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Human bioavailability and health protective effects of soy isoflavones

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INTRODUCTION

Flavonoids are ubiquitously present in the plant kingdom (Ho 1992). They are phenolic compounds (Figure 1). The basic structure of these compounds consists of two aromatic rings linked by a three carbon aliphatic chain which normally has been condensed to form a pyran or, less commonly, a furan ring. Based on the oxidation state of the aliphatic fragment and the position of C ring attached to the B ring, they can be subdivided into: flavones, flavanones and isoflavones.

Flavonoids are ingested in daily quantities of about 1 g by humans who eat diets typical of those found in the Western world (Kuhnan 1976). They are present in tea, coffee, cereal grains, beans and a variety of fruits and vegetables (Huang and Ferraro, 1992). Isoflavones are present in large quantities (0.2-1.5 mg/g) in soybean foods (Wang and Murphy 1994), which are almost the only source of isoflavones in the American diet (Messina and Barnes, 1991).

Although high intake of fruits and vegetables has been associated with lower cancer risk (Willett 1994), evidence also suggests that higher intake of isoflavones from a soy rich diet in eastern Asia is associated with much lower incidence of breast, colon and prostate cancer than in those who eat a typical Western diet (Adlercreutz et al., 1991 and 1995). Therefore, understanding the mechanism of potential health protective effects by characterizing isoflavone bioavailability and specific cancer-preventive effects is important.

Variety and content of isoflavones in soybean foods

Soybeans contain three types of isoflavones which are present as four chemical forms: 1) the aglycones: daidzein, genistein and glycitein; 2) the glucosides: daidzin, genistin

and glycitin; 3) the acetylglucosides: 6"-*O*-acetyldaidzin, 6"-*O*-acetylgenistin, 6"-*O*-acetylglycitin; and 4) the malonylglucosides: 6"-*O*-malonyldaidzin, 6"-*O*-malonylgenistin, 6"-*O*-malonylglycitin (Kudou et al. 1991).

By high-performance liquid chromatography and photodiode array detection, Wang and Murphy (1994) characterized the concentration and distribution of all twelve isoflavone isomers in 29 commercial soybean foods which were categorized into soy ingredients, traditional and second-generation soy foods. Compared with unprocessed soybeans (total isoflavone concentration: 1.2-4.2 mg/g), high-protein soy ingredients such as soy flour and texturized vegetable protein contained similar isoflavone concentrations (total isoflavone concentration: 1.1-1.4 mg/g), except soy concentrate. Soy concentrate was produced by a water or alcohol wash of soy flakes to remove soluble carbohydrates and improve functionality. It had extremely low isoflavone concentration because alcohol washing removed most of the isoflavones. With minimal procession soy flour had 6"-*O*-malonyldaidzin and 6"-*O*-malonylgenistin as its major isomer. In contrast, due to heat treatment during extrusion processing which transformed malonyl isoflavones to acetyl forms, texturized vegetable protein had appreciable amount of 6"-*O*-acetyldaidzin and 6"-*O*-acetylgenistin. Traditional soybean foods showed differences between nonfermented foods (e.g. tofu) and fermented foods (e.g. tempeh). Nonfermented foods had greater levels of glucosides, and greater levels of aglycones were present in fermented foods due to enzymatic hydrolysis during fermentation. Second-generation soy foods such as tofu yogurt and tempeh burger were made by adding soy ingredients to a variety of foods to replace animal protein and/or to reduce fat. They contained only 6-20% of the isoflavones found in whole soybeans, since most of the food matrices in these foods were nonsoybean constituents.

Barnes et al. (1994) examined the extraction of isoflavones from several commercial soy foods and ingredients with particular reference to the effect of heating during this process. Extraction of isoflavones from soy products with 80% aqueous methanol at room temperature was just as efficient as at 60-80 °C, but extraction at higher temperatures (60-80 °C) caused changes in isoflavone composition. To facilitate the identification of the isoflavone glycosides in the extracts, heated nebulizer-atmospheric pressure chemical ionization (HN-APCI) and modified electrospray interfaces (ESI) were used to introduce the isoflavone conjugates directly from HPLC into the mass spectrometer without the necessity of hydrolysis or derivatization. They found that soybeans and defatted soy flour (which had been minimally heated during their extraction) contained mostly isoflavone 6"-*O*-malonylglucosides, with lesser quantities of the β-glucosides and only trace amounts of 6"-*O*-acetylglucosides. Soy milk, tofu, and soy molasses, each of which are heated to 100 °C during their manufacture, contained mostly isoflavone β-glucosides. Toasted soy flour and an isolated soy protein had moderate amounts of each of the isoflavone glycosides.

In general, variety and content of isoflavones in soy foods depend upon their procession such as heat treatment, washing, fermentation and dilution by nonsoy ingredients even soy foods were made by soy ingredients from same environment condition (location and corp year).

Estrogenic and antiestrogenic effect of isoflavones

Estrogen is defined as the substance capable of stimulating the growth of vagina, uterus and the mammary gland and responsible for the development of female secondary sex characteristics (Clark et al., 1985). Weak estrogenic effect of isoflavones has been extensively reviewed by Molteni et al.

(1995).

The estrogen receptor binding affinity of natural and synthetic estrogens compared with plant estrogens (mainly isoflavones, lignans and flavones) was studied by Shutt and Cox (1972) in sheep uterine cytosol. The relative nuclear binding affinities in vitro of daidzein, genistein, equol and O-desmethylangolensin were only 0.1%, 0.9%, 0.4% and 0.05% of estradiol-17β, respectively. However, at high concentrations such as 10^2-10^3 times of estradiol (close to the levels of soy isoflavones in human plasma after soybean foods consumption) these isoflavones could compete effectively with the endogenous mammalian estrogens by competitive inhibition and suppress estrogen stimulated growth in mammalian (Adlercreutz et al., 1995).

In vivo genistein and daidzein are roughly 10^5 times less effective than estrogens and DES as shown by mice uterine enlargement assays (Bickoff et al., 1962). In a uterine growth assay reported by Farmakalidis et al. (1985), Inbred B6D2F1 mice were assigned to the dose of 0.12 μg of diethylstilbesterol, 8 mg of genistein or 12 mg each of genistin and daidzin in a completely randomized design. The testing compounds were suspended in 5% Tween 80 and were administered to the mice by stomach intubation in four daily doses of 0.1 ml per day. The estrogenic response to 1.5 mg of genistein was equivalent to 1 mg of genistein, giving a 1:1 molar relationship in estrogenic activity between genistin and genistein. The estrogenic response to 3.8 mg of daidzin was equivalent to 1 mg of genistein.

Flavonoids may be able to reduce estrogen synthesis. In vitro α-naphthoflavone (synthetic flavonoid), chrysin, flavone and biochanin A can inhibit the activity of human preadipocyte aromatase with IC_{50} values of 0.5, 4.6, 68 and 113 μM, respectively (Campbell and Kurzer, 1993). In another cell culture study, isoflavones and their metabolites, daidzein,

equol and O-desmethylangolensin inhibit human placental aromatase with IC_{50} of <1 mM, 150 μM and 160 μM, respectively (Adlercreutz et al., 1993 a). Flavonoids may also decrease free estrogens in circulation system through the stimulation of sex hormone binding globulin synthesis in the liver. Enterolactone (1-10 μM) can increase sex hormone binding globulin synthesis by HepG2 cells in culture (Adlercreutz et al., 1991).

Isoflavone antiestrogenic activity was also studied in neoplastic cells. Verdeal et al. (1980) demonstrated the inhibition of binding of [^{3}H]Estradiol to estrogen receptors in rat mammary tumors when genistein was added. In addition, isoflavones can inhibit the estrogen-receptor complex bind to nuclear type II binding site and reduce the estrogen-stimulated cell proliferation. Daidzein and equol can compete with [^{3}H]Estradiol to rat uterine estrogen nuclear type II binding sites at IC_{50} of 1 and 9 μM, respectively (bioflavonoid receptor) (Adlercreutz et al., 1992).

Therefore, weak estrogenic isoflavones from soy food consumption most likely exert their antiestrogenic activity rather than estrogenic activity to humans.

Antioxidant activity of isoflavones

Naim et al. (1976) reported that isoflavones inhibited lipoxygenase action and prevented peroxidative hemolysis of sheep erythrocytes in vitro. Pratt and Birac (1979) found that phenolic compounds in soybeans, defatted soy flour, soy protein concentrates and soy isolates have appreciable antioxidant activity detected by the rate of β-carotene bleaching in a lipid-aqueous system. Rat hepatic cumene hydroperoxidase activity was increased significantly after feeding a soybean isoflavone extract (at 240 mg isoflavones/kg diet) for one week (Hendrich et al., 1994).

In a recent study reported by Wei et al. (1995), effects of structurally related flavones and isoflavones on hydrogen peroxide (H_2O_2) production by 12-O-tetradecanonylphorbol-13-acetate (TPA)-activated HL-60 cells and superoxide anion ($O_2^{\cdot -}$) generation by xanthine/xanthine oxidase were compared. Among the tested flavones and isoflavones at concentrations of 10, 50, 100 and 200 μM, genistein is the most potent inhibitor (IC_{50}= 25 μM) for TPA-induced H_2O_2 formation by dimethyl sulfoxide differentiated HL-60 cells, daidzein is second (IC_{50}= 150 μM), and apigenin and biochanin A show little effect. In contrast, genistein, apigenin and prunectin are equally potent in inhibiting $O_2^{\cdot -}$ production (IC_{50}= 1-2.5 μM), with daidzein showing a moderate inhibitory effect (IC_{50}= 5 μM) and biochanin A exhibiting no effect.

In an animal study (Wei et al., 1995), dietary feeding of 250 ppm genistein to 6-7 week old female CD-1 mice for 30 days significantly enhances the activities of antioxidant enzymes including catalase, SOD, GSH-PX and GSSG-R in skin and small intestine by 10-30 %. However, no bioavailability data was presented, and authors did not compare the effect of genistein with other flavones or isoflavones.

In conclusion, soy isoflavones can act as antioxidants directly or indirectly through the enhancement of antioxidant enzyme activities.

Anticarcinogenic effect of isoflavones

The epidemiologic association between low incidence of breast, colon and prostate cancer and high isoflavone intake from soy rich diets has been reviewed (Adlercreutz et al., 1995). Some of their key findings are summarized in later part of introduction. In animal studies, feeding a 30% soybean diet to mouse can protect liver from cancer development induced by nitrosamine precursors, dibutylamine

and nitrite (Fitzsimons et al., 1989). Rats consuming a soy-based diet develop fewer mammary tumors following administration of the carcinogens N-methylnitrosourea and 7,12-dimethylbenz[a]-anthracene than rats on isonitrogenous and isocaloric diets without soy (Barnes et al., 1990). In a hepatocarcinogenesis model with female F344/N rats initiated by 15 mg/kg body wt diethylnitrosamine at 10 days of age and promoted by 500 mg/kg diet of phenobarbital, soybean isoflavone extract at 240 or 480 mg /kg diet dose levels normalized the total hepatic glutathione peroxidase activity suppressed by phenobarbital, and inhibited phenobarbital promotion as reflected in volume of gama-glutamyltransferase and placental glutathione transferase positive altered hepatic foci after 3 month of feeding (Lee et al., 1995).

Genistein inhibits the growth of estrogen receptor-negative or -positive human breast cancer cell lines (concentration for 50% inhibition of cell growth (IC_{50}) =24-44 µmol/L; Peterson and Barnes 1991). Genistein and daidzein (IC_{50} of 2.2 µmol/L and 8 µmol/L respectively) can inhibit production of inositol phosphates, key intracellular signals of proliferation stimulated by aluminum tetrafluoride in 3T3 cells (Higashi and Ogawara 1992). In addition, inhibition of phosphatidyl inositol 4,5-bisphosphate hydrolysis by daidzein and genistein may also reduce prostaglandin production and further inhibit cell proliferation, since the 2-position on the glycerol moiety of PIP_2 was usually occupied by arachidonic acid. Genistein inhibits the autophosphorylation of the epidermal growth factor receptor in the A431 human epidermoid carcinoma cells (IC_{50}= 2.6 µmol/L, Akiyama 1987) and DNA topoisomerase II of human leukemic MOLT-4 and HL-60 cells (IC_{50}=31.5 and 48 µmol/L, respectively) but not the normal human proliferating lymphocytes (Traganos et al., 1992). Genistein also blocks endothelial cell proliferation and in vitro angiogenesis, another proposed mechanism of

anticarcinogenesis (IC_{50} of 5 and 150 μmol/L respectively, Fotsis et al., 1993). Genistein suppressed TPA-induced overexpression of proto-oncogene c-*fos*, but not c-*jun* in mice fed 250 ppm genistein for 30 days (Wei et al., 1995).

These findings suggest that anticarcinogenic activities of soy isoflavones are probably due to their profound impact on control of cell growth and proliferation at molecular levels. Based on our human bioavailability and Wang and Murphy's soy food isoflavone data, it seems that only isoflavones' normalizing the total hepatic glutathione peroxidase activity, suppression of hepatocarcinogenesis, phosphatidyl inositol 4,5-bisphosphate (PIP_2) hydrolysis and proto-oncogene c-*fos* overexpression at above in vivo and in vitro studies may be relevant to the actual isoflavone levels in human diet and/or in body general circulation (less or equal to 6 and 5.5 μM for daidzein and genistein, respectively) from physiological achievable amounts of soy food consumptions (maximum dose we used in human feeding was 2.7 mg/kg body wt). It is reasonable to propose that a dose of soy isoflavones ranging from 1.5 to 2 mg isoflavones/ kg body wt per day may exert anticarcinogenic effects in humans (Hendrich et al., 1994).

Modulation of NK cell by flavonoids in cancer prevention

Natural killer (NK) cells represent a subset of lymphocytes (up to 15%), distinguishable from T and B lymphocytes by their morphology, phenotype, and functional capability to spontaneously kill tumor or virus-infected cells (Whiteside and Herberman, 1989). Morphologically, NK cells belong to a subset of large granular lymphocytes; phenotypically, they express a characteristic array of surface markers ($CD3^-$, $NKH1^+$ and $CD16^+$); and functionally, they can act as effector cells that mediate natural immunity, which plays

an essential role in immune surveillance against tumors and infectious agents. NK cells are not restricted by the major histocompatibility complex (MHC) in their function, and they are capable of killing a broad range of human solid tumor, leukemic, and virus-infected target cells, including cells with no detectable MHC expression.

As reviewed by Whiteside and Herberman (1989), decreased natural immunity, as measured in vitro by NK activity and/or absolute numbers of circulating NK cells has been found to be associated with the development and progression of cancer, with acute and chronic viral infections including AIDS, chronic fatigue syndrome, psychiatric depression, various immunodeficiency syndromes, and certain autoimmune diseases.

Finlay et al. (1988) proposed that the pronounced antitumor effects achieved in vivo against the Lewis Lung carcinoma in mice with flavone acetic acid, a synthetic flavonoid, was not mediated by direct cytotoxicity, but because of its ability to augment natural killer activity. This conclusion is based on several pieces of evidence. First, the tumoricidal activity of flavone acetic acid was more pronounced and occurred more rapidly in vivo than direct cytotoxic effects observed in vitro. Second, Lewis lung carcinoma cells that were implanted in diffusion chambers in the peritoneum of (DBA/6J× C57BL/6J) F1 mice were not susceptible to the effects of flavone acetic acid administered intraperitoneally. Wiltrout and Hornung (1989) demonstrated that a dose of flavone acetic acid (at 220 mg/kg body wt) administered intraperitoneally can increase natural killer activity in nonlymphoid organs (liver and lung) of (DBA/6J× C57BL/6J) F1 mice as well as in their spleen at 12 h after dosing, and that flavone acetic acid plus recombinant interleukin 2 (rIL 2) synergistically augmented natural killer activity in normal and tumor-bearing mice.

Soybean isoflavone extracts can significantly increase NK

cell activity in rats (Cunnick and Hendrich, unpublished data). After rats were fed 240 mg isoflavones/kg diet for 10 days, liver associated NK cell activity was two fold greater than that in the control group. However, rats receiving a dose of 480 mg isoflavones/ kg diet did not differ in NK cell activity compared with the control group.

Theoretically, isoflavones acting as antioxidants can enhance glutathione peroxidase activity (Hendrich et al., 1994) which constrains prostaglandin production (Marshall et al., 1988). In an interesting study reported by Baxevanis et al., 1993), a comparison of natural killer and lymphokine-activated killer (LAK) cell activity was undertaken in 85 preoperative patients with breast cancer and 75 healthy donors. Natural killer cell activity tested in 18-hour cultures of effector peripheral blood mononuclear cells with K562 or MOLT-4 tumor target cells was significantly diminished in these breast cancer patients, and the reduced natural killer cell activity was due to abnormally high levels of prostaglandin E_2 produced by monocytes in culture. It is reasonable to hypothesize that soy isoflavones can enhance natural killer activity through its inhibition of prostaglandin production. However, high levels of genistein (20-110 μmol/L) can inhibit tyrosine kinase activity which is necessary for the activation of natural killer cells (Borrego et al., 1993). Therefore, the effect of isoflavones on NK cell activity may at least depend upon the balance between their inhibition of tyrosine kinase activity and suppression of prostaglandin production.

Role of bioavailability in action of health protectants

Effects of dietary health protectants require a sufficient systemic bioavailability except for those substances which can perform their health protective effects

at local gastrointestinal tract. Unfortunately, many current studies regarding dietary substances' health protective effects did not have solid support of bioavailability data.

From nutritionist point of view, bioavailability of dietary health protective substances refers to the proportion of these substances absorbed and entered the systemic circulation. Except for those had lymphatic vessels as their main absorption route, bioavailable health protective substances should be not only absorbed but also survived first-pass effect which refers to biotransformation by the gastrointestinal cells or extraction by the liver and excretion into bile with or without prior biotransformation (Klaassen and Rozman, 1991). Therefore, urinary recovery of ingested health protective substance could be a reasonable indicator for its bioavailability. In addition, health protective effects of dietary substances depend on their active forms which may be parent compounds and/or their metabolites. Establishment of bioavailability and kinetics model for those active forms will be crucial.

