Identification of urinary isoflavone excretion phenotypes related to the cholesterol lowering ability of soy protein in Golden Syrian hamsters

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Identification of urinary isoflavone excretion phenotypes related to the cholesterol lowering ability of soy protein in Golden Syrian hamsters

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Toxicology

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ABSTRACT

Apparent absorption of isoflavones varies greatly among individuals, but is relatively stable within an individual. We hypothesized that high urinary isoflavone excreters would show less plasma non-HDL cholesterol than low isoflavone excreters after soy protein feeding. Fifty Golden Syrian hamsters were fed a high fat/casein diet (n = 10) or a high fat/soy protein diet (n = 40) for 4 wk. Two distinct urinary isoflavone excretion phenotypes were identified using a pairwise correlation plots analysis, or using a hierarchical cluster test. High isoflavone excreters showed significantly greater urinary isoflavones (p<0.05) than did low isoflavone excreters. High urinary isoflavone excreters had significantly less non-HDL cholesterol than did the low isoflavone excreters or casein-fed controls (p < 0.05). Urinary isoflavone excretion phenotypes predicted the cholesterol-lowering efficacy of soy protein. Isoflavone absorbability, probably due to gut microbial ecology, is an important controllable variable in studies of effects of soy protein on blood lipids.
GENERAL INTRODUCTION

Introduction

Isoflavones are a class of phytoestrogens including genistein, daidzein, glycitein, and their various glucoside derivatives, found mainly in soybeans. Because isoflavones are structurally similar to mammalian estrogens, interest has focused primarily on their effects on hormone-dependent conditions. Some studies have provided evidence for a potentially protective role for isoflavones in cardiovascular disease, breast cancer, prostate cancer, osteoporosis, and menopausal symptoms.

Isoflavone bioavailability is complex and shows a wide range of interindividual variability. Many human studies and animal models have been established to study bioavailability of isoflavones. Some focused on understanding how isoflavones are metabolized in the body including plasma kinetics as well as urinary and fecal excretion. We know that isoflavone glucosides undergo cleavage of their sugar moiety by intestinal β-glucosidases to be absorbed mostly in the small intestine in humans—not sure about animal models. After absorption, isoflavones undergo intestinal and hepatic biotransformation to mainly glucuronide conjugates. Most of the conjugates are excreted through bile back into the intestine. Deconjugation is necessary for reabsorption. Bacteria are also responsible for degrading and producing metabolites of isoflavones. Establishment of phenotypes of isoflavone bioavailability is important to understand the specific roles that isoflavones may play in human health.

Soy isoflavones have gained considerable attention for their potential role preventing the development of atherosclerosis and subsequent cardiovascular disease (CVD). Potential mechanisms by which soy isoflavones might prevent atherosclerosis include beneficial effects on serum lipid profiles, antioxidant properties, blood vessel function, blood pressure, inhibiting thrombus formation and suppressing smooth muscle cell proliferation and migration as well as improving vascular reactivity.

The component or components of soy that are responsible for improvements in lipid metabolism have been investigated and their specific actions debated. A significant
reduction in LDL cholesterol in hypercholesterolemics is not evident among some recent trials. A recent review did not support the current research on the effect of soy protein or isoflavone supplements on the beneficial effect of isoflavones on human plasma lipoproteins.

Furthermore, the mechanism for the cholesterol lowering ability of soy protein and isoflavone is the topic of many hypotheses and the mechanisms by which isoflavones alter plasma lipoprotein concentrations are still matters of debate. It is known that reduced LDL-cholesterol concentrations associated with estrogen intake are a direct result of increased LDL receptor activity, decreased intestinal absorption of cholesterol as well as affecting sterol regulatory element binding proteins which regulate genes related to LDL receptor expression.

The objective of this study was to identify urinary isoflavone excretion phenotypes in a hamster model using two statistical methods and to characterize phenotypic stability over 4 wks. Secondly, we investigated the effects of the distinct isoflavone phenotypes on the level of plasma lipids. Third, we also quantified the relationships between the level of urinary and fecal isoflavone excretions and plasma lipids.

**Thesis Organization**

This thesis contains a general introduction, a literature review focusing mainly on health-effects, bioavailability and metabolism of isoflavones, and mechanism of lowering cholesterol of isoflavone. The paper entitled “High urinary isoflavone excretion phenotype decreases plasma cholesterol in golden Syrian hamsters fed soy protein” has been published by the *Journal of Nutrition*. 
LITERATURE REVIEW

A. Molecular Structure and biological properties of isoflavone

Isoflavones are a class of phytoestrogens in soybeans: including genistein, daidzein, and glycine. They are present as β-glucosides, 6”-O-malonyl-β-glucosides, and 6”-O-acetyl-β-glucosides as well as the aglucon forms. Genistein and daidzein have been found in relatively high concentration in soybeans and most soy-protein products, whereas much lower amounts of glycine are present in soybeans (Wang and Murphy. 1994 and 1996). The basic structural feature of isoflavone compounds is the flavone nucleus, which is composed of two benzene rings (Figure 1). The chemical structures of 17β-estradiol and equol (a daidzein metabolite) are so similar that they superimpose (Figure 2).

Figure 1. Molecular structure of soy isoflavone
Isoflavones have an intermediate solubility meaning they are not soluble in water or in very hydrophobic solvents. They are soluble in alcoholic solvents but the solubility is low. Also isoflavones can be ionized at high pH which affects their recovery from certain foods (Rickert et al. 2004). Soy protein concentrates contain insignificant amounts of isoflavones because they are obtained through alcohol extraction which removes isoflavones from soy (Wang and Murphy. 1994). The aqueous processing of tofu and the dilution of soy protein with other ingredients in soy milk and second-generation soy products explain the reduced isoflavone content present in these products (Anderson et al. 1995).

Isoflavones are traditionally consumed in relatively high amounts by some Chinese populations. Liu et al. (2004) reported average intake of soy foods and isoflavones was 23.5 g/d and 8.9 mg/d by 1,188 subjects in Gansu Province and Hebei Province of China using food frequency questionnaires (FFQ). In contrast, while it was reported that the intake of soy isoflavones by women in the Netherlands (aged 50–69 y, n = 17,357) and United States of
America (964 postmenopausal Caucasian women in the Framingham Offspring Study using the Willett food-frequency questionnaire) was estimated to be less than 1 mg/d (De Kleijn et al. 2001; Boker et al. 2002). Because isoflavones are structurally similar to the mammalian estrogens, interests have focused primarily on their effects on hormone-dependent conditions. Epidemiologic studies show potentially protective roles for isoflavones in cardiovascular disease, breast cancer, prostate cancer, osteoporosis, and menopausal symptoms (Duncan et al. 2003).

B. Metabolism of Isoflavones

Several studies have been conducted to investigate the metabolism of isoflavones. The actual process of isoflavone absorption is still somewhat obscure. The molecular weights of isoflavone aglycones are 254, 270, and 284 g/mol for daidzein, genistein, and glycine, respectively, which are neither hydrophilic nor lipophilic. Due to their low molecular weight, isoflavones could be absorbed through diffusion (Birt et al. 2001) and converted to aglycones by membrane-bound or bacterial β-glucosidases in the intestinal tract (Day et al. 1998). Day et al. (1998) investigated the ability of human small intestine and liver extracts to flavonoid and isoflavonoid glycosides. Samples of small intestine and liver were surgically obtained from patients undergoing gastrointestinal surgery. These compounds were deglycosylated by extracts from both tissues at 37°C incubation for up to 90 min.

The absorption and degradation processes of isoflavones may be influenced by the presence of the gut microflora. Bacteria play an important role in this cleavage process through β-glucosidase or β-glucuronidase activity. In an early study, Hawksworth et al. (1971) showed that a variety of bacteria possess these enzymatic activities using in vitro incubation of groups of bacteria with various sugars, all differing in their glycosidic bonds from one another and measuring the different sugar breakdown over time. β-glucosidases have been found in Enterococci and to a lesser extent in Lactobacilli, Clostridia, Bacteroides and Bifidobacteria strains. β-glucuronidase activity is mostly due to Enterobacteria and to a much lesser extent by Clostridia and Bacteroides. Day et al. (2000) indicated that lactase phlorizin hydrolase (LPH), a membrane-bound, β–glucosidase purified from mammalian
(sheep) small intestine was able to cleave the sugar moiety of daidzin and genistin with a greater Vmax for genistin (~2.8 U/mg LPH) than daidzin (~0.9 U/mg LPH). This cleavage process apparently delayed the absorption compared to ingested isoflavone aglycones.

In in vitro studies, Schoefer et al. (2002) showed that the incubation of *E. ramulus* with 0.5 mM genistein-7-*O*-glucoside yielded two metabolites with retention times of 26.0 min (Gg1) and 34.7 min (Gg2). Gg1 was identified by HPLC and LC–MS as 2-(4-hydroxyphenyl)-propionic acid. Comparison of the retention time and UV-spectrum of Gg2 with the reference substance revealed its identity with genistein. The ability of *E. ramulus* to hydrolyze the glycosidic bond of genistein-7-*O*-glucoside is in agreement with data collected by Schneider & Blaut (2000). *E. ramulus* was reported to cleave off the sugar moiety of flavone and flavonol glycosides in positions 3 and 7 of the ring system. The ability of an isolated bacterial species to hydrolyze the glycosidic bond of genistein-7-*O*-glucoside and daidzein-7-*O*-glucoside has been also reported for *Escherichia coli* HGH21 and the newly isolated strain HGH6 (Hur et al. 2000). Furthermore, Tsangalis et al. (2002) tested five strains of *Bifidobacterium* to screen β-glucosidases activity using p-nitrophenyl-β-D-glucopyranoside as the substrate, and using selected strains to ferment soymilk. Enumeration of viable *Bifidobacteria* and quantification of isoflavones using HPLC were performed at 0, 12, 24, 36, and 48 h of incubation. Four strains produced β-glucosidase. *B. pseudolongum* and *B. longum-a* displayed the best growth in soymilk, with an increase of 1.3 log10 CFU/mL after 12 h. *B. animalis, B. longum-a, and B. pseudolongum* caused hydrolysis of isoflavone malonyl-, acetyl- and β-glucosides to form aglycones. Fermentation of soymilk with *Bifidobacterium sp.* resulted in a significant increase in the concentration of aglycones. In conclusion, the cleavage of isoflavone glucosidic form was required for the absorption in the intestinal tract. After absorption the predominant forms of isoflavones in plasma and urine are the conjugates isoflavone sulfates and glucuronides.

Isoflavones undergo endogenous biotransformation by UDP-glucuronosyltransferase (UGTs) and sulfotransferases in the intestinal mucosa, liver, and other organs, which modify their chemical structure and solubility, thereby influencing distribution, storage, and excretion (Birt et al. 2004). Glucuronide and sulfate conjugates made up about 70-80% and 20% of total isoflavone in urine, respectively and 50-60% and
20-30% in plasma, respectively in women fed soymilk (n=6) (Zhang, 2000). In addition, greater amounts of isoflavone aglucon were recovered from 0-24 h plasma samples (~20-25% ingested dose) compared to 0-24 h urinary excretion (<10% ingested dose). Sfakianos et al. (1997) reported the more than half of ingested genistein was excreted in the bile after conjugation. Sfakianos et al. (1997) used anesthetized female rats with biliary cannulas. Genistein infused into the duodenum was recovered as 7-O-β-genistein glucuronide and 75% of the infused dose was recovered within 4 h. Moreover infusion of genistein glucuronide in the duodenum resulted in a slower biliary excretion of the same compound, indicating that like glucosides, isoflavones endogenously conjugated have to be cleaved prior to reabsorption, either by intestinal or microbial enzymes.

In order to be reabsorbed after biliary excretion, isoflavone conjugates must be cleaved. The same applies to any isoflavone glucosides that might have arrived intact in the ileum or large intestine. Isoflavones which are excreted back into the intestine will be deconjugated by gut microbial glucuronidase and sulfatase to release the aglycone form, part of it being possibly reabsorbed through enterohepatic circulation (Sfakianos et al. 1997). Isoflavones not undergoing this process are subject to microbial degradation and metabolite formation, while the rest undergoes fecal excretion.

In a summary about the data described above, humans and animals share many similarities in absorption, biotransformation, and excretion of isoflavones. Gut microorganisms play an important part in isoflavone metabolism. Furthermore, gut microflora are also responsible for converting isoflavones into metabolites.

Several candidate bacteria for daidzein metabolism have been suggested, for example, a Clostridium sp. (Hur et al. 2002) and E. ramulus (Schoefer et al. 2002) metabolized daidzein to O-DMA in vitro, and equol has been found in soymilk fermented with some strains of Bifidobacterium (Tsangalis et al. 2002).

Hur et al. (2002) has reported that anaerobic bacterium was involved in the ring cleavage of daidzein to produce O-DMA. A gram-positive anaerobic bacterium, strain HGH 136, capable of conversion of the isoflavonoid daidzein, was isolated and identified as a Clostridium sp. The bacterium cleaved the C-ring of daidzein to produce O-demethylandolensin (O-DMA). The identity of the metabolite was confirmed by liquid
chroomatography-mass spectrometry and NMR using synthetic O-DMA as a standard. The bacterium incubated with synthetic dihydrodaidzein also produced O-DMA. After 3 days of incubation, 28% of added daidzein and 12% of added dihydrodaidzein were converted to O-DMA. *E. ramulus*, a flavonoid-degrading anaerobic bacterium from the human gastrointestinal tract, was tested by Schoefer et al. (2002) for its ability to transform the isoflavonoids daidzein, genistein-7-O-glucoside (genistin), genistein. As a result, daidzein was also degraded to O-desmethylangolensin, the corresponding metabolite to 6'-hydroxy-O-desmethylangolensin. Genistein was completely degraded by *E. ramulus* via 6'-hydroxy-O-desmethylangolensin to 2-(4-hydroxyphenyl)-propionic acid. Dihydrogenistein was neither observed as an intermediate in this transformation nor converted itself by growing cells or cell-free extracts of *E. ramulus*. Genistein-7-O-glucoside was partially transformed by way of genistein to the product 2-(4-hydroxyphenyl)-propionic acid. They suggested the hydroxyl group in position 6' of O-desmethylangolensin is crucial for further degradation. Although in Tsangalis et al. (2002) study, five strains of *Bifidobacterium* were screened for β-glucosidase activity, they also found daidzein was transformed to equol using selected strains to ferment soymilk. It has been suggested that other bacteria, including *Escherichia coli*, *Bacteroides ovatus*, *Ruminococcus productus*, or *Streptococcus intermedius* (Hur et al. 2000; Ueno et al. 2002) could be involved in daidzein metabolism. Recently, specific bacteria responsible for isoflavone metabolism in humans have been identified by Renouf et al. (2005). They established that Golden Syrian hamsters may be a good model to study bioavailability of isoflavones and their possible health promoting effects. They showed that high fecal isoflavone degradation rate was related with distinct fecal bacterial species. Thirty three healthy adult subjects’ fresh feces were incubated anaerobically with isoflavones to identify degradation rates using HPLC and fecal bacterial 16S rDNA sequences were performed by PCR-DGGE. Cluster analysis and DGGE analysis indicated that high genistein and glycitein degraders shared 5 DNA bands of greater intensity than found in feces of low degraders. Three species, *Bacteroides*, *Prevotella* genus and *Clostridiales* were identified by sequencing of 16S rDNA from the interest bands. Furthermore *Bacteroides ovatus*, *Bacteroides acidifaciens*, *Eubacterium ramulus*, *Clostridium orbiscindens*, and *Tannerella forsythensis* were identified as the major human gut microbial species that degraded isoflavones under
both nutrient rich and poor in vitro systems. They concluded that bacterial species with greater amounts in high degraders which also existed in low degraders may be predictors for gut microbial degradation and overall bioavailability of isoflavones.

In a summary, gut microflora play a crucial role in isoflavone metabolism. The absorption and degradation of isoflavones are very complicated. Some specific fecal bacterial species may be responsible for these processes. The main reason for apparent variability in fecal isoflavone degradation among individuals is the different amount of bacterial population. However, the mechanism how these bacterial species convert isoflavones to metabolites remains further attention.

C. Isoflavones and cardiovascular disease risk

Isoflavones have gained considerable attention for their potential role in preventing the development of atherosclerosis and subsequent cardiovascular disease (CVD). Atherosclerosis is a main cause of human disease and death worldwide. Atherogenesis is a complex process, with multiple mechanisms contributing to its initiation and progression including infections, inflammation, or autoimmunity in pathogenesis of the disease (Leinonen et al. 2002). Susceptibility to atherosclerosis is determined by a combination of genetic and environmental factors, including diet. Consumption of diets rich in soy protein has been claimed to protect against the development of atherosclerosis (Anthony et al. 1997). Isoflavones exert several anti-atherogenic effects through which soy protein and say isoflavones may protect against CVD have been proposed (Anthony et al. 1997; Yamakoshi et al. 2000). In a cohort study of ~75,000 Chinese women aged 40–70 y at the baseline survey from 1997 to 2000, the clear inverse dose-response relationship between soy food intake and risk of total CHD observed for women in the highest intake (≥11.2 g/d) vs. the lowest (<4.5 g/d) quartile of total soy protein has been reported (Zhang et al. 2003). A recent analysis assessing eight different randomized controlled trials using human subjects indicated that intake of isoflavones has LDL cholesterol-lowering effects independent of soy protein intake (Zhuo et al. 2004).
a. Effects of isoflavones on serum lipid profiles

High total cholesterol and LDL cholesterol levels correlate with cardiovascular disease. Elevated cholesterol levels contribute to the formation of atherosclerotic plaques and eventually to thrombosis or myocardial infarction. Management of cholesterol levels is an essential part of treating cardiovascular disease. Soy intake has been shown to lower cholesterol levels and thereby reduce the risk of developing atherosclerosis. A meta-analysis of 38 controlled clinical trials reported that intake of soy protein was associated with a significant hypocholesterolemic effect which included a reduction in serum concentrations of total cholesterol, LDL cholesterol and triacylglycerols, and a nonsignificant increase in HDL cholesterol (Anderson et al. 1995). Subsequently, many well-controlled studies explored the soy protein hypothesis with greater specificity (Crouse et al. 1999; Lichtenstein et al. 2000; Merz-Demlow et al. 2000; Gardner et al. 2001 and Wangen et al. 2001) and found more LDL cholesterol reduction in hypercholesterolemic subjects than in those with lower LDL cholesterol levels. On the other hand, the component or components of soy that are responsible for improvements in lipid metabolism have been investigated and their specific actions debated. A meta-analysis concluded that isoflavones did not affect plasma lipid concentrations (Weggemans et al. 2003). A recent review from American Heart Association Science Advisory did not support the current research on the effect of isolated soy protein with isoflavones on LDL cholesterol concentrations (Sacks et al. 2006).

