Throughout the day, you will receive a background diet containing 1077 calories, for which you will have 20 minutes to consume at each feeding point, for the duration of the feeding study.

The diet will consist of:

"lunch" at hour 6
2 slices white bread
28g swiss cheese
mustard
one medium apple
15 potato chips (28 g)

"snack" at hour 9
2 slices white bread
22 honey-flavored Teddy Grahams (28g)
14g Colby-jack cheese
one medium apple
mustard
15 tortilla chips (12 g)
250 mL unsweetened apple juice

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 the catheter in your forearm every hour following the consumption of the dose meal for a total of 156 mL of blood. The RN will remain present for the duration of the study.

RISKS:
An intravenous needle will be placed in your arm for the removal of blood samples. The catheter will be left in for 12 hours. Approximately 2 teaspoons of blood will be removed on 12 occasions. There is momentary pain at the time the needle is inserted into the vein for catheter placement. In addition to this momentary pain, there will be the minor discomfort of having the catheter taped to your skin. In about 10% of the cases, a small amount of bleeding under the skin will produce a bruise. The risk of temporary clotting of the vein is about 1%, while the risk of infection or significant external blood loss 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.

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: _____ Date:_____

Witness: _____

Investigator: _____

APPENDIX C

DIET RECORD FORM

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

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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				

Vol. 29, No. 6 Supplement Official Journal of the American College of Sports Medicine

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

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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 - ice hockey - boxing - basketball - mountain-climbing - football - squash - racquetball - cross-country skiing	1.95

APPENDIX E

PROTEIN TECHNOLOGIES INTERNATIONAL

PROTEIN TECHNOLOGIES INTERNATIONAL
A DuPont Business — DUPONT

Soy and Placebo CI Nutritional
Vanilla Beverage Powder
Sample 363 & 658

The Soy and Placebo Nutritional Vanilla Beverage Powders have been formulated for maximum protein delivery with minimum caloric content. Isolated soy protein or milk protein isolate contributes 25 g of protein per serving size of 36.5 grams. The product is sweetened with aspartame and has a mild vanilla flavor. The product is standardized to meet an aglycone genistein target of 1.2 mg per gram of soy protein.

Typical Nutrition Information

Serving Size 36.5 grams

Amount Per Serving:

Calories	130	
Calories from Fat	5 – 10	
		% Daily Value
Total Fat	0.5 – 1 g	1 – 2%
Saturated Fat	0 g	0%
Cholesterol	0 – 5 mg	0 – 2%
Sodium	150 - 240 mg	6 – 10%
Potassium	10 - 200 mg	0 – 6%
Total Carbohydrate	5 – 6g	2%
Dietary Fiber	0g	0%
Sugars	3g	
Protein	25g	50%

	Typical Value	% RDI		Typical Value	% RDI
Vitamin A	625 IU	10%	Vitamin C	0 mg	0%
Calcium	900 mg	90%	Iron	1 – 4 mg	5 – 20%
Vitamin D	125 IU	30%	Riboflavin	0.5 mg	30%
Folate	35-75 mcg	5 – 20 %	Vitamin B12	1 mcg	15%
Phosphorus	625 mg	60%	Magnesium	50 – 140 mg	10 – 35%

Ingredients: Isolated soy protein or milk protein isolate; maltodextrin, sucrose, fructose, aspartame*, artificial flavor.

Vitamins & Minerals: Calcium phosphate, magnesium phosphate, riboflavin, vitamin A palmitate, folic acid, vitamin D3, vitamin B12.

*Phenylketonurics: Contains Phenylalanine

Storage Conditions: Store at room temperature or below

**Analytical data for the production lots utilized in your study will be provided, upon completion of the study, at the request of the researcher.

P.O. Box 88940
St. Louis, MO 63188 USA

Soy and Placebo CI Nutritional
Vanilla Beverage Powder
Sample 363 & 658

DIRECTIONS FOR USE:
Individual Serving Packet:

Add contents of packet to 10 - 12 oz. of water or other beverage and stir.

Additional serving suggestions:

- For hot beverages, add contents of packet to lukewarm water and heat for 1 ½ to 2 minutes in microwave.
- Use blender for shake-like consistency.
- To make a fruit shake, add contents of packet to 10 - 12 oz. of water and your favorite fresh fruit. Mix in blender. Try banana, strawberries, raspberries, blueberries, sliced peaches or pineapple.

