

**THE EFFECTS OF SOY PROTEIN AND ISOFLAVONES ON LIPID
OXIDATION AND BLOOD LIPID PROFILE ON HUMANS PARTICIPATING
IN MODERATE PHYSICAL ACTIVITY**

by

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ABSTRACT

The purpose of our study was to compare the effects of dietary soy protein and animal protein (casein) on plasma lipoprotein concentrations, and exercise induced oxidation in human subjects. Sixteen normocholesterolemic young men participated in 30 min of cycling at 70% $\text{VO}_{2\text{pk}}$ to induce plasma oxidation. Each subject then followed a 4wk dietary treatment replacing 33g animal protein in a self-selected solid food diet with either soy protein or casein. The exercise was then repeated and plasma lipoproteins and oxidation were compared. Soy protein and casein dietary treatments did not affect plasma concentrations. Our study therefore, suggests that in healthy normocholesterolemic individuals, 33g of soy protein does not effectively reduce plasma lipoprotein concentrations or exercise induced oxidation.

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CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death for both males and females in the United States. Extensive research has identified several factors that increase the risk of cardiovascular disease and myocardial infarction. Major risk factors have been shown by medical research to be definitely associated with a significant increase in the risk of cardiovascular disease (Stone, 1996). Some factors are unchangeable such as age, sex and heredity. Other factors can be modified to reduce risk such as high blood cholesterol levels, elevated blood triglyceride levels, high blood pressure, smoking and inactivity. Atherosclerosis is a systemic disease process that affects the entire arterial tree, especially coronary and cerebral circulation.

In October 1999 the FDA approved a claim stating that 25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease. Soy protein has been shown to reduce plasma lipid levels in studies with human and animal models (Carroll, 1991). Recent research shows that the effects of soy protein include decreases in plasma total cholesterol (TC) concentrations and low-density-lipoprotein (LDL)-cholesterol with an increase in high-density-lipoprotein (HDL)-cholesterol levels when compared to an animal protein diet. Isoflavones found in soy have been shown to decrease plasma and LDL oxidation levels which is also beneficial for a reduction in cardiovascular disease (CVD) risk.

In previous studies soy protein containing isoflavones has been incorporated into baked products such as muffins and cookies to replace animal proteins in the subjects' diet. These foods contained 5-6g isolated soy protein (ISP) which required high quantities to be eaten daily to meet the desired 25-50g/d level. Products with higher ISP content are preferable for long term compliance in human studies. Our goal was to evaluate the blood lipid and oxidative response to high ISP, high isoflavone food products.

CHAPTER 2

LITERATURE REVIEW

This chapter will review the role of soy and its components to help reduce the risk of CVD.

Historical Background

Investigations into the connection between dietary protein source and the causes of CVD and atherosclerosis began in the early 1900's. This early research provided an important foundation for research that is much more detailed and advanced, but uses the same scientific principles. Ignatowski began investigating diet and its relation to atherosclerosis in 1908 (Kritchevsky, 1995). By 1909 he was studying the effects of dietary proteins from different sources on changes in the arterial wall and formation of atherosclerotic lesions. A diet containing meat, milk and eggs that he fed to groups of rabbits, led to the development of pronounced atherosclerosis in some of the rabbits. He attributed this formation to the toxins released during the digestion of animal products (Ignatowski, 1909). In 1912 Fahr successfully repeated Ignatowski's experiments using the same protocol. He documented observable atherosclerosis in 5-8 months and severe atherosclerosis and elevated blood pressure in 9-10 months (Fahr, 1912).

In 1911, Stuckey performed an experiment with rabbits, feeding them milk plus egg white, milk plus meat juice, milk plus egg yolk, egg plus meat juice and milk or a

control diet. One rabbit in each group was killed at 2, 4 and 6 months. He concluded that the egg yolk was the atherogenic agent (Stuckey, 1911). In 1912 he did a follow-up experiment using rabbits fed one of four types of fat: tallow, cod liver oil, sunflower seed oil or ox brain. After 4 months, the ox brain group formed aortic fatty plaques, but no changes occurred in the other groups. Stuckey concluded that a non-protein component common to egg yolk and ox brain was the responsible atherogenic agent (Stuckey, 1912).

Chalatow (1912) studied the livers from the animals in Stuckey's experiments and suggested that the atherogenic agent was a mixture of cholesterol, lethicin and fatty acids (Chalatow, 1912). Wesselkin then tested whether cholesterol or lethicin could be the contributing factor. Only the egg-yolk fed rabbits exhibited atherosclerosis and fatty livers. The rabbits fed milk or lethicin were normal. Wesselkin concluded that cholesterol, not lethicin was the atherogenic factor (Wesselkin, 1913). Later in the same year, two separate studies (Wacker et al., 1913, Anitschkow et al., 1913) independently confirmed this cholesterol theory.

In 1919 Newberg studied the effects of high protein diets on atherosclerosis. By feeding rabbits 15 or 30g/d of casein protein or soy protein. The rabbits fed casein developed atherosclerosis of the aorta while the soy group did not (Newberg, 1919). The significance of these findings went unnoticed until the 1940's when researchers (Meeker et al., 1941) compared the effects of animal and plant protein on rabbits that were fed a stock diet plus an animal protein (defatted casein), a soy protein (white soybean flour), or a control (white bread flour). Some of the rabbits were given daily doses of 60 or 250mg

cholesterol dissolved in 1mL olive oil and added to the diet (+cholesterol). The casein diet was much more atherogenic. The soy and control diets produced no atherosclerosis in the groups free of added cholesterol. Cholesterol levels of the rabbits fed the cholesterol-free diets were (mmol/L) basal 1.37, casein 3.23 and soy 1.66. In the +cholesterol groups, the soy group showed less atherosclerosis than the control group with both daily doses of cholesterol. The researchers attributed the results to the differences in amino acid composition of the individual proteins (Meeker et al., 1941).

This early research established a relationship between atherosclerosis, cholesterol and dietary protein. It has been followed with a wealth of research on the hypocholesterolemic effect of plant proteins, specifically soy protein studies using human and animal subjects.

Soy Protein Overview

Soybeans have been a major agricultural crop in the United States since the early 1900s. It was discovered that soybeans could provide a useful, edible oil with a by-product, defatted soybean meal, which was a good feed for livestock and poultry. In recent years the use of soybean oil has increased, while soy protein is still not a significant part of the Western diet. If plant and legume sources of protein replaced animal sources in the Western diet, it would be possible for people to control their intake

of total fat, saturated fatty acids, cholesterol, starch and fiber to maintain a desirable weight (Erdman, 1989).

Soy food-protein products are being developed to help increase the use of these foods in typical diets. Whole soybeans contain about 40% protein. Soy flour (SF), produced from soybean flakes after most of the oil has been removed, is the most basic and widely used soy-protein product and it contains at least 50% protein on a moisture-free basis. Soy protein concentrate (SPC), a further refined product prepared from high-quality dehulled soybeans by removing most of the oil and water-soluble non-protein components, contains at least 65% protein on a moisture-free basis. Isolated soy protein (ISP) has the highest protein fraction and is prepared from high-quality, dehulled soybeans by removing most non-protein components. It contains at least 90% protein on a moisture-free basis. The fat content of soy protein products is low because the soybean oil has been removed. The carbohydrate content of SF is 32% and SPC is 25% while ISP is virtually free of carbohydrate (Klein, 1995).

Soy foods contain a good amino acid profile, and comparison with ideal patterns published by the Food and Nutrition Board (RDA, 1989) shows adequate quantities of the essential amino acids histidine, isoleucine, leucine, lysine, phenylalanine plus tyrosine, threonine, tryptophan, and valine. Lysine concentration is especially high, and is important for world nutrition, because soy protein is an inexpensive protein source (Erdman, 1989).

Many published studies investigate soy protein as the main source of dietary protein. Istfan et al. (1983) looked at the nutritional value of soy protein concentrate on nutritional maintenance in young men. Six college-aged men participated in an 82 day study, where each subjects ingested 0.8g protein per kilogram body weight (pro/kg bw) after a 9 day control diet of 1.5g pro/kg bw of animal protein. Soy protein was the only source of protein for the 82 day diet. Nitrogen balance was measured weekly and every 3-4 weeks blood chemistry and physical performance tests were completed. Zinc absorption and iron balance was also measured throughout the study. Blood urea nitrogen declined significantly due to the reduction in daily protein intake. Other biochemical measurements did not show any significant change. Nitrogen balances varied between slightly negative and slightly positive throughout the study, without consistent changes over time. Zinc and iron absorption was not affected by the soy based diet. The authors conclude that this study suggests soy concentrate can serve as the main source of protein for the long term.

Young et al. (1984) followed up with another long term study on soy protein to maintain nutritional status. Eight young males participated in 84 days of formula diets containing isolated soy protein as the sole protein source. Urine samples were used to measure nitrogen, urea, creatinine. A similar protocol was followed using beef protein in place of the soy protein. Fecal samples were used to determine the total nitrogen content so that the nitrogen balance could be calculated. Body nitrogen balances were within the equilibrium in both diet trials. Other variables did not change significantly from the baseline. Based on the results from this study, the authors suggest that the nutritional

quality of isolated soy protein can serve as the sole source of amino acids and nitrogen for health maintenance in adults.

These studies conclude that soy protein is a safe alternative to animal proteins as the sole protein source in the human diet.

Effects of Soy Protein on Blood Cholesterol

Van Raaij et al. (1981) investigated the effects of soy and casein protein on the cholesterol level and lipoprotein composition in 69 healthy volunteers with strict diet control. Baseline total cholesterol was 152 ± 27 mg/dL. Subjects were fed a diet consisting of 13% protein, 38% fat, 49% carbohydrate, and 380 mg cholesterol. Protein was either provided by 65% soy protein or casein protein or by 2:1 mixture of casein and soy protein, which also was used as the basal diet. All subjects began on the basal diet for 10 d. Then 20 subjects continued as a control group, while the remaining subjects were split into casein or soy groups for four weeks. Results show that there were no changes in the casein or basal group. The soy group had a significant decrease in LDL-C and significant increase in HDL-C. This change suggests that soy protein could have a beneficial effect on multiple lipoprotein fractions and total cholesterol concentration.

A follow-up study by Van Raaij et al. (1982) used a design similar to their previous study (Van Raaij et al., 1981) but compared the effects of casein, soy protein isolate, and soy protein concentrate. Subjects were 57 healthy volunteers with baseline

cholesterol levels of 207 ± 36 mg/dL divided into 3 groups, casein (n=17), soy isolate (n=20) and soy concentrate (n=20). Diets for all groups were identical except for protein source. Sixty percent of daily protein intake was provided by casein, soy isolate or soy concentrate for 4 weeks after the 17d control period on the casein diet. The soy isolate group had a small, non significant decrease in LDL-C and a significant increase in HDL-C, while there were no changes in the soy concentrate and casein groups. Results suggest that more refined soy proteins may be more beneficial with regard to cholesterol and lipoprotein distribution.

Nilausen et al. (1998) studied the effects of casein versus soy protein in nine normolipidemic men. The subjects were fed a liquid-formula diet of identical composition except that the protein was either soy protein or casein. Subjects were studied during 3 dietary periods. In the first dietary period, the subjects ate their usual self-selected solid foods. During the second and third dietary period, they consumed only the liquid formula diet randomized for soy or casein diets first and the other diet during the last dietary period. Each dietary period lasted 45 days and was separated by an interval of 53 days where each subject ate a self-selected solid food diet. Fasting samples for blood lipid profile were taken throughout the study. Results show that soy protein affected the major lipid risk factors favorably while the casein had no effect. The soy protein diet caused a significant decrease in LDL-C and increase in HDL-C and apo A-I.

Grundy et al. (1983) measured the effects of soy protein and casein on metabolism and cholesterol in human subjects. Four of the fourteen subjects had

hypertriglyceridemia and one had hypercholesterolemia. The subjects lived in a medical ward and consumed liquid formula diets. Each diet contained 30 % fat (as lard), 55% carbohydrate (as glucose), and 15% protein, which was provided by either casein or soy protein. Half began with the casein diet while the other half consumed soy. After 1 month the diets switched for another 30 days. Blood was taken 2x weekly after an overnight fast. Plasma was analyzed for TC, TG, and HDL-C biochemically and VLDL-C, LDL-C was calculated. Fecal samples were analyzed to determine cholesterol balance.