The topic of cholesterol lowering of soy protein and soy isoflavone is controversial and a subject of current debate. Some studies have demonstrated different results when intake of intact high isoflavone-containing soy protein was compared with intake of low isoflavone-containing soy supplemented with added isoflavones (Erdman et al. 2004). Some studies have mentioned that isoflavone-rich soy protein did not improve blood lipid profile (Dent et al. 2001; Blair et al. 2002; Lin et al. 2004). These studies have been conducted to investigate whether the hypocholesterolemic effect is attributable to the soy protein or the high content of the phytoestrogenic isoflavones in soy. Results did not show a specific effect of isoflavones on LDL or HDL cholesterol.
Dent et al. (2001) reported that a dietary intake of soy protein for six months did not improve circulating lipid and lipoprotein concentrations for either isoflavone-rich soy (80.4 mg/d aglycone components; n=24) or isoflavone-poor soy (4.4 mg/d aglycone components; n=24) in normocholesterolemic perimenopausal women or in perimenopausal women (n=30) who were mildly hypercholesterolemic.

Several studies showed isoflavones had no effect on cholesterol lowering in some animal trials. A meta-analysis reported that isoflavones did not affect blood lipid concentrations. Weggemans et al. (2003) investigated the specific effect of soy-associated isoflavones on cholesterol concentrations in well-controlled trials substituting soy protein with dairy or animal protein. They collected the data from MEDLINE searches (1995 - 1996 June 2002) and reviewing reference lists. Studies were included if they had a control group or treatment, experimental diets only differed in the amounts of soy protein and isoflavones and were each fed for at least 14 days. A total of 10 studies met these criteria, providing 21 dietary comparisons, including 959 subjects (336 men and 623 women), average age from 41 to 67 y and baseline cholesterol concentration from 5.42 to 6.60 mmol/l. The intake of soy-associated isoflavones was 1-95 mg/day and the intake of soy protein was 19-60 g/day. No definite evidence was found in feeding daily 36 g soy protein with 52 mg soy-associated isoflavones on average to decrease low-density lipoprotein (LDL) cholesterol and increase high-density lipoprotein (HDL) cholesterol compared with control group. No dose-response relation existed between soy-associated isoflavones intake and changes in LDL cholesterol or HDL cholesterol.

Recently, a review did not support the current research on the effect of soy protein and isoflavones on plasma LDL cholesterol concentrations from American Heart Association Science Advisory. Sacks et al. (2006) assessed the more recent work published on isolated soy protein with isoflavones in the majority of 22 randomized trials as compared with milk or other proteins. They showed no significant effects on HDL cholesterol, triglycerides, lipoprotein, or blood pressure. Especially among 19 studies of soy isoflavones, the average effect on LDL cholesterol and other lipid risk factors was very small. Also, soy protein and isoflavones have not been shown to lessen vasomotor symptoms of menopause, blood pressure and postmenopausal bone loss. The efficacy of soy isoflavones for preventing or
treating cancer of the breast, endometrium, or prostate was not evident. These studies concluded consumption of soy-associated isoflavones was not related to changes in LDL or HDL cholesterol and the data raised some questions about the clinical importance of the hypocholesterolemic effects observed.

In conclusion, there are many factors that influence the extent to which isoflavones play an efficacy of protecting against CVD. Basic diet ingredients, food matrix, cholesterol baseline or isoflavone dosage, experimental duration, subject age and sex and gut transit time may be involved in modulating processes related to CVD. The most important factor which these studies did not attempt to control was the interindividual variablility in isoflavone bioavailability. A lack of efficacy of isoflavones and soy protein feeding may occur in populations that had lesser absorption of isoflavones while the apparent protective effects of isoflavones on cholesterol-lowering may tend to have occurred in individual with higher absorption of isoflavones.

On the other hand, soy intake has been shown to lower cholesterol levels and thereby reduce the risk of developing atherosclerosis. The beneficial effects of soy intake on plasma lipoprotein levels led to FDA approval of a health claim that "25 g of soy per day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease." This health claim and other media publicity are promoting intake of both soy food products and isoflavone-containing soy supplements. Many studies have provided substantial evidence to confirm the cholesterol lowering effect of soy protein and soy isoflavone (Potter et al. 1998; Anthony et al. 1997; Lucas et al. 2001; Lukaczer et al. 2005).

Potter et al. (1998) investigated the effects of soy protein (40 g/d) containing moderate and higher concentrations of isoflavones on blood lipid profiles, mononuclear cell LDL receptor messenger RNA, and bone mineral density and content in 66 free-living, hypercholesterolemic, postmenopausal women during a 6-mo, parallel-group, double-blind trial. They reported non-HDL cholesterol for both groups was reduced compared with the control group besides significant increasing bone mineral content and density in the lumbar spine. They concluded that intake of soy protein at both isoflavone concentrations for 6 mo may decrease the risk factors associated with cardiovascular disease in postmenopausal women.
To distinguish the relative contributions of the protein moiety versus the alcohol-extractable phytoestrogens for cardiovascular protection, Anthony et al. (1997) studied a group of young male cynomolgus macaques fed a moderately atherogenic diet. The groups differed only in the source of dietary protein, which was either casein/lactalbumin (casein, n=27), soy protein with the phytoestrogens intact (soy+, n=27), or soy protein with the phytoestrogens mostly extracted (soy-, n=28). The diets were fed for 14 months. Animals fed soy+ had significantly lower total and LDL plus VLDL cholesterol concentrations compared with the other two groups. They indicated the beneficial effects of soy protein on atherosclerosis appear to be mediated primarily by the phytoestrogen component.

In a hamster study, Lucas et al. (2001) reported ethanol-extracted soy protein isolate alone does not lower serum cholesterol. Ethanol-extracted soy protein isolate (SPE) was fed to both sham-operated and ovariectomized 48 six-month-old female Golden Syrian hamsters for 70 d. Ovariectomy significantly increased total serum cholesterol and non-HDL cholesterol concentrations in casein-based or SPE-based diets compared with sham hamsters. Still, ovariectomy SPE-based diets did not decrease serum total cholesterol (7.3 ± 0.3 vs. 6.6 ± 0.3 mmol/L) and non-HDL cholesterol (3.6 ± 0.2 vs. 3.3 ± 0.2 mmol/L) compared to casein-based diets. Although these findings confirmed the ovariectomized hamster as a model of postmenopausal hypercholesterolemia, the components of protein fractions does not play cholesterol lowering role in soy protein. These data were in agreement with the data observed by Anthony et al. (1997) in which soy protein with the phytoestrogens mostly extracted (soy-) did not mediate beneficial effects of soy protein on cholesterol lowering. In another animal study, Song et al. (2003) fed sixty male and 60 female golden Syrian hamsters in six treatments for 10 weeks with dietary isolated soy protein (ISP), ethanol-extracted ISP (ISP (-)), soygerm or soygerm extract (containing large amounts of daidzein and glycitein and little genistein), daidzein and casein diet group. Hamsters fed ISP, ISP (-), daidzein, soygerm, and soygerm extract had significantly less plasma total cholesterol, less non-HDL cholesterol and less non-HDL/HDL cholesterol ratios compared with hamsters fed casein. They concluded that soy protein with or without isoflavones, soygerm and soygerm extract, and daidzein lessened plasma cholesterol to an approximately equal extent. Soy protein alone, varying mixtures of isoflavones, and other extractable components of soy were
responsible for cholesterol-lessening effects of soy foods, mainly due to their effects to lessen LDL cholesterol.

Furthermore, isoflavones from kudzu and red clover also were investigated by Lukaczer et al. (2005) in a pilot study to assess the effect of an isoflavone nutritional supplement on menopausal symptoms and markers of breast cancer and CVD risk. Twenty-five menopausal women suffering from severe hot flushes and night sweats completed a 12-week intervention using this combination isoflavone nutritional supplement. They observed a 46% decrease in reported hot flushes. Two markers of CVD risk, the ratio of total cholesterol to high-density lipoprotein (HDL) cholesterol and homocysteine, showed modest improvement. A proposed marker of breast cancer risk, the ratio of 2-hydroxyestronc to 16 alpha-hydroxyestrone, also showed a statistically significant improvement. They reported from this pilot trial that with isoflavone nutritional supplementation may significantly relieve the most troubling symptoms of menopause, as well as confer some chemopreventive and cardioprotective benefits.

In order to distinguish the main component of the cholesterol-lowering effect of soy protein, a greater effect of cholesterol-lowering was seen with increased isoflavone intake. Several studies focused on the threshold dose of the isoflavone effect in humans and animals models (Crouse et al. 1999, Merz-Demlow et al. 2000, Wangen et al. 2001, Lucas et al. 2003, Zhuo et al. 2004). Crouse et al. (1999) fed beverages containing either 25g casein/day, 25g soy protein/day at: 3, 27, 37, or 62 mg total isoflavones (0.15, 1.4, 1.95 and 3.3µmol/kg BW/d) for 9 weeks in moderately hypercholesterolemic men and women. The highest isoflavone group fed 62 mg total isoflavones/d significantly decreased the total and LDL cholesterol (4% and 6% reduction) compared with the casein group. This study determined a threshold effect of isoflavones on cholesterol-lowering dose-response. Afterwards, Merz-Demlow et al. (2000) used three isolated soy protein (ISP) consumption doses: <10mg/d (control; 0.5µmol/kg BW/d), ~65mg/d (low isoflavone; 3.4µmol/kg BW/d), or ~130mg/d (high isoflavone; 6.8µmol/kg BW/d) in different phases of a women’s menstrual cycle (early follicular, midfollicular, periovulatory and midluteal phases). LDL-C concentrations were lowered only in high isoflavone diet compared to control in the midfollicular (2.20±0.04 vs. 2.38±0.04mmol/L) and periovulatory (2.07±0.05 vs.
2.3±0.05mmol/L) phases. The same doses made by Merz-Demlow et al. (2000) were investigated in 18 postmenopausal women with a 93-d crossover study by Wangen et al. (2001). Subsequently, the concentrations of LDL-cholesterol in three groups were lowered gradually (3.22±0.05mmol/L vs. 3.05±0.05mmol/L vs. 3.01±0.05mmol/L). The results showed that the high isoflavone significantly lowered LDL-C cholesterol compared to control while the low isoflavone was no different from control or high isoflavone.

Similar results were obtained from six-month-old female Golden Syrian hamsters, in ovariectomized (ovx) hamsters, fed three different isoflavone doses compared with control, by Lucas et al. (2003) the cholesterol-lowering effects of isoflavones became more significant (7.8%, 11.8%, and 19.6% reductions in total cholesterol) for ovx + 9.5 (low-dose), 19 (medium-dose), or 38 (high-dose) mg isoflavones/kg diet, whereas the doses were less than that of Wangen et al. (2001). Alternatively, this study also explained how isoflavones play a role in soy protein’s effect.

Not only may isoflavones have an important role, but it was also observed that only a minimal dose of 96 mg isoflavone/d (~5µmol/kg BW/d) was required to observe an effect in a meta-analysis of 8 human studies by Zhuo et al. (2004). LDL-C levels were significantly decreased in both normo- and hypercholesterolemic subjects at 96mg isoflavone (0.14 and 0.18mmol/L decrease, respectively) while baseline hypercholesterolemia was lowered more than normocholesterolemia (p=0.0008 vs. 0.03) by the effect of isoflavones.

More recently, a review also supported the current research on the effect of soy protein and isoflavones on plasma LDL cholesterol concentrations. Reynolds et al. (2006) collected English language articles by searching MEDLINE (1966 to February 2005) and the bibliographies of the retrieved articles to examine the effect of soy protein supplementation on serum lipid levels in adults. They found 41 randomized controlled trials in which isolated soy protein supplementation was the only intervention and assessed the net changes in serum lipids during intervention. Information on study design, sample size, participant characteristics, intervention, follow-up duration, and treatment outcomes was independently abstracted using a standardized protocol. Data from each study were pooled using a random-effects model. They concluded soy protein supplementation was associated with a significant reduction in mean serum total cholesterol (−5.26 mg/dl), low-density lipoprotein cholesterol
(−4.25 mg/dl), and triglycerides (−6.26 mg/dl) and a significant increase in high-density lipoprotein cholesterol (0.77 mg/dl). At meantime, meta-regression analyses showed a dose-response relation between soy protein and isoflavone supplementation and net changes in serum lipids.

In conclusion, hypercholesterolemia is a major modifiable risk factor for cardiovascular disease. Most current studies have shown that intake of soy protein including isoflavones decreases total and low-density lipoprotein cholesterol and triglycerides and increases high-density lipoprotein cholesterol among adults with or without hypercholesterolemia. Replacing foods high in saturated fat and cholesterol with soy protein may have a beneficial effect on cardiovascular risk factors.

b. Antioxidant activity in antiatherogenic effect of soy protein or soy isoflavone

Oxidative processes are thought to be important in the initiation and progression of atherosclerosis. The oxidation hypothesis of atherosclerosis is based on the oxidative modification of LDL and phospholipids, which leads to foam cell formation and proliferation, which create an inflammatory state. Prevention of lipid oxidation and, specifically, LDL oxidation is thought to be potentially important for the prevention of atherosclerosis and ischemic heart disease (Gutteridge et al. 1993). Current thinking is that antioxidants inhibit lipid peroxidation and, thus, protect against CVD. Many reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed from physiological processes in the body, which contribute to aging, mutagenesis, carcinogenesis, DNA damage, and cardiovascular disease (Croft, 1998). Dietary isoflavones are also thought to protect against cardiovascular disease by the antioxidant properties which have been one important focus of particular. Potential mechanisms by which soy isoflavones might prevent atherosclerosis include a beneficial effect on these antioxidant effects, either in antiatherogenic effect of soy protein with intact isoflavones, or the effects of isoflavones without soy protein (Diaz et al. 1997). Yamakoshi et al. (2000) investigated the antiatherogenic effects of isoflavones (containing 429.4 mg/g isoflavone aglycones) without
soy protein from fermented soy in cholesterol-fed rabbits. The atherosclerotic lesion area of the aortic arch was significantly lower in the isoflavone fed groups than in the cholesterol fed control group. They suggested that the antioxidative action of isoflavones inhibited the oxidation of LDL because immunohistochemical analysis showed fewer oxidized LDL-positive macrophage-derived foam cells in atherosclerotic lesions in the aortic arch of isoflavone groups. Also Mahn et al. (2005) suggested that a soy protein diet increased mRNA levels for the antioxidant genes cytochrome c oxidase and γ-glutamylcysteine synthetase (catalytic unit) in vasculature, resulting in reduced oxidative stress and increased nitric oxide bioavailability in an animal study.

Several authors have reported on *in vivo* and *in vitro* soy isoflavone studies for antioxidant activity (Jenkins et al. 2000; Hwang et al. 2003; Jiang et al. 2003; Yousef et al. 2004; Park et al. 2005). Dietary isoflavones may confer significant health advantages including cholesterol reduction, antioxidant activity, and possibly a reduced cancer risk. However, the concern has also been raised that phytoestrogens may be endocrine disrupters and major health hazards. Jenkins et al. (2000) assessed the effects of soy protein as a rich source of isoflavonoid phytoestrogens on LDL oxidation and sex hormone receptor activity. Thirty-one hyperlipidemic subjects underwent two 1-month low-fat metabolic diets in a randomized crossover study. The major differences between the test and control diets were an increase in soy protein foods (33 g/d soy protein) providing 86 mg isoflavones/d. No significant difference was detected in *ex vivo* sex hormone activity between urine samples from the test and control periods. Soy consumption did not increase the risk for hormone-dependent cancers. However, in this study, the main point was that the test diet decreased both oxidized LDL measured as conjugated dienes in the LDL fraction and the ratio of conjugated dienes to LDL cholesterol. In conclusion, consumption of high-isoflavone foods was associated with reduced levels of circulating oxidized LDL which may become a cardiovascular disease risk and mediate the development of atherosclerosis by inflammatory processes.

Hwang et al. (2003) indicated the inhibition of superoxide radical (O$_2^•$) production and enhanced levels of free nitric oxide (NO) prevented LDL modification in an equol pretreatment of J774 monocyte/macrophages culture, apparently via the inactivation of the
reduced nicotinamide adenine dinucleotide phosphate oxidase complex. Conversely, inhibition of NO production enhanced LDL oxidative modification; and the combination of reduced NO and increased $O_2^{-}\cdot$ production yielded maximum LDL formation (Hwang et al. 2003).

Jiang et al. (2003) also investigated the effects of bioactive isoflavone metabolites on hyperlipidemia, endothelial dysfunction and the development of atherosclerotic lesions in apolipoprotein E-deficient (apoE (0)) mice fed a Western high-fat diet. Supplementation with dihydrodaidzein (DiD), dehydroequol (DeE) (both 25 mg kg (-1) x day (-1)) and their combination (D/D; 12.5 mg kg(-1) x day(-1) for each) for 24 weeks reduced plasma high-density lipoprotein (HDL) and non-HDL cholesterol. D/D also reduced the triglyceride level. In the abdominal aorta of apoE(0) mice, these compounds significantly increased endothelial nitric oxide (NO)-mediated vasorelaxations induced by acetylcholine, but had a minor effect on relaxations induced by the NO donor S-nitroso-N-acetylpenicillamine. Isoflavone treatment for 24 weeks had no effect on the total area of atherosclerotic plaques in the whole aorta. However, DeE reduced the plaque thickness in the aortic arch by 29%, although this did not reach statistical significance. The endothelial dysfunction in apoE (0) mice was associated with hyperlipidemia and increased vascular oxidative stress measured as increased superoxide production. Both isoflavones have superoxide-scavenging activities in vitro. They suggested that chronic supplementation with bioactive isoflavone metabolites may protect endothelial NO function in apoE(0) mice, through both lipid-lowering and antioxidant actions.