STORAGE CONDITIONS AFFECTING SHELF-LIFE
Shelf life is a minimum of 12 months from the date of manufacture, if the product is stored in accordance with the following conditions:

1. Product is to be stored out of contact with the floor, ceiling and walls.
2. Product is to be stored in a well-ventilated area, free from strong, pungent and objectionable odors.
3. Product is stored in unopened product packages.

Optimum storage conditions are 70 °F (20°C) or below and less than 50% relative humidity.

ISOFLAVONE PROFILE *

	mg/g Soy Protein (aglycone units) **	mg/g Soy Protein (all forms)***
Genistein	1.2 ±0.2	2.1
Daidzein	0.6	1.1
Glycitein	0.1	0.2
Total Isoflavones	2.0	3.4

* Isoflavone profile represents typical values. The product is standardized to meet an aglycone genistein target of 1.2 mg per gram of soy protein. Analysis is performed on each product and can be made available to the researcher.

** Individual forms of isoflavones are corrected to reflect only the weight of the aglycone within that isoflavone family.

*** Aglycones, glycosides, glycoside esters.

Isoflavone content of the isolated soy protein has been shown to be stable for 2 years at room temperature.

APPENDIX F

DOSE AND BACKGROUND DIET ANALYSIS

Client Diet Record Report

First:
Middle:
Last: soy study
Company:

Identification Number:
Date of Birth:
Height: Weight:

Total Days: 1
Total Foods: 13
Avg. Daily Kcals: 1429.251

Diet Name: soy study

Name	Amount	Unit	Kcal kcal	Protein g	Carb g	Fat g	Vit A (IU) IU	Vit C mg	Diet Fiber g	Beta-C µg	Vit E (mg) mg
soy study diet(dose & backgrd)			1429.251	72.283	205.824	38.379	1962.015	31.548	15.015	92.760	1.562
Breakfast			352.000	51.030	35.430	2.480	1331.000	9.100	2.400	21.000	0.270
Protein Technologies Beverage Powder(363&658)	36.500	g	130.000	25.000	6.000	1.000	625.000	0.000			
Protein Technologies Beverage Powder(363&658)	36.500	g	130.000	25.000	6.000	1.000	625.000	0.000			
Banana	100.000	g	92.000	1.030	23.430	0.480	81.000	9.100	2.400	21.000	0.270
Lunch			477.569	10.754	63.002	20.604	392.718	11.673	5.934	35.880	0.633
White Bread	2.000	sl.	133.500	4.100	24.750	1.800	0.000	0.000	1.150	0.000	0.191
KRAFT Singles Pasteurized Process Swiss Cheese Food Slices	28.000	g	93.333	5.333	1.333	6.667	266.667	0.000	0.000	0.000	
Apple	1.000	item	81.420	0.262	21.045	0.497	73.140	7.866	3.726	35.880	0.442
PRINGLES Original Potato Crisps	15.000	item	169.315	1.058	15.873	11.640	52.911	3.807	1.058		
Afternoon Snack			599.682	10.500	107.393	15.295	238.297	10.775	6.681	35.880	0.659
White Bread	2.000	sl.	133.500	4.100	24.750	1.800	0.000	0.000	1.150	0.000	0.191
SARGENTO MOOTOWN SNACKS Colby-Jack Cheese Sticks	14.000	g	46.667	2.917	0.292	4.083	116.667	0.000	0.000		
TOSTITOS Bite Size Tortilla Chips	15.000	item	86.668	1.238	10.524	4.952	0.000	0.000	0.619	0.000	
TEDDY GRAHAMS Honey Graham Snacks	22.000	item	128.260	1.826	20.174	3.674	45.870	0.550	0.924		
Apple Juice, Unsweetened, Canned	250.000	mL	123.168	0.157	30.608	0.288	2.621	2.359	0.262	0.000	0.026
Apple	1.000	item	81.420	0.262	21.045	0.497	73.140	7.866	3.726	35.880	0.442

Client Diet Record Report

First:
Middle:
Last: soy study
Company:

Identification Number:
Date of Birth:
Height: Weight:

