The effect of isoflavone supplementation on cardiovascular disease parameters in men undergoing 80% VO_2pk exercise.

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Thesis submitted to the Faculty of the department of Human Nutrition, Foods, and Exercise at theVirginia Polytechnic Institute and State University in partial fulfillment of the requirements for the completion of the degree of

Masters of Science

in

Nutrition

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May 28, 2002

Blacksburg, Virginia

Funded by the Virginia Soybean Board

Keywords: cardiovascular disease, soy, isoflavones, antioxidants, LDL oxidation, oxidative stress

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Abstract

Atherosclerosis, one of the major causative factors of cardiovascular diseases (CVD), is thought to be initiated by oxidative stress. Particular attention has been paid to the atherogenic effects of oxidative damage on low density lipoproteins (LDL). Current research shows that dietary antioxidant supplementation protects against oxidative stress, and therefore may present preventative measures and treatments for patients with diseases influenced by oxidative stress. Isoflavones found in soy, such as genistein and daidzein are reported to have potent antioxidant properties and have been shown to inhibit LDL oxidation *in vitro*. Although there is a strong base of data that supports the correlation between soy consumption, cholesterol reduction and cardiovascular protection, it remains to be elucidated whether it is the soy protein, the isoflavone, or a combination of both that confers benefits. This study investigated the effect of isoflavone supplementation on the following parameters: plasma genestein levels, oxidized LDL levels, plasma cholesterol, vitamin E, and C-reactive protein. Elevated serum cholesterol and C-reactive protein (CRP) have been identified as risk factors for cardiovascular disease. In a randomized, double-blind, placebo-controlled study, 150 mg/d isoflavone was supplemented for four weeks by 30 healthy, yet sedentary male subjects who underwent 30 minute exercise sessions at 80% VO_2pk before and after a 28 day period of supplementation. The purpose of the exercise was to induce oxidative stress. The average plasma genistein and daidzein concentrations increased significantly after isoflavone supplementation from 0 ng/ml to 561.6 ± 39.3 and 466.3 ± 35.5 ng/ml (SE) respectively ($P < 0.0001$), compared to 0 ng/ml in the placebo group throughout the study. There was no significant beneficial effect of isoflavone supplementation on oxidized LDL, plasma vitamin E concentrations, total cholesterol, LDL, HDL, or triglycerides. Isoflavone supplementation resulted in an average increase in CRP levels by 44% ($P = 0.014$), which was opposite from expectations. This study supports the theory that it may not be soy isoflavones alone that benefit lipid profiles, or offer protection from oxidative stress.

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Chapter 1

Introduction

Cardiovascular diseases (CVD) are the leading causes of death in the United States. CVD include coronary heart disease, stroke, and peripheral vascular disease, all of which are clinical expressions of advanced atherosclerosis. The oxidation of unsaturated lipids and cholesterol esters in the arterial epithelium and in the low density lipoprotein (LDL) particle is a substantial contributor to the pathogenesis of atherosclerosis (Esterbauer, 1992). Evidence suggests that antioxidants protect against oxidative damage in humans, thereby decreasing atherosclerotic development and lowering the risk of CVD (Esterbauer, 1992). Due to their antioxidant qualities, soy isoflavones have recently been a topic of research in this area and studies show promise for CVD protection.

The human body is well protected against normal oxidation with an antioxidant defense system comprised of enzymes such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), and also of numerous endogenous and exogenous antioxidant compounds such as glutathione (GTH), vitamin E (Vit E), and vitamin C (Vit C). Antioxidants are compounds that scavenge free electrons and reduce dangerous and reactive free radical molecules without becoming reactive themselves (Esterbauer, 1992). However, if the body's defenses are compromised such as in illness, chronic inflammation, sedentary lifestyles, strenuous exercise, stress, or old age, the body's antioxidant defenses may become overwhelmed, creating a situation termed oxidative stress. In times of oxidative stress, arterial tissues may be damaged, and oxidized molecules and particles, such as oxidized LDL (ox-LDL) will accumulate, initiate an inflammatory response, and lead to atherosclerotic lesions in the arteries (Hornstra, 1998).

Not surprisingly, antioxidants demonstrate interlocked, or synergistic reactions, meaning that one antioxidant is able to rejuvenate another upon depletion. Vitamin C for example, can reduce the oxidized vitamin E radical back to the antioxidant form, thereby sparing the *in vivo* supply of vitamin E and demonstrating that the effectiveness of one antioxidant may be modulated by other internal and dietary antioxidants (Kagan, 1998). Consequently, antioxidant power may be enhanced through dietary antioxidant intake; antioxidants will work together to reduce oxidation in their respective compartments as well as through mutual rejuvenation.

Vitamin E is thought to be the most effective lipid-soluble antioxidant in our cells and many studies have demonstrated its ability to reduce LDL oxidation (Meydani, 1993). Therefore, while it is important to increase all antioxidant levels, it is also essential to maintain adequate vitamin E levels. This may be accomplished not only by vitamin E supplementation, but by antioxidant supplementation in general due to antioxidant recycling.

Antioxidants have been identified in a number of foods, including fruits, vegetables, and whole grains. One subclass of dietary antioxidants are the isoflavones, for which the most significant dietary source is the soybean. Soybean isoflavones have been of special interest in current research due to their potency and evidence for disease prevention (Gooderham, 1996). Genistein has been shown to be the most potent soy isoflavone antioxidant. This fact, along with the synergistic interactions of antioxidants makes genistein supplementation a prime candidate for prevention and reduction of atherosclerosis development. Antioxidant activity of genistein has been observed in humans *in vitro* and in animals, the *in vivo* antioxidative capability of genistein in humans still lacks solid evidence.

Several markers indicate increased risk of CVD or of a cardiovascular event. These include elevated cholesterol levels, and C-reactive protein (CRP) levels. Elevated total cholesterol (TC), LDL, and CRP levels have all been identified as causing oxidative damage, thereby promoting atherosclerosis development. Conversely, elevated high density lipoprotein (HDL) levels exhibit a protective effect against atherosclerosis development. Current research is attempting to demonstrate that reduction of CVD risk factors through antioxidant supplementation will reduce atherosclerosis development and will therefore reduce the risk of CVD.

Hypotheses

This study investigated the effect of soy isoflavone supplementation on CVD risk parameters on an exercise induced oxidative stress state. The healthy, yet sedentary male subjects consumed isoflavone tablets for 4 weeks. The following CVD risk parameters were measured: serum Genistein levels, oxidized LDL levels, ferric reducing ability of plasma (FRAP), serum cholesterol, vitamin E, and C-reactive Protein. The hypothesis is that isoflavone supplementation will protect against *in vivo* oxidation due to a 30-minute session of exercise at 80% VO_2pk. Specific hypotheses are stated below:

1. One 30 minute exercise session at 80% VO_2pk will induce oxidation of plasma LDL

2. One 30 minute exercise session at 80% VO_2pk will elevate cholesterol, and CRP levels.

3. One 30 minute exercise session at 80% VO_2pk will reduce plasma antioxidant levels.

4. Isoflavone supplementation will increase plasma genistein concentration.

5. Isoflavone supplementation will reduce the oxidation of plasma LDL.

6. Isoflavone supplementation will prevent the reduction of antioxidants in the blood, such as vitamin E.

7. Isoflavone supplementation will reduce heart disease risk factors such as plasma cholesterol, C-reactive protein (CRP), and FRAP.

8. Isoflavone supplementation will prevent the increase of CVD risk factors after one 30 minute exercise session at 80% VO_2pk.

Assumptions

1. Bioavailability of isoflavones will not vary among the subjects.

2. The distribution of VO_2pk levels of the subjects between genistein and placebo groups will be homogeneous (controlled for by matched pairs).

3. The subjects will have similar antioxidant defense systems against oxidation.

4. All subjects will maintain their dietary habits and life styles throughout the study.

Limitations:

1. Daily food consumption will not be controlled in the subjects.
2. Bioavailability of isoflavones may vary in humans depending on gastrointestinal bacteria.
3. Actual reactive oxygen species (ROS) production/levels cannot be measured.

Chapter 2

Literature Review

Atherosclerosis: Development and Consequences

Heart Disease and Atherosclerosis

Advanced atherosclerosis is one of the major causative factors of cardiovascular diseases (CVD). The major initiator of atherosclerosis is oxidative stress by way of endothelial injury and LDL oxidation; ox-LDL is the major pathologic player in atherogenesis. LDL is oxidized, deposited and retained in the arterial intima where it is ingested by macrophages and by some smooth muscle cells (SMC). These lipid laden cells lose their characteristic properties and become foam cells. Concurrently, the injured endothelium changes its morphology and produces cytokines, adhesion factors, growth factors, and other molecules that encourage SMC, platelet, and monocyte migration into the growing lesion. This combination of alterations in the intima stimulates an inflammatory response and becomes a fatty streak that characterizes atherosclerotic lesions (Anderson, 1999). The lesion initially grows toward the adventitia to a critical point, where it expands into the lumen, becomes fibrous and calcified and eventually forms a hardened plaque, presenting a site for platelet aggregation and consequential clotting (Berliner, 1995). These lesions may either cause blockage in the artery at the site of build up, or the plaque may rupture causing a thrombus that could cause infarctions in other areas such as in the brain or heart causing stroke or heart attack respectively. Therefore, the development of atherosclerotic plaque increases the risk of thrombosis and results in CVD and possibly death. An abundance of research today focuses on the prevention of atherosclerosis via protection of the arteries from oxidation, monocyte aggregation and consequent plaque development.

Atherosclerosis and Oxidative Stress:

Atherosclerosis is generally recognized as being initiated by oxidative stress, a biological state in which oxidants abound, antioxidant defenses are overwhelmed, and essential macromolecules become oxidatively damaged (Naito, 1993). Oxidants may result from an overworked metabolism, from activation of specialized enzymes, or from free metal ion catalysts. During normal, aerobic metabolism, a small percentage of the electron flow through the electron

transport chain becomes uncoupled and reacts directly with oxygen. This leads to the production of oxygen centered free radicals, or reactive oxygen species (ROS) (Acworth, 1995). Proteins in the plasma membranes of cells such as NADPH oxidase have also been found to produce ROS (Meyer, 2000). Also, free metal ions may be released from their carrier proteins and consequently catalyze ROS production. For example, ferritin has been shown to release free ferric iron, catalyzing hydroxide ion production in the endothelial cell (EC) (Toshimi, 2002). ROS are highly reactive, reduced, usually free radical compounds that include superoxide (O_2°-), hydroxide ion (OH°-), and hydrogen peroxide (H_2O_2). Proteins, lipids, and DNA are all subject to oxidation, mostly by one-electron ROS attack on double bonds. It is the oxidation of endothelial cell molecules and of LDL particles that initiate and promote atherogenesis.

Endothelial Injury

Atherosclerosis is a cascade of events that is initiated by oxidative stress, resulting in oxidized low density lipoprotein (ox-LDL) particles and endothelial dysfunction or injury. Specific areas of the endothelium appear to be more injury and lesion prone than others, such as branching points in the arteries, which are approximately four times more permeable to LDL than non-branched areas (Schwenke and Carew, 1989). These focal leaky spots attract inflammatory proteins and prolonged leakiness can cause impaired tissue functioning (van Hinsbergh, 1997).

Ross (1986) presents a hypotheses in his review that there are two major pathways to endothelial injury and that the endothelial cells (EC) responds to this injury. The first pathway is that of obvious or direct injury to the EC. This would include denudation, or destruction of the EC layer by mechanical means, high LDL levels, elevated homocysteine levels, immunologic factors, toxins or viruses. The second pathway to endothelial injury is EC stimulation. The EC remain intact, yet are stimulated to change their chemical and protein expression by atherogenic levels of circulating LDL (Ross, 1986). Atherogenic levels of LDL stimulate the production of growth factors from the EC, causing changes in the EC morphology, including alteration of their plasma membrane content. Increased production of growth factors by the EC also stimulates smooth muscle cell (SMC) migration into the arterial intima (Ross, 1986). Furthermore, atherogenic levels of LDL have been found to affect arterial smooth muscle relaxation and

contraction both directly and indirectly. Ox-LDL has been suggested to impair the activity of nitric oxide (NO) synthase and to inactivate NO, the SMC relaxing factor (Luscher, 1997).

It is hypothesized that atherogenic levels of LDL stimulate NADPH oxidase (and other superoxide producing oxidases) in the EC plasma membrane via a cell signaling pathway that includes the LDL-receptor and arachadonic acid (Meyer, 2000). At low levels, superoxide and other ROS produced may act as second messengers, regulating gene expression. However high levels of ROS create oxidative stress and oxidative damage (Trupaev, 2002). A prime example is the reaction between superoxide and nitric oxide (NO). The superoxide produced by NADPH oxidase may react with NO produced by the EC to decrease arterial smooth muscle relaxation. In abundance, this may contribute to the stiffening seen in atherosclerotic arteries (Harrison, 1997).

LDL Uptake into the Arterial Intima

The arterial endothelial cells work to maintain circulating LDL homeostasis. When atherogenic levels of LDL are present, the endothelium deposits large amounts of LDL in the arterial intima. LDL deposition in the intima seems to be due to a combination of factors, including increased permeability of the EC, retention of the LDL in the intima, and the low degradation rate of LDL. Studies have indirectly and directly proven that the majority of circulating LDL is vesicularly transcytosed across the endothelial cell, independent of the LDL receptor (LDL-R) pathway (Born, 1994; Snelting-Havinga, 1989). Vasile et al. (1983) demonstrated duel mechanisms for LDL uptake and deposition by the EC. i) The receptor mediated transport describes the high affinity uptake of LDL by the LDL-R on the surface of the EC. This pathway is not affected by increased LDL concentration and it is therefore hypothesized that this pathway works to secure the EC need for cholesterol. ii) Fluid-phase endocytosis is a low affinity, receptor independent transcytosis by EC plasmalemmal vesicles. This non-saturable transport delivers the LDL to the arterial intima, allowing other cells access to LDL and cholesterol. The authors hypothesize that LDL-R independent transport also monitors and regulates plasma LDL accumulation. The increased permeability by receptor independent transport is supported by van Hinsbergh (1997); endothelial permeability is directly related to hydrostatic and osmotic pressure.

LDL Retention and Oxidation

During the traverse across the EC, the LDL may be exposed to ROS produced by mitochondrial metabolism leakage, to hydroxal radical whose formation is catalyzed by free metals released from their carrier protein or especially to superoxide that is produced from NADPH oxidase in the EC plasma membrane (Meyer, 2000; Toshim, 2002). Consequently, the LDL deposited in the arterial intima may be partially or fully oxidized.

Once in the intima, Schwenke and Carew (1989) demonstrated that LDL's retention time and rate of degradation account for the large accumulation in the intima. Further, LDL has been observed to bind to collagen fibers in the intima, where it becomes trapped (Nievelstein-Post, 1994). In the intima, LDL may be oxidized by endothelial, smooth muscle, and macrophage cells in co-operation with transition metals such as copper and iron. Free metal oxidation is not typically seen as a feasible method of *in vivo* oxidation, due to the small amount of free metal in circulation. However, recent research has shown that not only do plasma proteins, such as ceruloplasmin display pro-oxidant activities with the transition metal bound to specific sites (Mukhopadhyay, 1997), but these same proteins may release metals in a redox-active form under certain conditions (Patel, 1999). Thus, once in the intima, LDL are fully oxidized by EC, SMC, and macrophages in concert with free and bound metal ions.

Monocyte Recruitment

The recruitment of monocytes into the arterial intima is one of the most influential promoters of atherosclerotic lesion development. Monocytes are the major and often final oxidant for LDL, as well as being the major consumer of ox-LDL and consequently, are the major cell for foam cell formation. Monocytes are recruited to the arterial lesion by many factors. Upon injury, endothelial cells express monocyte receptors suggesting that they play an active role in the progression of atherosclerosis (DeCorleto, 1986). An injured or stimulated EC produces chemoattractant proteins for monocytes (Figure 1) (Meyer, 2000). However, possibly the most influential attractant for monocytes are the ox-LDL that are deposited in the arterial intima. The bulk of the macrophages are attracted after a significant amount of LDL oxidation and deposition in the intima (Berliner, 1995). Furthermore, the inflammatory process that is activated by ox-LDL deposition in the intima and endothelial injury, results in increased macrophage emigration

into the lesion (Ross, 1999). CRP that becomes deposited in the arterial intima also acts as a chemoattractant for the macrophage, due to the CRP receptors that are present on the surface of the macrophage (Torzewski, 2000).

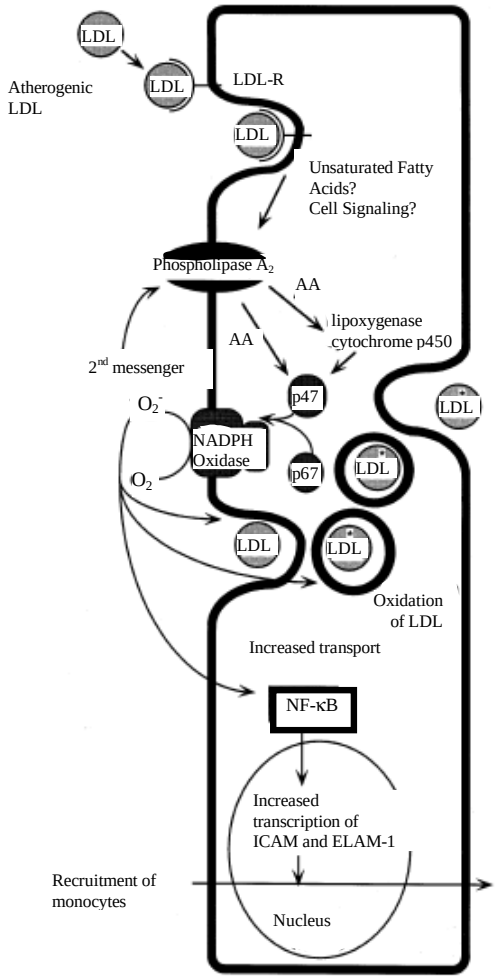

Figure 1. Model for the central involvement of NADPH oxidase in generation of an atherogenic EC. The ultimate result of atherogenic levels of circulating LDL is increased production of O_2^- by the EC-specific NADPH oxidase. The O_2^- then acts as a second messenger to further stimulate NADPH oxidase and NF-κB. Stimulation of the transcription factor NF-κB results in up-regulation of genes such as ICAM and ELAM-1 that serve to recruit monocytes to the site of injury.