Yousef et al. (2004) investigated the effects of either 2.5 or 5 mg/kg B.W. doses of isoflavones on the levels of free radicals in male New Zealand White rabbits. Treatment with isoflavones caused significant (P<0.05) decrease in the concentrations of free radicals in plasma, liver, brain and kidney by two doses, respectively, as compared to the control. On the other hand, the activity of glutathione S-transferase (GST) did not change in treated animals as compared to control. However, Mahn et al. (2005) showed mitochondrial levels of glutathione (GSH) were significantly higher feeding of rats with a soy protein-rich diet during gestation and adult life, suggesting that production of reactive oxygen species (ROS) was decreased. Furthermore, in Mahn et al. (2005) study, increased antioxidant enzymes
manganese superoxide dismutase and cytochrome c oxidase in aortic tissue were also 2- to 3-fold higher feeding the soy protein-rich diet.

In a spontaneously hypertensive rat (SHR) study, investigator aimed the protective effect of isoflavone against hypertension. Both the mitigation of oxidative stress and prevention of nitric oxide (NO, a potent vasodilator) were reported. Park et al. (2005) fed the 8 wk-old male SHR with a casein-based high fat diet (120 g fat, 1 g cholesterol/kg diet) for 30 d, either with or without 10 g of soy powder (containing 31.2% of isoflavones)/kg. During the 30-d study period, tail systolic blood pressures (BP) in the control SHR group increased, while the isoflavone-supplemented group benefited from a clear antihypertensive effect. The serum NO and total radical trapping antioxidant potential (TRAP) were elevated in the isoflavone group. The isoflavone group also experienced a significant decrease in oxidative DNA damage in leukocytes, using comet assay. DNA damage correlated positively with incremental BP during the study, and systolic BP at the end of the study. They indicated that soy isoflavone has an antihypertensive effect, possibly through the amelioration of oxidative stress, and the augmentation of NO production in SHR.

On the other hand, atherogenesis is also a complex process, including infections, inflammation, and autoimmunity (Leinonen et al. 2002). Heat shock proteins (HSP) were identified as possible autoantigenic determinants acting as immune targets in the atherosclerotic process (Pockley et al. 2002). In a previous study, the increase of *NO production may enhance HSP expression in some cell types (Hirvonen et al. 1996). Soy isoflavones may affect several biochemical pathways both the synthesis of nitric oxide (NO) and heat shock proteins (HSP) that are important factors for atherosclerosis development. More recently, Rosier et al. (2006) investigated the influence of soy isoflavones on NO production and HSP in experimental atherosclerosis in New Zealand rabbits with casein and soy isoflavones (5 mg/kg/day) (ISO). The ISO group showed the concentration of (*) NO metabolites (NO x)(*NO production) in plasma and the levels of reactive antibodies to HSP in aortic tissue were significantly decreased, as well as, a significant reduction of cholesterol in LDL and in aorta compared with the CAS group.

As a general conclusion from these studies, decreasing in the concentrations of oxidized LDL and free radicals and superoxide radical production in plasma, and enhancing
endothelial nitric oxide synthase and the antioxidant enzymes manganese superoxide dismutase and cytochrome c oxidase and total radical trapping antioxidant potential (TRAP) are the main issues in isoflavone antioxidant properties.

c. Effects of soy isoflavones on blood pressure

Washburn et al. (1999) and Teede et al. (2001) found that soy protein supplementation significantly reduced blood pressure in men and perimenopausal or postmenopausal women. Nevala et al. (2000) found that a soy-based diet attenuated the development of hypertension in spontaneously hypertensive rats. In contrast with the above studies, Hodgson et al. (1999) reported that soy isoflavonoids do not reduce blood pressure in hypertensive humans. Therefore, Rivas et al. (2002) conducted a randomized, double-blind comparative study of soy milk vs. cow’s milk in people with mild-to-moderate hypertension to corroborate the effects of soy isoflavone on blood pressure.

Washburn et al. (1999) investigated the effect of soy protein supplementation with known levels of phytoestrogens on cardiovascular disease risk factors and menopausal symptoms in 51 perimenopausal women in a randomized, double-blind crossover trial for 6-week periods. Women were randomly assigned to one of the three diets: 20 g of complex carbohydrates (comparison diet), 20 g of soy protein containing 34 mg of phytoestrogens given in a single dose, and 20 g of soy protein containing 34 mg of phytoestrogens split into two doses. A significant decline in diastolic blood pressure (5 mm Hg lower) was noted in the twice-daily soy diet, compared with the placebo diet. Significant declines in total cholesterol (6% lower) and low density lipoprotein cholesterol (7% lower) were observed in both soy diets compared with the placebo diet. Although nonsignificant effects were noted for a number of measures of quality of life, a significant improvement was observed for the severity of vasomotor symptoms and for hypoestrogenic symptoms in the twice-daily group compared with the placebo group. Soy supplementation in the diet of nonhypercholesterolemic, nonhypertensive, perimenopausal women resulted in significant improvements in lipid and lipoprotein levels, blood pressure, and perceived severity of vasomotor symptoms. These data confirmed the potential importance of soy supplementation
in reducing chronic disease risk in Western populations. Also, Teede et al. (2001) performed soy dietary containing phytoestrogens (40 g soy protein, 118 mg isoflavones) to measure blood pressure (BP), lipids, vascular function (systemic arterial compliance and pulse wave velocity), and endothelial function (flow-mediated vasodilation) in a randomized, double-blind trial in two hundred thirteen healthy subjects (108 men and 105 postmenopausal women) for 3 months. Although, in this study, Teede et al. (2001) did not show improved vascular function after soy intervention compared with casein placebo, urinary phytoestrogens increased, accompanied by a significant fall in BP reflected in systolic, diastolic, and mean BP. In the lipid model, soy induced greater changes, compared with placebo. On individual analysis, significant contributors included a reduction in the low- to high-density lipoprotein ratio and triglycerides and peripheral PWV (reflecting peripheral vascular resistance) improved with soy, whereas flow-mediated vasodilation (reflecting endothelial function) declined (in males only), compared with casein placebo. In normotensive men and postmenopausal women, soy improved BP and lipids. On the other hand, Hodgson et al. (1999) indicated that soy isoflavonoids do not reduce blood pressure in hypertensive humans. These different results may be induced by different subjects. Fifty-nine subjects with high-normal range systolic BP completed a randomized, double blind, placebo-controlled trial of two-way parallel design and 8 weeks duration to assess the effect of isoflavonoid supplementation on BP. One tablet containing 55 mg of isoflavonoids, including 30 mg of genistein, 16 mg of biochanin A (a genistein precursor), 1 mg of daidzein, and 8 mg of formononetin (a daidzein precursor), or one placebo tablet, was taken daily with the evening meal. Significant increases in urinary excretion of genistein and daidzein were observed in the group taking the isoflavonoid supplement. BP was measured at two visits, and ambulatory BP monitoring was performed over one 24-h period, at baseline and postintervention. There was no significant difference between groups, after adjustment for baseline values, in postintervention supine BP, erect BP, or 24-h ambulatory BP. Adjustment for age, sex and weight change did not alter the result. Therefore, these results do not support the hypothesis that isoflavonoids, and genistein in particular, are major contributors to the BP lowering effect of vegetarian diets. Subsequently, Rivas et al. (2002) also conducted the soy-based diets study in 40 men and women with mild-to-moderate hypertension. In this study,
the antihypertensive potential of soy milk (500 mL twice daily) compared with cow’s milk was investigated in a 3-mo double-blind randomized trial. Before initiation of the study, urinary isoflavonoids (measured by HPLC) were undetectable in most cases. After 3 mo of soy milk consumption, systolic blood pressure decreased by 18.4 ± 10.7 mmHg compared with 1.4 ± 7.2 mmHg in the cow’s milk group (P < 0.0001), diastolic blood pressure decreased by 15.9 ± 9.8 mmHg vs. 3.7 ± 5.0 mmHg in the cow’s milk group (P < 0.0001) and mean blood pressure decreased by 16.7 ± 9.0 mmHg compared with 3.0 ± 4.6 mmHg in the cow’s milk group (P< 0.0001). Urinary genistein was strongly (r = -0.588) and significantly (P = 0.002) correlated with the decrease in blood pressure, particularly for diastolic values. Chronic soy milk consumption had modest, but significant hypotensive action in essential hypertensive subjects. This hypotensive action was correlated with the urinary excretion of the isoflavonoid genistein.

In summary, the effect of soy isoflavone on blood pressure is also controversial, maybe due to the subjects’ basal level of blood pressure, age, diet and isoflavone dosages. Some current research focuses on soy protein supplementation in perimenopausal or postmenopausal women and devised animal hypertension models. The apparent decreased blood pressure is evident. However, in this research field, they also did not try to control the relationship between interindividual variability in isoflavone bioavailability and blood pressure levels.

d. Insulin sensitivity and Cardiovascular Risk

The metabolic syndrome (MS) which includes hypertension, dyslipidemia, increased prothrombic state, and central obesity may precede development of Type 2 diabetes mellitus (DM) and cardiovascular disease (Reaven et al. 2004). The primary factor underlying progression of MS is development of insulin resistance and hyperinsulinemia (Reaven et al. 2004). To investigate the effects of soybean protein on glucose tolerance and insulin receptor gene expression, Iritani et al. (1997) fed Wistar fatty rats (genetically obese, noninsulin-dependent diabetes mellitus) and their lean littermates (8 wk old) using a casein or soybean protein diet containing 9% partially saturated beef tallow (plus 1% corn oil), 10%
corn oil or 10% fish oil for 3 wk. In glucose tolerance tests, plasma insulin concentrations were significantly higher in obese rats fed corn oil or fish oil than in those fed partially saturated beef tallow, particularly in the soybean protein groups. Although, plasma glucose concentrations were not significantly affected by dietary protein or fat, soy protein-fed rats had lower fasting plasma glucose compared with casein-fed animals in Lavigne et al. (2000) study. Iritani et al. (1997) also reported the insulin receptor mRNA concentrations in livers and adipose tissues were higher in rats fed soybean protein/partially saturated beef tallow than in those fed any other protein/fat combination. Dietary soybean protein may help to reduce the insulin resistance, but only when a diet low in polyunsaturated fatty acids is consumed. On the other hand, the insulin receptor mRNA concentrations in adipose tissue were generally lower in the obese rats of all dietary groups than in the lean rats, suggesting that insulin resistance may be due to a defect of insulin receptor gene expression.

Lavigne et al. (2000) also determined the effects of feeding various dietary proteins on insulin sensitivity and glucose tolerance in rats. They fed male Wistar rats for 28 days with isoenergetic diets containing either casein, or soy protein, or cod protein. Cod protein-fed and soy protein-fed rats had lower insulin concentrations compared with casein-fed animals. After intravenous glucose bolus, cod protein- and soy protein-fed rats induced lower incremental areas under glucose curves compared with casein-fed animals. Improved peripheral insulin sensitivity was confirmed by higher glucose disposal rates in cod protein- and soy protein-fed rats compared with casein-fed animals. Moreover, test meal experiments revealed the lower plasma insulin concentrations in cod protein- and soy protein-fed animals could be also due to decreased pancreatic insulin release and increased hepatic insulin removal. They concluded the metabolic responses to three common dietary proteins indicate that cod and soy proteins, when compared with casein, improve fasting glucose tolerance and peripheral insulin sensitivity in rats.

Furthermore, Davis et al. (2005) focused on physiological effects of soy-based diets with varying isoflavone content on pathogenesis of insulin-resistance and cardiovascular risk using a rodent model that reflects early stages of insulin resistance and MS. In this study, lean male SHHF (+/cp) rats were randomly assigned to the several treatment groups: casein (control, C); low-isoﬂavone (LIS) soy protein isolate; high-
isoflavone (HIS) soy protein isolate; or C+ 0.01 % rosiglitazone (CR) for thirty-six weeks. Liver weight, heart weight, total plasma cholesterol, fasting blood glucose were lower in soy-fed animals compared to control. Body weight, kidney weight, alanine aminotransferase (ALT), fasting plasma insulin, and homeostasis model assessment (HOMA) score were also lower in LIS-fed rodents compared to casein treatment. All diet groups exhibited lower urine protein and small arteriole content compared to controls.

In conclusion, insulin sensitivity related to CVD risk factors are also interested isoflavone studies recently. Concern regarding the safety and efficacy of isoflavones which necessitate discovering alternative therapies for prevention and treatment of insulin-resistance still remains unknown. However, more research on interindividual variability in insulin resistance of isoflavone trials may help explain the importance of interindividual variability in isoflavone bioavailability.

e. Effects of soy isoflavones on endothelial function

The endothelium is crucial for the modulation of vessel tone and for the control of platelet adhesion and aggregation. Endothelial dysfunction, defined as an imbalance of endothelial-derived vasoactive factors leading to vasoconstriction and structural changes in the vessel wall, is an early event in the pathophysiology of atherosclerosis and hypertension, and it is an independent predictor of poor prognosis (Schachinger et al. 2000). Preliminary reports in female monkeys show enhancement of endothelium-dependent vasodilatation (Honore et al. 1995). Soybeans contain a number of compounds that have weak estrogenic activity (Adlercreutz et al. 1995). Of these, the isoflavonoids are especially attractive, possessing antioxidant property (Jha et al. 1985) as well as the capacity to occupy estrogen receptors.

Some studies focused on the favorable effects on blood vessel function and improving vascular reactivity (Nestel et al. 1997; Walker et al. 2001; Yildirir et al. 2001; van der Schouw et al. 2002). Nestel et al. (1997) investigated a pure preparation of isoflavones from soybean on several important biomarkers of cardiovascular health in 21 menopause women. They found systemic arterial compliance was significantly improved in
perimenopausal and menopausal women taking soy isoflavones to about the same extent as is achieved with conventional hormone replacement therapy. These included systemic arterial compliance, a measure of elasticity of the major conduit arteries such as the aorta, the vasodilatory capacity of the microcirculation in the forearm, plasma lipid concentrations, and the oxidizability of LDL. The possibility that the heightened cardiovascular risk associated with the menopause, which is said to be ameliorated by soybeans, can be reduced with soy isoflavones was tested in a placebo-controlled crossover trial with 80-mg daily isoflavones (45 mg genistein) over 5- to 10-week periods. Although arterial pressure and plasma lipids and LDL oxidizability in vitro were unaffected, it believed that systemic arterial compliance (arterial elasticity) improved compared with placebo. The vasodilatory capacity of the microcirculation was measured in nine women; high acetylcholine-mediated dilation in the forearm vasculature was similar with active and placebo treatments. Also Walker et al. (2001) investigated whether genistein influences endothelium-dependent vasodilation in forearm vasculature of healthy human subjects and compared the effects of genistein with those of 17ß-estradiol. It is thought that genistein causes L-arginine/NO-dependent vasodilation in forearm vasculature of human subjects with similar potency to 17ß-estradiol and potentiates endothelium-dependent vasodilation to acetylcholine. In this study, forearm blood flow responses were measured with strain-gauge plethysmography. Genistein (10 to 300nmol/min, each dose for 6 minutes) significantly increased a dose-dependent increase in forearm blood flow. Daidzein, another phytoestrogen, was ineffective, but equimolar concentrations of 17ß-estradiol caused similar vasodilation to genistein. Responses to genistein and 17ß-estradiol were inhibited to the same degree by the NO synthase inhibitor N\textsuperscript{G}-monomethyl-L-arginine. A threshold dose of genistein potentiated the endothelium-dependent vasodilator acetylcholine. Furthermore, Yildirir et al. (2001) investigated the effects of soy protein diet on endothelial function and plasma lipids parameters assessed by two different methods in twenty hypercholesterolemic, nonsmoker male patients with a normal body mass index. They showed soy protein diet significantly improves plasma lipid profile in patients with hypercholesterolemia. Furthermore, the endothelial function, as judged by two different methods (EDD and plasma TM levels), also improves with soy protein diet. After calculating their daily requirements, a diet with 25-30% of energy from
fats. 10-12% from proteins and the rest from carbohydrates were instituted. Sixty percent of
the animal source proteins of the diet were substituted by soy. The anthropometric measures,
lipid parameters, and endothelial functions of the subjects were assessed at baseline and 6
weeks after soy protein diet. Flow-mediated endothelium-dependent dilatation (EDD) and
plasma thrombomodulin (TM) levels were evaluated as endothelial function parameters. The
mean plasma TM levels were also significantly reduced with diet. Studies of the brachial
artery indicated a borderline dilatation in baseline brachial artery diameter, however the
diameter at reactive hyperemia was significantly larger after diet, resulting in a significant
improvement of EDD. After soy protein substitution, plasma total cholesterol, low-density
lipoprotein cholesterol, apolipoprotein B, and triglyceride levels decreased significantly
compared to animal protein-based diet. On the other hand, van der Schouw et al. (2002) have
focused the effect of Western dietary phytosterolate intake on the risk of atherosclerosis and
arterial degeneration through an effect on arterial walls, especially among older women. Four
hundred three women with natural menopause either between 1987 and 1989 or between
1969 and 1979 were selected from the baseline data of the PROSPECT study (n=17395).
Isoflavone and lignan intake was calculated from a food-frequency questionnaire. Aortic
stiffness was noninvasively assessed by pulse-wave velocity measurement of the aorta. After
adjustment for age, body mass index, smoking, physical activity, mean arterial pressure,
follow-up time, energy intake, dietary fiber intake, glucose, and high density lipoprotein
cholesterol, increasing dietary isoflavone intake was associated with decreased aortic
stiffness. Increasing dietary intake of lignans was also associated with decreased aortic pulse-
wave velocity.