Total Days: 3
Total Foods: 13
Avg. Daily Kcals: 476.417

Diet Name: soy study

Name	Amount Unit	Kcal kcal	Protein g	Carb g	Fat g	Vit A (IU) IU	Vit C mg	Diet Fiber g	Beta-C µg	Vit E (mg) mg
Dose Meal		352.000	51.030	35.430	2.480	1331.000	9.100	2.400	21.000	0.270
Breakfast		352.000	51.030	35.430	2.480	1331.000	9.100	2.400	21.000	0.270
Protein Technologies Beverage Powder(363&658)	36.500 g	130.000	25.000	6.000	1.000	625.000	0.000			
Protein Technologies Beverage Powder(363&658)	36.500 g	130.000	25.000	6.000	1.000	625.000	0.000			
Banana	100.000 g	92.000	1.030	23.430	0.480	81.000	9.100	2.400	21.000	0.270
Background Meal (6 hr)		477.569	10.754	63.002	20.604	392.718	11.673	5.934	35.880	0.633
Lunch		477.569	10.754	63.002	20.604	392.718	11.673	5.934	35.880	0.633
White Bread	2.000 sl.	133.500	4.100	24.750	1.800	0.000	0.000	1.150	0.000	0.191
KRAFT Singles Pasteurized Process Swiss Cheese Food Slices	28.000 g	93.333	5.333	1.333	6.667	266.667	0.000	0.000	0.000	
Apple	1.000 item	81.420	0.262	21.045	0.497	73.140	7.866	3.726	35.880	0.442
PRINGLES Original Potato Crisps	15.000 item	169.315	1.058	15.873	11.640	52.911	3.807	1.058		
Background Meal (9hr)		599.682	10.500	107.393	15.295	238.297	10.775	6.681	35.880	0.659
Afternoon Snack		599.682	10.500	107.393	15.295	238.297	10.775	6.681	35.880	0.659
White Bread	2.000 sl.	133.500	4.100	24.750	1.800	0.000	0.000	1.150	0.000	0.191
SARGENTO MOOTOWN SNACKS Colby-Jack Cheese Sticks	14.000 g	46.667	2.917	0.292	4.083	116.667	0.000	0.000		
TOSTITOS Bite Size Tortilla Chips	15.000 item	86.668	1.238	10.524	4.952	0.000	0.000	0.619	0.000	
TEDDY GRAHAMS Honey Graham Snacks	22.000 item	128.260	1.826	20.174	3.674	45.870	0.550	0.924		
Apple Juice, Unsweetened, Canned	250.000 mL	123.168	0.157	30.608	0.288	2.621	2.359	0.262	0.000	0.026
Apple	1.000 item	81.420	0.262	21.045	0.497	73.140	7.866	3.726	35.880	0.442

Client Diet Record Intake

First:
Middle:
Last: soy study
Company:

Identification Number:
Date of Birth:
Height: Weight:

Total Days: 1 Total Foods: 13
Avg. Daily Kcals: 1429.251 Diet Name: soy study

Percentage of Kcals

Protein	19.8%
Carbohydrate	56.5%
Fat, total	23.7%
Alcohol	0.0%

Food Item	Amount	Unit	Day	Meal
Protein Technologies Beverage Powder(363&658)	36.500	gram(s)	soy study diet(dose & backgrd)	Breakfast
Protein Technologies Beverage Powder(363&658)	36.500	gram(s)	soy study diet(dose & backgrd)	Breakfast
Banana	100.000	gram(s)	soy study diet(dose & backgrd)	Breakfast
White Bread	2.000	slice(s)	soy study diet(dose & backgrd)	Lunch
KRAFT Singles Pasteurized Process Swiss Cheese Food Slices	28.000	gram(s)	soy study diet(dose & backgrd)	Lunch
Apple	1.000	item(s)	soy study diet(dose & backgrd)	Lunch
PRINGLES Original Potato Crisps	15.000	item(s)	soy study diet(dose & backgrd)	Lunch
White Bread	2.000	slice(s)	soy study diet(dose & backgrd)	Afternoon Snack
SARGENTO MOOTOWN SNACKS Colby-Jack Cheese Sticks	14.000	gram(s)	soy study diet(dose & backgrd)	Afternoon Snack
TOSTITOS Bite Size Tortilla Chips	15.000	item(s)	soy study diet(dose & backgrd)	Afternoon Snack
TEDDY GRAHAMS Honey Graham Snacks	22.000	item(s)	soy study diet(dose & backgrd)	Afternoon Snack
Apple Juice, Unsweetened, Canned	250.000	milliliter(s)	soy study diet(dose & backgrd)	Afternoon Snack
Apple	1.000	item(s)	soy study diet(dose & backgrd)	Afternoon Snack