Meyer and Schmitt, FEBS Letters. 2000;472: 1-4

Oxidized LDL:

Native LDL is comprised of an inner core and an outer core. The inner core consists mainly of the lipophilic components, cholesterol esters (CE) and triglycerides (TG), and some unesterified cholesterols (UC). The outer core that faces the plasma includes the amphiphilic components, namely sphingomyelin (SM), phosphotidylcholine (PC) and lyso-PC, some UC, and the one large apolipoprotein B (apo-B) (Figure 2) (Hevonoja, 2000)

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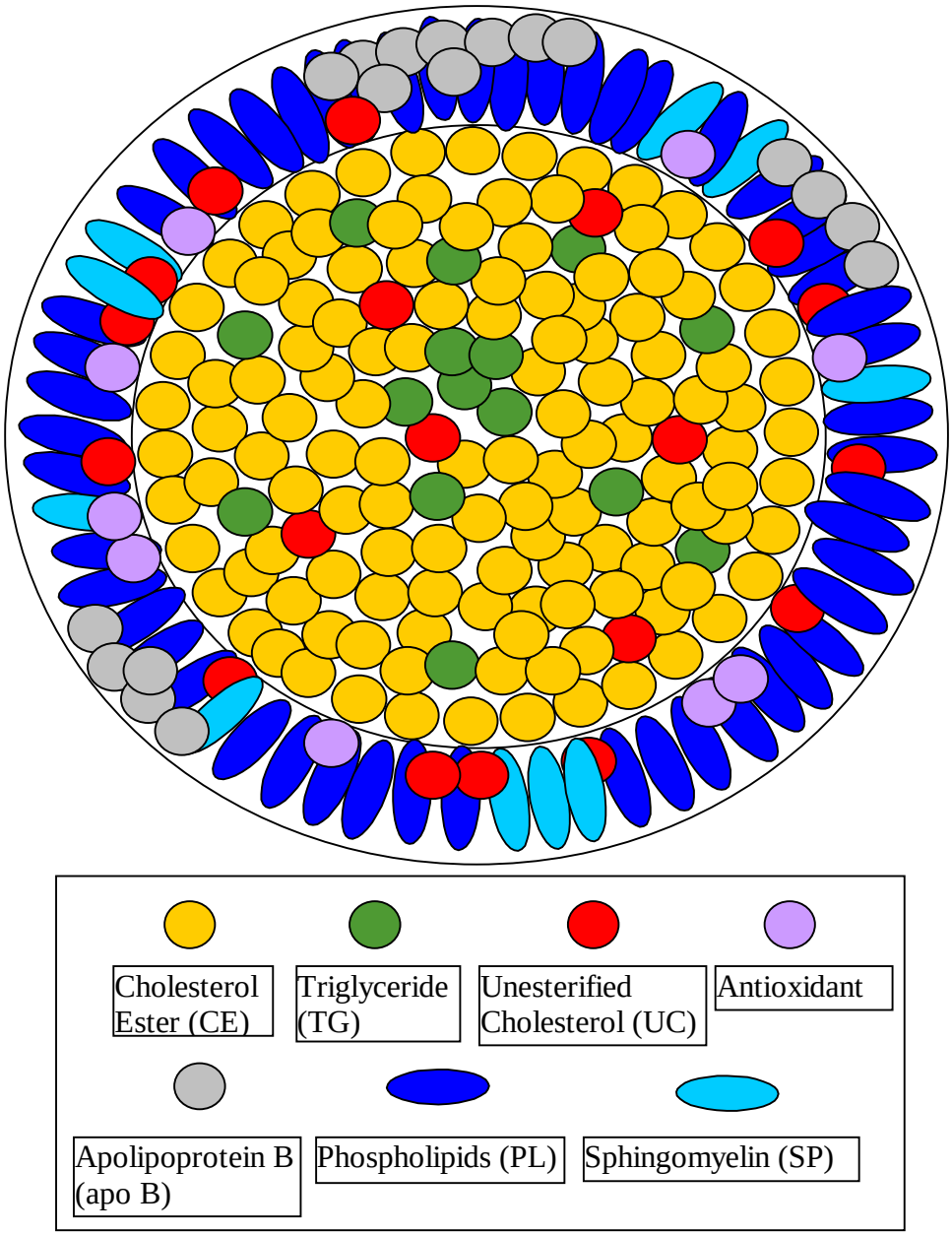

Figure 2. Schematic molecular model of an LDL particle at around body temperature (above the phase transition of core CEs). The depicted particle has a diameter of 20 nm, including a surface monolayer of 2 nm (yellowish background), and an average composition of 20% protein, 20% phospholipids, 40% CEs, 10% UC, and 5% TGs. The molecular components of the particle are drawn in both the correct percentages and size ratios. Note the different domains illustrated at the particle surface and the interpenetration of core and surface lipids. The individual molecules were built using Cerius 2 software (MSI Molecular Simulations Inc.). The chain compositions are illustrated as follows: SM (16:0) ; PC (16:0/18:2 v9;12) ; TG (16:0/18:2 v9;12 /14:0) ; CE (18:2 v9;12).
(Modified from Hevonoja, 2000)

It is well proven that ox-LDL plays a central role in the pathogenesis of atherosclerosis (Naito, 1993). Oxidation of LDL results in changes in its structure, function, and receptor recognition. After full oxidation of LDL antioxidants, its polyunsaturated fatty acids (PUFAs) are attacked and degraded to form aldehydes that then bind to the amino acid side chains of the apo B protein. Physiochemical changes present in ox-LDL include a total loss of endogenous antioxidants and PUFAs, a partial loss of phosphatidyl choline, cholesterol esters, and free amino groups on apo B. Oxidized LDL is further characterized by increased lysophosphatidyl choline, oxysterols, hydroxy- and hydroperoxy- PUFA's, conjugated dienes, maolndialdehyde and other aldehyde formation, and by increased electrophoretic mobility (Yuan, 1998).

Once oxidized, LDL is able to stimulate changes in the EC and SMC that surround it. A positive correlation has been found between the degree of atherosclerotic changes and the amount of lipoperoxides in the aortic walls (Naito, 1993). Ox-LDL contributes to macrophage growth and foam cell formation via multiple mechanisms. It displays chemoattractant properties for monocytes, and smooth muscle cells. In the arterial intima, ox-LDL is associated with an increase of endothelial cell adhesion molecules, an induction of macrophage signaling pathways, cytotoxicity, inhibition of anti-inflammatory cytokines, and stimulation of pro-inflammatory cytokines (Guy, 2000). Martens et al. (1999) have shown that modifications of the apo B protein stimulate macrophage growth.

The oxidation of LDL results in drastic changes in receptor recognition. Native LDL particles in plasma are recognized by multiple receptors on cells, especially by the LDL receptor (LDL-R) on macrophage cells. These regulated receptors allow macrophages to effectively rid the plasma of the LDL particles. However, once oxidized, the modified apo B protein in the ox-LDL particle is no longer recognized by the apo B/E receptor, but is recognized and taken up by the unregulated scavenger receptor on macrophage cells (Martens, 1999). This phenomenon results in ox-LDL and cholesterol ester-rich macrophages that develop into foam cells in the arterial intima.

Antioxidants:

Antioxidant Defense:

The body is well equipped with an antioxidant defense system comprised of enzymes (GPx, SOD, CAT) and antioxidant compounds (GTH, Vit E, Vit C, uric acid, and others) that serve to protect and repair the body's tissues as well as to render ROS inactive by scavenging their reactive, lone electron (Acworth, 1995). Antioxidant compounds, more than the antioxidant enzymes, regulate highly reactive radicals and oxygen species through the following one-electron transfer reaction to form an oxidized radical of low reactivity (Kagan, 1998):

$$ROO^{\circ} + AH \rightarrow ROOH + A^{\circ}$$

(ROO° = reactive radical; AH = antioxidant compound; ROOH = reduced, stable compound; A° = oxidized antioxidant, low reactivity)

Research has indicated that every antioxidant's physiological role is different according to its structural properties and its effectiveness depends on its reactivity towards ROS. Lipid soluble vitamin E for example, plays a large role in hydrogen atom donation to free radicals to protect cellular membranes, while water soluble vitamin C is an important general reducing agent toward a variety of oxidants (Pryor, 2000). Thus, protection of LDL from oxidation depends on the antioxidant's hydrogen donating potential, partition coefficient, and accessibility to the lipophilic phase of the LDL particle (Rice-Evans, 1993).

Antioxidants incorporated into the LDL particle are most likely found in its outer monolayer oriented towards the aqueous phase. Vitamin E (α-tocopherol) is the most abundant and most important antioxidant found in LDL in a ratio of about 5-9 molecules Vit E/ LDL particle; providing most of LDL's protection from oxidation (Meydani, 2001). The rest of the antioxidants in the LDL particle seem to proportionally represent the antioxidants found in plasma (Esterbauer, 1991).

Studies show that oxidation of the LDL PUFA's does not occur until after the oxidation of endogenous antioxidants (Esterbauer, 1989). Hence, dietary supplementation of antioxidants is a rapidly increasing area of research that is attempting to identify dietary antioxidants that

protect against LDL oxidation, thereby protecting against atherosclerosis progression (Esterbauer, 1992).

Antioxidant Regeneration

All antioxidants are instrumental in increasing oxidative resistance, either by direct interaction with oxidant species, or by synergistic/recycling actions between antioxidants. Compounds such as vitamin C, ubiquinone Q, and coenzyme Q are able to reduce oxidized α-tocopherol to its antioxidant state, extending its effectiveness (Kagan, 1998). Furthermore, the effectiveness of endogenous antioxidants is modulated by dietary antioxidant intake, highlighting the importance of total antioxidant supplementation. Vitamin C supplementation for example, has been shown to increase the function of vitamin E (Pryor, 2000; Hamilton, 2000). It has also been suggested that the soy isoflavones (genistein, daidzein, and equol) act synergistically with vitamin C *in vivo* (Hwang, 2000).

Vitamin E and LDL protection

Studies have shown a clear association between a decrease in CVD mortality and oxidative stress with increasing plasma α-tocopherol (vitamin E) concentration. Decreased CVD risk is associated with plasma α-tocopherol levels of 27-30 μM (Gey, 1993). This protection is most often attributed to α-tocopherol's effectiveness as a lipid soluble antioxidant. Its lipid solubility allows α-tocopherol to become incorporated into, and therefore to protect the LDL particle. Vitamin E is the major antioxidant found in the LDL particle (6 moles vitamin E/mole LDL) and is thought to be located in LDL's outer core where it interacts with oxidants in its environment as a chain-breaking antioxidant (Carr, 2000).

Vitamin E also offers protection against atherogenesis progression through stabilization and prevention of atherosclerotic plaque rupture. These mechanisms include inhibition of smooth muscle cell proliferation, modulation of platelet function, inhibition of thrombin generation, down-regulation of adhesion molecule expression, reduction of monocyte superoxide production, modulation of cytokine production, and mediation of NO through action on protein kinase C (Traber, 2001). Therefore, the maintenance of plasma α-tocopherol levels is ostensibly protective against atherosclerosis progression and CVD.

Isoflavones

Isoflavones are plant chemicals; a subclass of the flavonoids from the phytoestrogen group. Many biological properties have been attributed to the isoflavones, including estrogenic, antioxidant, antiallergenic, antiviral, anti-inflammatory, and vasodilation properties (Pietta, 2000). This study focused on their antioxidant and anti-inflammatory properties. Isoflavones have a polyphenol structure that enables them to perform multiple antioxidant functions. All polyphenols are reducing agents, hydrogen donating antioxidants, single oxygen quenchers, and possible transition metal chelators (Rice-Evans, 1996).

Isoflavones are found in soybeans, other legumes, soy foods, chickpeas, and clover. However, the most concentrated source of isoflavones is the soybean; soy foods contain ~0.1 - 3.0 mg isoflavone/g soy food (Setchell, 1999). Soy food consumption has been associated with favorable effects on lipids, lipoproteins, and possibly vascular function (Davis, 1999). This may be largely attributed to their potent antioxidant properties.

In the plant, the isoflavones genistin, daidzin, and in lesser amounts glycitin, are found in their glycoside forms (bound to a sugar). Research has shown however, that isoflavones are only absorbed in the gastrointestinal tract in their aglycone forms (genistein, daidzein, glycitein) (Figure 3) (Izumi, 2000; Sfakianos, 1997). Although the aglycones are the only form absorbed, the glycosides have proven to be very bioavailable (Setchell, 2001). Most of the ingested glycosides are hydrolyzed by glycosidases along the small intestine to their aglycone form (genistein, daidzein, glycitein), which are then absorbed. Once absorbed, the isoflavones undergo enterohepatic recycling and are glucuronidated in the intestine and in the liver (Setchell, 2001). The conjugated or glucuronidated isoflavones are thought to have little biological activity. The conjugation conversely, keeps the isoflavones in the enterohepatic cycle until intestinal glucuronidases hydrolyze and release them as biologically active aglycones. This allows a constant level of aglycones to be maintained for an extended period of time after ingestions (Setchell, 2001).

Isoflavones may also be metabolized further by gut microflora to phenolic acids, which may be reabsorbed in the intestines and which have shown antioxidant properties of their own (Pietta, 2000). Equol is an important biologically active isoflavone metabolite of daidzein.

Unfortunately, only about 1/3-2/3 of the population are able to convert daidzein to equol. In these equol converters, daidzein is modified to equol by microflora in the lower intestine and colon. The equol is then absorbed from the colon and is biologically active in the body. Recent research suggests that high dietary fat intake may decrease the capacity of the GI microflora to synthesize equol (Rowland, 2000).

The isoflavone is differentiated from the flavonoid by its location of the B-ring at the 3-position on the heterocyclic ring. This conformation has a great impact on the isoflavone's radical scavenging capacities. Of the isoflavones, genistein has displayed the greatest biological activity, as well as being twice as potent in its antioxidant properties as its flavone relatives (Pietta, 2000). Studies indicate that genistein inhibits LDL oxidation in a similar manner to vitamin E. Genistein supplementation inhibited isolated LDL oxidation *in vitro*, which suggests that it is incorporated into the LDL particle (Anderson, 1999, Tikkanen, 1998). Genistein also exhibits effective hydrogen peroxide (H_2O_2) scavenging capabilities, enhances antioxidant enzyme activities, and stabilizes lipid membranes thereby protecting against oxidation (Wiseman, 1996). In addition to its antioxidant properties, genistein has been found to inhibit endothelial cell proliferation, modulate transforming growth factor β, inhibit tyrosine kinase (and therefore inhibit growth factor production), interfere with platelet and thrombin action, and inhibit cell adhesion (Kim, 1998). Kirk et al (1998) suggest that genistein also increases LDL-receptor activity, thereby lowering cholesterol levels. Evaluating the role of genistein in the reduction of oxidative stress through antioxidant action is important in CVD risk factor reduction research.

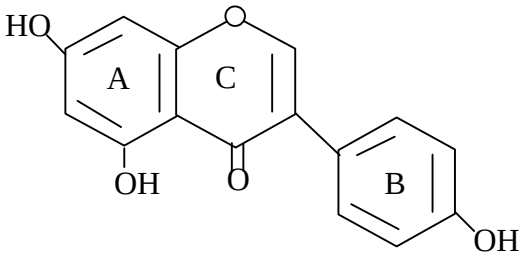

Figure 3. Chemical structure of isoflavone genistein, (aglycone form).

Risk Factors:

Cholesterol:

It is well proven that there is a direct association between CVD and elevated levels of total cholesterol (TC), elevated levels of low density lipoprotein cholesterol (LDL-C), and low levels of high density lipoprotein cholesterol (HDL-C) (Dwyer, 1995; Benlian, 2000; Law, 1994). Elevated TC and LDL-C indicate a significant increase in risk for CVD and has been deemed the primary risk factor for CVD (Wong, 1998). Conversely, studies have shown that HDL-C has an inverse association to heart disease, leading to the statement that a high TC:HDL-C ratio is indicative of increased risk of CVD (Sacks, 1998). Furthermore, research has demonstrated a causal relationship between CVD and LDL-C. Modified and ox-LDL have been shown to initiate and participate in atherosclerosis development (Dwyer, 1995).

Fortunately, there is strong evidence that low TC:HDL-C ratios contribute to decreased CVD risk, and lipid lowering interventions have proven to decrease CVD incidences (Hornstra, 1998; Kreisberg, 2002). Long term reduction in serum cholesterol concentration of 0.6 mmol/L (10%) lowers the risk of ischemic heart disease by 50% at age 40, and 20% at age 70 in men; similar results are seen in women (Law, 1994). Lipid lowering interventions usually begin with dietary interventions, and may progress to drug treatments, or even surgery. Dietary interventions include replacement of dietary saturated fats with carbohydrates and unsaturated fats, very low fat diets, increases in fruit and vegetable intake, and supplementation of antioxidants (Sacks, 1998). Research demonstrates that antioxidants prevent the oxidation of LDL, the main contributor to atherosclerosis development as well as attenuating NF-κB, which is stimulated in oxidative stress to increase gene expression (Rodriguez-Porcel, 2002).

As previously mentioned, soy foods are a rich source of the antioxidant isoflavones. Further, soy consumption in general has proven to be hypocholesterolemic, especially when accompanied by a diet low in saturated fat (Wong, 1998). Bakhit et al. (1994) demonstrated that as little as 25 g of soybean protein/day is associated with lower TC in individuals with initial cholesterol concentrations >5.7 mmol/L. Studies show that the isoflavones migrate with the protein fraction in soy foods (Setchell, 1999). Therefore, it has been difficult to study the individual effects of soy protein and soy isoflavones. Current research is attempting to delineate the functions of the two.

C-reactive protein:

Recent studies indicate that C-reactive protein (CRP) is one of the strongest univariate predictors for the risk of a cardiovascular event (Ridkir, 2000). CRP is a member of a unique plasma protein family, the pentraxin family, that includes CRP and serum amyloid P component (SAP). These proteins have been highly conserved throughout vertebrate evolution (Pepys, 1981). CRP is produced during the acute phase response to aid in detoxification of harmful substances in the blood (Figure 4). It specifically binds to phosphocholine-containing molecules (including plasma lipoproteins and microbial products) consequently neutralizing and detoxifying them via opsonization (Vigushin, 1993). CRP is consequently deemed one of the acute phase proteins. It is synthesized in the liver by hepatocytes upon stimulation by interleukin-6 (IL-6) (Heinrich, 1990). CRP is reportedly the best indicator of cardiovascular diseases and atherosclerotic development due to its high sensitivity to systemic inflammation. Consequently, high CRP levels persist in chronic inflammatory states, such as in atherosclerosis (Vigushin, 1993). The advancement of the atherosclerosis then correlates with CRP production (Libby, 2002). However, a review of the literature indicates that repeated CRP measures over time in the same patient would have a more highly correlated predictive value for CVD than one measure. This is due to the high potential for CRP to be elevated as a result of an unrelated inflammatory state such as a viral or bacterial infection (Levinson, 2002).

Interleukin-6, one of the pro-inflammatory cytokines, has been identified *in vitro* and *in vivo* as the major hepatocyte stimulating factor (Heinrich, 1998). It is synthesized by many different cells such as monocytes, endothelial cells, or fibroblasts that are at the site of infection or inflammation. IL-6 is released into the blood stream and binds to hepatocyte plasma membrane receptor complexes. The binding of IL-6 to the hepatocyte stimulates a very efficient amplification system, including stimulation of nuclear factor kappa B (NFκB) that induces CRP synthesis, allowing for a large amount of CRP production from a small amount of stimulus by IL-6 (Heinrich, 1990). NF-κB, constitutively inactive in the cytoplasm of cells, is activated by messengers such as cytokines and ROS. ROS produced during the inflammatory response activate NF-κB, which migrates to the nucleus and transcriptionally up-regulates genes for pro-inflammatory cytokine and acute phase protein production (Grimble, 1998).

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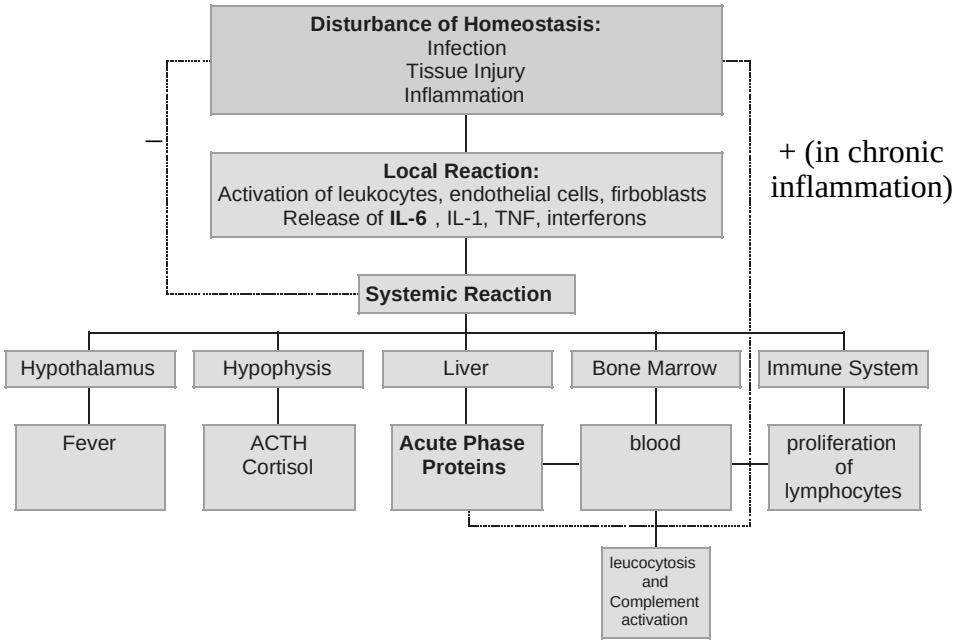

Figure 4. The acute phase response mechanism is shown here in this diagram. It is a nonspecific systemic immune reaction to any disturbance in homeostasis such as infection, injury, or inflammation (Heinrich, 1990).