The hypertriglyceridemic patients had a significant decrease in TG levels and no consistent change in any other variable. In the normal patients, there were no changes with the soy feeding in any lipoprotein fraction. Neutral, acidic, and total steroid concentrations went unchanged in response to diet in both the hypertriglyceridemic and normotriglyceridemic patients. The authors conclude that the only significant change induced by soy protein was a decrease in TG when baseline levels are abnormally high, with no noticeable effects on the metabolism of cholesterol.

Meinertz et al. (1988) studied dietary protein origin and its effects on lipoprotein levels in normolipidemic subjects. Five men and five women participated in a crossover design study by consuming liquid formula diets containing 20% of calories as casein or soy protein, 28% fat (mainly monounsaturated), and 52% as carbohydrate. Cholesterol consumption was less than 100 mg per day. Each of the two dietary periods lasted 1 month separated by a 1 month self selected solid food diet. Plasma cholesterol

concentrations were measured at baseline and on day 24, 28 and 32 of each dietary treatment.

The transfer from solid foods to the formula diet caused a 30% reduction in TC and LDL-C for both the casein and soy protein diets. This was primarily attributed to the limited cholesterol intake and a low saturated fat content of the formula diet. Plasma levels stabilized after 2 weeks on the new liquid formula diet. During the washout period plasma measures returned to pre-study values. Comparing plasma lipid and lipoprotein values between the soy and casein treatment groups there were no differences detected. Apo B and Apo A-I were also identical between groups. Body weights were also unchanged throughout the treatments. Authors conclude that dietary casein and soy protein could not be distinguished in their effects on the plasma concentrations and chemical composition of the major lipoprotein classes in normolipidemic subjects.

Another study by Meinertz et al. (1989) examined the effects of soy protein and casein with cholesterol enriched diets in normolipidemic subjects. Eleven subjects consumed a diet of 20% soy protein or casein, 27% fat and 53% carbohydrate with an average cholesterol intake of 500 mg/d. Half of the subjects began on each diet for 31 days. A 30 day washout period of self chosen foods followed, and then each subject consumed the alternate diet for 31 days. The results show a significant decrease (15%) in LDL-C and increase (16%) in HDL-C with soy diet than with the casein diet. Large differences between diets may be due to a relatively high intake of cholesterol, which is not recommended for heart healthy diets.

A study by Wong et al. (1998) compared the effects of soy protein on cholesterol levels in 13 normocholesterolemic (average 4.32 ± 0.49 mmol/L TC) and 13 hypercholesterolemic (average 7.06 ± 0.52 mmol/L TC) men. The subjects consumed a diet of 20% protein, 30% fat and 50% carbohydrate in a crossover design. Soy or animal protein (50 g/d) provided $\geq 75\%$ of total daily protein intake. Cholesterol intake was limited to 300 mg/d. Diets were consumed for 5 weeks with a washout period of 10-15 weeks followed by an alternate diet for 5 weeks. The soy diet showed a significant decrease in LDL-C (6%), and LDL-C:HDL-C ratio in both normocholesterolemic and hypercholesterolemic subjects.

Potter et al. (1993) investigated 26 mildly hypercholesterolemic men during four dietary treatments. Each diet was consumed for 4 weeks and included 50g protein and 20g dietary fiber from soy flour (SF), isolated soy protein/soy cotyledon fiber (ISP/SCF), ISP/cellulose (ISP/C), or nonfat dry milk/C (NFDM/C) along with a low-fat, low cholesterol diet. Both ISP groups had a significant reduction in TC and LDL-C compared to the baseline and NFDM/C. The SF group also had a reduction in TC compared to NFDM/C. Triglycerides and HDL-C were not affected by dietary interventions. This shows that replacing 50% of dietary protein with soy protein reduces TC and LDL-C in hypercholesterolemic men may be seen.

Verrillo et al. (1985) studied the effects of soybean protein treatment on subjects with stable type II hyperlipoproteinaemia. The subjects were separated into two groups: the soyrep group (n=10) substituted soy protein for animal protein, and the soyadd group

added soy protein (60g/d) to a low-fat diet. Treatment diets lasted for 16 weeks after an 8 week baseline period of low-fat diet. The soyrep group had a 29.5% reduction in TC, a 39% reduction in LDL-C and an 11.8% reduction in triglycerides. In the soyadd group TC was reduced by 29.9%, LDL-C by 36% and triglycerides by 18.2%. HDL-C did not change significantly in either group. The insignificant differences between both groups suggests that the addition of soy to a standard low-fat diet is effective in inducing a significant cholesterol decrease in patients with type II hyperlipoproteinaemia.

Mechanisms of Soy Protein Effects

Different dietary proteins affect plasma cholesterol concentrations differently. Soy protein has been shown previously to be hypocholesterolemic compared to animal proteins in many models. The mechanism of action by soy protein has not yet been specifically identified, although many recent studies suggest the components responsible for observed beneficial effects.

Metabolism of Cholesterol

The effects of the metabolism of cholesterol by the liver may be noted when a soy protein diet is consumed. Lovati et al. (1987) have compared the effects of textured vegetable protein (TVP) from soy to those of animal proteins on LDL receptor activity. Twelve subjects with severe Type II hyperlipoproteinemia began with plasma cholesterol concentrations > 300 mg/dL. A baseline low saturated fat diet was consumed for one

month prior to the experimental period. The subjects were then instructed to eat a diet consisting of 20% protein, 26% fat and 54% carbohydrate with a high P:S ratio of 2.0 and 150 mg cholesterol daily for 4 weeks. Protein was provided by TVP or animal protein. A washout period of 3-4 weeks was followed by the alternate diet for 4 weeks. Measurement of receptor activity at the beginning and end of each diet regimen was used to determine LDL degradation. The soy diet significantly lowered TC and LDL-C as well as greatly increased LDL degradation by 8-fold compared to the animal protein diet. This suggests that some component of soy protein may exert an up-regulation of LDL receptors.

Nagata et al. (1982) performed a series of studies using male Wistar rats to determine the effects of soy protein and amino acid mixtures simulating soy protein on cholesterol and to identify possible mechanisms. For four weeks the rats were fed a series of diets including intravenous cholesterol, soy protein (SP) and casein (C) diets and amino acid mixtures simulating soy (SAA) and casein (CAA).

Serum cholesterol was lowest in the SP group compared to the C, group although the specific lipoprotein fractions were unchanged. In the SAA group, serum cholesterol was lower than in the CAA group and lipoprotein fractions were altered with an increase in VLDL and a decrease in HDL. Cholesterol absorption was significantly lower in the SP group than in the C group. Absorption between the SAA and CAA groups was not different. Intact soy protein also showed an increase in HMG CoA reductase activity compared to casein while SAA caused it to decrease. This finding indicated that soy

protein may cause a decrease in intestinal absorption of cholesterol and an increase in fecal steroid excretion.

Sirtori et al. (1984) studied 27 female Sprague-Dawley rats and their response to dietary modification. The rats were separated into three groups: soy, casein, and control. Both experimental diets were hypercholesterolemic, and the control was a standard Charles River laboratory diet for rats. The casein group had a significant increase in TC and VLDL. Soy protein counteracted any cholesterol increases. HMG CoA reductase activity was significantly higher in the soy group compared to casein and control groups. These findings suggest that the liver regulation of cholesterol metabolism is affected differently by animal and plant proteins.

Tasker et al. (1993) have studied how feeding gerbils soy protein affects TC, LDL-C and HMG CoA reductase after 4 wk experimental period. HMG CoA is the rate limiting enzyme that is present in cholesterol synthesis. Each diet was identical except for the protein source, which came from soy protein isolate (SPI), casein (C), l-amino acids simulating soy protein (SAA) or l-amino acids simulating casein (CAA). The results show that TC was significantly lower in the SPI group compared to that of the C or CAA group. LDL-C was lowest in the SPI group compared to all the other groups. HDL-C was highest in the CAA group while TG was unaffected by dietary treatment. HMG CoA reductase activity was highest in the SPI group compared to C group. The SAA group surprisingly had a lower HMG CoA reductase activity than the CAA group, which suggests that intact proteins may be responsible for beneficial action.

This overview of mechanisms relation to soy protein action suggests that intact soy protein may have a hypocholesterolemic effect due to reduced absorption and increased excretion. A non-protein component found in soy protein may be at least partially responsible for the hypocholesterolemic effect since the SAA did not produce the same favorable effects.

Enhancement of bile acid excretion

When compared to casein, soy protein causes fecal excretion of bile acids to increase, which causes cholesterol to be drawn out of the body (Potter 1995). The liver metabolizes cholesterol to provide it for increased bile acid synthesis. LDL receptors and cholesterol biosynthesis are increased thereby causing an overall increase in removal of cholesterol, specifically LDL-C from the blood.

Tanaka et al. (1984) have studied the effects of soy protein on bile acid excretion in Wistar male rats by using diets contained intact soy protein, casein and amino acid simulators of both (SAA and CAA). He supplemented some of the rats' diets with added fat or cholesterol to determine if any occurred. The results show that the added fat (5%) and cholesterol did effect bile secretion by increasing it. The soy group had a significantly higher output of biliary cholesterol and bile acids than the casein group. There was a significant negative correlation between serum cholesterol and biliary cholesterol levels. The SAA group showed similar but fewer notable effects. This

suggests that soy protein may stimulate cholesterol mobilization from the liver to bile, thereby causing enhanced excretion.

Sugano et al (1988) used rats to determine the action of an undigested fraction of soy protein with a series of experiments. A 10% solution of SPI was hydrolyzed by microbial proteases and then centrifuged. The sediment which consisted of the high molecular weight fraction (HMF) which is thought to be responsible for cholesterol lowering, soy protein (SP), or low molecular weight fraction (LMF) was freeze dried. Five groups were given a combination of the soy digest for 14 or 28 days, and were all analyzed for body growth, serum and liver cholesterol, and fecal steroid excretion. Growth for the SP and HMF groups was comparable but was significantly lower for the LMF group. The latter was attributed to the highly hygroscopic property that caused the rats to eat less initially, but their weight increased after the diet became more familiar and eating increased to meet other group intakes. Serum cholesterol was significantly lower in the HMF group than in the SP group, while LMF caused an increase in cholesterol levels. Liver cholesterol levels were also significantly lower in HMF group. Comparison to the intact SP group showed an overall increase of excretion by the HMF group along with neutral and acidic steroids. This suggests that an indigestible peptide fragment of soy protein is responsible for a strong hypocholesterolemic effect by interfering in the steroid absorption process.

Jaskiewicz et al. (1987) have studied the mechanisms of the hypocholesterolemic effects of soy in Vervet monkeys. Ten mature male and ten mature female monkeys were

split into 2 groups of 5 animals of each sex. Each group was fed identical high cholesterol diets but with a different protein source. Diets were fed ad libitum for 12 months and ended with a 12 h fast before the monkeys were killed. Tests for TC, HDL-C, VLDL-C and TG were performed every 3 months and at the end of the experimental period. Bile was analyzed for bile acid and the phospholipid level was also measured. No differences were found in any lipoprotein fraction due to the dietary treatment group. There was also no significant change in bile constituents in both groups. This study suggests that soy protein does not beneficially effect cholesterol metabolism in monkeys fed long-term soy diets.

Studies on patients with type II hypercholesterolemia have also looked at the effects and possible mechanism of soy protein on cholesterol. A study by Fumagalli et al., (1982) studied seven subjects (5 male, 2 female) with type II hyperlipoproteinemia. Subjects were admitted to a Metabolic Ward to participate in this study. Four subjects were given either a low-lipid-low-cholesterol diet with animal proteins or soy protein diet for 3 weeks in a cross-over design. Fecal neutral steroids and bile acids were analyzed by chromatography during each dietary period. For the other three subjects a chromatographic+isotopic method was used, with feeding of a low-lipid-low-cholesterol diet with animal proteins for 10 days followed by the soy protein diet for 2 weeks. Blood and fecal samples were taken 3 times per week. Six of the seven patients had a significant hypocholesterolemic effect quickly after the soy protein diet began. No differences in fecal steroid excretion were seen in either group when fed soy. This study does not clarify a mechanism but suggests that the non-protein component is not

responsible for the cholesterol lowering effects of soy in patients with type II hyperlipoproteinemia.