Squadrito et al. (2003) also evaluated genistein effects on endothelial function in
79 healthy postmenopausal women using a double-blind, controlled, randomized design.
Subjects randomly were assigned to receive continuous estrogen/progestin therapy (n = 26;
17β-estradiol [1 mg/d] combined with norethisterone acetate [0.5 mg/d]), genistein (n = 27;
54 mg/d), or placebo (n = 26). Brachial artery flow–mediated, endothelium-dependent
vasodilation and plasma levels of nitrites/nitrates (a marker of nitric oxide metabolism) and
endothelin-1 were measured at baseline and after 1 year of therapy. Treatment with genistein
increased levels of nitrites/nitrates; estrogen/progestin therapy caused similar changes.
Plasma endothelin-1 levels decreased following 12 months of genistein and after 12 months of estrogen/progestin. Brachial artery flow–mediated dilation was improved by genistein and by estrogen/progestin. There were no significant differences between estrogen and genistein for any of these parameters.

Colacurci et al. (2005) evaluated the effects of soy isoflavone administration on endothelial function in healthy sixty postmenopausal women assigned to receive isoflavone or placebo tablets for 6 months. Endothelium-dependent vasodilatation was measured by brachial reactivity technique along with levels of plasma soluble intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin. Differences between endothelium-dependent and endothelium-independent vasodilatation were assessed by evaluating brachial reactivity parameters after reactive hyperemia and after sublingual administration of nitroglycerin; furthermore, in the active group, the effect of isoflavones was also evaluated during the intra-arterial infusion of N-monomethyl-L-arginine. Serum levels of lipids [high-density lipoprotein and low-density lipoprotein cholesterol, triglycerides and lipoprotein (a)] and hemostatic factors (prothrombin, fibrinogen, plasminogen activator inhibitor-1, and fibrin D-dimer) were also measured. Isoflavone treatment versus placebo was associated with a significant improvement in endothelium-dependent vasodilatation. Intra-arterial infusion of N-monomethyl-L-arginine inhibited the significant effect of isoflavones on endothelium-mediated vasodilatation. Furthermore, isoflavone group experienced statistically significant reductions in plasma concentrations of ICAM-1, VCAM-1, and E-selectin. These findings suggest a positive influence of soy isoflavones on endothelial function in healthy postmenopausal women as evidenced by an improvement in endothelium-dependent vasodilatation and a reduction in plasma adhesion molecule levels.

In conclusion, isoflavone may cause several alterations in vasoactive factors characteristic of endothelial dysfunction including systemic arterial compliance, bioavailability of nitric oxide (NO), vasoconstrictor prostanoids, endothelin-1, E-selectin, ROS, ICAM-1, VCAM-1. But interindividual variability in isoflavone bioavailability might influence such findings.

f. **Homocysteine and the risk of vascular disease**
Several studies have shown elevated homocysteine levels in patients with coronary, cerebrovascular, or peripheral arterial diseases; this association is frequent and independent of most other risk factors for atherosclerosis (Malinow et al. 1994). Suggested mechanisms include a direct effect on the vascular endothelium (Tsai et al. 1994) and a role in enhancing the risk of thrombosis (Fryer et al. 1993).

Hermansen et al. (2001) evaluated if a dietary supplement of soy protein, isoflavones, and cotyledon fiber (Abalon) affects cardiovascular risk markers in type 2 diabetic subjects. Twenty type 2 diabetic subjects participated in a crossover trial. They were randomized to double-blind supplementation for 6 weeks with Abalon (soy protein [50 g/day] with high levels of isoflavones [minimum 165 mg/day] and cotyledon fiber [20 g/day]) or placebo (casein [50 g/day] and cellulose [20 g/day]), separated by a 3-week wash-out period. This study demonstrated significantly lower mean values after Abalon intake than after placebo treatment for LDL cholesterol, LDL/HDL ratio, triglycerides, and homocysteine.

Also, Jenkins et al. (2002) investigated the effects of high- and low-isoflavone soy-protein foods on both lipid and nonlipid risk factors for coronary artery disease (CAD) in forty-one hyperlipidemic men and postmenopausal women. Three 1-mo diets: a low-fat dairy food control diet and high- (50 g soy protein and 73 mg isoflavones daily) and low- (52 g soy protein and 10 mg isoflavones daily) isoflavone soyfood diets were chosen in this study. Fasting blood samples were drawn and blood pressure was measured at the start and end of each diet. Compared with the control diet, both soy diets resulted in significantly lower total cholesterol, and ratios of total to HDL cholesterol, LDL to HDL cholesterol, and estimated CAD risk including reductions in oxidized LDL, homocysteine, and blood pressure.

Nagata et al. (2003) examined a cross-sectional relationship between soy product intake and serum homocysteine level in 201 premenopausal Japanese women. Intakes of soy products, folate, methionine and vitamins B-6 and B-12 were estimated by a semiquantitative food frequency questionnaire. Folate status was also assessed by measuring serum folate. Soy product intake in terms of soy protein as well as soy isoflavone intake was modestly but significantly inversely associated with serum homocysteine level after controlling for covariates. Soy product intake was also significantly positively correlated with serum folate.
Although it is unclear the extent to which each component of soy, such as folate and isoflavones, is associated with the serum homocysteine concentration, this biochemical complex appears to have a favorable effect on homocysteine metabolism in premenopausal women.

In conclusion, homocysteine levels related to CVD risk factors are also of recent interest with respect to isoflavone. However, more research on interindividual variability in homocysteine levels of during isoflavone trials may be helpful.

g. Atherosclerotic thrombus or plaques formation and the risk of vascular disease

To address its effects on clinical cardiovascular endpoints such as incidence of coronary heart disease or atherosclerosis progression in animal or human subjects, some studies focused on the effects of soy consumption on indices of endothelium-dependent and endothelium-independent vascular reactivity in men. A randomized, double-blind trial demonstrated a decline in flow-mediated (endothelium-dependent) peripheral vasodilatory function in healthy men who had consumed isoflavone-containing soy protein supplements for 3 mo compared with men who had consumed a casein placebo (Teede et al. 2001). Also, a previous study found a tendency toward a decline in endothelium-dependent vasodilatory function of coronary arteries in male monkeys fed isoflavone-replete soy protein for 6 mo compared with monkeys fed isoflavone-deficient soy protein (Honoré et al. 1997). In contrast with the above studies, Lissin et al. (2004) reported these findings conflict with those from studies of women and female monkeys, in which isoflavone-containing soy supplements seemed to improve vascular reactivity.

Two studies with monkeys found an inhibitory effect of isoflavone-containing dietary soy protein on atherosclerosis in juvenile male and ovariectomized female monkeys, an effect that was partially lost when the isoflavones were removed from the soy protein by alcohol washing (Anthony et al. 1997; Wagner et al. 2003). Previously, Wilcox et al. 1995) reported genistein, an isoflavonoid derived from soy products, inhibited thrombin formation and platelet activation in vivo in addition to its antigrowth factor activity. This compound
had the potential to affect the progression of atherosclerotic disease by modifying coagulation responses. In this study, they did not establish its effect on experimental vascular lesion formation to assess the potential of genistein as a therapeutic for vascular disease. However, Raines et al. (1995) indicated that the increased levels of isoflavonoids, in particular genistein, which are associated with consumption of soy-based diets, inhibited cell adhesion, altered growth factor activity and inhibited cell proliferation involved in lesion formation. Furthermore, Adams et al. (2005) determined the effects of the long-term (31 mo) consumption of commercially available soy protein containing 2 concentrations of isoflavones on the development of atherosclerotic plaques and vascular reactivity in coronary arteries of adult male monkeys. The monkeys were fed atherogenic diets that differed only in the source of protein: Control (n = 30), casein and lactalbumin; low-isoflavone soy (n = 30), a mixture of unmodified soy protein isolate and isoflavone-depleted soy protein isolate containing 0.94 mg of isoflavones/g protein; and high-isoflavone soy (n = 31), unmodified soy protein isolate containing 1.88 mg of isoflavone/g protein. Although there were no effects of dietary soy on endothelium-dependent or -independent reactivity of coronary arteries, atherosclerosis (mean plaque size in the coronary arteries) was reduced by ~34% in both groups fed soy protein. Plasma LDL cholesterol was reduced, whereas HDL cholesterol and apolipoprotein A-1 were increased in both groups that consumed soy protein.

In conclusion, potential factors by which soy isoflavones might prevent atherosclerosis include beneficial effects of soy and say isoflavones on serum lipid profiles. Isoflavones may decrease LDL-cholesterol levels and increase HDL-cholesterol levels and also have antioxidant properties which protect LDL from oxidation. These compounds also have some favorable effects on blood vessel function; lowering blood pressure; inhibiting thrombus formation; suppressing smooth muscle cell proliferation and migration and improving vascular reactivity.

h. Cholesterol lowering mechanism of isoflavone

High total cholesterol and LDL cholesterol levels correlate with cardiovascular disease. Elevated cholesterol levels contribute to the formation of atherosclerotic plaques and
eventually to thrombosis or myocardial infarction (Anderson et al. 1995). The mechanism by which soy isoflavone exerts its effects is unknown and a matter of debate. But it is known that reduced LDL-cholesterol concentrations associated with estrogen intake are a direct result of increased LDL receptor activity (Kovanen et al. 1979). Although some studies reported that LDL receptor wasn’t required for the role of the atheroprotective action of soy isoflavones in prevention of cardiovascular disease for postmenopausal women (Adams et al. 2002; Mortensen et al. 2004), recently, soy isoflavones were reported to affect sterol regulatory element binding proteins (SREBPs) and SREBP regulated genes related to LDL receptor expression in HepG2 cells (Mullen et al. 2004).

Baum et al. (1998) investigated the effect of soy protein and isoflavones on mononuclear cell LDL receptor messenger RNA (mRNA) in postmenopausal women over a 6-mo feeding period to determine the possible role of isoflavones in lipid metabolism. Sixty-six hypercholesterolemic, free-living, postmenopausal women were investigated in parallel-group, double-blind trial with 3 interventions. After a control period of 14 d, all subjects were randomly assigned to 1 of 3 dietary groups (all with 40 g protein): a National Cholesterol Education Program (NCEP) Step 1 diet with protein from casein and nonfat dry milk (control), an NCEP Step 1 diet with protein from isolated soy protein containing moderate amounts of isoflavones (ISP56), or an NCEP Step 1 diet with protein from isolated soy protein containing high amounts of isoflavones (ISP90). Reverse transcription–polymerase chain reaction (RT-PCR) was used to quantitate LDL receptor mRNA concentrations. Mononuclear cell LDL receptor messenger RNA concentrations increased in subjects consuming ISP56 or ISP90 compared with the control (P < 0.05). Non-HDL cholesterol in both the ISP56 and ISP90 groups was reduced compared with the control group (P < 0.05). HDL cholesterol increased in both the ISP56 and ISP90 groups (P < 0.05), and the ratio of total to HDL cholesterol decreased significantly in both groups compared with the control (P < 0.05). This study indicated that soy protein, with different amounts of isoflavones, decreased the risk of cardiovascular disease via improved blood lipid profiles, and that the mechanism by which apolipoprotein B-containing lipoproteins were depressed may be via alterations in LDL receptor quantity or activity.
Almost at the same time, two other authors (Kirk et al. 1998; Potter et al. 1998) also reported that isoflavones increased LDL receptor activity in C57BL/6 mice or postmenopausal women. Kirk et al. (1998) determined whether soy isoflavones had effect on cholesterol levels or on susceptibility of LDL to oxidative modification in LDLR-null mice and whether dietary isoflavones conferred protection against atherosclerosis in mice. C57BL/6 and LDL receptor-deficient (LDLR-null) mice were fed soy protein-based, high fat diets with high isoflavones (IF+) or diets from which isoflavones had been extracted (IF−). Because LDLR-null mice develop extensive atherosclerosis and hypercholesterolemia after minimal time on a high fat diet, they were fed the diets for 6 wk, whereas C57BL/6 mice were fed the diets for 10 wk. Plasma cholesterol levels did not differ between LDLR-null mice fed IF− and those fed IF+, but were 30% lower in C57BL/6 mice fed the IF+ diet than in those fed the IF− diet. Susceptibility of LDL to oxidative modification, measured as the lag phase of conjugated diene formation in LDLR-null mice, was not altered by isoflavone consumption. All LDLR-null mice developed atherosclerosis, and the presence or deficiency of dietary isoflavones did not influence atherosclerotic lesion area. In contrast, atherosclerotic lesion area was significantly reduced in C57BL/6 mice fed IF+ compared with those fed IF−. Thus, these findings suggest that soy isoflavones might lower cholesterol levels by increasing LDL receptor activity, and the reduction in cholesterol may offer some protection against atherosclerosis. Potter et al. (1998) investigated in 66 free-living, hypercholesterolemic, postmenopausal women during a 6-mo, parallel-group, double-blind trial with 3 interventions to determine effects of soy protein (40 g/d) containing moderate and higher concentrations of isoflavones on blood lipid profiles, mononuclear cell LDL receptor messenger RNA, and bone mineral density and content. All subjects were randomly assigned to 1 of 3 dietary groups: Step I diet with 40 g protein/d obtained from casein and nonfat dry milk (CNFDM), Step I diet with 40 g protein/d from isolated soy protein containing 1.39 mg isoflavones/g protein (ISP56), or Step I diet with 40 g protein/d from isolated soy protein containing 2.25 mg isoflavones/g protein (ISP90). Mononuclear cell LDL receptor mRNA was increased in subjects consuming ISP56 or ISP90 compared with those consuming CNFDM (P < 0.05). Total and regional bone mineral content and density were assessed. Non-HDL cholesterol for both ISP56 and ISP90 groups was reduced compared with the CNFDM group (P < 0.05).
HDL cholesterol increased in both ISP56 and ISP90 groups (P < 0.05). Significant increases occurred in both bone mineral content and density in the lumbar spine. Intake of soy protein at both isoflavone concentrations for 6 mo may decrease the risk factors associated with cardiovascular disease in postmenopausal women.

Furthermore, soy isoflavones were reported to affect sterol regulatory element binding proteins (SREBPs) and SREBP regulated genes related to LDL receptor expression in HepG2 cells (Mullen et al. 2004). These genes regulated by the SREBPs include LDL receptor (Tontonoz et al. 1993), HMG CoA reductase (Millinder-Vallett et al. 1996), 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase (Dooley et al. 1999) and fatty acid synthase (Magana et al. 2000). SREBP-2 is encoded on by its own gene and is primarily responsible for the regulation of genes involved in cholesterol biosynthesis and metabolism (Hua et al. 1993). Because SREBPs are critical for the regulation of intracellular sterol and lipid homoeostasis, Mullen et al. (2004) examined the effects of isoflavone-containing soy extract and individual isoflavones on SREBP levels and maturation as well as their effects on the SRE-regulated genes HMG CoA synthase, HMG CoA reductase, and the LDL receptor.

Understanding the metabolic effects of the various components of soy is an important goal to help guide or design the modification of cholesterol-lowering soyfoods or dietary supplements.

Mullen et al. (2004) proposed that the isoflavone component of soy mediates this effect, at least in part, by affecting cellular sterol homeostasis. They investigated the effects of an isoflavone-containing soy extract and the individual isoflavones on the maturation of the sterol regulatory element binding proteins (SREBP) and the expression of SRE-regulated genes controlling lipid metabolism and found a corresponding increase in the mature form of SREBP-2 in both soy extract– and isoflavone-treated human hepatoma cell line (HepG2). 3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase protein and HMG CoA synthase mRNA levels also increased. When HepG2 cells were transiently transfected with HMG CoA synthase and LDL receptor reporter plasmids there was an increase in expression in response to soy extract or isoflavone treatment from both of these promoters. The mechanism responsible for this effect may be via a statin-like inhibition of HMG CoA reductase enzyme activity or by enhanced SREBP processing via the SREBP cleavage
activating protein. They concluded that maturation of SREBP and induction of SRE-regulated genes produced an increase in surface LDL receptor expression that increased the clearance of plasma cholesterol, thus decreasing plasma cholesterol levels.

Another topic about the mechanism for the cholesterol lowering ability of soy protein and isoflavone was reported the peroxisome proliferator activator receptor (PPAR)\(\alpha\)-dependent and -independent pathways. Recent evidence suggests that isoflavones along with other botanical compounds may be agonists or activators of the "promiscuous" nuclear receptors regulating cellular lipid metabolism, most notably the PPARs (Mezei et al. 2003; Kim et al. 2004). The three different isoforms of PPARs (\(\alpha\), \(\gamma\), and \(\delta\)) have overlapping tissue distributions and functions associated with lipid metabolism. Results of in vitro studies demonstrate that the soy isoflavones, particularly genistein and daidzein, were able to activate both PPAR\(\alpha\) and PPAR\(\gamma\)-mediated gene expressions (Mezei et al. 2003). The soy isoflavone genistein has been identified as a ligand of the PPAR\(\gamma\) receptor (Dang et al. 2003). Gene profiling suggests that genistein is regulating gene expression through PPAR\(\alpha\), which acts to stimulate mitochondrial fatty acid oxidation (Kim et al. 2004). Recently, Mezei et al. (2006) reported that dietary isoflavone supplementation modulated lipid metabolism via PPAR\(\alpha\)-dependent and -independent mechanisms. In this study, both male and female mice were studied to determine whether the estrogen receptor and high circulating estrogen levels would be sufficient to produce changes in lipid levels associated with isoflavone consumption. Alternately, changes in lipid levels might be independent of sex, or there may be an interaction between sex and the presence of the PPAR\(\alpha\) receptor.