Bioavailability data are the "bridges" between dietary doses and their health protective effects (end-points). Based on the review of data from doses, end-point effects and bioavailability, we can assess what will be the optimal dose range. If we also know the content of those health protective substances in our foods which is another important aspect of research, we will be able to determine whether their health protective activities can be achieved through human food consumption, and to propose better dietary recommendations.

Plasma and urinary isoflavones in humans and animals

So far the most detailed characterization of isoflavones and lignans in human plasma was reported by Adlercreutz et al. in 1993 b. In this study, two fractions (free + sulfate, and

glucuronide) of four isoflavones (daidzein, O-desmethylangolensin, equol and genistein), and three lignans (matairesinol, enterodiol and enterolactone) were measured. Subjects in the plasma experiment included 28 Finnish women (14 omnivores and 14 vegetarians) and six Japanese men who eat a traditional Japanese diet. Three day plasma was pooled for analysis. Among Finnish women, except for both fractions (free + sulfate, and glucuronide) of matairesinol and equol, as well as glucuronide fraction of genistein, all the mean values of plasma isoflavones and lignans are significantly greater in the vegetarians. Among female vegetarian subjects, mean values of total daidzein, genistein, equol and O-desmethylangolensin are 18.5 nmol/L, 17.1 nmol/L, 0.7 nmol/L, and 0.8 nmol/L, respectively. For six men who eat a traditional Japanese diet, their plasma concentrations of total daidzein, genistein, equol and O-desmethylangolensin vary from 60-924 nmol/L, 90-1204 nmol/L, 0.54-24.6 nmol/L, and 0.98-223 nmol/L, respectively. In both studies, fraction of free and sulfate isoflavones count for about 20% of total isoflavones. Since phenolic sulfates are easily to be used by cells and function as their free forms (Pasqualini et al., 1989), these provide an important clue for further understanding the relationship between isoflavone bioavailability and potential health protective effects.

Adlercreutz et al. (1995) also summarized their data regarding urinary excretion of lignans and isoflavones in various populations (Finnish women, American women, oriental immigrant women on Hawaii, breast cancer patients, and Japanese women and men), and dietary groups (omnivores, vegetarians, lacto-ovovegetarians, macrobiotics and traditional Japanese diet). American macrobiotics, lacto-ovovegetarians and Japanese men have the greatest amount of urinary isoflavone excretions which are 3412-8770 nmol/day, 885-2188 nmol/day, and 1820-3630 nmol/day, respectively.

Japanese women have the greatest variation (347-6610 nmol/day). Finnish breast cancer patients have the lowest urinary isoflavone excretion (67.5-324 nmol/day). However, no dietary isoflavone intake data were presented in these studies.

Equol is the bacterial metabolite of daidzein in animals and humans (Setchell et al., 1984). P-ethyl phenol is proposed to the major metabolite of genistein by gut bacteria (Setchell and Adlercreutz, 1988). Equol has attracted attention in the past because it was implicated as the main cause for permanent and temporary infertility in sheep grazing on certain cultivars of subterranean clover which contains phytoestrogens (Shutt and Cox, 1972). Ruminant animals showed great phase II biotransformation ability outside liver, by gastrointestinal epithelium. Small intestine homogenate of cows has 70-100 % greater conjugative activity than that of sheep. This difference was used to explain the much lower susceptibility of phytoestrogen toxicities in cows which also ingest great amounts of isoflavones from subterranean clover as sheep do (Lundh, 1990). The implications of this finding for human isoflavone metabolism is not known.

In recent years, bioavailability of soy isoflavones has been intensively studied by our laboratory. Tew et al. (1995 a, b) investigated the effects of dietary fat and fiber content upon human soy isoflavone bioavailability. In the study of effect of dietary fat, seven healthy women were randomly assigned to low or high fat diet (20% or 40% of Kcalories from fat, respectively) containing a single dose of 0.9 mg isoflavones /kg body wt from tofu or texturized vegetable protein for one day. Bioavailability as reflected on their 24 h urinary isoflavone recoveries was not influenced by the amount of diet fat intake. In a similar cross-over design experiment, a controlled diet or a wheat fiber supplemented diet (contain 15 g and 40 g of dietary fiber,

respectively) was assigned to the subjects with the same dosage as fat study. Plasma concentration of genistein at 24 h after dose and 24 h urinary recovery of genistein among subjects had wheat fiber supplemented diet were significantly less than those in controlled diet. These results suggested that majority of isoflavones are less likely absorbed at proximal small intestine as aglycones, and gut bacteria may involved in the absorption process and play an important role after proximal small intestine.

In addition to human feeding studies, our laboratory has also characterized disposition of the same doses (1 mmol/ kg diet) of pure isoflavones (daidzein and genistein) or flavone (apigenin) with basal diet in rats after four day feeding in the metabolic cages, as well as isoflavones and flavone's effect on glutathione peroxidase activity in peripheral blood lymphocytes and liver cytosol (Lin, 1994). As reflected on their urinary recoveries, daidzein had greater bioavailability than genistein, and apigenin has lower bioavailability than both isoflavones. Both isoflavones reached steady state after 3 day feeding in rats. Plasma concentrations of daidzein and genistein in fourth day were about 1 and 0.8 μM, respectively. Glutathione peroxidase activity in peripheral blood lymphocytes and liver cytosol among isoflavones and flavone treatments were not significantly different from basal diet control.

In conclusion, higher plasma concentrations and urinary excretion of isoflavones were associated with soy food intakes in the populations had lower breast, colon and prostate cancer risk. In contrast, much lower urinary excretion of isoflavones was found in breast cancer patients. Dietary fiber but not fat content could affect human bioavailability of isoflavones. Furthermore, isoflavones seem to have better bioavailability than flavone (apigenin).

Analytical methods for isoflavones in urine, plasma and feces

Isotope dilution gas chromatography-mass spectrometry methods for urine and plasma analysis of conjugated and unconjugated daidzein, genistein, equol and O-desmethylangolensin as well as lignans were developed by Dr. Adlercreutz's lab (Adlercreutz et al., 1991b and 1993). This method has provided detailed characterization of isoflavones and lignans in plasma and urine.

In this method, conjugated and unconjugated isoflavones and lignans in plasma and urine samples were first extracted by Sep-Pak C_{18} reverse-phase column following by ion-exchange chromatography on DEAE-Sephadex column (acetate form) to clean-up and concentrate analytes. Free diphenols and solvolyzed mono- and disulfated diphenols were washed out by methanol. Fractions of glucuronides washed out from DEAE-Sephadex column by 0.3 M lithium chloride and 0.1 M of formic acid in 70 % methanol in water (v/v) were hydrolyzed by glucuronidase. Both fractions were further purified and concentrated on a QAE-Sephadex column (acetate form). Estrogens, lignans and equol were first eluted with methanol from the column, and then further extracted on carbonate form QAE-Sephadex column. Daidzein, genistein and O-desmethylangolensin were washed out from acetate form QAE-Sephadex column with 0.2 M acetic acid in methanol. Isoflavones and lignans were separated on a 0.2 mm × 12.5 m bonded phase BP-1 (SGE) vitreous silica column with Helium as the mobile phase. The temperature of the oven was first kept at 100°C for 1 min and then increased by 30°C/min to 280°C. The column was directly connected to the ionization chamber. After chemical ionization, isoflavones and lignans were identified in mass spectrometer by monitoring representive ion mass and compared with their standards mass spectrum. The approximate retention times of the 7 diphenols and the ions

monitored were 7.6, 7.9, 8.6, 9.0, 9.5, 9.8 and 11.2 min, respectively, for O-desmethylangolensin, equol, enterodiol, enterolactone, daidzein, genistein and matairesinol. Radiolabeled isoflavones and lignans were used as internal standards to assess loss during the extractions. Recoveries varied between 85.6 and 99.0%. The minimum concentrations that can be detected varied between 0.2 and 1.0 nmol/L depending on the form of isoflavone and lignan by this method.

High performance liquid chromatography with UV detection method which we are currently using for analysis of hydrolyzed isoflavones in plasma and urine was modified from the method described by Lundh et al. (1988). Compared with GC-MS method, HPLC analysis cost less, require shorter sample preparation time, and well serve the purpose for routine analysis of large sample quantity.

In this method, conjugated isoflavones were first hydrolyzed by β-glucuronidase and sulfatase to produce their aglycones. Isoflavones were extracted by Extrelut column following by liquid-liquid solvent extraction in plasma to clean-up and concentrate, or Sep-Pak C_{18} cartridge to achieve the same objective. Gradient elution from 40% methanol in water (v/v) to 70% methanol in water (v/v) can separate daidzein, equol and genistein on a C_{18} reverse-phase column within 30 min. Recoveries of daidzein, genistein and equol were 84-92%, 76-84% and 62-74%, respectively. Detection limit of our system is about 0.2-0.4 μM.

Fecal sample preparation method was modified from soy food analysis described by Murphy (1982). Isoflavones were then partly purified and concentrated by Sep-Pak C_{18} cartridge and analyzed by the same system as urine samples.

Effort to detect both isoflavone aglycones and conjugates at same time by HPLC and photodiode array detector has been made by our laboratory. Briefly, 2 ml of urine or plasma samples were mixed with 1 ml of 1.5 M sodium acetate buffer

(PH=3), then samples were further purified and concentrated on Sep-Pak C_{18} cartridge, and plasma samples were further concentrated under N_2 gas. HPLC conditions were the same as described by Wang and Murphy (1994). This method works well for urine samples although it was difficult to detect aglycones from plasma. Using more sensitive detection and/or increasing sample size would enhance the sensitivity of this method.

Currently Barnes et al. (1994) has reported a HPLC- mass spectrometry method for detection of unconjugated and conjugated isoflavones and their major metabolites in human serum. The sensitivity was increased about 50-fold by capillary reversed-phase HPLC column (0.3 mm i.d.) and electrospray ionization compared to the reversed-phase column with 2.1 mm i.d.. By this method the smallest isoflavone concentrations that can be detected were 1-2 nmol/L. It introduces the isoflavone conjugates directly into the mass spectrometer without the necessity of hydrolysis or derivatization as GC-MS methods. However, further development of this method is essential before its broad use in routine analysis.

Compared with GC-MS and HPLC-MS methods, our HPLC-UV detection method is less expensive, requires shorter sample preparation time and has sufficient sensitivity for routine analysis of isoflavones in physiological fluids of feeding studies. The GC-MS and HPLC-MS methods described here are relatively more sensitive than our method (detection limit is about 100 times smaller than that of HPLC-UV method). They are capable of detecting and quantifying isoflavone aglycones in plasma for epidemiological studies, and can provide more detailed characterization of isoflavones and their metabolites in plasma and urine.

Based on this literature review we know that soybean and soybean foods contain substantial amounts of isoflavones. The amounts of isoflavone isomers are different among soy foods. Isoflavones have broad biological functions and many of them could be beneficial in maintaining human health. Modern analytical technologies have enabled us to quantify trace amount of isoflavones in both soybean foods and physiological fluids. From many in vitro and in vivo studies, we can hypothesize that isoflavones from soy food consumption may perform a health protective effect when they reach certain concentrations in the systemic circulation and at target tissues. Soybean foods under certain dietary pattern with different isoflavone isomers and nutrient content might yield different extent of biological functions due to variation of isoflavone bioavailability profile. To test our hypothesis we conducted a series of human feedings. Our research aims are: 1) to further characterize the human disposition and bioavailability of soy isoflavones under controlled condition; 2) to evaluate the effect of soy food type and background diet upon isoflavone bioavailability; 3) to assess potential health protective roles of isoflavones from physiologically achievable amounts of soy food consumption.

Dissertation organization

The dissertation is composed of four complete papers, already published in (I), accepted for publication in (II), or to be submitted to (III and IV) professional journals. The first paper examines the bioavailability of single dose soy milk isoflavones in adult women after a controlled liquid diet. The second paper investigates isoflavone bioavailability after female subjects ingested three meals of soy milk for a day in controlled liquid diet. The third paper examines the effect of diet selection and type of soy foods

upon the human bioavailability of isoflavones. The fourth paper examines the effect of isoflavones bioavailability upon blood natural killer cell and glutathione peroxidase activities as well as prostaglandin level after seven days soy milk feeding in both male and female subjects. Following the fourth paper is a general conclusion chapter.

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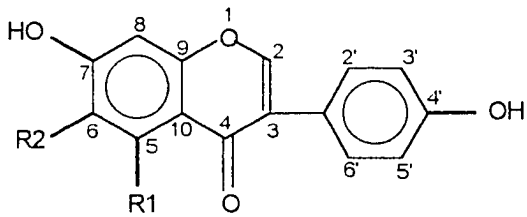
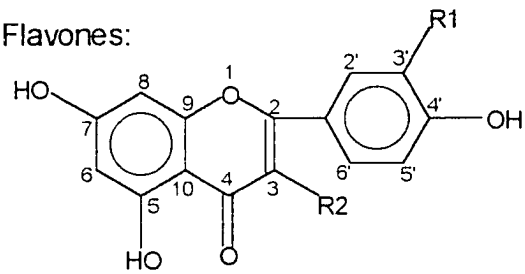
Isoflavones:



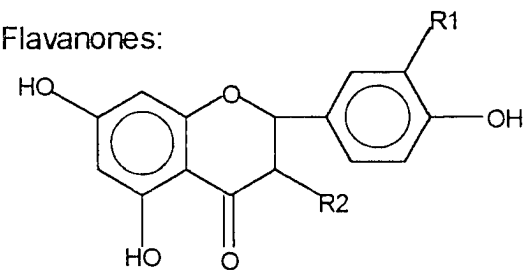
R1= H, R2= H, Daidzein
R1= OH, R2= H, Genistein
R1= H, R2= OCH3, Glycitein

Flavones:



R1= H, R2= H, Apigenin
R1= OH, R2= OH, Quercetin

Flavanones:



R1= H, R2= H, Naringenin
R1= OH, R2= OH, Taxifolin

Figure 1: Types and structures of common flavonoids

I. DAIDZEIN IS A MORE BIOAVAILABLE SOYMILK ISOFLAVONE THAN IS GENISTEIN IN ADULT WOMEN[1,2]

A paper published in the Journal of Nutrition

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Abstract

Soybean isoflavones are proposed to be anticarcinogenic. In order to study their disposition in human body, twelve young adult human females were fed single doses of 0.7, 1.3 and 2.0 mg isoflavones/kg body weight in soybean milk as part of a liquid diet. Plasma, urine and fecal isoflavones were measured by reverse phase HPLC. Average 24 h urinary recovery of daidzein and genistein was about 21% and 9% respectively at all three doses. Urinary recovery of daidzein was significantly greater than that of genistein ($p<0.001$). Total fecal excretion of isoflavones was only 1-2% of the ingested amount. Plasma total isoflavone concentration was significantly increased to 4.4±2.5 μmol/L at 6.5 hours after a dose of 2.0 mg/kg. The plasma concentrations of daidzein and genistein were approximately equal. Twenty-four hours after dosing, both plasma and urine isoflavone concentrations were nearly nil. Although soybean milk isoflavones seem to be 85% degraded in the intestine, the systemic bioavailability, especially of daidzein, may be sufficient to exert some health-protective effects.

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Introduction

Genistin, daidzin and their aglycones, genistein and daidzein are the major isoflavones in soybean foods (Murphy 1981 and 1982). In addition to their estrogenic activity, ten to one-thousandfold less than estradiol in the mouse uterine growth assay (Bickoff et al. 1962, Farmakalidis et al. 1985), and their antiestrogenic activity (Adlercreutz et al. 1986, Setchell and Adlercreutz 1988), they also possess antifungal (Naim et al. 1974), antiviral (MacRae et al. 1989, Markkanen et al. 1981,) and antioxidant activities (Naim et al. 1976). Antiestrogenic and antioxidant activities are proposed to be anticarcinogenic (Adlercreutz et al. 1987, Adlercreutz 1988, Fisher et al.1988). Genistein also inhibits tyrosine kinases (Akiyama et al. 1987), and blocks angiogenesis in vitro (Fotsis et al. 1993) which are also proposed mechanisms of anticarcinogenesis. Because of their biological effects and their abundance in soybean foods (0.1-1.5 mg/g, Wang and Murphy 1993), the isoflavones may be of great potential benefit to human health maintenance.

The incidence of and mortality from breast cancer in the Western world are much higher than in Asia where soybeans are an important part of the diet (Armstrong and Doll 1975, Dunn 1975, Rose et al. 1986). Dietary factors are suggested as a cause of the increasing breast cancer rate among successive generations of Japanese in California as compared with the rate in Japan (Dunn 1975). Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the traditional Japanese diet have low breast cancer incidence and mortality (Nomura et al. 1978, Smith 1956). Breast cancer patients excrete about 21% less of the isoflavone metabolite, equol, when compared with either vegetarian or omnivorous controls (Adlercreutz et al. 1982). Urinary isoflavone excretion was 20-30 times greater among Japanese women and men consuming a typical traditional diet compared with the values

obtained from women living in Boston and Helsinki. Soybean product consumption was the source of urinary isoflavones in Japan (Adlercreutz et al. 1991a).

Feeding a 30% soybean diet protects mouse liver from nitrosamine-induced cancer (Fitzsimmons et al. 1989), although this protective effect was not specifically attributable to isoflavones. Rats consuming a soybean-based diet develop fewer mammary tumors following administration of the carcinogens N-methylnitrosourea and 7,12-dimethylbenz[a]-anthracene than rats on isonitrogenous and isocaloric diets without soybeans (Barnes et al. 1990). The anticarcinogenic effect of soy was probably due to isoflavones. Genistein inhibits the growth of estrogen receptor-negative or -positive human breast cancer cell lines (concentration for inhibition of 50% cell growth, IC_{50}=24.1-44.4 µmol/L; Peterson and Barnes 1991). Genistein also inhibits tyrosine kinases, a major class of oncogene products regulating tumor cell growth, and DNA topoisomerase II, an enzyme needed for DNA replication (Traganos et al. 1992).

These findings suggest that a Western diet may lack certain cancer-protective factors which are present in soybeans. The antiestrogenic and antioxidant isoflavones, genistin and daidzin may be the most significant of these factors. The isoflavone aglycones, daidzein, genistein and metabolites, have been detected and identified in human biological fluids, especially in urine (Adlercreutz et al. 1982 and 1991b, Axelson et al. 1982, Bannwart et al. 1984a and 1984b). Urinary excretion of the isoflavone metabolites, equol and *O*-desmethylangolensin, was compared among women consuming omnivorous, lactovegetarian, or vegetarian diets. The largest amounts of equol and *O*-desmethylangolensin were found in the urine of the vegetarian group (Adlercreutz et al. 1986).