Studies with animal models have concluded that the hypocholesterolemic effects of intact soy protein is accompanied by enhanced bile secretion (Tanaka et al., 1984; Van der Meer et al., 1987; Sugano et al., 1988). In human subjects the hypocholesterolemic effect was not accompanied by any differences in the excretion of bile acids (Jaskiewicz et al., 1987; Fumagalli et al., 1982).

The reasons for inconsistency with varying species are unclear. Several theories to explain the inconsistency have been put forth:

- There are differences in protocol, number of subjects, different amounts and sources of soy protein.
- Hypocholesterolemic effects of soy protein in human subjects does not occur through bile excretion and is mediated by another mechanism.
- Current methods for bile excretion measurement are not optimal for human models.

Hormonal effects

Hormones and cholesterol metabolism are closely linked and may be altered by the addition of soy protein to the diet. Several hormones such as insulin, glucagon and thyroxin may be involved in the synthesis, metabolism or regulation of cholesterol in the

body. A closer look at the effects of soy protein on these hormones in conjunction with hypocholesterolemic effects is necessary to determine if a mechanism can be identified.

Forsythe et al. (1986) have attempted to identify possible mechanisms for the hypocholesterolemic effect of soy proteins. Male Mongolian gerbils were used because of their responsiveness to high fat, high cholesterol diets to increase LDL-C. Plasma concentrations of insulin, glucagon, thyroxine (T4), triiodothyronine (T3) and thyroid stimulating hormone (TSH) were measured pre and post treatment to determine differences between a soy based or casein based diet.

Plasma cholesterol concentrations were significantly lower for the soy group than for the casein group. The soy group had a decrease in LDL-C with no change in HDL-C. Plasma insulin concentrations were higher and plasma glucagon concentrations were lower in gerbils fed the soy protein than in those fed casein. Plasma glucose was also higher in the soy fed gerbils. Plasma T4 and TSH levels were significantly higher in gerbils fed the soy diet. T3 levels were unaffected by either the soy or casein diet. These results begin to suggest that the response of hormone levels to the soy protein may contribute to its hypocholesterolemic effect.

A similar study by Scholz-Ahrens et al. (1989) has looked at the hormonal effects of soy protein versus casein in minipigs. The pigs were separated into two groups (soy and casein) with a crossover design lasting a total of 14 weeks. The diets were identical except for 20% protein provided by either casein or soy protein isolate. Six hormones

were measured in 22 blood samples taken throughout each measurement day 2, 42, and 49. The soy diet group had a lower level of cholesterol but it was not significant. There was no difference in insulin, glucagon, the insulin/glucagon ratio, hydrocortisone or T3 concentrations due to the dietary group. Total and free T4 concentrations were higher throughout the day after soy feeding than after casein feeding (34% and 26% respectively). This suggests that since a hypocholesterolemic effect was still seen with the difference in just T4 concentrations, the T4 may be the important mediator that accounts for these beneficial effects.

Conclusions

There are many different theories for the hypocholesterolemic effects of soy protein and its specific mechanism. It is still not known whether it is a protein or non-protein component and how that component mediates cholesterol uptake and metabolism. Available research provides evidence that human and animal (rats, gerbils, rabbits, minipigs) responses may be different or altered by some protocol differences in the research studies. Many variables including the source of soy protein (soy protein isolate, soy protein concentrate, textured vegetable proteins) may skew the results that pooling the findings of current research gives.

Oxidation Overview

A growing amount of current research suggests that oxidation of lipoproteins is a key component in the formation of atherosclerotic plaques. Oxidative changes occurring in serum LDL over time may accelerate atherosclerosis. LDL that has been modified by a free radical process leads to oxidized LDL. This form of LDL is picked up by endothelial cells and acts as a chemical attractant for monocytes by the process illustrated in Figure 1. The enhanced susceptibility of oxidized LDL to uptake by macrophages may ultimately result in the formation of atherosclerotic plaques and vascular disease (Patel, 1998).

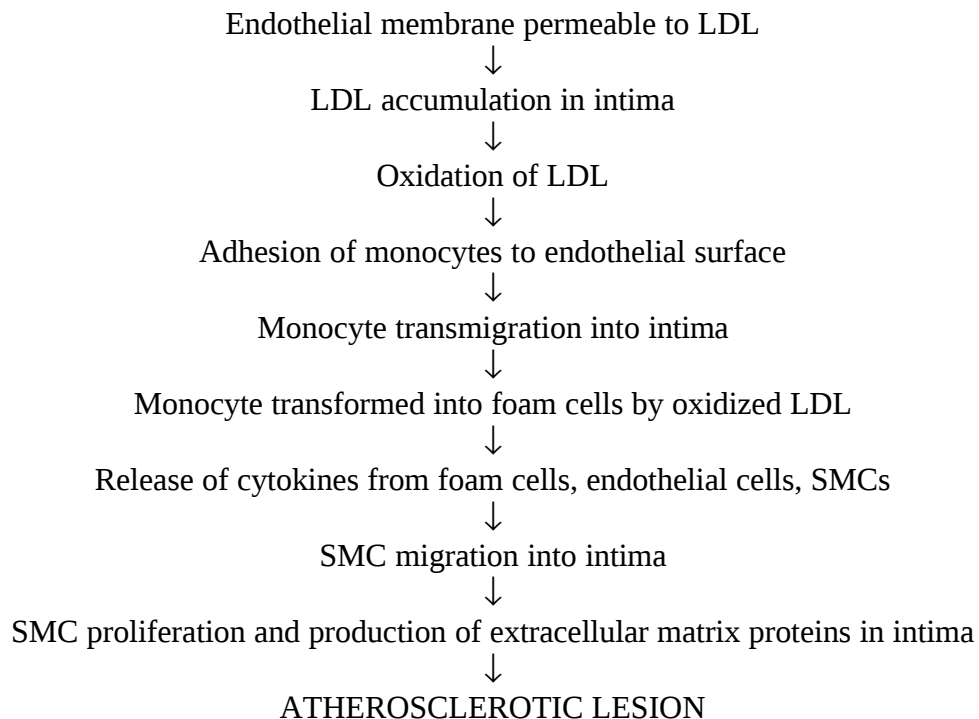


Fig 1. Steps in formation of atherosclerotic plaque in vivo. SMC, smooth muscle cell. (Patel, 1998)

Exercise to Induce Oxidation

Exercise is often recommended for the prevention and management of CVD. Although exercise is intended to be a positive influence, the increased energy demand may play a role in increased tissue oxidation. During physical exercise, especially of the aerobic type, there is a multifold increase in the supply of oxygen to the active tissues. Oxygen uptake may increase 15 to 20 fold with an up to 30 fold increase in blood flow (Viguie, 1993). Delivery of increased amounts of oxygen to active tissues during exercise fuels oxidative metabolism. This produces reactive oxygen species (ROS) which can lead to the accumulation of oxidation products that exceed the body's natural antioxidant defenses (Sen, 1995).

Vasankari et al. (1996) studied the effects of acute prolonged exercise on serum and LDL oxidation and antioxidant defenses. Eight male runners participated in a 31 km run and 22 males participated in a marathon run. Blood samples were taken before warm-up and immediately after exercise. Measurements were taken for LDL oxidation (LDL-ox), serum oxidation (S-ox), total peroxyl radical trapping antioxidant potential in LDL and serum (LDL-TRAP, S-TRAP). The results show that there were no changes in LDL-ox or LDL-TRAP during the 31 km run or the marathon. S-ox increased significantly during the 31 km run (9%) but not during the marathon. This may be due to marathon runners being permitted to eat and drink during the race while 31 km runners were only given water. S-TRAP increased by 22% in 31 km run and 16% in the marathon. The authors conclude that prolonged exercise raises S-ox. Differences

between runners in the two events may be due to training history, food ingestion pattern or increased antioxidant defenses to longer exercise in the marathon runners.

Sanchez-Quesada et al. (1995) have studied the increase in LDL susceptibility to oxidation after intense, long duration aerobic exercise. Six well-trained males ran for 4 hours on an indoor track at an average of 11.95 km/h. They were given orange juice, a carbohydrate (100% CHO) drink, and biscuits if desired. All consumption was recorded. Mean energy expenditure was calculated at 3400 Kcal. Total and LDL triglyceride increased significantly with running compared to pre-exercise values. LDL susceptibility to oxidation was increased with the long duration running. No variable was statistically associated with food consumption. The authors conclude that the LDL changes seen in these exercise conditions show an unfavorable effect of intense exercise on lipoprotein metabolism.

Ayres et al. (1998) have studied the effects of exercise-induced changes in lipid peroxidation in amenorrheic female athletes. The subjects were seven eumenorrheic (normally menstruating) and seven amenorrheic (<3 menses/year) female athletes that had been in a running program of at least 20 miles/week for the past 2 years. Testing included pre-exercise and post-exercise blood samples and an exercise session of running on the treadmill in a graded exercise session that increased speed and grade every 3 minutes until the subjects could no longer continue. Measurements for blood lipid profile and LDL-ox by conjugated dienes were performed on all samples. Both amenorrheic and eumenorrheic athletes showed a significant decrease in lag time of conjugated diene formation after exercise. Amenorrheic athletes had a greater magnitude

of change. They also had a significant increase in oxysterol formation and baseline creatine kinase activity compared to the eumenorrheic subjects. This study concludes that amenorrheic athletes demonstrate an increased potential for lipid peroxidation after exercise. Both groups showed that running was effective in causing an increase of lipid peroxides in blood.

Many researchers have looked at long duration acute exercise to cause oxidative stress, but we need to look at more realistic levels for the general population. Wetzstein et al. (1998) have looked at low levels of acute exercise and their effect on oxidation. Subjects were separated into two groups: sedentary (n=12) and exercise (n=11). All subjects participated in a $\text{VO}_{2\text{pk}}$ test on a treadmill and a continuous progressive protocol. After $\text{VO}_{2\text{pk}}$ was determined subjects returned to the lab and performed a 30 min submaximal exercise bout at 55% $\text{VO}_{2\text{pk}}$ for the sedentary group and 70% $\text{VO}_{2\text{pk}}$ for the exercise group. Blood was taken immediately before and after a submaximal exercise bout following an overnight fast. Measurements were taken for TC, LDL-C, TG, LDL-ox and myeloperoxidase (MPO) protein levels. A statistically significant decrease in lag time for LDL-ox was observed in all subject groups after exercise compared to the baseline. There was also a significant increase in MPO levels following exercise. This suggests that 30 minutes of submaximal exercise is sufficient to induce oxidative stress to increase the susceptibility of LDL to in vitro oxidation.

Since it is apparent that exercise does cause oxidative stress, studies have recently begun to look at the resistance of certain groups to oxidative stress. Another study by

Sanchez-Quesada et al. (1997) has examined the resistance to LDL oxidative modification in trained versus sedentary subjects. A group of 38 well-trained athletes was compared to a group of 38 age-BMI-matched sedentary individuals. Blood samples were taken after a 12 h fast and analyzed for plasma lipids, LDL-ox susceptibility, LDL subfraction, electronegative LDL (-LDL) and antioxidant resistance measured by antioxidant content of plasma and LDL. The trained individuals showed a higher concentration of HDL-C compared to the sedentary group. The lag phase of conjugated dienes formation was significantly higher in athletes, indicating a higher resistance to oxidation of LDL. Both groups had similar -LDL, and LDL subfraction. None of the antioxidants measured correlated with lag phase time. The authors conclude that LDL from trained subjects is more resistant to oxidative modification than it is from sedentary subjects.