Client Diet Record Intake

First:
Middle:
Last: soy study
Company:

Identification Number:
Date of Birth:
Height: Weight:

Total Days: 1
Avg. Daily Kcals: 352

Total Foods: 3
Diet Name: dose meal

Percentage of Kcals

Protein	55.4%
Carbohydrate	38.5%
Fat, total	6.1%
Alcohol	0.0%

Food Item	Amount	Unit	Day	Meal
Protein Technologies Beverage Powder(363&658)	36.500	gram(s)	New Diet Day	Breakfast
Protein Technologies Beverage Powder(363&658)	36.500	gram(s)	New Diet Day	Breakfast
Banana	100.000	gram(s)	New Diet Day	Breakfast

Client Diet Record Intake

First:
Middle:
Last: soy study
Company:

Identification Number:
Date of Birth:
Height: Weight:

Total Days: 1 Total Foods: 4
Avg. Daily Kcals: 477.5685 Diet Name: 6 hour

Percentage of Kcals

Protein	9.0%
Carbohydrate	52.5%
Fat, total	38.6%
Alcohol	0.0%

Food Item	Amount	Unit	Day	Meal
White Bread	2.000	slice(s)	New Diet Day	Lunch
KRAFT Singles Pasteurized Process Swiss Cheese Food Slices	28.000	gram(s)	New Diet Day	Lunch
Apple	1.000	item(s)	New Diet Day	Lunch
PRINGLES Original Potato Crisps	15.000	item(s)	New Diet Day	Lunch

Client Diet Record Intake

First:
Middle:
Last: soy study
Company:

Identification Number:
Date of Birth:
Height: Weight:

Total Days: 1
Avg. Daily Kcals: 599.6824

Total Foods: 6
Diet Name: 9 hour

Percentage of Kcals

Protein	6.9%
Carbohydrate	70.5%
Fat, total	22.6%
Alcohol	0.0%

Food Item	Amount	Unit	Day	Meal
White Bread	2.000	slice(s)	New Diet Day	Afternoon Snack
SARGENTO MOOTOWN SNACKS Colby-Jack Cheese Sticks	14.000	gram(s)	New Diet Day	Afternoon Snack
TOSTITOS Bite Size Tortilla Chips	15.000	item(s)	New Diet Day	Afternoon Snack
TEDDY GRAHAMS Honey Graham Snacks	22.000	item(s)	New Diet Day	Afternoon Snack
Apple Juice, Unsweetened, Canned	250.000	milliliter(s)	New Diet Day	Afternoon Snack
Apple	1.000	item(s)	New Diet Day	Afternoon Snack

APPENDIX G

DIET RECORD DIRECTIONS

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!!!

APPENDIX H

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. Isolation (ref. Lipoprotein Separations Using TL-100 Tabletop Ultracentrifuge, H.K. Naito)

1. Remove samples from -80°C freezer (if necessary) and allow to defrost.
2. Prepare 0.9% NaCl solution (weigh 900mg NaCl and 100mg EDTA on 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
3. To Beckman Centrifuge Tubes (Polyallomer, 7/16 x 1 3/8 in; reorder # 347287) add 500µl of the 0.9% NaCl solution and 500µl of plasma (EDTA); repeat this step into a second centrifuge tube for each sample (optional)
4. Centrifuge (Door-Enter/Display-Start if on correct program) at 100,000 rpm for 2 ½ 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)]
5. While samples are centrifuging, 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]
6. When step 3 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.

7. Centrifuge again as in step 3 of Isolation
8. While samples are centrifuging:
    a. Prepare PBS buffer (St. Lukes procedure): 22.8mg Sodium Phosphate Monobasic + 115mg Sodium Phosphate Dibasic + 935mg 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]
    b. 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)
    c. Turn on the plate shaker incubator and incubator containing plate reader, set both at 37°C
    d. Prepare Cupric Chloride Dihydrate solution (weigh 85.2mg 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
    e. Wash centrifuge slicer with KimWipe and water
    f. Optional – Can prep protein standards at this time (section IV, 1)
9. When step 6 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 (if followed option to use 2) 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
10. MIX the isolated LDL thoroughly!