Urinary isoflavones and their metabolites were measured

in a group of Japanese women and men who consumed a typical traditional diet (Adlercreutz et al. 1991a). Three-day food intake records were made to estimate their food consumption. Very high levels of urinary daidzein, equol and *O*-desmethylangolensin were found, although the interindividual variation was great. Urinary isoflavone excretion correlated closely with soybean and soybean product intake (r=0.75, p<0.001).

Previous studies of human isoflavone metabolism have not fed carefully controlled diets. No human isoflavone dose/response studies have been performed. When urinary isoflavones were compared among different dietary groups, the amount of isoflavones ingested was not measured. Because of the possible anticarcinogenicity of isoflavones, human absorption and excretion of soyfood isoflavones is of interest. This information will be useful in determining the anticarcinogenic potency of isoflavones, using a variety of endpoints.

In this study, a group of young female adults were fed three single doses of isoflavones from soymilk in a well controlled liquid diet. Plasma, urine and fecal isoflavone content were measured after feeding.

Subjects and methods

The subjects were twelve young adult females between 19 and 33 years of age, with body weight of 63.2±9.4 kg and body mass index of 22.5±2.2 kg/m² (**Table 1**).

All the subjects were in good health, based on medical histories and physical examinations performed by Student Health Center physicians at Iowa State University (ISU). The procedures for this feeding study were approved by the Human Subjects Committee of ISU, with prior approval of the U.S. Food and Drug Administration. All the subjects were omnivorous and most had never eaten soybean foods before the feeding

started.

The study consisted of three feeding days, each separated by a two-week washout period (**Table 1**). On each feeding day, subjects were fed three nutritionally complete liquid meals. Breakfast contained soy milk powder reconstituted with distilled water (Now Foods, Inc., Glendale Hts., IL) mixed with chocolate-flavored Carnation Instant Breakfast® (Nestle Food Company, Glendale, CA) diluted with whole milk. Lunch and dinner consisted of chocolate-flavored Carnation Instant Breakfast® reconstituted with whole milk. Subjects' energy requirements were estimated based on their age, body weight, and physical activity levels according to the Recommended Dietary Allowances tables (Food and Nutrition Board, National Research Council, 1989). Subjects were asked not to consume anything for 10 h before dosing. Lunch and dinner were fed at five hour intervals after dosing. Subjects were instructed not to eat anything other than what was given on the feeding day. They were also asked not to eat any foods containing texturized vegetable protein, hydrolyzed vegetable protein, and soy protein isolate during the washout periods. No adverse effects such as diarrhea were reported after soybean food dosing.

Biological Sample Collection

Blood samples. Five mL venous blood samples were collected in heparinized vacutainers by medical technologists under stringent aseptic conditions at the Student Health Center, ISU. A blood sample was collected within 1 h before dosing (Time "0"). Blood samples were also collected at 6.5 and 24 h after dosing. Samples were centrifuged within one hour after collection at 3000 × g for 20 minutes at 4°C (Model 4D; International Equipment Co., Needham Hts., MA), and plasma was stored in a -20°C freezer.

Urine samples. For each subject, a urine sample was collected in the morning before dosing (Time "0"). All urine was collected and pooled in two 12 h increments during the first 24 h after dosing. The first urination of the second day after dosing was also collected. After recording the total volume, 50 mL of each of the four samples was stored in a -20°C freezer.

Fecal samples. Capsules containing 1 g carmine red (Pharmaceutical Service, University of Iowa, Iowa City, IA) were given with the soymilk. One fecal sample from each subject was collected before soymilk feeding (Time "0"). After dosing, all feces excreted before and during fecal marker excretion were collected. Usually, the fecal marker appeared in feces one or two days after dosing. Fecal sample collection was stopped when fecal marker no longer appeared in feces. After collecting the fecal samples, feces were freeze dried. After recording all the weights of dry samples, feces were ground to a fine powder in a coffee mill (Braun Company Inc., Lynnfield, MA). Ten g of each dry fecal sample was stored in a -20°C freezer until analysis.

Soybean Milk Powder Analysis

Two g samples in triplicate of both soymilk powder and chocolate-flavored Carnation Instant Breakfast® were used for analysis. Total isoflavones were measured as free isoflavones after hydrolysis in 1 mol/L HCl. Sample preparation followed the method described by Wang et al. (1990). Reverse phase HPLC analysis was performed in a 4.6 mm i.d. × 25 cm length, 5 μ C_{18} ultrasphere column (Beckman Instruments, Inc., Berkeley, CA). Gradient elution as described by Murphy (1981) was achieved by two pumps, ALTEX model 110A (ALTEX Scientific, Inc. Berkeley, CA) and Beckman model 110A, controlled by a ALTEX model 420 microprocessor solvent flow controller. Hydrolyzed daidzein and genistein in soymilk powder were identified and quantified

by a Waters 991 photodiode array detector connected with a NEC computer system (Millipore Corporation, Milford, MA).

Biological Sample Analysis

After metabolism, in human plasma and urine about 90% of isoflavones and their major metabolites are present as glucuronides, and about 3-5% as sulfates (Adlercreutz et al. 1991b, Axelson et al. 1982 and 1984, Setchell et al. 1984). Plasma and urine samples were treated with glucuronidase/ sulfatase (Sigma Chemicals Company, St. Louis, MO) to produce the parent isoflavones (Lundh et al. 1988).

Plasma and urine samples. Sample preparation for plasma and urine isoflavone analysis was performed according to the methods described by Lundh et al. (1988). One mL of plasma and 2.5 mL of urine were prepared for analysis. Plasma samples were dissolved in 150 µL and urine samples in 2 mL of 80% methanol in H_2O. A 200 µL insert into a 2 mL vial (National Scientific Company, Lawrenceville, GA) was used for autosampling of plasma. Samples were automatically injected with a Beckman Model 502 Autosampler. Isoflavones were separated in a Waters 3.9 mm i.d. × 30 cm length µ-Bondapak C_{18} reverse phase column with gradient elution at ambient temperature. Solvent flow controller and pumps were the same as used for soymilk powder analysis. The mobile phase included methanol, HPLC grade (Fisher brand) and ultrapure Nalgene water (Nalge Company, Rochester, NY). A linear gradient was run from 40% to 70% methanol in 30 min at a flow rate of 1 mL/min. Waters 991 Photodiode Array Detector was used to quantitate spectral data from 210 nm-400 nm for each peak. In this reverse phase HPLC system, daidzein, equol and genistein eluted at 18.4, 20.8 and 23.4 min respectively. Daidzein has a maximum absorption at 254 nm, genistein at 263 nm and equol at 280 nm.

Daidzein (4',7-dihydroxyisoflavone) was obtained from

Life Science Group, ICN Pharmaceuticals, Inc., Plainview, NY. Genistein (4',5,7-trihydroxyisoflavone) was obtained from Calbiochem Corporation, La Jolla, CA. Equol (4',7-dihydroxyisoflavondiol) was a generous gift from Dr. H. Adlercreutz (Department of Clinical Chemistry, University of Helsinki, Finland).

A series of standards of 1, 2, 4, 8, 12, 16, 24 μmol/L, were assayed to quantify isoflavones. For each subject, plasma and urine samples were spiked randomly with daidzein, genistein and equol standards (0.15 μg isoflavone/100 μL added to each sample) to measure recovery.

Fecal samples. Two g ground, freeze-dried fecal samples were extracted with 10 mL acetonitrile mixed with 2 mL 0.1 mol/L HCl, and then filtered through No.1 Whatman filter paper. Samples were collected in 20 mL vials and dried under N_2. The residues were dissolved in 10 mL 20% ethanol/distilled, deionized water. The samples were acidified with 200 μL 1 mol/L HCl. Five mL of extract was slowly injected onto an activated Waters Sep-Pak C_{18} cartridge. Cartridges were washed with 2 mL ultrapure deionized water followed by 2 mL 20% methanol/water. The cartridge was washed with 2 mL 80% methanol to elute the isoflavones.

Reverse phase HPLC analysis was performed with two Beckman Model 110B pumps,and one Beckman Model 420 Microprocessor solvent flow controller to produce a gradient of 40% to 65% methanol over 30 minutes at flow rate 1 mL/minute. The same column was used as for plasma and urine analysis. Spectra-Physics Autosampler Model 8780XR (Spectra-Physics, Fremont, CA) was used to inject samples. Variable wavelength detector Beckman Model 163 was connected with Beckman Model 427 Integrator by which chromatographic data were calculated and plotted.

To ensure the quality of analysis, a series of standards (1, 2, 4, 8, 12, 16, 24 μmol/L) and fecal samples spiked with

500 µL of 32 µmol/L daidzein, genistein and equol standards were run to measure recovery.

Statistical Methods

The statistical analysis was performed with the Statistical Analysis System (SAS Institute, Inc., Cary, NC) version 6.06 on the Iowa State University mainframe computer. Data were tested for heterogeneity of variance. If necessary, data were transformed to homogeneity before ANOVA (Snedecor and Cochran 1967). Analysis of variance (GLM) was performed on the data obtained from this crossover design experiment. Subjects and feeding times were treated as blocks. The effects of dosages, measuring times, and kinds of isoflavone upon plasma concentration and urinary excretion were determined. If the effect from any one of these factors was significant, Tukey's test was used for comparison within the factor. A p value of 0.05 or less was considered to be significant.

Results

Soymilk powder contained 1.25±0.08 mg/g total isoflavones; 44% genistein and 56% daidzein. No isoflavones were detected in chocolate-flavored Carnation Instant Breakfast®.

At all three doses of isoflavones, 15-20% of the ingested total dose was excreted (**Table 2**). Average 24 h urinary recovery of daidzein and genistein was about 21% and 9% respectively at each dose. Urinary recovery of daidzein was significantly greater than that of genistein ($p<0.001$). Total fecal excretion of isoflavones was 1-2% of the ingested amount. No equol was found in any sample although the recovery of equol was between 62-74% in all spiked samples. The average amount of total urinary isoflavones increased significantly with increasing dose ($p<0.001$).

Variation in isoflavone content of biological samples was

great among the subjects ($p<0.05$), especially for fecal samples. Subject Eight excreted ten times more fecal isoflavones than the other subjects at all three dosages.

At all three doses, the amount of urinary isoflavones was about two times greater in the first 12 h after dosing than in the second 12 h after dosing ($p<0.001$)(**Table 3**). After 24 h, urinary excretion of isoflavones was nearly nil. The amount excreted in the first twelve hours increased significantly with the increase in dosage ($p<0.001$). There was a strong relationship between dose and amount excreted ($r=0.88$, $p<0.001$). At all three doses of isoflavones, the amount of urinary daidzein was significantly greater than that of genistein during the first 24 h after dosing ($p<0.001$).

Plasma isoflavone concentration was significantly increased at 6.5 h after dosing (**Table 4**). The plasma concentrations of daidzein and genistein were not significantly different at this time. Twenty-four hours after dosing, plasma isoflavone concentration was nearly nil. At 6.5 h after dosing, a strong correlation was found between plasma isoflavone concentration and ingested doses ($r=0.87$, $p<0.001$).

Discussion

In this study, only 1-2% of ingested isoflavones were excreted intact in feces (**Table 2**). Average 24 h urinary recovery of daidzein and genistein was about 21% and 9% respectively (**Table 2**). Because daidzein and genistein in both plasma and urine were nearly nil 24 h after dosing, the bioavailability of daidzein and genistein at all three doses seemed to be about 21% and 9% respectively.

The urinary recovery of isoflavones depends upon absorption from the gastrointestinal tract (Klaassen and Rozman 1991, Welling 1986). Human absorption of isoflavones after oral administration may be moderate. In healthy male volunteers, 40-50% of an ingested single dose of 200 mg

ipriflavone was absorbed (Shino 1985). (Ipriflavone is a synthetic isoflavone recently introduced as antiosteoporotic agent because of its estrogenic activity.) In dogs, only about 24% of 20 mg ^{14}C-ipriflavone/kg body weight oral dose was considered to be absorbed (Yoshida et al. 1985). The urinary recovery is also affected by the first-pass effect: the phenomenon of removal of xenobiotics before entrance to the systemic circulation. This may be due to biotranformation by the gastrointestinal mucosa, extraction by the liver and excretion into bile with or without hepatic biotransformation (Klaassen and Rozman 1991, Welling 1986). Any substance extensively metabolized and excreted in bile is unlikely to exhibit good systemic availability regardless of the extent of absorption (Welling 1986). Finally, the urinary recovery of isoflavones is also influenced by retention of the compounds within tissues. Because isoflavones are extensively transformed by phase II enzymes (Adlercreutz et al. 1991b, Axelson et al. 1982 and 1984, Lundh et al. 1988, Setchell et al. 1984), their retention in the human body becomes very unlikely.

Significant retention of isoflavones in the human body is improbable. After rats and dogs were orally dosed with 20mg ^{14}C-ipriflavone/kg body weight, it was eliminated mostly as metabolites within 48 and 72 h respectively. Rats excreted more ^{14}C-ipriflavone and its metabolites in urine than in feces, whereas the opposite was observed in dogs (Yoshida et al. 1985). Seemingly, animal species affects the route of excretion. In human studies, when a single 200 mg dose was administered orally to healthy volunteers, ipriflavone was rapidly absorbed (T_{max}=1.8±0.4 h) and extensively metabolized. In plasma, only small quantities of the parent compound were detected, together with seven metabolites (Shino 1985). A multiple-dose study in healthy volunteers (200 mg × 3/day) showed no accumulation of ipriflavone (Suda 1987). Hydroxyl

groups of isoflavones provide sites for phase II biotransformation by UDP-glucuronosyltransferases and sulfotransferases. Sulfation has a low capacity for conjugation of phenols, whereas glucuronidation has a much higher capacity. Therefore, when large doses of phenolic compounds are given, glucuronidation is the major pathway of biotransformation (Sipes and Gandolfi 1991). When rats were orally dosed with 50 mg/kg (0.3 mmol/kg) of ^{14}C-2-hydroxybiphenyl, almost 90% of the dose was recovered in urine within 24 h and about 75% of the dose was excreted as the glucuronides or sulfate esters of the hydroxybiphenyl and its metabolites (Reitz et al. 1983). As doses were increased from 5 mg/kg to 500 mg/kg, the proportion of glucuronides in urine was increased by 50%. Human phenol UDP-glucuronosyltransferase shows 76% amino acid sequence homology with phenol UDP-glucuronosyltransferases isolated from rat liver and kidney (Harding et al. 1988). Therefore, our study suggests that human subjects have a sufficient capacity to perform glucuronidation of isoflavones and their metabolites, because the subjects were given less than 0.01 mmol total isoflavones/kg body weight.

Although sulfate conjugates of xenobiotics are excreted mainly in urine, glucuronides can be excreted from the body in either the bile or urine, depending on the molecular weight of the aglycone. In the rat, the glucuronide will be cleared by renal tubular organic acid secretion into urine, if the aglycone has a molecular weight below about 250 (Sipes and Gandolfi 1991). If the aglycone has a molecular weight greater than 350, the conjugate is often secreted into bile. From molecular weights of 250-350 the conjugate may be excreted by either pathway (Sipes and Gandolfi 1991). Daidzein and genistein have molecular weights of 254 and 270 respectively. Isoflavones undergo urinary as well as biliary excretion in rats (Axelson and Setchell 1981), dogs (Yoshida et al. 1985)

and humans (Rondelli et al. 1991). These studies suggest that in humans the molecular weights of isoflavones are very likely within the range which enables their conjugates to undergo partitioning between bile and urine. This is also true of estradiol excretion (molecular weight, 272). After liver biotransformation about 50% of estradiol conjugates are excreted in bile (Gorbach 1984). Isoflavones differ from estradiol in humans in that about 85% of estradiol conjugate in bile is hydrolyzed by deconjugating enzymes (i.e. β-glucuronidases and arylsulfatases) produced by the gastrointestinal microbes to form the free hormone which is then reabsorbed in the intestinal tract, whereas isoflavones are extensively metabolized and degraded by intestinal bacteria (Adlercreutz et al. 1986 and 1991, Axelson et al. 1984, Setchell and Adlercreutz 1988, Setchell et al. 1984).

The molecular weight cutoffs in humans may be different from rats. But, the lesser molecular weight and greater water-solubility of daidzein might partly explain its greater presence in urine than genistein. The greater molecular weight and less water-solubility of genistein may promote excretion of its conjugate in bile. But, bacterial degradation of isoflavones seemingly occurs to a great extent at the same time. This may be why little genistein is recovered in urine and feces.

The bacteria in the gastrointestinal tract play an important role in metabolism of isoflavones in humans and animals. The condition of the intestinal microflora affects the amount and pattern of isoflavone excretion which was also extensively changed by antibiotics treatment (Adlercreutz 1991). The major metabolite of isoflavones, equol, is formed by bacteria in humans (Setchell et al. 1984) and animals (Batterham et al. 1965, Dickinson et al. 1988, Nilsson et al. 1967). Besides forming equol, bacteria also produce *O*-desmethylangolensin from daidzein (Adlercreutz et al. 1986,

Bannwart et al. 1984b) in humans, and produce p-ethylphenol from genistein in sheep (Batterham et al. 1965). Intestinal bacteria modified 70% of the 9 g/day isoflavones ingested by sheep (Shutt et al. 1970). Unlike normal rats, germfree rats do not excrete equol when given a soy-containing food (Axelson and Setchell 1981). In this study, no equol was found in any biological samples. Human gastrointestinal flora can form equol from soybean isoflavones both in vivo and in vitro (Setchell et al. 1984). One male and one young adult female were fed 40 g of commercial textured soybean as a meat substitute in lunch for five days. More than 0.1 mg of urinary equol was produced per gram of soybean (Axelson et al. 1984). About 1 mg equol/day appeared in urine 24 h after ingesting the textured soy and reached a maximum of 4-5 mg/day after three days, but only three out of six subjects were equol producers (Setchell et al. 1984). This may explain why no equol could be found in urine of subjects who ingested only a single dose of soymilk.