Soy Isoflavones Overview

Soy research over the past few decades has concentrated on the effects of soy protein on blood lipid lowering. More recently, it has focused on mechanisms of action, including protein and non-protein components of soy. Isoflavones are an indigestible non-protein constituent that may play a role in the beneficial effects of soy protein. Phytochemicals are plant-derived chemicals that play a role in the biological activity in the body. A subclass, called phytoestrogens have a nonsteroidal structure that can behave similarly to estrogen. The two major classes are lignans and isoflavones. Isoflavones are

found in relatively large concentrations in soy foods (Mitchell et al., 1998). Isoflavones have a signature phenolic ring, necessary for binding to the estrogen receptor.

Some phenolic compounds have the ability to donate H atoms/electrons from their hydroxyl groups to free radicals, suggesting a strong antioxidant capacity (Scott, 1997). This can aid in the protection against free radical damage in the body that has overcome natural system defenses.

In soy foods, isoflavones such as genistein and daidzein show the most promise as antioxidants and possibly the component partially responsible for the beneficial effects of soy on body systems.

Effects of Isoflavones

The effects of isoflavones to reduce cardiac risk factors have been studied by Anthony et al. (1996). Fourteen female and 13 male prepubertal rhesus monkeys were fed a diet of casein and lactalbumin as the main protein source for 3 weeks before experimental diets began. Experimental diets were either soy+, which was a moderately atherogenic diet with ISP and isoflavones intact or the soy- group which had the same diet composition with alcohol extracted ISP to remove isoflavones. Each diet period lasted 6 months in a cross-over design. Plasma lipid and lipoprotein concentrations were measured at baseline and throughout treatment periods. The soy+ group had significantly lower TC and LDL+VLDL-C concentrations in both male and female monkeys. HDL-C

was also higher in soy+group. This suggests that the isoflavones found in soy protein reduce cardiovascular risk in monkeys.

A follow-up study (Anthony et al., 1997) has examined the efficacy of soy protein amino acids and soy isoflavones on plasma lipids and lipoprotein concentrations, including atherosclerosis in cynomolgus monkeys. Eighty five male and 75 female monkeys were assigned to 1 of 3 treatment groups: casein (C), SPI with isoflavone extracted with ethanol (SPI-), and SPI with isoflavones intact (SPI+). The diets lasted for 14 months and provided identical percentages of energy from protein (18.5%), fat (40.6%), and carbohydrate (40.9%), and they contained the same amount of cholesterol (0.31mg/Kcal). There were no isoflavones in the C diet, low amounts in the SPI- diet (equivalent to 16 mg/person/day), and high amounts of isoflavones in the SPI+ group (equivalent to 143 mg/person/day). Human equivalencies were calculated based on a 200 Kcal/day diet.

The results show that the LDL+VLDL concentration was only slightly lower (8%) in the SPI- group than in the C group. The SPI+ group had a significantly lower LDL+VLDL (40%) compared to C. Beneficial effects on HDL cholesterol were also seen with the SPI+ (50% higher) and SPI- (20% higher) groups compared to the C group, which showed the lowest values. The effects of the diets on coronary artery atherosclerosis were measured by killing 11 male monkeys from each group and evaluating them for atherosclerosis. Overall, the C group had 73% of its monkeys showing atherosclerotic plaques >50% of the thickness of the media while the SPI- and

SPI+ groups showed 64% and 45% respectively. The authors conclude that these data suggest that alcohol extractable components found in soy, such as soy isoflavones, contribute in a major way to the regulation of plasma lipid concentrations and the prevalence of atherosclerosis. It is not certain whether the effects seen in the SPI- group could be attributed to residual isoflavones or amino acids.

Gooderham et al. (1996) have studied SPI rich in genistein and daidzein and its effects on plasma isoflavone concentrations, platelet aggregation, blood lipids, and fatty acid composition of plasma phospholipid in normal male subjects. Twenty healthy college-aged males were separated into experimental and control groups. The experimental group was supplemented with 60 g/d soy protein in the form of a beverage powder. The soy protein contained 78.6 mg total isoflavones per day including 48 mg genistein, 21 mg daidzein, and 9 mg glycitein. The control group consumed 60 g/day of calcium caseinate powder that was free of isoflavones. Supplementation lasted for 28 days and was followed by a 28 d washout period with no supplementation. Blood was drawn on day 0, 28 and 56.

Plasma genistein and daidzein concentrations were not different on day 0 between groups, although a dramatic increase was observed in the experimental group at the end of the soy supplementation with no change in the control group. At the end of the washout period the level of isoflavones in the experimental subjects returned to basal levels. There were no significant differences seen in platelet aggregation, HDL or TC between groups on any measurement day. This study suggests that although soy protein

can increase plasma concentrations of isoflavones, this level may not be sufficient to decrease cholesterol and reduce CVD risk.

Tikkanen et al. (1998) have studied soybean phytoestrogen intake and its effects on low density lipoprotein oxidation resistance. Six healthy subjects (3 men and 3 women) consumed one soy bar 3 times a day for two weeks after a two week baseline diet. During the baseline diet, the subjects eliminated all soy products, beans, peas, nuts, sprouts, seeds and any vitamin supplements or fortified foods. Soy bars gave a total daily supply of 36 mg genistein and 21 mg daidzein without any other antioxidants or vitamins. After the soy supplementation, the baseline diet was followed for another two weeks. Blood was taken during the last 2 days of each 2 week treatment. Measurements for TC, TG, VLDL, LDL, HDL, and protein in LDL did not change during the administration of the soy bars. After the soy feeding, there were large increases in plasma genistein and daidzein. LDL was subjected to copper-mediated oxidation in vitro. Compared with values obtained during baseline diet periods, lag phases of LDL oxidation curves were an average of 20 min longer during soy intake, thus suggesting a reduced susceptibility to oxidation. The authors conclude that an intake of soy antioxidants such as genistein and daidzein may provide protection against oxidative modification of LDL.

Mechanisms for Isoflavone Action

Kaplotis et al. (1997) have looked at the effects of in vitro genistein on LDL oxidation in and vascular cells using bovine and human endothelial cells. LDL oxidation was measured by thiobarbituric acid assay (TBARS), lipid hydroperoxide assay, lipid electrophoresis, cytotoxic assay, and western blot analysis of tyrosine phosphorylated proteins. When genistein was added to the LDL oxidation system (50 ug/mL of LDL, 10umol/L Cu^{2+}), formation of TBARS was greatly reduced. Measurement of total lipid peroxides and electrophoresis of LDL also revealed a substantial decrease in oxidative modification with added genistein. Bovine aortic endothelial cell and human endothelial cell-mediated LDL oxidation was inhibited with genistein.

Genistein also lessened damage to endothelial cells by oxidized LDL because it provided a protective effect. Identical methods were used replacing genistein with daidzein or genistin. Daidzein showed mild inhibition of LDL oxidation but to a much less extent than genistein. Genistin was much less effective than either genistein or daidzein. The authors propose that genistin was a less effective inhibitor of oxidation, possibly due to the loss of the polyphenolic character by the o-glucoside formation or to the hydrophilic property of genistin making access to the lipid environment more difficult. This environment is also the site of oxidative damage. The slight effectiveness of daidzein suggests that the polyphenolic molecular structure plays a role in the antioxidant capabilities of these compounds.

Hogsdon et al. (1996) have looked at the isoflavones found in soy and their metabolic products that inhibit in vitro LDL oxidation in serum. Blood was taken from 6 healthy volunteers and centrifuged to obtain serum. Genistein and daidzein along with daidzein metabolites equol and O-desmethylangolensin were tested on copper induced oxidation of lipoproteins in serum. Concentrations of 0.1 μ M, 1 μ M and 10 μ M were tested for each compound with each serum sample. The results show that all compounds inhibited lipoprotein oxidation to some extent. The concentrations needed to be protective are 1 μ M for genistein and daidzein and 0.1 μ M for both daidzein metabolites. This study has demonstrated that the daidzein metabolites are more potent in their antioxidant ability in vitro and more human studies need to be done to determine in vivo effects.

Kerry et al. (1998) have conducted a study investigating the effects of genistein on LDL oxidation in vitro. All LDL was provided by a single human subject at one blood draw. The in vitro genistein inhibited copper mediated oxidation was measured by an increase in lag time in conjugated diene formation. The peroxy radical oxidation of LDL was also measured with differing concentrations of genistin and ethanol determined by the formation of MDA. Significant decreases in oxidation were seen with genistein treatment compared to ethanol. 32, 44, and 46% decreases in MDA were seen at incubation times of 3, 4, and 5 hours respectively. Another trial included plasma that was incubated with 25, 50, or 100 μ mol/L genistein for up to 24 hours in a water bath to determine if the genistein would be incorporated into the LDL. Control plasma was incubated with ethanol at the same concentrations. The concentrations of genistein in

plasma and LDL was determined by HPLC. Approximately 3-4% of genistein concentration that was present in plasma was incorporated into LDL but oxidation of ethanol and genistein-treated LDL did not differ significantly.

The authors conclude that the mechanism of the action of genistein is not an effective physiological antioxidant in LDL, since it is not readily incorporated as demonstrated. The antioxidant properties may be limited to plasma where higher levels are seen. The levels of genistein used in this experiment were higher than those seen in Japanese men (Aldercruz, 1993) consuming a traditional soy diet or a subject receiving 80 mg genistein per day from a soy supplement. The amounts used in vitro may not be realistic for a subject who wishes to obtain the benefits seen in this study.

Conclusions

These studies presented demonstrate the different aspects of soy protein and its effects to show that it has the potential to decrease CVD risk. Specific mechanisms of action have been studied but not ultimately identified. It is still not clear whether the protein or non-protein component or a combination of both is responsible for the activity. Differences between animal and human models suggest different mechanisms of action with subject type. In humans, studies have given variable results possibly due to differences in baseline measurements such as hypocholesterolemia, normocholesterolemia, and hypercholesterolemia. Continuing research to determine a

clear mechanism of the beneficial effects of soy, soy protein and soy isoflavones would be most effective if humans were used as the model. With similar protocols and varied measurements a more reliable conclusion may be reached.

CHAPTER 3

MATERIALS AND METHODS

This project was approved by the Institutional Review Board for Research Involving Human Subjects at Virginia Polytechnic Institute and State University.

Subjects

Sixteen healthy men participating in this study were recruited through postings around the Virginia Tech campus. All subjects signed an informed consent before any procedures began (Appendix I). They were all normolipidemic, and of healthy body weight (**Table 1**). Subjects were evaluated using a medical health history questionnaire (Appendix II) and self-reported physical activity. Exclusion criteria included hyperlipidemia, hypertension, cardiovascular or pulmonary disease, current medications, and documented abnormal thyroid function. Each subject chosen for the study reported participation in moderate (50-70% $\text{VO}_{2\text{pk}}$) cardiorespiratory physical activity 2-3 times per week for 1 year prior to study. Subjects were randomly assigned to either soy or casein treatment groups. There were no subject dropouts during this study period.

Diets

Soy protein and casein diets were used during this study. Proteins were baked into blueberry muffins and snickerdoodle cookies to be consumed daily by subjects (**Table 2**). ISP (Protein Technologies International, St. Louis, MO) provided 33g/d

protein, which replaced an equivalent amount of animal protein in the subjects' normal self-selected solid food diet. Calcium Caseinate (New Zealand Milk Products, Inc., Santa Rosa, CA) provided 33g/d casein protein as control and was incorporated identically in subjects' diet.

Participants were asked to consume 2 muffins and 3 cookies daily (total 33 g/d either soy or casein protein) for the 28 day treatment period.

Study Design

After recruitment subjects reported to the lab for a 28 ml blood draw after overnight fast so baseline plasma lipids could be measured (day1). The next morning (day2) subjects reported to the Human Performance Laboratory to complete a $\text{VO}_{2\text{pk}}$ test using a Monark cycle ergometer and Medgraphics CPX/D metabolic cart. This test was used to determine individual exercise capacity. Before the session, subjects were interviewed to establish estimated capacity for cycling so progression of workloads could be provided to achieve $\text{VO}_{2\text{pk}}$ within 8-14 minutes. This allowed the body time to reach peak performance before the legs fatigued.