Chronically elevated CRP levels enhance the development of atherosclerosis via two mechanisms. i) Recent research has demonstrated that the deposition of native CRP in arterial lesions creates a chemotactic gradient that draws monocytes into the intima. Monocytes and macrophages express a specific CRP-receptor (CRP-R) on their surfaces. Once they enter the endothelium of the artery, they migrate to and bind to the CRP within the intima (Torzewski, 2000). ii) CRP opsonizes ox-LDL. Tissue-deposited, extracellular LDL may be enzymatically modified to yield lipid droplets with a high content of free cholesterol (E-LDL) that have intrinsic complement-activating capacity. Each of the 5 subunits of CRP will bind to one of these E-LDL particles forming a stable CRP-5(E-LDL) complex. Once bound to the E-LDL, the complex is able to bind to the CRP-R on the monocytes. Monocytes ingest the CRP-5(E-LDL) complex, recycle the CRP-R and then bind to native CRP in the arterial intima (Bhakdi, 1999).

Finally there is an exacerbation of CRP's effect on atherogenesis by the complement system. Bhakdi et al. (1999) demonstrate production enhancement of one of the complement factors (C5) in response to elevated CRP levels. C5 then promotes up-regulation of CRP synthesis (Bhakdi,

1999; Szalai, 2000). Certainly, reduction of CRP synthesis and plasma levels in chronic inflammation would be a beneficial preventative strategy for reducing atherosclerosis.

Most successful nutrition interventions for improvement of immune function are antioxidant in nature (Meydani, 1998). Consequently, some studies have looked at modulations in CRP levels provoked by antioxidant supplementation; results seem promising. It is hypothesized that antioxidants may interfere with NFκB activation by ROS, thereby reducing cytokine and acute phase protein production. (Figure 5) Studies have demonstrated an inverse relation between plasma vitamins E and C and IL-6 levels (Grimble, 1998).

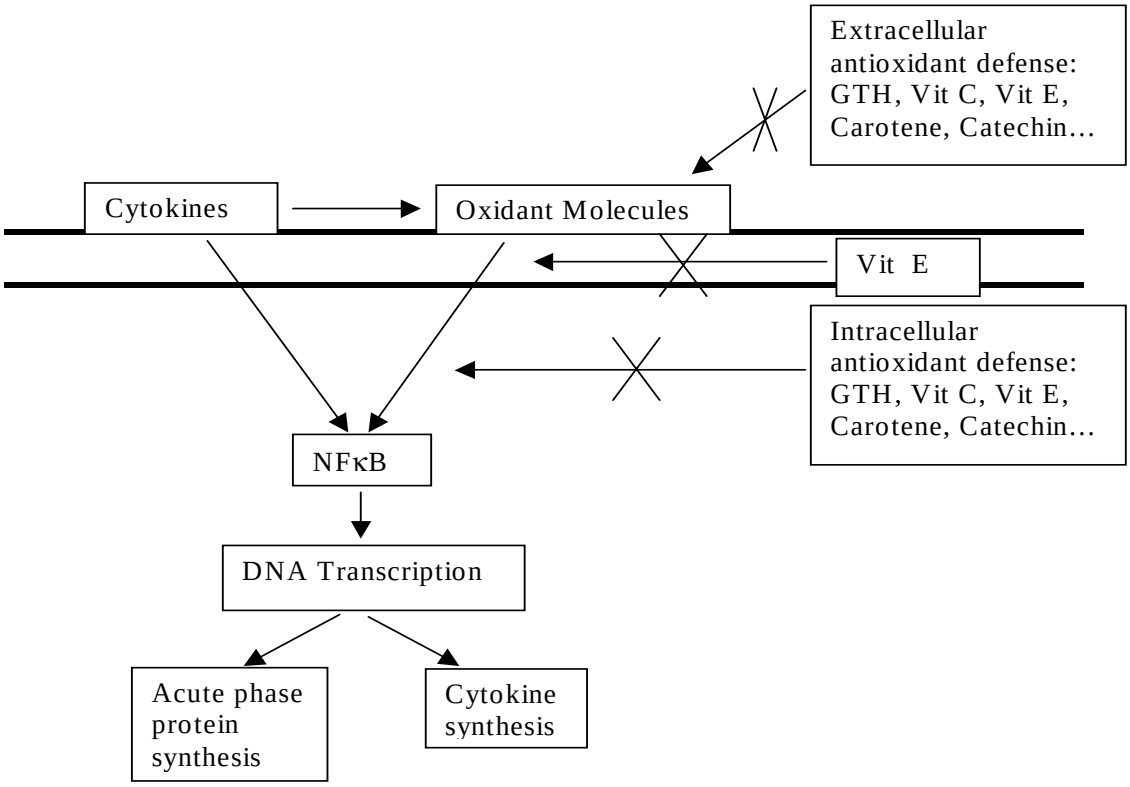

Figure 5. The interaction between pro-inflammatory cytokines, oxidants, and anti-oxidants, and their role in modulating the inflammatory response. (Modified from Grimble, 1998)

Elevated IL-6 has been noted in vitamin E and ascorbic-acid deficient rats (Grimble, 1998, Ikeda, 1998). Further, IL-6 and CRP levels have been found to decrease with various antioxidant supplementation. Upritchard et al. (2000) found that supplementation with 500 ml/d of tomato juice or 800 U/d of vitamin E for 4 weeks yielded a 49% decrease in plasma CRP levels in type 2

diabetes patients (p=0.004). In another study, α-tocopherol supplementation significantly reduced CRP and IL-6 levels in type 2 diabetes patients and in control subjects (Jialal, 2000). Intense exercise has been found to produce a mild inflammatory response in man that yields an increase in plasma IL-6 and acute phase protein concentrations. Studies demonstrate that this effect is inhibited by vitamin E supplementation (Grimble, 1998). No studies to date have looked at modulation of CRP levels by antioxidants from soy. However, as mentioned previously, the soy isoflavone, genistein, appears to have a similar mode of action as vitamin E and therefore may be an effective nutritional supplement for plasma CRP reduction, general systemic inflammation reduction, and atherosclerosis prevention.

Exercise:

Exercise as a model for oxidative stress:

It is well proven that a lifestyle rich in exercise decreases overall mortality, especially from heart disease (Myers, 2002). However, an acute session of physical exercise may be associated with a 10- to 20-fold increase in body oxygen uptake, causing antioxidant defenses may become overwhelmed, creating oxidative stress (Sen, 2001). The idea that strenuous exercise may be an acceptable model of oxidative stress began in 1978 with the observation by Dillard et al, that expired pentane increased after 70% VO_2pk exercise (Dillard, 1978). Due to the strong correlation between many diseases and oxidative stress, this model of exercise is thought to be a significant model for oxidative stress (Sen, 1995, Jenkins, 2000).

Studies suggest that increased oxygen consumption during exercise yields superoxide radicals and other ROS that result in lipid peroxidation and other damaging effects associated with oxidative stress (Evans, 2000). After one session of strenuous exercise, oxidative stress has been documented by fingerprint parameters such as lipid peroxidation and antioxidant status. Research has repeatedly demonstrated the increase in lipid peroxidation after acute strenuous exercise (Jenkins, 2000; Chen, 2002). Results of endogenous plasma antioxidant stores after exercise have been mixed. Hinchcliff et al. (2000) report a significant decrease in serum vitamin E concentration after an exercise run in sled dogs. However, Duthie et al. (1990) report transient increases in plasma uric acid, vitamin A, and vitamin C immediately post-exercise – increases in vitamin E did not reach significance. Supplementation with antioxidants such as vitamin C and

vitamin E has proven to decrease the lipid peroxidation and antioxidant store depletion seen after a bout of strenuous exercise (Evans, 2000).

Exercise and CVD Parameters:

Associations have been made between exercise and CVD parameters such as cholesterol, CRP, and homocysteine levels. Both cholesterol levels and CRP levels have been studied with the exercise model for oxidative stress. After prolonged strenuous exercise, triglycerides decreased by 39%, total cholesterol decreased by 9%, LDL cholesterol decreased by 11%, and apolipoprotein B decreased by 10%, whereas HDL cholesterol increased insignificantly in a study by Ginsburg et al. (1996). Studies show that sessions of strenuous exercise create an acute phase response (APR) similar to the APR seen in trauma or septic patients (Pedersen, 2000). This effect was also seen after a standard bicycle ergometric exercise (Ernst, 1991). After a marathon race, Weight et al. (1991) found a significant increase in plasma CRP immediately following, 24 h after, and 48 h after the race. In another study, IL-6, the cytokine responsible for the stimulation of CRP production, increased after a 30 minute run (Pedersen, 2000). However, the CRP response to short term, strenuous exercise with untrained subjects is little studied.

Chapter 3

Study Design and Methods

(Chen, 2001)

Study design:

This study examined CVD risk parameters in blood samples that were donated from subjects from a previous study completed in our lab, "Isoflavone supplementation modulated erythrocyte antioxidant enzyme activities and glutathione homeostasis in healthy young men undergoing 80% VO_2pk exercise," Chung-Yen Chen, 2001. The study, completed in the fall of 2000, followed the design of a double blind intervention with two supplementation groups, soy isoflavones and placebo (Chen, 2001). Thirty non-smoking, healthy, yet sedentary (less than 3 hours exercise / week) male subjects, aged 18-30 years, were paired according to VO_2pk as a measure of fitness level, then randomly assigned to each group (isoflavone supplement/placebo). The subjects' VO_2pks were determined at the beginning of the study. During a four-week supplementation period, the subjects consumed either 150 mg of high genistein soy isoflavone extract tablets or placebo tablets. They also underwent two sessions of 80% VO_2pk exercise on a cycle ergometer, once before and once after the isoflavone supplementation, with accompanying blood draws (before and after each exercise session). This study was approved by the Virginia Tech human studies review board.

Study Timeline:

The duration of the study was 35 days (Figure 6). All necessary information, and informed consent forms were presented to the subjects in an orientation meeting two weeks prior to the commencement of the study. The subjects were informed about possible risks and benefits of the study, dietary and physical activity records, the peak oxygen consumption (VO_2pk) test, submaximal exercises on the cycle ergometer, supplementation of isoflavone/placebo tablets, and blood collections. Prior to day one of the study, each subject had a baseline blood draw of 35 ml. Day one of the study commenced with the VO_2pk exercise test. In the first week, each subject completed the first set of 3-day dietary and physical activity records (two week days and one

weekend day). Isoflavone/placebo supplementation began on day 1 of the study, the same day as the first 30 minute 80% VO_2pk submaximal exercise on the cycle ergometer. The supplementation continued for four weeks. In the final week of the study, all subjects completed the second set of 3-day dietary and physical activity records. Blood samples were collected from each subject before exercise and five minutes after each submaximal exercise. Data collection from the subjects was complete after the final collection of blood samples.

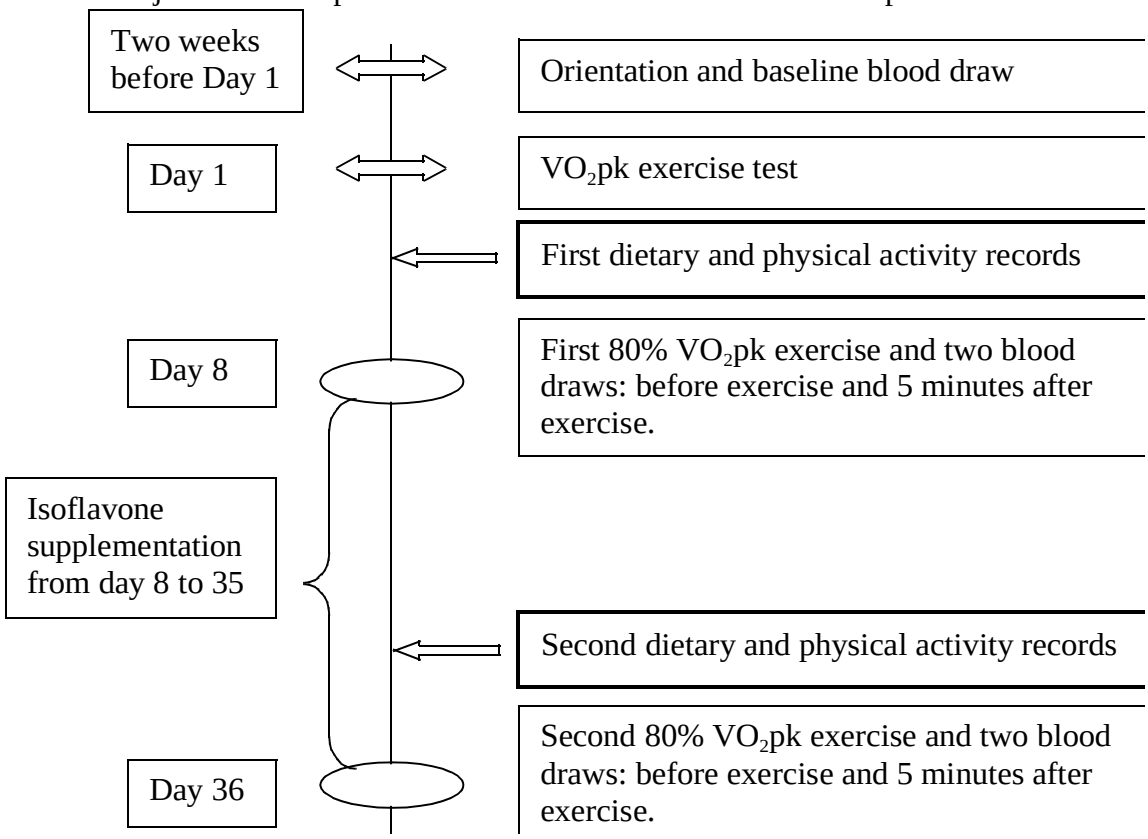

Figure 6. Timeline of Study.

Subjects (Chen, 2001):

Hormonal fluctuations found in women, such as estrogen with antioxidative activity, may affect the extent of oxidation of LDL and plasma (Tiidus, 1995). Therefore, men were selected as subjects due to the stability of their hormonal status. Thirty healthy, non-smoking, male college students, aged 18-30 years old, were recruited from the Virginia Tech campus area. A food and physical activity questionnaire was used to eliminate potential subjects consuming antioxidant supplements such as vitamins A, C, or E, and those who were physically active (>3 hours/wk of

exercise). A list of food items containing high isoflavone amounts was handed to all participants at the orientation meeting. They were instructed to abstain from eating high isoflavone food items during the study; these were all soy products, beans, peas, nuts, sprouts, seeds, and any other food that may contain any of these components (Tikkanen et al., 1998). A medical history questionnaire was used to eliminate potential subjects with any diseases such as asthma, arthritis, cardiovascular disease, diabetes, gastrointestinal problems, or thyroid disorders. All subjects were asked to sign an informed consent form before the beginning of the study. During the study, all subjects maintained their regular dietary and physical lifestyles.

Isoflavone supplementation (Chen, 2001):

All subjects were given two tablets of either isoflavone or placebo each day. The isoflavone tablets contained 75 mg of a high-genistein isoflavone extract supplied by Archer Daniels Midland (ADM), resulting in a daily intake of 150 mg of isoflavones. The isoflavone tablet was comprised of 50.8% genistin, 39.1% daidzin, and 10.1% glycitin. The level of supplemented genistein in this study was comparable to the 80.3 mg of genistein given per day in a study by Gooderham et al. (1996), which was found to significantly enhance plasma genistein concentration. Participant compliance was monitored by a self-marked calendar, in addition to regular contact via phone or e-mail, and delivery of tablets on a bi-weekly schedule.

Dietary and Physical Activity Records (Chen, 2001):

Subjects were asked to maintain their dietary and physical activity habits throughout the study, by consuming similar types and amounts of foods, and by maintaining a similar level of physical activity. In order to understand nutrient and food energy intake and physical activity, all subjects completed 3-day dietary food records and 24-hour physical activity records in the first and final weeks of the study. In both sets of records, the subjects recorded food intake and 24 hour physical activity for any two week days and one weekend day. The diets were analyzed by Nutritionist V (First DataBank Inc. San Bruno, CA.). Each subject was given a copy of his own first dietary and physical activity records to follow during the supplementation period.

Blood Collection (Chen, 2001):

There were a total of five 35-ml blood draws during the study (Figure 6). A baseline blood sample was taken before the commencement of the study and blood samples were collected from each subject before and five minutes after each submaximal exercise (before and after isoflavone/ placebo supplementation). Blood was drawn from an antecubital vein into four EDTA and one heparin containing tubes that were immediately placed in ice.

Plasma separation:

Plasma was separated from the whole blood by centrifugation at 3000 g at 4°C for 10 minutes. Plasma will be used for oxidized LDL, isoflavone, vitamin E, cholesterol, C-reactive protein, and FRAP determinations.

LDL separation from plasma (Chen, 2001):

The separation of LDL followed the method developed by Havel et al. (1955). The density of plasma was adjusted to 1.063 g/ml by the addition of 0.6530g potassium bromide to 2 ml of plasma. Two ml of density adjusted plasma was overlaid with 2.8 ml of 0.254 M NaCl in a centrifuge tube. Subsequently, all tubes were centrifuged at 800,000 rpm at 7°C for 45 minutes using a Beckman ultracentrifuge (Optima L-90K, Beckman Inc., Palo Alto, CA.). After centrifugation, the isolated LDL layer is visible as a yellow band in the upper portion of the tube. This fraction was isolated by a glass pipette, and was retained for further measurement of LDL oxidation.

Sample Storage:

All samples were kept in 1.5 ml microtubes and are frozen at –80°C until further analyses.

Parameters Measured in a Previous Experiment:

In vivo changes in various antioxidants and antioxidant enzymes due to isoflavone supplementation were studied to present a picture of the incurring oxidative stress from exercise and the subsequent oxidative protection by genistein supplementation. The following parameters

were measured: glutathione, vitamin C, catalase, superoxide dismutase, glutathione peroxidase, malondialdehyde, LDL oxidation, and isoflavone levels

Measurements

To generate a more comprehensive report of the *in vivo* happenings *in vivo* before and after exercise with genistein supplementation, this study measured a select number of oxidative stress parameters and known risk factors for CVD. The parameters include plasma isoflavone levels, ox-LDL, plasma vitamin E, FRAP, lipid profile (TC, HDL, and triglycerides), and C-reactive protein. Plasma isoflavone and vitamin E levels were measured by HPLC. LDL oxidation was performed on by agarose gel electrophoresis. FRAP was measured by a simple colorometric assay. Plasma cholesterol levels were determined by a well known enzymatic assay (Allain, 1974), and plasma C-reactive protein levels were determined using an in house ELISA.