Isoflavones may be degraded as well as metabolized in the intestine. The C-ring of flavonoids can be cleavaged by human intestinal bacteria and 5 mg of quercetin, one of the flavonoids, can be completely converted to a monophenolic compound, 3,4-dihydroxyphenylacetic acid within about 3-4 hours in a prereduced brain-heart infusion broth under anaerobic conditions (Winter et al. 1989). Isoflavones are analogous to the flavonoids with the aromatic B-ring linked to carbon 3 of the C-ring instead of carbon 2. Therefore, it is very likely that human intestinal bacteria can also metabolize ingested isoflavones to a great extent. This may be why only 15-20% of isoflavones were recovered intact in urine and feces.

Developing methods to measure monophenolic and other diphenolic isoflavone breakdown products may be important because some monophenolic compounds like methyl p-

hydroxyphenylacetate, a metabolite of both exogenous flavonoids and tyrosine are inhibitors of hormone-dependent cancer cell growth and proliferation. This compound has higher affinity for the nuclear type II binding site than estradiol. This type II binding site has been suggested to be involved in cell growth regulation by estrogenic hormones. This metabolite can block estradiol stimulation of uterine growth in vivo and inhibit the growth of MCF-7 human breast cancer cells in vitro (Markaverich et al. 1988).

In the future, the cancer-protective potential of isoflavones may be estimated from plasma concentrations. Much work remains to be done to determine effective plasma isoflavone levels. Average plasma concentration of total isoflavones reached 4.4 μmol/L at 6.5 h after a dose of 2.0 mg isoflavones/kg body weight (**Table 4**). A study of 3T3 cell inhibition of production of inositol phosphates (IP), key intracellular signals which mediate tumor promotion, showed an IC_{50} for daidzein of 8 μmol/L and for genistein of 2.2 μmol/L, when IP production was stimulated by AlF_4^- (Higashi and Ogawara 1992). Thus, our data suggest that human dietary exposure to isoflavones could provide circulating levels of isoflavones that might be of potential benefit with respect to cancer development.

From this study, it was concluded that human absorption, excretion and plasma concentration of isoflavones depended upon the dose given. Systemic bioavailability of daidzein is greater than that of genistein, which suggests that a greater focus on the biological effects of daidzein may be warranted. Although bioavailability of isoflavones may be, on average, only 15%, probably due to a first-pass effect and subsequent intestinal bacterial degradation, isoflavones may still be enough absorbed from soymilk to exert potentially beneficial biological effects.

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Table 1. Experimental Design

Subject				Feeding 1 (1 day)	Washout (2 weeks)	Feeding 2 (1 day)	Washout (2 weeks)	Feeding 3 (1 day)
No.	Age	Weight (kg)	Body mass index (kg/m²)	Dose mg/kg body weight isoflavones		Dose mg/kg body weight isoflavones		Dose mg/kg body weight isoflavones
1	20	59.1	20.45	2		1.3		0.7
2	21	60.9	20.35	1.3		0.7		2
3	41	84.1	26.84	0.7		2		1.3
4	29	56.4	22.31	2		1.3		0.7
5	22	53.2	19.78	1.3		0.7		2
6	21	65.9	24.21	0.7		2		1.3
7	20	52.3	21.77	2		1.3		0.7
8	23	54.5	20.77	1.3		0.7		2
9	19	65.4	21.36	1.3		0.7		2
10	32	72.9	23.01	0.7		2		1.3
11	19	59.1	22.52	2		1.3		0.7
12	22	74.5	26.40	1.3		0.7		2
$\bar{x}$	24	63.2	22.48					
SD	6.4	9.4	2.20					

Table 2. Human isoflavone excretion after a single dose of 0.7 mg/kg, 1.3 mg/kg and 2 g/kg[1]

Isoflavones	Dosages		
	0.7 mg/kg	1.3 mg/kg	2 mg/kg
Intake			
Daidzein(mg)	24.73 ± 3.66	45.93 ± 6.80	70.67 ± 10.46
Genistein(mg)	19.29 ± 2.89	36.21 ± 5.36	55.70 ± 8.25
Total intake(mg)	44.28 ± 6.61	82.24 ± 12.28	126.50 ± 18.89
Urinary excretion(mg)[2,3]	5.93 ± 3.47[c]	14.87 ± 7.65[b]	20.26 ± 10.33[a]
Daidzein recovery (%)[4]	19.85 ± 6.35	23.69 ± 9.71	20.76 ± 8.12
Genistein recovery (%)	5.29 ± 3.74	11.02 ± 6.54	10.04 ± 6.31
Fecal excretion(mg)[5]	0.79 ± 0.97	0.96 ± 1.55	2.57 ± 4.78
% Isoflavone excreted in urine and feces	15.20 ± 5.82	19.31 ± 6.97	18.05 ± 6.73

[1]Values are means and SD; n= 12; mg/kg = mg of isoflavones per kg body weight.

[2]Urine samples included all the urine in the first 24 hours after dosing and the first urination of the second day.

[3]For the amount of total urinary isoflavones, the data were log-transformed to homogeneity variance before ANOVA. Means and SD of the untransformed data are presented in table. Effect of dosage in ANOVA(GLM) was significant ($p<0.001$). Values in a row with different superscripts are significantly different ($p<0.05$, tukey's test).

[4]For urinary recovery of isoflavones, effect of kind of isoflavone in ANOVA(GLM) was significant ($p<0.001$). Amount of daidzein urinary recovery was significantly greater than that of genistein at each dosage ($p<0.05$,tukey's test).

[5]Fecal samples included all feces excreted before and during fecal marker excretion.

Table 3. Urinary excretion of isoflavones at doses of 0.7 mg/kg, 1.3 mg/kg and 2 mg/kg[1]

Dosage	Isoflavone	Time "0"	0-12 Hours	12-24 Hours	>24 Hours
			µmol		
0.7 mg/kg	Daidzein[2]	0.04 ± 0.08	14.48 ± 7.79	4.52 ± 3.11	0.31 ± 0.28
	Genistein	0.00 ± 0.04	2.70 ± 2.96	0.93 ± 1.55	0.15 ± 0.15
	Total[3]	0.04 ± 0.12^{c}	17.20 ± 10.74^{a}	5.45 ± 4.65^{b}	0.46 ± 0.42^{c}
1.3 mg/kg	Daidzein[2]	0.08 ± 0.17	30.44 ± 10.42	11.45 ± 9.99	0.83 ± 1.06
	Genistein	ND	10.44 ± 6.70	3.89 ± 6.07	0.44 ± 0.93
	Total[3]	0.08 ± 0.17^{c}	40.88 ± 17.10^{a}	15.34 ± 17.00^{b}	1.27 ± 1.98^{c}
2 mg/kg	Daidzein[2]	0.31 ± 0.67	42.21 ± 17.35	14.20 ± 12.00	0.94 ± 1.02
	Genistein	0.11 ± 0.19	13.77 ± 9.88	6.18 ± 9.77	0.67 ± 1.15
	Total[3]	0.42 ± 0.85^{c}	55.98 ± 27.15^{a}	20.38 ± 21.76^{b}	1.61 ± 2.15^{c}

[1]Values are means and SD; n = 12; mg/kg = mg of isoflavones per kg body weight; ND = Not detectable, at a detection limit of 2 ng per 20 µL injected.

[2]For the amount of daidzein and genistein urinary excretion, the data were log-transformed to homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Effect of kind of isoflavones in ANOVA(GLM) was significant ($p<0.001$). The amount of daidzein was significantly greater than that of genistein in the same period ($p<0.05$, Tukey's test).

[3]For the total amount of urinary excretion of isoflavones, data were log-transformed to homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Effect of measuring time in ANOVA(GLM) was significant ($p<0.001$). Values in a row with different superscripts are significantly different ($p<0.05$, Tukey's test).

Table 4. Plasma concentration of isoflavones at doses of 0.7 mg/kg, 1.3 mg/kg and 2 kg/mg[1]

Dosages	Isoflavone	Time "0"	6.5 Hours[2]	24 Hours
			µmol/L	
0.7 mg/kg	Daidzein	ND	0.79 ± 0.04	0.04 ± 0.08
	Genistein	ND	0.74 ± 0.44	0.07 ± 0.10
	Total[3]	ND	1.53 ± 0.47	0.11 ± 0.17
1.3 mg/kg	Daidzein	ND	1.22 ± 0.67	0.08 ± 0.08
	Genistein	ND	1.07 ± 0.63	0.19 ± 0.37
	Total[3]	ND	2.29 ± 1.30	0.27 ± 0.45
2 mg/kg	Daidzein	ND	2.24 ± 1.18	0.12 ± 0.08
	Genistein	ND	2.15 ± 1.33	0.26 ± 0.22
	Total[3]	ND	4.39 ± 2.50	0.38 ± 0.30

[1]Values are means and SD; n = 12; mg/kg = mg of isoflavones per kg body weight; ND = Not detectable, at a detection limit of 2 ng per 20 µL injected.

[2]For plasma concentration of daidzein and genistein at 6.5 h, data were log-transformed to homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Effect of kinds of isoflavone in ANOVA(GLM) was not significant ($p>0.1$).

[3]For concentration of total plasma isoflavones, data were transformed to homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Effect of measuring time in ANOVA(GLM) was significant ($p<0.01$).

II. BIOAVAILABILITY OF SOYBEAN ISOFLAVONES DEPENDS UPON GUT MICROFLORA IN WOMEN[1]

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Abstract

Soybean isoflavones are proposed to be anticarcinogenic, but their effective doses have not been established. To study their bioavailability, seven women were fed 3.4, 6.9, 10.3 μmol isoflavones/kg body wt in soy milk in each of three meals of a liquid diet at one of three feeding days. Plasma, urine and fecal isoflavones were measured by reverse phase HPLC. In two subjects, fecal isoflavone recovery was 10-20 times that in the other five subjects. Average 48 h urinary recovery of ingested daidzein and genistein was 16±4% and 10±4%, respectively, at all three doses among the five subjects excreting only small amounts of isoflavones in feces, whereas urinary recovery of daidzein and genistein in the two subjects who excreted large amounts of fecal isoflavones was 32±5% and 37±6%, respectively. Urinary isoflavone excretion was nearly nil in all subjects at 48 h after dosing. Average plasma concentration of genistein at 24 h after the first isoflavone dose in subjects excreting large

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amounts of fecal isoflavones was significantly greater by 2.5-fold than in subjects who excreted small amounts of fecalisoflavones ($p<0.05$). In vitro anaerobic incubation of isoflavones with human feces showed that intestinal half-life of daidzein and genistein may be as little as 7.5 h and 3.3 h respectively. These data suggest that human isoflavone bioavailability depends upon the relative ability of gut microflora to degrade these compounds.

Introduction

The isoflavones, daidzin, genistin and their aglycones daidzein and genistein, are abundant in soy foods (0.2-1.5 mg/g) (Wang and Murphy 1994). Isoflavones are antioxidants in vitro and in vivo, inhibiting β-carotene bleaching and fat rancidity (Pratt and Birac 1979), and increasing rat hepatic cytosolic glutathione peroxidase activity (Hendrich et al. 1994). Isoflavones are also antiestrogens, enhancing sex hormone binding globulin (Adlercreutz et al. 1987) and inhibiting human preadipocyte aromatase (an estrogen synthase) (Adlercreutz et al. 1993). Dietary isoflavone intake might partly explain why Japanese consuming a traditional diet rich in soyfoods have low breast cancer incidence and mortality (Adlercreutz et al. 1991). Rats consuming a soy-based diet develop fewer mammary tumors following administration of the carcinogens N-methylnitrosourea and 7,12-dimethylbenz[a]-anthracene than rats on isonitrogenous and isocaloric diets without soy (Barnes et al. 1990).

The anticarcinogenic effect of soy may be due to isoflavones. Genistein inhibits the growth of estrogen receptor-negative or -positive human breast cancer cell lines (concentration for 50% inhibition of cell growth (IC_{50}) =24-44 μmol/L; Peterson and Barnes 1991). Genistein and daidzein (IC_{50} of 2.2 μmol/L and 8 μmol/L respectively) can inhibit production of inositol phosphates, key intracellular signals

of proliferation stimulated by aluminum tetrafluoride in 3T3 cells (Higashi and Ogawara 1992). Genistein also blocks in vitro angiogenesis, another proposed mechanism of anticarcinogenesis (IC_{50} of 150 μmol/L, Fotsis et al. 1993).

These findings suggest that isoflavones in soybean foods might play an important role in human health maintenance. A previous human feeding study showed that absorption of a single dose of isoflavones from soy milk fed in a controlled liquid diet was dose dependent (Xu et al. 1994). Average 24 h urinary recovery of daidzein and genistein was 21% and 9%, respectively, at doses of 2.7, 5.0 and 7.7 μmol isoflavones/kg body weight. Average plasma total isoflavone concentration was 4.4 μmol/L at 6.5 h after a dose of 7.7 μmol isoflavones/kg body wt. Soymilk isoflavones seemed to be 85% degraded in the intestine. To further characterize the bioavailability of soy isoflavones and assess the potential for human gut microflora metabolism and degradation of isoflavones after ingestion, a group of adult females received three soymilk meals per day in a well-controlled liquid diet. In vitro anaerobic incubation of isoflavones with human feces was also performed. Isoflavone content of plasma, urine, fecal samples were measured.

Materials and methods

Subjects and Protocol

The subjects were seven adult females between 20 and 41 years of age, with body weight of 61.9 ± 11.3 kg and body mass index (BMI) of 22.0 ± 2.4 kg/m^2.

All the subjects were in good health, based on examinations performed by Student Health Center physicians at Iowa State University (ISU). Subjects did not use medication before and during the study. The procedures for this feeding study were approved by the Human Subjects Committee of ISU. Informed consent of subjects was obtained in writing. All subjects were omnivorous.

The study consisted of three feeding days, each separated by a two-week washout period. Each subject consumed 3.4, 6.9 and 10.3 μmol/kg body wt total isoflavones from soy milk powder. Each dose was consumed three times in nutritionally complete liquid meals on each feeding day (10.2, 20.7 and 30.9 μmol total isoflavones/ kg body wt per day) beginning at 7:00 am, with lunch and dinner fed at five hour intervals after breakfast. Subjects were randomly assigned to doses in a cross-over design. Each meal contained soy milk powder reconstituted with distilled water (Now Foods, Inc., Glendale Hts., IL) mixed with chocolate-flavored Carnation Instant Breakfast® (Nestle Food Company, Glendale, CA) diluted with whole cow's milk. Total caloric intakes fed were based upon subjects' energy requirements, according to the Recommended Dietary Allowances tables (Food and Nutrition Board, National Research Council, 1989). Subjects were instructed not to consume anything for 10 h before dosing, and not to eat anything other than what was given on the feeding day. They were also asked not to eat any soybean foods or foods containing texturized vegetable protein, hydrolyzed vegetable protein, or soy protein isolate during the washout periods. A list of such foods was provided. No adverse effects such as diarrhea were reported after soymilk dosing.

Biological Sample Collection

Blood samples. Venous blood samples (5 mL) were collected in heparinized vacuum containers by medical technologists under stringent aseptic conditions at the Student Health Center, ISU. A blood sample was collected within 1 h before dosing (Time "0"). Blood samples were also collected at 6.5 and 24 h after dosing. Samples were centrifuged within one hour after collection at 3000 × g for 20 minutes at 4°C (Model 4D; International Equipment Co., Needham Hts., MA), and plasma was stored in a -20°C freezer.

Urine samples. For each subject, a urine sample was collected in the morning before dosing (Time "0"). All urine was collected and pooled in four 12 h increments during the first 48 h after dosing. The first urination of the third day after dosing was also collected. After recording the total volume, 50 mL of each of the six samples was stored in a -20°C freezer.

Fecal samples. Capsules containing 1 g carmine red (Pharmaceutical Service, University of Iowa, Iowa City, IA) were given with the soymilk. One fecal sample from each subject was collected before soymilk feeding (Time "0"). After dosing, all feces excreted before and during fecal marker excretion were collected. Usually, the fecal marker appeared in feces one or two days after dosing. Fecal sample collection was stopped when fecal marker no longer appeared in feces. After collecting the fecal samples, feces were freeze dried. After recording the weights of dry samples, feces were ground to a fine powder in a coffee mill (Braun Company Inc., Lynnfield, MA). Ten g of each dry fecal sample was stored in a -20°C freezer until analysis.

Soybean Milk Powder Analysis

The concentrations of total isoflavones in both soymilk powder and chocolate-flavored Carnation Instant Breakfast® were measured as free isoflavones after hydrolysis in 1 mol/L HCL at 98°C following the method of Wang et al. (1990). The method of HPLC analysis was as described by Wang and Murphy (1994).

Biological Sample Analysis

Plasma and urine samples. Sample preparation for plasma and urine isoflavone analysis was performed according to the methods described by Lundh et al. (1988). Plasma and urine samples were treated with glucuronidase/ sulfatase (Sigma

Chemicals Company, St. Louis, MO) to produce the parent isoflavones. Chromatographic analysis followed the conditions described by Xu et al.(1994). Samples were automatically injected with a Spectra-Physics Autosampler Model 8780XR (Spectra-Physics, Fremont, CA). Two Beckman Model 110B pumps (Beckman Instruments, Inc., Berkeley, CA), and one Beckman Model 420 Microprocessor solvent flow controller were used. Beckman Model 163 variable wavelength detector set at 254 nm was connected with Beckman Model 427 Integrator by which chromatographic data were calculated and plotted.

Daidzein (4',7-dihydroxyisoflavone) was obtained from Life Science Group, ICN Pharmaceuticals, Inc., Plainview, NY. Genistein (4',5,7-trihydroxyisoflavone) was obtained from Calbiochem Corporation, La Jolla, CA. Equol (4',7-dihydroxyisoflavondiol) was a generous gift from Dr. H. Adlercreutz (Department of Clinical Chemistry, University of Helsinki, Finland).

A series of standards (1, 2, 4, 8, 12, 16 and 24 μmol/L) were assayed to quantify isoflavones. For each subject, plasma and urine samples were spiked randomly with daidzein, genistein and equol standards (0.15 μg isoflavone/100 μL added to each sample) to measure recovery.

Fecal samples. Sample preparation and analysis were performed as described by Xu et al.(1994). To ensure the quality of analysis, a series of standards (1, 2, 4, 8, 12, 16 and 24 μmol/L) and fecal samples spiked with 500 μL of 32 μmol/L daidzein, genistein and equol standards were analyzed to measure recovery.