Subjects pedaled on a cycle ergometer for two minutes as a warm-up to familiarize them with the bike and cadence of 60rpm. After initial warm-up subjects cycled at 60rpm with an increase in resistance every 1-2 minutes until they could no longer maintain cadence or were unwilling to continue. HR, BP and RPE as well as VO_2 were monitored and recorded throughout session (Appendix III).

$\text{VO}_{2\text{pk}}$ measurement was used to calculate individual 70% $\text{VO}_{2\text{pk}}$ to be used in future exercise sessions. After 1 week of rest (day 9) subjects returned after overnight

fasting for sustained exercise bout #1 which consisted of 30 minutes of cycling at 70% VO₂pk. HR was monitored using 3-lead ECG system. Blood was drawn 5 minutes post exercise to measure baseline exercise induced changes. The next morning protein supplementation began and lasted for 28d (day 10-38). Day 39 subjects returned and completed sustained exercise bout #2 which was identical to previous bout with blood draw 5 minutes post exercise. This allowed a comparison of blood measurements pre- and post-supplementation.

Schedule	
Day 1	Fasting after 9pm, subject may consume only water
Day 2	Subjects will arrive at the lab in the morning for blood draw #1
Day 3	VO ₂ pk test
Day 9	Fast after 9pm
Day 10	Sustained exercise bout (30 min @ 70% VO ₂ pk), Blood draw #2
Days 11-39	Soy/casein supplementation from bakery products
Day 39	Fast after 9pm
Day 40	Sustained exercise bout (30 min @ 70% VO ₂ pk), Blood draw #3

Food Analysis

Six batches of blueberry muffin samples were analyzed for descriptive measures and consistency. Fresh samples were tested on the day they were baked after cooling to room temperature to determine:

- weight/height/diameter

- total kcal
- % protein
- % fat
- % moisture
- water activity
- compression
- soy protein (g)
- isoflavones (mg)

Recipes and detailed food analysis methods are illustrated in Appendix IV.

Sensory Evaluation

Acceptability of high SPI blueberry muffin was determined by public response using a simple consumer scorecard (Appendix V). Questions were based on overall opinion, appearance, texture, and flavor of the muffin. Participants were adults from the general Southwest Virginia population (n=170).

Biochemical Measurements

Blood was collected in tubes containing EDTA and placed on ice until analysis. Plasma was separated by centrifugation and HDL was separated and measured immediately using enzymatic procedure (Stanbio, San Antonio TX). Plasma was also used to determine TC and triglycerides (TG) enzymatically (Stanbio, San Antonio TX). LDL-C was calculated according to Friedewald et al (1972) and was stored at -80°C before testing. Plasma was incubated at 37°C for 24 hours with 10 uM CuSo₄.

Oxidation was measured by means of a modified thiobarbituric acid reactive substances (TBARS) method as described by Naito et al (1993). Identical procedures were followed for each blood draw. All biochemical measurements were duplicated for accuracy and re-measured if trials were $\geq 10\%$ difference.

Detailed Biochemical procedures are shown in Appendix VI.

Records

Subjects completed a 3-day dietary record (Appendix VII) at the initiation and termination of supplemental period. This was analyzed using *Diet Analysis +* (ESHA Research, Salem, OR) to detect any changes in diet throughout treatment period. Two 24-hour physical activity records (Appendix VIII) were kept during the same 3-day period as dietary intakes. Body height and weight were measured to calculate BMI and weight changes across treatment periods.

Statistical Analysis

A 2-factor (group, stage) ANOVA with repeated measures was used to evaluate the effects of dietary proteins on plasma lipids and oxidation. A paired t-test was used to determine any differences between the two diet and physical activity records. The *Statistical Analysis System* (SAS Institute, Cary, NC) was used to analyze all data. Significance was assessed at the $p < 0.05$ level.

CHAPTER 4

RESULTS

Baseline characteristics for subjects who completed the study is summarized in **Table 1**. Mean body mass index (BMI; kg/m²) for subjects at baseline was 23.8 ± 3.6 kg/m² which is in the desirable range for adult men (ACSM, 1995). Relative VO₂pk measured on a cycle ergometer measured a mean of 40.6 ± 7.2 ml/kg which is in the excellent category (80th percentile) for age and gender (ACSM, 1995). Baseline plasma lipid profile was measured after 24 hours of rest and a 12 hour fasting period. Total cholesterol, LDL-C and TG levels were 144.4 ± 19.5 mg/dL, 76.9 ± 20.2 mg/dL, 86.4 ± 23.4 mg/dL respectively and are all within the desired ranges for reduced risk of CVD. HDL-C of the subjects was 50.1 ± 9.4 which is greater than the minimum desired level of 35 mg/dL (NCEP, 1993). There were no significant changes in body weight or BMI throughout the study.

TABLE 1

Baseline characteristics of subjects completing the study*

	Value
Age (y)	25 ± 3.9
Body Weight (kg)	77.3 ± 14.8
Body mass index (kg/m ²)	23.8 ± 3.6
Relative VO ₂ pk (ml/kg)	40.6 ± 7.2
Plasma lipids (mg/dL)	
Total cholesterol	144.4 ± 19.5
HDL cholesterol	50.1 ± 9.4
LDL cholesterol	76.9 ± 20.2
Triglycerides	86.4 ± 23.4
Plasma TBARS (nmoles/mg protein)	2.41 ± 0.69

*Mean $\pm$ SD, n=16

Dietary intake is summarized in **Table 2** for baseline and treatment periods. The subjects' diets were within the Dietary Guidelines for Americans (USDA 1995). There were no statistically significant differences between the two dietary treatment periods.

TABLE 2

Actual dietary intake

	Baseline	Treatment
Kcal	2879 $\pm$ 812	2948 $\pm$ 504
Protein (% energy)	13.5 $\pm$ 4.3	13.8 $\pm$ 4.8
Fat (% energy)	25.7 $\pm$ 4.7	22.9 $\pm$ 4.8
Carbohydrate (% energy)	59.5 $\pm$ 6.4	61.7 $\pm$ 7.5
Cholesterol (mg)	244 $\pm$ 26	221 $\pm$ 24

*Mean $\pm$ SD, n=16

Mean plasma concentrations are summarized in **Table 3** after 30 minute sustained exercise bout before and after dietary treatment. Pre supplementation values are higher than baseline values (table1) due to decreased plasma volume and increased LDL circulating in the blood immediately following exercise. All plasma lipid measurements were unaffected by dietary treatment. Both casein and soy groups had a slight but not significant decrease in TG levels. TBARS were also minimally decreased with dietary treatment while the casein group showed a slight increase.

TABLE 3

Summary of plasma lipid profile

	Diet			
	Casein		Soy	
	Pre-suppl	Post-suppl	Pre-suppl	Post-suppl
Total cholesterol (mmol/L)	156.9 $\pm$ 8.9	160.1 $\pm$ 12.7	171.9 $\pm$ 20.8	169.0 $\pm$ 22.2
HDL cholesterol (mmol/L)	51.6 $\pm$ 13.2	50.1 $\pm$ 12.6	49.3 $\pm$ 10.8	44.8 $\pm$ 9.3
LDL cholesterol (mmol/L)	84.8 $\pm$ 11.0	90.9 $\pm$ 13.3	99.1 $\pm$ 20.4	102.4 $\pm$ 17.2
Triglyceride (mmol/L)	102.4 $\pm$ 29.2	95.8 $\pm$ 25.1	117.3 $\pm$ 66.8	108.3 $\pm$ 42.1
TBARS (nmoles/mg protein)	2.10 $\pm$ 0.54	2.23 $\pm$ 0.41	2.43 $\pm$ 0.59	2.36 $\pm$ 0.38

Pre-suppl blood draw 5 minutes post sustained exercise bout #1
Post-suppl blood draw 5 minutes post sustained exercise bout #2
*Mean $\pm$ SD, n=16

Food Analysis

Extensive development and testing was done to ensure that an acceptable product was being used for clinical research purposes. A blueberry muffin containing SPI from Protein Technologies International was made to contain high levels of soy protein and isoflavones. A breakdown summary of the SPI is shown in **Table 4** and **Table 5**. A convenient and tasty product was desired to ensure subject compliance for treatment periods with minimal preparation at home. Food analysis of the soy blueberry muffin is summarized in **Table 6**.

TABLE 4
Composition of SPI as is (Protein Technologies International)

Nutrient	%
Protein	79.70%
Moisture	4.20%
Fat (PE extract)	0.60%
Fat (Acid Hydrolysis)	4.80%
Ash	11.20%
Sodium	0.80%
Calcium	3.10%
Potassium	0.70%

TABLE 5
Isoflavone analysis mg/g protein

Aglycone components	mg/g protein
Genistein	1.27
Daidzein	0.58
Glycitein	0.09
Total aglycone components	1.94

TABLE 6
Characteristics of bakery products*

	Soy muffin	Casein muffin	Soy cookie	Casein cookie
ISP (g)	9.3	0	4.7	0
Casein (g)	0	9.1	0	4.8
Genistein (mg)	11.6	0	6.0	0
Daidzein (mg)	5.3	0	2.7	0
Kcal	180	180	160	160
Protein (g)	11.3	11.0	6.25	6.3
Fat (g)	5.8	5.8	7.1	7.1

*Subjects consumed 2 muffins and 3 cookies daily throughout the treatment period

The study design contained daily supplementation of 33 g of soy protein daily so another product was needed in addition to the blueberry muffins for variety throughout the day. Since we asked subjects to spread the consumption of these products throughout the day, we wanted a snack type product that may be desired later in the day. A snickerdoodle cookie was therefore developed. Subjects were asked to consume 2 muffins and 3 cookies each day for a total soy protein intake of 33 g and 64 mg of isoflavones. Casein was used in the control products to provide animal protein that was free of isoflavones. The control and treatment foods had the same dietary composition except for protein source. A detailed breakdown the soy and casein cookies and muffins is shown in **Table 6**. Food analysis results of the high ISP blueberry muffin used in the sensory evaluation is summarized in **Table 7**.

TABLE 7Analysis of high SPI blueberry muffin recipe mean $\pm$ SD

Measurement	1 muffin
Weight (g)	78.4 $\pm$ 2.8
Height (mm)	50.6 $\pm$ 4.2
Diameter (mm)	65.5 $\pm$ 1.1
Total kcal	183
Protein (%)	14.8 $\pm$ 0.6
Fat (%)	28
Moisture (%)	43.2 $\pm$ 1.1
Water activity @22°C	0.954 $\pm$ 0.01
Compression displacement (mm)	13.0 $\pm$ 0.5
Compression load at max (kg)	1.7 $\pm$.3
Soy protein calculated (g)	9.1
Isoflavones calculated (mg)	18.0

Sensory evaluation

After the initial soy blueberry muffin was developed it was taken out into the general public for sensory evaluation. Survey responses were collected from nutritional professionals and general adult and elderly participants attending health fairs throughout Southwest Virginia. Results are summarized in **Figure 1**.

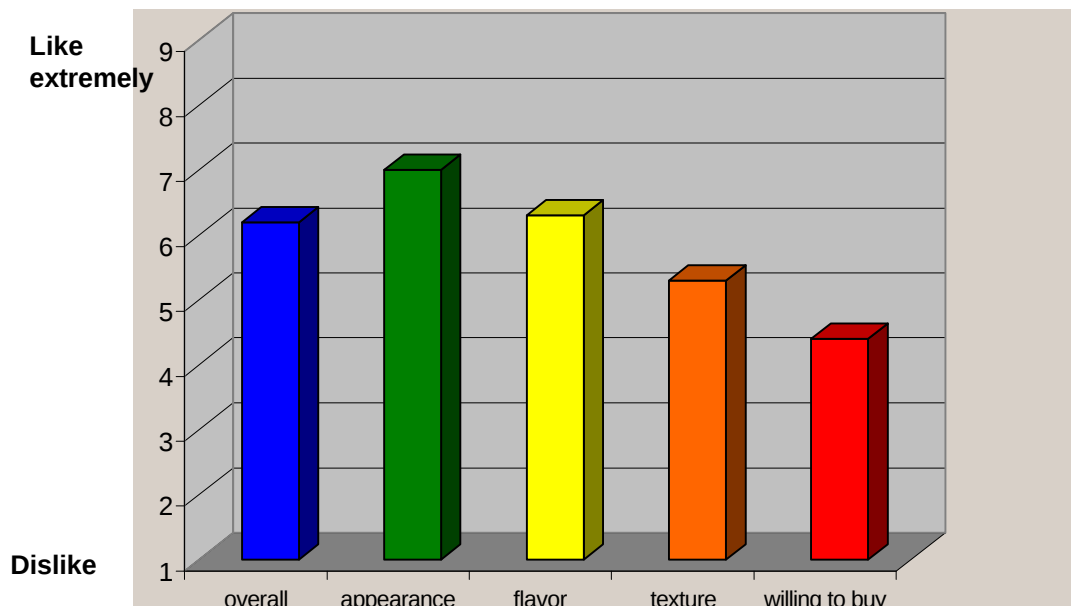
FIGURE 1

Sensory characteristics of high SPI blueberry muffin evaluated by the general public population.