1. Plasma Volume Change (Hematocrit)

A measure of hematocrit (Hct) at all blood draws was used to account for plasma volume change (% ΔPV) after exercise. Two capillary tubes were filled with whole blood. They were microcentrifuged for 15 minutes before the hematocrit values were read against the centrifuge's ruler. Plasma volume change between pre- and post-exercise was calculated by the following equation (Van Beaumont, 1973):

$$\% \Delta PV = 100/(100-Hct_{pre})*100*(Hct_{post}-Hct_{pre})/Hct_{post}$$

The concentrations of plasma parameter volumes were adjusted by plasma volume change before statistical analyses.

2. Plasma Genistein and Daidzein:

The determination of genistein and daidzein in plasma by HPLC was modified from a method by Wang and Murphy (1994) and Xu et al. (1994). One half ml of plasma and 19 µl was mixed with 10 ml of methanol. The mixture was centrifuged at 5000 x g for 10 minutes at 10°C. The methanol supernatant was decanted into a rotary evaporated flask. The plasma residue was re-dissolved in 10 ml of methanol and centrifuged again at 5000 x g for 10 minutes at 10°C. The

total 20 ml methanol supernatant, harvested from all extractions, was evaporated to complete dryness using a rotary evaporator at 37°C. Seven ml of 1 M sodium acetate buffer (pH 5.5) was added to rinse down the sides of the flask. The solution was transferred to a capped glass test tube and 50 µl of β-glucuronidase/sulfatase was added. The solution was then incubated at 37°C overnight (16-20 hours) in a dry heating block. After incubation, the solution was filtered through a solid phase extraction cartridge (J&W Scientific, Folsum, CA) and the isoflavones were eluted with methanol and dried under nitrogen. The residue was re-dissolved in 180 µL of 80% methanol and 100 µl of the extract was injected into HPLC for genistein and daidzein determination. The total recovery of isoflavone from plasma was 97.4±5.9% according to the HPLC fluorescin values. Unknown concentrations of plasma genistein and daidzein were calculated from the standard curves of genistein and daidzein.

3. Plasma LDL Oxidation:

The method of determining the relative electrophoretic mobility (REM) of low density lipoprotein (LDL) and oxidized LDL (ox-LDL) on an agarose gel was modified from Vieira et al. (1996) and Greenspan et al. (1993). Due to its more negative charge, ox-LDL has a greater REM than LDL. This method then allows the comparison of *in vitro* and *in vivo* oxidation of LDL. First, LDL was dialyzed with 10 µM EDTA phosphate buffer solution at 4°C for 24 hours. Then, 200 µl of 100 µM $CuSO_4$ and 600 µl distilled water was added to all tubes containing 200 µl of dialyzed LDL (approximately 1 mg/ml protein). These tubes were incubated at 37°C for 24 hours. After incubation, 23 µl of sample was mixed with 2.3 µl of 30% sucrose solution and loaded into a 0.5% agarose gel. The gel was run at a constant voltage of 125 V for 1 hour, stained with 0.1% Sudan black in 70% ethanol for 6 minutes, and destained with 1:1 ethanol: water solution overnight.

4. Plasma Vitamin E:

Plasma Vitamin E levels were determined using a kit from Chromsystems Instruments and Chemicals (Fraunhoferstrasse, Martinsried, Germany). Two hundred µl plasma was pipetted into a light protected reaction vial, along with 20 µl internal standard and 25 µl precipitation reagent 1. Each vial was vortexed for 30 seconds before adding 400 µl precipitation reagent 2. The vials were then centrifuged for 10 minutes at 9000 x g. 50 µl of the supernatant was injected into the

HPLC system for vitamin E determination. Unknown concentrations were calculated using the standard curve.

5. Ferric Reducing Ability of Plasma (FRAP)

Ferric reducing ability of plasma (FRAP) was measured according to the methods of Benzie and Strain (1996) and Langley-Evans (2000). A FRAP cocktail solution was made in a 10:1:1 ratio of the following reagents: 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), and 20 mM $FeCl_3*6H_20$. The cocktail solution was pre-heated to 37ºC. Nine hundred µl cocktail solution and 90 µl water was added to 30 µl sample or standard. The mixture was incubated at 37ºC for 4 minutes before the absorbance was read against a reagent blank at 593 nm. Antioxidant power of plasma was determined by a standard curve of ferrous sulphate.

6. Cholesterol Levels:

Cholesterol levels were measured using a well-known enzymatic assay developed by Allain et al. (1974). The lipid profile reagent kits were obtained from StanBio (Boerne, Texas). Total cholesterol was measured by incubating 10 µl serum with 1.0 ml of reagent for 15 minutes at room temperature. The reagent consists of cholesterol ester hydrolase and cholesterol oxidase. The absorbance was read at 500 nm against a reagent blank and concentrations of unknown samples were determined from a standard curve. HDL cholesterol was measured by precipitating out LDL and VLDL with Mg^{++}/dextran sulfate reagent according to Finley et al. (1978). The absorbance of the remaining supernatant was measured according to the previous Allain method. LDL was determined by a calculation from the total and HDL cholesterol measurements. Plasma triglycerides were measured by incubating 10 µl serum with 1.0 ml of reagent for 15 minutes at room temperature. The reagent consists of 4-aminoantipyrine, 4-chlorophenol, ATP, lipases, glycerol-kinase, glycerol-3-phosphate oxidase, peroxidase, and PIPES buffer solution, pH 7.5. The absorbance was read at 500 nm against a reagent blank and concentrations of unknown samples were determined from a standard curve.

7. C-reactive Protein:

CRP levels were analyzed by a sandwich ELISA reagent kit that was obtained from Alpha Diagnostic International, Inc. (San Antonio, TX). Plasma samples were diluted 1:100; 25 µl of sample was diluted with 2.5 ml of diluent or PBS buffer. Diluted samples were pipetted (10 µl) into CRP-Ab coated wells. One hundred µl of Ab-enzyme conjugate was added to each well and mixed gently. The plate was incubated at room temperature for 30 minutes. The wells were then aspirated and manually washed with dH_2O five times and were tapped over paper towels between washings. After washing, the 2 chromogenic substrates were added; 100 µl HRP-substrate solution A was added to each well, followed by 100µl HRP-substrate solution B in the same timed intervals. The wells were mixed gently before incubation for 10 minutes at room temperature. In the same timed intervals as above, 50 µl of stop solution (1N H_2SO_4) was added to all wells, which were mixed gently by swirling. The absorbance was read in a microplate reader at 450 nm and the concentrations of the unknown samples were calculated against a standard curve of pure CRP.

Statistical Analyses:

The effects of supplementation (isoflavone vs. placebo), exercise (pre- vs. post-) and time (day 1 vs. day 28) and their interactions were assessed by analysis of variance of a matched-pair repeated-measure (split-plot) mixed-model in two stages, and the statistical significance level was set at $P = 0.05$ (Sall et al, 2001).

The first stage was used to determine (i) whether there is a change in pre-exercise response level over the four-week period of supplementation and (ii) whether there is a change in response level due to exercise (pre vs. post). The linear model for the first-stage analysis used matched pairs of subjects as blocks and supplementation as the between-subjects (whole-plot) factor. The interaction between matched pairs and supplementation were the between-subjects (whole-plot) error term. Exercise (pre- vs. post-) and time (week 0 vs. week 4) were within-subject (split-plot) factors. The three two-way interactions and the three-way interactions were included in the first-stage model. A linear contrast was used to make the determination (i) above.

The second-stage was used to determine (i) the main effects of supplementation (isoflavone vs. placebo) and (ii) the interactions between supplementation and time (week 0 vs. week 4). The

linear model that was used for the second-stage analysis is a simplification of the first-stage model achieved by analyzing the post-exercise minus pre-exercise difference and thereby eliminating exercise as a factor. As in the first-stage model, matched pairs of subjects were used as blocks, supplementation was the between-subjects factor, time as the within-subjects factor, and the interaction between supplementation and time was included. Tukey's HSD procedure was used for multiple comparison (Sall, 2001).

Chapter 4

Results

General subject characteristics

Thirty healthy, college age, non-smoking, males participated in this study. They were divided into two groups and matched according to their fitness levels (VO_2pk). The two groups did not differ significantly in their mean age, BMI, or fitness levels ($P > 0.10$). They had a mean age of 20.7 years, mean BMI of 23.5 kg/m^2 and mean fitness level of 40.2 mg/min/kg (Table 1).

TABLE 1. Subject characteristics.

	Placebo			Isoflavone		
	N	**Mean**	**SE**	**N**	**Mean**	**SE**
Age (years)	15	20.7	0.19	15	20.7	0.30
BMI (kg/m^2)	15	23.1	0.35	15	23.9	0.30
VO_2pk (ml/min/kg)	15	40.2	1.32	15	40.1	1.32

Nutrient intake and plasma isoflavones:

Three-day dietary records were collected from the subjects before the first exercise session on day 1 and before the second exercise session on day 28 of the study. Nutrient composition of the diets was analyzed by Nutritionist V. The subjects in the isoflavone group consumed significantly less macronutrients and micronutrients in the fourth week of the study than they did in the first week (Table 3). This trend was not seen in the placebo group.

Exercise intensities of initial and final submaximal exercises:

There was a statistically significant increase between the intensities of the first and second submaximal exercise sessions ($P = 0.03$) (Table 2). Variation in exercise intensity is attributed to the individuals physical and mental exercise capabilities. Isoflavone supplementation had no effect on exercise intensities ($P > 0.10$).

TABLE 2. Difference in exercise intensities between first (Day 1) and second (Day 28) exercise sessions. (P = 0.03)

Exercise Session	N	Mean (% VO_2pk)	SE
Day 1	30	80.0	0.82
Day 28	30	81.4	0.79

TABLE 3. Dietary intake comparisons before each exercise session (Day 1 vs Day 28).

		Placebo					Isoflavone			
		Day 1		Day 28			Day 1		Day 28	
Nutrient	N	Mean	SE	Mean	SE	N	Mean	SE	Mean	SE
Calorie, kcal/d	15	2726[a]	220	2588[a]	245	15	2633[a]	196	2209[b]	208
Protein, g/d	15	108.0[a]	12.5	101.8[a]	10.8	15	110.3[a]	10.9	89.0[b]	8.9
CHO, g/d	15	374.8[a]	33.4	358.9[a]	37.1	15	348.1[a]	31.1	298.2[b]	31.6
Fat, g/d	15	85.1[a]	7.6	83.7[a]	9.3	15	91.2[a]	8.01	76.8[b]	9.5
Fiber, g/d	15	17.0[a]	2.2	22.6[a]	4.2	15	20.6[a]	2.9	17.0[b]	2.2
Vitamin A, RE/d	15	1184[a]	179	1199[a]	206	15	1314[a]	140	997[b]	175
Vitamin C, mg/d	15	163.4[a]	38.9	146.2[a]	29.6	15	169.5[a]	29.5	128.3[b]	24.7
Vitamin E, mg/d	15	9.8[a]	2.5	6.4[a]	1.1	15	8.6[a]	1.5	5.5[b]	1.0

[ab]Means in the same group with different letters were significantly different between the first and second exercises analyzed by a paired t-test, P<0.05

Isoflavones

Genistein and Daidzein

Plasma genistein and daidzein concentrations significantly increased in those subjects that were on the high genistein isoflavone (HGI) supplement for 4 weeks. The two-way interaction between time (day 1, 28) and supplementation (placebo, isoflavone), as well as the main effects of both time and supplementation alone, were highly statistically significant ($P < 0.0001$) (Table 4, Figure 7). The average plasma genistein and daidzein concentrations increased significantly after isoflavone supplementation from 0 ng/ml to 561.6 ± 39.3 and 466.3 ± 35.5 ng/ml (SE) respectively ($P < 0.0001$), compared to 0 ng/ml in the placebo group throughout the study. Neither the three-way interactions among exercise (pre, post), time (day 1, 28), and

supplementation (placebo, isoflavone), the two-way interaction between exercise and time, the two-way interaction between exercise and supplementation, nor the main effects of exercise were statistically significant (P > 0.10). Although the effects of exercise were not significant, the average isoflavone concentration did increase slightly after exercise from 499.3 ng/ml to 623.9 ng/ml and from 414.9 ng/ml to 517.6 ng/ml for genistein and daidzein respectively (Figure 7).

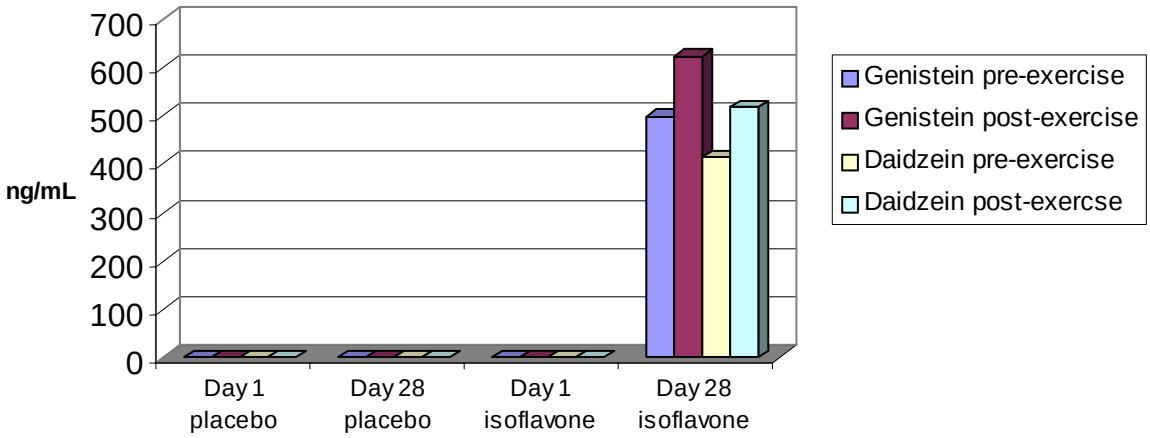

Figure 7. Isoflavone concentrations before and after supplementation (P < 0.0001)

Study Parameters

The means and pooled standard errors for all study parameters are shown in Table 14. Table 15 summarizes the results of the repeated-measures analysis of variance. For the statistically significant main effects and interactions, *post hoc* analysis of the relevant means are discussed in greater detail below. Any values that were effected by dehydration were adjusted according to plasma volume change using the equation (VanBeaumont, 1973):

$$\% \Delta PV = 100/(100-Hct_{pre})*100*(Hct_{post}-Hct_{pre})/Hct_{post}$$

Oxidative Stress Parameters

Oxidized LDL

The resistance of isolated LDL from plasma to *ex vivo* oxidation was analyzed by agarose gel electrophoresis. The main of effects of exercise (pre, post) were highly statistically significant (P = 0.0003) (Table 4). There were no statistically significant interactions or main effects of supplementation (placebo, isoflavone), or time (day 1,28) (P > 0.10) (Tables 14 & 15, Figure 9).

TABLE 4. Oxidized LDL's Relative Electrophoretic Mobility (REM) before and after exercise.

Exercise	N	Mean (REM)	Pooled SE
Pre	30	2.53	0.061
Post	30	2.85	0.061

Ferric Reducing Ability of Plasma (FRAP)

Total antioxidant potential of plasma was measured by a simple colorometric FRAP assay. The main effects of exercise (pre, post) were statistically significant (P = 0.0048) (Table 5). There were no statistically significant interactions or main effects of supplementation (placebo, isoflavone), or time (day 1, 28) (P > 0.10), nor was there a three-way interaction among exercise (pre, post), supplementation (placebo, isoflavone), and time (day 1, 28) (P > 0.10) (Tables 14 & 15, Figure 10).

TABLE 5. Plasma FRAP concentrations before and after exercise (P = 0.0048).

Exercise	N	Mean (μM)	Pooled SE
Pre	30	858.0	18.1
Post	30	932.0	18.1

The second stage of statistical analysis for FRAP showed a trend with the two-way interaction among supplementation and time (P = 0.081). The pre-exercise FRAP values were subtracted from the post-exercise values, thereby removing exercise as a factor from the analysis. This demonstrated that with the removal of exercise as a factor, the placebo group demonstrated decreased FRAP values while the isoflavone group demonstrated increased FRAP values after supplementation (Table 6, Figure 8).

TABLE 6. The effect of supplementation on the change in FRAP due to exercise (P = 0.081)

Supplementation	Time	N	Mean Change (post - pre-exercise) (μM)	Pooled SE
Placebo	Day 1	15	108.0	34.2
Placebo	Day 28	15	9.38	34.2
Isoflavone	Day 1	15	76.7	34.2
Isoflavone	Day 28	15	102.1	34.2

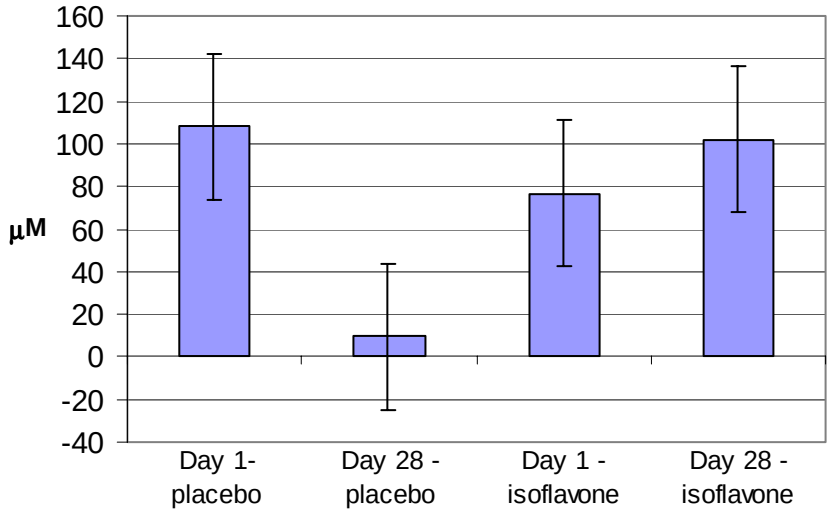

Figure 8. Effect of supplementation on the change in FRAP due to exercise. (P = 0.081)

Vitamin E

There were no statistically significant interactions or main effects of supplementation (placebo, isoflavone), time (day 1, 28), or exercise (pre, post) on plasma vitamin E concentrations (P > 0.10) (Tables 14 & 15, Figure 11).

Cardiovascular Disease Parameters

Lipid Profile

1. Total Cholesterol

There were no statistically significant interactions or main effects of supplementation (placebo, isoflavone), time (day 1, 28), or exercise (pre, post) on plasma total cholesterol levels (P > 0.10) (Tables 14 & 15, Figure 12). However, there was a trend toward increased plasma total cholesterol with exercise (P = 0.11) (Table 7).

TABLE 7. Effect of exercise on total cholesterol (P = 0.11).

Exercise	N	Mean (mg/dL)	Pooled SE
Pre-exercise	30	152.8	1.9
Post-exercise	30	148.4	1.9

2. LDL Cholesterol

There were no statistically significant interactions or main effects of supplementation, time (day 1, 28), or exercise (pre, post) on plasma LDL concentrations (P > 0.10) (Tables 14 & 15, Figure 13). There was a tendency for LDL concentrations to increase with time in all treatments (P =0.074) (Table 8).

TABLE 8. Effect of time on LDL concentrations (P = 0.074)

Time	N	Mean (mg/dL)	Pooled SE
Day 1	30	76.8	1.5
Day 28	30	80.6	1.5

3. HDL Cholesterol

There were no statistically significant interactions, or main effects among exercise (pre/post), time (day 1, 28), or supplementation (placebo, isoflavone) on HDL concentrations in the phase one analysis (P > 0.10) (Tables 14 & 15, Figure 14). There was a tendency for HDL concentrations to increase with exercise alone (pre vs. post) in all treatments (P = 0.072) (Table 9). There was also a tendency for HDL concentrations to change with time and supplementation (P = 0.094) (Table 10). The placebo group exhibited an increase HDL concentration (54.3 - 56.9 mg/dL) from day 1 to day 28, where the isoflavone group decreased their HDL concentrations (48.5 - 47.1 mg/dL) from day 1 to day 28.