In vitro incubation of isoflavones with human fecal flora

Daidzein and genistein standards were dissolved in sterilized brain-heart infusion broth (DIFCO Laboratories, Detroit, MI) at concentrations of 600 μmol/L. The solution was kept at 4°C until use. Twice within a 4 wk period, 20 g of

freshly voided feces from one 31 year-old, 76 kg male subject (BMI=22.5) collected in a sterile container was added to 180 mL of sterilized brain-heart infusion broth, and then homogenized by Stomacher Lab-Blender 400 (Tekmar® Company, Cincinnati, OH) under anaerobic conditions. Homogenate was quickly transferred to 50 mL sterile tubes and centrifuged at 3000 × g for 5 minutes at ambient temperature. The supernatant was used for the experiment. Four mL of freshly made supernatant was added to sterile tubes containing 1 mL of 600 µmol/L daidzein or genistein. Four ml sterile brain-heart infusion broth was added to two sterile tubes containing 1 ml isoflavone standard solution as controls. Final concentration of both daidzein and genistein in each tube was 120 µmol/L. Tubes were incubated at 37°C in a Gas-Pak® anaerobic incubation jar (Fisher Scientific, Pittsburgh, PA) filled with N_2 gas. Two tubes were taken out at 6, 12, 18 and 24 h and at 8 h interval until 96 h. The two control samples were taken out after 96 h incubation. The incubator was recharged with N_2 gas each time samples were taken. Two ml samples were injected onto pre-wetted Waters Sep-Pak C_{18} cartridges (Millipore, Milford, MA). The cartridges were washed with 2 ml Waters milli-Q water twice, and 2 ml 80% methanol to elute the isoflavones. All samples were analyzed by the same reverse phase HPLC system as for fecal sample analysis (Xu et al. 1994). Four ml of freshly made homogenate supernatant was also added to four sterilized tubes containing 1 ml isoflavone standard solution and assayed for isoflavones immediately to measure recovery.

Statistical Methods

Statistical analysis was performed with the Statistical Analysis System (SAS Institute, Inc., Cary, NC) version 6.06 on the Iowa State University mainframe computer. Data were tested for heterogeneity of variance. If necessary, data were

transformed to homogeneity before ANOVA (Snedecor and Cochran 1989). Analysis of variance (general linear models) was performed on the data obtained from the human feeding experiment. Subjects and feeding times were treated as blocks. The effects of dosage, time, and type of isoflavones as well as subject and feeding time upon plasma concentration, urinary and fecal excretion of isoflavones were determined. If the effect from any one of these factors other than the type of isoflavones was significant, Tukey's test was used for comparison of means within the factor. Meanwhile, the difference of isoflavone plasma concentration, urinary and fecal recovery between low and high isoflavone fecal excretors was tested by using the mean square for subjects within the type of fecal excretion as the error term. A p value of 0.05 or less was considered to be significant.

Results

Soymilk powder contained 4.79±0.31 μmol total isoflavones/g (44% genistein and 56% daidzein). No isoflavones were detected in chocolate-flavored Carnation Instant Breakfast®.

Fecal recoveries of daidzein and genistein were only 0.7% and 0.4% of the ingested amount, respectively, among five subjects (**Table 1**). In contrast, fecal recoveries of daidzein and genistein were about 6% and 7% of the ingested amount, respectively, in subjects 3 and 5. Their average fecal recoveries of daidzein and genistein at all three isoflavone doses were significantly greater than those among the five subjects excreting small amounts of isoflavones ($p<0.01$).

For five subjects excreting small amounts of fecal isoflavones (**Table 2**), average 48 h urinary recovery of daidzein and genistein was about 16% and 10%, respectively, at each dose. Urinary recovery of daidzein was significantly greater than that of genistein ($p<0.01$). For two subjects

excreting large amounts of fecal isoflavones, average 48 h urinary recovery of daidzein and genistein was about 34% and 38%, respectively, at each dose. Urinary daidzein and genistein recoveries in subjects excreting large amounts of fecal isoflavones was significantly greater than those among subjects excreting small amounts of fecal isoflavones at each dosage ($p<0.05$). No equol was found in plasma, urine and fecal samples although the recovery of equol was between 64-75% in all samples tested.

At all three doses, the amount of urinary isoflavones among subjects excreting small amounts of fecal isoflavones was significantly greater in the first and second 12 h after dosing than at other time points ($p<0.05$) (**Table 3**). After 48 h, urinary excretion of isoflavones was nearly nil. The amount of urinary daidzein was significantly greater than that of genistein during the first 24 h ($p<0.001$). Among the two subjects excreting large amounts of fecal isoflavones, the greatest amount of urinary isoflavones was found in the second 12 h (**Table 4**). Although the amount of urinary daidzein was greater than that of genistein in the first 24 h after dose, in the second 24 h, the amount of urinary genistein was greater than that of daidzein. In these two subjects, urinary excretion of isoflavones was also almost nil at 48 h after dosing.

Among the five subjects excreting small amounts of fecal isoflavones, plasma concentration of daidzein and genistein was significantly increased at 6.5 h after dosing compared with the concentration at other times ($p<0.05$), although at twenty-four hours after dosing plasma concentration of isoflavones was still about half or two-thirds of the level at 6.5 h (**Table 5**). The plasma concentration of daidzein was significantly greater than that of genistein ($p<0.05$) at 6.5 and 24 h after dosing. The average plasma concentration of both daidzein and genistein increased significantly with

increasing dose ($p<0.05$). Among the two subjects excreting large amounts of fecal isoflavones, plasma concentration of genistein at 24 h was significantly greater than that among the five subjects excreting small amounts of fecal isoflavones at each dosage ($p<0.05$) (**Table 5**).

During four days of anaerobic incubation of human feces, daidzein and genistein completely disappeared after 72 h and 18 h respectively (**Figure 1**). The loss of isoflavones seemed to follow first-order kinetics, with the half-lives of daidzein and genistein estimated at 7.5 h and 3.3 h respectively. After 18 h incubation some new peaks appeared, accompanying the loss of genistein and daidzein (**Figure 2**). No loss of isoflavones was observed in control samples. Isoflavone recoveries in samples tested ranged from 93-98%.

Discussion

This study may provide insight into the nature of gastrointestinal absorption of soy isoflavones. On the average, soybean milk powder only contains about 4-5% of total isoflavones as the aglycones, daidzein and genistein (Wang and Murphy 1994). The glycosides of flavonoids and isoflavones are very poorly absorbed in the small intestine compared with their aglycones because of the glycosides' higher hydrophilicity and greater molecular weight (Brown 1988). On the average, gastric emptying time after 500-600 mL of a complete liquid meal which has similar volume and content as our subjects received is about 1 h in humans (Davenport 1982). The average length of transit time of such a meal is about 5 min in the duodenum, 2 h in the jejunum, and 3-6 h in the ileum (Welling 1986). In our study, average plasma concentrations of isoflavones at 6.5 h after breakfast dose (1.5 h after lunch dose) were 3.6 and 6.7 μmol/L, respectively, at doses of 3.4 and 6.9 μmol isoflavones/kg body weight per meal (**Table 5**). However, at 6.5 h after a single

breakfast dose of 5.0 and 7.7 μmol isoflavones/kg body weight, average plasma concentration of isoflavones was only 2.3 and 4.4 μmol/L, respectively, in our previous report (Xu et al. 1994). Since human subjects in both studies were given the same controlled liquid diet, some of the isoflavones consumed at lunch very likely already entered the general circulation at 1.5 h after lunch. This suggested that the absorption of soybean isoflavones in humans might begin in the proximal small intestine since surface area and blood perfusion rate strongly favor more efficient absorption from the proximal small intestine than from the stomach (Welling 1986). This is problematic because isoflavones are present primarily as β-glucosides in soybean foods, which are poorly hydrolysed by mammalian intestinal digestive enzymes (Brown 1988). Glucosidases of intestinal microflora in lower bowel can liberate aglycones and promote their absorption (Friend and Chang 1984). But gastric hydrochloric acid might also produce isoflavone aglycones by the same mechanism used in soybean food analysis. Thus, absorption of soymilk isoflavones as aglycones might begin in the proximal small intestine.

Human intestinal bacteria can metabolize and degrade isoflavones as well as liberate isoflavone aglycones. Although several groups of bacteria possess β-glucosidase activity, *Lactobacilli*, *Bacteroides* and *Bifidobacteria* play major roles in the intestinal hydrolysis of numerous plant β-glucosides present in the human diet such as glucosides of flavonoids and isoflavones to produce aglycones (Hawksworth et al. 1971). From proximal to distal small intestine, the amount of bacteria is increased from very low bacterial numbers in the upper 1/3 of small intestine to almost "colon-like" resident flora in the lower 1/3 of small intestine (Heneghan 1988). Contents of both distal small intestine and large intestine in humans have substantial amount of *Lactobacilli*, *Bacteroides* and *Bifidobacteria*, and the number of each of three bacterial

groups is about 10^4-10^7 bacteria per g wet weight (Gorbach et al. 1967 and Mitsuoka 1982). Whereas, contents of the human proximal small intestine contain almost none of these bacteria (Mitsuoka 1982). Some bacteria in human large intestine also have β-glucuronidase and arylsulfatase which can liberate aglycones from conjugates of liver phase II biotransformation (Heneghan 1988). On the other hand, certain strains of *Clostridia* in human lower gastrointestinal tract can cleave the C-ring of many flavonoids, such as quercetin (3,3',4',5,7-pentahydroxyflavone), kaempferol (3,4',5,7-tetrahydroxyflavone), naringenin (4',5,7-trihydroxy flavanone), and produce monophenolic compounds anaerobically (Winter et al. 1989). Flavonoids are analogous to the isoflavones, with the aromatic B-ring attached to carbon 2 of the C-ring instead of carbon 3. Interestingly, *Clostridia* are not present in all individuals' feces although their average number is about 10^9-10^{10} bacteria per g wet feces (Mitsuoka 1982). Rat intestinal microflora can also cleave the C-ring of flavonoids and isoflavones in vitro (Griffiths and Smith 1972), and germ-free rats did not excrete C-ring fission products in urine compared with conventional rats after aglycones were fed (Griffiths and Barrow 1972).

The bacteria in the gastrointestinal tract play an important role in determining the magnitude and pattern of isoflavone bioavailability. When four human subjects orally received 4 g quercetin, about 50% of the dose was not recovered in urine and feces after 72 h, and the remainder of the dose was thought to have been degraded microbially in the human lower bowel (Gugler et al. 1975). Human intestinal bacteria can completely convert 5 mg of quercetin to a monophenolic compound, 3,4-dihydroxyphenylacetic acid within about 3-4 hours anaerobically in a prereduced brain-heart infusion broth (Winter et al. 1989). Our in vitro fecal incubation (**Figure 1**) also suggested that human intestinal

bacteria might metabolize and degrade ingested soybean isoflavones to a great extent. This may be why only 14-43% of isoflavones were recovered intact in urine and feces (**Tables 1, 2**).

In our study, five of the seven subjects only excreted 0.4-0.7% of ingested isoflavones intact in feces (**Table 1**), whereas 6-8% of ingested isoflavones were excreted intact in feces in the other two subjects. This difference in fecal excretion of isoflavones seemed to profoundly alter isoflavone bioavailability reflected in urinary recovery of ingested isoflavones, which was more than twice as great in high versus low fecal excretors (**Table 2**). A model of human isoflavone disposition following oral dosing was proposed by Xu et al. (1994). After absorption isoflavones are extensively transformed by phase II enzymes, especially, by glucuronidation (Axelson et al. 1984). Isoflavones then undergo rapid urinary and biliary excretion in rats (Axelson and Setchell 1981), and humans (Rondelli et al. 1991). In humans the molecular weights of isoflavones are probably within the range which enables their conjugates to undergo partitioning between bile and urine. Gut bacterial deconjugating enzymes (i.e., β-glucuronidases and arylsulfatases) can hydrolyze the isoflavone conjugates to produce aglycones. Isoflavones also are extensively metabolized and degraded by intestinal microflora, which prohibit their reabsorption from lower bowel. If for any reason these bacteria were not effective in degradation and metabolism of isoflavones, more isoflavones would be absorbed. These might explain the strong association between high fecal isoflavones and greater total urinary recovery of isoflavones.

Isoflavones and flavonoids which possess a 5-OH group, such as genistein and not daidzein, are much more susceptible to C-ring cleavage by rat intestinal bacteria (Griffiths and Smith 1972). Consistent with this, our in vitro incubation

study indicates that the human intestinal half-life of genistein may be less than half of that for daidzein (**Figure 1**). Therefore, less genistein than daidzein can be absorbed. This may be why subjects excreting small amounts of fecal isoflavones had less urinary recovery and lower plasma concentration of genistein than those of daidzein (**Tables 2, 5**), although the soymilk fed contained similar amounts of daidzein and genistein. When intestinal bacterial degradation of isoflavones, especially of genistein is limited, greater amounts of genistein than daidzein can be found in urine and plasma of the two subjects excreting relatively large amounts of fecal isoflavones (**Tables 4, 5**).

Cancer protective effects of isoflavones may be contributed by isoflavones and their metabolites, because some monophenolic compounds like methyl p-hydroxyphenylacetate, a metabolite of both exogenous flavonoids and tyrosine are inhibitors of hormone-dependent neoplastic cell proliferation. This compound has higher affinity for the nuclear type II binding site than estradiol. This type II binding site has been suggested to be involved in cell growth regulation by estrogenic hormones. This metabolite can block estradiol stimulation of uterine growth in vivo and inhibit the growth of MCF-7 human breast cancer cells in vitro (Markaverich et al. 1988). In our in vitro incubation, the appearance of new peaks in the chromatogram after 18 h of incubation (**Figure 2**), accompanying the loss of genistein and daidzein indicate the possible existence of isoflavone metabolites that deserve further chemical and biological characterization. These new peaks are not equol because the retention time for equol in our reverse phase HPLC system is between daidzein and genistein (Xu et al. 1994). Developing methods to measure monophenolic and other diphenolic isoflavone breakdown products may be important.

In the two subjects excreting large amounts of fecal

isoflavones, plasma concentration of genistein at 24 h after 3 doses of 10.3 μmol/kg isoflavones were fed was still about 5.07 μmol/L, about twice as much as that among subjects excreting small amounts of fecal isoflavones (**Table 5**). A study of 3T3 cell inhibition of production of inositol phosphates, key intracellular signals which mediate tumor promotion, showed an IC_{50} for daidzein of 8 μmol/L and for genistein of 2.2 μmol/L, when inositol phosphate production was stimulated by aluminum tetrafluoride (Higashi and Ogawara 1992). Thus, our data suggest that enhancement of anticarcinogenic activity of isoflavones may be expected among subjects with relatively great fecal recovery of ingested isoflavones or exhibiting longer isoflavone intestinal half-life. Alteration of gut microbial function to prevent isoflavone degradation might enhance the anticarcinogenic potential of isoflavones.

From this study, it was concluded that human absorption, excretion and plasma concentration of isoflavones depended upon dose. It is reasonable to infer that the condition of intestinal microflora profoundly influences isoflavone bioavailability. Although bioavailability of isoflavones may, on average, vary from 13% to 35% depending upon the individual's gut microflora, isoflavones may still be absorbed enough from soymilk to exert potentially beneficial biological effects.

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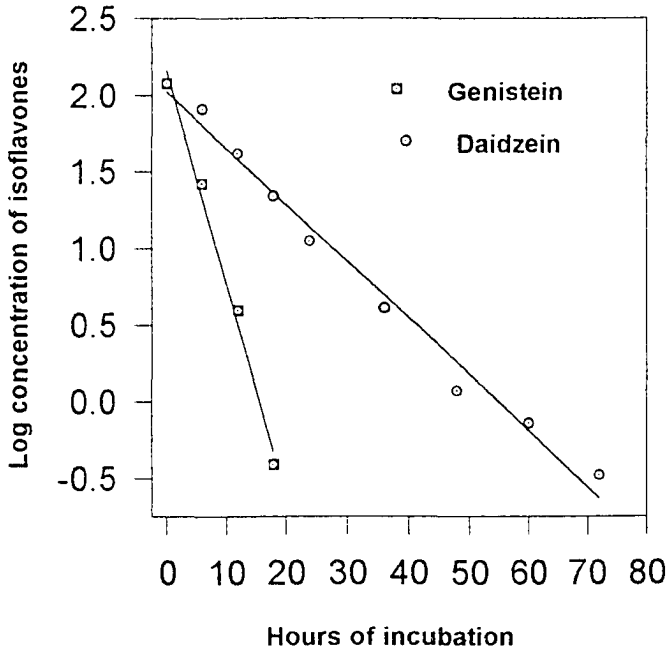

Figure 1: In vitro incubation of isoflavones with human feces

Anaerobic incubation of 1 mL 600 µmol/L daidzein and genistein with 4 mL supernatant from homogenate of prereduced brain-heart infusion broth and freshly voided feces of a healthy human volunteer at 37°C. Concentration of daidzein and genistein was measured by a reverse phase HPLC system at 6, 12, 18, 24 h and 8 h interval until 96 h after incubation. Data of Log concentration and hours of incubation were plotted. The loss of isoflavones seem to follow first-order kinetics, with the loss of genistein occurring at a much faster rate than daidzein. Half-lives of daidzein and genistein were about 7.5 h and 3.3 h respectively.