Max score =9 “like extremely”, 6= “like”

Willing to buy: max =9 “every chance I get”, 6= 2-3x per week

n=170



CHAPTER 5

CONCLUSIONS

The present study demonstrates that soy protein with high isoflavone content does not have a marked effect on plasma lipoprotein levels or plasma oxidation when compared to casein in healthy college-aged males. Other studies with normal healthy subjects have shown a marginal decrease in LDL-C (Van Raaij, 1981, Wong, 1998) and increase in HDL-C (Van Raaij, 1981, 1982) with no change in TC. Other studies including healthy volunteers with normal cholesterol levels showed no difference in response to the two types of dietary protein (Grundy 1983, Meinertz 1988). More marked changes in response to soy protein were seen with hypercholesterolemic patients (Potter, 1993, Wong, 1998). There was a direct relationship to the degree of hypercholesterolemia and the beneficial effect of the soy protein treatment

Subjects continued on a self selected solid food diet throughout the study, replacing only part of their daily protein intake with either soy protein or casein with no added cholesterol. The average cholesterol intake of the subjects in this study was 221 mg $\pm$ 24 mg daily. Other studies that have seen an effect of dietary protein in normocholesterolemic subjects used cholesterol-enriched diets (Meinertz, 1989). Meinertz et al, 1989 added 500 mg of cholesterol to each diet, which is much higher than the daily value of 300 mg for dietary cholesterol and the value of 221mg dietary cholesterol consumed by our subjects throughout the study.

Formula diets have also shown an effect with soy protein compared to casein diets in normal subjects (Grundy, 1983, Nilausen 1998). This data may not be representative

of actual responses of soy protein on lipoprotein levels due to drastic dietary changes. Weight loss occurred when changing from solid food to formula diets, which may have played a role in altering lipoprotein fractions.

Exercise induced oxidation was also not affected by dietary protein. The level of exercise administered during the sustained exercise bouts may not have caused enough oxidative stress to overcome the natural antioxidant defenses of the healthy, moderately active subjects. This is shown by a lack of difference between baseline TBARS measures with no exercise and the TBARS after sustained exercise bout #1. Wetzstein et al. (1998) used the same level of exercise for 30 minutes and did see an increase in oxidative stress. Our subjects may be more fit and used to this level of exercise stress than the subjects in Wetzsteins study.

A follow-up study using higher levels of exercise for a longer duration and alternate levels of daily protein replacement, and a subject pool to include healthy women would be beneficial to gain a better understanding of soy protein replacement in healthy subjects.

The soy protein products used in this study were effectively incorporated into bakery products that were well liked and convenient to add to the subjects diet. The blueberry muffins provided 9.3g soy protein per muffin which is much higher than the 5-6g found in bakery products used in other studies (Klein, 1995, Potter, 1993).

In conclusion, these data indicate that individuals with normal cholesterol levels that participate in moderate physical activity may not benefit from short-term partial substitution of soy protein incorporation in their diets. The soy products used in this study provided > 25g of soy protein per day, which the newly approved FDA claim states

may reduce the risk of heart disease as part of a diet low in saturated fat and cholesterol. Our study did not show beneficial effects at the lipoprotein or plasma oxidation level but may be benefiting our subjects by maintaining their healthy cholesterol levels and lack of LDL oxidation.

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APPENDICES

APPENDIX I

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Informed Consent for Participants of Investigative Projects

Title of Project: Study to Test the Beneficial Effects of High Genistein Soy Protein on Humans

Investigators: Sandra Shehadeh, Raga Bakhit, Ph.D., Oliver Chen

I. Purpose of the Research/Project:

I am being asked to participate in a research study to determine if soy protein has any positive effects on risk factors for heart disease, specifically LDL cholesterol and blood lipids. It will involve periodic exercise and blood draws to get adequate data. Fourteen healthy college aged males are being asked to participate in this research study.

II. Procedures:

Prior to being included in the research study, I will complete a health history that will help determine if there may be reasons why I should not participate in this study. If the results of the health history indicate that I am an appropriate subject for this study, 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 to determine the maximal amount of exercise that I can perform. This will be on the first day of the research study and I will need to report to the lab in the morning without eating or drinking 2 hours prior to arrival. During this test I will be connected by electrodes and cables to an electrocardiograph recorder which will enable the technician to monitor my heart rate. I will also be fitted with a breathing apparatus (mouthpiece and nose clip) which will measure my exhaled gases to properly measure oxygen consumption. This test will be on a cycle ergometer with the amount of effort gradually increased. As I understand it, the increase in effort will continue until I report to the technician that I am unable to continue or the pedal rate falls below 40 revolutions per minute. I will decide when I am unable to continue and the technician will stop the test when I so request.

The second and third exercise sessions will last 30 minutes. I will be connected to the electrocardiograph for heart rate but not the breathing apparatus. I will cycle on the ergometer for 30 minutes at a constant moderate intensity. It is important that I continue cycling for all 30 minutes but the session will stop at my request.

Blood will be taken before the first exercise session and 24 hours after the second and third exercise session. The total amount will be 28 ml each time and will be done by a Licensed Medical Technician.

During the study I understand that I am to consume bakery products that the investigators will provide. It is important that I follow instructions and eat the foods each day for 28 days.

III. Risks:

It is my understanding and I have been informed that there exists the possibility during exercise of adverse physiologic responses during the tests. I have been informed that these changes could include abnormal blood pressure, fainting, disorders of the heart beat, and in rare instances, heart attack, stroke, or death. Every effort will be made to minimize these risks by evaluation of the preliminary information relating to your health and by observations during testing. Other possible discomforts I may experience in this study include leg fatigue, muscle soreness, a dry mouth (from the mouthpiece), pain, bleeding and local bruising at the site the blood was taken. I understand that the registered nurse or 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 registered nurse or licensed medical technician, certified exercise specialist and other support personnel will be present during all exercise testing to minimize the risks during exercise. I also understand that there is also a working telephone in the exercise testing area that can be used to alert the emergency rescue squad on the campus of Virginia Tech. Their average response time in getting to the Laboratory for Health and Exercise Science 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 protein for reducing risk factors for cardiovascular disease. In addition I will receive information about my physical fitness level and outcomes of the study, if desired.

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 your 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.

V. Compensation:

I understand that there is no monetary or course credit compensation available for participating in this project.

VI. Freedom to Withdraw:

I understand that I may withdraw from this study at any time. There will be no penalty or loss of benefits to which I am otherwise entitled.

VII. Approval of Research:

This project has been approved by the Institutional Review Board.

VIII. Subjects Responsibilities:

I know of no reason that I cannot participate in this study. I have the following responsibilities:

- Accurately report my medical history.
- Arrive at the laboratory at my scheduled for each exercise session.

- Arrive at the laboratory at least 2 hours after waiting or drinking.
- Arrive to give blood after fasting since 10pm the previous night.
- Eat bakery products following instructions daily.
- 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:

<u>Sandra Shehadeh</u>	<u>961-0627</u>
Investigator	Phone

<u>Dr. Raga Bakhit</u>	<u>231-6784</u>
Faculty Advisor	Phone

<u>E. R. Stout</u>	<u>231-9359</u>
Chairman, IRB Research Division	Phone

APPENDIX II

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Medical Health History Questionnaire

Name: _____ Age: _____

Date of Birth: _____ 19 _____

Address:

Phone: (540) _____ (work)

Phone: (540) _____ (home)

Please complete the following questionnaire as accurately as possible.

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	Short of Breath	Dizziness/Fainting	Joint Soreness

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 III

VO₂PK FORM

Name:_____

Subject #:_____

Age:_____

Height:_____

Predicted HR Max:_____

Weight:_____

Resting BP:_____

Seat Height:_____

RPM:_____

Minute	Work Load	Heart Rate	RPE	VO ₂
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				

APPENDIX IV

FOOD METHODS

Snickerdoodles- SOY

16 Cookies

4.7g isolated soy protein per cookie

Ingredients

½ C	Vegetable Butter Flavored Shortening
1 C	Isolated Soy Protein
1/3 C	All Purpose Flour
1 C	Sugar
1	Egg
½ tsp	Vanilla
½ tsp	Almond Extract
¼ tsp	Baking Soda
½ tsp	Baking Powder
1/3 C	Wheat Flour, Whole Grain
1 tsp	Water
4 T	Apple Sauce
2 T	Orange Juice
TOPPING	
2 T	Sugar
1 tsp	Cinnamon

1. Mix Margarine on high speed for 30 seconds.
2. Add isolated soy protein, all-purpose flour, sugar, egg, vanilla, almond extract, baking soda and baking powder. Mix.
3. Slowly add wheat flour, water and applesauce and OJ to moisten. Mix.
4. Chill 1 hour.
5. Shape 55 g batter into 1 ½ inch balls.
6. Roll the balls cinnamon/sugar mixture and place on greased cookie sheet.
7. Flatten balls slightly to shape.
8. Bake at 375°F for ~12 minutes.
9. Cool on wire rack.

Snickerdoodles- Casein
16 Cookies
4.8g isolated casein protein per cookie

Ingredients

½ C	Vegetable Butter Flavored Shortening
¾ C + 2 T	Isolated Casein Protein
1/3 C	All Purpose Flour
1C	Sugar
1	Egg
½ tsp	Vanilla
½ tsp	Almond Extract
¼ tsp	Baking Soda
½ tsp	Baking Powder
1/3 C	Wheat Flour, Whole Grain
4 T	Apple Sauce
2 T	OJ
TOPPING	
2 T	Sugar
1 tsp	Cinnamon

1. Mix Margarine on high speed for 30 seconds.
2. Add isolated casein protein, all-purpose flour, sugar, eggs, vanilla, almond extract, baking soda and baking powder. Mix.
3. Slowly add wheat flour and apple sauce to moisten. Mix.
4. Chill 1 hour.
5. Shape 55 g batter into 1 ½ inch balls.
6. Roll the balls cinnamon/sugar mixture and place on greased cookie sheet.
7. Flatten slightly to shape.
8. Bake at 375°F for ~12 minutes.
9. Cool on wire rack.

Blueberry Muffins – SOY
 12 Muffins
 9.3 Grams Isolated Soy Protein Per Muffin

Ingredients

1 ½ C	All Purpose Flour
1 ½ C	Isolated Soy Protein
1 ½ tsp	Baking Powder
¼ tsp	Baking Soda
½ tsp	Salt
½ tsp	Cream of Tartar
1/3 C	Granulated Sugar
1/3 C	Vegetable Butter Flavored Shortening
¾ C	Canned Blueberries, rinsed
4	Egg Whites
1 C	Orange Juice
2 tsp	Vanilla Extract
4 T	Apple Sauce
¼ C	All purpose flour
¼ C	Sugar
¼ tsp	cinnamon
1 tsp	Vegetable Butter Flavored Shortening

1. Preheat oven to 375°F.
2. Fill muffin pans with paper liners.
3. In large bowl combine the dry ingredients. Blend well.
4. With a pastry blender cut in shortening until evenly blended.
5. In another bowl beat egg whites. Beat in orange juice, applesauce and vanilla.
6. Add liquid mixture to dry mixture, and stir with spoon until moistened. About 25 strokes.
7. Fold in blueberries.
8. Spoon into muffin pans using ice cream scoop, filling close to rim.
9. Mix sugar, flour and cinnamon. Cut in shortening until even.
10. Spoon ½ tsp topping onto each muffin.
11. Bake for 20 minutes.
12. Cool to room temperature in muffin pans.