The second stage of statistical analysis for HDL revealed a slightly statistically significant decrease in HDL concentration with time (P = 0.043). The pre-exercise HDL values were subtracted from the post-exercise values, thereby removing exercise as a factor from the analysis. This established that at the second exercise session (day 28 of study), all subjects had a smaller difference in HDL concentrations with exercise than at the first exercise session (day 1 of the study) (Table 11).

TABLE 9. Effect of exercise on HDL concentrations (P = 0.072)

Exercise	N	Mean (mg/dL)	Pooled SE
Pre-exercise	30	50.7	0.84
Post-exercise	30	52.8	0.84

TABLE 10. HDL concentrations before and after supplementation (P = 0.094)

Supplementation	Time	N	Mean (mg/dL)	Pooled SE
Placebo	Day 1	15	54.3	1.19
Placebo	Day 28	15	56.9	1.19
Isoflavone	Day 1	15	48.5	1.19
Isoflavone	Day 28	15	47.1	1.19

TABLE 11. Effect of time on change in HDL due to exercise (P = 0.043)

Time	N	Mean (mg/dL)	Pooled SE
Day 1	30	3.92	1.17
Day 28	30	0.42	1.17

4. Triglycerides

There were no statistically significant two- or three-way interactions or main effects of supplementation, time (day 1, 28), or exercise (pre, post) on plasma triglyceride (TG) concentrations (P > 0.10) (Tables 14 & 15, Figure 15). There was a tendency for TG concentrations to decrease with time in all treatments (P = 0.063) (Table 12).

TABLE 12. Effect of time on triglyceride concentrations (P = 0.063)

Time	N	Mean (mg/dL)	Pooled SE
Day 1	30	105.5	3.35
Day 28	30	96.6	3.35

C-Reactive Protein

Analysis of C-reactive protein revealed statistically significant interactions between supplement and time (P = 0.013) (Table 15). Linear contrasts revealed the difference of 0.426 mg/L between supplementation at day 1 was not statistically significant (t = 1.1593, P = 0.11), the mean *increase* from day 1 to day 28 of 0.505 mg/L within the isoflavone group was not statistically significant (t = 1.889, P = 0.062), and the mean *decrease* from day 1 to day 28 of 0.452 mg/L within the placebo group was not statistically significant (t = 1.691, P = 0.095) (Tables 14 & 15, Figure 16). The statistical significance of the interactions appears to be an artifact of the statistically significant (t = 2.352, P = 0.013) differences between the isoflavone and

placebo groups in the changes from day 1 to day 28 [+0.505 - (-0.452) = 0.956 mg/L] (Table 13). That is, the not-significant *increase* among isoflavone subjects and the not-significant *decrease* among placebo subjects added up to a significant difference in the differences.

TABLE 13. C-Reactive protein before and after supplementation (P = 0.013)

Supplementation	Time	N	Mean (mg/L)	Pooled SE
Placebo	Day 1	15	1.08	0.19
Placebo	Day 28	15	0.625	0.19
Isoflavone	Day 1	15	0.651	0.19
Isoflavone	Day 28	15	1.16	0.19

Table 14. Treatment means of all response variables.

Supplement	Time	Exercise	Hct	LDL - ox (REM)	Vitamin E (μM)	FRAP (μM)	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)	CRP (mg/L)
Placebo	Day 1	Pre	45.9	2.62	23.3	855.0	152.8	79.9	52.3	102.3	1.05
Placebo	Day 1	Post	47.1	2.95	25.4	963.0	155.4	76.8	56.3	102.2	1.10
Placebo	Day 28	Pre	46.2	2.47	22.4	867.5	153.5	77.0	56.4	100.8	0.59
Placebo	Day 28	Post	47.2	2.82	21.9	876.9	159.3	81.3	57.5	110.1	0.70
Isoflavone	Day 1	Pre	46.1	2.52	22.6	857.1	141.8	74.3	46.6	104.5	0.72
Isoflavone	Day 1	Post	47.2	2.81	22.8	933.8	147.4	76.0	50.5	104.7	0.59
Isoflavone	Day 28	Pre	46.0	2.51	22.7	852.4	145.6	80.1	47.3	91.1	1.03
Isoflavone	Day 28	Post	47.4	2.84	21.8	954.5	149.3	83.9	47.0	92.3	1.28
Pooled SE			0.455	0.122	1.96	36.2	3.75	2.98	1.69	6.70	0.268

Note: All treatment groups have an N of 15.

Hct = Hematocrit, LDL-ox = LDL oxidation resistance, FRAP = ferric reducing ability of plasma, TC = total cholesterol, LDL = low density lipoprotein cholesterol, HDL = high density lipoprotein cholesterol, TG = triglyceride, CRP = C-reactive protein

Table 15. ANOVA results for all response variables.

Main Effects	Interactions	Hct	LDL - ox (REM)	Vit E (μM)	FRAP (μM)	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)	CRP (mg/L)
Supplement		NS	NS	NS	NS	NS	NS	NS (P = 0.094)	NS	NS
Time		NS	NS	NS	NS	NS	NS (P = 0.074)	NS	NS (P = 0.063)	NS
Exercise		P = 0.0006	P = 0.0003	NS	P = 0.0048	NS (P = 0.11)	NS	NS (P = 0.072)	NS	NS
	Two-way S*T	NS	NS	NS	NS	NS	NS (P = 0.16)	NS (P = 0.094)	NS	P = 0.013
	S*E	NS	NS	NS	NS	NS	NS	NS	NS	NS
	T*E	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Three-way S*T*E	NS	NS	NS	NS	NS	NS	NS	NS	NS

Note: All treatment groups have an N of 15; NS = P > 0.05, however, P-value given if P < 0.20.

NS = Not significant, Hct = Hematocrit, LDL-ox = LDL oxidation resistance, FRAP = ferric reducing ability of plasma, TC = total cholesterol, LDL = low density lipoprotein cholesterol, HDL = high density lipoprotein cholesterol, TG = triglyceride, CRP = C-reactive protein

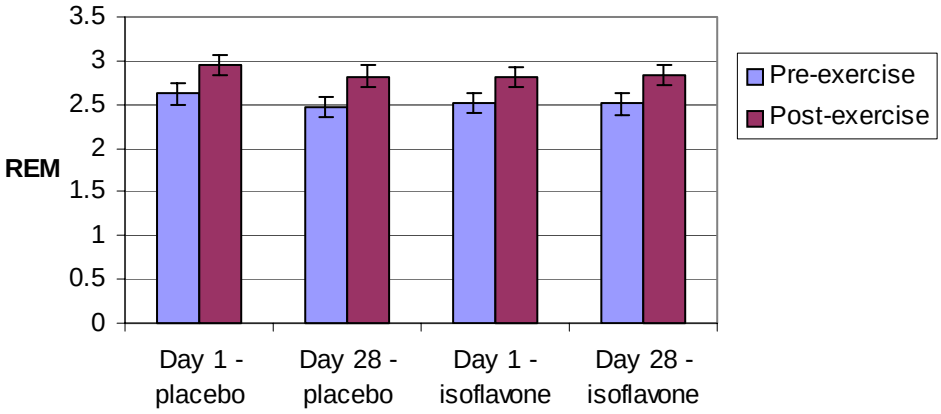

Figure 9. LDL's *ex vivo* oxidation (P > 0.10)

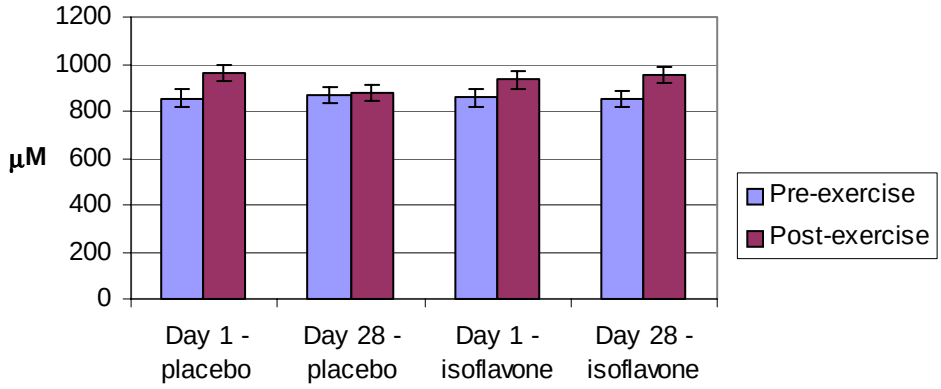

Figure 10. FRAP values throughout the study (P > 0.10)

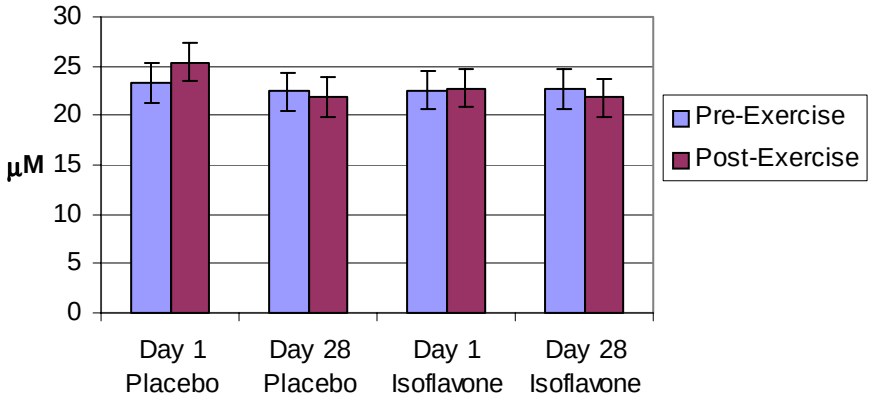

Figure 11. Vitamin E concentrations throughout the study (P > 0.10)

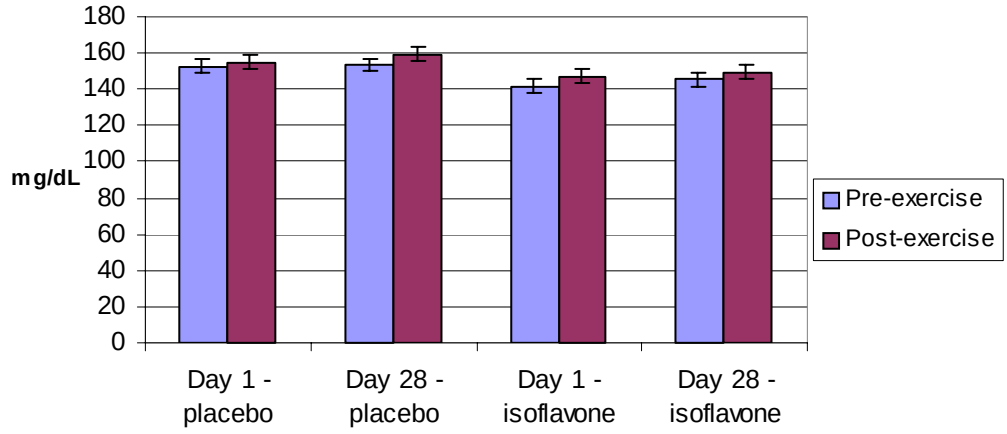

Figure 12. Total Cholesterol Concentrations throughout the study (P > 0.10)

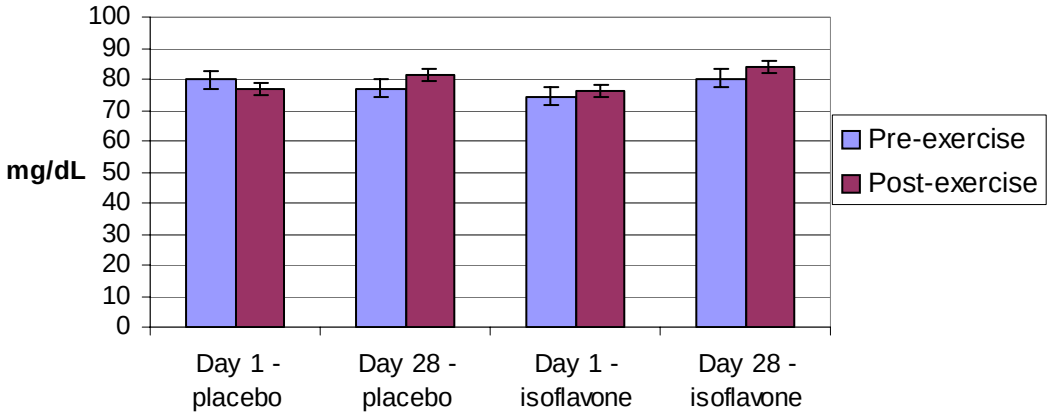

Figure 13. LDL concentrations throughout the study. (P > 0.10)

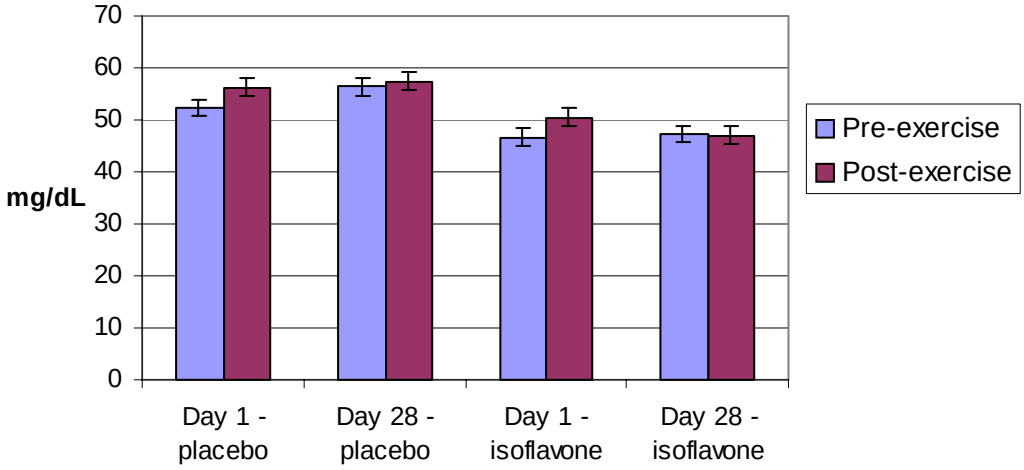

Figure 14. HDL Concentrations throughout the study (P > 0.10)

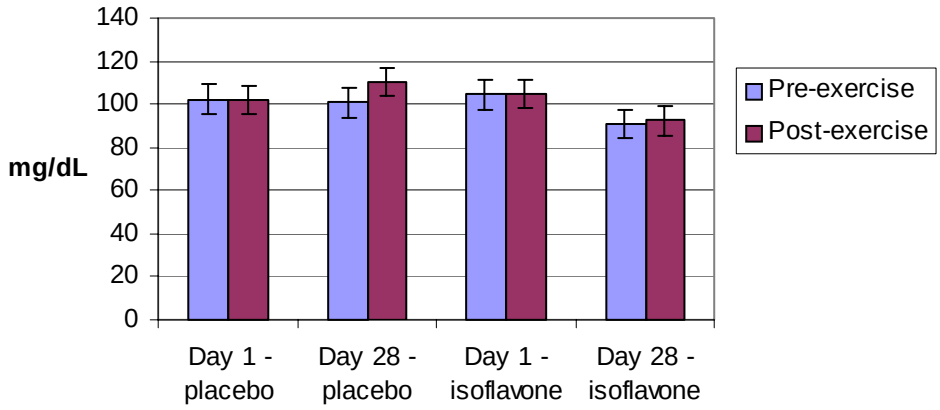

Figure 15. Triglyceride concentrations throughout the study (P > 0.10)

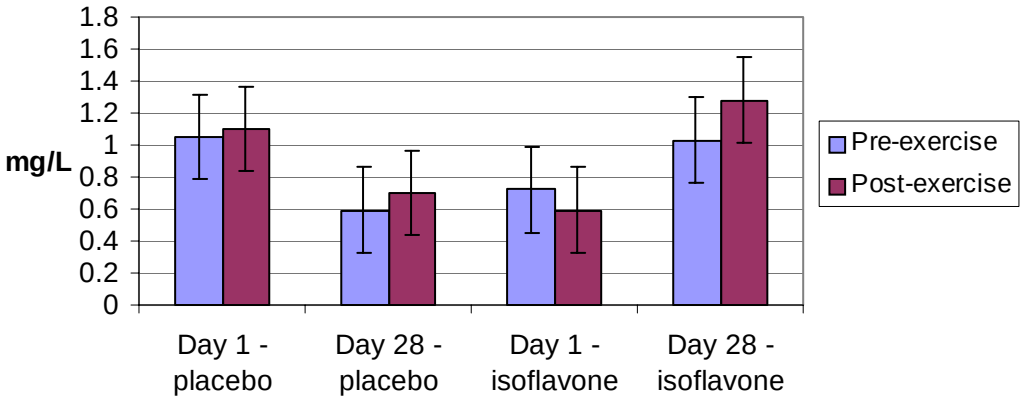

Figure 16. CRP concentrations throughout the study (P > 0.10)

Chapter 5

Discussion

Soybean research has grown profoundly in recent years in areas such as cancer, heart diseases, osteoporosis, cognitive function, menopausal symptoms, and renal function. This is mainly attributed to the plentiful health benefits that soy protein and soy isoflavones provide. Many biological properties have been attributed to the isoflavones, including estrogenic, antioxidant, antiallergenic, antiviral, anti-inflammatory, and vasodilation properties (Pietta, 2000). Although the positive health benefits of soy are little disputed, there are conflicting results as to the extent and mechanisms of soy's action (Messina, 2002). Most studies have demonstrated positive effects of soy on atherosclerosis and cardiovascular diseases. The best documented effect is LDL reduction of ~10%, and small increases in HDL concentrations (~2%) (Clarkson, 2002). The cholesterol lowering effect of soy has become so well documented that the FDA issued a health claim in 1999, which states that "25g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease," (FDA, 1999) However, the bioactive component of soy, whether it be soy protein alone, soy isoflavones alone, or a combination of the two, is still unclear.

Since the identification of oxidized LDL's role in atherosclerosis initiation and progression, soy isoflavone's potential to prevent LDL oxidation has received much attention. Many of the studies that investigated the antioxidative properties of soy isoflavones have been *in vitro* studies. This study tested the *in vivo* antioxidant properties of soy isoflavones in healthy males.

Soy isoflavones have proven to be very bioavailable in humans, even though only the aglycone forms are absorbed. The aglycone isoflavone genistein is the most biologically active isoflavone with the greatest antioxidant potential (Pietta, 2000). In light of these two facts, our study supplemented healthy, college-age, non-smoking, sedentary males with either placebo, or 150 mg/d of high genistein isoflavone tablets (all isoflavones in their aglycone forms). After 4 weeks of supplementation, plasma genistein and daidzein concentrations rose dramatically, from 0 ng/ml to 561.6 ± 39.3 and 466.3 ± 35.5 ng/ml (SE) respectively ($P < 0.0001$), compared to 0 ng/ml in the placebo group throughout the study. Although not significant, a trend of increasing plasma isoflavone concentration after exercise was observed, from 499.3 ng/ml to 623.9 ng/ml

and from 414.9 ng/ml to 517.6 ng/ml for genistein and daidzein respectively (Figure 7). This suggests that isoflavones may be stored in the body and released when needed. Isoflavones have been found in substantial amounts in animal tissues such as the liver, mammary glands, prostate, testes, ovaries, thyroid gland, brain, and muscle (Chang, 2000). It has also been suggested that isoflavones undergo enterohepatic cycling in a glucuronidated form until they are hydrolyzed by glucuronidases in the intestine or liver, and are thereby released as their biologically active aglycone form. Under normal, physically unstressful conditions, this serves to maintain a constant plasma concentration of isoflavones after ingestion (Setchell, 2001). However, it may be possible that when oxidation occurs, the body will hydrolyze a greater amount of conjugated isoflavones accounting for the increase in plasma concentration of aglycones after exercise.