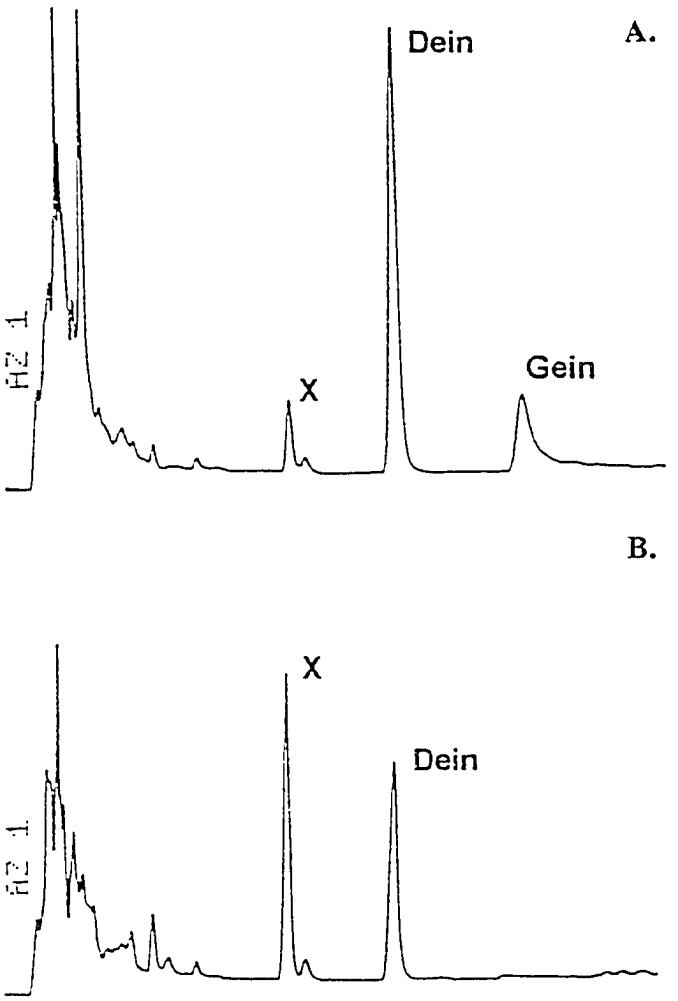

Figure 2: HPLC chromatogram of incubated samples

HPLC chromatogram after 12 h (A) and 18 h (B) of anaerobic incubation as described in **Figure 1**. Dein= daidzein, Gein= genistein, and X= unknown peak. The same reverse phase HPLC system as for fecal sample analysis (Xu et al. 1994) was used to determine and quantify isoflavones. Comparing A and B, after 18 h incubation unknown peak X was increased, accompanying the loss of genistein and daidzein.

Table 1. Human fecal isoflavone recovery after three doses of 3.4, 6.9 or 10.3 μmol/kg body wt[1]

Isoflavones	3.4 μmol/kg	6.9 μmol/kg	10.3 μmol/kg
		%	
Subjects excreting small amounts of isoflavones[2]			
Daidzein recovery	0.55 ± 0.22	0.72 ± 0.18	0.82 ± 0.41
Genistein recovery	0.29 ± 0.16	0.46 ± 0.29	0.49 ± 0.31
Total recovery	0.44 ± 0.18	0.61 ± 0.22	0.69 ± 0.36
Subjects excreting large amounts of isoflavones[3]			
Daidzein recovery[4]	5.98 ± 2.67	5.02 ± 2.13	5.50 ± 2.78
Genistein recovery[5]	8.59 ± 0.32	5.92 ± 0.21	6.39 ± 1.02
Total recovery	7.62 ± 1.51	5.50 ± 1.17	6.01 ± 1.89

[1]Fecal samples included all feces excreted before and during fecal marker excretion.

[2]Subjects excreting small amounts of isoflavones included subjects 1, 2, 4, 6 and 7. Values are means and SD; n = 5.

[3]Subjects excreting large amounts of isoflavones included subjects 3 and 5. Values are means and SD; n = 2.

[4 5]Data were log-transformed to achieve homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Fecal recoveries of daidzein and genistein in two subjects excreting large amounts of fecal isoflavones was significantly greater than those of daidzein and genistein among five subjects excreting small amounts of fecal isoflavones at each dosage, by ANOVA (general linear models) with mean square for subjects within the type of fecal excretion as error term ($p < 0.01$).

Table 2. Human 48 h urinary isoflavone recovery after three doses of 3.4, 6.9 or 10.3 μmol/kg body wt[1]

Isoflavones	3.4 μmol/kg	6.9 μmol/kg	10.3 μmol/kg
		%	
Subjects excreting small amounts of isoflavones[2]			
Daidzein recovery[3]	17.22 ± 4.19	15.21 ± 3.04	15.97 ± 4.26
Genistein recovery	10.31 ± 4.08	9.96 ± 4.14	10.49 ± 4.03
Total recovery	14.17 ± 4.13	12.90 ± 3.87	13.56 ± 4.16
Subjects excreting large amounts of isoflavones[4]			
Daidzein recovery[5]	33.06 ± 5.65	31.46 ± 4.56	30.31 ± 6.05
Genistein recovery[6]	38.68 ± 4.08	36.77 ± 6.14	35.61 ± 6.29
Total recovery	35.53 ± 4.87	33.80 ± 5.52	33.04 ± 6.23

[1]Urine samples included all the urine excreted during the first 48 hours after dosing and the first urination of the third day.

[2]Subjects excreting small amounts of isoflavones included subjects 1, 2, 4, 6 and 7. Values are means and SD; n = 5.

[3]The urinary recovery of daidzein was significantly greater than that of genistein at each dosage by ANOVA (general linear models) ($p<0.01$).

[4]Subjects excreting large amounts of isoflavones included subjects 3 and 5. Values are means and SD; n = 2.

[5 6]Urinary recoveries of daidzein and genistein in two subjects excreting large amounts of fecal isoflavones was significantly greater than those of daidzein and genistein among five subjects excreting small amounts of fecal isoflavones at each dosage, by ANOVA (general linear models) with mean square for subjects within the type of fecal excretion as error term ($p< 0.05$).

Table 3. Urinary excretion of isoflavones during and after three doses of 3.4, 6.9 and 10.3 μmol/kg body wt in subjects excreting small amounts of fecal isoflavones[1]

	Time "0"	0-12 h	12-24 h	24-36 h	36-48 h	>48 h
				μmol		
3.4 μmol/kg						
Daidzein[2]	0.12±0.16	30.46± 7.39	27.16± 9.35	2.04± 1.89	0.47±0.94	0.16±0.16
Genistein	ND	10.88± 4.51	12.91± 4.48	2.04± 1.81	0.89±1.15	0.07±0.07
Total[3]	0.12±0.16[b]	41.34±11.90[a]	40.07±13.83[a]	4.08± 3.70[b]	1.36±2.09[b]	0.23±0.23[b]
6.9 μmol/kg						
Daidzein[2]	0.43±0.75	65.16±20.32	34.51±11.12	5.19± 5.42	1.18±1.14	0.12±0.12
Genistein	0.59±0.78	24.57± 9.62	20.76±10.18	4.66± 3.37	0.96±0.70	0.07±0.07
Total[3]	1.02±1.53[b]	89.73±29.94[a]	55.23±21.30[a]	9.85± 8.79[b]	2.14±1.84[b]	0.19±0.19[b]
10.3 μmol/kg						
Daidzein[2]	0.75±1.38	85.24±33.92	60.84±27.98	16.07±11.32	4.60±5.34	0.35±1.10
Genistein	0.37±0.48	30.45±11.69	34.78±26.83	9.29± 7.18	5.77±9.03	0.89±0.93
Total[3]	1.12±1.86[b]	115.69±45.61[a]	95.62±54.81[a]	25.36±18.50[b]	10.37±14.37[b]	1.24±2.03[b]

[1]Values are means and SD; n = 5; μmol/kg = μmol of isoflavones per kg body weight; ND = Not detectable, at a detection limit of 2 ng per 20 μL injected.

[2]To compare excretion of the two isoflavones, the data were log-transformed to achieve homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. The amount of daidzein was significantly greater than that of genistein at first 24 h, by ANOVA(general linear models) ($p<0.001$).

[3]To compare excretion of total isoflavones at different times after dosing, data were log-transformed to achieve homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Effect of time was significant by ANOVA (general linear models) ($p<0.001$), and values noted by different superscripts are significantly different ($p<0.05$, Tukey's test).

Table 4. Urinary excretion of isoflavones during and after three doses of 3.4, 6.9 and 10.3 μmol/kg body wt in subjects excreting large amounts of fecal isoflavones[1]

	Time "0"	0-12 h	12-24 h	24-36 h	36-48 h	>48 h
				μmol		
3.4 μmol/kg						
Daidzein	0.43±0.43	43.47± 4.44	68.46±13.79	18.90± 2.40	4.60± 1.85	0.47±0.12
Genistein	ND	32.38± 7.96	49.21±20.28	25.23±11.43	10.03± 6.36	1.15±0.85
Total	0.43±0.43	75.85±12.40	117.67±34.07	44.13±13.87	14.63± 8.21	1.62±0.97
6.9 μmol/kg						
Daidzein	ND	94.04± 8.53	114.36±18.98	38.99±13.13	11.83± 7.51	0.24±0.24
Genistein	ND	61.16± 8.33	91.76±30.56	47.62±19.76	23.79± 8.10	ND
Total	ND	155.20±16.86	206.12±49.54	86.61±32.89	35.62±15.61	0.24±0.24
10.3 μmol/kg						
Daidzein	1.53±1.53	125.84±29.51	166.00±37.30	61.82±14.66	18.75±11.24	1.10±0.55
Genistein	ND	92.02±25.75	124.28±40.55	70.63±14.80	36.78±25.42	2.18±0.33
Total	1.53±1.53	217.86±55.26	290.28±77.85	132.45±29.46	55.53±36.66	3.28±0.88

[1]Values are means and SD; n = 2; μmol/kg = μmol of isoflavones per kg body weight; ND = Not detectable, at a detection limit of 2 ng per 20 μL injected.

Table 5. Plasma concentration of isoflavones during and after three doses of 3.4, 6.9 and 10.3 µmol/kg body wt

		Time "0"	6.5 h	24 h
Subjects excreting small amounts of isoflavones[1]			µmol/L	
Dose				
3.4 µmol/kg	Daidzein[2]	ND	1.88 ± 0.46	0.94 ± 0.34
	Genistein	ND	1.43 ± 0.41	0.53 ± 0.30
6.9 µmol/kg	Daidzein[2]	ND	3.62 ± 0.72	2.04 ± 0.63
	Genistein	ND	2.78 ± 0.75	1.15 ± 0.46
10.3 µmol/kg	Daidzein[2]	ND	5.28 ± 1.07	3.04 ± 0.80
	Genistein	ND	4.59 ± 1.35	2.17 ± 0.75
Subjects excreting large amounts of isoflavones[3]				
Dose				
3.4 µmol/kg	Daidzein[4]	ND	1.92	1.16
	Genistein[5]	ND	2.03	1.92
6.9 µmol/kg	Daidzein[4]	ND	3.84	3.00
	Genistein[5]	ND	3.15	3.00
10.3 µmol/kg	Daidzein[4]	ND	6.08	3.60
	Genistein[5]	ND	6.00	5.07

[1]Subjects excreting small amount of isoflavones included subjects 1, 2, 4, 6 and 7. Values are means and SD; n = 5; µmol/kg = µmol of isoflavones per kg body weight; ND = Not detectable, at a detection limit of 2 ng per 20 µL injected.

continued

continued, Table 5

[2]To compare plasma concentration of two isoflavones, data were log-transformed to achieve homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Plasma concentration of daidzein was significantly greater than that of genistein at 6.5 and 24 h after dosing, by ANOVA (general linear models) ($p<0.05$).

[3]Subjects excreting large amount of isoflavones included subjects 3 and 5. Values are means; n=2.

[4]To compare plasma concentration of daidzein between low and high fecal isoflavone excretors at each dosage, data were log-transformed to achieve homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Plasma concentrations of daidzein between low and high fecal isoflavone excretors were not significantly different at both 6.5 h and 24 h after dosing, by ANOVA (general linear models) with mean square for subjects within the type of fecal excretion as error term ($p>0.1$).

[5]To compare plasma concentration of genistein between low and high fecal isoflavone excretors at each dosage, data were log-transformed to achieve homogeneity of variance before ANOVA. Means and SD of the untransformed data are presented in the table. Plasma concentrations of genistein between low and high fecal isoflavone excretors were significantly different at 24 h after dosing ($p<0.05$), but not at 6.5 h after dosing ($p>0.1$) by ANOVA (general linear models) with mean square for subjects within the type of fecal excretion as error term.

III. NEITHER DIET SELECTION NOR TYPE OF SOY FOOD SIGNIFICANTLY AFFECT ISOFLAVONE BIOAVAILABILITY[1]

A paper to be submitted to the Journal of Nutrition

Xia Xu, Huei-Ju Wang, Patricia A. Murphy, and Suzanne Hendrich[2]

Abstract

To characterize the effect of background diet and soyfood form upon bioavailability of soybean isoflavones, proposed anticarcinogenic food components, eight adult women, ages 20-41, were fed 0.9 mg isoflavones/kg body wt from soymilk in three meals for a day. Each subject consumed a basic foods diet at 7:30 am, 12:30 pm and 5:30 pm, a self-selected diet at these same times, or an ad libitum diet with the soymilk. In a second study, single isoflavone doses of 0.8-1.4 mg/kg were fed in breakfast casseroles containing tofu, tempeh, soybeans or texturized vegetable protein. Both studies employed a randomized cross-over design. There was a one-week washout period between soy doses. Plasma, urine and fecal isoflavones were measured by reverse-phase HPLC. With basic, self-selected, and ad libitum diets, 48 h urinary recovery of daidzein was 27±8%, 26±10% and 26±9%, respectively, and of genistein, 20±7%, 18±9%, and 18±8%, respectively. Recoveries of daidzein in urine over 24 h from cooked soybean, texturized vegetable protein, tofu, and tempeh were 46±16%, 51±10%, 50±10%, and 38±18%, respectively, and of

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genistein, 13±6%, 14±8%, 16±5%, and 9±4%, respectively. In both studies, urinary recovery of daidzein was greater than that of genistein, and the recovery did not vary with soyfood or diet selection. Plasma concentration of isoflavones was 2.7±1.3 μmol/L 24 h after soymilk feeding. Plasma daidzein and genistein levels were approximately equal. Only a few percent of the total isoflavone dose was recovered in feces, probably due to bacterial breakdown of these compounds. Isoflavone bioavailability did not vary with the type of soyfood or background diet.

Introduction

Isoflavones are abundant in soy foods (0.2-1.5 mg/g) (Wang and Murphy 1994) with various amounts of isomers, three types (daidzein, genistein and glycitein) in four chemical forms (aglycone, glucoside, acetylglucoside, and malonylglucosides) (Kudou et al. 1991). Although biological activity of glycitein was not known, two major types of soy isoflavones, daidzein and genistein are proposed anticarcinogenic agents (Messina and Barnes, 1991).

Substantial dietary isoflavone intake has been linked to the population who showed much lower breast and prostatic cancer incidence and mortality (Adlercreutz et al. 1995). In a rat study, hepatic cumene hydroperoxidase, an antioxidant enzyme, activity was significantly increased by feeding a diet containing soybean isoflavones extract (at 240 mg isoflavones /kg diet) for seven days (Hendrich et al. 1994). Although mechanism of antiestrogenic activity of isoflavones has not been entirely clear yet, they can compete with estradiol for the nuclear type II binding sites (Adlercreutz et al. 1992), and inhibit human aromatase (an estrogen synthase) (Adlercreutz et al. 1993). Rats consuming a soy-based diet develop fewer mammary tumors following administration of the carcinogens N-methylnitrosourea and 7,12-dimethylbenz[a]-

anthracene than rats on isonitrogenous and isocaloric diets without soy (Barnes et al. 1990).

Isoflavones also demonstrated abilities to suppress tumor cell growth in vitro. Genistein inhibits the growth of estrogen receptor-negative or -positive human breast cancer cell lines (IC_{50}=24-44 μmol/L; Peterson and Barnes 1991). Daidzein and genistein (IC_{50} of 8 μmol/L and 2.2 μmol/L, respectively) can inhibit production of inositol phosphates, key intracellular signals of proliferation stimulated by AlF_4 in 3T3 cells (Higashi and Ogawara 1992). Genistein also inhibits activation of tyrosine kinases which are a major class of oncogene products (IC_{50}=2.6 μmol/L, Akiyama et al. 1987), and topoisomerase II of human leukemic MOLT-4 and HL-60 cells (IC_{50}=31.5 and 48 μmol/L, respectively) but not the normal proliferating lymphocytes (Traganos et al. 1992).

These findings suggest that isoflavones in soybean foods might play an important role in human health maintenance. Previous human feeding studies showed that absorption of isoflavones from soy milk with controlled liquid diet was dose dependent (Xu et al. 1994 and 1995). However, in the real life situation people eat a variety of soy foods with different diet selections. These variations may affect soy isoflavone absorption and disposition among individuals. Since current clinical trial is hard to afford pure isoflavone standards and entirely controlled diet, investigating the effect of diet selections and type of soybean foods upon human isoflavone absorption and excretion are the purpose of following two studies.

Subjects and methods

Subjects and Protocol

The procedures for this feeding study were approved by the Human Subjects Committee of ISU. Informed consent of subjects was obtained in writing. All the subjects were

omnivorous, and in good health, based on medical histories and physical examinations performed by Student Health Center physicians at Iowa State University (ISU). Subjects did not take any medication such as antibiotics during entire feeding studies.

Experiment One

The subjects were eight adult women between 20 and 41 years of age, with body weight of 58.35±10.49 kg and body mass index of 21.43±2.15 kg/m^2 (**Table 1**).

The study consisted of three feeding days, each separated by a one-week washout period (**Table 1**). On each feeding day, all subjects were fed three doses of isoflavones (0.9 mg/kg body weight in each meal) from soymilk powder (Now Foods, Inc., Glendale Hts.,IL) reconstituted with distilled water. The three doses of soymilk were served at 7:30 am, 12:30 am and 5:30 pm.

A basic food diet (**Table 2**) was provided and consumed at 7:30 am, 12:30 am and 5:30 pm, with the soymilk doses. When the subjects ate a self-selected diet, they chose their own foods but consumed at the same time as the soymilk doses. When the subjects ate ad libitum, they chose their own foods and consumed them at non-controlled times, but soymilk was consumed at the same times as with the other feeding regimens. Subjects consuming self-selected and ad libitum diets recorded their daily food intakes which were then analyzed by Nutritionist IV version 2.0 (N-SQUARED COMPUTING, Salem, OR). The subjects were also instructed not to eat any soybean foods or foods containing texturized vegetable protein, hydrolyzed vegetable protein, and soy protein isolate. A list of such foods was provided. Subjects were asked not to consume anything for 10 h before dosing. Subjects eating the basic food diet were instructed not to eat anything other than what was given on the feeding day.