Blueberry Muffins – Casein
 12 Muffins
 9.1 Grams Isolated Casein Protein Per Muffin

Ingredients

1 ½ C	All Purpose Flour
1 1/4 C	Isolated Casein Protein
2 ½ tsp	Baking Powder
¼ tsp	Baking Soda
½ tsp	Salt
½ tsp	Cream of Tartar
1/3 C	Granulated Sugar
1/3 C	Vegetable Butter Flavored Shortening
¾ C	Canned Blueberries, rinsed
4	Egg Whites
1 C	Orange Juice
2 tsp	Vanilla Extract
4 T	Apple Sauce
¼ C	All purpose flour
¼ C	Sugar
¼ tsp	cinnamon
1 tsp	Vegetable Butter Flavored Shortening

1. Preheat standard oven to 375°F. Convection oven 315°F.
2. Fill muffin pans with paper liners.
3. In large bowl combine the dry ingredients. Blend well.
4. With a pastry blender cut in shortening until evenly blended.
5. In another bowl beat egg whites. Beat in orange juice, applesauce and vanilla.
6. Add liquid mixture to dry mixture, and stir with spoon until moistened, about 25 strokes.
7. Fold in blueberries.
8. Spoon into paper lined muffin pans (76g batter) using ice cream scoop, filling close to rim.
9. Mix sugar, flour and cinnamon. Cut in shortening until even.
10. Spoon ½ tsp topping onto each muffin.
11. Bake for 20 minutes in standard oven, 14 minutes in convection oven.
12. Cool to room temperature in muffin pans.

FOOD ANALYSIS METHODS

Analysis of six batches of soy blueberry muffin samples was performed for descriptive measures and consistency. Fresh samples were tested on the day they were baked after cooling to room temperature to determine:

Standing Measurements

- Weight of each sample was measured in grams.
- Height was measured using Vernier calipers with the pointer end through the center of each muffin.
- Diameter was determined with Vernier calipers and measured at two sites on each sample.

Total kcal

Determined using Nutritionist V (San Bruno, CA).

% Protein

Kjeldahl procedure

Digestion

The catalyst is copper sulfate.

Sodium sulfate is added to elevate the boiling point.

Sulfuric acid aids in the digestion process.

Samples are heated at 450 degrees until there is a clear green color.

During the heating and boiling process the organic and nitrogenous compounds are converted to ammonium sulfate which produces a clear green color.

1. Grind sample in food processor until uniform.
2. Weigh out sample 0.3-0.5g. Record and place in labeled digestion tubes.
3. Add 1/2 scoop catalyst (sodium sulfate/copper sulfate)
4. Add 15ml sulfuric acid
5. Secure tubes in digester.
6. Attach manifolds to tubes. Use clamps and attach them one at a time turning the tube while pushing up to get a good seal on the o-ring and tube. Once all are secure connect the rubber tubing from scrubber bottle to each manifold. Turn on scrubber and hood. Place cotton in the end of each manifold.
7. Power on. Turn on both heaters to a setting of 5. After 15 minutes raise the setting to 10 for both heaters. Allow digestion to continue until all samples turn a green color. Continue digestion for an additional 15 minutes. Turn all settings back to 0 and power off on the digestion blocks.
8. Allow samples to cool for 30 minutes in the block. Remove clamps and manifold tubes and place samples into a holding rack. Let cool an additional 30 minutes.
9. Add 25ml water to each tube slowly to prevent crystallization.

Distillation

The addition of excess sodium hydroxide causes displacement of free ammonia. The steam distillation causes the free ammonia to move into a solution of 4% boric acid where free ammonia is trapped. When ammonia reaches the boric acid the indicator

changes in color from red to greenish-blue indicating a change in pH due to addition of free ammonia.

Titration

During titration a standard solution of 0.1N HCl is added slowly until the color of the boric acid changes from the greenish-blue color back to red. This estimates the amount of ammonia trapped in the boric acid during distillation. This is the endpoint of titration.

1. Turn on cold tap water for condenser.
2. Turn on distillation unit controller and printer.
3. Enter date and mode.
4. Set parameters.
5. Do a pH calibration with 4.0 and 7.0 buffers.
6. Place the electrode into distillation boric acid collection cup. Place in the opening at the front of the boric acid collection cup.
7. Set the dosimat speed between 6-7 dv/dt
8. Preheat steam generator by placing 50-60ml distilled water in a clean digestion tube. Place water under rubber bung and into holder. Lower shield and press preheat. Preheat until distillate begins to collect in the boric acid collection cup. Press preheat again and to begin aspiration of sample. Repeat until there is a good steady stream of distillate coming down the collection tube.
9. Press balance program. Enter sample code and weight of sample in mg. Change to run mode.

10. Place sample #1 in holder by removing preheated tubes and secure with gray lever.

Shield down.

11. Press start. When distillation and titration is complete, aspiration will begin and result is printed.

12. Place sample #2 tube into clamp. Run. Press end after last sample has been tested.

13. Wash all tubes and with tap and distilled water. Run water through distillation unit.

14. Shut down controller, printer and dosimat. Turn off water and place electrode back into KCl solution and recap.

To convert the % Kjeldahl nitrogen to % protein:

$$\%N = \frac{(V1 - V2) \times (N \times f)}{E} \times 1400$$

E(mg)

$$\% \text{ protein} = \%N \times CF$$

V1= volume of acid used during titration

V2= volume of acid used to titrate blank

N= normality of the acid (0.1)

f= factor of the acid (1)

E= quantity of sample used in mg

CF= conversion factor

Fat %

Determined using Nutritionist V (San Bruno, CA).

Moisture %

1. Weigh thin heating pan and record weight and pan number.
2. Weigh ~10g ground sample, record. Place sample in pan.
3. Place in dryer oven for 1 hour at 130 °C.
4. Remove and place in moisture free cooling bin for 15 minutes.
5. Weigh dry sample in pan.
6. Calculate % moisture:

$$(\text{Water weight loss} / \text{sample start weight}) * 100$$

Water activity

1. Press ~3g finely ground sample into plastic disk.
2. Insert disk into Aqua Lab water activity analyzer.
3. Record water activity and temperature reading.

Compression

1. Calibrate Instron machine for test compression displacement and max load setting at distance equal to 25% of total sample height.
2. Initiate procedure by pressing the test sample.
3. Return the cross-head to its upward position and remove the first sample.
4. Repeat steps with each sample.
5. Print all compression graphs and save measurement data.

Soy protein (g) and isoflavones (mg)

- Calculated from Protein Technologies International SPI analysis.

Sensory evaluation

- Acceptability of high SPI blueberry muffin was determined by public response using a simple consumer scorecard.
- Survey questions were based on overall opinion, appearance, texture, and flavor of the muffin (Appendix V).
- Participants were adults from the general Southwest Virginia population (n=170).

APPENDIX V

SENSORY EVALUATION SCORECARD

Product _____

Date _____

Please evaluate the product by looking at it and tasting it.

Considering ALL characteristics, please indicate your overall opinion by checking one box:

☐	☐	☐	☐	☐	☐	☐	☐	☐
Dislike extremely			Neither like nor dislike			Like extremely		

Taste the product as many times as needed and indicate how much you LIKE or DISLIKE the following:

I. OVERALL APPEARANCE

☐	☐	☐	☐	☐	☐	☐	☐	☐
Dislike extremely			Neither like nor dislike			Like extremely		

OVERALL FLAVOR

☐	☐	☐	☐	☐	☐	☐	☐	☐
Dislike extremely			Neither like nor dislike			Like extremely		

II. OVERALL TEXTURE

☐	☐	☐	☐	☐	☐	☐	☐	☐
Dislike extremely			Neither like nor dislike			Like extremely		

Does this product make you feel:

☐	☐	☐	☐	☐	☐	☐	☐	☐
Not very full			About right			Extremely full		

Would you choose to eat this product again?

☐	☐	☐	☐	☐	☐	☐	☐	☐
Never again			At least twice per week			Every chance I get		

Please share any comments about this product on the back of the form. THANK YOU!!

APPENDIX VI
BIOCHEMICAL PROCEDURES

Plasma separation for blood by centrifugation

1. Collect blood samples from antecubital vein in EDTA-tubes
2. Swirl to mix and place on ice.
3. Spin at 600 x G (2000 rpm) for 20 minutes.
4. Collect plasma (upper yellow layer) and place in microtubes for immediate use or storage at -80°C for later measurement.

Plasma lipid measures

Total cholesterol analysis (Cholesterol LiquiColor, Stanbio Lab, Inc. San Antonio, TX)

Reagents

4-Aminophenazone	0.25 mmol/L
Phenol	25.0 mmol/L
Peroxidase	5.0 U/mL
Cholesterol Esterase	0.15 U/mL
Cholesterol Oxidase	0.2 U/mL

Buffers and Stabilizers

Chemical Principles:

CE

Cholesterol Esters ----> Cholesterol + Fatty Acids

COx

Cholesterol + O₂ ----> Cholset-4-en-3-en-one + H₂O₂

POD



Cholesterol esterase (CE) hydrolyzes esters to free cholesterol and fatty acids. The free cholesterol so produced plus the preformed cholesterol are then oxidized in the presence of cholesterol oxidase (COx) to Cholesterol-4-en-3-one and hydrogen peroxide. A quinoneimine chromogen, with absorption maxima at 500nm, is produced when phenol is oxidatively coupled with 4-Aminophenazone in the presence of peroxidase (POD) with hydrogen peroxide. The intensity of the final red color is proportional to total cholesterol concentration.

Procedure:

1. Set spectrometer to 500 nm and set absorbance to zero with water as reference.
2. Set up cuvettes for blank (B), standard (S) and sample (U).
3. Pipet 1.0 mL reagent into each cuvette
4. Pipet 0.01 mL standard (200 mg/dl), or sample into appropriate cuvettes.
5. Incubate at room temperature (25°C) for 10 minutes.
6. Read at 500 nm within 60 minutes.

Calculation:

$$\text{Serum Total Cholesterol (mg/dL)} = \text{Au/As} \times 200$$

HDL Cholesterol Analysis (Stanbio Lab, Inc. San Antonio, TX)

Reagents

Magnesium chloride, 1 mol/L in aqueous 1% (W/V) Dextran sulfate, stabilizers and preservative.

Chemical principles:

LDL cholesterol and VLDL cholesterol fractions are precipitated from plasma by means of Magnesium chloride/dextran sulfate reagent (Finley, 1978). HDL cholesterol is then determined in the supernatant fluid, using a derived dilution factor in the calculation.

Procedure:

HDL Separation Procedure:

1. Pipet 0.5mL plasma into centrifuge tube.
2. Add 0.05mL HDL Precipitating Reagent.
3. Mix well (vortex) and allow to stand 5 minutes at room temperature.
4. Centrifuge 10 minutes at 1000 xG.
5. Use clear supernatant, which contains HDL cholesterol as sample.

Manual Procedure:

1. Pipet 0.025mL standard or sample into appropriate tubes.
2. Add 1.0mL reagent to standard (S), sample (U), and blank (B) tubes.
3. Mix well (vortex).