Acute sessions of strenuous exercise have proven to create a short term state of oxidative stress (Sen, 2001). The oxidative stress produced by intense, short-term exercise sessions has been deemed a possible model for studying disease parameters that would arise in a long-term oxidative stress state, such as CVD (Sen, 1995; Jenkins, 2000).

To test the antioxidant capacities of the isoflavones *in vivo,* the subjects underwent intense exercise sessions at 80% of their personal VO_2pk for 30 minutes before and after the 4 week supplementation. Increased oxygen consumption during exercise yields superoxide radicals and other ROS that result in lipid peroxidation and other damaging effects associated with oxidative stress (Evans, 2000). The results of this study combined with a study completed previously in our laboratory demonstrate that a state of oxidative stress was achieved in the circulating blood compartment after the 30 minute 80% VO_2pk exercise. This study observed an increase in LDL oxidation with exercise, indicating a decrease in LDL's resistance to oxidation from exercise ($P = 0.0003$) (Tables 14 & 15, Figure 9). When analyzed by agarose gel electrophoresis, the relative electrophoretic mobility of *ex vivo* ox-LDL increased by 11.2%. Furthermore, plasma malondialdahyde (MDA) levels, an indicator of lipid oxidative damage increased after exercise.

The antioxidant defense system in the human body is designed to staunch the creation of oxidative stress by scavenging the damaging ROS. Therefore, the rise in oxidative stress in the plasma fraction due to 80 % VO_2pk exercise would be expected to cause a decrease in antioxidant status. Exercise increased erythrocyte SOD activity and decreased GPx activity. The plasma

fraction antioxidant compounds glutathione (GSH), uric acid, and vitamin C were measured previously, and vitamin E was measured in this study. Exercise decreased blood glutathione (GSH), total glutathione (TGSH), and the GSH/TGSH ratio, and increased blood oxidized glutathione (GSSG). Although vitamin C did not change, plasma uric acid levels did increase after exercise (Chen, 2001). There was no significant effect on plasma vitamin E concentrations by exercise (Tables 14 & 15, Figure 11). This is a surprising result, due to the expectation that oxidative stress caused by exercise would diminish vitamin E stores. The total antioxidant potential, or FRAP values were significantly increased after exercise ($P = 0.0048$) (Table 5), which may be due to the increase in uric acid concentration after exercise (Chen, 2001). The lipid oxidation and antioxidant status results indicate an overall increase in oxidation and oxidative stress after exercise.

The present study observed no significant interactions or main effects of exercise on plasma total cholesterol concentrations ($P > 0.10$) (Tables 14 & 15, Figure 12). However there was a trend toward increased plasma total cholesterol (TC) concentrations after 30 minute exercise sessions at 80% VO_2pk ($P = 0.11$) (Table 8). A tendency for HDL concentrations to increase (~4%) was observed after exercise ($P = 0.072$) (Table 7). Although slight, these findings aid in the confirmation of oxidative stress after an acute session of intensive exercise.

Studies have affirmed that sessions of strenuous exercise create an acute phase response (APR) similar to the APR seen in trauma or septic patients (Pedersen, 2000). Moreover, the APR may be induced by oxidative stress (Grimble, 1998). C-reactive protein, one of the acute phase proteins, would hence be expected to increase its plasma levels after exercise. As anticipated, CRP concentrations have been found to increase after strenuous exercise sessions (Weight, 1991, Pederson, 2000). This study observed no statistically significant change in CRP levels with exercise ($P > 0.1$) (Tables 14 & 15, Figure 16). However, it must be noted that our subjects were young, non-smoking, healthy males, with no indication of heart disease or any other disease. Therefore, they would not be expected to show very high systemic inflammation levels with accompanying high plasma CRP concentrations unless they were ill. The lack of statistical significance among the vitamin E data, cholesterol data and CRP data suggests that a 30 minute exercise session at 80% VO_2pk in healthy, young males does not induce measureable

inflammation and may not be the best model for studying isoflavone supplementation effects on CVD parameters in this population.

Previous work on this same study established that the isoflavones did not provide antioxidative protection against the oxidative stress induced by intensive exercise. However, isoflavone supplementation did exert antioxidant activity under unstressed, pre-exercise conditions (data not shown) (Chen, 2001). Isoflavone supplementation significantly decreased pre-exercise plasma MDA ($P < 0.05$), demonstrating protection from oxidation. Supplementation significantly increased pre-exercise blood TGSH ($P = 0.01$), and lead to a significantly higher utilization of blood TGSH and GSH during exercise, possibly indicating effects by antioxidant recycling. Pre-exercise erythrocyte SOD activity was significantly increased ($P = 0.0006$) and pre-exercise GPx levels were maintained by isoflavone supplementation, which suggests an increase in antioxidant defense. Isoflavone supplementation had no statistically significant effects on LDL-MDA. Further, isoflavone supplementation had no effect on pre-exercise values of any parameter examined in this study (LDL oxidation, FRAP, vitamin E, TC, LDL, HDL, TG, CRP).

Interest has been aroused in the potential for isoflavones to inhibit the oxidation of LDL. Isoflavones have inhibited LDL oxidation *in vitro*, which suggests that they are incorporated into the LDL particle (Anderson, 1999; Tikkanen, 1998). Helisten et al. (2001) identified the fatty acid esterification of soy isoflavones, which allows their incorporation into the LDL particle and consequently confers protection on LDL from oxidation. The present study observed that although ox-LDL was significantly increased after exercise ($P = 0.0003$), isoflavone supplementation offered LDL no further resistance to oxidation (Tables 14 & 15, Figure 9). This is consistent with our previous findings (Chen, 2001), yet it suggests that either the isoflavones were not incorporated into the LDL particle *in vivo*, or they were not incorporated into the LDL particle in large enough amounts to offer protection from *ex-vivo* oxidation.

Isoflavone supplementation also had no boosting or recycling effects on plasma vitamin E levels, further supporting the absence or miniscule amount of isoflavones in the LDL particle (Tables 14 & 15, Figure 11). While there were no significant effects of isoflavone supplementation on FRAP values (Tables 14 & 15, Figure 10), the phase 2 statistical analysis demonstrated a trend for decreased FRAP with time in the placebo group, and increased FRAP

with time in the isoflavone group (P = 0.081) (Table 6, Figure 8). Although the lack of statistical significance is an unexpected result, the lack of supplementation effects may be due to the fact that the FRAP assay does not account for all antioxidants in the plasma. While the FRAP assay has shown linearity with total antioxidant concentration *in vitro*, the assay depends on the antioxidant's ability to reduce Fe^{3+}. It is known for example, that GSH is unable to reduce Fe^{3+} to Fe^{2+} (Prior, 1999). The isoflavones may also be unable to reduce Fe^{3+}. This question must be answered by further research.

Numerous studies have demonstrated beneficial effects to the lipid profile from soy consumption (Bakhit, 1994; Setchell, 1999; Wong 1998). Initially, research replaced dietary protein with soy protein and monitored its effects on cholesterol levels. Many of these studies observed significantly decreased total and LDL cholesterol concentrations with slight increases in HDL concentrations (Clarkson, 2002). The trend to discover the mechanism of action lead to the investigation of the role of soy isoflavones in the hypocholesterolemic effect. Also, many studies have observed beneficial effects of the isoflavones on the lipid profile. However, there are a number of studies that identified no significant effects on cholesterol from soy isoflavones (Clarkson, 2002). Hence, the role of soy protein and soy isoflavones in cholesterol homeostasis has yet to be elucidated.

The findings from this study confirm the evidence that soy isoflavone supplementation alone has little or no effect on cholesterol concentrations in normo-cholesterolemic subjects. Isoflavone supplementation of 150 mg/d for four weeks did not present any statistically significant changes in TC concentrations pre- or post-exercise (Tables 14 & 15, Figure 12). Soy isoflavones conferred no benefit to plasma LDL concentrations; there was no statistical difference in LDL concentrations after 4 weeks of isoflavone supplementation pre- or post-exercise (P > 0.1) (Tables 14 & 15, Figure 13). LDL concentrations in all treatments exhibited a tendancy to increase over the course of the study, yet this effect did not reach significance (P = 0.074) (Table 8).

As mentioned earlier, many studies have shown a slight increase in HDL concentrations with soy supplementation. In this study, there was a tendency for HDL concentrations to decrease (2.9%) with isoflavone supplementation, while there was a 4.6% increase in HDL concentrations after 4 weeks of placebo, although this effect did not reach significance (P = 0.094) (Table 10). It

is possible that the variation among subjects was too great to detect any meaningful effects of isoflavones. There were no statistically significant interactions or main effects of isoflavone supplementation on plasma TG concentrations ($P > 0.1$) (Tables 14 & 15, Figure 15). Plasma TG concentrations tended to decrease over the 4 weeks of supplementation in all treatments (placebo and isoflavone supplementation), yet again, this effect did not reach significance ($P = 0.063$) (Table 12). Isoflavones also had no significant protective effects on any CVD parameters due to induced oxidative stress by exercise.

Research indicates that the greatest effect of soy in any combination of protein or isoflavone is seen in hypercholesterolemic populations (Bakhit, 1994, Wong, 1998). The subjects who participated in this study were all young (~20.7 years), healthy males and 83.3% of them had total cholesterol concentrations below 200 mg/dL (the recognized cut-off point for hypercholesterolemia). Furthermore, the subjects' TC levels ranged from 89.0 - 279.4 mg/dL, which implies large variation in the population and may have hindered the revelation of any effects from isoflavone supplementation. Nonetheless, these results support the mounting evidence that soy isoflavone extracts alone offer little cardiovascular benefits.

Although the influence of soy isoflavone supplementation on CRP concentrations has not been studied to date, decreased CRP concentrations have been manifested with antioxidant supplementation, namely vitamin E and vitamin C supplementation (Gribmle, 1998; Upritchard, 2000; Patrick, 2001). Isoflavones appear to have similar antioxidant action as vitamin E and therefore have the potential to effectively reduce plasma CRP concentrations. Plasma CRP concentrations decreased with time in the placebo group by 42% and increased with time in the isoflavone supplemented group by 44%, although these changes were not significant (Tables 13, 14, & 15). Albeit, it is possible that the soy isoflavone supplementation lead to an over abundance of antioxidants in the body, causing them to become pro-oxidant, this is an unlikely circumstance since the level of isoflavone administered was consistent with levels consumed daily in a typically soy-rich Asian diet, and the observed changes with supplementation did not reach significance.

Chapter 6

Conclusions

Copius research on soy has been done in the past decade with many studies supporting the beneficial CVD protective effects from soy consumption (Bakhit, 1994; Wong, 1998; Clarkson, 2002). Yet there are many studies that reported equivocal results with soy supplementation (Clarkson, 2002). There have also been ambivalent results concerning the use of soy protein, soy isoflavones, or a combination of the two against CVD. The present study investigated the possible *in vivo* antioxidant effects from soy isoflavone supplementation in humans against induced oxidative stress from intensive exercise sessions.

Four weeks of 150 mg/d isoflavone supplementation resulted in a dramatic increase in plasma genistein and daidzein concentrations ($P < 0.0001$). However, the increase did not serve to influence any of the oxidative stress parameters investigated. Even though oxidized LDL and FRAP levels were significantly increased after exercise as expected ($P = 0.0003$ and 0.0048 respectively), no protection was offered by isoflavone supplementation. Similarly, isoflavone supplementation had no significant effect on plasma vitamin E levels, or lipid profiles. Although plasma CRP concentrations decreased with time in the placebo group by 42% and increased with time in the isoflavone supplemented group by 44%, the changes did not reach statistical significance.

Research indicates that the greatest effect of soy in any combination of protein or isoflavone is seen in hypercholesterolemic populations (Bakhit, 1994, Wong, 1998). The subjects who participated in this study were all young (~20.7 years), healthy males and 83.3% of them had total cholesterol concentrations below 200 mg/dL, with a variation in TC from 89.0 - 279.4 mg/dL. This may have hindered any effects from isoflavone supplementation that may have been seen in an older at risk population. Furthermore, the level of supplementation is relatively high for an American population and may have resulted in more pro-oxidant activity that increased general inflammation, consequently increasing CRP levels.

The results of this study, in addition to our previous findings (Chen, 2001) demonstrate that although isoflavones exhibit antioxidant activity *in vivo* under normal physiological conditions in

healthy young men, they do not confer protection against induced oxidative stress, nor do they confer benefits to CVD risk parameters to this population. This substantiates the hypothesis that it may not be soy isoflavones alone that improve lipid profiles, or offer protection from oxidative stress.

Chapter 7

Future Studies

Cardiovascular diseases are still the leading cause of death in the United States. Consequently, there is a considerable amount of soy research focuses on its role in the prevention of CVD. Epidemiologic studies have shown that populations with high soy intake, such as Asian populations have lower incidences of CVD (Wong, 1998). Furthermore, research has demonstrated hypocholesterolemic effects of soy consumption, and the FDA issued a health proclamation for soy's possible prevention of CVD (FDA, 1999). However, there are still many unanswered questions about soy's role in protection against CVD. Outcomes are not conclusive of the mechanism of soy's hypocholesterolemic and CVD protective actions. Many studies have shown both positive and neutral effects of soy protein, soy isoflavones, andcombinations of the two (Clarkson, 2002)

This study analyzed the antioxidant effects of soy isoflavones in healthy young men undergoing induced oxidative stress by exercise. We observed no effect by isoflavones on the extent of *ex-vivo* oxidation of LDL particles, which suggests that either isoflavones are not incorporated into the LDL particle, or they are not incorporated in high enough amounts to offer protection from oxidation. Since other studies have demonstrated isoflavone incorporation into the LDL particle, more research is needed to deliniate the actual involvement of isoflavones with LDL.

The FRAP assay also indicated no antioxidative effects of soy isoflavones. FRAP values were not significantly increased after soy isoflavone supplementaton. This was a surprising result since other studies have demonstrated that soy isoflavones have potent antioxidant actions (Pietta, 2000). However, it is possible that isoflavones are not able to reduce Fe^{3+}, which would then render them undetectable by the FRAP assay. To answer this question, the FRAP results need to be compared with other antioxidant potential assays.

This study observed little to no effect on lipid profiles by soy isoflavone supplementation. Since many studies have demonstrated hypocholesterolemic effects of soy consumption, research needs to focus on the mechanisms by which soy operates. More studies are needed to deliniate

whether it is soy isoflavones, protein, or a combination of the two that has the cholesterol lowering effect. Furthermore, whether soy has the potential to reduce cholesterol levels in normo-cholesterolemic individuals must be examined. It may be that soy is only able to reduce cholesterol in hypercholesterolemic subjects. However, it may also be possible that soy is capable of maintaining reduced, or normal cholesterol levels in normo-cholesterolemic individuals as they age, or enter an oxidatively stressed state. This is a question that has yet to be addressed by research.

It would be interesting to investigate the effect of soy isoflavone supplementation on these same parameters in an older population that has greater risk of being under oxidative stress, or in an older hypercholesterolemic population. In these populations, soy isoflavone supplementation may result in greater effects on the CVD parameters that were measured in this study, due to the greater risk of the population and to the less control of oxidative damage. It would also be interesting to investigate the effect of soy protein supplementation without isoflavones in similar older, at risk populations.

Finally, the role of C-reactive protein in heart disease must be more fully examined. High CRP levels highly correlate with CVD, however, individual measurements have not proven to be valuable predictive measures. This is the first study to look at the effects of soy on CRP. No effect of isoflavone supplementation on CRP levels was seen in this study. However, it is possible that soy consumption may play a role in reduction of high CRP levels, or maintenance of low CRP levels.

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Appendix A

Progressive Exercise Test (VO_2pk test) on Cycle Ergometer-Method

(Chen,2001)

Principle

Peak oxygen consumption (VO_2pk) is a measurement of dynamic exercise capacity that defines cardiorespiratory fitness, the potential for high level physical performance, and is the definitive laboratory indicator for individual aerobic exercise capacity (Wareham, 1998). Its measurement is identified as the highest value in a progressive exercise that no longer increases despite increased external work rate or exercise intensity. VO_2pk is expressed in ml/kg body mass/min, and is attained under conditions of continuously increasing loads that begin with a warm-up stage and increase to a maximal effort within an 8-12 minute period. Response criteria that accompany VO_2pk include a peak heart rate of >90% of the age-adjusted predicted maximal value, the appearance of an R value (VCO_2/VO_2) > 1.1-1.2 units; or a rating of perceived exertion (RPE) (Borg Scale (Borg, 1982) to indicate mental fatigue) > 18-19.

Equipment

Monark cycle ergometer (Monark 818E, Monark, Stockholm, Sweden)

MedGraphics CPX/D VO_2 System (St Paul, MN) automated metabolic cart

ECG system (Quinton: ECG: Models Q5000)

Mouthpieces and noseclip

Borg scale card

Procedure

Before the VO_2pk test, the subject is interviewed to establish and estimated exercise capacity for cycling, to help ensure achievement of peak oxygen consumption within 8-12 min before muscle fatigue. The subject is then fitted with a breathing apparatus (mouthpiece, noseclip, and three-way breathing tube). One end of the breathing tube is in the subject's mouth, one end is open so the subject can breathe freely, and the third end is connected to the MedGraphics machine to analyze expired air during exercise. Software programs that drive the MedGraphics card via desktop computer automatically store and record the responses for VO_2pk, maximal pulmonary

ventilation, and the respiratory exchange ratio. Heart rate is measured by an ECG system with 3 CC5 leads. The LL lead is placed on the fourth left intercostal space, below and lateral to the nipple (Positive electrode). The RA lead is placed on the fourth right intercostal space, lateral to the nipple (negative electrode). The third RL lead is placed on the lower right quadrant of the anterior thorax.

Subjects warm up on the cycle ergometer for two minutes to familiarize themselves with the equipment and the speed of 60 rpm. Every 1-2 minutes, the workload is gradually increased by 30-60 watts. Workload increase depends on the subject's heart rate response and estimated exercise capacity, until the subject is unwilling to continue or unable to maintain 55 rpm. The test is complete after all criteria accompanying the VO_2pk are achieved.

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Appendix B

Eighty Percent VO_2pk Exercise on Cycle Ergometer - Method (Chen, 2001)

Principle

Eighty percent of each subject's VO_2pk was calculated from their individual VO_2pk values. They were then exercised at this 80% VO_2pk for 30 minutes to induce a state of oxidative stress. The induced oxidative would allow for the examination of disease state parameters in healthy individuals.

Equipment

Monark cycle ergometer (Monark 818E, Monark, Stockholm, Sweden)

MedGraphics CPX/D VO_2 System (St Paul, MN) automated metabolic cart

ECG system (Quinton: ECG: Models Q5000)

Mouthpieces and noseclip

Borg scale card

Timer

Procedure

The subject is instructed not to participate in any exercise or sport two days prior to the submaximal exercise. Once in the laboratory, the subject rests for 10 minutes before a venous blood sample is drawn from and antecubital vein. The subject then warms up on the cycle ergometer until he reaches his personal 80% VO_2pk. The subject is then maintained at 80% VO_2pk for 30 minutes. During the exercise, heart rate and rate of perceived exertion are monitored continuously. Every 5 minutes oxygen consumption is measured to check for 80% VO_2pk maintenance, the workload is adjusted according to the subject's 80% VO_2pk, and oxygen consumptions are recorded. The average exercise intensity throughout the submaximal exercise is calculated by the recorded oxygen consumptions of the subject.