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

1. Remove upper cap of Econo-Pac column, save cap
2. Add 20ml PBS buffer (from step 8a of Isolation) to column, snap of tip at the bottom of the column (save tip), allow column to drain into a small beaker and discard

3. Add ≤ 3ml of isolated LDL to the column (250-500µl), allow to enter column
4. Add PBS to achieve a total volume of 3ml:
    i. 3ml – volume of isolated LDL = volume of PBS to be added to the column
5. Use a 10ml graduated cylinder to measure the first 3ml of effluent and discard (solution should stop flowing at about this point)
6. Add [1.5 X volume of isolated LDL] *or* 4 ml of PBS (whichever is less) to elute the higher molecular weight components, collect in test tube and cover, MIX
7. Store column in PBS, replace cap and tip
8. Turn on plate reader and allow to warm up (~30 minutes)

IV. Protein Concentration (ref. Pierce BCA Protein Assay Kit 23225, microwell plate protocol)

1. Prepare standard solutions with bovine serum albumin (BSA) standard provided according to Pierce table 1 by pipeting into separate microcentrifuge tubes and mixing :

ID	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	25	100µl F	400

2. 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 Nunc microwell plate; Prepare in duplicate (optional)
3. Add 200µl of WR (from step 8b of Isolation)to each well
4. Shake plate for 30 seconds on a plate shaker
5. Cover plate with a plate sealer (Seal Plate NonSterile 100-THER-PLT, Excel scientific 760-249-6371)
6. Incubate for 30 minutes at 37°C in plate shaker/incubator
7. Allow plate to cool to room temperature
8. Read absorbance at 562 nm on KC Junior plate reader (p. 6-10 of User's Guide)
9. Open existing protocol for protein assay by going to Protocol, then Open Protocol, then selecting BCAprotein

10. 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)
11. 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)
12. Click OK to exit the Modify Protocol Screen
13. 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
14. When the read is complete, results are automatically displayed
15. 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

V. LDL oxidation (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 achieve a concentration of 0.10 mg protein/ml (or 100 µg/ml):

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

2. Dilute as calculated above into microcentrifuge tubes and mix
3. 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
4. Pipet 10µl of the Cupric Chloride Dihydrate solution (step 8d of Isolation) into each well; seal with plate sealer
5. 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
f. When the read is complete, results are automatically displayed
g. 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
h. 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

VI. Data:

- Enter values into Excel program via KCjr
- Open KC Junior (open protocol, open results)
- Go to Results→Export data (click on), select directly to Microsoft Excel (be sure selection is raw data in column on right)
- Start Excel now (on bottom of export data screen)
- Go back to KC Junior(on bottom of screen), click on to maximize
- Click on export data (top right of export data screen), be sure to wait for all of the files to be loaded, can go to Excel to watch the progress

- Then, in Excel
- 1st go to save as, file name will be: filename.xls → to change to CSV, pull down under "save as type" and select CSV→file will change to filename.csv (at this time you can change the file name to whatever you want)
- 2nd click save (after "filename.csv" has been changed to 1010.csv for example) be sure to save under the folder, drive, etc of your choice : SAVE → (The selected file type.....) OK → ("X" may contain features not compatible....)YES

- Close file in Excel: (Save changes?) NO
- Minimize KC junior
- Click to open icon for "Dr.King2.exe" →select drive csv file is save on (a drive if on disk)→open→ select the number of hours
- In Excel, open new file (be sure looking in "all files") that has been created ("filenamez.txt" or 1010z.txt)
- Click on finish

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

VII. Supplies

Beckman (1-800-742-2345):
Centrifuge Tubes, Polyallomer, 7/16 x 1 3/8 in, reorder # 347287 ($94/100tubes)

BioRad (1-800-424-6723):
Econo-Pac 10 DG Columns, #732-2010 ($124/30columns)