4. Incubate at room temperature for 10 minutes.
5. Read S and U vs. B at 500 nm within 60 minutes.

Calculation:

$$\text{HDL cholesterol (mg/dL)} = \text{Au/As} \times 55$$

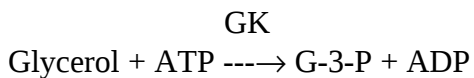
Triglycerides Determination (Stanbio Lab, Inc. San Antonio, TX)

Reagents:

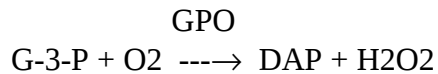
4-aminoantipyrine	0.4 mmol/L
4-chlorophenol	5.0 mmol/L
ATP	1.0 mmol/L
Lipases	150 U/mL
Glycerol-kinase	0.4 U/mL
Glycerol-3-phosphate oxidase	1.5 U/mL
Peroxidase	0.5 U/mL
PIPES buffer solution (pH 7.5)	mmol/L

Chemical Principles:

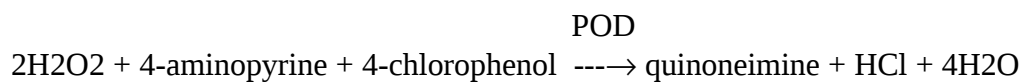
1. Glycerol and fatty acids are first formed by lipase action on the triglycerides.
2. Glycerol is then phosphorylated by adenosine-5-triphosphate (ATP) to produce glycerol-3-phosphate (G-3-P) and adenosine-5-diphosphate (ADP) in a reaction catalyzed by glycerol kinase (GK):



3. The G-3-P is oxidized by glycylphosphate oxidase (GPO) producing dihydroxyacetone phosphate (DAP) and hydrogen peroxide:



4. Peroxide reacts with a 4-aminoantipyrine and 4-chlorophenol under the catalytic influence of peroxidase (POD) to form quinoneimine:



Procedure:

1. Pipet 0.01mL standard or sample into appropriate tubes.
2. Add 1.0mL reagent to standard (S), sample (U), and blank (B) tubes.
3. Mix well (vortex).
4. Incubate at room temperature for 10 minutes.
5. Read S and U vs. B at 500 nm within 60 minutes.

Calculation:

$$\text{Serum triglycerides (mg/dL)} = \text{Au/As} \times 200$$

Method for Determining Plasma Oxidation by TBARS

Sample Preparation:

Dialysis:

1. Pipet 0.5mL plasma into membrane tubing.
2. Clip both ends and place in 1.5L 10 uM EDTA solution.
3. Place in refrigerator with magnetic stirrer for 24 hours.
4. Change solution after 8 and 16 hours.
5. Remove clips and transfer plasma to microtubes for immediate use or storage at -80°C.

Copper Oxidation:

1. Pipet 100 uL dialyzed plasma sample into four small test tubes.
2. Add 100 uL 1000uM CuSO₄ to 2 tubes containing sample.
3. Dilute all tubes to 1.0mL with H₂O.
4. Mix Well (vortex).
5. Cover with parafilm.
6. Place in oven at 37°C for exactly 24 hours.

TBARS Determination:

Reagents:

HCl	0.05 M
Thiobarbituric acid (TBA) solution	0.67 (w/vol)
Methanol / butanol mixture	3:17

Chemical Principles:

Malondialdehyde ** produces as a result of lipid peroxidation reacts with thiobarbituric acid to produce a red color absorbing at 535 nm.

Procedure:

1. Label screw top test tubes.
2. Add 3.0mL 0.05M HCl solution to each tube.
3. Add 0.3 mL sample after Cu oxidation.
4. Add 1.0mL 0.67% TBA solution, freshly prepared.
5. Mix well (vortex).
6. Place tubes in heat safe test tube rack and cover with foil.
7. Heat in waterbath at 95°C for exactly 30 minutes.
8. Remove and cool immediately with tap water.
9. Add 4mL methanol/butanol mixture.
10. Mix well (vortex).
11. Centrifuge at 3000 rpm at room temperature for 20 minutes.
12. Measure upper layer at 535 nm.

Calculation:

Protein Assay (Sigma Diagnostics, St. Louis, MO)

Reagents:

Lowry Reagent, Modified

Folin & Ciocalteu's Phenol Reagent

Protein Standard: prepared from bovine serum albumin

Chemical Principle:

An alkaline cupric tartrate reagent complexes with the peptide bonds and forms a purple color when the phenol reagent is added. Absorbance is read at a suitable wavelength between 500 nm and 800 nm. The protein concentration is determined from a calibration curve.

Procedure:

1. Prepare standard tubes by diluting Protein Standard Solution in water to a volume of 1.0mL in appropriately labeled test tubes:

Protein Standard Sol (mL)	Water (mL)	Protein Concentration (ug/mL)
0	1.0	0 (BLANK)
0.125	0.875	50
0.250	0.750	100
0.500	0.500	200
0.750	0.250	300

1.000	0	400
-------	---	-----

2. Add 5uL sample to labeled tube and dilute to 1.0mL with water.
3. Add 1.0mL Lowry Reagent Solution to standard, blank and sample tubes.
4. Mix well (vortex).
5. Allow to stand at room temperature for 20 minutes.
6. With rapid and immediate mixing, add 0.5mL Folin and Ciocalteu's Phenol Reagent Working Solution to each tube.
7. Allow color to develop for 30 minutes.
8. Transfer solutions to cuvetts and measure the absorbance of standards and samples vs. the blank at 750 nm. Complete readings within 30 minutes.
9. Plot the absorbance values of the standards vs. their corresponding protein concentrations to prepare a calibration curve.
10. Determine the protein concentrations of the samples using the calibration curve. Multiply the results by the appropriate dilution factor (200) to obtain the protein concentration in the original sample.

APPENDIX VIII

ACTIVITY DIARY

A. Sleep / Lying Down

B. Sitting Activities (little or no arm movement)

- B1 Eating
- B2 Reading / Writing
- B3 Watching TV
- B4 Driving
- 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 (dressing / showering)
- 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 (golf, bowling)
- D4 Other

E. Hard Activities

- E1 Physical (lifting)
- E2 Vigorous Sports
- E3 Aerobics
- E4 Dancing, Cycling
- E5 Swimming
- E6 Snow Shoveling
- E7 Other

F. Very Hard Activities

- F1 Strenuous Physical Labor
- F2 Exertive sports (running)
- F3 Singles Tennis
- F4 Skiing
- F5 Basketball
- F6 Swimming Laps
- F5 Other

	:00	:15	:30	:45
5:00 AM				
6:00				
7:00				
8:00				
9:00				
10:00				
11:00				
12:00 NOON				
1:00 PM				
2:00				
3:00				
4:00				
5:00				
6:00				
7:00				
8:00				
9:00				
10:00				
11:00				
12:00 MID				
1:00 AM				
2:00				
3:00				
4:00				

RAW DATA

Subject	Stage	Group	HDL	TC	LDL	TG	Oxidation
3	1	2	46	122	55	103	2.568
6	1	2	41	156	97	87	1.774
7	1	2	46	138	76	79	1.843
9	1	2	31	143	87	126	2.854
10	1	2	54	160	94	60	1.783
14	1	2	58	168	94	82	2.157
15	1	2	63	183	96	120	2.24
16	1	2	40	150	86	119	2.693
1	1	1	58	134	54	111	2.452
2	1	1	55	124	58	54	2.203
4	1	1	57	117	45	73	2.18
5	1	1	62	160	82	78	2.357
8	1	1	46	120	61	63	2.731
11	1	1	42	157	103	58	2.154
12	1	1	43	153	95	77	4.671
13	1	1	60	126	47	93	1.897
3	2	2	42	151	90	93	2.148
6	2	2	47	208	134	136	3.082
7	2	2	52	169	95	109	1.786
9	2	2	32	150	63	273	3.326
10	2	2	48	156	97	57	1.634
14	2	2	63	174	94	85	2.435
15	2	2	65	195	113	85	2.299
16	2	2	45	172	107	100	2.741
1	2	1	32	156	92	158	2.334
2	2	1	75	158	71	62	1.161
4	2	1	52	165	93	102	1.768
5	2	1	50	165	90	126	1.736
8	2	1	54	139	64	105	1.956
11	2	1	54	158	87	84	2.628
12	2	1	37	150	93	98	2.479
13	2	1	59	164	88	84	2.768
3	3	2	42	148	91	69	2.904
6	3	2	39	179	115	121	2.129
7	3	2	42	157	96	96	1.832
9	3	2	33	155	83	198	2.458
10	3	2	41	148	94	66	2.53
14	3	2	60	174	95	95	2.345
15	3	2	58	214	137	94	1.912
16	3	2	43	177	108	127	2.739
1	3	1	34	158	95	149	2.236
2	3	1	75	162	73	69	1.485
4	3	1	51	171	104	79	2.363
5	3	1	56	184	108	101	1.77
8	3	1	45	146	81	101	2.456
11	3	1	51	150	84	76	2.601
12	3	1	37	161	103	104	2.678
13	3	1	52	149	79	87	2.288
Group1= casein			stage1= baseline		stage2=exercise 1, no supplementation		
Group2= soy			stage3=exercise 2, supplementation				

CURRICULUM VITAE

Sandra C. Shehadeh

5065 Queens Wood Drive
Burke, VA 22015
(703) 978-4888
sshehade@vt.edu

Objective: Researcher; Special interest in project planning and monitoring

Education: **M.S. Human Nutrition, Foods and Exercise**
Virginia Polytechnic Institute and State University, Blacksburg VA
Graduation Date: December 17, 1999
Thesis Topic: Effects of High Genistein Soy Protein on Blood Lipid Profile and LDL Oxidation with Moderate Physical Activity

B.S. Human Nutrition and Foods, August 1997
Virginia Polytechnic Institute and State University, Blacksburg VA
Specialization: Exercise Science

Related Courses:

- Research Design in Health Science
- Statistics and Biometry
- Pharmacology
- Nutrition Counseling
- Human Nutrition Seminar
- Nutrition and Physical Activity
- Epidemiology
- Research and Thesis

Computer Knowledge:

- Mac/PC based system maintenance
- MS Word, PowerPoint, Excel
- Statistical programs including JMP and SAS
- Web page design including HTML
- Advanced graphics and scanning
- Internet browsers and advanced search methods including Medline

Experience: **Research Assistant**, August 1998 – August 1999
Virginia Polytechnic Institute and State University, Blacksburg VA
20 hours per week

- Developed human research studies for Nutrition Department
- Recruited and communicated with subjects throughout studies
- Completed all biochemical procedures including plasma profile, LDL separation, TBARS and HPLC
- Analyzed data for statistical significance using SAS
- Maintained lab inventory of glassware and chemicals

Nutrition Counselor, August 1998 – May 1999
Virginia Polytechnic Institute and State University, Blacksburg VA
On an as need basis, volunteer position

- Met with clients weekly to promote nutrition education
- Communicated the importance of healthy eating and exercise to lay public populations
- Administered questionnaires and surveys to determine individual needs
- Maintained contact and retention of clients

Teaching Assistant, August 1997 – August 1999

Virginia Polytechnic Institute and State University, Blacksburg VA
20 hours per week

- Responsible for assisting instructional designer team in course development
- Revised course lectures for content and design
- Developed and maintained course web site

Exercise Physiologist, January 1997 – January 1998

Virginia Tech Cardiac Intervention Program, Blacksburg VA
10 hours per week

- Conducted maximal and sub-maximal exercise stress testing
- Developed and re-evaluated personal exercise prescriptions
- Performed personal monitoring of clients during exercise
- Monitored administration of emergency procedures

Honors and Activities:

Phi Omicron Nu, National Honor Society, January 1997- Present
Completed ACLS course, December 1998
CPR/First Aid Certification, September 1998

Abstracts and Presentations:

Shehadeh S, Chen C, Bakhit R; The effect of soy protein and antioxidants on exercise induced oxidation in healthy college-aged males. Institute of Food Technologists, National conference 2000: Abstract submitted December 1999.

Shehadeh S, Bakhit R; Development of a Well-Liked Muffin Containing High Levels of Soy Proteins and Isoflavones. Third International Symposium on the Role of Soy in Preventing and Treating Chronic Disease: Poster presentation, November 1999.

Shehadeh S, Bakhit R; Soy protein and isoflavone effect on lipid oxidation and blood lipid profile in humans participating in moderate physical activity. FASEB 2000: Abstract submitted.

Shehadeh SC, Bakhit RM, Chen C; The effect of soy proteins and isoflavones on lipid oxidation and blood lipid profile in humans participating in moderate physical activity. Am J Clin Nut: Journal article submitted for publication, December 1999.