Appendix C

LDL separation from plasma - Method (Chen, 2001):

Principle

Human LDL may be isolated from plasma by ultracentrifugation within a density gradient of 1.019 - 1.063 g/ml (Esterbauer, 1991).

Reagents

Potassium bromide

0.154 M NaCL

Procedure

The separation of LDL followed the method developed by Havel et al. (1955). The density of plasma was adjusted to 1.063 g/ml by the addition of 0.6530g potassium bromide to 2 ml of plasma. Two ml of density adjusted plasma was overlaid with 2.8 ml of 0.254 M NaCl in a centrifuge tube. Subsequently, all tubes were centrifuged at 800,000 rpm at 7°C for 45 minutes using a Beckman ultracentrifuge (Optima L-90K, Beckman Inc., Palo Alto, CA.). After centrifugation, the isolated LDL layer is visible as a yellow band in the upper portion of the tube. This fraction was isolated by a glass pipette, and was retained for further measurement of LDL oxidation.

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Appendix D

Hematocrit - Method (Chen, 2001)

Principle

Plasma volume changes with exercise are accounted for by hematocrit measurements. Hematocrit was analyzed by microcentrifugation and measurement against the hematocrit ruler. The percent change in plasma volume (% ΔPV) was calculated using the equation from Van Beaumont (1973):

$$\% \Delta PV = 100/(100-Hct_{pre})*100*(Hct_{post}-Hct_{pre})/Hct_{post}$$

Procedure

Two capillary tubes were filled with whole blood and microcentrifuged for 15 minutes in the capillary tube microcentrifuge. The hematocrit was read against the ruler. Plasma volume changes between pre- and post-exercise were calculated using the above equation.

Reference

Van Beaumont W. et al, Changes in total plasma content of electrolytes and proteins with maximal exercise. J Appl Physiol 1973 Jan;34(1):102-106

Appendix E

Plasma Genistein and Daidzein Assay - Method (Chen, 2001)

Principle

Prior to HPLC detection, plasma isoflavones must undergo condensation and glucuronidase digestion. Inter-individual variation in plasma isoflavone concentrations (10-2000 ng/ml) necessitates condensation of plasma isoflavones for accurate detection by HPLC. Furthermore, the glucuronide link in the glucuronidated isoflavones must be cleaved for accurate HPLC detection. The amphiphilicity of isoflavones allows them to be extracted by methanol and subsequently, easily detected by the HPLC.

Reagents

Methanol

1 M sodium acetate buffer, pH 5.5

Beta-glucuronidase/sulfatase

80% acetonitrile

Fluorescein, 199.541 ng/µl (internal standard)

Standards:

Genistein: 12.5, 62.5, 125, 250, 500 ng

Daidzein: 12.5, 62.5, 125, 250, 500 ng

Flurescein: 140, 700, 1400, 2800, 5600 ng

Procedure

The determination of genistein and daidzein in plasma by HPLC was modified from a method by Wang and Murphy (1994) and Xu et al. (1994). One half ml of plasma and 19 µl was mixed with 10 ml of methanol. The mixture was centrifuged at 5000 x g for 10 minutes at 10°C. The methanol supernatant was decanted into a rotary evaporated flask. The plasma residue was re-dissolved in 10 ml of methanol and centrifuged again at 5000 x g for 10 minutes at 10°C. The total 20 ml methanol supernatant, harvested from all extractions, was evaporated to complete dryness using a rotary evaporator at 37°C. Seven ml of 1 M sodium acetate buffer (pH 5.5) was added to rinse down the sides of the flask. The solution was transferred to a capped glass test tube

and 50 µl of β-glucuronidase/sulfatase was added. The solution was then incubated at 37°C overnight (16-20 hours) in a dry heating block. After incubation, the solution was filtered with solid phase extraction cartridge (J&W Scientific, Folsum, CA) and the isoflavones were eluted with methanol and dried under nitrogen. The residue was re-dissolved in 180 µL of 80% methanol and 100 µl of the extract was injected into HPLC for genistein and daidzein determination. The total recovery of isoflavone from plasma was 97.4±5.9% according to the HPLC fluorescin values. Unknown concentrations of plasma genistein and daidzein were calculated from the standard curves of genistein and daidzein.

References

Wang H. and Murphy P.A, Isoflavone content in commercial soybean foods. J Agric Food Chem 1994;42:1666-1673

Xu X. et al, Daidzein is a more bioavailable soy milk isoflavone than is genistein in adult women. J Nutr 1994;124:825-832

Appendix F

LDL Oxidation by Agarose Gel Electrophoresis - Method

Principle

The different sizes and charges of the various lipoproteins enable them to be separated from one another in an agarose gel by electrophoresis. Due to its more negative charge, ox-LDL has a greater relative electrophoretic mobility (REM) than native LDL and will therefore move farther on a gel than will native LDL. This method allows the comparison of *in vitro* and *in vivo* oxidation of LDL.

Reagents

10 µM EDTA phosphate buffer solution

100 µM $CuSO_4$

Barbital Buffer, pH 8.6 (Sigma)

0.5% Agarose

0.375 g Type I A agarose (Sigma) in 75 mL barbital buffer

30% sucrose solution

0.1% Sudan Black

0.5 g Sudan Black (Sigma) in 500 mL 90% ethanol (room temperature)

Destaining solution

1:1 (v/v) ethanol - water mixture

Procedure

The method of determining the relative electrophoretic mobility (REM) of low density lipoprotein (LDL) and oxidized LDL (ox-LDL) on an agarose gel was modified from Vieira et al. (1996) and Greenspan et al. (1993). First, LDL was dialyzed with 10 µM EDTA phosphate buffer solution at 4°C for 24 hours. Then, 200 µl of 100 µM $CuSO_4$ and 600 µl distilled water was added to all tubes containing 200 µl of dialyzed LDL (approximately 1 mg/ml protein). These tubes were incubated at 37°C for 24 hours. After incubation, 23 µl of sample was mixed with 2.3 µl of 30% sucrose solution and loaded into a 0.5% agarose gel. The gel was run at a constant

voltage of 125 V for 1 hour, stained with 0.1% Sudan black in 70% ethanol for 6 minutes, and destained with 1:1 ethanol: water solution overnight.

References

Greenspan P. and Gutman R.L, Detection by Nile Red of agarose gel electrophoresed native and modified low density lipoprotein. Electrophoresis 1993;14:65-68

Vieira O.V. et al, Rapid isolation of low density lipoproteins in a concentrated fraction free from water-soluble plasma antioxidants. J Lipid Res 1996;37:2715-2721

Appendix G

Plasma Vitamin E Assay - Method

Principle

Vitamin E (α-tocopherol) and vitamin A (retinol) are both fat-soluble vitamins that play important antioxidant roles in the body. This reagent kit allows for the simultaneous analysis of both vitamins in one HPLC run.

Reagents

Reagent Kit from Chromsystems Instruments and Chemicals

Mobile Phase

Precipitation Reagent 1

Precipitation Reagent 2

Internal standard

Serum Controls levels I and II

Standards

Procedure

Plasma Vitamin E levels were determined using a kit from Chromsystems Instruments and Chemicals (Fraunhoferstrasse, Martinsried, Germany). Two hundred µl plasma was pipetted into a light protected reaction vial, along with 20 µl internal standard and 25 µl precipitation reagent 1. Each vial was vortexed for 30 seconds before adding 400 µl precipitation reagent 2. The vials were then centrifuged for 10 minutes at 9000 x g. 50 µl of the supernatant was injected into the HPLC system for vitamin E determination. Two channels were run simultaneously in the HPLC, one at 295 nm to detect vitamin E, and one at 325 nm to detect vitamin A. Unknown concentrations were calculated using the standard curve.

Appendix H

Ferric Reducing Antioxidant Ability of Plasma (FRAP) Assay - Method (Chen, 2001)

Principle

The FRAP assay provides an index of general resistance to oxidative damage by providing a measure of general non-enzymatic antioxidant protection. At low pH, ferric-tripyridyltriazine complex is reduced to the ferrous form by reductants (antioxidants) in the plasma solution, and an intense blue color forms with optimal absorption at 593 nm. Excess ferric ion is used in this assay, allowing the ferrous-tripyridyltriazine formation to be the rate-limiting step. The color development therefore is directly proportional to the antioxidant capacity of the plasma.

Reagents

40 mM HCl

300 mM acetate buffer, pH 3.6 (3.1 g $C_2H_3NaO_2$-$3H_2O$, 16 ml glacial acetic acid, fill to 1 L with dH_2O)

10 mM 2,4,6-tripyridyl-s-triazine (TPTZ)

20 mM $FeCl_3$-$6H_2O$

FRAP Cocktail Solution:

Acetate buffer: 10 mM TPTZ: 20 mM $FeCl_3$-$6H_2O$ in a 10:1:1 ratio

Standards using $FeSO_4$-$7H_2O$

50,100,250,500,750,1000 µM

Procedure

Ferric reducing ability of plasma (FRAP) was measured according to the methods of Benzie and Strain (1996) and Langley-Evans (2000). A FRAP cocktail solution was made in a 10:1:1 ratio of the following reagents: 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), and 20 mM $FeCl_3$*$6H_20$. The cocktail solution was pre-heated to 37ºC. Nine hundred µl cocktail solution and 90 µl water was added to 30 µl sample or standard. The mixture was incubated at 37ºC for 4 minutes before the absorbance was read against a reagent blank at 593 nm. Antioxidant power of plasma was determined by a standard curve of ferrous sulphate.

References

Benzie I.F.F. and Strain J.J, The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. Anal Biochem 1996;239:70-76

Langley-Evans S.C, Consumption of black tea elicits an increase in plasma antioxidant potential in humans. Inter J Food Sci Nutri 2000;51:309-315

Appendix I

Cholesterol Assay (Lipid Profile) - Method

Principle

Plasma lipid profiles are attained by simple enzymatic colorometric assays. Total cholesterol concentration is ultimately proportional to the red color that develops when the enzymatic cholesterol reagent is mixed with plasma. The cholesterol reagent contains aminophenazone, phenol, peroxidase (POD), cholesterol esterase (CE), cholesterol oxidase (COX), buffers and stabilizers. When it is mixed with plasma, the following reactions ensue:

cholesterol esters → *(CE) → cholesterol + fatty acids

cholesterol + O_2 → *(COX) → cholest-4-en-3-en-one + H_2O_2

H_2O_2 + 4-aminophenazone + phenol → *(POD) → H_2O + O-quinoeimine dye

* (note: CE, COX, and POD are enzymes that catalyze reactions, but do not change in course of reaction)

High density lipoprotein (HDL) cholesterol can be measured by the above reactions if the low density lipoproteins (LDL) and very low density lipoproteins (VLDL) are precipitated out of the solution. This is done by using the HDL precipitating reagent from StanBio (Boerne, Texas) that contains magnesium chloride in dextran sulfate.

The measurement of plasma triglycerides is not only useful for the calculation of LDL, but also proves useful in the diagnosis of hyperlipoproteinemia. The method is identical to the total cholesterol method, except that the enzymatic triglyceride reagent is used instead of the cholesterol reagent. The triglyceride reagent includes 4-aminoantipyrine, 4-chlorophenol, ATP, lipases, glycerol-kinase, glycerol-3-phosphate oxidase, peroxidase, and PIPES buffer solution, pH 7.5. Similar to the total cholesterol assay, a colored quinoneimine results in direct proportion to the concentration of triglycerides.

Reagents/Kits

LiquiColor Cholesterol Reagent Kit (StanBio No. 1010)

Enzymatic cholesterol reagent and cholesterol standard (200 mg/dL)

HDL-Cholesterol Precipitating reagent (StanBio No. 0599)

includes HDL standard (50 mg/dL)

LiquiColor Triglycerides Enzymatic Reagent Kit (StanBio No. 2000)

Enzymatic triglyceride reagent and activator

Triglyceride standard (200 mg/dL)

Procedure

Cholesterol levels were measured using a well-known enzymatic assay developed by Allain et al. (1974). The lipid profile reagent kits were obtained from StanBio (Boerne, Texas). Total cholesterol was measured by incubating 10 µl serum with 1.0 ml of reagent for 15 minutes at room temperature. The absorbance was read at 500 nm against a reagent blank and concentrations of unknown samples were determined from a standard curve. HDL cholesterol was measured by precipitating out LDL and VLDL with Mg^{++}/dextran sulfate reagent according to Finley et al. (1978). The absorbance of the remaining supernatant was measured according to the previous Allain method. LDL was determined by a calculation from the total and HDL cholesterol measurements. Plasma triglycerides were measured by incubating 10 µl serum with 1.0 ml of reagent for 15 minutes at room temperature. The absorbance was read at 500 nm against a reagent blank and concentrations of unknown samples were determined from a standard curve.

References

Allain C.C. et al, Enzymatic determination of total serum cholesterol. Clin Chem 1974;20(4):470-475

Finley P.R. et al, Cholesterol in high-density lipoprotein: Use of Mg^{++}/dextran sulfate in its enzymic measurement. Clin Chem 1978;24:931-933

Appendix J

C-Reactive Protein Assay - Method

Principle

Plasma C-reactive protein (CRP) was analyzed by a sandwich ELISA kit received from Alpha Diagnostic International. In the ELISA assay, CRP binds to the human CRP antibody that is immobilized on the microtiter well plates. The second human CRP antibody that is conjugated to the enzyme horseradish peroxidase is added to the wells and binds to the CRP, sandwiching it between the two antibodies. Chromogenic substrates A and B are added after washing and color develops. The color is directly proportional to the concentration of CRP in the samples.

Reagents

Human C-Reactive Protein (CRP) ELISA KIT (ADI, No. 1000)

PBS buffer

Procedure

CRP levels were analyzed by a sandwich ELISA reagent kit that was obtained from Alpha Diagnostic International, Inc. (San Antonio, TX). Plasma samples were diluted 1:100; 25 µl of sample was diluted with 2.5 ml of diluent or PBS buffer. Diluted samples were pipetted (10 µl) into CRP-Ab coated wells. One hundred µl of Ab-enzyme conjugate was added to each well and mixed gently. The plate was incubated at room temperature for 30 minutes. The wells were then aspirated and manually washed with dH_2O five times and were tapped over paper towels between washings. After washing, the 2 chromogenic substrates were added; 100 µl HRP-substrate solution A was added to each well, followed by 100µl HRP-substrate solution B in the same timed intervals. The wells were mixed gently before incubation for 10 minutes at room temperature. In the same timed intervals as above, 50 µl of stop solution (1N H_2SO_4) was added to all wells, which were mixed gently by swirling. The absorbance was read in a microplate reader at 450 nm and the concentrations of the unknown samples were calculated against a standard curve of pure CRP.

Appendix K

The Study In a Nutshell

Your Responsibilities:

1. Attend an information session about the study
2. Attend two 30 minute exercise tests and give maximal effort on the VO_2 maximum test
   a. Do not consume alcohol or caffeine after 10pm the previous night
   b. Bring or wear exercise clothing to the gym
3. Take isoflavone or placebo tablet 2 times daily for 4 weeks
4. Donate blood 5 times (35 ml each time)
5. Record daily food intake six times
6. Fast overnight before each blood draw (only water allowed)

Schedule:

Day 1	Orientation
Night 1	Fast overnight (10-12 h before blood draw)
Day 2	Baseline Blood Draw
Day 3	VO_2 maximal test
Night 6	Fast overnight
Day 7	80 % VO2 max exercise test (30 min) and blood draw before and after exercise
Day 8	Start Isoflavone supplementation
Day 9-36	Take Isoflavone supplementation as directed
Night 36	Fast overnight
Day 37	80 % VO2 max exercise test (30 min) and blood draw before and after exercise... END OF STUDY ☺

Benefits:

You will know about:

1. your fitness level (Are you in shape?)
2. your dietary habits (Do you eat a healthy diet?)
3. your antioxidant defense levels (Are you able to fight chronic diseases such as cancers, heart diseases, cataracts, arthritis, Parkinson's disease, and so on.)

Appendix L

Volunter Information (Subject Questionnaire)

Instruction:

1. Please answer the following questions.
2. Please list a phone number where you can be reached.
3. Please send the completed form to me by attachment through e-mail.

Name: **Age:**

Phone number (home):

Phone number (office):

E-mail address:

How many hours do you exercise per week generally?

Do you smoke?

Do you take any vitamin supplement? If you do, how many tablets do you take a week?

Have you eaten any foods made of soy, such as soy burger, tofu, soy milk, and soy cheese? If you do, how often do you consume these foods?

Are you on any medication for any disease? If yes, please explain.

Appendix M

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Informed Consent for Participants of investigative project

Title of Project: The Effect of Isoflavone Supplementation on the Modulation of Antioxidant Defense systems in Men Undergoing 80% VO_2pk Exercise

Investigators: Raga Bakhit, Ph.D., Oliver Chen, and Vanessa Rogowsky

I. Purpose of the Research/Project:
I am being asked to participate in a study that aspires to identify the positive effects of isoflavones from soy on risk factors for heart disease, specifically the effects that antioxidants have on overcoming the deleterious results of oxidation that can lead to atherosclerosis. It will involve periodic exercise and blood draws to get adequate data. I am being asked to participate in this research study along with 29 other college age males.

II. Procedures: Prior to being included in the research study, I will complete a health history questionnaire that will help to identify possible reasons that I should not participate in this study. If the results of the questionnaire indicate that I am an appropriate subject, then I will be informed of when I should report to the Human Performance Laboratory at 230 War memorial Hall for initial exercise testing.

Exercise sessions: I understand that I will perform 1 maximal exercise test on a cycle ergometer on the third day of the study to determine my personal maximum exercise level. I understand that my heart rate will be monitored via an electrocardiograph recorder during this exercise test. Non-piercing, stick-on electrodes will be placed on my skin and cables that are attached to the electrocardiograph recorder will be snapped onto the electrodes. I also understand that I will also be fitted with a breathing apparatus (mouthpiece and nose clip) that will measure my oxygen consumption. This test will be performed on a cycle ergometer, during which the workload will be gradually increased until either I report to the technician that I am unable to continue pedaling, or until the rpm's fall below 50. I will decide when I am unable to continue and the technician will stop the test when I so request.

I understand that the second exercise session will be 30 minutes in duration at 80% of my personal maximal effort and that my heart rate will again be monitored via the electrocardiograph recorder. I also understand that I will not be required to use the breathing apparatus (mouthpiece and nose clip) continuously throughout the exercise. However, to maintain 80% maximal effort it will be necessary to periodically monitor my oxygen consumption and therefore necessary to periodically use the breathing apparatus. I agree to pedal the ergometer for 30 minutes at a constant 60 rpm. I agree to continue cycling continuously for 30 minutes unless I request to stop prematurely. I understand that five tubes of blood will be taken by a Licensed Medical Technician immediately before and immediately after this exercise test.

During the following 4 weeks, I understand that I am to take the isoflavone/placebo supplement tablets that the investigators will give me. I agree to follow the instructions and consume the supplement pills each day for 28 days.

At the end of the 4 weeks of supplementation, I understand that I am expected to return once again to the lab for a final exercise test and an accompanying blood draw. I agree to fast the night before all blood draws. I understand that my blood will be drawn by a Licensed Medical Technician immediately before and immediately after this exercise test. I also understand that I will perform this last 80% maximal effort exercise test in the exact way that I performed the previous 80% maximal effort exercise test.