Experiment Two

The subjects were ten young adult female between 20 and 35 years of age, with body weight of 59.58±5.97 kg and body mass index of 21.60±1.25 kg/m^2 (**Table 3**). Subjects were fed four types of soybean foods: tofu, texturized vegetable protein (TVP), tempeh, or cooked soybeans. Soyfood was given in the breakfast of each feeding day with a controlled diet (**Table 4**) for one day. The controlled diet was designed to meet subjects' average energy requirement based on their ages, body weights, and physical activity levels according to the Recommended Dietary Allowances tables (Food and Nutrition Board, National Research Council, 1989). Feeding days were separated by one-week washout periods (**Table 3**). Isoflavone dose ranged from 0.8-1.4 mg/kg body weight based on HPLC analysis of the soybean foods ingested. Subjects were instructed not to eat anything other than what was given on the feeding day.

In both studies, subjects were asked not to eat any foods from a list of items which may contain isoflavones during the washout periods. No adverse effects such as diarrhea were reported after soy food dosing.

Biological Sample Collection

Blood samples. In both experiments, five mL venous blood samples were collected in heparinized vacuum containers by medical technologists under stringent aseptic condition at the Student Health Center, ISU. A blood sample was collected within one h before dosing (Time "0"). Blood samples were also collected at 6.5 and 24 h after dosing. Samples were centrifuged within one h after collection at 3000 × g for 20 minutes at 4°C (Model 4D; International Equipment Co., Needham Hts., MA), and plasma was stored in a -20°C freezer.

Urine samples. In Experiment One, for each subject, a urine sample was collected in the morning, before dosing (Time "0"). All urine was collected and pooled in four 12 h increments

over the first 48 h after dosing. The first urination of the third day after dosing was also collected. In Experiment Two, a urine sample was collected in the morning, before dosing (Time "0"). All urine in 24 h and first urination of the second day after dosing were collected. Urine samples were pooled during the following time intervals: Time "0", 0-12 h, 12-24 h, and >24 h. After recording the total volume, 50 mL of each of the six samples was stored in a -20°C freezer.

Fecal samples. In both experiments, capsules containing 1 g carmine red (Pharmaceutical Service, University of Iowa, Iowa City, IA) were given with the soymilk or soyfoods in the morning. One fecal sample from each subject was collected before soymilk feeding (Time "0"). After dosing, all feces excreted before and during fecal marker excretion were collected. Usually, the fecal marker appeared in feces one or two days after dosing. Fecal sample collection was stopped when the fecal marker no longer appeared in feces. After collecting the fecal samples, feces were freeze dried. After recording all the weights of dry samples, feces were ground to a fine powder in a coffee mill (Braun Company Inc., Lynnfield, MA). Ten g of each dry fecal sample was stored in a -20°C freezer until analysis.

Soy Milk Powder and Soy Foods Analysis

The concentrations of total isoflavones in soymilk powder and four soy food were measured as free isoflavones after hydrolysis in 1 mol/L HCl at 98°C following the method of Wang et al. (1990). The method of HPLC analysis was as described by Wang and Murphy (1994).

Biological Sample Analysis

Plasma and urine samples. Sample preparation for plasma and urine isoflavone analysis was performed according to the methods described by Lundh et al. (1988). Plasma and urine samples were treated with glucuronidase/sulfatase (Sigma Chemicals Company, St. Louis, MO) to produce the parent

isoflavones. Chromatographic analysis followed the conditions described by Xu et al. (1994). Samples were automatically injected with a Spectra-Physics Autosampler Model 8780XR (Spectra-Physics, Fremont, CA). Isoflavones were separated in a Waters 3.9 mm i.d. × 30 cm length μ-Bondapak C_{18} reverse phase column with gradient elution at ambient temperature. Two Beckman Model 110B pumps,and one Beckman Model 420 Microprocessor solvent flow controller were used. Beckman Model 163 variable wavelength detector set at 254 nm was connected with Beckman Model 427 Integrator by which chromatographic data were calculated and plotted.

Daidzein (4',7-dihydroxyisoflavone) was obtained from Life Science Group, ICN Pharmaceuticals, Inc., Plainview, NY. Genistein (4',5,7-trihydroxyisoflavone) was obtained from Calbiochem Corporation, La Jolla, CA. Equol (4',7-dihydroxyisoflavondiol) was a generous gift from Dr. H. Adlercreutz (Department of Clinical Chemistry, University of Helsinki, Finland).

A series of standards of 0.25, 0.5, 1, 2, 3, 4, 6 μg/mL, were run to quantify isoflavones. For each subject, plasma and urine samples were spiked randomly with daidzein, genistein and equol standards (100 μL 1.5 μg/mL isoflavone added to each sample) to measure recovery.

Fecal samples. Sample preparation and analysis were performed as described by Xu et al.(1994). To ensure the quality of analysis, a series of standards (0.25, 0.5, 1, 2, 3, 4, 6 μg/mL) and fecal samples spiked with 500 μL of 8 μg/mL daidzein, genistein and equol standards were run to measure recovery.

Statistical Methods

Analysis of variance (General Linear Model) was used. Subjects and feeding times were treated as blocks. Effects of diet selection, time and type of isoflavones and soy foods as

well as subject and feeding time upon plasma concentration and urinary and fecal excretion were determined. Unweighted means were used for analysis of variance in Experiment Two (Snedecor and Cochran 1989). If the effect from any one of these factors other than type of isoflavones was significant, Tukey's test was used for comparison of means within the factor. The statistical analysis was performed with the Statistical Analysis System (SAS Institute, Inc., Cary, NC) version 6.06 on the Iowa State University mainframe computer. A p value of 0.05 or less was considered to be significant.

Results

Isoflavone concentration in soymilk powder was 1.25±0.08 mg/g, daidzein and genistein were 44.0% and 56.0% respectively. Isoflavone concentrations of tofu, tempeh, texturized vegetable protein and cooked soybean were 0.42-0.58 mg/g, 39.7-46.4% daidzein and 53.6-60.3% genistein (**Table 5**).

Experiment One

Average intakes of energy, carbohydrate and fiber were not significantly different among the three background diet, but the average intakes of fat, protein, and cholesterol from self-selected and ad libitum diets were significantly greater than those on the basic food diet ($p<0.05$) (**Table 6**). After consuming basic, self-selected, and ad libitum diets, 48 h urinary recovery of daidzein was 27±8%, 26±10% and 26±9%, respectively, and of genistein, 20±7%, 18±9%, and 18±8%, respectively (**Table 7**). Urinary recovery of daidzein was greater than that of genistein ($p<0.05$). Total fecal excretion of isoflavones was 4% of the ingested amount. No equol was found in any sample although the recovery of equol was between 62-74% in all spiked samples.

The amount of urinary isoflavones under all dietary conditions was about three times greater in the first 24 h after dosing than in the second 24 h after dosing ($p<0.05$)

(**Table 8**). After 48 h, urinary excretion of isoflavones was nearly nil. The amount excreted at each time point was similar among the three dietary conditions. The amount of urinary daidzein was twice that of genistein during the first 24 h after dosing ($p<0.01$).

Variation in isoflavone content of biological samples was great among the subjects, especially for fecal samples. Subject Three excreted ten times more fecal isoflavones than the other subjects under all three dietary conditions.

Plasma isoflavone concentration was significantly increased to an average of 1.3±0.5 μg/mL at 6.5 h after dosing compared with Time "0" (**Table 9**). The plasma concentrations of daidzein and genistein were not significantly different at this time.

Experiment Two

Amounts of total fat, fiber, and carbohydrate intake were not significantly different, but the amount of protein intake when subjects consumed texturized vegetable protein was significantly greater than from the other three soy foods (**Table 10**). During feeding of the four soybean foods, 21-34% of the ingested total isoflavone dose was excreted (**Table 11**). Urinary recoveries of daidzein collected over 24 h after soy dosing from cooked soybean, texturized vegetable protein, tofu, and tempeh were 46±16%, 51±10%, 50±10%, and 38±18%, respectively, and of genistein, 13±6%, 14±8%, 16±5%, and 9±4%, respectively. Regardless of type of soy food, urinary recovery of daidzein was, on the average, 2.5 times greater than that of genistein ($p<0.01$). No significant variation of urinary recovery of isoflavones were observed among the soyfoods ($p>0.1$). Total fecal excretion of isoflavones was 2% of the ingested amount. Types of soybean foods and variation of dosages did not significantly alter plasma concentration of isoflavones ($p>0.1$) (**Table 12**).

Discussion

On the average, cooked soybean, texturized vegetable protein, and soy milk powder contain more than 95% of total isoflavones as glycosides, whereas, tofu has about 20% of its isoflavones as aglycones, and tempeh has almost 40% of its isoflavones as aglycones (Wang and Murphy 1994). Glycosides of isoflavones are very poorly hydrolysed by mammalian intestinal digestive enzymes. Isoflavone glycosides are not favorable for absorption in the small intestine compared with their aglycones because of the higher hydrophilicity and greater molecular weight of the glycosides (Brown 1988). Average amounts of urinary isoflavones in both studies were 20-35% of ingested doses (**Tables 7, 11**), which were much greater than the amount of isoflavone aglycones in cooked soybean and TVP, and did not vary with the varying content of aglycones among soy foods. This supports gut microfloral hydrolysis of glycosides to their isoflavone aglycones.

Human intestinal bacteria especially, *Lactobacilli*, *Bacteroides* and *Bifidobacteria*, possess β-glucosidase activity (Hawksworth et al. 1971). They may play a major role in liberating isoflavone aglycones and promoting isoflavone absorption. From proximal to distal small intestine, the amount of bacteria is increased from very low bacterial numbers in the upper 1/3 of small intestine to almost "colon-like" resident flora in the lower 1/3 of small intestine (Heneghan 1988). Contents of both distal small intestine and large intestine in humans have substantial amount of *Lactobacilli*, *Bacteroides* and *Bifidobacteria*, and the number of each of three bacterium groups is about 10^4-10^7 bacteria per gram wet weight (Gorbach et al. 1967 and Mitsuoka 1982), whereas, contents of the human proximal small intestine contain almost none of these bacteria (Mitsuoka 1982). In addition, some bacteria in human large intestine also have β-glucuronidase and arylsulfatase which can liberate aglycones

from conjugates derived from tissue phase II biotransformation (Sipes and Gandolfi 1991). This may facilitate reabsorption of isoflavones.

Furthermore, the average length of time during which material stays in the intestine is about 5 min in the duodenum, 2 h in the jejunum, 3-6 h in the ileum, and 24 h or longer in the large intestine (Welling 1986). Bacteria in distal small intestine and colon have much longer time to interact with isoflavones and perform their hydrolysis activities.

In healthy human volunteers dietary fat absorption is practically completed in proximal small intestine after a meal containing 75 g of fat (Linscheer and Vergroesen 1988). Fat might facilitate the absorption of isoflavone aglycones in proximal small intestine. However, free forms of isoflavone from soy milk powder were only 2-5% of ingested doses. Therefore, it is very unlikely that more than 95% of soy milk isoflavones will be absorbed along with dietary fat. In another study, seven healthy women were randomly assigned to low or high fat diet (20% or 40% of Kcalories from fat, respectively) containing a single dose of 0.9 mg isoflavones/ kg body wt from tofu or texturized vegetable protein for one day. Bioavailability of isoflavones as reflected on their 24 h urinary recoveries was not affect by the level of dietary fat intake (Tew et al., submitted). In this study, although the amount of fat intake from self-selected or ad libitum diet was 30%-50% greater than that from the controlled basic foods diet, it did not alter isoflavone bioavailability among the three diets.

Fat might affect bioavailability of isoflavones in relatively long-term feeding studies. After rats were fed a diet containing 12% corn oil for 4 weeks, the activity of phenol uridine diphosphate-glucuronyltransferase, a key enzyme for isoflavone biotransformation was significantly enhanced

(Nanbo et al. 1993). This can further alter isoflavone disposition in humans. In addition, dietary fat and protein might influence bioavailability of isoflavones through their effect on gut microflora. In a rat study, β-glucosidase and β-glucuronidase activities of caecal bacteria were increased with increasing dietary protein intake from 0% to 40% of diet for 10 days (Wise et al. 1983). Rats fed a diet containing 35% beef fat or olive oil in purified diet for 30 days can significantly decrease their total caecum bacterial β-glucosidase activity and increase total caecum bacteria β-glucuronidase activity by two fold in comparison to low fat diet containing 1% of safflower oil (Mallett et al. 1984). It is very possible that dietary fat and protein intake might also affect these enzyme activities of human gut bacteria. Since these bacterial enzymes are also important for isoflavone absorption and reabsorption, dietary effect on isoflavone bioavailability may be expected. In our study, differences in dietary protein and fat intake among three regimens had no effect upon isoflavone bioavailability after only one day feeding.

Dietary insoluble fiber can bind to estrogens, especially, their aglycones in vitro (Whitten and Shultz 1988). Because of structural similarity of isoflavones and estrogens, dietary water-insoluble fiber may also bind to free form isoflavones in intestine and decrease their absorption. Furthermore, dietary insoluble fiber can hold water and cause bulking effect of intestinal content in low bowel (Adlercreutz 1984). This may reduce bacterium enzyme activities such as β-glucosidase and β-glucuronidase, withine the same volume by dilution and prevent isoflavones from absorption and reabsorption. In another study conduct by our laboratory, seven healthy female human subjects were randomly assigned in a crossover design to a control diet or a wheat fiber supplemented diet (contain 15 g and 40 g of dietary fiber,

respectively). Each diet contained a single dose of 0.9 mg isoflavones/kg body wt from tofu or texturized vegetable protein. At 24 h after soy dosing plasma concentration of genistein in fiber rich diet groups was two fold lower than that in control diet. In addition, total 24 h urinary genistein in wheat fiber supplemented diet was 20% lower than that in control diet (Tew et al. b, in press). In this study, dietary intake of insoluble fiber was not significantly different among three diet selections. This may be one of main reasons why there was no significant difference in bioavailability reflected on 48 h urinary recoveries of isoflavones.

From experiment one, it was concluded that human absorption, excretion and plasma concentration of isoflavones do not depend upon diet selections with different amounts of fat and protein but similar amounts of dietary fiber. This suggests that in future studies, ad libitum diets may be appropriate in short-term isoflavone feeding studies in humans.

Although the four soy foods studied contained various amounts of isoflavone glycosidic conjugates and aglycones, urinary recoveries of both daidzein and genistein were not significantly different among four soy food treatments (**Table 11**). Almond β-glycosidases can hydrolyze the β-glycosidic bond between the carbohydrate moiety and the isoflavonoid nucleus regardless of the difference in carbohydrate group (Farmakalidis and Murphy 1985). If β-glucosidase of gut bacteria act similarly to almond β-glycosidase, urinary recoveries of daidzein and genistein may not be affected by differences in isoflavone glycoside forms in soybean and soy foods. Our data do support this concept.

Four soybean foods provided similar human bioavailability. Average isoflavone urinary recovery of daidzein was about 10 - 15 % higher than that of soymilk.

These certainly suggested that common soybean foods might provide more daidzein than genistein and further investigation of biological activity of daidzein might be important.

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Table 1. Experiment 1: Design[1]

Subject				Feeding 1 (1 day)	Washout (1 week)	Feeding 2 (1 day)	Washout (1 week)	Feeding 3 (1 day)
No.	Age	Weight (kg)	Body mass index (kg/m²)	Dietary Assignment		Dietary Assignment		Dietary Assignment
1	20	59.1	20.45	BF		AL		SS
2	21	60.0	20.14	SS		BF		AL
3	41	84.4	26.90	BF		SS		AL
4	29	51.1	21.71	BF		AL		SS
5	22	54.4	20.08	SS		AL		BF
6	21	52.2	19.93	AL		BF		SS
7	20	56.7	21.49	AL		SS		BF
8	23	48.9	20.76	SS		BF		AL
$\bar{x}$	24	58.35	21.43					
SD	6.4	10.49	2.15					

[1]All subjects were fed 0.9 mg isoflavones/kg body wt in three meals for one day. BF = timed basic foods diet, SS = timed self-selected foods diet, AL = Ad Libitum diet.

Table 2. Basic foods diet

Food	Approximate time	Portion size
Toasted plain bagel	7:30 AM	1 medium (3" diameter)
Grape jam	7:30 AM	1 Tbsp. (0.7 oz.)
Unsweetened orange juice	7:30 AM	1 Cup (8 fl. oz.)
Soybean milk		
Sandwich	12:30 AM	
White bread		2 slices (3.6 oz.)
Swiss cheese		1 oz.
Mustard		
Apple	12:30 AM	1 medium (4.9 oz.)
Pringles's chip (regular)	12:30 AM	15 (1.05 oz.)
Water	12:30 AM	250 ml
Sandwich	5:30 PM	
White bread		2 slices (3.6 oz.)
Colby Jack cheese		1 oz.
Mustard		
Tostitos tortilla chips (regular)	5:30 PM	15 (0.9 oz.)
Unsweetened orange juice	5:30 PM	1 Cup (8 fl. oz.)
Teddy Grahams-honey	7:30 PM	22 (1 oz.)
Apple	7:30 PM	1 medium (4.9 oz.)

Table 3. Experiment 2: Design[1] [2]

Subject				Feeding 1 (1 day)	Feeding 2 (1 day)	Feeding 3 (1 day)	Feeding 4 (1 day)
No.	Age	Weight (kg)	Body Mass index (kg/m^2)	Soy Food	Soy Food	Soy Food	Soy Food
1	20	59.1	20.45	CS	TVP	TF	TP
2	24	50.9	20.13	CS	*	TVP	TP
3	27	61.4	21.46	*	TVP	CS	TP
4	32	70.9	24.31	*	*	CS	TVP
5	20	55.9	21.32	*	*	CS	TVP
6	23	50.9	19.87	TVP	CS	TP	TF
7	25	55.9	22.10	TVP	CS	TP	TF
8	32	63.6	22.12	*	*	TVP	CS
9	35	63.6	21.56	*	TVP	TF	CS
10	34	63.6	22.68	*	*	TVP	CS
$\bar{x}$	24	58.35	21.43				
SD	6.4	10.49	2.15				

[1]CS= cooked soybean, TVP= texturized vegetable protein, TF= tofu, TP= tempeh. Data of subject with * was not used because consumed soybean foods were not available for isoflavone analysis.