In the study by Mezei et al. (2006), male and female 129/Sv mice were obtained, including both wild-type and genetically altered PPAR\(\alpha\) knockout mice. Groups of mice were fed high-fat atherogenic diets containing soy protein +/- isoflavones and PPAR\(\alpha\) agonist fenofibrate for 6 wk. At the end of 6 wk, serum and tissue lipid levels were measured along with hepatic gene expression. Most notably, serum triglycerides were reduced by isoflavone consumption. Compared with intake of a low-isoflavone basal diet, isoflavone intake reduced serum triglyceride levels by 36 and 52% in female and male wild-type mice, respectively, compared with 55 and 52% in fenofibrate-treated mice. The wild-type mice contributed to reduced serum triglyceride levels due to isoflavone intake. And loss of the PPAR\(\alpha\) receptor in
soy isoflavone fed mice decreased less serum triglyceride levels than wild-type mice. However, soy isoflavones also improved serum triglyceride levels in knockout mice because female and male PPAR_α_/– mice showed reductions in serum triglyceride in the soy isoflavone fed group, whereas fenofibrate did not, suggesting that two different regulatory mechanisms may be affected by isoflavone intake. Thus there was a positive effect of isoflavone intake on serum triglyceride levels in the absence of PPAR_α_. Isoflavone intake resembled action of fenofibrate on PPAR_α_-regulated gene expression, although less robustly compared with fenofibrate. They suggested that, at the levels consumed in this study, isoflavone intake was altering lipid metabolism in a manner consistent with activation of PPAR_α_ and also via a PPAR_α_-independent mechanism as well.

In conclusion, the mechanism for the cholesterol lowering ability of soy protein and isoflavone is the topic of many hypotheses. Although the mechanism by which isoflavone alters plasma lipoprotein concentrations is still a matter of debate, besides increase in hepatic LDL-receptor activity, change in hepatic biotransformation of cholesterol and decreasing intestinal absorption of cholesterol might be factors. We now have much more interesting progress in this field including effects of isoflavones on sterol regulatory element binding proteins (SREBPs), SREBP regulated genes related to LDL receptor expression and the peroxisome proliferator activator receptor (PPAR)_α_-dependent and –independent pathways.

D. Saponins and lessening of plasma cholesterol

Saponins, which are one kind of steroid or triterpene glucoside compounds associated with soy protein in addition to isoflavone, were also studied to see whether they had a cholesterol-lowering effect. Sugano et al. (1990) fed rat diets with the undigested high-molecular-weight fraction of soybean protein (HMF) or soybean saponin diets also containing cholesterol (0.5%) and sodium cholate (0.125%). Soybean saponin equaled to that contained in HMF, which can significantly decrease serum cholesterol levels, did not lower serum cholesterol. Only the addition of 1.3% of saponin to the soy protein diet decreased serum cholesterol level. Yamakoshi et al. (2000) showed that saponin-rich extract diets containing 1% cholesterol with 1.09% saponin did not affect the serum lipid profile in 12 -
wk-old New Zealand white male rabbits compared to controls which did not consume cholesterol, isoﬂavone or saponins.

On the other hand, several studies on the effect of saponins on improving lipid profiles have also been reported. To investigate the potency of saponin with respect to its hypoglycaemic and hypolipidaemic action, and the association of these effects with oxidative stress, Rodrigues et al. (2005) used two groups (n =6): control group and saponin-treated group (60 mg/kg) during 30 days in male Wistar rats. They reported saponin-treated animals had increased low-density lipoprotein-cholesterol/triacylglycerol ratio and decreased triacylglycerol, very low-density lipoprotein-cholesterol and total/high-density lipoprotein-cholesterol ratio than the control group. Saponin-treated rats showed lower lipid hydroperoxide than control rats, indicating decreased potential to atherosclerosis. No alterations were observed in antioxidant enzymes, superoxide dismutase and glutathione peroxidase, while lipid hydroperoxide were decreased in saponin-treated rats. They concluded the beneficial effects of saponin on serum lipids were related to a direct saponin antioxidant activity.

Furthermore, Lee et al. (2005) showed that a diet containing 2.2 mmol/kg group B soyasaponins caused signiﬁcantly lower plasma total cholesterol and non-HDL cholesterol (by 20 and 33%) in ten 11–12 wk old female golden Syrian hamsters compared with those fed casein. On the other hand, high and low producers of a putative saponin metabolite, divided by ranking peak area of this fecal metabolite, did not have signiﬁcant differences in plasma total and non-HDL cholesterol, whereas high saponin metabolite producers had greater ratios of total cholesterol to HDL cholesterol compared with low producers. Thus, saponins which vary among soy protein sources were also modiﬁers of plasma lipids and may contribute to lessening of plasma cholesterol.

E. Isoﬂavones and cancer

Among the numerous dietary factors, soy isoﬂavones have received much attention as nutritional components because of their inhibitory role in the growth of cancer cells in vitro and cancer development in vivo. The high level of soy isoﬂavones in Japanese
and Chinese diets and the low incidence of hormone-dependent neoplasms, including breast and prostate cancers, in these countries suggest a protective role of soy-derived substances against cancer (Adlercreutz et al. 1995; Hebert et al. 1998). High consumption of soy milk has also been associated with reduced risk of prostate cancer (Jacobsen et al. 1998). The fact that soy isoflavone—particularly, genistein—can inhibit the carcinogenesis and the growth of cancers makes it a promising cancer preventive and/or therapeutic agent for various cancers. The inhibitory effects of soy isoflavones on cancer cell growth and the comprehensive view on molecular mechanism(s) for the role of isoflavone, particularly genistein, in cancer chemoprevention and/or treatment were summarized below.

Genistein, one of the predominant soy isoflavones, has been shown to compete with 17beta-estradiol for estrogen receptor binding because of its structural similarity. It causes inhibition of cell growth in breast and prostate cancers in vivo and in vitro. From gene expression profiles, genistein has been found to regulate the genes that are critical for the control of cell proliferation (Wei et al. 1995; Zhou et al. 1999), cell cycle (Davis et al. 1998), apoptosis (Lian et al. 1998), oncogenesis (Onozawa et al. 1999), and cell signal transduction pathways. It has been reported that genistein induces apoptosis and inhibits activation of Akt signaling pathways (Li et al. 2002) and NF-kappaB (Li et al. 2005), both of which are known to maintain a balance between cell survival and apoptosis.

Wei et al. (1995) investigated the antiproliferative effects and antioxidant properties of the soybean isoflavone genistein in HL-60 cells and the mouse skin tumorigenesis model. Effects of structure-related flavone/isoflavones on hydrogen peroxide ($H_2O_2$) production by 12-O-tetradecanoylphorbol-13-acetate (TPA)-activated HL-60 cells and superoxide anion ($O_2^-$) generation by xanthine/xanthine oxidase were compared. Studies showed that genistein significantly inhibits TPA-induced $H_2O_2$ formation by (dimethyl sulfoxide) DMSO-differentiated HL-60 cells. In contrast, genistein, apigenin, and prunectin are equally potent in inhibiting $O_2^-$ generation by xanthine/xanthine oxidase, with daidzein showing a moderate inhibitory.
Although these results suggested that the antioxidant properties of isoflavones were structurally related and the hydroxy group at Position 4’ was crucial in both systems and dietary administration of 250 ppm genistein for 30 days significantly enhances the activities of antioxidant enzymes in the skin and small intestine of mice. It was confirmed that genistein's antiproliferative effects and antioxidant properties may be responsible for its anticarcinogenic effect. Its high content in soybeans and relatively high bioavailability favor genistein as a promising candidate for the prevention of human cancers. In another experimental prostate tumor study, Zhou et al. (1999) characterized the ability of dietary soybean components to inhibit growth of transplantable prostate adenocarcinomas and tumor cell proliferation and angiogenesis of transplantable prostate cancer in immunodeficient mice. Soy isoflavones (genistein or daidzein) or soy phytochemical concentrate inhibited the growth of prostate cancer cells LNCaP, DU 145 and PC-3 in vitro. G2-M arrest and DNA fragmentation consistent with apoptosis of prostate cancer cells were also observed at concentrations causing growth inhibition. In contrast, the in vitro proliferation of vascular endothelial cells was inhibited by soy phytochemicals at much lower concentrations. Mice inoculated subcutaneously with LNCaP cells (2 x 10⁶) were randomly assigned to one of the six dietary groups based on the AIN-76A formulation for 3 wk. A 2 x 3 factorial design was employed with two protein sources (20%, casein vs. soy protein) and three levels of soy phytochemical concentrate (0, 0.2 and 1.0% of the diet). The ability of dietary soy phytochemical concentrate and soy protein isolate to inhibit the growth of the LNCaP human prostate cancer in severe combined immune-deficient mice was evaluated. Compared with casein-fed controls, the tumor volumes after 3 wk were reduced by 28% by soy protein with 0.2% soy phytochemical concentrate (P < 0.05), 30% by 1.0% soy phytochemical concentrate (P < 0.05) and 40% by soy protein with 1.0% soy phytochemical concentrate (P < 0.005). Histologic examination of tumor tissue showed that consumption of soy products significantly reduced tumor cell proliferation, increased apoptosis and reduced microvessel density. These data suggested that dietary soy products may inhibit experimental prostate tumor growth through a combination of direct effects on tumor cells and indirect effects on tumor neovasculature.
Furthermore, the growth inhibition of cancer cells could be due to the cell-cycle arrest, which ultimately results in ceasing cell proliferation. Davis et al. (1998) also showed that genistein induced a G2/M cell-cycle arrest in PC3 and LNCaP prostate cancer cells. The inhibition of cell proliferation by genistein could also be due to increased apoptosis. Lian et al. (1998) & Li et al. (1999) showed that genistein induced apoptosis in MDA-MB-231, MDA-MB-435, and MCF-7 breast cancer cells; PC3 and LNCaP prostate cancer cells; H460 and H322 non-small-cell lung cancer cells; and HN4 head and neck squamous carcinoma cells. Onozawa et al. (1999) reported soy isoflavone-supplemented diets prevented the development of adenocarcinomas in the prostate and seminal vesicles in a rat carcinogenesis model.

Akt signaling pathway also plays an important role in cell growth and apoptosis. Akt, also referred to as PKB, contains an amino-terminal pleckstrin homology domain that binds phosphorylated lipids at the membrane in response to activation of phosphatidylinositol 3 kinase (PI3 kinase) (Franke et al. 1997). Because genistein is a protein-tyrosine kinase inhibitor but not reported to be a serine kinase inhibitor, Li et al. (2002) examined whether Akt is phosphorylated and, thereby, activated in PC3 cells under normal growth conditions and whether the phosphorylation of Akt is affected in genistein-treated cells. Western blot, immunoprecipitation, and kinase assays were conducted. They demonstrated that genistein inhibited the activation of Akt kinase activity, which may result in the inhibition of survival signals and thereby induce the apoptotic processes.

Nuclear factor kappaB (NF-kappaB) signaling pathway is one of cell signaling pathways that plays an important role in cell proliferation. The NF-kappaB family, one of the transcription factors, is composed of five proteins—RelA (p65), RelB, Rel, NF-kappaB1 (p50), and NF-kappaB2 (p52)—each of which may form homo- or heterodimers (Verma et al. 1995). Chemotherapeutic agents are known to induce NF-kappaB activity in tumor cells, resulting in lower cell killing and drug resistance. In contrast, genistein has been shown to inhibit the activity of NF-kappaB and the growth of various cancer cells without causing systemic toxicity. Li et al. (2005) reported inactivation of nuclear factor-kappaB by soy isoflavone genistein contributed to increase apoptosis induced by chemotherapeutic agents in human cancer cells. They investigated whether the inactivation of NF-kappaB by genistein
before treatment of various cancer cells with chemotherapeutic agents could lead to better tumor cell killing as tested by animal studies and by in vitro studies using gene transfections. PC-3 (prostate), MDA-MB-231 (breast), H460 (lung), and BxPC-3 (pancreas) cancer cells were pretreated with 15 to 30 micromol/L genistein for 24 hours and then exposed to low doses of chemotherapeutic agents for an additional 48 to 72 hours. The animal experiments of p65 cDNA transfection and p65 small interfering RNA studies clearly showed that a specific target (NF-kappaB) was affected in vivo. In in vitro studies, they found that 15 to 30 micromol/L genistein combined with 100 to 500 nmol/L cisplatin, 0.5 to 2 nmol/L docetaxel, or 50ng/mL doxorubicin resulted in significantly greater inhibition of cell growth and induction of apoptosis compared with either agent alone. Moreover, the NF-kappaB activity was significantly increased within 2 hours of cisplatin and docetaxel treatment and the NF-kappaB inducing activity of these agents was completely abrogated in cells pretreated with genistein were found. Collectively, these results clearly suggested that genistein pretreatment inactivates NF-kappaB and may contribute to increased growth inhibition and apoptosis induced by cisplatin, docetaxel, and doxorubicin in prostate, breast, lung, and pancreatic cancer cells. Theses results warranted carefully designed clinical studies investigating the combination of soy isoflavones and commonly used chemotherapeutic agents for the treatment of human cancers.

The effects of dietary flavonoids on cytochrome P450 (CYP) enzymes related to carcinogen metabolism also is related to cancer development. The flavonoid effects on CYP enzymes involved in the activation of procarcinogens (phase I monooxygenase enzymes) and phase II enzymes, largely responsible for the detoxification of carcinogens. A number of naturally occurring flavonoids have been shown to modulate the CYP450 system, including the induction of specific CYP isozymes, and the activation or inhibition of these enzymes (Conney 2003).

CYP enzymes (phase I monooxygenase enzymes) are widely known for their role in the metabolism of drugs and other foreign compounds. Thus, modulation of this enzyme system can influence the metabolism of xenobiotics, producing effects of pharmacological and toxicological importance. Many carcinogens are metabolized by CYP enzymes to either biologically inactive metabolites or to chemically reactive electrophilic
metabolites that covalently bind to DNA producing carcinogenicity. Therefore, induction of either phase I or phase II enzymes can result in increased detoxification of carcinogens (Conney 2003). CYP19, also known as aromatase (11β-hydroxysteroid dehydrogenase), is one member of the cytochrome P450 enzyme superfamily. This enzyme represents a crucial enzyme of estrogen biosynthesis. Increased expression of aromatase has been observed in breast cancer tissue (Miller et al. 1990 & Zhou et al. 1996). Flavones (chrysin, baicalein, and galangin), flavanone (naringenin) and isoflavones (biochanin A) inhibited the activity of human aromatase in Chinese hamster ovary (CHO) cells, thus decreasing estrogen biosynthesis and circulating estrogen levels (Kao et al. 1998). Isoflavone, equol was potent inhibitor of the ovarian aromatase activity in rainbow trout and also showed inhibitory effect on human placental aromatase (Pelissero et al. 1996).

Kao et al. (1998) investigated flavone and isoflavone phytoestrogens whether to act as competitive inhibitors of cytochrome P450 aromatase with respect to the androgen substrate in site-directed mutagenesis study. Aromatase is the enzyme that converts androgen to estrogen; therefore, these plant chemicals are thought to be capable of modifying the estrogen level in women. In this study, the inhibition profiles of four flavones [chrysin (5, 7-dihydroxyflavone), 7,8-dihydroxyflavone, baicalein (5,6,7-trihydroxyflavone), and galangin (3,5,7-trihydroxyflavone)], two isoflavones [genistein (4,5,7-trihydroxyisoflavone) and biochanin A (5,7-dihydroxy-4-methoxyisoflavone)], one flavanone [naringenin (4, 5,7-trihydroxyflavanone)], and one naphthoflavone (alpha-naphthoflavone) on the wild-type and six human aromatase mutants (I133Y, P308F, D309A, T310S, I395F, and I474Y) were determined. In combination with computer modeling, the binding characteristics and the structure requirement for flavone and isoflavone phytoestrogens to inhibit human aromatase were obtained. These compounds were found to bind to the active site of aromatase in an orientation in which rings A and C mimic rings D and C of the androgen substrate, respectively.

Activation of phase II detoxifying enzymes, such as UDP-glucuronyl transferase (UGT), glutathione S-transferase (GST), and NAD(P)H, quinone oxidoreductase (QR) by flavonoids results in the detoxification of carcinogens and represents one mechanism of their anticarcinogenic effects (Chen at al. 2004). A significant negative correlation was
demonstrated between GST enzyme activity and tumor incidence in the mucosa along the human gastrointestinal tract, suggesting the importance of GSTs in cancer prevention (Peters et al. 1993). GSTs have a considerably important role in the detoxification of carcinogens (Hayes et al. 1995). Genistein and daidzein were found to increase QR activities in Hepa1c1c7 cells (Yannai et al. 1998).

F. Bioavailability of isoflavones

Bioavailability is defined as the proportion of a compound that appears in plasma over time and the proportion excreted in the urine and feces compared to the amount ingested, when the compound is administered orally. From a toxicological perspective then, bioavailability is a measure of the potential for entry of a chemical into sites of action and implies movement of a chemical into the systemic circulation because this is a good indication of the biologically effective dose. Casarett and Doull (2001) define bioavailability as the fraction of the oral dose that is absorbed. That means that bioavailability equals to mass of chemical absorbed compared with mass of chemical administered. Food matrices in which isoflavones are found and chemical differences that separate daidzein, genistein and glycitein have also been studied with respect to possible variations in isoflavone. Mostly, bioavailability of isoflavones focuses on plasma and urinary kinetics as well as microbial metabolism (Birt et al. 2001; Xu et al 2002).

The area of isoflavone bioavailability is complex and wide range of variability in response to dietary isoflavones. Many human studies and animal models have been established to study bioavailability of isoflavones. Some focused on understanding how isoflavones are metabolized in the body including plasma kinetics as well as urinary and fecal excretion. Hendrich and co-workers indicated that a variety of factors affected bioavailability of soybean isoflavones.

