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Influence of fecal isoflavone degradation phenotype on bioavailability of soybean isoflavones in women

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**Influence of fecal isoflavone degradation phenotype on bioavailability of soybean
isoflavones in women**

by

Yan Zheng

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

DOCTOR OF PHILOSOPHY

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For the Major Program

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ABSTRACT

Soybean isoflavones are one of major phytoestrogens in the human food supply. The proposed health effects of soy consumption include preventing hormone-dependent cancers and cardiovascular disease and lowering the risk of osteoporosis. But there also exist health concerns such as the potential hormonal effects in infants fed soy-based infant formula, and anti-thyroid effects in humans. Bioavailability of isoflavones must be better understood in order to better assess their potential health and adverse effects. Isoflavone degradation phenotype and gut transit time (GTT) may have great influence on their bioavailability. To characterize what forms have optimal bioavailability, isoflavone aglucons and glucosides were compared between high and low daidzein degradation phenotypes with various GTT in women. Daily urinary isoflavone excretion was similar for aglucons and glucosides (51.5 ± 6.1 % and 50.8 ± 5.6 % of ingested amount, respectively). Apparent absorption of isoflavones was significantly greater in the low daidzein degradation phenotype coupled with shorter GTT than in the high phenotype with longer GTT as reflected in total urinary isoflavone excretion.

To study the influence of dose on isoflavones bioavailability, high and low doses of isoflavone were fed crossover for 7 days in women of both high and low daidzein degradation phenotype with varying GTT. As reflected in urinary isoflavone excretion, dose-dependent response was significant and expected (high vs. low dose: 197 ± 11 v. 72 ± 11 $\mu\text{mol/d}$, $P < 0.05$); phenotypic difference coupled with GTT difference greatly influenced isoflavone bioavailability (low vs. high daidzein degraders: 189.0 ± 16.3 v. 119.0 ± 5.0 $\mu\text{mol/d}$, $P < 0.05$). Repeated dosing decreased urinary isoflavone excretion by 20~30%.

Higher intakes of dietary fiber may accelerate GTT, therefore may increase apparent isoflavone absorption. High (45g/d) or moderate fiber (15g/d) diets were given to women of the low daidzein degraders with longer GTT (> 84 h) for 7 days as well as isoflavones in a randomized crossover feeding trial. Since GTT were decreased after both high fiber and moderate fiber diets, the apparent absorptions of isoflavones did not differ between the two fiber treatments. Repeated isoflavone feeding showed lower total urinary isoflavone excretion (week 1 vs. 2, 9.0 ± 0.9 vs. 4.8 ± 0.9 $\mu\text{mol/kg}$, $P = 0.02$) and plasma isoflavone (week 1 vs. 2, 4.8 ± 0.7 vs. 1.8 ± 0.7 $\mu\text{mol/L}$, $P = 0.005$). These data suggested that isoflavone degradation phenotype coupled with GTT is crucial in determining isoflavone bioavailability. Repeated dosing lowered isoflavone bioavailability.

GENERAL INTRODUCTION

Introduction

Soybeans are a major plant seed in human diets, the main source of isoflavones containing amounts of these compounds ranging from 1 to 3 mg/g. Due to the structural similarity to mammalian estrogen, 17 β -estradiol, isoflavones exert weak estrogenic effect and are called "phytoestrogens". Two chemical forms of isoflavones are found in soybeans: the isoflavone aglucons, daidzein, genistein and glycitein, exist only in minor amount in soybeans; the isoflavone glucosides are predominant in soybeans, which include malonyl-, glucosyl- and acetyl- β -glucosides. More than 90% of isoflavones in soy products are in the glucosidic form except fermented soy products that contain considerable amounts of aglucons. The isoflavone contents in soy products and isoflavone supplements vary product by product. The consumption of soy products has been associated with decreased risk of hormone-dependent cancers, coronary heart disease, osteoporosis and menopausal symptoms. But there are health concerns about the consumption of large amounts of isoflavones. Among the potential risks and adverse effects, possible endocrine effects in infants fed soy-based infant formula and anti-thyroid effects have been targeted.

The bioavailability of isoflavones is clearly important in assessing the likely importance of these compounds to human health. Bioavailability has different interpretations from different research perspectives. It is not indicated entirely by the extent of absorption. Absorption, distribution, metabolism (biotransformation in the gut, liver and other tissues) and elimination all contribute to bioavailability following ingestion. Bioavailability thus quantifies the exposure of the body to the compound. Many studies suggest that

bioavailability of dietary isoflavones depends upon their chemical structures, dose and time course. Recently, it is also suggested that the interactions of isoflavones with gut microflora and gut transit time are important in determining their bioavailability. The activities of gut microorganisms play an important role in isoflavone metabolism and bioavailability because considerable interindividual variation of fecal isoflavone degradation was reported in a human feeding study in which the variation of urinary isoflavone excretion was 10- to 20-fold within the same dose of dietary isoflavone treatment. *In vitro* human fecal isoflavone degradation phenotypes have been identified based on isoflavone degradation half-lives or degradation rate constants. Greater urinary isoflavone excretion has been associated with lower fecal isoflavone degradation rate constants and shorter gut transit time. Gut transit time reflects the residence time of substances passing through the gut and may be a crucial factor in influencing isoflavone bioavailability because with shorter GTT, the less isoflavones can be degraded, therefore the more isoflavones are absorbed.

Isoflavone aglucons are suggested to be absorbed directly in the stomach and intestine. But isoflavone glucosides must undergo the hydrolysis of β -glucosidic bond by the action of mammalian or gut microbial β -glucosidases before absorption. Seemingly, whether dietary isoflavones were present as aglucons or glucosides, the overall bioavailability of isoflavone was not affected. Chronic exposure to isoflavones, e. g., feeding soymilk for one month, decreased the urinary isoflavone excretion, which suggested the induction of isoflavone degrading gut microbial enzyme activities after frequent consumption of soy foods; the more isoflavone being degraded, the lower the isoflavone bioavailability. Studies regarding the

influence of dietary factors on isoflavone bioavailability suggested neither soy food types nor background diet affect short-term isoflavone bioavailability.

The objective of my research is to characterize the factors that influence isoflavone bioavailability. In a series of isoflavone feeding studies in women, I compared isoflavone bioavailability as reflected in urinary isoflavone excretion between aglucons and glucosides, between dietary isoflavones rich in glycitein and rich in genistein and between high dose and low dose of dietary isoflavones; the influence of isoflavone degradation phenotype coupled with GTT on isoflavone bioavailability was characterized in all the studies, especially in the last one, in which I attempted to modify GTT by increasing intake of dietary fiber to see if it would change urinary isoflavone excretion.

Dissertation Organization

This dissertation contains a general introduction, a literature review, three papers, a general conclusion and acknowledgements. All three papers “Comparative bioavailability of isoflavone glucosides and aglucons in women”, “Gut transit time and gut microbial isoflavone degradation influence apparent isoflavone absorption in women” and “Confounding effects of caloric intake and time course on modulation of apparent absorption of isoflavones by dietary fiber intake in women” will be submitted to Journal of Nutrition. The papers are followed the format of the journal to which will be submitted.

LITERATURE REVIEW

Source, Chemistry, and Isoflavone Analysis

Chemistry of isoflavones

Isoflavones are phytochemicals mainly found in soybeans and other legume species, such as clover, peas and alfalfa. Soybeans are a major plant seed in human diets, which contain the highest amounts of isoflavones ranging from 1 to 3 mg/g (Wang and Murphy, 1994). There are over 350 different naturally occurring isoflavones (Harborne and Baxter, 1999), and soybeans contain 12 forms of isoflavones. These are aglucons daidzein, genistein, glycitein; glucosyl- β -glucosides, daidzin, genistin and glycitin (the glucosylation occurs at the 7-*O*-position of aglucons); acetyl- and malonyl- β -glucosides (the acetylation and malonylation occur at the 6 position of the glucose moiety). The aglucons, daidzein and genistein were first isolated and identified in the soybean by Walter (1941). Glycitein was first isolated by Naim et al. (1973). Kudou et al. (1991) reported the nine predominant β -glucosides isolated from soybean hypocotyls.

Dietary sources and estimated intake of isoflavones

Soy products and isoflavone supplements are the major dietary sources of isoflavones and the isoflavone contents vary product by product. For those soy products in which soy is used as an ingredient, such as soy flour, the isoflavone contents normalized to aglucons ranges from 1~2 mg/g (USDA Iowa State University isoflavone database). In soy-based infant formula, in which soy protein is used as a functional ingredient, isoflavones are ~250 μ g/g in the non-reconstituted formulas (USDA Iowa State University isoflavone database). For those foods directly made from soy, such as soymilk, tofu, tempeh, and miso, consumed

as staple foods mainly in China and Japan, the isoflavone contents range from 400 to 2000 $\mu\text{g/g}$ (USDA Iowa State University isoflavone database). In the newly generated soy foods such as soy-based burger, hot dog, bacon, and soy cheeses, the isoflavone contents are as low as $< 100 \mu\text{g/g}$ (USDA Iowa State University isoflavone database). Isoflavones in soy products mainly exist as glucosides except in fermented soy foods such as tempeh and miso, which contain considerable amounts of aglucons since fermentation increases the aglucon contents. The distributions of individual isoflavones in soy foods depend on the natural distribution and the fractions used from the soybeans; genistein usually is in highest amount.

Recently many kinds of isoflavone supplements are commercially available. Setchell et al. (2001) reported isoflavone contents in 33 of these products by HPLC and electrospray ionization-mass spectrometry (ESI-MS) analysis. A large variation was found in isoflavone contents in these supplements when the sum of individual aglucons and glucosides was calculated, ranging from 1,700 to 96,000 $\mu\text{g/g}$ of the supplement. The distributions of each aglucon and glucoside differed product by product. Overall, they grouped these supplements into four categories: 1) those in which daidzein plus glycitein is 2-fold more than genistein, such as the products made from soy germ; 2) those products with this ratio lower than 1.5-fold and only a small portion of glycitein is present; 3) those made from extracts of clover containing mainly methoxylated isoflavones formononetin and biochinin A; 4) those containing daidzein and puerarin made from extracts of kudzu (an Asian legume plant and noxious weed introduced into Southern US) (Setchell et al., 2001).

The estimated dietary intakes of isoflavone differ significantly among populations. In adults eating typical Western diets without isoflavone supplements, the intakes are as low as

1~3 mg/d (Jones et al., 1989, Tham et al., 1998). In typical Eastern Asian diets, the intakes are 15~50 mg/d (Wakai et al., 1999, Chen et al., 1999). For example, in Japanese diets, it is estimated at 30 mg/d (Watanabe et al., 2000). For isoflavone supplements users, the intakes would depend on isoflavone concentrations in individual products and compliance with instructions for daily use, probably ranging from 0.5 to 2 mg/kg body weight per day. The isoflavone contents in soy-based infant formulas range from 214 to 285 $\mu\text{g/g}$ in the dry formulas, and from 25 to 30 $\mu\text{g/mL}$ in the reconstituted formulas (Murphy et al., 1997). The estimated intakes in infants (0~4-month of age) are 6~11 mg/kg body weight per day when they only consume soy-based formulas (Setchell et al., 1998).

Isoflavone analysis

The methods for isoflavone analysis have developed rapidly in recent years because the qualitative and quantitative data are greatly important for isoflavone research. Ideally, the method should have high sensitivity and selectivity; the equipment should be easily installed and the clean-up procedure should be relatively simple. But achieving all optimal conditions may not be easy; the choice of method is highly dependent on the research purpose and the nature of sample to be analyzed.

The methods for isoflavone analysis include chromatographic and non-chromatographic methods. Of chromatographic methods, gas chromatography-mass spectrometry (GC-MS) was the first one used for isoflavone analysis. GC-MS not only provides high sensitivity and resolution in analysis, but also is used as a tool to identify new peaks in chromatograms. It is particularly good for determining isoflavone content in biological samples such as blood, feces, urine and tissue where only low concentration of

isoflavones and their metabolites are present. But due to the nature of this analysis, a complicated clean-up using internal standard is needed before the injections. Adlercreutz et al. (1991a, 1991b, 1995) developed an isotope dilution GC-MS method, which had been used in detecting and determining isoflavones or their metabolites in biological samples: plasma, urine and feces. The detection limit of this method is in the range of ng/mL. For example, in feces, the limit of detection (LOD) was about 1 to 14 nmol/24h fecal sample (Adlercreutz et al., 1995). This multi-step method usually starts with a solid-phase extraction (SPE) after enzymatic hydrolysis of conjugated isoflavones, especially for biological samples urine and plasma; then followed by adsorption chromatography (Sephadex LH-20) to get the fraction of interest; derivatization of isoflavones to volatile analytes is necessary for GC methods. Each step could introduce errors and thus internal standard is highly recommended to monitor the loss during clean-up procedure (Heinonen et al., 1999). Due to these limitations, GC-MS may be applied to only a limited percentage of isoflavone analysis, mainly focused on the identification of isoflavones and their metabolites.

Compared with GC-MS, another chromatographic method, high-performance liquid chromatography (HPLC) has been extensively used in isoflavone analysis with the characteristics of being less expensive, requiring no or simple clean-up procedure (usually SPE). In addition, HPLC can be combined with multi-detection methods, including ultraviolet (UV) detection, fluorescence detection, electrochemical detection (ECD), MS, and recently reported coulometric detection. Solid-phase extraction followed by reverse-phase HPLC-UV detection modified from Lundh et al. (1988) is used in our laboratory for isoflavone analysis in urine, plasma and feces with 2, 4, 4'-trihydroxydeoxybenzoic acid (THB, a

precursor of isoflavone synthesis) used as internal standard (Song et al., 1998). UV detection is monitored at 254 nm, the LOD is about 20~100 ng/mL, and the analytical recoveries from the spiked isoflavones range from 65 to 87% in the biological samples. Photodiode array detection (PDA) is applied to identify individual isoflavones and their metabolites through spectra under different wavelength (190~350 nm) for maximum UV absorption. One potential problem of UV detection is that it is not sensitive for some isoflavone metabolites such as equol and *O*-desmethylangolensin (ODMA). For example, the LOD for equol and ODMA was 151~201 ng/mL, respectively, but for daidzein and genistein, it was 1.3~2.4 ng/mL, respectively in a SPE-HPLC-UV method (Franke and Custer, 1994). This problem could be solved by using HPLC-MS, HPLC with coulometric detection or HPLC-ECD, the detection limits of these methods are in the range of ng/mL to pg/mL (Franke et al., 2002, Nurmi et al., 2002, Setchell et al., 1987). Fluorescence detection is more sensitive than UV, but since not all isoflavones may have natural fluorescence response, this method is not widely used. One study showed that only daidzein and formononetin but not genistein and biochanin A had fluorescence response, and the LOD for UV and fluorescence detection was 2 and 0.5 ppm (mg/kg), respectively, in food samples (Wang et al., 1990). Now HPLC coupled with plenty of choices of detection is widely accepted and applied in isoflavone analysis. Among these methods, UV detection is less expensive and easily installed when working with soy food samples or biological samples after soy consumption.

The non-chromatographic methods such as immunoassay methods include radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and time-resolved fluoroimmunoassay (TR-FIA). Immunoassay methods offer a rapid analysis with high

sensitivity, specificity and relatively less expense. The detection limits of these assays reported in urine or plasma are 0.1~50 ng/mL (Lapcik et al., 1998, Uehara et al., 2000). It is suggested that these methods are suitable for large-scale studies or studies needing rapid scanning.

Isoflavones are phytochemicals synthesized by plants. Over the past 15 years there has been a dramatic increase in isoflavone research due to the specific beneficial and toxicity-related issues. This in turn encourages the development of new methods for the qualitative and quantitative measurement of isoflavones. In conclusion, various analytical methods have been used in isoflavone analysis based on different purpose of the study. Among these methods, HPLC-UV method is low-cost, but may be less sensitive especially for detection of isoflavone metabolites compared to other methods. HPLC with coulometric detection may be a better choice because it has the same cost but higher sensitivity than UV detection and much less expensive than GC-MS and ECD. Immunoassay is ideal for epidemiological studies with a large number of samples requiring high specificity.

Phytoestrogen Isoflavones and Hormone-dependent Cancers

Estrogens are steroidal hormones that play an important role in maintenance of good health in all vertebrates. Their biological effects are mainly found in influencing the growth, differentiation and functioning of target tissue or organs. In addition, estrogens may influence the cardiovascular system and bone health. The principal estrogen in animals is 17 β -estradiol (E₂), which interacts with the estrogen receptors (ERs) to exert its estrogenic effects in the body. Two subtypes of ERs, ER α and ER β have been identified. ER α is predominant in the testis, kidney, adrenal and non-pregnant myometrium; ER β is distributed

in bone, brain, thymus, prostate, lung, vascular endothelia, bladder and pregnant term myometrium (Kuiper et al., 1997). The binding affinity of E_2 to the ERs is different in that the affinity of E_2 to $ER\beta$ is one-quarter of that to $ER\alpha$ in synthesized human $ER\alpha$ and rat $ER\beta$ protein (Kuiper et al., 1997).

Phytoestrogens refer to the compounds derived from plants that exert estrogenic effects. Isoflavones are one major class of phytoestrogens and their estrogenic effects were first reported in the 1940s when these compounds were found to cause infertility in Australian sheep eating red clover (Bennets et al., 1946). The major isoflavones found in red clover were biochanin A and formononetin (Shutt, 1976).

Epidemiology of hormone-dependent cancers and soy consumption

Hormone-dependent cancers, such as those of breast, prostate and colon cancers, have higher incidence in Western countries. The relation between soy consumption and hormone-dependent cancers was reported in several epidemiological studies in which the intake of soy foods shown to be inversely correlated with breast and prostate cancer incidence (Adlercreutz et al., 1991b, Lee et al., 1991, Yuan et al., 1995, Hirose et al., 1995, Zheng et al., 1999). Soy consumption is very high in Eastern Asia compared to the North America and Europe. In several breast cancer case-control studies done in Singapore, China and Japan, soy food intakes were estimated by soy-food frequency questionnaires or evaluated by urinary isoflavone excretions, the urinary isoflavone excretions were substantially lower in breast cancer cases compared to their control counterparts (Lee et al., 1991, Yuan et al., 1995, Hirose et al., 1995, Zheng et al., 1999). The factors associated with the decreased risk of hormone-dependent cancers include lower circulating reproductive hormones, higher level of

sex hormone binding-globulin (SHBG), longer menstrual cycle (for breast cancer, especially the length of follicular phase), and higher urinary isoflavone excretion. Soy isoflavones have been suggested to have estrogenic and anti-estrogenic effects *in vivo*, *in vitro* and in humans. They may modify these risk factors, and therefore, exert cancer prevention effects.

Estrogenic effects of isoflavone *in vivo* and *in vitro*

In classic estrogenicity assay, both *in vitro* and *in vivo*, isoflavones show weak estrogenic effect compared with 17 β -estradiol or synthetic estrogen diethylstilbestrol (DES). These assays include E-screen (the proliferation of ER α -positive cell lines), competitive binding of ERs, expressions of ER-regulated genes and proteins, rodent uterotrophic assay and the responses to the estrogen sensitive tissues such as ovary, vagina, uterus and mammary gland. For example, genistein has been shown to induce the proliferation of estrogen-dependent human breast cancer (MCF-7) cells *in vitro* at a concentration as low as 10 nM, and at 100 nM, the proliferation effect was similar to that of 1 nM 17 β -estradiol (Hsieh et al., 1998). The expression of the estrogen-responsive gene pS2 was also induced in this cell line by 1 μ M genistein (Hsieh et al., 1998). This *in vitro* estrogenic effect found in MCF-7 cells was also reproduced *in vivo* in ovariectomized athymic mice when MCF-7 cells were implanted subcutaneously (under the skin). When the athymic mice were fed either 750 ppm of genistein or 1200 ppm of genistin (providing equal molar concentrations of aglucon equivalents) for 11 weeks, dietary genistein induced cell proliferations, pS2 gene expressions and tumor growth (Hsieh et al., 1998, Allred et al. 2001). Later the tumors regressed when these mice were switched to genistein-free diet for 9 weeks (Allred et al. 2001). Dose-dependent (\geq 250 ppm) cell proliferation, pS2 gene expressions and tumor growth were also

reported with this model when mice were fed 125, 250, 500 and 1000 ppm of genistein in diet (Ju et al., 2001). Furthermore, dietary genistein at 750 ppm for 5 days enhanced mammary gland growth in 28-day-old mice of this type (Hsieh et al., 1998).

In competitive binding of estrogen receptors assay, the relative binding affinity of isoflavones was 0.01~0.9% of 17β -estradiol (Shutt and Cox, 1972, Santell et al., 1997, Song et al., 1999). In addition, the binding affinity of genistein toward *in vitro* synthesized ER β protein was 8-fold higher than for ER α protein (Kuiper et al., 1997). When genistein and 17β -estradiol were injected subcutaneously into ovariectomized female Wistar rats at doses of 2.5, 0.25, 0.025, 0.0025 mg/kg body weight per day for 7 days, there was a 20-fold higher binding affinity to ER β than to ER α for genistein, whereas for 17β -estradiol, there was no difference between the two receptors within this dose range (Mäkelä et al., 1999). In the rodent uterotrophic assay, genistein, daidzein and glycitein were roughly 10^3 - to 10^5 -fold less effective than 17β -estradiol or synthetic estrogen diethylstilbestrol (DES), but genistein and glycitein significantly increased rodent uterine wet and dry weight compared with the control group (Farmakalidis et al., 1985, Santell et al., 1997, Song et al., 1999). Results from these studies suggested that although isoflavones have weak estrogenic activity, they could act as estrogen agonists *in vivo* and *in vitro*.

Early exposure to estrogens and progesterone has been suggested to induce differentiation in mammary tissue and reduce subsequent susceptibility to chemically induced mammary cancer (Grubbs et al., 1985, Russo et al., 1979). Isoflavones, acting as estrogen agonists, would be expected to have similar effects. Lamartiniere's research group initiated this type of study. In 1992, they reported that neonatal diethylstilbestrol treatment resulted in

reducing the incidence of spontaneous development of mammary tumors in Sprague-Dawley CD rats (Lamartiniere and Holland, 1992). Then they began to investigate the potential of genistein as a substitute for DES to exert a similar chemopreventive effect. Female Sprague-Dawley CD rats were injected subcutaneously with 5 mg genistein, or 20 μ L of the vehicle, dimethylsulfoxide (DMSO), on days 2, 4 and 6 postpartum. At day 50, rats were exposed to 80 mg dimethylbenz[a]anthracene (DMBA)/kg body weight to induce mammary cancer. They found that neonatal injections of genistein suppressed the development of DMBA-induced mammary adenocarcinomas in rats (Lamartiniere et al., 1995a, 1995b). Neonatal treatment reduced the incidence and the number of tumors per rat. In addition, the subsequent studies showed that when genistein was injected into prepubertal rats at 500 μ g/g body weight on days 16, 18 and 20 postpartum, genistein also produced a protective effect against DMBA-induced mammary cancer (Murrill et al., 1996). Later, they gave dietary genistein to rats perinatally in amounts likely to be found in diets and found that genistein protected against DMBA-induced mammary cancer in their offspring. At concentrations of 25 and 250 mg genistein/kg diet (AIN-76A), genistein reduced the number of mammary tumors by 20% and 50%, respectively in dose-dependent manner (Fritz et al., 1998). These data are consistent with earlier data using pharmacologic doses of genistein (Lamartiniere et al., 1995a, 1995b, Murrill et al., 1996). Thus, early exposure of rats to nutritional concentrations of genistein exert a permanent protective effect against breast cancer, which suggests that genistein, like estrogen, enhances the differentiation and programming of mammary gland cells, resulting in reducing the susceptibility to mammary cancer.

Anti-estrogenic effects of isoflavones *in vivo* and *in vitro*

Isoflavones could compete with endogenous estrogens in binding of ERs and act as estrogen agonist or antagonist *in vivo* and *in vitro* depending on many factors such as the presence of endogenous estrogens, the dose of isoflavones and the status of ERs. The preferential binding of genistein to ER β , may be important in exerting biological effects of isoflavones. Since the circulating concentrations of isoflavones may be 100~1000-fold greater than that of 17 β -estradiol in premenopausal women after consuming moderate amount of soy foods (Watanabe et al., 2000), isoflavones could have significant biological roles in humans. As discussed before, *in vitro* at low concentrations, 10~100 nM, genistein acted as estrogen agonist to stimulate estrogen-dependent MCF-7 cells (Hsieh et al., 1998). However, at high concentration (> 20 μ M) or in the presence of 17 β - estradiol, genistein significantly inhibited cell proliferations (Hsieh et al., 1998). This anti-estrogenic effect may due to the competition of isoflavones with endogenous estrogens for ERs binding sites, hence, preventing estrogen-stimulated cell proliferation. *In vivo*, Fritz et al. (2002) reported that male Sprague-Dawley rats when fed 25 and 250 mg genistein/kg diet from conception to 70 day postpartum, androgen receptor (AR), and ER α and β mRNA expressions in dorsolateral prostate were decreased in dose-dependent manner. Later when genistein was fed to adult rats (from day 56 to 70 postpartum) at 250 and 1000 mg/kg diet, similar results were found. In addition, ER α protein levels were significantly reduced in rats fed 1000 mg genistein /kg diet compared to the control animals. This down-regulation of ER and AR expression in prostate could be due to the anti-estrogenic effects of isoflavones and explain the lower incidence of prostate cancer in the countries where soy is consumed as a staple food.