Fisher (1-800-766-7000):
Pierce BCA Protein Assay Kit(23225), cat# PI-23225 ($116/kit)
µcentrifuge tubes, cat# 05-669-32 ($51.64/1000)
Nunc µWell Plate, flat, 400µl, cat#12-565-226 ($70.95/cs)
CoStar 96well UV flat bottom plate(370µl, 3635, down to 220nm),
cat# 07-200-623 ($187.50/25plates)
Gloves, cat #19-050-173B ($16.50/100pk)
Needles,21Gx1', cat#02-665-20 ($18.20/100pk)
Vaccutainer (EDTA/purple top),cat #22-040-034 ($7.20/50pk)
Luer adapters,Ndl holder, cat#22-658-210 ($122/1000)
Fisherbrand Redi-Tips, cat# 21-197-8F

Sigma (1-800-325-3010):
Sodium Chloride, #S9888 ($30.10/kg)
EDTA, #ED2SS ($14.20/50g)
Sodium Phosphate Monobasic, Anhydrous #S0751 ($11.70/100g)
Sodium Phosphare Dibasic, Anhydrous #S0876 ($12.20/100g)
Cupric Chloride Dihydrate, #C6917 ($25.80/100g)

Excel Scientific (1-760-249-6371):
Seal Plate, Non Sterile, 100-THER-PLT

ST 5/2/02
(saved as LDLox2 in My Documents, S.Tate)

APPENDIX I

STATISTICAL DATA

Lag Time Data:

STATISTIX 7.0
PPSOY, 4/4/02, 8:27:01 AM

ANALYSIS OF VARIANCE TABLE FOR LDLOX

SOURCE	DF	SS	MS	F	P
SUBJ (A)	9	345.328	38.3698	2.78	0.0716
PROTIEN (B)	1	3.53440	3.53440	0.26	0.6247
A*B	9	124.044	13.7826		
TIME (C)	4	90.8650	22.7163	1.77	0.1565
A*C	36	462.267	12.8407		
B*C	4	49.5966	12.3992	1.70	0.1721
A*B*C	36	263.015	7.30598		
TOTAL	99	1338.65			

STATISTIX 7.0
PPSOY, 4/4/02, 8:30:39 AM

TUKEY (HSD) COMPARISON OF MEANS OF LDLOX BY PROTIEN*TIME

PROTIEN	TIME	MEAN	HOMOGENEOUS GROUPS
2	5	0.8400	I
1	3	0.4000	I
2	1	0.0000	I I
1	1	0.0000	I I
1	2	-0.3900	I I
1	5	-0.4200	I I
2	3	-0.4900	I I
2	4	-1.0400	I I
2	2	-1.5200	I I
1	4	-3.6800	.. I

THERE ARE 2 GROUPS IN WHICH THE MEANS ARE
NOT SIGNIFICANTLY DIFFERENT FROM ONE ANOTHER.

CRITICAL Q VALUE		4.763
REJECTION LEVEL	0.050	
CRITICAL VALUE FOR COMPARISON		4.0710
STANDARD ERROR FOR COMPARISON		1.2088

ERROR TERM USED: SUBJ*PROTIEN*TIME, 36 DF

STATISTIX 7.0
PPSOY, 4/4/02, 8:33:49 AM

TUKEY (HSD) COMPARISON OF MEANS OF LDLOX BY TIME

TIME	MEAN	HOMOGENEOUS GROUPS
5	0.2100	I
1	0.0000	I
3	-0.0450	I
2	-0.9550	I
4	-2.3600	I

THERE ARE NO SIGNIFICANT PAIRWISE DIFFERENCES AMONG THE MEANS.

CRITICAL Q VALUE		4.060
REJECTION LEVEL	0.050	
CRITICAL VALUE FOR COMPARISON		3.2535
STANDARD ERROR FOR COMPARISON		1.1332

ERROR TERM USED: SUBJ*TIME, 36 DF

STATISTIX 7.0
PPSOY, 4/4/02, 8:57:30 AM

ANALYSIS OF VARIANCE TABLE FOR LDLPCNT

SOURCE	DF	SS	MS	F	P
SUBJ (A)	9	486029	54003.2	2.30	0.1156
PROTIEN (B)	1	1510.92	1510.92	0.06	0.8056
A*B	9	211544	23504.9		
TIME (C)	4	107611	26902.6	1.57	0.2041
A*C	36	618050	17168.1		
B*C	4	48453.1	12113.3	1.49	0.2246
A*B*C	36	291966	8110.17		
TOTAL	99	1765164			

STATISTIX 7.0
PPSOY, 4/4/02, 8:58:09 AM

TUKEY (HSD) COMPARISON OF MEANS OF LDLPCNT BY PROTIEN*TIME

PROTIEN	TIME	MEAN	HOMOGENEOUS GROUPS
2	2	32.285	I
1	4	19.899	I I
2	1	0.0000	I I
1	1	0.0000	I I
2	3	-18.385	I I
1	3	-23.257	I I
1	2	-28.876	I I
2	4	-51.606	I I
1	5	-71.088	I I
2	5	-104.49	.. I

THERE ARE 2 GROUPS IN WHICH THE MEANS ARE
NOT SIGNIFICANTLY DIFFERENT FROM ONE ANOTHER.