Xu et al. (1994) showed that daidzein is a more bioavailable soymilk isoflavone than genistein in adult women while glycitein was not measured. Xu et al. (1994) fed 12 women 3 doses of isoflavones (2.5, 4.8 and 7.4 µmol/kg BW from soymilk powder; 44% genistein and 56% daidzein). Plasma levels of daidzein and genistein were similar to each other at time 6.5
and 24h following ingestion at all 3 doses. Urinary recovery of daidzein and genistein were
21% and 9% of the ingested dose, respectively and fecal excretion was 1-2% ingested dose.
The amounts of isoflavone recovered in the urine were proportional to the ingested dose
(dose-response between amounts fed and those recovered in the urine). Total isoflavone
excreted in the urine was significantly higher in 0-12 h compared to 12-24 h following
ingestion by a factor of 2 to 3, meaning that isoflavone absorption and metabolism is a fast
process, occurring mostly in the first 12 h after ingestion and disappearing almost completely
after 24 h. Based on urinary excretion, Xu et al. (1994) concluded that daidzein was more
bioavailable than genistein. Soybean milk isoflavones seem to be 85% degraded in the
intestine, daidzein may be sufficient to exert some health-protective effects.

Xu et al. (1995) found that the efficiency of absorption of soymilk isoflavones
varied from 13 to 35%, depending on individual gut microflora. Xu et al. (1995) performed a
similar study to that of Xu et al. (1994) with three doses of isoflavones fed (3.4, 6.9 and 10.3
µmol isoflavones/kg body weight; n=7 women), but looked at the individual results and
found that 2 subjects had significantly higher fecal excretion of isoflavones compare to the 5
others (about 6% vs. 0.6% of the ingested dose, respectively and regardless of the dose fed).
48 h urinary recovery was 16±4% and 10±4% of the ingested dose for daidzein and genistein,
respectively in the 5 subjects with low fecal excretion. The two subjects with high fecal
excretion had 32±5% and 37±6% urinary recovery expressed as a % ingested dose. As for
plasma, subjects with high fecal and urinary excretion had plasma isoflavone level 2.5-fold
higher compare to subjects who were low isoflavone excreters. This study established the
principles of phenotypes of isoflavone bioavailability, in which people can be grouped as
high apparent absorbers (high urinary, plasma and fecal isoflavone contents) or low apparent
absorbers. Moreover, high apparent absorbers do not seem to have as much gut microbial
activity degrading isoflavones, because they are excreted intact and in greater level in the
feces those subjects. The role of the gut microflora in determining the extent of isoflavone
bioavailability became then a factor that could not be overlooked. These two studies
performed by Xu et al. (1994 & 1995) did not consider bioavailability of glycinein, a minor,
but still important soy isoflavones.
Zhang et al. (1999; erratum, 2001) indicated urinary daidzein excretion was the greatest among that of three isoflavones in moderate fecal degraders and glycitein bioavailability was similar to daidzein in humans. On the other hand, comparing with this present study with two other studies in our laboratory, glycitein bioavailability was greater than daidzein and genistein excretion in hamsters.

Hendrich et al. (2001) found that plasma daidzein and genistein concentration was negatively correlated with in vitro fecal daidzein and genistein disappearance rate constant ($r = -0.74$, $P = 0.04$; $r = -0.88$, $P = 0.01$, respectively), supporting an important role for gut microbial activity in isoflavone bioavailability. Recently Zheng et al. (2003) demonstrated a relationship between isoflavone disappearance phenotypes and GTT and suggested that gut microorganisms may affect GTT. Among 35 Chinese vs. 33 Caucasian women, Chinese subjects who were low degraders of genistein had threefold greater bioavailability of genistein than Chinese high degraders. The Chinese who were low isoflavone degraders had the average GTT of 40 h vs. 65 h for Chinese high degraders. Caucasian subjects, regardless of isoflavone degradation phenotypes, had GTT > 80 h, and less apparent isoflavone absorption than did the Chinese subjects who were low degraders of isoflavones. GTT may be a crucial determinant of human differences in isoflavone bioavailability.

In an animal study, King (1998) compared the bioavailability of daidzein and genistein in male Wistar rats fed a soy extract providing 74 µmol genistein and 77 µmol daidzein/kg body weight. 48h urinary excretion of daidzein and genistein were 17.4±1.2% and 11.9±1.1% of the ingested dose, respectively, while fecal excretion was 2.3±0.5% and 3.4±0.4%, respectively. As observed in humans (Xu et al. 1994 & 1995; Setchell et al. 2003), daidzein was more bioavailable than genistein and the range of urinary excretion is comparable to human data. Therefore, not only are rats an interesting model to study bioavailability, but also the gut microbial metabolism of humans and rats may share some similarities in degrading or metabolizing isoflavones.

Using isolated rat small intestine as an *ex-vivo* animal model, Andlauer et al. (2000b), studied the absorption rate and biotransformation of isoflavones daidzin and genistin derived from pre-digested tofu (1184 nmol genistein expressed as aglycone/g tofu and 572 nmol daidzein expressed as aglycone/g tofu). Tofu also contained small amounts of
malonyl-isoflavone and isoflavone aglycone. Of these compounds, 8% genistein and 8.9% daidzein appeared at the vascular side, either as aglycone, glucuronide and glucoside. A 3 and 2-fold increase in the aglycone genistein and daidzein, respectively was found in the luminal side. However, no sulfate conjugate were found in this experiment. In another experiment with only genistin applied in an isolated rat small intestine, Andlauer et al. (2000b) found an absorption rate of about 15%, with glucuronides>aglycones>glucosides found on the vascular side. These two studies showed that daidzein and genistein absorption rate are similar and that biotransformation occur during the absorption process. However, the absence of sulfate conjugate indicated that rat may not be able to produce this conjugate, or this process is restricted exclusively to the liver. The presence of isoflavone glucoside on the vascular side was surprising and lead to the conclusion that rat may be capable of absorbing isoflavone glucoside without cleavage. On the other hand, the very small proportion of isoflavone glucosides found may have been related to a leakage in this ex-vivo system.

Lee et al. (2005) fed pure synthetic daidzein, genistein, or glycitein to female Golden Syrian hamsters (11-12 weeks of age, 10 hamsters/treatment) for 4 weeks and reported the urinary isoflavone excretion was glycitein> daidzein>genistein (32.2%>4.6%>2.2%). Meanwhile, Renouf et al. (2006) showed similar data in feeding either 1.18 or 1.77 mmol total isoflavon es/kg diet to 19 one-year old hamsters for 10 d in both males and females. These results indicated that the microbes in hamsters seem to differ from those in humans. They reported similar isoflavone urinary excretion and gut microbial degradation patterns compare to humans in Golden Syrian hamster fecal and cecal microbial degradation of isoflavones. Daidzein excretion was significantly greater than glycitein and genistein excretion in urine and female urinary excretion was significantly greater than male. Therefore, this study established Golden Syrian hamsters as a potential animal model to be used instead of humans to study some chronic diseases which could not be induced in humans, such as cancer.

Renouf et al. (2005) conducted two separate studies, one focusing on fecal (study #1, n=20/sex) and the other on cecal (study #2, n=10/sex) microbial degradation of isoflavones in Golden Syrian hamsters. They reported that urinary excretion was significantly lower by 2-4 fold in males compared to females in both studies. In addition, females from study #1 had
significantly greater urinary excretion levels of daidzein (44.2 ± 13.7% vs. 29.6 ± 13.4%),
glycitein (31.4 ± 11.2% vs. 18.2 ± 8.0%) and genistein (26.7 ± 11.5% vs. 15.8 ± 9.4%
ingested dose) compared to cecal study #2, respectively. Fecal isoflavone excretion was not
significantly different between sexes or isoflavones (study #1) and showed extremely low
levels of excretion (<0.5% ingested dose). *In vitro* fecal degradation rates from study #1
showed low degradation levels and no significant correlation between urinary and fecal
isoflavone excretion. The most importance finding was that cecal isoflavone degradation
rates (study #2) were much higher than fecal isoflavone degradation rates (study #1) and
were statistical correlated with urinary excretion of daidzein (R=0.90; p=0.01) and genistein
(R=0.93; p=0.004). They concluded that Golden Syrian hamsters displayed similar patterns
of bioavailability of isoflavones compared to humans in terms of apparent absorption, urinary
excretion and gut microbial degradation.

Recently, Renouf et al. (2005) showed *Bacteroides ovatus, Bacteroides acidifaciens, Eubacterium ramulus, Clostridium orbiscindens* and *Tannerella forsythensis* were the major
human gut microbial species that degraded isoflavones and established that Golden Syrian
hamsters may be good models to study bioavailability of isoflavones and their possible health
promoting effects. Renouf et al. (2005) identified high fecal isoflavone degradation rate to
coincide with distinct fecal bacterial species. Fresh feces from 33 healthy adult subjects (20
men, 13 women) were incubated anaerobically with isoflavones to assess degradation rates
using HPLC. Fecal DNA was extracted, bacterial 16S rDNA sequences amplified by
polymerase chain reaction (PCR) and separated by denaturing gradient gel electrophoresis
(DGGE). Cluster analysis identified high and low degraders of daidzein, genistein and
glycitein. DGGE analysis showed that high genistein degraders (n=4; fecal degradation rate
1.47 ± 0.14h\(^{-1}\)) shared 5 bands of greater intensity than found in feces of low genistein
degraders (n=4; fecal degradation rate 0.146 ± 0.034 h\(^{-1}\)) high glycitein degraders (n=4; 0.574 ± 0.299h\(^{-1}\)) also shared 5 bands of greater intensity than found in feces of low glycitein
degraders (n=4; 0.146 ± 0.034 h\(^{-1}\)). They also showed concordance with known species from
the *Bacteroides* and *Prevotella genus* as well as the *Clostridiales* order using sequencing of
16S rDNA from the bands of interest. After developing two in vitro systems, one rich (rumen
fluid based brain heart infusion media) and one poor in nutrients (feces incubated overnight
in brain heart infusion media), they identified *Bacteroides ovatus, Bacteroides acidifaciens, Eubacterium ramulus, Clostridium orbiscindens* and *Tannerella forsythensis* as the major human gut microbial species that degraded isoflavones under both nutrient rich and poor conditions, thus these species may be the most significant ones in degrading isoflavone in the human gut. They also concluded that bacterial species shared by both high and low degraders with greater amounts in high degraders may be predictors of gut microbial degradation and overall bioavailability of isoflavones. Secondary species that may be specific to each individual fecal isoflavone degradation rate may be of importance for assessing microbial activity and will deserve further attention.

In summary, bioavailability of isoflavones on plasma kinetics, urinary and fecal excretion is now well understood. Isoflavone absorption is a fast process with a maximum plasma peak occurring within 12 h after ingestion. Only traces of isoflavone are excreted in the urine after 48 h; most of the isoflavones ingested are being excreted within 24-48h. From animal and human studies obtained until now, we believed that potential animal models could be used to study isoflavone bioavailability. At meantime, in both humans and animals, urinary together with fecal excretions are less to 100% of the ingested dose and most of the studies have found a cumulative urinary and fecal excretion less 60%, suggesting that a great amount of isoflavones disappear in metabolism. There is now good evidence that gut microorganisms play an important role in the process of bioavailability. We now know that isoflavone bioavailability varies greatly among individuals in terms of the amount of isoflavone degradation microbial species which are relatively stable within an individual. Interindividual variability in isoflavone bioavailability needs more investigation.

**G. Isoflavone toxicity**

Together with the safety of soy isoflavone products use, the toxicity of isoflavone is also considered. Some toxicological findings in relation to endocrine disrupters including reproductive effects, infertility and thyroid disease due to dietary intake of isoflavones had been observed in animals. Hughes et al. (1991a&b) indicated that acute and subacute administration of the phytoestrogen genistein blocked the gonadotropin-releasing hormone-
induced rise of luteinizing hormone in rats. The prenatal exposure to genistein in the environment could influence sexual differentiation in pregnant rats (Levy et al. 1995).

Yellayi et al. (2002) conducted an ovariectomized C57BL/6 mouse experiment with subcutaneous injection of genistein (2, 8, 20, 80, 200 mg/kg body weight) and indicated that isoflavones may have a negative impact on thymus. They found that thymus weight was decreased compared to control in a dose response manner with the 4 highest doses in the 8, 20, 80 and 200 mg/kg per day genistein groups being significantly different from the control (17 to 78%) which had a subsequent negative effect on immune function, including keyhole limpet haemocyanin (KLH)-specific antibody titers, CD4⁺CD8⁻ T cells as a percentage of splenic lymphocytes and relative percentages of lymphocytes. To compare serum genistein levels in this injected mice with those reported for soy-fed human infants which plasma isoflavone levels of 2.0–6.6 μmol/liter and genistein levels of 1.5–4.4 μmol/liter have been reported in 4-month-old human infants (Setchell et al. 1997), serum genistein in mice dosed with 8 mg/kg were comparable to those in soy-fed infants at 0.5, 1, and 2 h; and the 20 mg/kg dose produced serum genistein levels 2- to 3-fold greater than maximal levels reported in soy-fed infants. Because the peak levels of maximal serum genistein in soy-fed infants were similar to that in mice after 8 mg/kg genistein injection, the possibility that serum genistein concentrations found in soy-fed infants may be capable of producing thymic and immune abnormalities. However, it is still problematic to compare effects of specific doses of genistein on mice and humans because ingestion of a compound normally tends to lower blood levels than injection and mice may be different with humans in genistein metabolism.

Hughes et al. (1991a) compared tonic luteinizing hormone (LH) secretion and gonadotropin releasing hormone-induced LH responses following high intravenous (iv) dose pretreatments with genistein or estradiol and determined effects of genistein or estradiol pretreatments on progesterone (P)-induced secretion of LH. Mature Charles River CD rats were ovariectomized and intraatrial cannulae were placed after 2 to 5 weeks later. Serial blood samples were drawn and LH was measured. In three experiments, genistein or estradiol was administered acutely by gavage or iv, and subcutaneously (sc) oil 3 days prior to cannulation and sampling. Acute po administration of vehicle or genistein (0.1, 1.0, and 10 mg/kg BW) had no effect on tonic LH while estradiol suppressed LH at all doses (0.1, 1.0,
and 10 mg/kg BW). Acute iv administration of vehicle and higher doses of genistein (1 and 10 mg/kg BW) had no effect on tonic LH, while the lowest dose genistein (0.1 mg/kg BW) and all doses of estradiol (0.1, 1, and 10 mg/kg BW) suppressed tonic LH. They concluded that acute intravenous administration of the phytoestrogen genistein blocked the gonadotropin-releasing hormone-induced rise of luteinizing hormone in ovariectomized rats.

In the second study, Hughes et al. (1991b) determined whether subacute administration of genistein or the mycoestrogens zearalenone and zearalenol would affect GnRH-induced or progesterone-induced LH secretion in ovariectomized rats. The same animal species and similar feeding doses were used in this study. They found that subacute administration of genistein, zearalenone, or zearalenol did not inhibit tonic LH secretion in the ovariectomized rat and confirmed genistein and zearalenol did block GnRH-induced LH secretion. In Hughes et al. studies, because they did not measure the serum genistein concentrations, we can not compare with humans, at least soy-fed human infants as mentioned above.

Furthermore, Levy et al. (1995) determined whether exposure to genistein during critical periods of development could alter morphologic and physiologic markers of sexual differentiation. They characterized the effects of in utero treatment with genistein on birth weight and anogenital distance (AGD) at birth, gonadotropin releasing hormone (GnRH) stimulated luteinizing hormone (LH) secretion, volume of the sexually dimorphic nucleus in the preoptic area of the hypothalamus (SDN-POA), puberty onset, and vaginal cyclicity in pregnant Charles River CD rats. The rats were injected subcutaneously (sc) daily on gestation day 16-20 with either 25mg genistein (G25), 5mg genistein (G5), 5μg diethylstilbestrol (DES), 50μg estradiol benzoate (E), or corn oil alone for controls. Birth weights and anogenital distance were taken and exposed progeny were subsequently used in two experiments. In Experiment 1 intra-atrial catheters were placed in adult castrated rats, GnRH was given iv, serial blood samples were drawn and sera were assayed for LH by radioimmunoassay (RIA). Brains obtained by subsequent decapitation were saved for histology. In Experiment 2, females were monitored for timing of vaginal opening as a marker of puberty onset, and vaginal smears were taken to monitor cyclicity. G25-treated females and DES-and E-treated animals of both sexes had decreased weights at birth.
compared with controls. G5- and E-treated animals of both sexes and DES males had smaller AGD than controls. They found no significant differences in pituitary responsiveness to GnRH among treatment groups. G5-treated females had delayed puberty onset, and DES-treated females had atypical vaginal cycles in comparison with controls. The results concluded that prenatal exposure to genistein in the environment could influence sexual differentiation. Genistein did mimic other estrogens' effects on AGD and birth weight and had a unique influence on puberty onset. However, they also can not determine the relevance to humans because genistein injection is different with human ingestion of this compound.

Gametogenesis and reproductive efficiency of trout were decreased in feeding genistein-containing diets to rainbow trout (*Oncorhynchus mykiss*) for one year (Bennetau-Pelissero et al. 2001). In this study, they fed diets including 0, 500, or 1000 ppm genistein to groups of rainbow trout undergoing their first gametogenesis and until spawning. They found that growth performance of rainbow trout was not affected by dietary treatments. Plasma cholesterol levels were equivalent between groups. In males, a decrease in testosterone levels was observed. There was a significantly reduced 17alpha, 20beta-(OH)(2)-progesterone. Testicular development was accelerated in genistein-fed fish, and sperm motility and concentration were decreased in a dose-dependent manner at spawning. In females, a significant increase in plasma of vitellogenin (VTG) occurred only at the beginning and at the end of oogenesis. Testosterone levels were decreased at the beginning of oogenesis. Both betaFSH and betaLH were decreased by genistein, whereas spawning was delayed only in females fed the diet with 500 ppm of genistein. Gamete quality was impaired only in this group, as underlined by a lower percentage of ovulating females, a lower fertilization rate, and a lower viability of fry. These results may be explained by the agonistic/antagonistic effect of genistein on estrogen functions.

In human studies, there are very limited data on sexual and reproductive development or fertility related to isoflavone toxicity. Strom et al. (2001) investigated the association between infant exposure to soy formula and health in young adulthood, with an emphasis on reproductive health. The retrospective cohort study conducted from 248 adults aged 20 to 34 years were fed soy formula and 563 were fed cow milk formula during infancy. Self-reported pubertal maturation, menstrual and reproductive history, height and usual
weight, and current health, compared based on type of formula exposure during infancy. They did not find statistically significant differences between groups in either women or men for more than 30 outcomes except slightly longer duration of menstrual bleeding and discomfort with menstruation in women who had been fed soy formula. They concluded that exposure to soy formula did not appear to lead to different general health or reproductive outcomes than exposure to cow milk formula.