Hormonal effects of soy consumption and hormone-dependent cancer in human

Soy isoflavones exert hormonal effects in both men and women. But most of these types of studies were done in premenopausal women, focusing on the modification of risk factors related to breast cancer. The lower concentrations of blood reproductive hormone and the longer menstrual cycle especially the length of follicular phase have been associated with the lower risk of breast cancer. The influence of soy feeding on ovarian cycle was first reported by Cassidy et al. (1994). Soy protein (60 g/d) providing 45 mg total isoflavones for one cycle significantly ($P < 0.01$) increased follicular phase length and delayed menstruation about 1.5 days. In addition, the mid-cycle surges of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were significantly suppressed and follicular phase plasma estradiol increased. These responses are similar to the estrogen antagonist, tamoxifen, when given as an anti-breast cancer agent. Since then, many other feeding studies have been done but the results were not consistent. Lu et al. (1996a, 2000a) fed 6 or 10 healthy cycling women soymilk that contained 113~207 mg total isoflavone/d for one cycle. A significant reduction in plasma 17β -estradiol ($P < 0.01$), and progesterone ($P < 0.001$) were found, but no changes in LH and FSH were found. The cycle length was prolonged 3.5 day in the first study (Lu et al., 1996a) but only 0.6 day in the second one (Lu et al., 2000a). Duncan et al. (1999a) studied 3 cycles plus 9 days, which started on the 1st day of cycle. Fourteen subjects consumed soy protein powder in a randomized crossover design with low-isoflavone (64 mg/d) or high-isoflavone (128 mg/d) or a control diet (10 mg/d). The low-isoflavone diet significantly decreased plasma LH ($P = 0.009$) and FSH ($P = 0.04$) during the periovulatory phase. Only estrone was decreased during mid-follicular phase and the menstrual cycle length

did not change significantly. The results from these studies were in conflict because such studies must be very carefully controlled, i.e., if only one cycle is studied, it is not known if the soy is causing the effect or not. Therefore, multiple cycles, carefully controlled diets and control of cycle are recommended, i.e., feeding is begun on the same day of the cycle for all subjects, in order to sort this out. The characteristics of subjects and the bioavailability of isoflavones in the subjects may also be responsible for the observed differences.

McMichael-Phillips et al. (1998) reported that daily consumption of 60 g of soy protein containing 45 mg isoflavones for 14 days in 48 premenopausal women with benign or malignant breast disease resulted in significantly increased proliferation rate of lobular epithelium and the expression of progesterone receptor when biopsy samples were examined. This suggested that soy feeding might also exert estrogenic effect in women. But since this study only lasted for 2 weeks, the relatively short feeding period may not reflect the changes for long-term. As seen when tamoxifen was given as anti-cancer agent to breast cancer patients, the increased pS2 gene expression after 6 weeks administration was reversed after 6 months of treatment (Willsher et al., 1994). Petrakis et al. (1996) found that daily soy consumption (38 g of soy protein containing 38 mg genistein) for 6 months increased the secretion of breast nipple aspirate fluid (NAF) and breast cell hyperplasia in 7 of 24 premenopausal women with the subjects as their own controls. Results from those two studies raised some important questions about effects of soy consumption on breast tissue. More clinical studies are needed to answer these questions.

In postmenopausal women, the hormonal effects of soy consumption were not consistent. In one study (Duncan et al., 1999b), 18 postmenopausal women consumed control

(0.11 mg/kg body weight), low-isoflavone (1.0 mg/kg body weight) or high-isoflavone (2.0 mg/kg body weight) diet in crossover design for 1 month each; only the high-isoflavone diet decreased plasma estrone-sulfate significantly, and the decreases of estradiol and estrone were not significant. No vaginal cytology and endometrial biopsy changes were found in these women. On the other hand, Baird et al. (1995) found no changes in serum FSH, LH, and estradiol, but estrogenic effect on vaginal cytology in 48 postmenopausal women after daily consumption of 165 mg total isoflavone for 4 weeks. In the study mentioned above (Petrakis et al., 1996), the secretion of NAF and changes of breast cancer cells were not found in 24 postmenopausal women after the same treatment as was used in premenopausal women. These results suggested that soy isoflavone intake in postmenopausal women may exert estrogenic or anti-estrogenic effects, but the reasons for such effects were not very clear.

The studies about hormonal effects of soy consumption in men focused on both beneficial effects such as prostate cancer prevention and potential adverse effects such as the lower sperm quality. In 14 young men (18~35 y), daily intake of 40 mg total isoflavones from soy extract for 2 months had not effects on testicular volume, sperm concentration, count and motility, which suggested that isoflavone intake at physiological level, did not have adverse effect on sperm quality (Mitchell et al., 2001). Soy consumption has been suggested to reduce the risk of prostate cancer as seen in Eastern Asia and the lower incidence associated with lower reproductive hormones. Nagata et al. (2000) reported that the reproductive hormones, estradiol ($r = -0.32$, $P = 0.009$), estrone ($r = -0.24$, $P = 0.05$) and testosterone ($r = -0.25$, $P = 0.05$) were inversely correlated with soy food consumptions in 69 Japanese men (mean age 60 y) with intake of 22mg isoflavone/d estimated by a dietary questionnaire. But not all

studies showed similar reductions in these hormones. Habito et al. (2000) found no changes in blood concentrations of estradiol, testosterone, and dihydrotestosterone in 42 middle-aged men (mean age 45 y) when they switched from 150 g meat diet to 290 g tofu diet, providing 70 mg total isoflavones daily for 4 weeks, but the mean testosterone-estradiol ratio was decreased 10% ($P = 0.05$) after tofu consumption. The different results from the two studies may be due to the different study design in which the first one is a cross-sectional epidemiological study done in Eastern Asia and the second one is a short-term intervention study done in a Western country, ethnic difference also could be a confounding factor. The beneficial effect of soy consumption in prostate cancer prevention has not been fully understood. More solid evidence is needed.

Other factors related to soy consumption and hormone-dependent cancer

Higher levels of sex hormone binding globulin (SHBG) have been associated with decreased breast cancer risk. SHBG is a plasma protein that binds to steroid hormones (estrogen and androgens) in the circulating system. Since only free steroids exert their effects, the higher level of SHBG may lower the concentrations of the free form estrogens; hence lower the effects of estrogens. Adlercreutz et al. (1992) reported that significant positive correlation between urinary isoflavone excretions and plasma SHBG in 30 postmenopausal women. In the study mentioned above, Duncan et al. (1999a) found that 5 equol producers (equol is a metabolite of daidzein) among the 14 subjects had lower plasma estrone, estrone sulfate, androgens, and prolactin and higher SHBG, as well as trends toward longer menstrual cycle compared to non-equol producers. The observations of decreased estrogens and increased SHBG concentrations in the equol producers are consistent with *in vitro* data

showing that equol is a weak inhibitor of aromatase, an enzyme catalyzing estrogen synthesis (Adlercreutz et al., 1993), and a stimulus for SHBG synthesis in HepG2 liver carcinoma cells (Loukovaara et al., 1995). There are epidemiological data showing a positive association between urinary equol and plasma SHBG concentrations (Adlercreutz et al., 1987). In the clinical trials discussed above, SHBG was increased significantly ($P = 0.01$) in men when switched to 290 g tofu diet (containing 70 mg isoflavones) for 4 weeks (Habito et al. 2000) and in postmenopausal women ($P < 0.05$) after they consumed high isoflavone diet (2 mg/kg body weight per day) for 1 month (Duncan et al., 1999b). These results suggested that isoflavones and their metabolites might affect the uptake of sex hormones by regulating plasma level of SHBG, therefore lowered free estrogens in circulation.

The metabolites of estrogens may also play a role in hormone-dependent cancers. The two potentially carcinogenic metabolites of estradiol, 4-hydroxyestrogen and 16 α -hydroxyestrogen and the anti-carcinogenic metabolite 2-hydroxyestrogen have been implicated in breast cancer risk. In the study of Lu et al. (2000b), 8 women were given soymilk either containing 113~207 mg isoflavones/d or 4.5 mg isoflavones/d for a month with a 4-mo washout period in between. The high isoflavone soymilk increased mean daily urinary excretion of 2-hydroxyestrone by 47% ($P = 0.03$), but had no effect on 16 α -hydroxyestrone. Similar results were also reported by Xu et al. (1998). In this study, 14 premenopausal women consumed soy protein diet with low-isoflavone (64 mg/d), high-isoflavone diet (128 mg/d) or control diet (10 mg isoflavones/d) for 1 month in randomized crossover design. The ratios of 2:16 α -hydroxyestrogen and 2:4 α -hydroxyestrogen were increased by 67% and 33%, respectively after low- and high-isoflavone feeding compared to

control diet. This suggested that isoflavone consumption increased metabolism of endogenous estrogens to the protective 2-hydroxy form, therefore lowering cancer-promoting efficacy of 17β -estradiol.

In conclusion, soy consumption is associated with lower incidence of hormone-dependent cancers. Isoflavones found in soy foods have weak estrogenic activity compared to endogenous mammalian estrogens but their very high concentrations in circulation could cause estrogenic or antiestrogenic effects depending on the host hormonal milieu. Efficacy as estrogen agonists or antagonists is crucial in understanding effects of isoflavones on carcinogenesis.

The Role of Isoflavones in Cholesterol-lowering Effect of Soy Protein

The hypocholesterolemic effect of soy protein has been known for more than 60 years (Meeker and Kesten, 1941). Many species including rabbits, rats, hamsters, guinea pigs, pigs, and cynomolgus monkeys have been used to test this effect when animal protein replaced with soy protein (Carroll and Kurowska, 1995, Anthony et al., 1996). In human clinical trials, a meta-analysis showed that soy protein intake, reduced plasma LDL cholesterol and triglycerides by 13% and 10%, respectively, and increased HDL cholesterol by 2% (Anderson et al., 1995). Recently, research interest has focused on identifying the components of soy protein responsible for the observed hypocholesterolemic effect. The amino acid composition of soy protein, phytoestrogens isoflavones, and other component such as saponins and plant sterol have been targeted. Since the intact soy protein contains all of these components, studies using intact soy protein as the source of isoflavones may not be able to explain the

role of isoflavones in hypocholesterolemic effect of soy protein. But so far, there are not many studies using isoflavones alone to test these effects.

Effects of isoflavones in cholesterol-lowering in animals

The type of study with isoflavones added to the casein-based diet was first done by Balmir et al. (1996). In this study, four diets were fed to male Sprague-Dawley rats for 4 weeks in the first part of the study and in the second part, diets were fed to male Golden Syrian hamsters for 8 weeks. The diets were ethanol-acetone extracted isolated soy protein (ISP-), unextracted soy protein (ISP), casein (casein-), and casein with 0.36 mg ethanol-acetone extract/g protein added (casein+). The ethanol-acetone extract is primarily an isoflavone-containing component because sugars, proteins and saponins were precipitated after acetone was added. They found that rats fed ISP and casein+ diets had significantly lower LDL cholesterol concentrations ($P < 0.05$) than did the casein- group. In the second part of this study, hamsters were fed ISP, ISP with 0.36 mg ethanol-acetone extract/g protein added (ISP+), casein-, casein+, and casein++ (with 0.72 mg ethanol-acetone extract /g protein added) diets, and the decreased total cholesterol and LDL cholesterol ($P < 0.05$) were found in ISP, ISP+ and casein+ groups but not in casein++ group compared with casein-fed control group. These results suggested that the ethanol-acetone extract (isoflavones-containing extract) had hypocholesterolemic effects especially on LDL cholesterol concentration in both species and the higher dose of isoflavone may not exert further cholesterol-lowering effect. The potential problem of this type of study is that the ethanol extracts may contain other components from soy that are responsible for the cholesterol-lowering effect. For example, in the study discussed above, even after ethanol and acetone extraction, the isoflavone-rich

extract contained only 79% of isoflavones, the remaining 21% was unknown in composition, and therefore, the conclusion that the observed hypocholesterolemic effects were only due to the presence of isoflavones could not be drawn with certainty. To avoid these limitations, purified or pure isoflavones needed to be added to the experimental diets. Nogowsky et al. (1998) investigated the effects of genistein on cholesterol and triglyceride metabolism in ovariectomized female rats. Three experimental diets, animal protein diet (casein and fish meal) without isoflavones as the control diet, 0.01 and 0.1% genistein diet, were fed to rats for 14 days. Although no effect was found on the total cholesterolemia, rats fed genistein had lower plasma triglyceride concentration and increased hepatic cholesterol which suggested a long-term effect on the plasma lipids profile after isoflavone consumptions. The relatively short feeding period of this study may not be sufficient for genistein to exert effects on cholesterol. Song et al. (2003) studied the hypocholesterolemic effect of dietary isolated soy protein (ISP) with or without isoflavones (ISP-), daidzein along and soygerm in Golden Syrian hamsters. Sixty males and 60 females were fed six experimental diets for 10 weeks: casein control, ISP, ISP-, daidzein, soygerm and soygerm extract with 1.3 mmol total isoflavones /kg in the diet except for casein and ISP- groups which had 0.013 mmol total isoflavones /kg diet. Feeding ISP, ISP-, daidzein, soygerm and soygerm extract significantly lowered plasma total cholesterol by 16~28%, LDL cholesterol by 15~50% and the ratios of LDL/HDL compared with the casein control group ($P < 0.01$). These data suggested that soy protein, with or without isoflavones, daidzein and soy germ have cholesterol-lowering effects in hamsters. One potential problem in this study is that the relatively high dose of isoflavones used may not be relevant to physiological conditions (the isoflavone dose was similar to that

found in diet containing soy protein (25% of total diet) as the only protein source). But this is the first study to show that isoflavones alone have hypocholesterolemic effects in rodents.

Non-human primates are considered to be good models to study the hypocholesterolemic effects of diet because they are similar to humans in cholesterolemia profiles. One research group is particularly interested in investigating the role of isoflavones in cholesterol-lowering effects of soy protein in monkeys. Anthony et al. (1996, 1997) studied the effects of intact isolate soy protein (ISP), alcohol-washed ISP (ISP-), and casein diets on plasma cholesterol levels in male and female Rhesus monkeys (1996) and in young male cynomolgus monkeys (1997) for 14 months. Monkeys fed ISP had significantly lower total and LDL plus VLDL cholesterol concentrations and the decreased ratio of total cholesterol/HDL cholesterol compared with the other two groups. In the later study, monkeys fed ISP had the highest HDL cholesterol concentrations compared with the other groups; ISP-fed group had intermediate and casein-fed had the lowest HDL cholesterol. But not all studies in monkeys showed similar results. When the isoflavone-rich extract added to casein diet (casein+) was fed to ovariectomized cynomolgus female monkeys for 12 weeks, the addition of isoflavones to casein did not have effects on plasma cholesterol levels compared with casein control group. Whereas, monkeys fed intact soy protein had significantly lower plasma total cholesterol, VLDL plus IDL and LDL cholesterol, and higher HDL cholesterol (Greaves et al., 1999). Again, questions are raised from those studies, for example, the alcohol-washed soy protein or isoflavone-rich extracts may also contain other components such as saponins co-extracted with isoflavones which could also have effects on plasma cholesterol. The 12-week feeding in the later study compared with the 14-month feeding in

the former one may also result in differences when the two studies were compared. Results from animal studies might not support the independent role of isoflavones in lowering plasma cholesterol, but do suggest there may be some synergic effects or additive effects between soy proteins and isoflavones or other unidentified components in soy in modulating cholesterol profiles.

The role of isoflavone in hypocholesterolemic effects of soy protein in humans

Controlled human feeding studies have been performed to investigate the role of isoflavones in hypocholesterolemic effects of soy protein in humans. The results from these studies suggested that the effect of soy protein on lowering LDL cholesterol concentrations is related to the amount of isoflavones contained in soy protein. In a 9-week randomized feeding trial, 156 mildly hypercholesterolemic men and women were given 25 g soy protein daily which containing different amount of isoflavones from 3 to 62 mg, an increasing reduction in LDL cholesterol concentrations found to be related to the increasing isoflavone contents and only when soy protein containing isoflavones ≥ 37 mg/d, the reductions of LDL cholesterol were significant compared with that of baseline (Crouse et al., 1999). In 13 normocholesterolemic premenopausal women, 53 g soy protein, providing 129 mg total isoflavone daily for 3 months, significantly lowered the LDL cholesterol concentrations compared with the same amount of soy protein but containing 65 mg total isoflavones (Merz-Demlow et al., 2000). In 18 mildly hypercholesterolemic and normocholesterolemic postmenopausal women, 63 g isolated soy protein (ISP) containing either 7.1 mg (control), 65 mg (low isoflavone), or 132 mg (high isoflavone) isoflavones were given daily for 3 months, soy protein with 132 mg isoflavones was much better than that with 65 mg isoflavones in

lowering LDL cholesterol concentration (Wangen, et al., 2001). These results suggest that intact soy protein with considerable amount of isoflavones (depending on the cholesterolemia status of the subjects) has better hypocholesterolemic effect in humans. One clinical study failed to find the cholesterol-lowering effects of soy isoflavone extract. When peri- and postmenopausal women were given 80 mg/d isoflavone (from tablet) for 5 weeks, no effect was found on plasma HDL and LDL cholesterol (Nestel et al., 1997). The short feeding period of this study may have confounding effect on the expected results. Both animal studies and human clinical trails suggest that intact soy protein has the optimal plasma cholesterol-lowering effects and at this point, it is not clear whether the plasma cholesterol-lowering effects of soy protein are related primarily to the presence of the isoflavones or not. Other soy components such as saponins may also contribute to plasma lipid lowering effect since in most of studies, saponins are co-extracted with isoflavones, such that one cannot distinguish an isoflavone-specific effect. But it seems that several factors may influence the results in human feeding studies: the higher the lipoproteins are in the subjects pretreatment, the more likely isoflavones will benefit. Also the higher isoflavone content (≥ 37 mg isoflavone/d) and the longer feeding period (> 5 weeks), the better plasma lipids profile in humans.

Soy Isoflavones and Menopausal Symptoms Including Osteoporosis

Osteoporosis is a metabolic bone disease, which affects postmenopausal women due to ovarian hormone deficiency after menopause. Currently, the most effective therapy for treating osteoporosis is the estrogen or hormone replacement therapy (ERT or HRT) (Lindsay, 1998). But due to the possible side effects, such as increased incidence of mammary and endometrial cancers (Scharbo-Dehaan, 1996), the acceptances of ERT or HRT

are not as good as expected (Groeneveld et al., 1994). Research interest focuses on seeking a substitution to the traditional therapy.

Animal studies demonstrate that dietary isoflavones had a protective effect against bone loss in ovariectomized (OVX) animal models (Arjmandi et al., 1996, 1998a, 1998b). Arjmandi initiated this type of study by using female rats ovariectomized at 95 days old. In the first study (Arjmandi et al., 1996), 32 female Sprague-Dawley rats (SD rats) were divided into 4 groups: sham-operated group (SHAM), OVX group, OVX + E₂ group and OVX + soy protein group. The first three groups were fed a casein-based diet, whereas the last group fed soy protein for 30 days. The soy protein diet improved bone mineral density (BMD) by 15% in OVX rats compared with OVX control group and this effect was comparable with the positive control OVX + E₂ group, suggested that soy protein diet prevented bone loss in OVX rats. The subsequent study tried to determine the role of isoflavones in soy protein in this bone-protective effect (Arjmandi et al., 1998a). Forty-eight SD rats with a SHAM groups fed a casein-based diet and 3 OVX treated groups fed either casein-based diet (OVX+CASEIN), soy protein with normal (OVX+SOY) or reduced isoflavone (OVX+SOY-) diets for 65 days. The SOY diet contained 1462 mg genistin, 25 mg genistein, 590 mg daidzin, and 11 mg daidzein /kg soy protein isolate and the SOY- diet contained 10% that of the SOY diet. The OVX+SOY group but not OVX+SOY- had significantly greater femoral bone density than that of the OVX+CASEIN group. They concluded that isoflavones in soy protein was responsible for the bone-protective effect of soy protein (Arjmandi et al., 1998a).

Other studies by using either oral administration or subcutaneous injection of isoflavones to rodents showed that isoflavones improved BMD and the prevention of bone

loss was comparable to that of 17 β -estuarial. These studies are: Fanti et al. (1998) with subcutaneous injection of genistein at 1, 5, or 25 mg/kg body weight in 2-month-old OVX rats for 21 days; Ishida et al. (1998) with oral administration of daidzein at 50 mg/kg body weight per day in OVX rats for 4 weeks; Ishimi et al. (1999) with subcutaneous injection of genistein at 0.7 mg/d in OVX mice; Picherit et al. (2000) with oral administration of genistein and daidzein at 10 mg/kg body weight per day in 12-month-old OVX rats for 3 months; and Ishida et al. (2000) with oral administration of daidzein, genistein and glycitein at 50 mg/kg body weight per day in 11-week-old OVX rats for 4 weeks. The last two studies also suggested that daidzein and glycitein were more effective than genistein in protecting OVX rodents from bone loss.

Observational studies in humans also indicated that soy isoflavones in soy foods have a protective effect on bone. In one study (Mei et al., 2001), 650 Chinese women, ages 19–86 y, enrolled to determine the relationship of their dietary isoflavones intake and lumbar spine and hip bone mineral density (BMD). Among the 357 postmenopausal women, significant differences in lumbar spine BMD and Ward's triangle BMD were found between the highest (53.3 mg isoflavone/d) and lowest (3.4 mg isoflavone/d) intake of isoflavone after adjusting factors including age, height, weight, years since menopause, smoking and alcohol consumption, HRT usage, and daily calcium intake. Other parameters such as serum parathyroid hormone, osteocalcin, and urinary N-telopeptide were significantly lower in women with the highest intake of isoflavone compared with those with the lowest intake. But these were not seen in 293 premenopausal women.

Human intervention studies suggest the bone-protective effect of isoflavones as well. Alekel et al. (2000) determined the effects of 24-week consumption of isoflavone-rich soy protein with 80 mg isoflavone/d (n = 24) and isoflavone-poor soy protein with 4 mg isoflavone/d (n = 24) and whey protein diet (control, n = 21) in attenuating lumbar spine bone loss during the menopause transition. Isoflavone-rich soy protein treatment attenuated bone loss and maintained higher BMD and bone mineral content (BMC) compared with control group and isoflavone-poor soy protein group. The protective effect of soy protein may be isoflavones dose-dependent. In another clinical trial (Potter et al., 1998), 66 postmenopausal women consumed daily either 40 g casein-based diet, 40 g soy protein diet with 56 mg isoflavones or soy protein diet with 90 mg isoflavones for 6 months, lumbar spine BMD was significantly increased by 2.2% in 90 mg isoflavones group compared with casein group (decreased 0.6%), and no changes were found in 56 mg isoflavones group. But not all studies came up with similar results. In Gallagher et al. (2000) and Violins et al. (2002), as isoflavones dose increased, 40 g soy protein with 0, 52, and 96 mg isoflavones for 9 months in the first study; 25 g soy protein with 5, 42, and 58 mg isoflavones for 24 months in the second study, no significant changes were found in lumbar spine BMD or total-body BMD in 65 and 172 postmenopausal women, respectively. Compared with Alekel's and Potter's studies, the latter two studies lasted longer, and the doses of isoflavones in Violins's study was relatively lower, which suggested the dose of isoflavones is crucial in exerting the bone protecting effect and long term feeding of isoflavones may induce some effect counteract the protecting effect.

In addition to maintaining good bone health, intake of isoflavone-containing soy products may also improve other menopausal symptom such as hot flushes. For example, in a 3-month double-blind, parallel, randomized trial, 79 postmenopausal women were either given 60 g isolated soy protein (providing 76 mg isoflavone/d) or placebo. Soy treatment was significantly more effective than placebo in reducing the mean number of hot flushes per 24 h after 4, 8, and 12 weeks of treatment (Albertazzi et al., 1998). In the study mentioned above (Alekel et al., 2000), the frequency of hot flushes and night sweats declined significantly in all treatment groups compared with that of baseline. Messina et al. (2003) did a meta-analysis to sort out the relationship between soy isoflavones intake and frequency of hot flush. Based on 13 clinical trials, they found that initial hot flush frequency was positively and significantly correlated with treatment efficacy of soy foods ($r = 0.46$, $P = 0.01$). It is suggested that initial hot flush frequency explained 46% of the treatment effects. They also found that isoflavone supplementation had superior effect when given multiple times per day than given only once per day. A question raised from this analysis is that the estimated 20% of placebo effect in control group made the reduction of hot flush frequency in soy treatment group not statistically significantly different from that of control group, therefore, the placebo effect may lower the efficacy of soy when evaluating its influence on menopausal symptoms. Isoflavone supplementation may improve the menopausal symptoms and seemingly depend on the initial frequency of hot flushes.

Potential Toxicity of Soy Isoflavones

The beneficial effects of soy consumption, particularly isoflavones, have been widely publicized. But there are also health concerns about the consumption of large amounts of

isoflavones due to their hormonal and non-hormonal properties. Among these potential adverse effects, possible endocrine effects in infants fed soy-based infant formula (SBF), and effects on thyroid, immune system and carcinogenesis have raised attention.

Soy-based formula and infant health

Soy-based infant formula (SBF), the substitution of cow milk-based formula, has been used for more than 60 years (Klein, 1998), and now account for 25% of infant formulas sold in the United States (Klein, 1998, Lonnerdal, 1994). Initially, SBF was made from soy flours and later in 1970s, soy flours were replaced with soy protein isolates with methionine fortification (Fomon, 1991, Fomon et al., 1979). Isoflavone content was lower in soy protein isolate compared with those in soy flours (Anderson and Wolf, 1995). Setchell et al. (1997) analyzed the isoflavone concentrations in commercially available SBF. In powdered formulas, the total isoflavone was ~300 mg/kg, and in prepared infant food was ~42 mg/L. In liquid formula, total isoflavones ranged from 43-90 mg/L. Based on these data, the daily intake of isoflavones estimated in infants fed SBF is: 5.7~7.3 mg/kg body weight per day at 1-week, 6.0~11.9 mg/kg body weight per day at 1~4-month (Setchell et al., 1998). Compared with adults, infant exclusively fed SBF are exposed to 6~11-fold higher isoflavones relative to their weight.

