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
© Copyright by Sharon Jean Tate (2002)

Abstract:
no abstract found in this volume

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-BOZEMAN
Bozeman, Montana

May, 2002

© COPYRIGHT

by

Sharon Jean Tate

2002

All Rights Reserved

N378
T1879

APPROVAL

Of a thesis submitted by

Sharon Jean Tate

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

Dr. Christina Campbell Christina Campbell 5-16-02
(Signature) Date

Approved for the Department of Health and Human Development

Dr. Ellen Kreighbaum Ellen Kreighbaum 5/16/02
(Signature) Date

Approved for the College of Graduate Studies

Dr. Bruce McLeod Bruce R. McLeod 5-20-02
(Signature) Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature Shawn Tate

Date 5/17/02

ACKNOWLEDGMENTS

I would like to express my sincerest thanks to Dr. Christina Campbell, my advisor and friend, for her encouragement and support over the past two and a half years. I know that I would not be where I am today without you; you pushed me to dig deep within myself and do the best that I possibly could every step of the way, for that I am truly indebted to you.

I owe thanks to so many instructors here at Montana State University who have provided me with a base of knowledge that has allowed me to achieve my goals. But, I would especially like to thank Dr. Bettie Stanislao and Dr. Mary Miles for serving on my graduate committee and for their support and guidance. I also owe a special thank you to Dr. Daniel Heil and Dr. Deborah King for their assistance with data analysis. A sincere thank you is also owed to Pamela Harris for supporting and encouraging me throughout my education here.

Great appreciation goes to Kimmer Penniman, Kristen Tate, Sam Glueckert, Bobbi Miller, and Danielle Dufner for their assistance with study days. Also, thank you to all of the subjects, I could never have done this without you!

I am especially grateful to Shelly Hogan, if we were not going through this together, I don't what I would have done. Thank you for you friendship and understanding, not to mention the many cups of coffee!

Finally, a big thank you to Ted Freeman who took care of me (and my dog), supported me and put up with me throughout this process!

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).

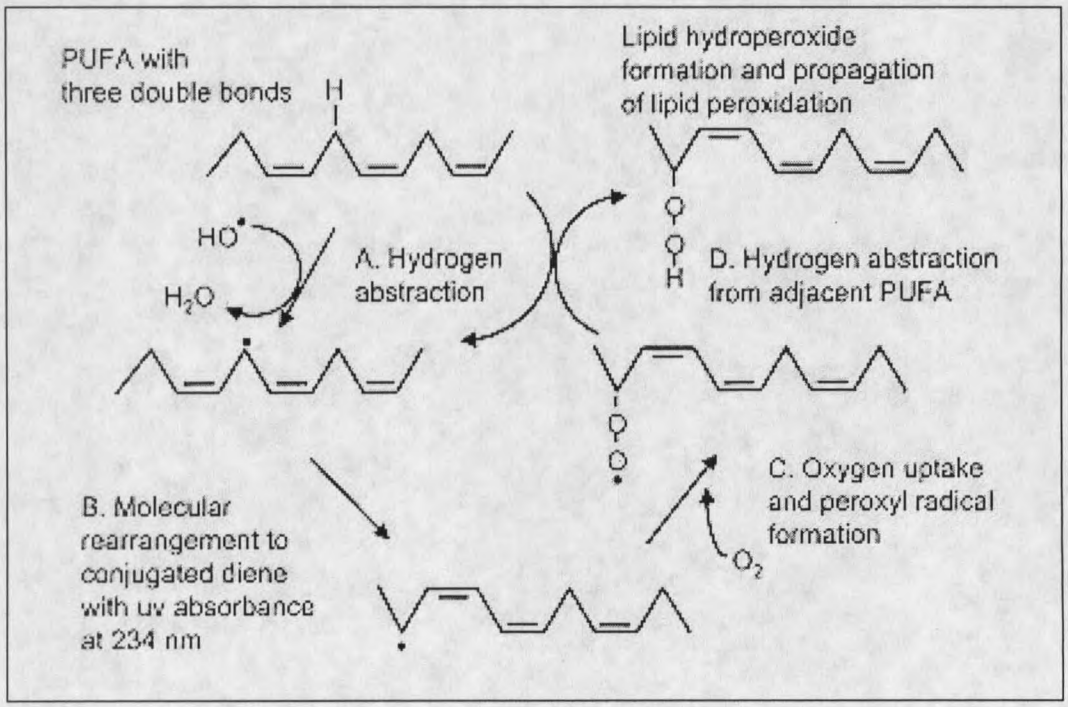

Figure 1. Lipid Peroxidation

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.

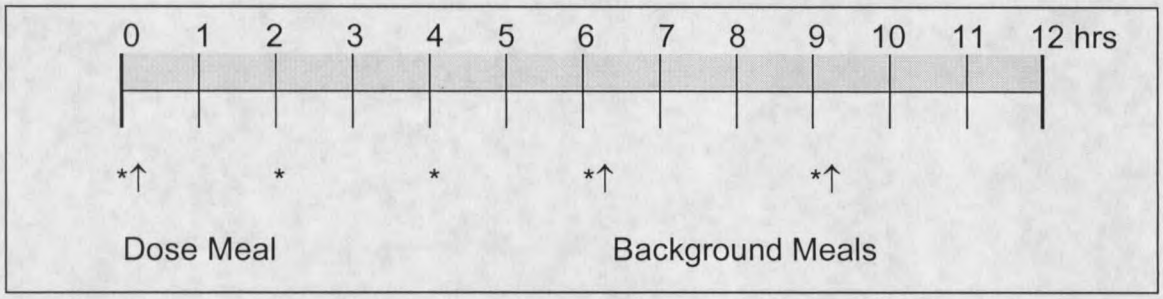

Figure 2. Timeline (* = point of analysis)

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