In a subsequent review, Mendez et al. (2002) conducted to evaluate various measures of infant health and development in clinical studies comparing modern soy-based formulae with other diets. They concluded that modern soy-based formulae supported normal growth and nutritional status in healthy term infants and did not provide evidence of meaningful differences in timing of maturation, sexual development or fertility in adolescents or adults. In another study, Takimoto et al. (2003) evaluated toxicity from pilot data on in vivo effects on protein-tyrosine phosphorylation. They conducted the pharmacokinetic parameters of two different preparations of unconjugated soy isoflavones, PTI G-2535 and PTI G-4660 (which contain 43% and 90% genistein, respectively) in human subjects with cancer. Four group patients were given single doses of each preparation. They received oral genistein at 2, 4, or 8 mg/kg sequentially. Pharmacokinetic sampling was performed after each dose, and tyrosine phosphorylation was measured in proteins extracted from peripheral blood mononuclear cells. Only one of 13 patients treated developed a treatment-related rash. No other toxicities were observed.

However, Bloedon et al. (2002) found a few symptom of nausea, pedal edema, and breast tenderness related to isoflavone toxicity in excessive intakes. They performed safety and pharmacokinetic studies of purified unconjugated isoflavone preparations containing genistein, daidzein, and glycitein in postmenopausal women. Twenty-four healthy postmenopausal women ingested a single dose of 1 of 2 purified isoflavone preparations. Toxicity studies were performed 24 h and 3, 6, 14, and 30 d after isoflavone administration. They observed a 7% decrease in systolic and diastolic blood pressure and a 32% decrease in the neutrophil count 24 h after treatment with formulation. No other toxicities were found from measurements of clinical signs. The terminal plasma half-lives for free genistein, daidzein, and glycitein averaged 3.8, 7.7, and 3.4 h, respectively. The terminal pseudo half-
lives for total genistein and total daidzein in plasma averaged 10.1 and 10.8 h, respectively. The estimated bioavailabilities of both total genistein and total daidzein from each of the 2 formulations were not significantly different. They indicated the pharmacokinetic data suggested that chronic dosing at 12–24-h intervals would not lead to progressive accumulation of these isoflavones.

In conclusion, with regard to isoflavone toxicity, although the studies described above demonstrated that isoflavone interfered with endocrine and reproductive processes in animals, very few toxicities have been found in human. There is no doubt that the dosage, route and timing of exposure during toxicological studies are very important factors in modifying end points associated with hormone disruption. However, most of the toxicological studies done were using injections not oral exposures; it is difficult to extrapolate those toxic doses to humans based on injection doses, thus more oral dose response of specific toxic doses for isoflavone in animal models are needed.

H. References


Bennetau-Pelissbero C, Breton B, Bennetau B, Corraze G, Le Menn F, Davail-Cuisset B, Helou C and Kaushik SJ. 2001 Effect of genistein-enriched diets on the endocrine process of


Renouf M. 2005 Ph.D. Dissertation, Iowa State University Library, Ames, IA


Xu X, Wang HJ, Murphy PA, Cook L & Hendrich S. 1994 Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J. Nutr. 124:825-832.


Zhang Y. 2000 Dissertation, Iowa State University Library, Ames, IA.


Zheng Y, Lee S-O, Verbruggen MA, Murphy PA, Hendrich S. 2004 The apparent absorptions of isoflavone glucosides and aglucons are similar in women and are increased by rapid gut transit time and low fecal isoflavone degradation. J. Nutr. 134: 2534-2539.


HIGH URINARY ISOFLAVONE EXCRETION PHENOTYPE DECREASES PLASMA CHOLESTEROL IN GOLDEN SYRIAN HAMSTERS FED SOY PROTEIN

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Abstract

Apparent absorption of isoflavones varies greatly among individuals, but is relatively stable within an individual. We hypothesized that high urinary isoflavone excreters would show less plasma non-HDL cholesterol than low isoflavone excreters after soy protein feeding. Fifty Golden Syrian hamsters were fed a high fat/casein diet (n = 10) or a high fat/soy protein diet (n = 40) for 4 wk. Two distinct urinary isoflavone excretion phenotypes were identified based upon HPLC analysis of urinary glycitein using a pairwise correlation plots analysis, or based upon total urinary isoflavone using a hierarchical cluster test. High isoflavone excreters showed significantly greater urinary isoflavones (p<0.05) than did low isoflavone excreters at w 1 and 4. The low urinary glycitein excretion phenotype was more stable than the high urinary glycitein excretion phenotype by McNemar’s test. High urinary isoflavone excreters had significantly less non-HDL cholesterol than did the low isoflavone excreters or casein-fed controls (p < 0.05). Plasma total and non-HDL cholesterol were significantly negatively correlated with urinary daidzein, glycitein and total isoflavone excretion (r between -0.45 and -0.58, p<0.05). Urinary isoflavone excretion phenotypes predicted the cholesterol-lowering efficacy of soy protein. Isoflavone absorbability, probably due to gut microbial ecology, is an important controllable variable in studies of effects of soy protein on blood lipids.

KEY WORDS: · isoflavone excretion, cholesterol, soy protein, hamster
Introduction

Increased intake of soy protein lowers blood cholesterol, as confirmed by a recent meta-analysis of 22 randomized human trials (1). The role of isoflavones in this effect of soy protein was determined to be negligible. But, moderately hypercholesterolemic men and women who ate 62 mg total isoflavones/d in soy protein had decreased total and LDL cholesterol (LDL-C) compared with casein-fed controls (2). LDL-C was less in premenopausal women who ate soy protein high in isoflavones diet compared with controls during the midfollicular and periovulatory phases of the menstrual cycle (3). These same soy foods and doses fed to postmenopausal women in a crossover study showed that only the greatest isoflavone intake caused significantly less LDL-C compared with controls (4). A minimal dose of 96 mg isoflavone/d was required for cholesterol-lowering in a meta-analysis of 8 human studies with no, low and high-isoflavone containing soy protein treatments (5).

The hamster model has also provided contrasting results across studies of isoflavones. Song et al. (2003) (6) fed male and female Golden Syrian hamsters (n=10/sex/group; 6-8 weeks of age). Total cholesterol and non-HDL cholesterol were significantly less in hamsters fed soy protein with or without isoflavones or fed 1.3 mmol daidzein/kg diet in a casein-based diet (~ content of total isoflavones in a 25% soy protein diet), compared with a high fat/casein control diet, illustrating similar efficacy for cholesterol lowering by soy protein and daidzein. The cholesterol-lowering effects of isoflavones increased with increasing isoflavone doses in six mo-old ovariectomized Golden Syrian hamsters (7). In another study, plasma isoflavones were positively correlated with plasma LDL+VLDL-C in female hamsters, and males fed isoflavones in a casein/lactalbumin diet showed no cholesterol-lowering compared with the control diet, but isoflavones within a soy diet increased cholesterol-lowering (8). The addition of lactalbumin to casein seemed to alter the effects of isoflavones on blood lipids.

Interindividual variability in apparent absorption of isoflavones may account for the observed negligible effects of isoflavones in meta-analyses of soy protein feeding trials (1). In a randomized crossover design, seven women fed various doses of soy milk showed distinct patterns of urinary isoflavone excretion: two women had significantly greater excretion of isoflavones than did the other five women (9). Human fecal isoflavone
disappearance phenotypes were observed in vitro (10). The phenotypes were stable in 12 of 15 subjects reexamined after 10 months, and fecal isoflavone disappearance was strongly inversely correlated with plasma isoflavones in 8 men fed a single isoflavone dose (10). Gut microbes seemed played an important role in the metabolism of isoflavones. Zheng et al. (11) identified fecal isoflavone disappearance phenotypes among 35 Chinese and 33 Caucasian women. The high urinary isoflavone excretion phenotype affected urinary excretion of genistein when accompanied by relatively rapid gut transit time. Low fecal isoflavone degradation rate in 12 women fed soy for 7 days was related to ~50% greater apparent absorption of isoflavones compared with 13 women with high fecal isoflavone degradation rates (12).

Golden Syrian hamsters may be good models to study the role of isoflavone bioavailability in the cholesterol-lowering efficacy of isoflavones because greater isoflavone bioavailability was associated with increased cholesterol-lowering by these compounds when isoflavones were fed in a casein-based diet (13). Interindividual variability in isoflavone bioavailability, which we think is a significant issue in understanding the health effects of these compounds in humans, seemed to be greater among female than among male hamsters (13), making female hamsters a particularly useful model for humans. The objectives of this study were to identify in a hamster model urinary isoflavone excretion phenotypes using statistical methods and to test the hypothesis that a high excreter isoflavone bioavailability phenotype was associated with cholesterol-lowering efficacy of dietary soy protein.

**Materials and Methods**

**Diets**

In the experimental period, hamsters were fed a hyperlipidemic casein-based control diet high in saturated fatty acids (14) or a treatment diet with soy protein (Supro 670®, Protein Technologies International, St. Louis, MO) substituted for casein. The isoflavone content of soy protein was analyzed by HPLC linear gradient with UV detection (15). The soy protein contained 405 µg of daidzein, 647 µg of genistein, and 72 µg of glycitein/g. The saponin fractions of soy protein were analyzed by HPLC (16); the soy diet contained 1.2 mg
saponin/g. Experimental diets were prepared and stored at 4°C. Food intake was measured weekly and over 2-3 consecutive days in metabolic cages.

**Animals and Sample Collection**

Fifty 8 wk old female golden Syrian hamsters, 118-128 g were obtained from Charles River Breeding Laboratories (St. Constant, Canada). Hamsters were fed AIN 93 M diet for 2 mo (17), then assigned to two groups randomly but to achieve similar mean body weight/group: 10 hamsters fed casein diet and 40 hamsters fed soy protein diet. The hamsters had free access to food and drinking water in a temperature-controlled room (23°C) with a 12 h light/dark cycle during the 4-week experimental period. Clinical observation and body weight measurements were conducted once a week. Urinary and fecal samples were collected over 24 h at the end of weeks 1 and 4 when hamsters were put in metabolic cages individually for 2-3 d. At the end of the feeding period, diets were withdrawn from hamsters 14-16 h before they were euthanized under CO₂. Blood samples were collected by cardiac puncture in EDTA tubes and centrifuged at 5000g for 10 min, 4°C. Plasma samples were then frozen at -20°C until analysis. The animal experimental protocol was approved by the Iowa State University Animal Use Committee.

**Plasma Lipid Analysis**

Plasma total cholesterol, HDL cholesterol, and triglyceride concentrations were measured with Infinity™ Cholesterol and Triglycerides Reagent (Thermo DMA, Inc. Louisville, CO) and HDL Cholesterol Reagent (PTA/MgCl₂) Diagnostics kits (Sigma Diagnostics, Inc., St. Louis, MO). Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol and represented LDL+ID+VLDL cholesterol.

**Chemicals**

Daidzein (4′,7-dihydroxyisoflavone), genistein (4′,5,7-trihydroxyisoflavone), glycine (4′,7-dihydroxy-6-methoxyisoflavone), and THB (2,4,4′-trihydroxybenzoin) were synthesized (18). Milli-Q (Millipore Co., Bedford, MA) HPLC grade water was used. Other HPLC solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA).
Analytical Methods

Fecal and urinary sample preparation and HPLC analysis

Isoflavone extraction from urine and fecal samples was performed as described previously (19). Fecal saponin metabolite analysis in wk 4 samples was as described (16, 20).

Statistical Analysis

A pairwise correlation plots analysis and hierarchical clustering test were conducted to classify isoflavone excretion phenotypes. One-way ANOVA followed by t-tests determined the differences in urinary and fecal isoflavones between two isoflavone excretion phenotypes at weeks 1 and 4, differences in excretion of daidzein, genistein and glycine within excretion phenotype, differences in the plasma lipid levels between the soy protein-fed and control groups, between isoflavone excretion phenotypes, and between isoflavone excretion phenotypes and the controls. McNemar’s test (21) was used to verify high and low isoflavone phenotype stability. Intraclass Correlation Analysis (22) was done to verify individual phenotype stability. Pearson correlation analysis determined the correlations between urinary and fecal isoflavone excretion and between isoflavone excretion and plasma lipids. All results were reported as mean ± SEM. All differences were considered significant at $P < 0.05$. Statistical analysis was performed with R software (23) and SAS (SAS Institute, version 8.1; 1998, Cary, NC).

Results

Identification of Urinary Isoflavone Excretion Phenotypes

Food intakes and body weights did not differ between the groups at w 1 and 4. For casein-fed hamsters, food intake was $7.7 \pm 0.5$ g/d (wk 1) and $7.1 \pm 0.6$ g/d (wk 4), whereas soy protein-fed hamsters had food intakes of $7.1 \pm 0.6$ and $7.0 \pm 0.5$ g/d (means ± SEM) at wk 1 and 4, respectively. Body weights for both groups (means ± SEM) were $123 \pm 3$ g initially; casein-fed hamsters had body weights of $130 \pm 5$ g (wk 1) and $134 \pm 7$ g (wk 4),
whereas soy protein-fed hamsters had body weights of 128 ± 6 g (wk 1) and 131 ± 6 g (wk 4).

Urinary isoflavone excretion phenotypes were classified on pairwise correlation plots. The plot of the ranked values showed a gap in urinary glycinein excretion between individuals at wk 1 (Fig. 1). From these rankings, hamster 2 had the maximum excretion of all isoflavones. Hamster 8 remained among the least in urinary excretion of all isoflavones. The highlighted data for these 2 individuals indicates the consistency of apparent absorption for all isoflavones, and the extreme difference among individuals. The number of observations in the two categories were great enough for inference, thus the statistical analysis showed a natural cut-off for ‘high’ and ‘low’ urinary glycitein excreters (Fig.1).

Another method to classify urinary isoflavone excretion phenotypes was based on hierarchical clustering using a complete linkage method (Fig. 2), which ensured that all items in a cluster were within some maximum distance of each other. Only hamsters in two of the four groups were used in further studies (Group 3 – High isoflavone excreters, Group 2 – Low isoflavone excreters) because they displayed more consistent characteristics within a cluster than did the two other groups. Hamster 2 was removed from this analysis because it consistently formed its own ‘cluster’. Hamster 1 had missing values for the clustering variables (no HPLC data). The full clustering tree for 38 hamsters indicated 4 excretion phenotypic clusters (Fig.3). Bivariate plots showed each observation’s categorization with the respective phenotypic cluster number. We chose to compare Clusters 2 and 3 for the effect of isoflavone excretion on plasma lipids because Clusters 1 and 4 had no ‘universal’ ordering (i.e., individuals in Clusters 2 and 3 remained grouped for their excretion of each of the isoflavones and for total isoflavone excretion). We classified urinary isoflavone excretion phenotypes for wk 4 by the same methods.

**Influence of Urinary Isoflavone Excretion Phenotypes on Isoflavone and Saponin Excretion**

Glycitein excretion (as percentage of ingested dose) was significantly greater than daidzein and genistein excretion in urine within urinary excretion phenotype (p<0.05, Table 1, 2).
According to high and low urinary glycitein excretion phenotypes identified by a natural break point, high excreters excreted significantly more urinary daidzein, genistein, glycitein and total isoflavone (p<0.05) than did individuals of the low excretion phenotype at wk 1 and 4 (Table 1). No differences were found in fecal daidzein, genistein, and total isoflavone excretion between excretion phenotypes at wk 1 and 4.

According to total urinary isoflavone excretion at wk 1 (Fig. 2 and 3), high and low urinary excretion phenotypes were identified by a hierarchical cluster test. Individuals of the high excretion phenotype (cluster 3) excreted more urinary daidzein, genistein, glycitein and total isoflavone (p<0.05) than did those of the low urinary excretion phenotype (cluster 2) at wk 1 and 4 (Table 3).

No differences were found in high and low fecal saponin metabolite (20) excretion phenotypes in wk 4 according to glycitein and total isoflavone phenotypes (data not shown).

**Stability of Urinary Excretion Phenotypes over 4 Weeks**

McNemar’s test showed the stability of the urinary excretion phenotypes between wk 1 and 4 (Table 2, 3). Only 4 of 39 individuals switched their urinary glycitein excretion phenotypes between wk 1 and 4. Twenty-six hamsters remained low excreters and 1 hamster became a high excreter. The low urinary glycitein excretion phenotype was more stable than the high glycitein urinary excretion phenotype. Three of 11 hamsters of the high glycitein excreter phenotype became low excreters. By the other classification method, the low excretion phenotype (cluster 2) was more stable (2 individuals switched) than the high excretion phenotype (cluster 3) in which 3 individuals switched.

Individual glycitein excretion phenotype stability from wk 1 to 4 (Fig. 4) was investigated using Intraclass Correlation Analysis, defined as $\frac{\delta_B^2}{(\delta_B^2 + \delta^2)}$, which compared the variability between hamsters to the total variability in the data. The 95% confidence interval estimate for this ratio was [0, 0.89]. This interval was wide due to high variability, or low precision, in the estimate of this ratio. However, the variability of the values within a hamster can be seen as the length of the lines connecting the two measurements. In general, there was less change from wk 1 to 4 when a hamster had a relatively low original glycitein excretion phenotype.
Plasma Lipids and Urinary Isoflavone Excretion Phenotypes

Compared with the control diet, the soy protein diet significantly lessened plasma total cholesterol by 18% (p < 0.05), non-HDL cholesterol (non-HDL-C) by 30% (p<0.05) and non-HDL-C/HDL cholesterol (HDL-C) (p<0.05, Table 3). No significant differences in plasma HDL-C and triglyceride concentrations were found between groups. Plasma total cholesterol (by 26% v. 14%, p<0.05), non-HDL-C (by 51% v. 36%, respectively, p<0.05) and non- HDL-C/HDL-C (p<0.05) were significantly less in individuals of the high urinary glycine excretion phenotype (identified at wk 1 or 4), compared with individuals of the low glycine excretion phenotype (Table 3). Individuals of the low glycine excretion phenotype did not differ from hamsters fed the casein diet in plasma lipid status. Individuals of the high isoflavone excretion phenotype at either wk 1 or 4 had significantly less plasma total cholesterol (by 18% and 28%, respectively, p<0.05), non-HDL-C (by 49% and 53%, respectively, p<0.05) and the ratio of non- HDL-C to HDL-C (p<0.05) compared with individuals of the low isoflavone excretion phenotype (Table 3). Individuals of the low excretion phenotype were similar to casein controls in plasma lipid status. Isoflavone excretion phenotypes did not significantly affect plasma HDL-C and triglycerides.