Due to the estrogenic properties of isoflavones, there is concern about the hormonal effects of isoflavone in infants. Several studies have examined the influence of modern SBF on infant growth and development (Hillman et al, 1988, Venkataraman et al., 1992, Giovannini et al., 1994, Mimouni et al., 1993, Lasekan et al., 1999). Generally speaking, the results from these studies suggested that SBF support the normal growth and development in

infants fed SBF and no adverse effect compared to infants fed cow's milk-based formula or human milk. The duration of these studies usually was less than 1 year, with SBF feeding exclusively at 0~4-month.

Two studies reported reproductive development in humans fed SBF as infants. One retrospective cohort study (Strom et al., 2001) followed up at age 20~34 y. SBF and control groups came up with similar outcomes in terms of pubertal development, menstrual history, fertility and pregnancy, hormonal disorders and sexual orientation. SBF-fed women reported longer menstrual bleeding and discomfort compared with controls. A case-control study of early breast development found that SBF feeding at early age could not be responsible for the premature breast development because only 20% of cases were fed SBF (Freni-Titulaer et al., 1986). In conclusion, there is no evidence that SBF feeding in infancy adversely affects human health. In addition, in light of the studies of Lamartiniere's laboratory (Lamartiniere et al., 1995a, 1995b, Murrill et al., 1996, Fritz et al., 1998), which found that early exposure to isoflavones might result in later protection from chemical-induced cancer development in rats, SBF feeding in infancy may actually be health beneficial, but this remains to be proven in humans.

Soy isoflavones and thyroid health

Soy consumption may influence thyroid health because goiters were seen in SBF-fed infants, and reversed when iodine supplemented to SBF or a switch to cow or human milk. Rats fed an iodine-deficient diet containing 20% defatted soybean diet developed hypothyroid condition compared with control group fed gluten as the protein source and the group fed 20% defatted soybean diet (Ikeda et al., 2000). Ishizuki et al. (1991) reported after consumed

30 g soybean/d for 1 month, thyroid hormones triiodothyronine, T3 and thyroxine, T4 did not change but thyroid stimulating hormone (TSH) was increased but still within the normal range in 37 healthy adults with iodine-sufficient diet. The elevated TSH decreased to the original levels one month after the experiment. Duncan et al. (1999a) also reported the decreased T3 in 14 premenopausal women, but not in 18 postmenopausal women after consuming 128 mg isoflavones/d for 3 months. Watanabe et al. (2001) found soy isoflavone supplementation resulted in decreased T3 and T4 levels in luteal phase, but increased in follicular phase of the menstrual cycle.

The effect of soy and isoflavones intake on thyroid function was not very clear. A research group has been working in this area using the model of inactivation of thyroid peroxidase (TPO) *in vivo* and *in vitro* (Chang and Doerge, D. R. 2000, Doerge and Chang, 2002). TPO, a heme-containing enzyme, is critical in synthesis of T4 and T3. *In vitro*, genistein at 10 μ M, inactivated microsomal rat TPO by 53%, porcine TPO by 62%, and human TPO by 66%, respectively (Chang and Doerge, 2000). *In vivo*, dose-dependent decreased microsomal TPO activity was found in male and female Sprague-Dawley rats fed soy-free basal diet supplemented with genistein at doses of 5, 100, and 500 ppm, which corresponded to 0.4, 8, 40 mg/kg body weight per day of intake. As much as 80% loss of TPO activity in thyroid was seen in female rats fed 500 ppm genistein and the loss was greater in females than in males but this difference was not significant. Interestingly, although TPO activity lost significantly, thyroid hormone levels, as well as the gland weights and histopathological examination of thyroid sections, did not change compared to the untreated

controls (Doerge and Chang, 2002). These findings were paradoxical in light of the dramatic effects of genistein administration on TPO activity.

The possible effects of soy intake on thyroid health may largely depend on iodine status. The above results suggest that iodine deficiency has synergic effect with soy consumption in inducing thyroid disease in rats (Ikeda et al., 2000). Peri- and postmenopausal women may benefit from using soy products to counteract the symptoms of menopause (Duncan et al., 1999b), but close attention should be paid to this group regarding hypothyroidism and a subclinical hypothyroid state, but so far, there are no clinical studies on the topic.

Effects of isoflavones on immune function

Yellayi et al. (2002, 2003) showed immuno-suppression in humoral and cell-mediated immunity and thymic atrophy when genistein was administered to ovariectomized mice by injection or in the diet. Daily subcutaneous injections of genistein (2~200 mg/kg body weight) for 21 days induced dose-dependent decreases of thymic weight and size in female C57BL/6 mice and the decreased thymocyte numbers was seen in mice fed 200 mg/kg for 21 days. Genistein treatment also impaired humoral immunity at 80 mg/kg per day as reflected in lowering antibody titers. When dietary genistein fed to mice at 1000 or 1500 ppm for 21 days, decreased thymic weight and cell-mediated immunity were found with plasma genistien in the physiological range compared to humans.

Zhang et al. (1999a) showed that daidzein, genistein and their metabolites, daidzein and genistein glucuronides (DG and GG), may activate or inhibit natural killer cell (NK) activity *in vitro*. In human peripheral blood NK cells, genistein at $< 0.5 \mu\text{M}$, DG and GG at

0.1~10 μM , enhanced NK cell-mediated K562 cancer cell killing significantly ($P < 0.05$). At $> 5.0 \mu\text{M}$, genistein inhibited NK cytotoxicity significantly ($P < 0.05$). The glucuronides only inhibited NK cytotoxicity at 50 μM . Although the plasma concentrations of isoflavones could reach 5 μM after soy consumption (Xu et al., 1994 and 1995), isoflavones would be present mainly as glucuronides, which is less toxic than parent compound. Results from *in vivo* and *in vitro* studies showed that isoflavone at physiological level may or may not cause adverse effects in immune system. Clinical studies regarding immune toxicity of isoflavones are limited; thusfar no report shows that isoflavone intake influences immune system health in humans.

Isoflavones and carcinogenesis

Research from our laboratory has shown tumor promotion effect of high dose dietary isoflavone in rats. Lee et al. (1995) found that after 3-month feeding of 920 or 1840 μmol total isoflavones/kg diet, soybean extract suppressed hepatocarcinogenesis promoted by phenobarbital in diethylnitrosamine-initiated rats. But when rats fed 1840 μmol total isoflavones/kg diet for 11 months, there was a cancer promotion effect induced by isoflavones feeding in this group. Rao et al. (1997) also reported that a diet containing 250 ppm genistein enhanced azoxymethane-induced (15 mg/kg body weight for 2 successive weeks) noninvasive adenocarcinomas in colon in male F344 rats. These studies suggested that soybean isoflavones may have cancer-protective effects, but the margin of safety (toxic dose/effective dose) may be low. As discussed before, Helferich's research group showed that *in vivo* and *in vitro*, genistein at low dose exert estrogenic effect, induced MCF-7 cells proliferation but at high dose, inhibited cell proliferation (Hsieh et al., 1998, Allred et al.

2001). Their studies suggested that genistein could be estrogen agonist or antagonist *in vivo* and *in vitro*, which may highly depend on its concentration. This is important in evaluating the potential carcinogenic or chemopreventive properties of genistein.

So far, there is no known evidence show that isoflavones in diet are harmful to humans; however high dose of isoflavones may induce adverse effects. Recently, in a safety-evaluation study by using purified unconjugated isoflavone, 24 postmenopausal women ingested a single dose of purified genistein at dose of 2, 4, 8, or 16 mg/kg body weight, and there was a 7% decrease in systolic and diastolic blood pressure and a 32% decrease in the neutrophil count at 24 h after treatment. Nausea, pedal edema, and breast tenderness were found in some individuals and could be possibly related to the genistein treatment (Bloedon et al. (2002). This study suggested that isoflavones had minimal clinical toxicity when given at high dose exceeding normal dietary intake. More clinical safety studies are needed to better understand isoflavone toxicity-related issue.

Metabolism of Isoflavones

Absorption of isoflavones after ingestion

Isoflavones occur mainly as glucosides in soybean and most of soy foods except fermented soy foods. The conjugated β -glucosides daidzin and genistin are still stable even after processing (Coward et al., 1998). Since isoflavone glucosides have never been found in human blood or urine using HPLC or even more sensitive ESI-MS techniques (Xu et al., 1994, 1995, Setchell et al., 2002), they seemingly cannot be absorbed by the intestine directly. Therefore, isoflavone glucosides must undergo a β -glucosidic bond cleavage to release aglucons before being absorbed by passive diffusion from the intestine (Setchell et al.,

2002). When rats were orally given aglucons and glucosides daidzein and genistein at 7.9 $\mu\text{mol/kg}$ body weight, the appearance of isoflavone glucosides metabolites in plasma was delayed compared to that of aglucons, which suggested that aglucons but not glucosides were absorbed in rat stomach and glucosides need undergo hydrolysis before absorption (Piskula et al., 1999). Both intestinal and bacterial β -glucosidases may be responsible for β -glucosidic bond cleavage. For example, bacterial β -glucosidases have been isolated from human feces and it is well known that gut microflora produce these enzymes (Rowland et al., 1985, Rowland, 1986). Daidzin shown to be metabolized to daidzein by the intestinal bacteria *Bacteroides* J-37 and *Eubacterium* A-44 (Kim et al., 1998). *Enterococci*, one of the predominant species in small intestine, display the highest activity of β -glucosidases, which implies that even in small intestine, gut microflora are involved in hydrolysis of β -glucosidic bound (Turner et al., 2003).

Four mammalian β -glucosidases have been purified in the small intestine. Glucocerebrosidase and lactase phlorizin hydrolase (LPH) are membrane-bound enzymes. Glucocerebrosidase is a lysosomal enzyme and LPH is found in the brush-border of the small intestine (Hays et al., 1996, Leese and Semenza 1973). The other two are cytosolic enzymes: broad-specificity β -glucosidases (Daniels et al., 1981) and pyridoxine glucoside hydrolase (McMahon et al., 1997). Among these enzymes, Day et al. (1998) showed that broad-specificity cytosolic β -glucosidases were responsible for hydrolysis of isoflavone β -glucosides, and later, Day et al. (2000) also found that LPH, purified from sheep small intestine, was capable of hydrolyzing isoflavone β -glucosides daidzin and genistin. The active site is the lactase but not phlorizin hydrolase.

It has not been determined yet whether intestinal or bacterial β -glucosidases are dominant in this β -glucosidic bond cleavage. Some human feeding studies found that the appearance of aglucons and glucosides metabolites in plasma with only a few minutes difference, but the t_{\max} (time needed to reach the highest concentration) of isoflavone glucosides was much longer than aglucons, which suggested that at least part of the hydrolysis is due to bacterial action. It seems like both mammalian intestinal and bacterial hydrolysis are involved in isoflavone absorption. No matter which one is dominant, isoflavone β -glucosides would be cleaved after ingestion.

Biotransformation of isoflavones

After isoflavone aglucons absorbed from the intestine, they are conjugated with glucuronic acid catalyzed by UDP-glucuronosyltransferases (UGTs). This has been shown both in humans and rats (Zhang et al., 2003, Sfakianos et al., 1997). Some of them may also be conjugated with phosphoadenosylphosphosulfate (PAPS) by the action of sulfotransferase (STs) (Sipes and Gandolfi, 1986). The ratio of isoflavone glucuronide versus sulfate conjugates is species-dependent. Zhang et al. (2003) showed that the major isoflavone metabolites found in urine and plasma are 7-*O*- glucuronides in women after consumption of soymilk, with the estimated percentages are 60% and 50% for plasma and urinary daidzein, respectively, and 70% for both plasma and urinary genistein. Sulfates were about 20% of the all metabolites found in plasma and urine for both daidzein and genistein. This phase II biotransformation of isoflavones by UGTs and STs seems to occur mainly in the intestine and liver. When rats infused intestinally with ^{14}C -genistein, the predominant metabolite was its glucuronide in portal vein blood (Sfakianos et al., 1997), which suggested in rats, the

intestinal mucosa was the major biotransformation site. Further glucuronidation may occur in the liver. Isoflavone metabolites may undergo biliary excretion because human feeding studies found that urinary excretions of isoflavone were only 10~50 % of ingested dose (Xu et al., 1994, 1995, Zhang et al., 1999b). In rats, 75% of ^{14}C -genistein was recovered from bile within 4 h after duodenal infusion (Sfakianos et al., 1997). This biliary excretion has significance in isoflavone metabolism: it allows isoflavone glucuronides back into the intestine after initial absorption; given the glucuronidase activity of gut microflora, the isoflavone glucuronides could be deconjugated and release aglucons again. These aglucons might either be reabsorbed via enterohepatic recirculation, or metabolized to other metabolites by gut, e.g., equol and ODMA from daidzein (Bannwart et al., 1984, Axelson et al., 1984) or degraded because the recovery of isoflavone from feces is very low (only a few percent of ingested dose) and less than 50% of ingested dose excreted from urine (Xu et al., 1994, 1995).

Equol, the metabolite of daidzein by the action of gut bacteria, is another estrogenic isoflavone and has been found in plasma and urine only in one-third to two-thirds of individuals after soy consumption which suggests not all individuals can produce equol (Setchell et al., 1984, Lu et al., 1996a, Hutchins et al., 1995). Some individuals seem to lack unidentified microbial species in the gut that catalyze this reaction (Setchell et al., 1984). This led to use of the term “producers” to describe people who have the bacterial enzymes or intestinal conditions for equal production.

Degradation of isoflavones

The degradation of isoflavones by human gut microflora had been shown (Xu et al., 1995). *In vitro* anaerobic incubation of isoflavones with human feces showed that half-life of daidzein and genistein was 7.5 and 3.3 h, respectively. Four *Clostridium* strains (257, 258, 264, and 265) capable of cleaving the C-ring of flavonoids quercetin, kaempferol, and naringenin were isolated from human feces (Winter et al., 1989). It is reasonable to assume that C-ring cleavage of isoflavones also occurs. When daidzein was incubated with human feces for 72 h, dihydrodaidzein, and benzopyran-4, 7-diol, 3-(4-hydroxyphenyl) were purified by TLC and HPLC but only dihydrogenistein was isolated from the fermentation broth of genistein incubated with human feces (Chang and Nair, 1995). The dihydroisoflavones may be an intermediate or a side product in isoflavone degradation by gut microorganisms or may be like equol, only produced by certain individuals because it was found in fermentations from some but not all individuals (Chang and Nair, 1995). Isoflavones can be broken down into other compounds, especially monophenolics, such as the non-estrogenic metabolite of genistein, p-ethylphenol (Verdeal and Ryan, 1979), but at this point, no biological role of this compound has been reported.

Studies have been done in our laboratory showing that gut microorganisms play an important role in metabolism and bioavailability of isoflavones. Eight male subjects provided fresh fecal samples for *in vitro* anaerobic incubation with isoflavones to measure isoflavone disappearance rate constant. These subjects consumed a single dose of soy isoflavones with breakfast (1.2 mg total isoflavones/kg body weight). Plasma daidzein and genistein concentration was negatively correlated with daidzein and genistein disappearance

rate constant ($r = -0.74$, $P = 0.04$; $r = -0.88$, $P = 0.01$, respectively) (Hendrich and Murphy, 2000). In 35 Asian (Chinese immigrant) women, low genistein degraders with short gut transit time (43 h) compared with high degraders with longer GTT (60 h), had significantly greater urinary genistein excretion (11 vs. 4% of ingested dose, respectively) after they consumed a single dose of isoflavone ($4.5 \mu\text{mol/kg}$ body weight), supporting an important role for gut microbial activity in isoflavone bioavailability (Zheng et al., 2003).

In conclusion, isoflavones can be metabolized extensively in some individuals. There is considerable evidence to show that the hydrolysis of the isoflavone glucosides is necessary for their absorption since glucosides have never been detected in plasma and urine. Although the glucosidase can be produced either by the small intestine or by gut microflora, the sites of hydrolysis have not been fully elucidated. Gut microflora are also involved in biotransformation and degradation steps which clearly have great influence on isoflavone bioavailability, therefore potentially affecting their biological effects.

Isoflavone Bioavailability and Kinetics

The bioavailability of isoflavones is clearly important in assessing the likely importance of these compounds to human health. Bioavailability has different interpretations from different research perspectives. From a pharmacological point of view, it is the proportion of the compound that appears in plasma over time (area under the curve) when administered orally compared with that when the compound is administered intravenously. From the nutritionist's point of view, it is the proportion of ingested compound reaching the sites of action. Therefore bioavailability is not indicated entirely by the extent of absorption. Absorption, distribution, metabolism (biotransformation in the gut, liver and other tissues)

and elimination all contribute to bioavailability following ingestion. Bioavailability and kinetics of dietary isoflavones depends upon their chemical structure, dose, and characteristics of their food matrices. The interactions of isoflavones with gut microflora may be also important in determining their bioavailability.

Influence of chemical structure on isoflavone kinetics and bioavailability

Isoflavones in most soy foods are more than 90% in glucosidic form, which must be hydrolyzed before absorption (Brown, 1988, Setchell et al., 2002). No matter which β -glucosidases, mammalian or gut microbial, dominates this hydrolysis, isoflavone aglucons seem to be more rapidly absorbed than glucosides. As discussed early, Piskula et al. (1999) observed that when daidzein, genistein, daidzin and genistin were orally administered to rats at a dose of $7.9 \mu\text{mol/kg}$ body weight in solution, isoflavone aglucons but not glucosides were already absorbed in the rat stomach. Human feeding studies showed similar results. Setchell et al. (2001) compared plasma kinetics of pure standardized 50 mg ($2.8\text{--}3.5 \mu\text{mol/kg}$ body weight) doses of daidzein, genistein and their β -glucosides administered as a single bolus to 19 healthy Caucasian women. The mean time to attain peak plasma concentration (t_{max}) for genistein and daidzein was 5.2 and 6.5 h, respectively, whereas for the corresponding β -glucosides, the t_{max} was delayed to 9.3 and 9.0 h, respectively, consistent with the time needed for hydrolysis before absorption. The mean maximum plasma concentration (C_{max}) was 0.76 and $1.26 \mu\text{mol/L}$ for daidzein and genistein, and was 1.55 and $1.22 \mu\text{mol/L}$ for daidzin and genistin, respectively. In other soy feeding studies in which predominantly glucosides isoflavone were fed, the results were similar to Setchell's isoflavone glucosides feeding study. King and Bursill (1998) gave 6 healthy men a soy flour-

based meal providing 2.7 μmol daidzein and 3.6 μmol genistein/kg body weight and reported that t_{max} was 7.4 and 8.4 h, C_{max} was 3.14 and 4.09 μM for daidzein and genistein, respectively. Watanabe et al. (1998) gave 7 males 60 g of kinako (baked soybean powder), containing 103 μmol daidzein and 112 μmol genistein, t_{max} was 6 h for both daidzein and genistein, C_{max} was 1.56 and 2.44 μM for daidzein and genistein, respectively. Izumi et al. (2000) further investigated the difference in the absorption of soy isoflavone aglucons and glucosides by using fermented and unfermented soybean extract in Japanese men and women at both low and high doses. In low dose feeding trial, 4 men and 4 women consumed 110 μmol isoflavone aglucons (48 μmol genistein and 62 μmol daidzein) or 110 μmol glucosides (60 μmol genistin and 50 μmol daidzin), the dosing of the two forms separated by 2 months. After consumption of aglucons, t_{max} was 2 h for both daidzein and genistein; C_{max} was 1.01 and 0.77 μM for genistein and daidzein, respectively. After consumption of glucosides, the t_{max} was 4 h and C_{max} was 0.40 and 0.20 μM for genistein and daidzein, respectively, which were significantly lower ($P < 0.05$) than that after aglucons intake. In high dose feeding trial (1700 μmol isoflavone aglucons or glucosides: 780 μmol genistein and 920 μmol daidzein; or 900 μmol genistin and 800 μmol daidzin), 4 men and 4 women had mean t_{max} 4 h for isoflavone aglucons daidzein and genistein, and the C_{max} was 21 and 16 μM for genistein and daidzein, respectively, whereas t_{max} was 6 and 4 h and C_{max} was 4.0 and 3.5 μM for isoflavone glucosides genistin and daidzin, respectively. Again C_{max} for glucosides was significantly lower than aglucons ($P < 0.05$). The plasma concentrations of isoflavone aglucons were significantly greater than that of isoflavones glucosides at 2, 4, and 6 h after dosing for both low dose and high dose groups. After 6 h, plasma concentrations of

isoflavone aglucons dropped rapidly from 0.9 to 0.2 μM within 24 h, whereas plasma concentrations of isoflavone glucosides were relatively stable at 0.3~0.4 μM within 24 h after dosing (Izumi et al., 2000). These studies suggested that no matter used as pure compound or in food matrices, consumed by males or females, isoflavone aglucons are absorbed more rapidly than glucosides.

Setchell et al. (2001) did not report significant differences in C_{max} between aglucons and glucosides as well as the apparent bioavailability calculated as AUC but Izumi et al. (2000) found that C_{max} was much higher after feeding aglucons than glucosides ($P < 0.05$), although the later study did not compare AUC between aglucons and glucosides directly. The reasons why there are differences in isoflavone bioavailability and kinetics between the two studies were not clear. One possible reason may be due to the different subjects used in the studies. Japanese are well known to consume soy foods more frequently than Western people, and they have different genetic characteristics and dietary habits compared to Western people as well. It is plausible that Japanese in this study had a somewhat different β -glucosidases enzyme activity compared to Western people, thus Japanese may have lower capacity to hydrolyze glucosides in the intestines than did the Westerns, e.g., Western people may have metabolized the glucoside forms of isoflavones more efficiently than did the Japanese. Due to the possible difference in isoflavone metabolism between the two populations, their isoflavone bioavailability may be different.

The overall bioavailability for isoflavone calculated as area under the curve (AUC) was not significantly different between aglucons and glucosides based on Setchell's study (daidzein:genistein vs. daidzin:genistin, 12:17 vs. 18:16 $\mu\text{mol/L}\cdot\text{h}$, respectively (Setchell et

al., 2001). Similar results were also reported by Richelle et al. (2002), where 6 European women were given a single dose of soy germ (Fujiflavone P 10) extract isoflavone glucosides and aglucons (enzymatic hydrolysis from glucosides) at 1 mg total isoflavone/kg body weight with 1-week between the two feedings. The AUC was not significantly different between aglucons and glucosides (daidzein:genistein:glycitein vs. daidzin:genistin:glycitin, 44:20:11 vs. 50:21:11 $\mu\text{mol/L}\cdot\text{h}$, respectively). Izumi et al. (2000), although not measuring AUC in their study, reported that both genistein and daidzein aglucons were absorbed significantly faster and greater as reflected in plasma concentration than that of glucosides within 0~24h after dosing in 8 Japanese. This difference was observable with either low or high doses of isoflavone. As discussed earlier, a variety of factors may contribute to the different results, including ethnic background, dietary habits and enzyme activities. Most of the studies done in Western people showed that whether dietary isoflavone are present as aglucons or glucosides, the overall bioavailability of isoflavone was not affected. But since this may not be found in Asians, the capacity for hydrolysis of isoflavone glucosides may be crucial in isoflavone absorption.

Influence of dose and time course on isoflavone bioavailability

Many studies have been done to assess the influence of isoflavone doses on urinary excretion and plasma concentrations of isoflavone. In a randomized crossover feeding trail, 12 women were given a single dose of 2.0, 4.0, and 8.0 μmol total isoflavone /kg body weight from soymilk, plasma concentrations of total isoflavone showed linearly dose-response at 6.5 h after doses (0.7, 1.1, and 2.1 μM , respectively) as well as urinary isoflavones (24, 58, and 79 μmol total isoflavone, respectively (Xu et al., 1994). Later, 7

women were given 3 isoflavone-containing meals per day with 5-h interval at doses of 10, 20, and 30 μmol isoflavone /kg body weight separated by 2 weeks, total urinary isoflavone excretion was linearly related to dose (88, 159, and 250 μmol total isoflavone, respectively (Xu et al., 1995). Izumi et al. (2000) also show similar dose-dependent results as reflected in plasma daidzein and genistein concentration at low and high doses. Not similar to these studies, in a randomized, crossover (with 1mo washout) feeding study, Setchell et al. (2003) gave 10 healthy women a single-bolus doses of 10, 20 or 40 g of soy nuts containing daidzin (6.6, 13.2 and 26.4 mg) and genistin (9.8, 19.6 and 39.2 mg), respectively, and found a curvilinear relationship between the plasma isoflavone AUC and the amount of isoflavones ingested. This nonlinear relationship was also found in urinary excretion of isoflavones: 63.2, 54.4 and 44.0 % of ingested dose, respectively, for daidzein; and correspondingly, 25.2, 13.4 and 15.8% of ingested dose for genistein, as dose increased. Although different percentage of plasma and urinary isoflavone were found between doses in this study, these differences were not significant. The 3 doses used in this study were relatively lower and within a narrow range compared to the other feeding studies, so that it may not have been able to distinguish the differences between the doses with the small number of subjects studied. In conclusion, human isoflavone bioavailability shows a dose-dependent response, in most studies, the plasma and urinary isoflavones were relatively proportional to the intake of isoflavones over a wide dose range of 2~30 $\mu\text{mol}/\text{kg}$ body weight.