III. Risks: I understand and agree that there is a possibility of adverse physiologic responses during the exercise tests. I understand that these responses could include abnormal blood pressure, fainting, disorders of the heartbeat, and in rare instances, heart attack, stroke, or death. I have every confidence that every effort will be made to minimize these risks through evaluation of my preliminary health questionnaire and through observations during the tests. Other possible discomforts I may experience in this study include leg fatigue, muscle soreness, dry mouth (from the mouthpiece), and pain, bleeding and local bruising at the site of the blood draw. I understand that the licensed medical technician collecting the blood samples, and that the technicians who may be handling the samples, will be wearing gloves at all times. I also understand that a licensed medical technician, certified exercise specialist and other support personnel will be present during all exercise testing to minimize risks. In the case of an emergency, the rescue squad (that is located on the Virginia Tech campus) will be immediately notified via the working telephone in the testing area. Their average response time is approximately 4 to 5 min.

IV. Benefits of the Project: My participation in this project will provide valuable information that will help clarify the benefits of soy isoflavone on reducing risk factors for cardiovascular disease. In addition I will receive information about my physical fitness level and outcomes of the study, if I so desire.

V. Confidentiality/Anonymity: I understand that the results of this study will be kept strictly confidential. At no time will the researchers release my results of this study without my written consent. The information I provide will have my name removed and only a subject number (not the social security number) will identify me during analysis and written reports of this research.

VI. Compensation: I understand that I will receive the $50.00 compensation only if I complete the study.

VII. Freedom to Withdraw: I understand that I may withdraw from this study at any time without penalty. The only loss of benefits accompanying my early withdraw will be that I will not receive the $50.00.

VIII. **Approval of Research:** The procedures of this research study were approved by the Virginia Tech Institutional Review Board (IRB) in August 2000.

IX. **Subjects Responsibilities:** I know of no reason that I cannot participate in this study. I understand that I have the responsibility to:

- Accurately report my medical history.
- Arrive at the laboratory at my scheduled time for each exercise session.
- Arrive at the laboratory after a 10-12 hour fast.
- Take the isoflavone tablet daily according to the given instructions.
- Report any unusual signs/symptoms during the study.

X. **Subjects Permission:** I have read and understand the informed consent and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent for participation in this project.
If I participate, I may withdraw at any time without penalty. I agree to abide by all the rules of this project.

_____ _____

Signature Date

Should I have any questions about this research or its conduct, I may contact:

Oliver Chen	961-0211
Investigator	Phone
Vanessa Rogowsky	951-1342
Investigator	Phone
Dr. Raga Bakhit	231-6784
Faculty Advisor	Phone
Dr. David Moore	231-9359
Chairman, IRB Research Division	Phone

Appendix N

Medical Health History Questionnaire

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Health History Questionnaire

Subject #: _____

Title of Project: The effect of isoflavone supplementation on the modulation of antioxidant defense systems in men undergoing 80% VO_2pk exercise

Investigators: Raga M. Bakhit, Ph.D.; Oliver Chen, M.S.; and Vanessa Rogowsky

Please complete the following questionnaire as accurately as possible.

Name: _____ Age: _____

Date of Birth: _____19_____

Address:

_____

_____

_____

Phone: (540) _____(work)

Phone: (540) _____(home)

E-mail address: _____

1. Do you have any known allergies? Y N

   If yes, please explain: (food, medical, other)

_____

_____

_____

_____

2. Do you currently take any medications? Y N

   If yes, please explain:

_____

_____

_____

3. Do you currently exercise? Y N

If yes, please explain: (what? how often?)

_____

_____

_____

_____

4. Please circle all that apply to you?

High Blood Pressure	Asthma	Smoking/Tobacco
Skipped Heart Beats	Chest Discomfort	Fast Heart Rate
Diabetes	Heart Murmur	Shortness of Breath
Dizziness/Fainting	Joint Soreness	Others

If circled, please explain:

_____

_____

_____

_____

_____

5. Is there any reason not mentioned above that would limit your ability to perform high-intensity exercise? Y N

If yes, please explain:

_____

_____

_____

_____

6. Has a physician ever told you to refrain from activity or exercise for an extended period of time? Y N

If yes, please explain:

_____

_____

_____

_____

7. Do you have any difficulty with getting your blood taken?

Y N

If yes, please explain:

_____

_____

_____

_____

Signature _____

Date _____

Thank you.

Appendix O

Foods Containing Isoflavones & their Alternatives

Food Categories	Foods to Avoid	Acceptable Alternatives
Bread and Starches	Any baked product containing soy (soy flour, soy fiber, soy protein, soy concentrate, etc.) Soy noodles Soy chips	All baked products made without soy All other noodles (rice, semolina, regular wheat, corn, etc.) Potato, corn, vegetable chips
Vegetables	Edemame (edible green soybeans) Soy-containing: Spinach nuggets Broccoli nuggets Veggie patties Clover sprouts (read labels)	All other fresh and canned and frozen vegetables
Fruits	Please try to avoid Vitamins A- and C- supplemented juices (limit to < 2C/day)	All fruits, canned and fresh
Milk	Soy Milk/ soy powder Soy yogurts, puddings, and ice creams Soy cheeses Soy infant formulas	All other types of milk and milk's products (cow, goat, rice…)
Meats, beans, and Meat Substitutes	Tempeh Natto (fermented soy beans) Tofu (soybean curd) Soy Protein Isolate or concentrate Veggie burgers w/ soy Meatless chicken nuggets USDA commodity beef patties with TVP Meatless bacon Soy link-sausages Soy/meatless franks or hotdogs	(Vegetarians, compliment grains with nuts, cheeses) -Pumpkin seeds -Sunflower seeds -Almonds, Pecans, Walnuts

Food Categories	Foods to Avoid	Acceptable Alternatives
Meat and Meat substitutes continued	Legumes: -soybeans -kidney beans -navy beans -Pinto beans -Red beans -White beans -Broad beans -Chick peas -Pigeon peas -Split beans (lentils, split peas) -Mung beans -Peanuts **Peanut Butter** Soybean Butter	Tahini/sesame butter Almond butter Hazelnut butter
Miscellaneous supplements	-Vitamins A, Beta-carotene, C, D, and E -Antioxidants supplements -Isoflavones -All Vit/Min supplements -Soy sauce -Soy paste and powder -most muscle/diet drinks,smoothies, shakes, etc. -most muscle/diet bars -Teas: Green tea Jasmine tea	

Extended List of Foods Containing Isoflavones

Forms of Soy Protein and Isoflavones

Tempeh	Natto (fermented soybeans)	Soy protein isolate
Soy sauce	Soybean chips	Soy protein concentrate
Soy cheese	Soy milk	Soy drink
Miso	Soy yogurt	Soy fiber
Soy flour	Soy noodles	Soy paste
Soy powder	**Soy infant formula**	Soy based liquid formulas
Soybeans	Soy bean butter	Soy pudding
Soy ice cream	Textured Vegetable Protein	Soy nuts

Soybeans: Products and Brand Names

Meat Substitutes:

- Green Giant Harvest Burger
- Meatless Chicken Nuggets (Frichick and other brands)
- Morning Star Products (eg. Breakfast soy links, mini corndogs, veggie burgers, etc.)
- Meatless franks/hot dogs
- Lunch "meats"
- USDA commodity, beef patties with TVP (remember school lunches?)
-
- Tofus
- Meat substitute instant meals
- Hamburger Helpers
- Betty Crocker meal "helpers"
- Chef Boy R D- ravioli

"Dairy" soy foods:

- soy milk
- soy yogurt
- soy/tofu puddings
- soy spread (butter replacer)
- soy cheese

Snack foods:

- Soy nuts
- Healthy Valley cookies, granola bars
- Soy nut butters (peanut butter replacers)
- Maruchan Instant soups
- Knorr Instant soups
- Raman Noodles

Sauces:

- Soy sauce
- Teriyaki sauce
- Soy salad dressings

Breakfast foods:

- Healthy Valley Scones and hot cereals
- Soy cereals (e.g. Nature's Path)

Soy Drinks/Supplements (nutrition, diet, muscle drinks/supplements)

- Slim fast drinks (powder drinks)
- Nestle sweet success
- Ensure
- Adnantage/10
- Naturade Total Soy powder drink
- GeniSoy powder drink
- Kashi Go Lean Powder drink
- Atkins diet shake mixes
- MLO Super high protein powder drink
- Antioxidant drinks/teas (and green teas)
- American Fair Nutritional supplement
- Metab-O-lite
- Weidermuscle builder
- Soy Sensations
- Jenny Craig
- Pounds off
- EAS-EDGE nutritious protein drink
- Carb Solutions
- Denise Austin's "Tasty meal to-go"
- Metabolife

Soy bars (nutrition, diet, muscle bars)

- Slim fast bars
- Luna bars
- Clif bars
- Ultimate LO Carb bars
- GeniSoy bars
- Balance and Balance plus bars
- Powerbar Harvest
  - Protein Plus bars
  - Essentials
  - Peanut butter flavors
- Kashi Go Lean bars
- Atkins diet advantage bars
- Twinlab Ironman bars
- MetRx Source One bars and Peanut butter flavors
- Viactiv bars
- Tiger milk bars
- Ensure bars
- Boost bars
- Science-labs soy protein
- EAS-EDGE nutritious bar
- BioxBioProtein
- Max-X-Bar
- Zone and Zone Perfect bars
- Your barbel

ALTERNATIVE Choices

All meats:

- Pure beef
- Chicken
- Fish
- Turkey
- Seafood
- Pork

Snack foods:

- Any non-soy nuts (walnuts, cashews, almonds...)
- BUT NO PEANUTS
- Any cookies, granola bars without soy protein
- Cashew butter, hazelnut butter, almond butter, sesame butter (NO PEANUT BUTTER)
- Any soups without soy protein

Dairy foods

- All non-soy milks (cow, goat, rice...)
- All non-soy yogurts (cow milk, rice)
- All non-soy puddings
- Butter, margarine
- All non-soy cheeses (cow milk, rice)

Sauces:

- Avoid soy based sauces
- Tomato/cream based sauces...

Breakfast foods

- Any cereals without soy
- Any breakfast foods without soy (nutragrain bars, oatmeal...)

Non-soy Nutrition/Diet/Muscle drinks/supplements:

- Slimfast pre-mixed can drinks (chocolate, strawberry, and capuchino)
- Boost drink
- MetRx powder drinks
  e.g. Total Nutrition Drink Mix
- MLO Musc-L Blast 2000 powder drinks
- MLO Milk and Egg protein powder drink
- Creatine Powder
- Fat Burner
- Twinlab drinks/supplements:
  -Thermogenic Formula Diet Fuel
  -Chitosan Formula diet fuel
  -Performance Enhancer Mega creatine fuel
  -Metabolift
  -Iron Man Energy Booster
  -Iron man Fast Burner
  -Iron Man Advanced Creatine
  Iron Man Protein Shake

Non-soy Nutrition/Diet/Muscle bars:

- Nutrigrain cereal bars
- Quaker Fruit bars
- Burnup bars
- Worldwide Pure Protein bars
- Selected Powerbars:
  Chocolate
  Vanilla Crisp
  Oatmeal raisin
- Cloud bars
- MetRx protein plus bars
- MetRx nutrition bars
  Fudge brownie
  Chocolate gram cracker chip
  Chocolate chip
  Extreme vanilla
- EAS myoplex deluxe
- MetRx Krunch
- Steel Bar

Appendix P

HOW TO COMPLETE FOOD INTAKE FORMS

1. On the top of each form, write the date and day on intake record in the spaces provided.
2. Please write as clearly as possible because we will have a large number of these to analyze.
3. List all foods and beverages (including water and condiments) consumed during the 24-hour period.
4. Please describe food precisely. For example, "2% milk" vs. "milk" or whole wheat bread-Wonder brand' vs. "bread".
5. Please specify quantities consumed. If you are not certain on amounts, use measuring cups or spoons whenever possible.
6. Remember to list all additions to food and beverages, such as cream, sugar, butter, jelly, lemon, salad dressing, artificial sweeteners, catsup, etc. Do not forget ingredients added in food preparation such as vinegar, oils, and if fat and salt were used.
7. Describe how foods are prepared. For example: ½ cup frozen broccoli, steamed.
8. For beverages, you may use fluid ounces instead of cups, For example: 12 oz Pepsi.
9. For foods that do not fit in a cup or spoon, use dimensions. For example: 1 corn tortilla, 6 inches across; 1 piece of cheddar cheese 3"x2"x1"; 1 banana nut muffin 2"x1".
10. For whole pieces of fruit or vegetables, you may use small, medium, or large. For example: one small Granny Smith apple.
11. List each food item, and the amount of each item, in mixed dishes. For example: 1 cup leaf lettuces, two ¼ "tomato slices, two 2" slices cucumber.
12. When including recipes, be sure to include how much the recipe yields in cups and your serving size. For example: Total recipe eight 1-cup servings. I had 1 cup.

AN EXAMPLE OF NOW TO FILL OUT YOUR FOOD INTAKE FORM.

Time	Food/Beverage	Amount (C., tbsp, tsp, oz., etc) Dimensions (5" banana)	Home/ out?
7:00	Water	c	Home
7:15	Coffee	2 c	"
	Creamer-Carnation	2 tsp	"
	Frosted mini wheats	1 c	"
	2% milk	2 c	"
8:30	Coffee-decaf brewed	1 c	School
	Creamer-Carnation	1 tsp	"
11:30	Bread-lite wheat-Nature Maid	2 slices	"
	Ham-92% fat free-Oscar Meyer	4 slices (4 oz)	"
	Mayo-Kraft Regular	1 tbsp	"
	Apple-red	1, 3-inch diameter	"
	Diet Pepsi	12 oz	"
3:00	Cookies-Chips Ahoy	6	"
	Iced tea-instant with lemon- Lipton	12 oz	"
6:15	McDonalds Quarter Pound w/Cheese	1	McDonalds
	French Fries	1 large order	"
	Vanilla Shakes	1 regular	"
	Side Salad	1	"
	Ranch Dressing	2 tbsp	"
8:00	Peach	1, 3-inch diameter	Home
	Cranapple Juice	10 oz	"

Measurement/Reporting Aids

NAME: _____

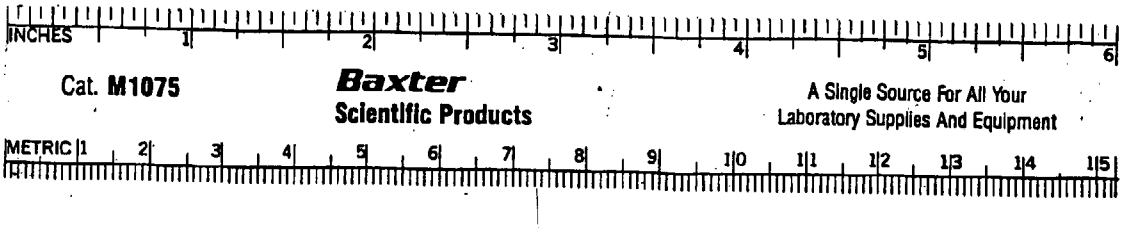
One handful = _____ Cups

One knuckle-to-knuckle measurement = _____ Inches

Personal mugs/bowls/etc:

Utensil	Usual Amount

Other notes:



Common Weights and Measures

Fluids:

1 cup = 8 ounces
1 ounce = 2 tablespoons
teaspoons

Dry and fluid foods:

1 Cup = 16 tablespoons
1 tablespoon = 3

Please use standard measuring cup to measure the food you record.

Abbreviations:

Tablespoon = T or Tspb
Teaspoon = t or tsp
Cup = C

Estimating Quantity of food:

1. For mixed dishes, please record approximate amounts of main ingredients eaten. For example, instead of "tuna casserole," list:
   2 oz. tuna fish, Bumble-bee, water-packed
   2 oz. cottage cheese, Breakshote's, 4% milk-fat
   1/2 C spiral noodles

2. For sandwiches, list ingredients separately. For example, a "turkey sandwich" is recorded as follows:
   2 slices bread, rye
   2 oz turkey
   1 leaf lettuce
   2 slices tomato
   1tsp. Mayonnaise, Weight-Watchers

3. All foods should be measured when ready for eating. Report the amount of meats, fruits and vegetables (etc) either raw or after cooking, depending on how they are served. For example, 3 cups of raw spinach may shrink to 3/4 cups cooked, so record 3/4 cups boiled spinach.

4. Please list whether the food was frozen, canned, or fresh, salted or unsalted.

5. Note the method of cooking: fried in peanut oil, boiled, baked, etc...

Appendix Q

FOOD INTAKE FORM

DAY_____ DATE _____ NAME _____

Time	Food/Beverage	Amount (cups, tbsp, oz., etc) Dimensions (5" banana)	Home/Out?

Appendix R

ACTIVITY DIARY

DAY_____ DATE _____ NAME _____

A. Sleep/Lying Down

B. Sitting Activities (little or not arm movement)
- B1 Eating
- B2 Reading/Writing
- B3 Watching television
- B4 Shaving
- B5 Driving
- B6 Typing/Computer work
- B7 Other

C. Light Activities (some arm movement while standing)
- C1 Office work
- C2 Standing/Slow walk
- C3 Housework
- C4 Personal Care (e.g. Dressing/Bathing)
- C5 Shopping
- C6 Light Gardening
- C7 Other

D. Moderate Activities (Moderate arm movement while standing or vigorous arm movement while sitting)
- D1 Walking (Vigorous pace)
- D2 Yardwork
- D3 Moderate Sports (e.g. Golf, Bowling)
- D4 Other

E. Hard Activities
- E1 Physical (e.g. Lifting/Carrying)
- E2 Vigorous Sports (e.g. Softball)
- E3 Aerobics
- E4 Dancing, Bicycling
- E5 Swimming
- E6 Snow Shoveling
- E7 Other

F. Very Hard Activities
- F1 Strenuous Physical Labor (e.g. Digging)
- F2 Exertive Sports (e.g. Running)
- F3 Single Tennis
- F4 Skiing
- F5 Basketball
- F6 Swimming Laps
- F7 Other

Write in the space provided the categorical value that corresponds best to the dominant activity of each 15-minute period.

Time	:00	:15	:30	:45
AM 5:00				
6:00				
7:00				
8:00				
9:00				
10:00				
11:00				
Noon 12:00				
1:00				
2:00				
3:00				
4:00				
5:00				
6:00				
7:00				
8:00				
9:00				
10:00				
11:00				
Midnite 12:00				
1:00				
2:00				
3:00				
4:00				

Adapted from: Bruce Elmore, An Evaluation of Five Physical Activity Assessment Methods in a Group of Women (Ph.D. Dissertation, 1989)

Appendix S

Compliance Survey (Subject Questionnaire)

Please honestly answer the following question (s). There is no penalty if you let us know how many tablets you did not take, if any. This is just to help us analyze your results more accurately.

Did you miss taking any tablet during the supplementation period?
Please circle the correct answer.

No Yes

If you answer is Yes, please fill in below the number of tablets you did not take?

_____ tablets

THANK YOU VERY MUCH FOR YOUR PARTICIPATION IN OUR STUDY.
WHITOUT YOU, WE COULD NOT HAVE FINISHED THIS EXPERIMENT.

Vita

Vanessa L. R. Hart

Vanessa Lynn Rogowsky Hart was born to Linda and Robert Rogowsky on July 14, 1977. She married Daniel Constantine Hart on October 14, 2000. She spent most of her formative years in Montgomery County, Maryland. In high school she moved to Virginia, where she has resided ever since. She got her bachelors degree in Chemistry with a specialization in Biochemistry, and a minor in Religion from the University of Virginia, in 1999. She has worked in heart disease research for 3 years; 1 year as an undergraduate at UVa under the supervision of Dr. G. Paul Matherne, MD, and 2 years as a graduate student at Virginia Tech under the supervision of Dr. Raga Bakhit, PhD.