Relation between Plasma Lipids and Isoflavone or Saponin Metabolite Excretion

Strong relationships existed between urinary and fecal isoflavone excretion within individuals at wk 1 and 4 according to correlation analysis (r = 0.79-0.93, p<0.05). Total cholesterol level was significantly positively correlated with non-HDL-C and the ratios of non- HDL-C to HDL-C (r = 0.87 and 0.79, p<0.05). Non- HDL-C was significantly positively correlated with non-HDL-C/HDL-C (r=0.97, p<0.05). There was no correlation of HDL-C or triglyceride with total or non-HDL-C.

Total and non-HDL-C were significantly negatively correlated with urinary daidzein, glycine and total isoflavone excretion (r = -0.45 to -0.58, p<0.05). HDL-C and triglycerides were not significantly correlated with urinary or fecal isoflavone excretion. Total and non-HDL-C were not significantly correlated with fecal saponin metabolite excretion.
Discussion

We identified two distinct urinary isoflavone excretion phenotypes based on urinary glycitein or total isoflavone excretion in hamsters using two statistical methods. Zheng et al. (12) showed that 13 women of a low in vitro fecal isoflavone disappearance phenotype and relatively rapid GTT showed ~50% greater urinary isoflavone excretion (apparent absorption) than did 12 women of a high fecal isoflavone disappearance phenotype and slow GTT when fed soy isoflavones for 7 days. Hamsters showed in vitro cecal isoflavone degradation rate phenotypic clusters (24) that could account for the urinary isoflavone excretion phenotypes observed in the present study, and suggesting that the isoflavone bioavailability phenotypes observed in both humans (11, 12) and hamsters (13) are due to gut microbial degradation of isoflavones that differs greatly and relatively stably among individuals.

In hamsters, urinary isoflavone excretion was significantly greater than fecal isoflavone excretion, presumably due to gut microbial degradation of these compounds, as observed in humans (9, 25, Tables 2, 3). Glycitein showed significantly greater excretion than daidzein or genistein in this study (Tables 2, 3), indicating its greater bioavailability than the other isoflavones. Feeding 1.18 or 1.77 mmol total isoflavones/kg diet to one-year old male (n = 10) and female (n=9) hamsters for 10 d produced similar 24-h urinary isoflavone excretion differences among the isoflavones (13). Daidzein was a more bioavailable isoflavone than genistein in women fed single doses of soymilk, as reflected in urinary excretion (9, 25). Urinary glycitein excretion was similar to that of daidzein and greater than was genistein in 7 women and 7 men fed single doses of soymilk (19). Not all human studies have shown greater bioavailability for daidzein than for genistein. Women who were “high excreters” of isoflavones did not differ in daidzein and genistein excretion, whereas “low excreters” showed greater daidzein than genistein excretion (9). We also found that the low glycitein urinary excretion phenotype was more stable and the high glycitein urinary excretion phenotype was less stable in hamsters (Fig. 4). In women, a high fecal daidzein isoflavone disappearance phenotype was not as stable as a low daidzein disappearance phenotype, but both high and low genistein disappearance phenotypes were relatively stable.
Thus, in a broad sense, hamsters may usefully model human isoflavone bioavailability and its stable phenotypic variations, to better study the health effects of these compounds.

In the current study, either glycitein or total isoflavone bioavailability as reflected in urinary excretion clusters determined at wk 1 or 4 predicted cholesterol-lowering efficacy of soy protein (Table 3). Hamsters fed 1.3 mmol daidzein/kg diet for 10 weeks had lesser plasma cholesterol compared with casein-fed controls (20). In part because of the apparent similarity in daidzein and glycitein bioavailability in humans (19), the present results are likely to be relevant to humans for cholesterol-lowering efficacy of soy proteins containing isoflavones. No studies of the effects of isoflavones on blood lipids have compared isoflavone bioavailability indices with cholesterol status to our knowledge, however Urban et al. (25) showed that serum isoflavones were increased as serum cholesterol was significantly decreased after isolated soy protein high in isoflavone content was eaten by 34 elderly men for 6 weeks in a randomized crossover design that compared the high isoflavone soy protein to a soy protein that had most of the isoflavones removed. These subjects showed serum isoflavone concentrations after eating the soy protein high in isoflavones that varied among subjects more than tenfold for genistein and more than thirtyfold for daidzein. This study along with several others (2-5) supported the role of isoflavones in cholesterol-lowering but did not attempt to relate individual isoflavone status to blood lipid status. Based on our hamster model and the wide range of human variability in isoflavone status after soy intake (25), controlling for the isoflavone bioavailability phenotypes of human subjects may optimize conditions to observe isoflavone effects.

Several studies have observed that isoflavones had no effect on improving blood lipid profile, including a recent meta-analysis (1). For example, dietary intake of isoflavone-rich (80.4 mg isoflavone aglucones/d; n=24) or isoflavone-poor soy protein (4.4 mg isoflavones/d; n=24) for 6 mo did not improve circulating lipid and lipoprotein concentrations in perimenopausal women or in subjects who were mildly hypercholesterolemic (n=30) (26). Hamsters fed isoflavones (0.02 g/100g diet) for 5 wk did not differ from the casein control in total cholesterol, triglycerides or HDL-C (p>0.05) (27). These studies did not attempt to control for interindividual variability in isoflavone bioavailability.
Soyasaponins, associated with soy protein in addition to isoflavones, were also assessed for cholesterol-lowering effect. A diet containing 2.4 g group B soyasaponins/kg (saponin concentration similar to that in 25% soy protein diet) caused significantly lesser plasma total cholesterol and non-HDL-C (by 20 and 33%) in ten 11–12 wk old female Golden Syrian hamsters compared with those fed casein (20). High producers of a putative fecal saponin metabolite had lesser total cholesterol/HDL-C compared with low producers. In the present study, high and low saponin metabolite producers did not differ in plasma lipid status (data not shown), and the dietary saponin content (1.1 g/kg) was ~half that shown to be effective in lessening cholesterol in a previous study (20). Thus, isoflavones and not saponins were the main modifiers of plasma lipids, but both components vary among soy protein sources and may contribute to lessening of plasma cholesterol by soy protein.

The cholesterol-lessening effect of soy protein in hamsters largely depended on the extent of apparent absorption of isoflavones, which varies according to distinct phenotypes. These urinary isoflavone excretion phenotypes may be defined according to glycine or total isoflavone excretion over a given period. These isoflavone absorption phenotypes are probably determined by gut microbial ecology, but isoflavone degrading microbial species and other factors determining this relatively stable variability in isoflavone absorption remain to be identified. Studies of effects of isoflavones in humans and animal models might be better served by taking interindividual variability in isoflavone absorption into account, as it seems to be a factor that could be controlled for.

Literature cited


2. Crouse JR III, Morgan T, Terry JG, Ellis J, Vitolins M, Burke GL. 1999 A randomized trial comparing the effect of casein with that of soy protein containing varying amounts of


FIGURES

FIGURE 1 Display of the ranked values of the percentage of ingested isoflavone dose excreted in hamster urine at w 1 for (A) Daidzein, (B) Glycitein, (C) Genistein, and (D) Total Isoflavone. Rankings on the x-axis were different for each isoflavone. According to the percentage of ingested dose of glycitein excreted in urine, high and low urinary excretion phenotypes were identified by a natural break point in (B) Glycitein.
FIGURE 2 Hierarchical clustering based on hamster urinary excretion of total isoflavone in w 1. A dendrogram of the urinary excretion of total isoflavone in w 1 was computed by complete-link clustering which divided all hamsters (except hamsters 1 and 2) into four clusters (as indicated by the dotted line). Dissimilarity between the clusters was defined as the maximum dissimilarities between members. The height at which two cases joined a single group indicated their dissimilarity.
FIGURE 3 Scatterplots of hamster urinary excretion of ingested dose of isoflavones for w 1. Pairwise relationships were compared among urinary excretion of glycitein, daidzein, genistein, and total isoflavones in bivariate scatterplots A-F. The numbers indicate groupings of individuals as defined in Fig. 2 (hierarchical clustering). Group 2 constituted low urinary excreters of all isoflavones whereas Group 3 were high urinary excreters of all isoflavones compared with the other groups. Groups 1 and 4 fell between groups 2 and 3 and had no ‘universal’ ordering.
FIGURE 4 Stability of urinary glycitein excretion within hamsters between w 1 and 4. The vertical axis is the hamster number (1-39) ranked in order by urinary glycitein excretion at w 1. The fraction of ingested dose of glycitein excreted in urine in each hamster is represented on the horizontal axis and the horizontal line connects w 1 and w 4 measurements for each hamster.
### TABLE 1

**Urinary Isoflavone Recoveries in Female Golden Syrian Hamsters According to Urinary Glycitein Excretion Phenotypes (high and low) in Weeks 1 and 4**

<table>
<thead>
<tr>
<th>Urinary excretion phenotype</th>
<th>% ingested dose recovered in urine over 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daidzein</td>
</tr>
<tr>
<td><strong>Week 1</strong></td>
<td></td>
</tr>
<tr>
<td>High (n=11)</td>
<td>27.7±5.9</td>
</tr>
<tr>
<td>Low (n=27)</td>
<td>8.8±3.8*</td>
</tr>
<tr>
<td>All (n=38)</td>
<td>14.3±4.8</td>
</tr>
<tr>
<td><strong>Week 4</strong></td>
<td></td>
</tr>
<tr>
<td>High (n=9)</td>
<td>25.9±6.8</td>
</tr>
<tr>
<td>Low (n=29)</td>
<td>9.1±4.1*</td>
</tr>
<tr>
<td>All (n=38)</td>
<td>13.1±5.2</td>
</tr>
</tbody>
</table>

1 Values represent means± SEM. * Different from high excreter, # different from daidzein and genistein, P <0.05.
### TABLE 2

**Urinary Isoflavone Recoveries in Female Golden Syrian Hamsters According to Urinary Total Isoflavone Excretion Phenotypes in Weeks 1 and 4**

<table>
<thead>
<tr>
<th>Urinary excretion phenotype</th>
<th>% ingested dose recovered in urine over 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daidzein</td>
</tr>
<tr>
<td><strong>Week 1</strong></td>
<td></td>
</tr>
<tr>
<td>High (n=7)</td>
<td>30.8±5.2</td>
</tr>
<tr>
<td>Low (n=23)</td>
<td>8.1±3.9*</td>
</tr>
<tr>
<td>All (n=30)</td>
<td>13.4±4.8</td>
</tr>
<tr>
<td><strong>Week 4</strong></td>
<td></td>
</tr>
<tr>
<td>High (n=9)</td>
<td>28.9±5.7</td>
</tr>
<tr>
<td>Low (n=21)</td>
<td>8.3±4.2*</td>
</tr>
<tr>
<td>All (n=30)</td>
<td>14.5±4.8</td>
</tr>
</tbody>
</table>

1 Values represent means± SEM. * Different from high excreter, # different from daidzein and genistein, P < 0.05.
**TABLE 3**

*Plasma Cholesterol Level in Female Golden Syrian Hamsters Fed Casein or Soy Protein with Different Urinary Isoflavone Excretion Phenotypes*\(^1,2\)

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Total cholesterol</th>
<th>HDL cholesterol</th>
<th>Non-HDL cholesterol</th>
<th>Triglyceride</th>
<th>Non-HDL/HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Casein</strong></td>
<td>10</td>
<td>5.71±0.88</td>
<td>3.02±0.42</td>
<td>2.41±0.91</td>
<td>1.19±0.32</td>
<td>0.79±0.28</td>
</tr>
<tr>
<td><strong>Soy Protein</strong></td>
<td>38</td>
<td>4.68±0.72*</td>
<td>2.79±0.57</td>
<td>1.67±0.69*</td>
<td>1.15±0.57</td>
<td>0.59±0.19*</td>
</tr>
</tbody>
</table>

**Week 1**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Total cholesterol</th>
<th>HDL cholesterol</th>
<th>Non-HDL cholesterol</th>
<th>Triglyceride</th>
<th>Non-HDL/HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>High glycitein</td>
<td>11</td>
<td>4.21±0.71*</td>
<td>2.81±0.63</td>
<td>1.18±0.75*</td>
<td>1.12±0.42</td>
<td>0.41±0.21</td>
</tr>
<tr>
<td>Low glycitein</td>
<td>27</td>
<td>4.88±0.81</td>
<td>2.78±0.37</td>
<td>1.87±0.88</td>
<td>1.16±0.31</td>
<td>0.67±0.33</td>
</tr>
</tbody>
</table>

**Week 4**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Total cholesterol</th>
<th>HDL cholesterol</th>
<th>Non-HDL cholesterol</th>
<th>Triglyceride</th>
<th>Non-HDL/HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>High glycitein</td>
<td>9</td>
<td>4.01±0.81*</td>
<td>2.61±0.69</td>
<td>1.09±0.65*</td>
<td>1.09±0.38</td>
<td>0.39±0.27</td>
</tr>
<tr>
<td>Low glycitein</td>
<td>29</td>
<td>4.93±0.79</td>
<td>2.92±0.48</td>
<td>1.92±0.83</td>
<td>1.13±0.36</td>
<td>0.69±0.38</td>
</tr>
</tbody>
</table>

**Week 1**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Total cholesterol</th>
<th>HDL cholesterol</th>
<th>Non-HDL cholesterol</th>
<th>Triglyceride</th>
<th>Non-HDL/HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>High total isoflavone</td>
<td>7</td>
<td>4.10±0.56*</td>
<td>2.75±0.51</td>
<td>1.12±0.62*</td>
<td>1.13±0.59</td>
<td>0.41±0.39</td>
</tr>
<tr>
<td>Low total isoflavone</td>
<td>23</td>
<td>5.01±0.83</td>
<td>2.51±0.48</td>
<td>2.23±0.87</td>
<td>1.17±0.31</td>
<td>0.88±0.37</td>
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</tbody>
</table>

**Week 4**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Total cholesterol</th>
<th>HDL cholesterol</th>
<th>Non-HDL cholesterol</th>
<th>Triglyceride</th>
<th>Non-HDL/HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>High total isoflavone</td>
<td>9</td>
<td>3.98±0.57*</td>
<td>2.65±0.39</td>
<td>1.11±0.52*</td>
<td>1.03±0.61</td>
<td>0.40±0.41</td>
</tr>
<tr>
<td>Low total isoflavone</td>
<td>21</td>
<td>5.21±0.73</td>
<td>2.62±0.50</td>
<td>2.33±0.77</td>
<td>1.12±0.35</td>
<td>0.87±0.39</td>
</tr>
</tbody>
</table>

\(^1\)Values represent means± SEM. * Different from casein, P <0.05.

\(^2\)Urinary isoflavone excretion clusters were determined by either glycitein (Fig. 1) or total isoflavone excretion (Fig. 2).

\(^3\)Represents the VLDL+IDL+LDL fractions (by difference: Total-HDL).
GENERAL CONCLUSIONS

Because much recent work published on soy protein and its component isoflavones did not support a significant role for soy isoflavones in cholesterol-lowering effects of soy protein, the more investigations which should be focused on bioavailability are needed. In addition to the valid animal model, hamster which has close cholesterol metabolism and similarities in bioavailability compared to humans, was established, the further study, especially for interindividual variability in isoflavone bioavailability, was considered deeply in our present project.

First of all, the cholesterol-lessening effect of soy protein in hamsters largely depended on the extent of apparent absorption of isoflavones, which varies according to distinct phenotypes. These urinary isoflavone excretion phenotypes may be defined according to glycitein or total isoflavone excretion over a given period. These isoflavone absorption phenotypes are probably determined by gut microbial ecology. At meantime, apparent absorbability of isoflavones varied stably among individuals. To identify urinary isoflavone excretion phenotypes and investigate the effect of these phenotypes on plasma lipids after soy protein feeding, we identified two distinct urinary isoflavone excretion phenotypes based on urinary glycitein or total isoflavone excretion in hamsters using pairwise correlation plots analysis and hierarchical cluster test. With either grouping method, the high isoflavone excretion phenotype showed significantly greater urinary daidzein, genistein, glycitein and total isoflavones and marginally greater fecal glycitein excretion than the low excretion phenotype at given period.

By McNemar’s test, we also found that the low glycitein urinary excretion phenotype was more stable and the high glycitein urinary excretion phenotype was less stable. More meaningfully, hamsters fed the soy protein diet had significantly lower total and non-HDL cholesterol and non-HDL/HDL ratios compared with the control. Hamsters of the high urinary isoflavone excretion phenotype had significantly lower total, non-HDL cholesterol and non-HDL/HDL ratios than did the low isoflavone excretion phenotype and the control group. Total and non-HDL cholesterol were significantly negatively correlated with urinary daidzein, glycitein and total isoflavone excretion.
These illustrated that the distinct urinary isoflavone excretion phenotypes are major determinants of cholesterol-lowering efficacy of soy protein and suggest that absorbability may be an important controllable variable in studies of effects of soy protein and isoflavones. Urinary isoflavone excretion phenotypes predicted the cholesterol-lowering efficacy of soy protein. Isoflavone absorbability, probably due to gut microbial ecology, is an important controllable variable in studies of effects of soy protein on blood lipids. Studies of effects of isoflavones in humans and animal models might be better served by taking interindividual variability in isoflavone absorption into account.
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