In longer term feeding studies (up to 1 month), plasma isoflavone concentrations and urinary excretion may be decreased compared with single isoflavone doses. Izumi et al. (2000) showed that when total isoflavone at 300 $\mu\text{mol}/\text{d}$ (3.7~5.2 $\mu\text{mol}/\text{kg}$ body weight), was

given to 8 Japanese men for 1 month, plasma daidzein and genistein were 10~15% lower after 4 weeks compared with that of after 2 weeks. Lu et al. (1995b, 1996b) reported after 1 month of a daily dose of 80~210 mg daidzein and genistein of each from soymilk, urinary isoflavone excretion was 10% lower than initial feeding in women but not in man. It is not clear why long term feeding may result in decreasing isoflavone bioavailability, but this may be due to induction of isoflavone degrading gut microbial enzyme activities after frequent consumption of soy foods; the more isoflavone being degraded, the lower the isoflavone bioavailability.

Influence of dietary factors on isoflavone bioavailability

Soy food type may not influence isoflavone bioavailability. As mentioned before, isoflavone in most soy foods are glucosides, but it seems like the chemical form of isoflavone does not affect their bioavailability based on the studies of Setchell and Richelle (Setchell et al., 2001, Richelle et al., 2002). The malonyl or acetyl glucosides may also have no effect because when isoflavone bioavailability of tofu (relatively high in acetyl glucosides) and texturized vegetable protein (TVP, relatively high in malonyl glucosides) were compared in women, urinary and plasma isoflavones did not differ between tofu and TVP, which contained the same amount of isoflavones (Tew et al., 1996). In another study, a single dose of isoflavones from tofu, cooked soybeans, TVP and tempeh was consumed by 10 women in a randomized crossover feeding study. No significant difference was found in urinary daidzein (38~50% of ingested dose) and genistein excretion (9~16% of ingested dose) (Xu et al., 2000). Other dietary interventions such as different background diets did not influence isoflavone bioavailability (Xu et al., 2000). Three diets, an *ad libitum* diet, basic foods diet

and self-selected diet were randomly assigned to 8 women in a crossover design with the same amount of isoflavone intake. Urinary and fecal isoflavone recoveries as a percentage of intakes were not significantly different among the three background diets (25, 27, and 25%, respectively). Although there are few studies regarding the influence of dietary factors on isoflavone bioavailability, seemingly, neither soy food types nor background diet affect short-term isoflavone bioavailability.

Influence of gut microflora on isoflavone bioavailability

The activities of gut microorganisms play an important role in isoflavone metabolism and bioavailability. Xu et al. (1995) reported that among 7 adult women who consumed a single isoflavone-containing meal at 3 doses of isoflavone from soymilk (2-week washout between each dose), two of the subjects consistently excreted significantly greater amounts of isoflavones in feces and had 2~3-fold greater urinary and plasma isoflavone compared with the other five subjects who excreted small amounts of fecal and urinary isoflavones. Considerable interindividual variation of isoflavone excretion was also reported in a 9-day feeding study with 3 doses of isoflavone from soy protein in which the variation of genistein excretion was 12-fold and that of daidzein was 15-fold even within the same dose treatment (Slavin et al., 1998).

In vitro human fecal isoflavone degradation phenotypes have been identified based on isoflavone degradation half-lives (Hendrich et al., 1998). In 20 human subjects, *in vitro* anaerobic incubation of daidzein and genistein with feces showed three distinct isoflavone degradation phenotypes with significantly different isoflavone degradation rate constant, k , (the negative slope of the regression line plotted for log concentration of isoflavone of each

sample over time). The three daidzein degradation phenotypes were: high ($n = 5$, $k = 0.299$), moderate ($n = 10$, $k = 0.055$) and low ($n = 5$, $k = 0.012$). The high and moderate phenotypes were stable when checked 10 months later but not for the low phenotype: high ($n = 5$, $k = 0.326$), moderate ($n = 4$, $k = 0.073$) and low ($n = 5$, $k = 0.053$). For genistein degradation, the rate constants were 0.299, 0.163 and 0.023 for high, moderate and low degradation phenotypes, and 10 months later, they were 0.400, 0.233 and 0.049 for high, moderate and low phenotypes, respectively. When 8 men of varying degradation phenotypes were fed soymilk, plasma isoflavones negatively correlated with fecal isoflavone degradation rate constant ($r = -0.88$, $P = 0.04$ and $r = -0.74$, $P = 0.01$ for daidzein and genistein, respectively) (Hendrich and Murphy, 2000). These data suggest that part of the interindividual variability in isoflavone bioavailability is due to the variation of gut microbial isoflavone degradation. When a bioavailability study only involved moderate isoflavone degraders, the inter-subject variation in urinary isoflavone excretion was 5~8-fold. Although this variation is much less than that reported when fecal isoflavone degradation phenotype was not controlled for, it may suggest that other unidentified factors may also affect isoflavone bioavailability (Zhang et al., 1999b).

In a subsequent study in which isoflavone degradation phenotypes were identified by *in vitro* anaerobic incubation of isoflavone with fresh human fecal sample (Zheng et al., 2003), three daidzein and two genistein degradation phenotypes were identified in 33 Caucasian and 35 Asian (Chinese immigrant) women based on their isoflavone degradation rate constant. Daidzein degradation rate constant was < 0.15 , $0.15\sim 0.30$ and > 0.30 for low, moderate and high phenotypes, respectively. Genistein degradation rate constant was ≤ 0.3

and > 0.3 for low and high phenotypes, respectively. Fecal incubations of isoflavones were repeated 5 months later to check the phenotypic stability, and gut transit time (GTT) was measured for each subject and a single meal feeding study was conducted at the same time, providing 1.2 mg total isoflavone (4.5 μ mol) /kg body weight. The results showed that the degradation phenotypes were stable except for high degradation phenotype; urinary genistein excretion was significantly higher in Asian low genistein degraders who had significantly shorter GTT (43 h) than did Asian high genistein degraders (60 h) and both Caucasian low and high degraders (~84 h) who had significantly longer GTT than did Asian subjects (Zheng et al., 2003). These results suggested that isoflavone degradation by gut microorganisms coupled with GTT had greater influence on isoflavone bioavailability. GTT may be a determinant of isoflavone bioavailability because the shorter residence time in the gut, the less isoflavone can be degraded, and the more isoflavone can be absorbed.

The other aspect of isoflavone bioavailability is the production of equol and other metabolites. Equol was the first isoflavone to be identified in human urine and blood (Axelson et al., 1982, Setchell et al., 1984). It is produced by the action of gut bacteria. Some evidence supported this view: equol was detected and identified when soy protein incubated with human fecal bacteria (Setchell et al., 1984); germ-free rats did not excrete equol in urine on a soy diet, whereas conventional rats did (Bowey et al., 2003); a human soy feeding study showed the appearance of equol in the plasma was delayed 6~8 h compared with other isoflavones, which suggested equol produced in the large intestine (Setchell et al., 2001). Because of the greater binding affinity of equol for the estrogen receptor than that of daidzein (Shutt 1976, Shutt and Cox 1972), and because its binding to serum SHBG is much lower

(Nagel et al., 1998), equol has greater estrogenic potency than daidzein. But at this point, there is limited evidence regarding the beneficial effect of equol. Other metabolites such as ODMA, has been identified in urine and plasma of human subjects who consumed soy products (Kelly et al., 1993), but whether they have benefits to human or not is not clear.

The bioavailability of dietary isoflavones is clearly crucial in assessing the importance of these compounds to human health. Bioavailability is also a major determinant of the safety and efficacy of dietary isoflavones. Bioavailability is the access of a compound to its sites of action. Gut microflora are involved in isoflavone absorption degradation and biotransformation to other metabolites, therefore influencing their bioavailability to a great extent.

Gut Microflora and Gut Transit Time (GTT): Factors Influencing the Environment for Isoflavones Metabolism and Absorption

The intestine, an extremely active and complicated system, plays an important role in living organisms. The basic functions of intestine include food digestion, nutrients absorption and defense against environmental adverse stimuli (Bourlious et al., 2003). The interactions between host, nutrients and microflora are dynamic, diverse and important in maintaining the normal functions of intestine. The gastrointestinal (GI) tract includes stomach, small intestine, and large intestine (colon). Small intestine can be subdivided into duodenum, jejunum and ileum, and is responsible for majority of digestion and absorption. The transit time of food through GI tract (from mouth to anus) is highly dependent on host status, usually 3~6 h from mouth to distal part of small intestine, and at least 12 h before elimination through rectum and anus. Greater inter-individual variation in gut microflora species has been

reported, but the population distribution and colonization of gut microflora usually are the same from stomach to large intestine (Turner et al., 2003). The stomach has very low population of bacteria ($10^2\sim 10^3$ colony forming units (CFU)/g or mL) because of the acidic environment. These species include facultative anaerobic *Streptococcus*, *Staphylococcus* and *Lactobacillus*. The small intestine contains $10^3\sim 10^9$ CFU/g bacteria with a dramatic increase in ileum ($10^5\sim 10^9$). The species are similar as found in stomach except in ileum, where a greater variety such as *Bifidobacterium*, *Bacteroides*, *Clostridium* and members of *Enterobacteriaceae* also can be found because the lower oxygen content allows obligatory anaerobes to grow. The largest bacteria population is found in colon ($10^9\sim 10^{14}$), more than 400 species have been isolated and the obligatory anaerobes including *Bifidobacterium* and *Eubacterium* are predominant. It has been estimated that one-third of fecal dry weight is viable bacteria (Turner et al. 2003).

Factors influence gut microbes colonization

Many factors influence microbial colonization including maternal flora, model of birth and feeding method, genetic background, diet composition, use of prebiotics, probiotics and antibiotic drugs, and age (Bourlouis et al., 2003). Based on these factors, colonization could be substantially divided into several stages: from birth to before introduction of solid foods, from solid foods introduction to ~2 y and then through adult and elderly. At birth, fetus is contaminated with mother's fecal and vaginal or obstetric care professional's skin microbes dependent on mode of birth, subsequently from air, food and environment such that this initial colonization develop rapidly. Then different feeding method results in different species distribution. In breastfed infants, *Bifidobacteria* are predominant, with only about 1%

of *E. coli*, *Enterococci* and *Lactobacilli*. In bottle-fed infants, species such as *E. coli*, *Streptococci*, *Bacteroides*, and *Eubacteria* are present in significantly greater amount than that of breastfed infants (Bourlious et al., 2003). After introduction of solid foods, the differences in species distribution between breastfed and bottle-fed infants are decreased and the species become rather stable up to 2 years of age. The microbes in children are similar to that in adults, which may be affected by many environmental factors. In elderly, *Bifidobacteria* decrease, but *Lactobacilli* and *Clostridium* increase (Rastall and Maitin, 2002).

Among those environmental factors, the diet composition and the use of probiotics, prebiotics and antibiotic drugs exert significant influence on distributions of species. For example, high-beef diet significantly introduced species of *Bacteroides* and *Clostridia*, but lowered *Bifidobacterium* counts (Hentges et al. 1977). High-carbohydrate diet resulted in larger proportion of *Eubacteria*, but fewer *Bacteroides* (Peach et al. 1974). Prebiotics such as fructooligosaccharides, added to the diet, significantly increase *Bifidobacterium*, and decrease *Bacteroides* (Roberfroid, 1996). Supplementation of probiotic *Bifidobacterium bifidum* has been shown to influence flora distribution and prevent the diarrheal disease in hospitalized children (Saavedra et al., 1994). *Lactobacillus rhamnosus* improves the digestion of lactose and eliminates symptoms in lacto-intolerance people. Antibiotic drugs often cause microflora disorder, resulting in diarrhea. Genetic background may also influence the distribution of species because similar species distribution was found between identical twins living apart, which implied that living environment was less important than host genotype in determining bacterial distribution (Zoetendal et al., 2001). Due to the nature of intestinal microflora being

diverse and dynamic, many factors may influence their colonization; but relatively, the whole system is stable.

Factors influencing gut transit time.

Gut transit time refers to the residence time in the GI tract, and reflects gut motility. The phases of gut motility include gastric, small intestinal and colonic. The transit time in the small intestine varies in individual, but usually within 3~6 h, food can be transported to cecum (Staniforth et al., 1991). The colonic phase of GTT is the longest one. Several methods have been used to determine GTT including visible dye, plastic pellets and radio-opaque pellets. GTT is affected by many factors include age, gender, physical activity level and most importantly, dietary fiber. Dietary fiber has a variety of effects on transit time. In the stomach, dietary fiber tends to delay the gastric emptying, therefore food is retained in the stomach; in the small intestine, the effect of dietary fiber on transit time is not fully understood. In the large intestine, dietary fiber softens and causes bulk effect on stool, hence decreases transit time. Many studies have shown that increased fiber intake is associated with increased stool frequency, stool weights, and decreased gut transit time. When 44 human subjects switched from low-fiber diet containing 6 g/1000 kcal to high-fiber diet containing 19 g/1000kcal, the transit time was decreased from more than 70 h to 45 h ($P < 0.05$), fresh fecal weight increased from 69 g to 184 g ($P < 0.05$) (Stasse-Wolthuis and Katan, 1978). In an observational study where 29 male and female subjects consumed self-selected diet containing 4~19 g dietary fiber, fecal weighs not only were correlated with fiber intake ($r = 0.524$, $P < 0.0001$), but also correlated with caloric intake ($r = 0.543$, $P < 0.0001$), number of stools was correlated with fecal weight ($r = 0.62$, $P < 0.0001$) (Kelsay and Clark, 1984).

Another intervention study showed in 5 young males, as the dietary fiber amount increased from 0 to 53 g, the frequency of evacuation increased from 2.8 to 4.4 times in 4 days (Saito et al., 1990). Gender differences regard to gut motility can be seen in stool weights (men 162 g, women 83 g) and this difference could be explained entirely by differences in transit time (men 44.8 h, women 91.7 h) in 19 men and 11 women when they consumed basal (white bread) diet (Stephen et al., 1986). Age-related gut motility change is seen in elderly. In a retirement home, 21 subjects (18 women and 3 men), aged 68~87 y (mean = 81 y), some of them using laxative drugs regularly, had average transit time 3.0 days which was much longer than young or middle-aged subjects (Mykkanen et al., 1994).

Physical activity level is another important factor that may influence GTT. High activity was associated with increase gut motility as seen in long distance runners, where diarrhea was reported (Sullivan, 1981). Increased mass movement after meals was seen in physically active individuals but not in sedentary counterparts (Holdstock et al., 1970). A study tried to characterize the effect of exercise on large intestine function by training 14 sedentary men and women for 7~9 weeks on a constant diet. The monitored physical fitness were improved significantly but no changed was observed overall in fecal weight (124 vs. 129 g/d), transit time (55 vs. 54 h) and fecal frequency between control and exercise period (Bingham and Cummings, 1989). The reason that results from this study are in conflict with the observational study may due to the relatively short period of training.

Changes of GTT may alter microbial growth in human colon. The human colon contains about 230 g of bacteria, and bacteria account for 55% of dry fecal weight in humans (Banwell et al., 1981). An intervention study showed that when GTT decreased from 64 h to

25 h by giving laxative drug senokot in 6 subjects, stool weight increased from 148 to 285 g/d (wet weight) and bacterial mass increased from 16.5 to 20.3 g/d (dry weight, $P < 0.025$). When GTT was prolonged by codeine from 47 to 87.6 h in 5 subjects, fecal weight decreased from 182 to 119 g/d, and bacterial mass decreased from 18.9 to 16.1 g/d. A significant correlation between transit time and bacterial mass was seen ($r = -0.77$, $P < 0.001$) (Stephen et al., 1987). This study suggested that GTT has a controlling influence over the colonic microflora and function. The difference in gut motility and microbial function in individuals on the similar diet may largely be explained by the difference of GTT. If the diet is important in determining the intestinal function through the influence on microflora, GTT does too.

The changes of GTT and gut microflora influence isoflavone metabolism and absorption

The subsequent changes of GTT and gut microflora may significantly influence isoflavone metabolism and absorption as reflected in the change of microbial enzyme activities. A small pilot study showed that feeding 10^{10} *L. acidophilus* daily reduced fecal β -glucuronidase in 7 human subjects (Goldin et al., 1980), then in 21 subjects, daily supplementation of milk with 2×10^6 per mL viable *L. acidophilus* for 4 weeks resulted in significant decrease of β -glucuronidase activity compared to that when the subjects consumed control diet and the enzyme activity rose up back within 30 days after stopping the supplementation (Goldin and Gorbach et al., 1984). When 4 healthy subjects (2 males and 2 females) consumed their regular diet supplemented with 18 g pectin, the fiber supplementation decreased the concentration of gut microbes in feces, in addition, fecal β -glucuronidase and β -glucosidase activities per g of feces were decreased 50% compare to that

of control diet (Mallett et al., 1987). Fiber supplementation results in decreased β -glucuronidase activity were also found in chicks (Indira et al., 1980) and rats (Shiau et al., 1983). β -glucosidase and β -glucuronidase are responsible for releasing isoflavone aglucons, therefore important in isoflavones absorption. It has been shown that *Enterococci*, *Lactobacillus*, *Bacteroides*, and *Bifidobacterium* have strong β -glucosidase activity and *Enterobacteria*, *Clostridium*, and *Bacteroides* have strong β -glucuronidase activity (Turner et al., 2003). Any changes that cause significant changes in these species distribution or enzyme activity would cause changes in isoflavone absorption.

The gut microflora is also important in isoflavones metabolism. Antibiotic use impaired the balance of gut microbes, hence when human subjects were administered antibiotic, the excretion of bacterial metabolites of isoflavone such as equol was decreased (Rowland et al., 1999). Infants fed with soy-based formula also showed lower excretion of equol maybe mainly due to the lack of gut bacteria during early life stage (Setchell et al., 1997).

It is well-known that gut microflora extensively metabolize isoflavone. GTT influence gut microflora species and their enzyme activity significantly. Therefore GTT and gut microflora play important roles in determining isoflavone bioavailability.

Soybeans are a major source of phytochemicals including phytoestrogenic isoflavones, which has been identified as having health protective effects to humans. The isoflavones occur predominantly as β -glucoside forms in soybeans and foods derived from soybeans, which affects their metabolism. The biological effects and some toxicity-related issue of isoflavones raised more attention and research interests. To better assess the potential

health effects of isoflavones as well as adverse effects, their bioavailability must be better understood. Optimizing isoflavone bioavailability is likely to permit more conclusive studies of these compounds.

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**COMPARATIVE BIOAVAILABILITY OF ISOFLAVONE GLUCOSIDES
AND AGLUCONS IN WOMEN**

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ABSTRACT

The bioavailability of isoflavone aglucons and glucosides were compared in 25 women from sources rich in glycitein versus a source rich in genistein. The effects of isoflavone degradation phenotype and gut transit time (GTT) on isoflavone bioavailability were determined as well. We hypothesized that individual with a high daidzein degradation phenotype (daidzein degradation rate constant, $D_k > 0.30 \text{ h}^{-1}$) and $\text{GTT} > 80 \text{ h}$ would show less apparent isoflavone absorption as reflected in urinary excretion. The ingestion of isoflavone glucosides and aglucons would lead to similar urinary excretion over 24 h after ingestion at breakfast. Thirteen subjects with $D_k > 0.30 \text{ h}^{-1}$ and mean $\text{GTT} 106 \pm 11 \text{ h}$ and 12 subjects with $D_k < 0.20 \text{ h}^{-1}$ and mean $\text{GTT} 71 \pm 12 \text{ h}$ consumed unfermented soygerm (1.1 μmol total isoflavones /kg body weight), fermented soygerm (3.3 $\mu\text{mol}/\text{kg}$) or soybean isoflavone extracts (1.5 $\mu\text{mol}/\text{kg}$) for 7 days with 4 or 5 subjects of each daidzein degradation phenotype per treatment. Urine and blood were collected on day 1 and 7. Reverse-phase HPLC analysis showed significant differences in urinary isoflavone excretion (% of daily ingested amount, mole based); between 0~12 h, fermented soygerm > soybean isoflavone extracts (40.2 ± 4.9 vs. 18.7 ± 4.6 , $P < 0.05$); between 12~24 h, unfermented soygerm > soybean isoflavone extracts (16.0 ± 1.8 vs. 7.1 ± 1.8 , $P < 0.05$). Over 24 h, urinary excretions of both types of soygerm were greater than soybean isoflavone extracts (51.5 ± 6.1 and $50.8 \pm$

5.6 vs. 25.7 ± 5.8 , $P < 0.05$). Subjects of the low daidzein degradation phenotype had significantly greater urinary isoflavone excretion than did subjects of the high daidzein degradation phenotype (51.6 ± 4.8 vs. 33.8 ± 4.7 , $P < 0.05$). Total estimated plasma isoflavone (% of daily ingested amount, mole based) was significantly different between fermented soygerm and unfermented soygerm at 3 h after feeding (1.8 ± 0.3 v. 0.5 ± 0.3 , $P < 0.05$). Daily urinary isoflavone excretion was similar for aglucons and glucosides, but a glycitein rich isoflavone source was apparently absorbed to a greater extent than an isoflavone source rich in genistein. Apparent absorption of isoflavones was significantly greater in subjects of the low daidzein degradation phenotype coupled with shorter GTT than in subjects of the high daidzein degradation phenotype with longer GTT as reflected in total urinary isoflavone excretion.

KEY WORDS: isoflavones · aglucons · glucosides · gut transit time (GTT) · bioavailability

Soybean isoflavones are major phytoestrogens in the human food supply. Isoflavones are implicated in cholesterol-lowering effects of soy protein, because soy protein containing ≥ 37 mg isoflavones/d lowered cholesterol in humans (1). Soy protein containing isoflavones also reduced bone loss from the lumbar spine in perimenopausal women (2), suggesting that these compounds may prevent osteoporosis. Soy intake is also positively associated with the lower risk of hormone-dependent cancer in premenopausal women (3).

To better assess the potential health effects of isoflavones, their bioavailability must be better understood. Optimizing isoflavone bioavailability is likely to permit more conclusive studies of these compounds. Isoflavones exist in most soy foods and ingredients mainly as glucosides. Fermentation releases aglucons. Controversy exists as to what forms of

isoflavones are absorbed quickly and retained the longest in the body. Previous work in our laboratory comparing soy foods rich in isoflavone aglucons (tempeh, a fermented product) with soy foods rich in glucosides (cooked soybeans or tofu) supported the view that the aglucons and glucosides were similar in bioavailability as reflected in urinary isoflavone excretion (4). On the other hand, some studies showed that isoflavone bioavailability was greater when fermented soy foods rich in aglucons consumed (5, 6). Recently Izumi et al. (7) reported that soy isoflavone aglucons were absorbed faster and in greater amounts than their glucosides in humans. But Setchell et al. (8) and Zubik and Meydani (9) showed little difference in plasma isoflavone concentrations over time in humans fed glucosides compared with aglucons. Furthermore, bioavailability of isoflavones was not altered when aglucons hydrolyzed enzymatically from glucosides were consumed compared with that of glucosides (10). More work is necessary to better understand the bioavailability of the two major forms of isoflavones.

Many isoflavone bioavailability studies have been done recently, and most studies focused on isoflavone glucosides (11, 12). King and Bursill (13) showed isoflavone excretion patterns and pharmacokinetics in humans given 2.7 μmol daidzein and 3.6 μmol genistein/kg body weight; of daidzein and genistein were similar in terms of plasma concentrations, notwithstanding the greater urinary excretion of daidzein than of genistein. Watanabe et al. (14) fed 60 g baked soybean powder (103 μmol daidzein and 112 μmol genistein) and concluded that genistein was the most important isoflavone in plasma due to its greater concentration and longer half-life compared with daidzein. But, insofar as urinary excretion reflects overall isoflavone absorption and disposition, daidzein has been shown in several studies to have

2~3 times greater apparent absorption than genistein (12, 15,16). Glycitein and its glucosides are minor component in most soy products, accounting for 5~10% of total isoflavones. But in soygerm, glycitein accounts for about 40~50% of total isoflavone. One bioavailability study showed that glycitein was equal to daidzein in bioavailability, and both were more available than genistein in terms of urinary excretion (16). Glycitein was more estrogenic than genistein in mice (17), so more studies of the effects and bioavailability of glycitein are in order.

Great interindividual variation of isoflavone bioavailability after consumption of isoflavone-containing products has been reported (12, 16) and gut microorganisms are suggested to play an important role in isoflavone degradation, hence preventing isoflavone reabsorption after initial rapid biliary excretion. High, moderate, and low isoflavone degradation phenotypes have been identified in humans (18). Gut transit time was positively correlated with daidzein degradation rate constant ($r = 0.35$, $P < 0.05$) and negatively correlated with total urinary isoflavone excretion ($r = - 0.24$, $P < 0.05$) in 35 Asian (Chinese immigrants) and 31 Caucasian (Iowan) women who were identified within high, moderate and low daidzein degradation phenotypes, and who consumed a single dose of soy isoflavone $4.6 \mu\text{mol/kg}$ in soybean powder (19). Thus GTT may affect isoflavone bioavailability. Differences in isoflavone degradation phenotype and GTT are suggested to be responsible for much of the variation in isoflavone bioavailability between individuals (19). Further work on isoflavone degradation phenotype, GTT and bioavailability of isoflavones may help in designing human feeding studies of health effects of these compounds.

In the present study, we compared bioavailability of isoflavones in their two major forms, aglucons and glucosides, and between two isoflavone sources, one rich in glycitein and one rich in genistein in women fed the isoflavone sources at breakfast for 7 days. We determined whether daidzein degradation phenotype and GTT influenced urinary excretion and plasma concentrations of isoflavones over time.