CRITICAL Q VALUE		4.763
REJECTION LEVEL	0.050	
CRITICAL VALUE FOR COMPARISON		135.64
STANDARD ERROR FOR COMPARISON		40.274

ERROR TERM USED: SUBJ*PROTIEN*TIME, 36 DF

STATISTIX 7.0
PPSOY, 4/4/02, 8:58:50 AM

TUKEY (HSD) COMPARISON OF MEANS OF LDLPCNT BY TIME

TIME	MEAN	HOMOGENEOUS GROUPS
2	1.7042	I
1	0.0000	I
4	-15.853	I
3	-20.821	I
5	-87.788	I

THERE ARE NO SIGNIFICANT PAIRWISE DIFFERENCES AMONG THE MEANS.

CRITICAL Q VALUE		4.060
REJECTION LEVEL	0.050	
CRITICAL VALUE FOR COMPARISON		118.96
STANDARD ERROR FOR COMPARISON		41.434

ERROR TERM USED: SUBJ*TIME, 36 DF

Initial Absorbance Data:

STATISTIX 7.0 SHARON_THESIS_DATA_V2,
5/14/2002, 8:09:18 AM

ANALYSIS OF VARIANCE TABLE FOR INIT

SOURCE	DF	SS	MS	F	P
SUBJ (A)	9	0.05484	0.00609	2.76	0.0731
DIET (B)	1	0.00228	0.00228	1.03	0.3364
A*B	9	0.01986	0.00221		
TIME (C)	4	0.00264	6.596E-04	0.82	0.5193
A*C	36	0.02886	8.017E-04		
B*C	4	0.00246	6.144E-04	1.87	0.1361
A*B*C	36	0.01180	3.277E-04		
TOTAL	99	0.12272			

SCHEFFE COMPARISON OF MEANS OF INIT BY DIET

DIET	MEAN	HOMOGENEOUS GROUPS
1	0.1932	I
2	0.1836	I

THERE ARE NO SIGNIFICANT PAIRWISE DIFFERENCES AMONG THE MEANS.

CRITICAL F VALUE 5.117
REJECTION LEVEL 0.050
CRITICAL VALUE FOR COMPARISON 0.0213
STANDARD ERROR FOR COMPARISON 9.394E-03

ERROR TERM USED: SUBJ*DIET, 9 DF

SCHEFFE COMPARISON OF MEANS OF INIT BY TIME

TIME	MEAN	HOMOGENEOUS GROUPS
0	0.1955	I
2	0.1933	I
4	0.1859	I
9	0.1852	I
6	0.1820	I

THERE ARE NO SIGNIFICANT PAIRWISE DIFFERENCES AMONG THE MEANS.

CRITICAL F VALUE 2.634
REJECTION LEVEL 0.050
CRITICAL VALUE FOR COMPARISON 0.0291
STANDARD ERROR FOR COMPARISON 8.954E-03

ERROR TERM USED: SUBJ*TIME, 36 DF

SCHEFFE COMPARISON OF MEANS OF INIT BY DIET*TIME

DIET	TIME	MEAN	HOMOGENEOUS GROUPS
2	0	0.1977	I
1	2	0.1964	I
1	9	0.1961	I
1	4	0.1959	I
1	0	0.1933	I
2	2	0.1903	I
1	6	0.1840	I
2	6	0.1800	I
2	4	0.1760	I
2	9	0.1743	I

THERE ARE NO SIGNIFICANT PAIRWISE DIFFERENCES AMONG THE MEANS.

CRITICAL F VALUE		2.153
REJECTION LEVEL	0.050	
CRITICAL VALUE FOR COMPARISON		0.0356
STANDARD ERROR FOR COMPARISON		8.096E-03