MATERIALS AND METHODS

Experimental Procedures

Chemicals. Isoflavone aglucons, daidzein, genistein, glycitein and 2, 4, 4'-trihydroxybeozoin (THB), used as internal standard, were synthesized in Dr. Murphy's laboratory, Iowa State University (20, 21). Brain-heart infusion (BHI) medium was purchased from DIFCO Laboratories (Detroit, MI). Cysteine and resazurin were purchased from Aldrich Chemical Company (Milwaukee, WI). Milli-Q HPLC grade water (Millipore Co., Bedford, MA) was used. Other HPLC solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Subjects. Subject prescreening was conducted by *in vitro* anaerobic fecal incubation to identify high and low daidzein degradation phenotypes. Briefly, a freshly voided fecal sample was incubated anaerobically with autoclaved brain-heart infusion (BHI) culture medium (DIFCO Laboratories, Detroit, MI) and daidzein (100 $\mu\text{mol/L}$). Daidzein degradation rate constant, D_k , was calculated as the negative slope of the regression line plotted for log concentration of isoflavone of each sample over time (19). The participants in this study were 25 healthy women, 20 Caucasians (Iowan) and 5 Chinese (immigrant); 13 of them of the high daidzein degradation phenotype (including 1 Chinese) ($D_k > 0.30 \text{ h}^{-1}$) and 12

(including 4 Chinese) of the low daidzein degradation phenotype ($D_k < 0.20 \text{ h}^{-1}$), 18~40 year of age, body mass index (BMI), $23.2 \pm 1.0 \text{ kg/m}^2$ (Table 1). The experimental procedures for this study were approved by the Human Subjects Committee of Iowa State University. Subjects gave their informed consent to the protocol.

Experimental design. Three sources of soy isoflavone were used in this study: unfermented soygerm (Soylife®, Acatris Holding BV, Giessen, The Netherlands), fermented soygerm (Soylife®, Acatris Holding BV, Giessen, The Netherlands), and soybean isoflavone extract (Novasoy®, ADM Nutraceutical, Decatur, IL). The soygerm-derived products were characterized by an isoflavone mole ratio, 3 daidzein: 1 genistein: 3 ~ 4.5 glycitein. The soybean-derived product showed the isoflavone mole ratio, 3 daidzein: 4.5 genistein: 1 glycitein (Table 2). Thirteen high daidzein degraders and 12 low daidzein degraders were randomly assigned to the three treatments, providing $1.1 \mu\text{mol}$ total isoflavones/kg body weight for unfermented soygerm, $3.3 \mu\text{mol/kg}$ for fermented soygerm, and $1.5 \mu\text{mol/kg}$ for soybean isoflavone extracts, respectively. Isoflavone-containing powder was mixed with orange or apple juice given for 7 days at breakfast between 7:30 and 8:30 am in the Human Metabolic Unit, Iowa State University. Two gelatin capsules contained 16 ceramic marker beads were given at the same time to measure GTT. Other foods were chosen from white bread, cereals, skim milk, peanut butter, jelly, apple and orange and consumed *ad libitum*. All subjects were instructed to consume only isoflavone-free foods for 1 week before and during the experiment. A list of isoflavone-containing foods was given to each subject (See Appendix C.).

Biological sample collection. After an overnight fast, 10 mL venous blood samples were collected into EDTA-containing vacuum containers by a licensed phlebotomist under stringent aseptic conditions before isoflavone feeding (time 0) and at 3 and 24 h after feeding on day 1 and 7. Blood samples were centrifuged within 1 h after collection at 3000 g for 30 min at 4° C (Model 4d, International Equipment Co., Needham Heights, MA). Plasma was stored at -20° C until analysis. Each subject gave a urine sample right before the breakfast test meal on the first day of the experiment (time 0). After the breakfast, urine from each subject was collected and pooled over the following time intervals: 0~12 h, 12~24 h on day 1 and 7. The volume of urine at each time interval was recorded and 50 mL aliquots were stored at -80° C until analysis. To measure GTT, after ingestion of 16 ceramic beads with breakfast on day 7, all feces were collected until 12 or more marker beads had been excreted.

Analytical Methods

Soy products analysis. Samples in duplicate from each source were extracted and isoflavone concentrations determined by HPLC (22, 23) with THB as internal standard. The total isoflavone content was the sum of mole amounts of total daidzein, genistein and glycitein normalized to the aglucon forms (Table 2).

Plasma and urine isoflavone analysis. Urine samples were prepared by methods modified from Lundh et al. (24) as previously described (19). For plasma samples, 1 mL was incubated with 1 mL 0.2 mol/L sodium acetate buffer (pH = 5.5), 50 μ L β -glucuronidase/sulfatase (H₂ type, Sigma Chemical, St. Louise, MO), and 50 μ L THB (2g/L) as internal standard in 37° C water bath for 18 h to release isoflavone aglucons. After incubation, 1 mL 10 μ mol/L sodium phosphate buffer (pH = 7.0) was added to the mixture,

and the mixture was loaded onto 5 mL Extrelut™ QE column (EM science, Gibbstown, NJ) and extracted with ethyl acetate. The eluents were dried under nitrogen. Extracts were reconstituted in 200 μ L methanol and water (80:20 v/v). The HPLC analysis followed Zhang et al. (16) with minor modification: THB and three isoflavone aglucons were separated and quantified on a YMC-Pack ODS-AM C₁₈ reverse-phase column (5 μ m, 25 cm \times 4.6 mm i.d.) (YMC, Wilmington, NC), the mobile phase was 1 mL glacial acetic acid /L in water (A) and methanol (B).

Plasma isoflavone contents determined as a percentage of ingested dose.

Because different doses were fed of each isoflavone source, plasma isoflavone content was expressed as an estimated percentage (mole based) of ingested dose present in plasma at the time point measured. Total plasma volume (mL) was estimated based on subject body weight and height (25), and multiplied by isoflavone content/mL; this total plasma isoflavone content was divided by the amount of isoflavone ingested at breakfast on day 1 or 7.

Recovery study. Synthesized daidzein, genistein, glycitein, THB and blank urine and plasma samples from one subject were used to determine analytical recoveries for each isoflavone. Fifty μ L of THB (2 g/L) and a series of isoflavone standards, between 6.25~100 μ mol/L for daidzein and genistein and 6.55~131.5 μ mol/L for glycitein, were added to urine and plasma samples. Each standard was run in duplicate using the same extraction and analysis method as above. Analytical recoveries for urinary daidzein, genistein, glycitein and THB were 82.2 %, 79.3 %, and 84.5%, and 86.5 %, respectively; for plasma daidzein, genistein, glycitein and THB were 75.2 %, 69.2 %, 70.2 %, and 79.5 %, respectively. Plasma

and urinary isoflavones from the feeding study were calculated according to the internal standard curves for each individual isoflavones, THB used as internal standard.

Statistical analysis. Statistical analysis was performed by SAS (SAS Institute, Inc., version 8.2, 2001, Carry, NC). Analysis of variance using general linear model was conducted to determine treatment and phenotypic differences. When treatment differences were found, multiple comparisons using Tukey's test was performed to identify the difference. Clustering test (based on the simple linkage method) was used to identify daidzein degradation phenotypes. Pearson's correlation analysis was used to determine the correlation in plasma and urinary isoflavone contents between day 1 and 7, and between GTT, D_k , and urinary total isoflavone excretion. All results were reported as mean \pm SEM, statistical significance at $P < 0.05$.

RESULTS

Urinary excretion of total isoflavone in the three treatments. The urinary isoflavone excretion expressed as % of ingested amount was calculated as the mean of day 1 and 7 for each subject because isoflavone excretions on the two days were highly correlated ($r = 0.94$, $P < 0.001$). Significant differences were found in urinary total isoflavone excretions among the three treatment groups (Table 3). Between 0~12 h, subjects who consumed fermented soygerm excreted significantly greater amounts of isoflavones than did subjects who consumed soybean isoflavone extract, ($P < 0.05$). Between 12~24 h, isoflavone excretion from subjects fed unfermented soygerm was significantly greater than that of soybean isoflavone extract ($P < 0.05$). Total 24 h urinary isoflavone excretion from both groups fed soygerm was significantly greater than that from subjects fed soybean isoflavone

extract ($P < 0.05$, Table 3). Twenty-four hour urinary excretion of isoflavones from the two soygerm-fed groups did not differ significantly. Urinary total isoflavone excretion was significantly greater between 0 and 12 h than between 12 and 24 h in all treatment groups ($P < 0.05$) (Table 3). The excretion during 0 and 12 h was highly correlated with 24 h excretion ($r = 0.97$, $P < 0.0001$).

Urinary excretion of isoflavone in high and low daidzein degraders. Significant phenotypic differences were found in overall urinary total isoflavone excretion between 0 and 12 and 0 and 24 h between high and low daidzein degradation phenotypes (Table 4). Subjects within the low degradation phenotype excreted significantly greater amounts of isoflavones than did subjects of the high degradation phenotype ($P < 0.05$). Between 12~24 h, urinary excretion did not differ between high and low degradation phenotypes.

For individual isoflavones, significant phenotypic differences in urinary daidzein and glycitein excretion were found between high and low daidzein degradation phenotypes (Table 5). Urinary excretion of daidzein and glycitein was significantly greater in subjects of the low daidzein degradation phenotype than in subjects of the high degradation phenotype ($P < 0.05$). Urinary daidzein and glycitein were excreted to a significantly greater extent than was genistein in all treatment groups, but excretion of daidzein and glycitein did not differ significantly (Table 5).

Plasma isoflavone contents in high and low phenotypes and treatments.

Plasma isoflavone expressed as % of ingested amount was calculated as the mean of day 1 and 7 for each subject. Intake of fermented soygerm caused greater plasma total isoflavone than did unfermented soygerm at 3 h after ingestion ($P < 0.05$) and greater plasma

daidzein than did both unfermented soygerm and soybean isoflavone extract ($P < 0.05$). No difference was found in total plasma isoflavone between high and low daidzein degradation phenotypes (Table 6). Total plasma isoflavone at 24 h after feeding was not detected. The main plasma isoflavone found at 3 h after feeding was daidzein. Genistein was only detected in 8 subjects (2~3/treatment) and no glycitein was detected in plasma at 3 h after feeding in any subject (Table 6).

Correlations between GTT, D_k , and urinary isoflavone excretion. There was no significant difference in GTT among the three treatments (Table 3). GTT was significantly different between high and low daidzein degradation phenotypes (Table 4). Subjects of the high daidzein degradation phenotype had significantly longer GTT than subjects of the low degradation phenotype ($P < 0.05$), and their total urinary isoflavone excretion was significantly less than subjects of the low daidzein degradation phenotype as well ($P < 0.05$, Table 4). Gut transit time was positively correlated with D_k ($r = 0.44$, $P < 0.01$), D_k was marginally negatively correlated with 0~12 h urinary total isoflavone excretion ($r = -0.29$, $P = 0.05$).

DISCUSSION

In the present study, urinary isoflavone excretion was significantly greater over 0~12h than over 12~24h. The total urinary isoflavone excretion between 0~12 h and 0~24 h was highly correlated ($r = 0.97$, $P < 0.001$). These results were consistent with previous work in our laboratory (12, 13).

The overall daily bioavailability of isoflavones as reflected in urinary total isoflavone excretion did not differ between fermented soygerm (mostly isoflavone aglucons) and

unfermented soygerm (mostly isoflavone glucosides) (Table 3). Between 0~12 h, urinary isoflavone excretion in subjects fed fermented soygerm was slightly greater than that of subjects fed unfermented soygerm but between 12~24 h, urinary excretion of unfermented soygerm was greater than fermented soygerm. This different absorption and excretion pattern between fermented and unfermented soygerms may be due to their different chemical structures. Glucosides have long residence time in the gut because the glucosidic bound must be hydrolyzed before absorption. Setchell et al. (8) compared plasma kinetics of 50 g of pure daidzein, genistein and their β -glucosides in 19 women, and found that aglucons were absorbed more quickly than glucosides as reflected in t_{max} (mean time to attain peak plasma concentrations), 5~6 h for aglucons, and ~9 h for glucosides, respectively. Izumi et al. (7) reported that soy isoflavone aglucons were absorbed faster and in greater amounts ($P < 0.05$) than isoflavone glucosides in 8 Japanese men and women at 2, 4, and 6 h after feeding, t_{max} was 2 h for aglucons, and 4 h for glucosides, respectively. At 24 h after feeding, plasma genistein after glucoside feeding was slightly greater than plasma daidzein after glucoside feeding and after feeding either aglucons. These findings are consistent with isoflavone glucosides needing to undergo hydrolysis before being absorbed; therefore the absorption of glucosides was delayed compared with aglucons. The delayed absorption seemed to delay excretion as well, hence the slightly greater urinary excretion of isoflavones between 12~24 h for unfermented soygerm compared with fermented soygerm. In agreement with these observations, our plasma data also showed that at 3 h after feeding, isoflavone aglucons were absorbed to a significantly greater extent than were isoflavone glucosides (Table 6). However, Richelle et al. (10) reported that t_{max} (7.2~8.3 h and 7.3~9.2 h for aglucons and

glucosides, respectively) did not differ between aglucons and glucosides in 6 European postmenopausal women after they consumed a single dose of either glucosidic isoflavone (soygerm-based extract contained 98% of glucosides) or isoflavone aglucons (enzymatically hydrolyzed from glucosides containing 57% aglucons), providing 3.6 μmol isoflavone aglucon equivalent /kg body weight. The enzymatic hydrolysis of isoflavone glucosides seems to have been incomplete, because only 57% of the isoflavones were in aglucon form. Thus Richelle et al. (10) observed effects of absorption of an aglucon/glucoside mixture. Recently Zubik and Meydani (9) also showed similar absorption pattern between aglucons and glucosides. In this study, 15 American women were given a single bolus dose of either aglucons (32 mg or 0.12 mmol) or glucosides (51.72 mg or 0.12 mmol) of isoflavones from tablets in a crossover. The mean t_{max} (4~5 h) for plasma daidzein and genistein after consumption of the aglucon tablets were not significantly different from the values after consumption of the glucoside tablets. They suggested that the ethnic background, intestinal bacterial composition and dietary habits were different compared with the study of Izumi et al. (7). American women, whose intestinal microfloral populations may differ from those of Japanese subjects, may have had a greater capacity to hydrolyze glucosides in the intestines than did the Japanese. But given the relative times of absorption according to Zubik and Meydani (9), the isoflavones would not have had time to reach the colon. This may suggest that a difference in human intestinal glucosidase activity but not gut microbial glucosidase activity was responsible for the most of the β -glucosidic bond hydrolysis during isoflavone absorption in this case. Thus, American women may metabolize the glucoside forms of isoflavones more efficiently than do the Japanese subjects. In our study, we had 20

Caucasians and 5 Chinese subjects and our absorption data seem to agree with Setchell's study (8), which showed that aglucons absorbed faster than glucosides.

Setchell et al. (8) also compared systemic bioavailability and C_{\max} (the maximum plasma concentration) between isoflavone aglucons and glucosides. The mean AUC (area under the curve) for genistein and daidzein were 4.54 and 2.94 $\mu\text{g}/(\text{mL}\cdot\text{h})$, whereas, for genistin and daidzin were 4.95 and 4.52 $\mu\text{g}/(\text{mL}\cdot\text{h})$, respectively, suggesting only slight difference in bioavailability between daidzein aglucons and glucosides but not for genistein. The C_{\max} for genistein and daidzein were 1.26 and 0.76 $\mu\text{mol}/\text{L}$, respectively; for genistin and daidzin were 1.22 and 1.55 $\mu\text{mol}/\text{L}$, respectively. Richelle et al. (10) also did not find differences in C_{\max} and AUC between aglucons and glucosides. Zubik and Meydani (9) found no difference in C_{\max} (0.5 vs. 0.5 $\mu\text{mol}/\text{L}$, respectively) and AUC over 48-h period (8.3 ± 4.2 vs. $8.9 \pm 4.7 \mu\text{mol}/(48\text{h}\cdot\text{L})$, respectively) between the aglucons and glucosides for genistein. But they found a significantly higher C_{\max} (0.5 vs. 0.4 $\mu\text{mol}/\text{L}$, respectively, $P < 0.05$) and a larger AUC (8.3 ± 2.6 vs. $6.2 \pm 1.7 \mu\text{mol}/(48\text{h}\cdot\text{L})$, respectively, $P < 0.05$) for plasma daidzein between aglucons and glucosides. But they pointed out this difference was partly due to the higher content of daidzein in the aglucon tablet compared with glucoside tablet (daidzein was 0.0624 mmol in the 3 aglucon tablets and 0.0492 mmol in the 3 glucoside tablets, respectively). Although there exists some conflict, based on our study, isoflavone aglucons, being absorbed quickly, appeared in circulation earlier and in greater amounts than glucosides; but glucosides were absorbed to a greater extent later and persisted in circulation such that the total bioavailability of aglucon and glucosides as reflected in total urinary isoflavone excretion did not differ.

In either the aglucon or glucosidic form, bioavailability of an isoflavone source rich in glycitein (unfermented soygerm and fermented soygerm) was significantly greater than that of an isoflavone source rich in genistein (soybean isoflavone extract) at all time intervals as reflected in urinary excretion (Table 3). Comparing urinary isoflavone excretion of each of the three isoflavones, significantly greater amounts of daidzein and glycitein were excreted than genistein in all treatments (Table 5). Less excretion of genistein in urine compared with daidzein and glycitein may be due to structural differences. Griffiths and Smith (26) reported that genistein's hydroxyl group at the 5 position of the A-ring is much more susceptible to gut microfloral degradation in rats than are compounds without this structure (e.g., daidzein, and glycitein). The more that genistein is being degraded, the less that is recovered in urine. Sfakianos et al. (27) evaluated the intestinal uptake and biliary excretion of the isoflavone genistein in rats and found that the main metabolite of genistein was 7-*O*- β glucuronide, which was excreted mainly into bile with only a small proportion into urine, therefore genistein may undergo more degradation by gut microflora. Zhang et al. (16) reported that after consumption of either soymilk (mole ratio of isoflavone was 5 daidzein: 5.5 genistein: 1 glycitein) or soygerm powder (mole ratio of isoflavone was 3 daidzein: 1 genistein: 2.6 glycitein) by seven men and seven women with moderate fecal isoflavone degradation, urinary excretion of genistein (as a percentage of ingested isoflavone) was significantly lower ($P < 0.001$) than daidzein and glycitein. Glycitein showed the same excretion pattern in urine as did daidzein. The current findings show that bioavailability of total isoflavone from a source as reflected in urinary excretion depends on the molar ratio of individual isoflavones present in the source.

Due to the small number of subjects, we did not find significant differences in GTT and urinary isoflavone excretion between high and low daidzein degradation phenotypes within each treatment. But when data from high and low daidzein degradation phenotypes were combined across the three treatments, urinary total isoflavone excretion was significantly greater in subjects of the low degradation phenotype who had significantly shorter GTT than in subjects of the high degradation phenotype with significantly longer GTT between 0~12 h and between 0~24 h time intervals (Table 4). Furthermore, the 0~12 h total urinary isoflavone excretion was marginally negatively correlated with D_k and GTT among all the subjects; urinary isoflavone excretion between 0~12 h contained the greater portion of isoflavones excreted over the day. Previous research (19) showed that bioavailability of genistein as reflected in urinary genistein excretion was significantly greater in subjects of a low genistein degradation phenotype with shorter gut transit time than in subjects of a high genistein degradation phenotype with longer gut transit time, after consumption of soybean powder providing 4.6 μmol total isoflavone/kg body weight. Gut transit time and gut microflora may play a crucial role in isoflavone degradation, and therefore in determining isoflavone bioavailability. Xu et al (12) reported that among seven adult women who consumed three doses of isoflavone in soymilk, two of them excreted significantly greater amounts of isoflavone in feces and also had greater isoflavone in urine and plasma compared with the other five subjects who excreted small amount isoflavone in feces and urine, suggesting an influence of gut microbial degradation on isoflavone bioavailability. The low isoflavone degradation phenotype coupled with shorter GTT seemed to prevent isoflavone degradation; therefore isoflavones are apparently absorbed to a greater

extent in such individuals. No phenotypic differences were found in total and individual plasma isoflavone either within each treatment or across the three treatments at 3 h after feeding. This observation was expected because during this short a time period, gut microflora would not exert an effect on isoflavone degradation or absorption.

We did not detect glycitein in plasma at 3 h or 24 h after feeding. Glycitein may be absorbed more slowly than daidzein such that it is undetectable at 3h after feeding. As observed in a previous study, at 6 h after soymilk and soygerm consumption, Zhang et al. (16) reported that plasma glycitein concentrations were significantly lower than that of daidzein. Genistein was detected in only 8 subjects in plasma at 3 h after feeding, and genistein intakes were less, especially in subjects fed soygerm, probably explaining why only a few subjects had detectable plasma genistein at 3 h after feeding. At 24 h after soy feeding, no isoflavones were detected in plasma probably due to the detection limit ($0.5 \mu\text{mol/L}$ or $\sim 150 \text{ ng/mL}$).

In this study, we showed that the daily bioavailability of the two major forms of isoflavone, aglucons and glucosides, derived from soygerm, did not differ as reflected in total urinary excretion over 24 h, but that genistein was less absorbed than glycitein or daidzein as reflected in total urinary isoflavone excretion. Isoflavone aglucons were absorbed more rapidly than glucosides as reflected in total and individual plasma isoflavone content at 3 h after feeding. Bioavailability of isoflavone was significantly greater in subjects of a low daidzein degradation phenotype with shorter GTT than in subjects of a high daidzein degradation phenotype with longer GTT as reflected in higher total urinary isoflavone

excretion. This phenomenon should be taken into account to better control human feeding studies of the effects of isoflavones.

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TABLE 1

Subject characteristics¹

Treatment	Daidzein degradation phenotype	n	Age	Weight	D _k	BMI
			Y	kg	h ⁻¹	kg/m ²
Unfermented soygerm	High	5	22.4 ± 2.3	61.3 ± 7.1	0.35 ± 0.02 ^a	22.1 ± 2.1
	Low	4	24.0 ± 2.6	71.4 ± 8.0	0.14 ± 0.02 ^b	26.2 ± 2.4
	Combined	9	23.2 ± 1.7	66.4 ± 5.4	0.26 ± 0.01	24.1 ± 1.6
Fermented soygerm	High	4	23.8 ± 2.6	74.6 ± 8.0	0.39 ± 0.02 ^a	25.7 ± 2.4
	Low	4	27.3 ± 2.6	59.4 ± 8.0	0.14 ± 0.02 ^b	21.9 ± 2.4
	Combined	8	25.5 ± 1.8	67.0 ± 5.7	0.27 ± 0.02	23.8 ± 1.7
Soybean isoflavone extract	High	4	21.2 ± 2.3	57.0 ± 7.1	0.36 ± 0.02 ^a	20.8 ± 2.1
	Low	4	22.0 ± 2.6	61.9 ± 8.0	0.13 ± 0.02 ^b	23.2 ± 2.4
	Combined	8	21.6 ± 1.7	59.4 ± 5.4	0.25 ± 0.01	22.0 ± 1.6
Mean	High	13	22.5 ± 1.4	64.3 ± 4.3	0.36 ± 0.01 ^a	22.9 ± 1.3
	Low	12	24.4 ± 1.5	64.2 ± 4.6	0.14 ± 0.01 ^b	23.8 ± 1.4
	Combined	25	23.3 ± 1.0	64.2 ± 3.1	0.26 ± 0.01	23.1 ± 2.4

¹ Values are mean ± SEM. Within a treatment, daidzein degradation rate constant, D_k, bearing different letters (a, b) was significantly different according to daidzein degradation phenotype, P < 0.05.

TABLE 2

Isoflavone composition of the three isoflavone-containing products¹

Source	Daidzein	Genistein	Glycitein	Total ¹
	$\mu\text{mol/g}$ % of total ² % aglucons ³	$\mu\text{mol/g}$ % of total % aglucons	$\mu\text{mol/g}$ % of total % aglucons	$\mu\text{mol/g}$ % of total % aglucons
Unfermented soy germ	20.0 ± 0.7 40.0 4	7.0 ± 0.2 14.0 5	23 ± 0.5 46.0 9	50 ± 0.6 100.0 6.4
Fermented soy germ	22.0 ± 0.6 50.0 91	7.0 ± 0.3 16.0 83	15.0 ± 0.5 34 84	44 ± 0.7 100.0 87.5
Soybean isoflavone extract	450.0 ± 5.2 38.0 3	617.0 ± 7.3 53.0 2	107.0 ± 3.5 9.0 11	1174.0 ± 5.5 100.0 3.2

¹ Values are mean ± SD. The total isoflavone content was the sum of mole amounts of total daidzein, genistein and glycitein.

² % of total = mole % of total isoflavones.

³ % of aglucons = mole % of total isoflavones.

TABLE 3

Urinary excretion of total isoflavones during 0~12, 12~24 h and over 24 h from three isoflavone sources fed to women for 7 days^{1,2}

Collection period ²	Unfermented soygerm	Fermented soygerm	Soybean isoflavone extract	Mean
	<i>mole % ingested dose</i>	<i>mole % ingested dose</i>	<i>mole % ingested dose</i>	<i>mole % ingested dose</i>
0~12 h	34.7 ± 4.5 ^{a, b}	40.2 ± 4.9 ^a	18.7 ± 4.6 ^b	30.4 ± 3.2
12~24 h	16.0 ± 1.8 ^a	11.3 ± 2.0 ^{a, b}	7.1 ± 1.8 ^b	11.7 ± 1.2
0~24h	50.8 ± 5.6 ^a	51.5 ± 6.1	25.7 ± 5.8 ^b	42.1 ± 3.9

¹ Values are mean ± SEM of day 1 and 7. Urinary excretion of total isoflavones was expressed as a mole percentage of ingested doses. Within a row, urinary excretion of total isoflavones with different superscripts (a, b) were significantly different.

² Urinary excretion of total isoflavones was significantly greater between 0~12 h than that of 12~24 h, $P < 0.05$.

TABLE 4

Urinary excretion of total isoflavones during 0~12, 12~24 h, and over 24 h in women of high and low daidzein degradation phenotypes fed isoflavone sources for 7 days¹

Treatment	Daidzein degradation phenotype	n	0~12 h	12~24 h	0~24 h	GTT
			<i>% ingested dose</i>	<i>% ingested dose</i>	<i>% ingested dose</i>	<i>h</i>
Unfermented soygerm	High	5	20.5 ± 6.0	17.2 ± 2.4	37.7 ± 7.5	130 ± 18
	Low	4	49.0 ± 6.7	14.9 ± 2.7	63.8 ± 8.4	67 ± 20
Fermented soygerm	High	4	33.0 ± 7.1	8.2 ± 2.9	41.1 ± 9.0	83 ± 20
	Low	4	47.5 ± 6.7	14.3 ± 2.7	61.8 ± 8.4	71 ± 20
Soybean isoflavone extract	High	4	15.1 ± 6.3	7.3 ± 2.5	22.4 ± 7.9	102 ± 20
	Low	4	22.2 ± 6.7	6.8 ± 2.7	29.0 ± 8.4	76 ± 20
Mean	High	13	22.8 ± 3.7 ^b	10.9 ± 1.5	33.8 ± 4.7 ^b	106 ± 11 ^a
	Low	12	39.6 ± 3.9 ^a	12.0 ± 1.6	51.6 ± 4.8 ^a	71 ± 12 ^b

¹ Values are mean ± SEM. Urinary excretion of total isoflavones was the mole % of ingested dose, mean of day 1 and 7. The urinary excretion of total isoflavone and GTT with different superscripts (a, b) were significantly different between high and low daidzein phenotypes, $P < 0.05$.

TABLE 5

Urinary excretion of individual isoflavone over 24 h in high and low daidzein phenotypes and among three treatments¹

Treatment	Daidzein degradation phenotype	n	Daidzein <i>% ingested dose</i>	Genistein <i>% ingested dose</i>	Glycitein <i>% ingested dose</i>
Unfermented soygerm	High	5	40.9 ± 7.9	10.1 ± 4.0	43.4 ± 10.2
	Low	4	64.0 ± 8.9	18.2 ± 4.5	77.6 ± 11.4
	Mean		52.5 ± 5.9	14.1 ± 3.0	60.5 ± 7.7
Fermented soygerm	High	4	51.7 ± 9.5	7.7 ± 4.8	41.2 ± 12.2
	Low	4	70.1 ± 8.9	14.2 ± 4.5	72.0 ± 11.4
	Mean		60.9 ± 6.5	10.9 ± 3.3	56.6 ± 0.4
Soybean isoflavone extract	High	4	36.8 ± 8.3	9.0 ± 4.3	39.7 ± 10.8
	Low	4	47.6 ± 8.9	10.7 ± 4.5	56.3 ± 11.4
	Mean		42.2 ± 6.1	9.8 ± 3.1	48.0 ± 7.8
Mean	High	13	43.1 ± 5.0 ^b	8.9 ± 2.5	41.4 ± 6.4 ^b
	Low	12	60.6 ± 5.1 ^a	14.3 ± 2.6	68.6 ± 6.6 ^a
	Mean		51.1 ± 3.8	11.6 ± 1.8	54.5 ± 4.8

¹ Values are mean ± SEM of day 1 and 7. Urinary excretion of individual isoflavone was the mole % of ingested dose. Urinary excretion of daidzein and glycitein were significantly greater than that of genistein, $P < 0.05$. Urinary excretion of daidzein and glycitein with different superscripts (a, b) were significantly different between high and low daidzein degradation phenotypes, $P < 0.05$.

TABLE 6

Estimated plasma total and individual isoflavone at 3 h after feeding in women of high and low daidzein degradation phenotypes fed three isoflavone sources for 7 days¹

Phenotype	Isoflavone	n	Unfermented soygerm	Fermented soygerm	Soybean isoflavone extract	Mean
			<i>% ingested dose</i>	<i>% ingested dose</i>	<i>% ingested dose</i>	<i>% ingested dose</i>
High	Daidzein	13	0.59 ± 0.47	3.43 ± 0.53	1.48 ± 0.50	1.83 ± 0.29
	Genistein		1.53 ± 0.95	0.28 ± 1.06	1.31 ± 1.0	1.04 ± 0.58
	Glycitein		ND ²	ND	ND	ND
	Total		0.51 ± 0.41	1.76 ± 0.45	1.26 ± 0.43	1.17 ± 0.25
Low	Daidzein	12	0.73 ± 0.53	3.13 ± 0.53	1.21 ± 0.53	1.69 ± 0.31
	Genistein		1.63 ± 1.06	1.50 ± 1.06	1.00 ± 1.06	1.37 ± 0.61
	Glycitein		ND	ND	ND	ND
	Total		0.52 ± 0.45	1.80 ± 0.45	0.99 ± 0.44	1.10 ± 0.26
Mean	Daidzein	25	0.66 ± 0.36 ^b	3.28 ± 0.38 ^a	1.34 ± 0.36 ^b	1.71 ± 0.25
	Genistein		1.58 ± 0.71	0.89 ± 0.75	1.16 ± 0.73	1.22 ± 0.40
	Glycitein		ND	ND	ND	ND
	Total		0.51 ± 0.30 ^b	1.78 ± 0.32 ^a	1.12 ± 0.31 ^{a,b}	1.12 ± 0.19

¹ Values are mean ± SEM of day 1 and 7. Plasma total and individual isoflavones was the mole % of ingested dose. Plasma volumes were estimated based on subject body weight and height (22). Within a row, plasma total isoflavone and plasma daidzein contents with different superscripts (a, b) were significantly different among treatment groups, $P < 0.05$.

² ND = not detected.

**GUT TRANSIT TIME AND GUT MICROBIAL ISOFLAVONE
DEGRADATION INFLUENCE APPARENT ISOFLAVONES ABSORPTION IN
WOMEN**

A paper submitted to Journal of Nutrition

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ABSTRACT

Greater apparent absorption of isoflavones from high and doses of isoflavone-containing supplements fed for 7 days was hypothesized to depend upon relatively rapid gut transit time (GTT) and low daidzein degradation rate, $D_k < 0.15 \text{ h}^{-1}$, as determined from anaerobic incubation of human feces with daidzein. Twenty Caucasian women, $21 \pm 1 \text{ y}$, $70 \pm 3 \text{ kg}$, and BMI of $24 \pm 1 \text{ kg/m}^2$ were studied: 10 of high daidzein degradation phenotype and GTT $130 \pm 9 \text{ h}$, 10 of low daidzein degradation phenotype and GTT $86 \pm 9 \text{ h}$. Isoflavone doses (54 or 163 μmol total isoflavones/d) were fed daily for 7 days at breakfast in a randomized crossover design with doses separated by a 1-week washout period. Reverse-phase HPLC analysis of urine samples from subjects indicated the expected significant dose response in 24-h urinary excretion of isoflavones (high vs. low dose: 197 ± 11 vs. $72 \pm 11 \mu\text{mol/day}$, $P < 0.05$). Degradation phenotype and GTT differences were significant among the 10 subjects who consumed the high isoflavone dose initially. Low daidzein degraders had greater total urinary isoflavone excretion than did high degraders (189.0 ± 16.3 vs. $119.0 \pm 5.0 \mu\text{mol/d}$, $P < 0.05$). Urinary isoflavone excretion decreased 20~30% in week 2 of isoflavone feeding compared with week 1. Plasma glycitein showed significant dose difference, phenotypic difference and repeated dosing difference. These data suggest that

microbial degradation of isoflavones is dose-dependent and inducible. Isoflavone degradation phenotype coupled with GTT and repeated dosing significantly affect apparent absorption of isoflavones, which is of importance for design of human feeding trials of isoflavones.

KEY WORDS: isoflavone bioavailability · gut transit time · microbial degradation

Isoflavones stimulate research interest in the field of nutraceuticals and functional foods because of the potential health benefits from consumption of these phytoestrogens found primarily in soybeans. Epidemiologic studies have suggested that the consumption of soy products lower the incidence of hormone-dependent cancer (1-3), prevent osteoporosis (4) as well as menopausal symptoms (5), and coronary heart disease (6). There is little evidence about isoflavone dose-response in exerting these effects. Messina et al. (7) has suggested that as little as 1 serving/d of soy products can protect against chronic diseases. A few randomized crossover dose-response feeding studies have been done in human subjects (8-10) with either soy protein isolate or soymilk powder as isoflavone sources. The doses ranged from 2~30 $\mu\text{mol/kg}$ body weight, and 1~9-day feeding periods. All these studies showed dose-response in isoflavone bioavailability as reflected in urinary excretion.

Urinary isoflavone excretion varies considerably among individuals. In one study, the variation of genistein excretion was ~12-fold and that of daidzein ~15-fold within the same dose after 9-day isoflavone feeding with different doses (8). This variability is probably due to individual differences in isoflavone absorption and degradation, which we propose to be influenced significantly by gut microorganisms and gut transit time (GTT) (11, 12).

Isoflavone degradation phenotypes have been identified in women based on the *in vitro* rate of fecal disappearance of these compounds (11, 13). More rapid gut transit time was one

factor identified to be related to increased apparent genistein absorption as reflected in urinary excretion in Asian and Caucasian women (11). Chronic soy product consumption may modulate the metabolism and disposition of ingested isoflavones, hence affecting the bioavailability of these compounds (14).

In the present study, we examined the bioavailability as a function of urinary and plasma content of high and low doses of isoflavone among individuals of varying GTT and isoflavone degradation phenotype as measured by isoflavone disappearance during anaerobic fecal incubations.

MATERIALS AND METHODS

Experimental Procedures

Subjects. Subjects were screened to identify high and low daidzein degradation phenotypes. Daidzein, at 100 $\mu\text{mol/L}$, was incubated in anaerobic Brain-Heart Infusion media for 12 h with a fresh fecal sample provided by each subject as described in Zheng et al. (11). Daidzein degradation rate constant $D_k < 0.15 \text{ h}^{-1}$ was the criterion for the low daidzein degradation phenotype; $D_k > 0.20 \text{ h}^{-1}$ was the criterion for the high daidzein degradation phenotype. Twenty healthy Caucasian women (Iowan), 18-30 y, $70.1 \pm 3.0 \text{ kg}$, body mass index (BMI) of $23.7 \pm 0.9 \text{ kg/m}^2$ were recruited for this study, 10 of each degradation phenotype (Table 1). The experimental procedures for this study were approved by the Human Subjects Committee of Iowa State University. Subjects gave their informed consent to the study.

Experimental design. The experiment was divided into two phases (week 1 and week 2) separated by a 1-week washout period. Low and high doses of isoflavones were

given for 7 days in a randomized crossover design (Table 2). The isoflavone source was a mixture of soy protein and dietary isoflavone supplement (General Nutrition Corporation, Pittsburgh, PA) (Table 3). The high dose contained 291 mg isoflavone supplement and 18 g soy protein which provided a total of 43 mg (163 μ mole) isoflavone; the low dose contained 97 mg supplement and 6 g soy protein providing 14 mg (54 μ mole) isoflavone, yielding a 3-fold dose difference between the two treatments. The daily dose of isoflavone was mixed with orange juice given at breakfast between 7:30 and 9:00 am in Human Metabolic Unit, Iowa State University. Other foods including white bread, bagel, cereal, skim milk, cream cheese, peanut butter, jelly, apple and orange were consumed *ad libitum*. On day 7 (the last day of feeding) of each feeding period, 2 gelatin capsules containing 16 marker beads were given to each subject with breakfast for measuring GTT (11). All subjects were asked to avoid any isoflavone-containing foods one week before and during the study including the washout period. A list of isoflavone-containing foods was given to each subject (See Appendix C.).

Biological sample collection. One blank urine sample (time 0) was collected from each subject in the morning on day 1 right before initial feeding in both feeding periods. Urine was collected for 24 h on day 6, 7 and 8 and the first urination on day 9 in both weeks. Subjects were instructed to keep the urine samples in the ice chests provided or in refrigerators on each collecting day. Immediately after collection, 24 h urine from each subject was mixed well, and after recording the volume, 50 mL aliquots were stored at -80° C until analysis. After ingesting marker beads at breakfast on day 7 in both feeding periods, feces were collected until 12 out of 16 beads were excreted (11). After an overnight fast, 10

mL venous blood samples were collected into EDTA-containing vacuum containers by a licensed phlebotomist under stringent aseptic conditions before isoflavone feeding (time 0) and at 24 h after feeding on day 7 and 8. Blood samples were centrifuged within 1 h after collection at 3000 g for 30 min at 4° C (Model 4d, International Equipment Co., Needham Heights, MA). Plasma was stored at -20° C until analysis.

Analytical Methods

Chemicals. Isoflavones, daidzein, genistein, glycitein and 2, 4, 4'-trihydroxybenzoic acid (THB), used as internal standard, were synthesized in Dr. Murphy's laboratory, Iowa State University (15, 16). Other HPLC solvents and extraction agents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Soy products analysis. Samples from each isoflavone source were analyzed in duplicate in Dr. Murphy's laboratory, Iowa State University. Isoflavone extraction and HPLC analysis were performed as described before (17, 18). The total isoflavone content was the sum of total daidzein, genistein and glycitein on a mole basis (Table 3). The overall mole ratio among the three isoflavones fed was 3 daidzein: 3.5 genistein: 1 glycitein.

Urinary and plasma isoflavone analysis. Details of isoflavone extraction and HPLC analysis have been described previously by Zheng et al. (11). Recovery studies were done as reported previously by Zheng et al. (11). The analytical recoveries of urinary daidzein, genistein, glycitein, and THB were 84.3%, 80.6%, 86.5%, and 88.6%, respectively. The recoveries of plasma daidzein, genistein, glycitein and THB were 79.2 %, 72.2 %, 74.0% and 82.5 %, respectively. Reported urinary and plasma isoflavones were calculated according to the internal standard curves of each individual isoflavone, THB used as internal standard.

Statistical analysis. Statistical analysis was conducted by SAS program (Version 8.2, 2001, SAS Institute, Carry, NC). Crossover effect was not significant after statistical examination; data were combined cross the two feeding periods. General linear model was used to test effects of treatment, phenotypic, repeat dosing and the difference of GTT. Pearson's correlation analysis was conducted to describe the correlation between urinary and plasma isoflavone. Clustering test (based on simple linkage method) was used to identify daidzein degradation phenotypes. All results were reported as mean \pm SEM, $P < 0.05$ for all analyses.

RESULTS

Urinary and plasma isoflavones in all subjects. Only daidzein degradation rate constant was significantly different between high and low phenotypes ($P < 0.001$, Table 1). Urinary excretions of individual and total isoflavones were the mean of day 6 and 7 because they were highly correlated ($r = 0.76$, $P < 0.0001$) and there was no significant difference between the two days. Urinary isoflavone excretion on day 8 was significantly less than that of day 6 and 7 ($P < 0.05$), representing a small amount of carryover from the last day of isoflavone dosing (data not shown), and isoflavones from the first urination on day 9 were undetectable in most subjects (data not shown). Therefore, only results from day 6 and 7 were used in analysis. Concentrations of plasma isoflavone were the mean of day 7 and 8 at 24 h after feeding. Urinary isoflavone excretion was slightly but significantly correlated with plasma isoflavone concentration ($r = 0.23$, $P < 0.05$).

Dose-response, phenotypic difference, GTT, and repeated dosing influence urinary excretion of isoflavones in all subjects. Urinary excretion of individual and total

isoflavones was shown according to dose, daidzein degradation phenotype, and feeding period (Table 4). As expected, significant dose effects were found in urinary excretion of individual and total isoflavones (Table 4). Urinary excretion of isoflavones was significantly greater (~3-fold) after the high dose than the low dose ($P < 0.0001$). Phenotypic differences were significant for urinary genistein excretion between the two phenotypes ($P = 0.01$) (Table 4), which excretion in subjects of the low daidzein degradation phenotype was greater than in subjects of the high phenotype. GTT (mean of week 1 and week 2) was positively correlated with D_k ($r = 0.71$, $P < 0.001$) and differed significantly between the two phenotypes, such that subjects within the low phenotype had shorter GTT than did subjects of the high phenotype ($P < 0.05$) (Table 4). Significant effects of repeated dosing were seen in urinary excretion of daidzein ($P = 0.002$) and total isoflavones ($P = 0.006$), where the excretions of isoflavones in week 1 were greater than in week 2. The decreases were 30%, 29%, 21%, and 29% for daidzein, genistein, glycitein and total isoflavone excretions in week 2 compared to week 1, respectively (Table 4).

Effects of phenotypic difference and GTT on urinary excretion of isoflavones in 10 subjects who were given the high dose during week 1. Among the 10 subjects (6 high and 4 low phenotype) who were given a high isoflavone dose in week 1, urinary excretion of total isoflavone ($P = 0.005$), daidzein ($P = 0.009$) and genistein ($P = 0.006$) were significantly greater in subjects of the low degradation phenotype than in subjects of the high degradation phenotype (Table 5), and GTT was significantly more rapid in subjects of the low degradation phenotype as well ($P < 0.05$). In addition, in this group, total urinary isoflavone excretion was negatively correlated with D_k ($r = -0.32$, $P < 0.05$). These

differences were not found in the 10 subjects who were given a low isoflavone dose in week 1 (Table 5).

Dose-response, phenotypic difference, GTT, and repeated dosing influence plasma isoflavones in subjects. Only plasma glycitein was found to be significantly different between high and low dose, high and low phenotype, and between week 1 and week 2 ($P < 0.05$) (Table 6). Plasma daidzein was significantly greater during week 1 than in week 2 ($P < 0.05$) (Table 6). In the 10 subjects who given a high isoflavone dose in week 1, plasma glycitein was significantly greater in low degradation phenotype compared with high degradation phenotype ($P < 0.05$) (Table 7). No difference was found in the 10 subjects given a low dose of isoflavone during week 1 (Table 7).

DISCUSSION

Our results showed that there was 3-fold greater urinary excretion of both individual and total isoflavones in subjects after consuming a high dose of isoflavones compared to a low dose, consistent with the 3-fold difference between the two doses (Table 4). This agreed with previous feeding studies (9, 10) that the apparent absorption and urinary excretion of isoflavones is dose-dependent.

Phenotypic and GTT differences found between high and low daidzein degradation phenotypes are important in determining isoflavone bioavailability as reflected in plasma isoflavone and urinary isoflavone excretion. In the current study, we examined the effects of daidzein degradation phenotype coupled with GTT on urinary isoflavone excretion and plasma isoflavone concentration. Among all subjects, D_k and GTT were positively correlated ($r = 0.71$, $P < 0.001$). Urinary genistein excretion, plasma glycitein and GTT were

significantly different between high and low daidzein degradation phenotypes ($P < 0.05$) (Tables 4 & 6). Furthermore, among the 10 subjects who were given a high dose of isoflavones during week 1, subjects of the low degradation phenotype with shorter GTT had greater urinary excretion of daidzein, genistein and total isoflavone and plasma glycitein compared with subjects of the high degradation phenotype and long GTT ($P < 0.05$) (Table 5 & 7). But we did not find similar results in the 10 subjects who were given a low dose of isoflavones during week 1 for several reasons (Table 5 & 7). First, GTT was not significantly different between the high and low degradation phenotypes in this group of subjects (Table 5), even though as a whole, GTT differed between two phenotypes (Table 4). This suggests that GTT is a crucial factor that influences isoflavone bioavailability. Secondly, the phenotypic differences may only be found under certain conditions (i. e., an isoflavone dose greater than a certain amount seemed to be needed to induce microbial degradation phenotypic effect), therefore, a low isoflavone dose during week 1 may not have “triggered” a phenotypic difference. This remains speculative because of the limited number of human feeding studies done with varying isoflavone doses and the lack of isoflavone degradation phenotypic data. Setchell et al. (19) determined bioavailability and dose-response effects of soy isoflavone in 10 healthy women after single bolus ingestion of 10, 20, or 40 g of soy nuts providing daidzein (6.6, 13.2, and 26.4 mg) and genistein (9.8, 19.6 and 39.2 mg), respectively, in a randomized crossover design. They found a nonlinear relationship between the plasma isoflavone AUC as well as urinary excretions of isoflavone and the amount of isoflavones ingested because the urinary excretions of isoflavone decreased with increasing intake when expressed as a percentage of the administered dose (63.2, 54.4 and 44.0%, respectively, for

daidzein, and correspondingly, 25.2, 13.4 and 15.8 % for genistein). They also found the great variability in urinary excretion among the subjects but they did not describe this interindividual variability. Although different percentage of plasma and urinary isoflavone content between doses were reported in Setchell's study, they were not significant, which still suggested the dose-dependent response in these subjects.

Xu et al. (9) showed that after 7 women consumed three doses of isoflavone-containing soymilk (3.4, 6.9, or 10.3 μmol isoflavones/ kg body weight) with 3 meals on each feeding day separated by 2-week washout periods, 2 of them excreted 10~20 times more fecal isoflavones and 2 ~ 3-fold greater urinary isoflavones than did the other five subjects: the urinary recoveries of ingested daidzein and genistein were 16% and 10% in the five subjects, and 32% and 37% in the other 2 subjects, respectively. This suggested that isoflavone bioavailability depended upon the ability of gut microorganisms to degrade isoflavones. Different isoflavone degradation phenotypes (high, moderate, and low) have been identified in humans (11, 13) based on in vitro anaerobic incubation degradation half-life or degradation rate constant. Zheng et al. (11) also reported that significant differences in degradation phenotype and GTT significantly affected urinary genistein excretion in 35 Asian (Chinese immigrant) women after a single dose of isoflavones (4.6 $\mu\text{mol}/\text{kg}$ body weight) (11). In another study (12), total urinary isoflavone recoveries differed significantly between subjects of high and low daidzein degradation phenotypes among 25 women after 7-day dosing of three sources of isoflavones with 8~9 subjects/ treatment (unfermented soygerm 1.1 $\mu\text{mol}/\text{kg}$ body weight, fermented soygerm: 3.3 $\mu\text{mol}/\text{kg}$, soybean extract: 1.5 $\mu\text{mol}/\text{kg}$). All these studies showed that low daidzein degradation phenotype coupled with more rapid GTT

resulted in greater urinary isoflavone excretion compared with a high degradation phenotype with long GTT.

We also found that repeated feeding of isoflavone with a short washout period intervening (1 week) may decrease isoflavone bioavailability as we observed in the current study: urinary isoflavone excretion was 20~30% less in week 2 (Table 4) compared with week 1 and plasma daidzein and glycitein were significantly less in week 2 compared to week 1 (Table 6). Similar results have been reported by Lu et al. (14). After daily doses to women of 80~210 mg of each isoflavone (daidzein and genistein) in soymilk for one month, urinary excretion of isoflavones was 10% less than during initial excretion in women. This may suggest that the ability of gut microorganisms to degrade isoflavones is inducible. Therefore after repeated exposures to isoflavones, even with a brief washout period, the host gut microorganisms retain greater ability to degrade isoflavones, hence decreasing isoflavone bioavailability. This finding may be important for designing clinical feeding trials and evaluating potential isoflavone toxicity *in vivo*, especially for chronic exposure.

Identification of isoflavone degrading microorganisms in the human gut and of the microbial ecologies and environmental factors that stimulate or inhibit isoflavone degradation is likely to be an important next step in understanding isoflavone bioavailability. This has implications for bioavailability of other dietary phenolics with potential health effects as well.

We observed that bioavailability of glycitein as reflected in urinary excretion was similar to daidzein, but was greater than genistein. The mole ratio of the three isoflavones was 3 daidzein: 3.5 genistein: 1 glycitein in the mixture of soy protein and isoflavone supplement, and was 4 daidzein: 1.5 genistein: 1 glycitein in urinary excretion. Urinary

excretion of genistein was much less than daidzein and glycitein, relative to dietary content. Daidzein and genistein were abundant in the isoflavone sources in the current study (Table 3); glycitein is a minor component of isoflavones in soy products except for soygerm. Because glycitein is more bioavailable than genistein, its biological effects may also be important.

Genistein may be more bioactive than the other isoflavones. For example, as cancer chemopreventive agents, genistein ($IC_{50} = 25 \mu\text{mol/L}$) and daidzein ($IC_{50} = 150 \mu\text{mol/L}$) inhibited H_2O_2 formation in 12-O-tetradecanoylphorbol-13-acetate (TPA)-activated HL-60 cells (20). Wang et al. (21) also demonstrated that genistein at $0.01 \mu\text{mol/L}$ and biochanin A, at $1.0 \mu\text{mol/L}$, induced quinone reductase, a phase II enzyme that detoxifies some chemical carcinogens, six fold and two fold, respectively. But genistein is also associated with potential toxicities such as thyroid peroxidase (TPO) inhibition in rats (22). Sprague-Dawley rats were exposed to genistein at doses of 0, 5, 100, and 500 ppm in soy-free basal diet, the estimated genistein intake was 0.04, 0.4, 8, 40 mg/kg body weight per day. In both male and female rats, dose-dependent decreases in microsomal TPO activity were observed with as much as 80% loss of TPO activity in thyroid in female rats fed 500 ppm genistein. Even at the lowest dose of 5 ppm, 40 ~ 55% of TPO activity in thyroid was lost. But this TPO inhibition had not effect on thyroid hormone status in these rats (22). Perhaps the lesser bioavailability of genistein is a health protective mechanism, showing symbiosis of gut microorganisms with respect to isoflavone metabolism.

The results from this study suggested that the effects of gut microflora on absorption of isoflavone probably relate strongly to gut transit time, i. e., the length of time gut microbial species have to exert their effects on isoflavones. Repeat feeding soy isoflavones for a period

of time may decrease their bioavailability as reflected in urinary excretion due to induced gut microbial degradation of these compounds. Isoflavone degradation phenotype coupled with GTT had significant influence on apparent isoflavone absorption, and GTT may be a crucial factor to control for in future clinical trials of isoflavone efficacy.

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TABLE 1

Subject characteristics¹

Daidzein degradation phenotype	n	D _k <i>h</i> ⁻¹	Age <i>Y</i>	Body weight <i>kg</i>	BMI <i>kg/m</i> ²
High	10	0.24 ± 0.01 ^a	20.9 ± 0.8	69.9 ± 4.3	24.1 ± 1.3
Low	10	0.07 ± 0.01 ^b	19.9 ± 0.9	70.2 ± 4.5	23.4 ± 1.4
Mean		0.15 ± 0.01	20.4 ± 0.6	70.1 ± 3.0	23.7 ± 0.9

¹ Values are mean ± SEM. Daidzein degradation rate constant, D_k, with different superscripts (a, b) were significantly different between the two phenotypes (P < 0.001).

TABLE 2

Experimental design¹

Daidzein degradation phenotype	Week 1		Week 2	
	High dose (n)	Low dose (n)	High dose (n)	Low dose (n)
High	6	4	4	6
Low	4	6	6	4
Total number of subjects	10	10	10	10
Dose of isoflavones/d	163 μ mol	54 μ mol	163 μ mol	54 μ mol

¹The subjects given high dose of isoflavones in the first week was switched to the low dose in the second week and vice versa.

TABLE 3

Isoflavone content of the two sources¹

Isoflavone source	Daidzein	Genistein	Glycitein	Total
	$\mu\text{mol/g}$ (mole% of total)	$\mu\text{mol/g}$ (mole% of total)	$\mu\text{mol/g}$ (mole% of total)	$\mu\text{mol/g}$ (mole% of total)
Soy protein	3.1 ± 0.2 (40.3)	3.6 ± 0.1 (46.8)	0.99 ± 0.05 (12.9)	7.6 ± 0.4 (100.0)
Supplement	29.9 ± 1.7 (36.2)	43.1 ± 2.5 (52.1)	9.7 ± 0.8 (11.7)	81.4 ± 6.9 (100.0)

¹ Values are mean \pm SD.

TABLE 4

Urinary excretion of total and individual isoflavones during high and low dose treatments, according to high and low daidzein degradation phenotypes, and during week 1 and 2¹

Isoflavone dose	obs	GTT	Daidzein	Genistein	Glycitein	Total
		<i>h</i>	$\mu\text{mol/d}$	$\mu\text{mol/d}$	$\mu\text{mol/d}$	$\mu\text{mol/d}$
High	20	105 ± 9	124.5 ± 6.7 ^a	44.0 ± 4.8 ^a	28.3 ± 1.9 ^a	196.8 ± 11.2 ^a
Low	20	111 ± 9	46.5 ± 6.7 ^b	14.8 ± 4.8 ^b	10.3 ± 1.9 ^b	71.5 ± 11.2 ^b

Daidzein degradation phenotype	obs	GTT	Daidzein	Genistein	Glycitein	Total
		<i>h</i>	$\mu\text{mol/d}$	$\mu\text{mol/d}$	$\mu\text{mol/d}$	$\mu\text{mol/d}$
High	20	130 ± 9 ^c	82.1 ± 6.8	20.5 ± 4.8 ^d	17.9 ± 1.9	120.5 ± 11.3
Low	20	86 ± 9 ^d	88.9 ± 6.6	38.3 ± 4.8 ^c	20.7 ± 1.9	147.9 ± 11.1

Week	obs	GTT	Daidzein	Genistein	Glycitein	Total
		<i>h</i>	$\mu\text{mol/d}$	$\mu\text{mol/d}$	$\mu\text{mol/d}$	$\mu\text{mol/d}$
1	20	108 ± 9	100.6 ± 6.7 ^e	34.4 ± 4.8	21.6 ± 1.9	156.5 ± 11.2 ^e
2	20	108 ± 9	70.4 ± 6.7 ^f	24.4 ± 4.8	17.0 ± 1.9	111.8 ± 11.2 ^f

¹ Values are mean ± SEM. Urinary excretion of isoflavones was the mean of day 6 and 7.

Urinary excretion of total and individual isoflavones with different superscripts (a, b) were significantly different between the high dose and low dose treatments, $P < 0.0001$. Urinary excretion of genistein and GTT with different superscripts (c, d) was significantly different between high and low phenotypes, $P < 0.05$. Urinary excretion of daidzein and total isoflavones with different superscripts (e, f) were significantly different between week 1 and 2, $P < 0.01$.

TABLE 5

Urinary excretion of total and individual isoflavones in the 10 subjects given the high dose of isoflavone in week 1 and low dose in week 2¹

Daidzein degradation phenotype	Dose	obs	GTT	Daidzein $\mu\text{mol/d}$	Genistein $\mu\text{mol/d}$	Glycitein $\mu\text{mol/d}$	Total $\mu\text{mol/d}$
			<i>H</i>				
High	High	6	126±15	130.3±11.6	29.2±9.4	31.0±3.3	190.6±21.2
High	Low	6	142±15	30.9±11.6	7.6±9.4	8.9±3.3	47.4±21.2
Low	High	4	69±19	174.4±13.6	76.9±11.0	35.9±3.8	287.2±25.9
Low	Low	4	61±19	57.5±13.6	20.3±11.0	12.8±3.8	90.6±24.9
Mean of high phenotype			134±14 ^a	80.6±8.2 ^b	18.4±6.6 ^b	20.0±2.3	119.0±5.0 ^b
Mean of low phenotype			65±18 ^b	116.0±9.6 ^a	48.6±7.8 ^a	24.3±2.7	189.0±16.3 ^a

10 subjects given the low dose of isoflavone in week 1 and high dose in week 2

Daidzein degradation phenotype	Dose	obs	GTT	Daidzein $\mu\text{mol/d}$	Genistein $\mu\text{mol/d}$	Glycitein $\mu\text{mol/d}$	Total $\mu\text{mol/d}$
			<i>H</i>				
High	High	4	138±19	116.3±15.3	32.8±9.9	22.7±4.3	171.8±23.9
High	Low	4	111±19	50.9±15.3	12.3±9.9	8.9±4.3	72.1±23.9
Low	High	6	87±15	77.0±12.5	36.9±8.1	23.5±3.5	137.5±19.5
Low	Low	6	113±15	46.8±12.5	19.0±8.1	10.5±3.5	76.1±19.5
Mean of high phenotype			125±18	83.6±10.9	22.5±7.0	15.8±3.0	121.9±16.9
Mean of low phenotype			100±16	61.9±8.9	27.9±5.7	17.0±2.5	106.8±13.8

¹ Values are mean \pm SEM. Urinary excretion of isoflavone was the mean of day 6 and 7. The urinary excretion of daidzein, genistein, total isoflavones and GTT with different superscripts (a, b) were significantly different between high and low daidzein degradation phenotype in the subjects who given high dose of isoflavone during week 1, $P < 0.05$.

TABLE 6

Concentration of plasma total and individual isoflavones during high and low dose treatments, according to high and low daidzein degradation phenotypes, and during week 1 and 2¹

Isoflavone dose	obs	GTT	Daidzein	Genistein	Glycitein	Total
		<i>H</i>	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$
High	20	105 ± 9	0.18 ± 0.05	2.05 ± 0.26	0.33 ± 0.09 ^a	2.57 ± 0.28
Low	20	111 ± 9	0.08 ± 0.05	1.73 ± 0.25	0.08 ± 0.08 ^b	1.88 ± 0.27

Daidzein degradation phenotype	obs	GTT	Daidzein	Genistein	Glycitein	Total
		<i>h</i>	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$
High	20	130 ± 9 ^c	0.16 ± 0.05	1.84 ± 0.27	0.08 ± 0.09 ^d	2.07 ± 0.28
Low	20	86 ± 9 ^d	0.09 ± 0.04	1.95 ± 0.25	0.33 ± 0.08 ^c	2.37 ± 0.26

Week	obs	GTT (h)	Daidzein	Genistein	Glycitein	Total
		<i>h</i>	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$
1	20	108 ± 9	0.18 ± 0.04 ^e	1.77 ± 0.25	0.32 ± 0.08 ^e	2.27 ± 0.26
2	20	108 ± 9	0.07 ± 0.05 ^f	2.01 ± 0.27	0.09 ± 0.09 ^f	2.17 ± 0.28

¹ Values are mean ± SEM. Concentration of plasma isoflavones was the mean of day 7 and 8 at 24 h after dose. Concentration of plasma total and individual isoflavones with different superscripts (a, b) was significantly different between the high dose and low dose treatments, $P < 0.05$. Concentrations of plasma glycitein and GTT with different superscripts (c, d) were significantly different between high and low phenotypes, $P < 0.05$. Concentration of plasma

daidzein and glycitein with different superscripts (e, f) were significantly different between week 1 and 2, $P < 0.05$.

TABLE 7

Concentration of plasma total and individual isoflavones in the 10 subjects given the high dose of isoflavone in week 1 and low dose inn week 2¹

Daidzein degradation phenotype	Dose	obs	GTT <i>h</i>	Daidzein $\mu\text{mol/L}$	Genistein $\mu\text{mol/L}$	Glycitein $\mu\text{mol/L}$	Total $\mu\text{mol/L}$
High	High	6	126±15	0.22±0.08	2.08±0.33	0.11±0.12	2.42±0.34
High	Low	6	142±15	0.06±0.09	1.98±0.35	0.00±0.12	2.04±0.36
Low	High	4	69±19	0.27±0.10	2.04±0.41	0.86±0.14	3.17±0.42
Low	Low	4	61±19	0.00±0.11	1.98±0.28	0.00±0.15	1.98±0.45
Mean of high phenotype			134±14 ^a	0.14±0.06	2.03±0.24	0.06±0.08 ^b	2.23±0.25
Mean of low phenotype			65.0±18 ^b	0.14±0.07	2.01±0.30	0.43±0.10 ^a	2.58±0.31

10 subjects given the low dose of isoflavone in week 1 and high dose in week 2

Daidzein degradation phenotype	Dose	obs	GTT <i>h</i>	Daidzein $\mu\text{mol/L}$	Genistein $\mu\text{mol/L}$	Glycitein $\mu\text{mol/L}$	Total $\mu\text{mol/L}$
High	High	4	138±19	0.22±0.11	1.80±0.80	0.00±0.23	2.02±0.85
High	Low	4	111±19	0.14±0.09	1.48±0.68	0.19±0.22	1.81±0.71
Low	High	6	87±15	0.00±0.07	2.31±0.52	0.34±0.11	2.65±0.55
Low	Low	6	113±15	0.10±0.07	1.47±0.52	0.11±0.17	1.69±0.55
Mean of high phenotype			125±18	0.18±0.07	1.64±0.52	0.10±0.17	1.92±0.55
Mean of low phenotype			100±16	0.05±0.05	1.89±0.37	0.23±0.12	2.17±0.39

¹ Values are mean \pm SEM. Concentration of plasma isoflavone was the mean of day 7 and 8 at 24 h after dose. Concentration of plasma glycitein and GTT with different superscripts (a, b) were significantly different between high and low phenotypes in the subjects given high dose of isoflavone during week 1, $P < 0.05$.

**CONFOUNDING EFFECTS OF CALORIC INTAKE AND TIME COURSE ON
MODULATION OF APPARENT ABSORPTION OF ISOFLAVONES BY DIETARY
FIBER INTAKE IN WOMEN**

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ABSTRACT

In this study we hypothesized that accelerating gut transit time (GTT) by feeding a high fiber diet would increase apparent isoflavone absorption. Nine Caucasian women, 18~35 year of age, identified by anaerobic incubation of feces with daidzein as low degraders (daidzein degradation rate constant, $D_k < 0.15 \text{ h}^{-1}$) with mean GTT $108 \pm 9 \text{ h}$, consumed either high fiber (45g/d) or moderate fiber (15g/d) diets for 7 days in a randomized crossover feeding trial with a 1-week washout period between treatments. Isoflavones ($4.5 \mu\text{mol/kg}$ body weight) were given in a drink with breakfast each of the 7 days. From fecal marker bead counts, GTT did not differ significantly after either high or moderate fiber diets were fed, perhaps because subjects fed moderate fiber in week 1 experienced increased caloric intake during treatment compared with pre-treatment which may have accelerated their GTT. No significant dietary fiber treatment effect was found in urinary isoflavone excretion over 24 h after the 6th and 7th daily dose of soy. Plasma daidzein was less at 24 h after the 6th and 7th daily doses of soy after high fiber compared with moderate fiber feeding. The second week of isoflavone feeding showed less total urinary isoflavone excretion (week 1 vs. week 2, 9.0 ± 0.9 vs. $4.8 \pm 0.9 \mu\text{mol/kg}$, mean of day 6 and 7, $P = 0.02$) and plasma isoflavone (week 1 vs. week 2, 4.8 ± 0.7 vs. $1.8 \pm 0.7 \mu\text{mol/L}$, mean of day 7 and 8, $P = 0.005$). The lack of

difference between moderate or high dietary fiber in effect on GTT coupled with the apparent decrease of isoflavone absorption with repeated feeding (week 2 < week 1) obscured the proposed ability of high dietary fiber intake to improve apparent isoflavone absorption by accelerating GTT. Randomization based on pre-treatment caloric intake and sufficient numbers of subjects to compensate for the apparently decreased absorption of isoflavones over time may be needed to effectively test the effects of modulation of GTT on isoflavone absorption.

KEY WORDS: isoflavone · bioavailability · gut transit time · dietary fiber

The health effects of consuming foods containing phytoestrogens, especially isoflavones, have been discussed widely (1-2). Soybean isoflavones raise considerable interest because epidemiological studies have shown that lower mortality from hormone-dependent cancer correlated with greater urinary isoflavone excretion in some populations (1, 3). The U.S. Food and Drug Administration also approved a health claim about soy protein intake and cardiovascular disease prevention, implicating isoflavones as a factor in this effect (4). But there is little evidence about required doses for isoflavones to exert their biological effects in humans. Considerable interindividual variation of urinary isoflavone excretion was reported in a 9-day feeding study with 3 doses of isoflavone from soy protein in which the variation of genistein excretion was 12-fold and that of daidzein was 15-fold even within the same dose treatment (5). Previous studies in our laboratory of Caucasian (Iowan) and Asian women (Chinese immigrant), providing 1~5 $\mu\text{mol/kg}$ body weight of total isoflavones per day, indicated that variability in isoflavone degradation phenotype (measured by assessing

disappearance of isoflavones from in vitro anaerobic incubations with feces) and gut transit time (GTT) were partly responsible for this interindividual variation (6-8).

Gut transit time is determined in part by age, sex and physical activity and can be modified by drugs or foods (9). Dietary fiber is thought to be one of the most significant determinants of GTT. In numerous studies, gut transit time was decreased by wheat bran supplementation and addition of fruits and vegetables to the diet (10). In the present study, we hypothesized that high dietary fiber intake could improve apparent isoflavone absorption by speeding GTT compared with moderate dietary fiber intake in women who were identified as the low daidzein degraders according to fecal daidzein degradation rate constant but who also had relatively slow GTT. The acceleration of GTT could prevent isoflavone degradation in such women, permitting them to absorb more isoflavones as reflected in urinary excretion and plasma concentrations.

MATERIALS AND METHODS

Experimental Procedures

Subjects. Female subjects were prescreened for GTT by marker bead counts and low daidzein degradation phenotype by anaerobic fecal incubation and HPLC analysis as described previously (6). The criteria for inclusion were daidzein degradation rate constant, $D_k < 0.15 \text{ h}^{-1}$ and $\text{GTT} > 84 \text{ h}$. Nine healthy Caucasian women (Iowan) 18 ~ 35 years of age, with body weight $71.5 \pm 3.3 \text{ kg}$, body mass index (BMI) $25.1 \pm 0.8 \text{ kg/m}^2$, $D_k 0.06 \pm 0.01 \text{ h}^{-1}$ and $\text{GTT} 108 \pm 9 \text{ h}$ were recruited for this study. The pretreatment diet information was collected from each subject based on 24-h Food Recall and analyzed by Nutritionist V™ (version 1.5, 1998, First DataBank Inc., San Bruno, CA). Based on whether high or moderate

fiber diet was fed during week 1, 9 subjects were randomly divided into two groups (Table 1). The subjects consuming a high fiber diet during week 1 were identified as group 1, and subjects consuming a moderate fiber diet during week 1 constituted group 2. The experimental procedures for this study were approved by the Human Subjects Committee of Iowa State University, subjects gave their informed consent to the study.

Experimental design. The experiment was divided into two 7-day phases separated by 1 week. With a randomized crossover design, either high or moderate fiber diets were randomly given to the 9 subjects for 7 days. During each feeding period, 1.2 mg (4.5 μmol) total isoflavones (Novasoy®, ADM Nutraceutical, Decatur, IL)/ kg body weight was given for 7 days. The isoflavone-containing supplement was mixed with orange juice given with breakfast between 7:30 and 8:30 am. The subjects consumed breakfast and dinner in the Human Metabolic Unit, Iowa State University, and carried out their lunch and snacks. All foods including three meals and three snacks /d were provided and the subjects were asked to finish all foods and not to consume any other foods except for water, which was consumed *ad libitum*. On day 7 (the last day of feeding) of each feeding period, 2 gelatin capsules containing 16 marker beads were given to each subject with breakfast for measuring GTT (6). All subjects were asked to maintain their regular diet during the one-week washout period and to avoid any isoflavone-containing foods one week before and during the entire study. A list of isoflavone-containing foods was given to each subject (see Appendix C.). Nutrition history was obtained from each subject at the beginning of the experiment.

Experimental diets. The experimental diets were two high fiber diets (type 1 and type 2) and two moderate fiber diets (type 1 and type 2) (Table 3) designed with

Nutritionist V™, (version 1.5, 1998, First DataBank Inc., San Bruno, CA) (see Appendix D.). All type 1 diets were consumed on Monday, Wednesday, Friday and Sunday; type 2 diets were consumed on Tuesday, Thursday and Saturday. Nutrient intakes/d were based on recommended intakes for 18 ~ 40 y females: 9196 kJ (2200 kcal) energy, 110 g protein, 275 g carbohydrate, 73 g total fat, respectively. For the fiber source in designed diets, 52 % of the fiber was from whole wheat, 40 % was from fruits and vegetables, and 8% from other fiber sources. The high and moderate fiber diets differed significantly only in dietary fiber content (Table 3).

Biological sample collection. One blank urine sample (time 0) was collected from each subject in the morning on day 1 before initial feeding in both feeding periods. Urine was collected for 24 h on day 6, 7 and 8, as well as the first urination on day 9 in both feeding periods. Subjects were instructed to keep the urine samples in ice chests or refrigerators. Immediately after collection, 24 h urine from each subject was mixed well, and 50 mL aliquots were transferred and stored at -80°C until analysis. After overnight fast, 10 mL venous blood was collected into EDTA-containing vacuum containers in the morning before initial feeding (time 0) and on day 7 and 8 before breakfast in both feeding periods. Blood samples were centrifuged within 0.5 h after collection at 3000 g, 30 min, and 4°C (Model 4D, International Equipment, Needham Heights., MA). Plasma was removed and stored at -20°C until analysis. After ingestion of marker beads with breakfast on day 7, feces were collected until 75% of beads were excreted (12 out of 16) during both feeding periods (6).

Analytical Methods

Chemicals. Daidzein, genistein, glycitein and 2, 4, 4'-trihydroxybeozoin (THB), used as internal standard, were synthesized in Dr. Murphy's laboratory, Iowa State University (11, 12). Other HPLC solvents and extraction agents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Soy product analysis. Novasoy® was analyzed for isoflavone content in Dr. Murphy's laboratory, Iowa State University. Isoflavone extraction and HPLC analysis were performed as described (13, 14). The isoflavone content was the sum of total daidzein, genistein and glycitein, expressed on a molar basis as the aglucon forms (450 ± 15.1 , 608 ± 20.3 , and $111 \pm 7.9 \mu\text{mol/g}$ for daidzein, genistein and glycitein, respectively).

Plasma and urinary isoflavone analysis. Urinary and plasma isoflavone extraction, HPLC analysis and recovery studies were performed as described previously (6). The analytical recoveries of urinary daidzein, genistein, glycitein and THB were 85.3%, 83.6% and 82.5%, and 86.0% respectively. The recoveries of plasma daidzein, genistein, glycitein and THB were 79.5, 75.2%, and 74.4%, and 80.6% respectively. Reported urinary and plasma isoflavones were calculated according to the internal standard curves of each individual isoflavone, THB used as internal standard.

Statistical analysis. Statistical analysis was conducted with SAS (Version 8.2, 2001, SAS Institute, Carry, NC). Crossover effect was not significant after statistical test; data were combined across the two feeding periods. General linear models, multiple comparisons with Tukey's test were used to test effects of fiber treatment and repeated dosing. All results were reported as mean \pm SEM, significance level $P < 0.05$ for all analyses.

RESULTS

Subject characteristics and diet comparisons before and during treatment.

There was no significant difference between the high fiber group (group 1) and moderate fiber group (group 2) fed during week 1 in age, D_k , body weight, BMI or pretreatment GTT (Table 1). But the pretreatment daily energy and total fat intake were significantly greater in group 1 than in group 2 ($P < 0.05$, Table 2). The pretreatment dietary fiber intake of both groups 1 and 2 was significantly less than the high fiber treatment diet ($P < 0.0001$), but not different from the moderate fiber treatment diet. In group 1, other pretreatment daily nutrient intakes were not significantly different from either high fiber or moderate fiber treatment diets except that carbohydrate intake was less compared with the high fiber diet may due to the fiber supplementation ($P < 0.05$) (Tables 2 & 3). In group 2, the pretreatment daily energy and carbohydrate intake were significantly less than both high fiber and moderate fiber dietary treatments ($P < 0.05$) (Tables 2 & 3).

GTT comparisons before and after treatment. Before dietary treatments, subjects' GTT was 108 ± 9 h (Table 1). After high fiber diet treatment, GTT was significantly less by 41% compared with pretreatment GTT (64 ± 9 vs. 108 ± 9 h, $P < 0.05$, respectively); after moderate fiber diet treatment, GTT was 19% less than pretreatment (87 ± 9 vs. 108 ± 9 h, respectively), but this difference was not significant ($P > 0.05$). Consuming the high fiber diet caused 26 % more rapid GTT compared with the moderate fiber diet, but this difference was not significant ($P > 0.05$) (Table 4). Pretreatment GTT was significantly longer than GTT measured after week 1 and week 2 (108 ± 9 , 77 ± 9 and 75 ± 9 h, $P < 0.05$, respectively) and there was no difference in GTT between week 1 and week 2 (Table 4).

Effects of dietary fiber and repeated isoflavone dosing on urinary isoflavones.

There were no significant differences in 24 h urinary isoflavone excretion between the high and moderate dietary fiber treatments. But urinary isoflavones were significantly greater in week 1 than in week 2 ($P < 0.05$) (Table 4).

Effects of dietary fiber and repeated isoflavone dosing on plasma isoflavones.

At 24 h after soy meal feeding, plasma daidzein was significantly greater after moderate fiber diet compared with high fiber diet ($P < 0.05$) (Table 5). Plasma genistein and total isoflavones were significantly greater in week 1 compared with week 2 ($P < 0.05$) (Table 5). No plasma glycitein was detected.

The details about the 24 h urinary excretion and concentration of plasma (24 h after dosing) total and individual isoflavones according to the order of fiber feeding were shown in Appendix A. by group.

DISCUSSION

Diet, GTT and isoflavone absorption. Previous studies showed that significant differences in GTT corresponded with significant differences in urinary isoflavone excretion (6). Among 35 Asian (Chinese immigrant) women, 25 high genistein degraders had GTT of 63 h, which was significantly longer than that of 10 low genistein degraders who had GTT of 40 h ($P < 0.05$). The 10 low degraders had significantly greater urinary genistein excretion than that of 25 high degraders after a single dose providing $4.6 \mu\text{mol}$ total isoflavone/kg. But Caucasian low and high genistein degraders did not differ in their GTTs or in urinary isoflavone excretion (6). In another study comparing isoflavone bioavailability between 6 high daidzein degraders and 4 low daidzein degraders after two 7-day feeding trials providing

either 54.3 μmol or 162.6 μmol total isoflavone daily, subjects who had a low daidzein degradation phenotype coupled with significantly shorter GTT, compared with subjects who had a high degradation phenotype with longer GTT (65 vs. 131 h, $P < 0.05$) had significantly greater total urinary isoflavone excretion (189 vs. 119 $\mu\text{mol/d}$, $P < 0.05$) (8). In a single day crossover study of the effects of dietary fiber intake on apparent isoflavone absorption in 7 women who consumed a single dose of total isoflavones (0.9 mg/kg body weight, high fiber intake (40 g/d) reduced total urinary genistein by 20% compared to fiber intake of 15 g/d ($P < 0.03$) as well as total plasma genistein by 55%, but only at 24 h after dosing ($P < 0.05$) when plasma levels were quite low ($< 0.2 \mu\text{mol/L}$). Urinary daidzein was not affected (15). Based on these studies, seemingly both GTT and increased dietary fiber intake influenced isoflavone bioavailability but in opposite ways. Increased dietary fiber intake over time decreases GTT to some extent. Theoretically, if GTT can be decreased significantly after high fiber intake for a period of time, isoflavone bioavailability should be increased as well. The previous observation of modestly decreased genistein bioavailability due to high fiber intake for a single day was probably not due to any effect of the dietary fiber on GTT but due to direct dietary fiber-isoflavone interactions in the gut. Such interactions would need to be accounted for in attempting to use dietary fiber as an approach to modifying GTT and isoflavone absorption.

We attempted to modify subjects' GTT in the present study. We gave subjects high fiber diets for a week, which reduced GTT significantly by 41% ($P < 0.05$) compared with their pretreatment GTT. During week 1 and week 2, GTT was reduced by 29% and 31%, respectively ($P < 0.05$), compared with pretreatment GTT. GTT after week 1 was not

different from that after week 2. Surprisingly, moderate fiber diet also decreased GTT (it was neither significantly different from pre-treatment nor high dietary fiber treatment). Especially subjects of group 2 who were fed moderate fiber during week 1 increased daily energy intake significantly by both dietary treatments compared with their pre-treatment energy intake (Table 2 & 3). Perhaps increased energy intake and increased fiber intake had similar effects to speed GTT.

Before treatment, subjects in the group 1 (fed high fiber during week 1) had the mean daily energy intake and total fiber intake of 8632 kJ and 19 g, respectively. When they consumed a high fiber diet in week 1, only the fiber intake was significantly different between their pre-treatment and high fiber diets. This difference in fiber intake resulted in a 32% more rapid GTT compared with their pretreatment GTT (72 ± 17 vs. 106 ± 13 h, $P < 0.05$), which indicated that 3-fold greater dietary fiber intake accelerated GTT based on ~ 8360 kJ (~ 2000 kcal) daily energy intake. When these subjects switched from high fiber during week 1 to moderate fiber during week 2 (i.e., back to their usual diet), their GTT was 24% slower than when they consumed high fiber diets (89 ± 18 vs. 72 ± 17 h), but still 16% more rapid than their pre-treatment GTT (89 ± 18 vs. 106 ± 13 h). There may be some carryover effect of the high fiber diet although it was not statistically significant ($P = 0.23$).

For subjects in the group 2 (fed moderate fiber diets during week 1), not only high fiber intake, but also the increased daily energy intake seemingly sped GTT. These subjects had mean daily energy intake of ~ 6300 kJ (~ 1600 kcal) before the treatments, which was significantly less than the energy content of both treatment diets. After 1-week moderate fiber treatment, these subjects' GTT was 25% faster compared with their pretreatment GTT

(84 ± 19 vs. 112 ± 14 h, $P > 0.05$), suggesting an effect of increased energy intake to speed GTT although this effect was not significant. The additional effect of high fiber diets in week 2 caused a 30% more rapid GTT than in week 1 (59 ± 20 vs. 84 ± 19 h, $P > 0.05$), again, this was significantly different compared with their pretreatment GTT but not significantly different from week 1, which may be due to the small number of subjects studied.

Seemingly both increased energy and fiber intakes may be important variables in attempting to better control GTT, which also suggested by previous studies. In one observational study, 13 male and 16 females consumed self-selected diets and collected duplicate portions of all food consumed for measuring fiber intake and all feces for 7 days four times during the year. Fecal weight was significantly correlated with fiber intake ($r = 0.524$, $P < 0.0001$), and they were also significantly correlated with caloric intake ($r = 0.543$, $P < 0.001$). Although this study did not collect GTT information from each subject, fecal weight (which is related to GTT) seemed to be related to total food intake as well as fiber intake (16). Another study used a crossover design to determine the relationship between fecal weight, GTT and fiber intake. Forty-four subjects consumed a high fiber diet containing 19 g of dietary fiber per 1000 kcal and a low fiber diet containing 6 g/1000 kcal for 3 weeks of each. About 55% of the dietary fiber was provided by fruits and vegetables and the rest by bread and other cereal products. Feces were collected for 4~5 days of each period. GTT were measured using radio-opaque plastic pellets. There was a strong influence of the amount of fiber intake on both GTT and fecal fresh weight. GTT went down as fecal weight went up and continued to decrease as fecal weight increased above 140 g/d (17). In a similar study, significantly linearly inverse relationship between GTT and stool weight was found ($r = 0.8$,

$P < 0.05$) in 19 men and 11 women when they consumed 30, 60, 110, or 170 g/d bran-enriched wholemeal bread for 3 weeks (9). From these studies, we know that fecal weight significantly positively correlated with caloric intake and fiber intake, and GTT significantly inversely correlated with fecal weight and fiber intake, therefore GTT is inversely related to caloric and fiber intakes. We suspect that pretreatment apparent isoflavone absorption would have been less than both dietary treatments based on the observed difference in GTT. As mentioned above, our previous studies have shown greater apparent isoflavone uptake to be related to more rapid GTT (6, 8).

Repeated isoflavone dosing decreased apparent isoflavone uptake. Apparent isoflavone uptake as reflected in urinary excretion was greater in week 1 than in week 2 of our study (Table 4). GTT in both week 1 and week 2 was significantly shorter than pre-treatment GTT in all subjects (77 ± 9 and 75 ± 9 vs. 108 ± 9 h, $P < 0.05$). Because GTT was significantly more rapid in week 1 and week 2 than pre-treatment, we would expect greater urinary isoflavone excretion after dietary treatments than before; that remains to be seen in future studies.

Lu et al. (18-19) showed that after daily dose of 80~210 mg of each isoflavone (daidzein and genistein) as in soymilk for one month, urinary excretion of isoflavone was about 10% lower than that of initial excretion in women but not in men. A previous 7-day crossover high and low dose of isoflavones study using a 1 week washout period between the two doses similar to the present study's washout period between the two dietary fiber treatments, also showed a significant 20~30% decrease in urinary isoflavone excretion from week 1 to week 2 across doses (8). We know that isoflavones can be degraded by gut

bacteria. It is possible that the isoflavone degrading microorganisms adapt over time to increase activity. This remains to be proven, and characterization of isoflavone metabolizing gut microflora is an important research need.

Plasma and urinary isoflavones. Concentrations of plasma isoflavone showed similar effect of repeated dosing as did urinary isoflavone excretion: plasma isoflavone contents were generally greater in week 1 than in week 2. As mentioned above, Tew et al. (15) found that the fiber-rich diet produced 55% lower plasma genistein at 24 h after soy dosing ($P < 0.05$). We also found decreased plasma isoflavone, but only daidzein, after high fiber diet compared with moderate fiber diet. The lesser plasma daidzein in the current study may be due to reduced β -glucuronidase activity caused by high fiber intake, therefore lessening daidzein reabsorption in the intestine. But there is no explanation as to why this effect should be selective for daidzein and not for genistein. Rose et al. (20) observed a suppression of estrogens bioavailability after doubled fiber intake of 62 premenopausal women from about 15 g to 30 g with wheat bran for 2 months: serum estrone ($P < 0.002$) and estradiol ($P < 0.02$) were significantly reduced by 15~20%. Isoflavones are structurally similar to estrogen, which high fiber intake may have the same suppression effect on isoflavone absorption. Similar to estrogens, isoflavones are conjugated with glucuronic acid in liver and intestine and then maybe 50~70% of conjugated isoflavones are excreted in bile. The reabsorption of isoflavones requires the hydrolysis of β -glucuronidase, releasing free form of isoflavones. Fiber intake has been shown to reduce β -glucuronidase activity in rats and chicks (21-22). Male F344 rats were fed either fiber-free basal diet or this basal diet mixed with 5 and 15% of fiber diet for 12 days. Specific activity and total output of β -

glucuronidase were significantly lower in those fed 15% fiber diet and higher in rats fed fiber-free diet (21). In another study, when dietary fiber was given at 30% level of diet to chicks for 2-week, significantly lowered bacterial β -glucuronidase activity (22). Thus we would expect this also to happen in humans; therefore high fiber intake may result in lower plasma isoflavone. Furthermore, the binding of fiber with unconjugated isoflavones may also prevent isoflavone reabsorption in the intestine.

We did not find urinary isoflavones to be different after high fiber diet compared with moderate fiber diet. This may have occurred because of opposing effects of high fiber treatment on urinary isoflavone excretion. First, high fiber intake decreased GTT, and increased fresh fecal weight (17), lowering bacterial β -glucuronidase and β -glucosidase concentrations (21-22, 15), resulting in lower opportunity for isoflavone reabsorptions. On the other hand, this greater excretion of intestinal bacteria and more rapid GTT may also decrease the degradation of isoflavones by these bacteria, therefore increasing the absorption and reabsorption of isoflavones. The overall influence of high fiber treatment on urinary isoflavone excretion may depend on which effect is significant, the lowering bacterial enzyme activities or the lowering degradation of isoflavones; and if GTT is accelerated significantly, which was not the case when the two treatments were compared.

In the present study, although GTT may be a determinant of isoflavone absorption, the strategy of speeding GTT by increasing dietary fiber intake must be done in a very carefully controlled manner, e.g. both treatment groups need to have similar total energy and other nutrient intakes before dietary treatment. Isoflavone uptake may be also affected by repeated dosing due to the induction of isoflavone degradation by the gut microflora. The probable

adaptation of isoflavone degrading microorganisms to increase their activity over time must also be taken into account in designing feeding trials.

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TABLE 1

Subject characteristics¹

Dietary fiber treatment	n	D_k h^{-1}	Age Y	Body weight kg	BMI kg/m^2	Initial GTT h
High in week 1 (group 1)	5	0.07 ± 0.02	22.4 ± 2.3	75.8 ± 4.0	25.8 ± 1.2	106 ± 13
Moderate in week 1 (group 2)	4	0.05 ± 0.02	21.5 ± 2.6	66.1 ± 4.5	24.0 ± 1.3	112 ± 14
Mean		0.06 ± 0.01	22.0 ± 1.6	71.5 ± 3.3	25.1 ± 0.8	108 ± 9

¹Subjects who consumed high fiber diets in week 1 were designated as group 1, subjects who consumed moderate fiber diets in week 1 were designated as group 2.

TABLE 2

Pre-treatment nutrient intakes of subjects based upon 24-h food records^{1,2}

Subject ID	Energy intakes <i>kJ (kcal)</i>	Protein <i>Gm</i>	Carbohydrate <i>gm</i>	Total fat <i>gm</i>	Total dietary fiber <i>gm</i>
1	8857 (2119)	127	232	77	13
4	8864 (2162)	96	286	69	14
6	8414 (2013)	64	276	77	20
7	10149 (2428)	147	272	84	15
9	6705 (1604)	56	250	47	32
Mean of group 1	8632 ± 598 ^b (2065 ± 143)	98 ± 14	263 ± 14	71 ± 8 ^b	19 ± 3
2	6492 (1553)	54	273	28	24
3	5204 (1245)	46	222	26	19
5	8515 (2037)	76	294	67	23
8	5693 (1362)	65	204	30	21
Mean of group 2	6475 ± 673 ^a (1549 ± 161)	60 ± 15	248 ± 16	38 ± 8 ^a	22 ± 3
Mean	8347 ± 564 (1836 ± 135)	81 ± 12	257 ± 10	56 ± 8	20 ± 2

¹Subjects # 1, 4, 6, 7 and 9 consumed high fiber diets in week 1 (group 1), subjects # 2, 3, 5, 8, consumed moderate fiber diets in week 1 (group 2).

² The energy intakes, and total fat intake with different superscripts (a, b) differed significantly between group 1 and group 2, $P < 0.05$.

TABLE 3

Nutrient composition of treatment diets ^{1,2,3}

Diet (Type)	Energy intakes	Protein	Carbohydrate	Total fat	Total dietary fiber
	<i>kJ (kcal)</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
High fiber (1)	8729 (2088)	77	345	56	45
High fiber (2)	8895 (2128)	113	338	48	45
Moderate fiber (1)	8966 (2145)	76	302	69	15
Moderate fiber (2)	8736 (2090)	100	298	59	15
Mean of high fiber diets	8811 ± 84 (2108 ± 20)	95 ± 18	342 ± 3.5	52 ± 4	45 ± 0 ^b
Mean of moderate fiber diets	8851 ± 117 (2118 ± 28)	88 ± 12	300 ± 2	64 ± 5	15 ± 0 ^a

¹ The diets were designed by using Nutritionist V (Nutritionist V TM, version 1.5, 1998, First DataBank Inc., San Bruno, CA) based on 18-40 y female, the recommended nutrients intakes are: energy: 9196 kJ/d (2200 kcal/d), protein: 110 gm/d, carbohydrate: 275 gm/d, total fat 73 gm/d, and total dietary fiber: 24 gm/d.

² All Type 1 diets were consumed on Monday, Wednesday, Friday and Sunday and all Type 2 diets were consumed on Tuesday, Thursday, and Saturday.

³ Dietary fiber bearing different superscripts (a, b) differed significantly between the high fiber and low fiber diets, $P < 0.05$.

TABLE 4

The 24-h urinary excretion of total and individual isoflavones during high and moderate fiber treatment, and during week 1 and 2, compared with gut transit time (GTT) ^{1,2}

Fiber treatment	GTT	Daidzein	Genistein	Glycitein	Total
	<i>h</i>	$\mu\text{mol/kg}$	$\mu\text{mol/kg}$	$\mu\text{mol/kg}$	$\mu\text{mol/kg}$
High	64 ± 9	4.21 ± 0.68	2.10 ± 0.40	0.79 ± 0.11	7.10 ± 1.16
Moderate	87 ± 9	4.20 ± 0.66	2.35 ± 0.39	0.60 ± 0.10	7.15 ± 1.13
Week	GTT	Daidzein	Genistein	Glycitein	Total
	<i>h</i>	$\mu\text{mol/kg}$	$\mu\text{mol/kg}$	$\mu\text{mol/kg}$	$\mu\text{mol/kg}$
1	77 ± 9	5.65 ± 0.56 ^b	2.85 ± 0.36 ^b	0.94 ± 0.09 ^b	9.43 ± 0.97 ^b
2	75 ± 9	2.68 ± 0.58 ^a	1.57 ± 0.37 ^a	0.43 ± 0.09 ^a	4.68 ± 1.00 ^a

¹ The urinary excretion of isoflavones was the mean of day 6 and d 7.

² The urinary excretions of total and individual isoflavones with different superscripts (a, b) were significantly different between the week 1 and week 2, $P < 0.05$.

TABLE 5

The plasma concentration of total and individual isoflavones during high and moderate fiber treatments, and during week 1 and 2^{1,2,3}

Fiber treatment	GTT	Daidzein	Genistein	Glycitein	Total
	<i>h</i>	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$
High	64 ± 9	0.0 ± 0.11 ^a	2.81 ± 0.82	ND ⁴	2.81 ± 0.86
Moderate	87 ± 9	0.24 ± 0.10 ^b	3.88 ± 0.80	ND	4.12 ± 0.83
Week	GTT	Daidzein	Genistein	Glycitein	Total
	<i>h</i>	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$
1	77 ± 9	0.22 ± 0.10	4.91 ± 0.74 ^d	ND	5.13 ± 0.78 ^d
2	75 ± 9	0.03 ± 0.11	1.89 ± 0.72 ^c	ND	1.93 ± 0.75 ^c

¹ The plasma concentration of isoflavones was the mean of day 7 and 8 at 24 h after dose.

² The plasma concentration of daidzein with different superscripts (a, b) was significantly different between high fiber and moderate fiber diet, $P < 0.05$.

³ The plasma concentration of genistein and total isoflavone with different superscripts (c, d) was significantly different between week 1 and week 2, $P < 0.05$.

⁴ ND = not detected.

GENERAL CONCLUSIONS

To better assess the potential health effects of isoflavones, their bioavailability must be better understood. Optimizing isoflavone bioavailability is likely to permit more conclusive studies of these compounds. All the three human feeding studies I have done demonstrated that isoflavone degradation phenotypes coupled with GTT significantly influence the bioavailability of isoflavone (apparent absorption) as reflected in urinary excretion and GTT is a crucial factor.

We compared bioavailability of isoflavones in their two major forms (aglucons vs. glucosides) and between two sources (rich in glycitein vs. genistein) in women fed the isoflavone for 7 days. We showed that the daily bioavailability of the two major isoflavone forms (aglucons vs. glucosides) did not differ as reflected in total urinary excretion over 24 h, but that genistein was less well absorbed than glycitein or daidzein as reflected in total urinary isoflavone excretion. Isoflavone aglucons were absorbed more rapidly than glucosides as reflected in total and individual plasma isoflavone content at 3 h after feeding, which might have implications for choice of isoflavone source when a rapid effect is desired.

We examined the bioavailability (urine and plasma content) of high and low doses of isoflavone among individuals of varying GTT and daidzein degradation phenotype. The results from this study suggested that the effects of gut microflora on absorption of isoflavone probably relate strongly to gut transit time, i. e., the length of time gut microbial species have to exert their effects on isoflavones. Repeated feeding soy isoflavones for a period of time may decrease their bioavailability due to induced gut microbial degradation of these compounds.

In order to further investigate the importance of GTT, in the third study, we hypothesized that high dietary fiber intake could improve apparent isoflavone absorption by speeding GTT compared with moderate dietary fiber intake in women who were identified as low daidzein degraders but also had relatively longer GTT. The acceleration of GTT could prevent isoflavone degradation in such women, permitting them to absorb more isoflavones as reflected in urinary excretion and plasma concentrations. We found that although GTT may be a determinant of isoflavone absorption, it can be modified by many factors. The strategy of speeding GTT by increasing dietary fiber intake must be done in a very carefully controlled manner, e.g. both treatment groups need to have similar total energy and other nutrient intakes before dietary treatment. Again we found isoflavone uptake may be affected by repeated dosing due to the induction of isoflavone degradation by the gut microflora.

In conclusion, bioavailability of isoflavone was significantly greater in subjects of a low isoflavone degradation phenotype with shorter GTT than in subjects of a high isoflavone degradation phenotype with longer GTT as reflected in total urinary isoflavone excretion. This phenomenon should be taken into account to better control human feeding studies of the effects of isoflavones. The probable adaptation of isoflavone degrading microorganisms to increase their activity over time must also be taken into account in designing feeding trials.

**APPENDIX A. URINARY EXCRETION OF ISOFLAVONES IN GROUP 1 AND
GROUP 2**

The 24-h urinary excretion of total and individual isoflavones according to order of feeding treatments and compared with gut transit time (GTT)^{1, 2, 3, 4}

Group	Week	Fiber treatment	GTT <i>h</i>	Daidzein $\mu\text{mol/kg}$	Genistein $\mu\text{mol/kg}$	Glycitein $\mu\text{mol/kg}$	Total $\mu\text{mol/kg}$
1	1	High	72 ± 17	5.66 ± 0.56 ^b	2.90 ± 0.42	1.09 ± 0.10 ^b	9.64 ± 1.02 ^b
	2	Moderate	89 ± 18	3.06 ± 0.56 ^a	2.00 ± 0.42	0.48 ± 0.10 ^a	5.54 ± 1.02 ^a
2	1	Moderate	84 ± 19	5.63 ± 1.10 ^d	2.78 ± 0.64	0.75 ± 0.14	9.16 ± 1.85
	2	High	59 ± 20	2.14 ± 1.18 ^c	0.95 ± 0.68	0.35 ± 0.15	3.45 ± 1.98
Mean of group 1			80 ± 16	4.36 ± 0.49	2.45 ± 0.30	0.78 ± 0.10	7.59 ± 0.84
Mean of group 2			72 ± 18	4.00 ± 0.90	1.93 ± 0.51	0.57 ± 0.11	6.50 ± 1.51

¹ The urinary excretion of isoflavones was the mean of day 6 and 7.

² Subjects consumed high fiber diet during week 1 was assigned to group 1, subjects consumed moderate fiber diet during week 1 was assigned to group 2.

³ The urinary excretion of daidzein, glycitein, and total isoflavones with different superscripts (a, b) were significantly different between high and moderate fiber diet in group 1, $P < 0.05$.

⁴ The urinary excretion of daidzein with different superscripts (c, d) was significantly different between high and moderate fiber diet in group 2, $P < 0.05$.

**APPENDIX B. PLASMA CONCENTRATION OF ISOFLAVONES IN GROUP 1 AND
GROUP 2**

The plasma concentration of total and individual isoflavones according to order of feeding treatments and compared with gut transit time (GTT) ^{1,2,3}

Group	Week	Fiber treatment	GTT <i>h</i>	Daidzein $\mu\text{mol/L}$	Genistein $\mu\text{mol/L}$	Glycitein $\mu\text{mol/L}$	Total $\mu\text{mol/L}$
1	1	High	72 ± 17	ND ⁴	3.74 ± 0.80	ND	3.74 ± 0.82
	2	Moderate	89 ± 18	0.06 ± 0.04	2.00 ± 0.76	ND	2.06 ± 0.76
2	1	Moderate	84 ± 19	0.64 ± 0.23	5.65 ± 1.39 ^b	ND	6.28 ± 1.42 ^b
	2	High	59 ± 20	0.0 ± 0.23	1.43 ± 1.39 ^a	ND	1.43 ± 1.42 ^a
Mean of group 1			80 ± 16	0.03 ± 0.03	2.82 ± 0.57	ND	2.85 ± 0.58
Mean of group 2			72 ± 18	0.32 ± 0.17	3.54 ± 1.09	ND	3.86 ± 1.16

¹ The plasma concentration of isoflavones was the mean of day 7 and 8 at 24 h after dose.

² Subjects consumed high fiber diets during week 1 was assigned to group 1, subjects consumed moderate fiber diets during week 1 was assigned to group 2.

³ The concentration of plasma genistein, and total isoflavones with different superscripts (a, b) was significantly different between high and moderate fiber diet in group 2, $P < 0.05$.

⁴ ND = not detected.

APPENDIX C. FOODS CONTAIN ISOFLAVONES

I. Soy Protein Isolate

Soybeans
 Tofu
 Soymilk, Soymilk powder
 Soybean sprouts
 Tempeh
 Miso soup
 Special K frozen waffles
 Carnation Instant Breakfast-chocolate malt flavored

II. Food Containing Texturized Vegetable Protein

Frozen pizza
 burritos
 Morningstar Farm breakfast links, patties, strips
 La Choy lobster egg rolls
 liquid non-dairy creamers

III. Foods Containing Hydrolyzed Vegetable Protein (HVP)

Most chip dips (French onion and some others)
 Garden vegetable flavored cheese spreads
 Many frozen entree's
Tombstone frozen pizza with meat
 Some franks (*John Morrel*, *Homel Light & Lean*, etc.)
 Many sauce mixes-gravy (usually brown), chill, etc.
Knorr soup mixes
Knorr dry sauce mixes except "Pesto"
 Some canned soups(usually those containing vegetable & meat, like chicken and mushroom)
 Many *La Choy* foods as well as oriental style mixes, etc. (containing HVP or soy sauce)
 Soy sauce has soybeans pr protein extracts from soybeans
Ramen noodles containing HVP and /or soy sauce powder
Heinz worestershire sause(containing HVP in soy sauce)
Hiland Red Hot Piplets(most other chips and snacks were fine)
Uncle Ben's Rice mix
 Some herbal magic salad dressing, Girad's salad dressing
 Most bacon flavored bits

APPENDIX D. TREATMENT DIETS

Type I high fiber diet:

Breakfast

Whole wheat bread: 2 slices
Jam: 2 tbsp
Fat free milk: 16 fl oz
Peanut butter: 2 tbsp
Orange juice: 8 fl oz
Apple: 1 item

Morning snack

Potato chips: 1 small package

Lunch

Smoked turkey breast: 2 slices
Whole wheat bread: 2 slices
Iceberg lettuce leaves: 1 piece
Tomato: 2 slices
Low calorie mayonnaise: 2 tbsp

Afternoon snack

Whole grain wheat crackers: 6 items

Dinner

Baby carrots: 0.5 cup
French onion dip: 2 tbsp
Whole wheat spaghetti: 2 cups
Tomato sauce: 1 cup
Orange: 1 item

Evening snack

Whole wheat onion cracker: 6 items

Type II high fiber diet:

Breakfast

Whole grain cereal: 1 cup
Fat free milk: 16 fl oz
Wheat bran muffin: 1 item
Orange juice: 8 fl oz
Apple: 1 item

Morning snack

Corn tortilla: 1 small package

Lunch

Smoked ham: 2 slices
Whole wheat bread: 2 slices
Tomato: 2 slices
Low calorie mayonnaise: 2 tbsp

Afternoon snack

Whole grain wheat crackers: 6 items

Dinner

Celery: 1 cup
Broccoli: 1 cup
French onion dip: 2 tbsp
Whole wheat pizza: 1 slice
Chicken noodle soup: 1 cup
Orange: 1 item

Evening snack

Whole wheat onion cracker: 6 items

Type I moderate fiber diet:

Breakfast

White bread: 2 slices
Jam: 2 tbsp
Fat free milk: 16 fl oz
Peanut butter: 2 tbsp
Orange juice: 8 fl oz

Morning snack

Milk chocolate bar: 1 item

Lunch

Smoked turkey breast: 2 slices
White bread: 2 slices
Iceberg lettuce leaves: 1 piece
Low calorie mayonnaise: 2 tbsp

Afternoon snack

Potato chips: 1 small package

Dinner

Spaghetti: 1 cup
Tomato sauce: 1 cup
Chocolate cake 1 slice

Evening snack

Nonfat plain yogurt: 1 cup

Type II moderate fiber diet:

Breakfast

Honey nut cereal: 1 cup
Small muffin: 1 item
Fat free milk: 16 fl oz
Orange juice: 8 fl oz

Morning snack

Potato chips: 1 small package

Lunch

Ham: 2 slices
White bread: 2 slices
Tomato: 2 slices
Low calorie mayonnaise: 2 tbsp

Afternoon snack

Vanilla Pudding: 1 cup

Dinner

Pizza: 1 slice
Chicken noodle soup: 1 cup
Banana 1 item

Evening snack

Nonfat plain yogurt: 1 cup

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