[2]There was one week washout period between feedings.

Table 4. Controlled diet of Experiment 2

Food	Approximate time	Portion size
Breakfast casserole	7:30 AM	
Soy foods		
1 large egg		
Kraft sharp cheddar cheese		40 g
Pace thick & chunky salsa		70 g
White sandwich bread		1 slice
Tropicana unsweetened orange juice		8 fl. oz.
distilled water		250 ml
Sandwich	12:30 AM	
White bread		2 slices (3.6 oz.)
Swiss cheese		1 oz.
Mustard		
Apple		1 medium (4.9 oz.)
Pringles's chips (regular)		20 (40 g)
Distilled water		250 ml
Sandwich	5:30 PM	
White bread		2 slices (3.6 oz.)
Colby Jack cheese		1 oz.
Mustard		
Tostitos tortilla chips (regular)	5:30 PM	17 (45 g)
Unsweetened orange juice	5:30 PM	8 fl. oz.
Teddy Grahams (honey flavor)	7:30 PM	40 g
Apple	7:30 PM	1 medium (4.9 oz.)

Table 5. Soy Food Analysis

Soy Foods	Isoflavone Concentration (mg/g)	& of Daidzein	% of Genistein
Soy milk powder	1.25±0.08	44.0	56.0
Tofu	0.46±0.09	42.6	57.4
Tempeh	0.64±0.02	39.7	60.3
Texturized Vegetable Protein	0.42±0.01	46.4	53.6
Cooked Soybean	0.58±0.02	46.4	53.6

Table 6. Nutritional Analysis of Basic Food Diet, Self-Selected Diet and Ad Lib Diet[1,2]

	Basic Food (n=8)	Self-Selected (n=8)	Ad Lib (n=8)
Kilocalories	1895 ± 18.97	1986 ± 34.67	2212 ± 32.14
Protein (g)	58.2 ± 3.12[a]	81.34 ± 6.78[b]	84.95 ± 7.91[b]
Carbohydrate(g)	326.1 ± 2.28	294.5 ± 7.10	347.3 ± 5.89
Total Fat (g)	44.06 ± 1.63[a]	56.93 ± 5.26[b]	62.99 ± 6.31[b]
Cholesterol(mg)	54.00 ± 0.00[a]	127.4 ± 7.43[b]	118.4 ± 5.82[b]
Saturated Fat(g)	15.17 ± 0.28[a]	23.58 ± 1.56[b]	25.36 ± 1.90[b]
Monounsaurated Fat(g)	22.81 ± 0.63[a]	26.89 ± 3.17[b]	30.67 ± 2.69[c]
Soluble Fiber(g)	2.47 ± 0.06	2.70 ± 0.68	2.47 ± 0.77
Insoluble Fiber(g)	9.20 ± 0.26	10.15 ± 2.87	9.69 ± 1.18

[1]Means and SD are presented in the table. Values bearing different letters in a row are significantly different ($p<0.05$). ANOVA were used for comparison of means. A p value of 0.05 or less was considered to be significant.

[2]Nutritional analysis was performed with Nutritionist IV version 2.0 (N-SQUARED COMPUTING, Salem, OR).

Table 7. Isoflavone excretion under three diet selection[1]

Isoflavones	Diet selection		
	Basic foods	Self-selected foods	Ad lid foods
Intake			
Daidzein(mg)	88.23 ± 15.87	88.23 ± 15.87	88.23 ± 15.87
Genistein(mg)	69.31 ± 12.47	69.31 ± 12.47	69.31 ± 12.47
Total intake(mg)	157.54 ± 28.34	157.54 ± 28.34	157.54 ± 28.34
Urinary excretion(mg)[2]	38.80 ± 11.72	35.30 ± 10.43	35.38 ± 9.17
Daidzein recovery (%)[3]	27.25 ± 7.58	25.68 ± 10.84	26.30 ± 8.63
Genistein recovery (%)	20.41 ± 10.18	18.23 ± 9.38	17.57 ± 8.40
Fecal excretion(mg)[4]	4.37 ± 3.95	4.48 ± 3.12	3.96 ± 3.08
% Isoflavone excreted in urine and feces	27.40 ± 9.64	25.25 ± 9.45	24.97 ± 8.72

[1]Values are means and standard deviations; n=8; mg/kg = mg of isoflavones per kg body weight. BF = basic foods, SS = self-select foods, AL = Ad Lib foods.

[2]Urine samples included all the urine in the first 24 hours after dosing and the first urination of the second day.

[3]The urinary recovery of daidzein was significantly greater than that of genistein in each diet selection ($p<0.05$).

[4]Fecal samples included all feces excreted before and during fecal marker excretion.

Table 8. Urinary excretion of isoflavones under three diet selection[1]

Isoflavone	Time "0"	0-12 Hours	12-24 Hours	24-36 Hours	36-48 Hours	>48 Hours
			mg			
Basic foods						
Daidzein	0.02±0.02	10.24±3.00	10.13± 7.06	2.41±1.28	0.91±0.89	0.18±0.34
Genistein	ND	5.02±2.32	5.56± 4.75	1.99±2.09	1.12±1.76	0.34±0.46
Total	0.02±0.02	15.26±5.32	15.69±11.81	4.40±3.37	2.03±2.65	0.52±0.80
Self-selected foods						
Daidzein	0.14±0.26	9.47±1.88	9.47± 4.25	2.77±3.55	0.66±0.78	0.09±0.11
Genistein	0.02±0.06	3.69±1.43	4.61± 3.17	3.19±5.01	0.98±1.95	0.14±0.30
Total	0.16±0.32	13.16±3.31	14.08± 7.42	5.96±8.56	1.64±2.73	0.23±0.41
Ad lid foods						
Daidzein	0.02±0.05	9.77±3.17	9.71± 4.29	2.53±2.04	1.04±0.92	0.12±0.13
Genistein	0.03±0.07	4.21±2.62	4.22± 5.44	2.45±2.86	1.13±1.59	0.14±0.21
Total	0.05±0.12	13.98±5.79	13.93± 9.73	4.98±4.90	2.17±2.51	0.26±0.34

[1]Values are means and standard deviations; mg/kg = mg of isoflavones per kg body weight; ND = Not detectable, at a detection limit of 2 ng per 20 µL injected.

Table 9. Plasma concentration of isoflavones under three diet selection[1]

Isoflavone	Time "0"	6.5 Hours	24 Hours
		µg/mL	
Basic foods			
Daidzein	ND	0.68±0.31	0.32±0.22
Genistein	ND	0.70±0.29	0.38±0.30
Total	ND	1.38±0.60	0.70±0.51
Self-selected foods			
Daidzein	ND	0.61±0.24	0.36±0.22
Genistein	ND	0.62±0.21	0.36±0.24
Total	ND	1.23±0.45	0.72±0.46
Ad lid foods			
Daidzein	ND	0.63±0.22	0.35±0.17
Genistein	ND	0.64±0.18	0.37±0.21
Total	ND	1.27±0.51	0.72±0.38

[1]Values are means and standard deviations; n=8; mg/kg = mg of isoflavones per kg body weight; ND = Not detectable, at a detection limit of 2 ng per 20 µL injected. Subjects were given 0.9 mg/kg body weight isoflavones in three meals.

Table 10. Nutritional Analysis of soybean foods Diet[12]

	Cooked soybean (n=10)	Texturized vegetable protein (n=10)	Tofu (n=5)	Tempeh (n=4)
Kilocalories	2156.86 ± 20.49	2315.71 ± 76.82	2165.25 ± 10.27	2136.54 ±22.10
Protein(g)	76.55 ± 2.02[b]	118.12 ± 14.57[a]	78.58 ± 2.09[b]	74.90 ± 2.21[b]
Carbohydrate(g)	296.91 ± 1.18	289.00 ± 0.00	292.90 ± 0.29	294.16 ± 0.98
Total Fat(g)	80.72 ± 1.07	74.69 ± 0.21	76.40 ± 0.68	79.42 ± 1.12
Cholesterol(mg)	307.00 ± 0.00	307.00 ± 0.00	307.00 ± 0.00	307.00 ± 0.00
Saturated fat(g)	30.13 ± 0.15	29.10 ± 0.00	30.30 ± 0.10	29.10 ± 0.00
Total fiber(g)	21.78 ± 0.52	20.77 ± 0.68	19.85 ± 0.08	20.52 ± 0.42

[1]Means and SD are presented in the table. Values bearing different letters in a row are significantly different ($p<0.05$). ANOVA were used for comparison of means. A p value of 0.05 or less was considered to be significant.

[2]Nutritional analysis was performed with Nutritionist IV version 2.0 (N-SQUARED COMPUTING, Salem, OR).

Table 11. Isoflavone excretion after feeding soybean foods[1]

	Soybean foods			
	Cooked soybean (n=10)	Texturized vegetable protein (n=10)	Tofu (n=5)	Tempeh (n=4)
Intake				
Daidzein(mg)	20.38 ± 1.84	27.75 ± 4.16	36.76 ± 9.43	21.77 ± 9.91
Genistein(mg)	24.02 ± 2.41	32.05 ± 4.34	42.82 ± 12.47	29.57 ± 10.22
Total intake(mg)	44.40 ± 3.23	59.80 ± 8.44	79.58 ± 21.89	51.34 ± 20.12
Urinary recovery(%)[2,3]				
Daidzein	45.45 ±15.90[a]	50.66 ± 10.17[a]	50.12 ± 10.02[a]	37.99 ± 18.90[a]
Genistein	12.75 ± 6.30[b]	13.45 ± 7.66[b]	16.07 ± 5.33[b]	8.51 ± 3.86[b]
Fecal excretion(mg)[4]	1.02 ± 0.66	1.11 ± 0.32	1.09 ± 0.24	1.20 ± 0.23
% Isoflavone excreted in urine and feces	30.27 ± 11.54	32.75 ± 8.89	34.01 ± 6.72	21.31 ± 9.83

[1]Values are means and standard deviations.
[2]Urine samples included all the urine in the first 24 h after dosing and the first urination of the second day.
[3]Values bearing different letters in column are significantly different from each other ($p<0.01$).
[4]Fecal samples included all feces excreted before and during fecal marker excretion.

Table 12. Plasma concentration of isoflavones after feeding soybean foods[1]

Soy foods	Isoflavone	Dosage (mg/kg)	Time "0"	6.5 Hours	24 Hours
			µg/ml		
Cooked soybean (n=10)	Daidzein	0.35 ± 0.03	ND	0.25 ± 0.09	0.03 ± 0.00
	Genistein	0.40 ± 0.02	ND	0.29 ± 0.09	0.03 ± 0.01
	Total	0.75 ± 0.04	ND	0.55 ± 0.18	0.06 ± 0.01
Texturized vegetable protein (n=10)	Daidzein	0.46 ± 0.03	ND	0.35 ± 0.12	0.04 ± 0.02
	Genistein	0.54 ± 0.03	ND	0.27 ± 0.06	0.06 ± 0.07
	Total	1.00 ± 0.05	ND	0.62 ± 0.17	0.10 ± 0.09
Tofu (n=4)	Daidzein	0.63 ± 0.12	ND	0.38 ± 0.15	0.04 ± 0.01
	Genistein	0.81 ± 0.17	ND	0.37 ± 0.10	0.04 ± 0.02
	Total	1.44 ± 0.21	ND	0.75 ± 0.25	0.08 ± 0.03
Tempeh (n=5)	Daidzein	0.39 ± 0.15	ND	0.28 ± 0.07	0.04 ± 0.01
	Genistein	0.53 ± 0.14	ND	0.28 ± 0.06	0.04 ± 0.03
	Total	0.92 ± 0.18	ND	0.56 ± 0.12	0.08 ± 0.04

[1]Values are means and standard deviations; mg/kg = mg of isoflavones per kg body weight; ND = Not detectable, at a detection limit of 2 ng per 20 µL injected.

IV. HUMAN ISOFLAVONE BIOAVAILABILITY AND NATURAL KILLER CELL ACTIVITY AFTER SEVEN DAY SOYMILK FEEDING [1]

A paper to be submitted to the Journal of Nutrition

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Abstract

To characterize the relationship between bioavailability of soybean isoflavones and a short-term indicator of their potential health protective effects in humans, eight women and eight men, ages 20-28, were fed 0, 0.8 or 1.6 mg isoflavones/kg body wt from soymilk in breakfast for seven days in a randomized cross-over design. Each subject consumed an ad libitum diet with the soymilk. There was a one-week washout period between soymilk doses. In each feeding week plasma samples were taken in the morning before the first soymilk dose, and 6.5 h after the last soymilk ingestion. Twenty-four h urine collections were conducted on the last two days of each feeding period. Fecal samples were collected over the last two days of each feeding week until fecal marker no longer appeared in feces. Plasma, urine and fecal isoflavones were measured by reverse-phase HPLC. Peripheral blood natural killer cell activities were also measured. Plasma concentrations of isoflavones were dose-dependent after soymilk feeding, however, blood natural killer cell activities were not affected by increasing of soy doses and plasma isoflavone concentrations ($p>0.1$). Plasma

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daidzein concentration in males was significantly greater than that in females after soymilk dosing ($p<0.01$). In all subjects, urinary excretion of isoflavones was nearly the same on day 6 and 7. Urinary daidzein was about two-fold greater than genistein, 10-13 times greater than equol on both feeding days. These data suggest that human disposition of daidzein after an oral dose differs between males and females, however, daidzein, genistein and equol seem to be at steady state by six days of feeding in our human subjects. The effect of isoflavones upon natural killer cell activities need to be further investigated.

Introduction

Isoflavones, daidzin, genistin and their aglycones, daidzein and genistein are abundant in soy foods (0.2-1.5 mg/g) (Wang and Murphy 1994). Substantial soybean isoflavones intake has been implicated as one of the important factors responsible for lower incidence and mortality of breast, prostatic and colorectal cancers in eastern Asia (Adlercreutz et al., 1995). Mechanism of isoflavone health protective effects are the topic of much current research. Isoflavones are antioxidants and can enhance activities of antioxidant enzymes in vivo (Hendrich et al., 1994) and in vitro (Wei et al., 1995). They are weakly estrogenic and also antiestrogenic (Adlercreutz et al., 1991). Genistein and daidzein have demonstrated many anticarcinogenic activities in vitro (Barnes 1995, Fotsis et al. 1993, and Higashi and Ogawara 1992).

In addition to direct tumor inhibitory effect, flavonoids may perform health protective function through their ability to enhance natural killer activity (Wiltrout and Hornung, 1989). In a preliminary study, soy isoflavone extract was found to significantly increase the activity of liver associated natural killer cells after feeding 240 mg

isoflavones/kg diet for 10 days (Cunnick and Hendrich, unpublished data). Natural killer activity plays an essential role in immune surveillance against tumor development and progression as well as infectious agents (Whiteside and Herberman, 1989). Isoflavones acting as antioxidants can enhance glutathione peroxidase activity (Hendrich et al., 1994) which constrains prostaglandin production (Marshall et al., 1988). Prostaglandins from monocytes inhibit blood natural killer activity in breast cancer patients (Baxevanis et al., 1993). On the other hand, isoflavones, especially, genistein is a specific inhibitor of tyrosine kinase. Since tyrosine kinase activity is crucial for the activation of natural killer cell, it is reasonable to hypothesize that soybean isoflavones can either enhance or inhibit natural killer activity, and natural killer cell activity varies with plasma isoflavone concentrations. The present soy milk human feeding study was conducted to investigate these hypotheses.

Our previous human studies demonstrate that bioavailability of soy isoflavones is dose dependent (Xu et al., 1994 and 1995). Subjects in those studies were only females and they received soy doses for no more than one day. In this study men and women received soy milk doses for seven days, and their isoflavone absorption and disposition pattern as well as their relationship with activities of natural killer cells were characterized.

Materials and methods

Subjects and Protocol.

The procedures for this feeding study were approved by the Human Subjects Committee of ISU. Informed consent of subjects was obtained in writing. All the subjects were omnivorous, and in good health, based on medical histories and physical examinations performed by Student Health Center physicians at Iowa State University (ISU). Subjects did not

take any medication such as antibiotics during the entire feeding studies.

The subjects were eight young adult women and eight young adult men between 20 and 28 years of age, with body weight of 63.0 ± 6.3 kg and 79.0 ± 9.4 kg for females and males, respectively, and body mass index of 22.8 ± 2.1 kg/m² and 22.6 ± 2.3 kg/m² for females and males, respectively.

The cross-over design experiment consisted of three seven-day feeding weeks, each separated by a one-week washout period. On each morning of the feeding week, every subject received 0, 0.8 or 1.6 mg isoflavones/kg body wt from soymilk powder (Now Foods, Inc., Glendale Hts.,IL) reconstituted with chocolate milk or 1% fat plain milk. Subjects ate an ad libitum diet during the study and recorded their food intake at last two days of each feeding. The subjects were also instructed not to eat any other soybean foods or foods containing texturized vegetable protein, hydrolyzed vegetable protein, and soy protein isolate during the entire study. A list of such foods was provided. Subjects were asked not to consume anything for 10 h before the first day of dosing. No adverse effects such as diarrhea were reported after soy food dosing.

Blood, Urine and Fecal Sample Collections.

Thirty mL venous blood samples were collected in heparinized vacuum containers by medical technologists under stringent aseptic condition. A blood sample was collected within one h before first dosing. Blood samples were also collected at 6.5 h after the last dosing. Ten ml of the sample was immediately processed for natural killer cell activity; another 10 ml sample was used for measurement of glutathione peroxidase activity and prostaglandin level (data not presented); the third 10 ml sample was centrifuged within one h after collection at 3000 × g for 20 minutes at 4°C

(Model 4D; International Equipment Co., Needham Hts., MA), and plasma was stored in a -20°C freezer.

All urine was collected and pooled in four 12 h increments over the last two days of feeding. The first urination of the eighth day was also collected. After recording the total volume, 50 mL of each of the five samples was stored in a -20°C freezer.

Capsules containing 1 g carmine red (Pharmaceutical Service, University of Iowa, Iowa City, IA) were given with the soymilk in the morning of last two days of feeding. All feces excreted before and during fecal marker excretion were collected. Fecal sample collection was stopped when the fecal marker no longer appeared in feces. After collecting the fecal samples, feces were freeze dried. After recording all the weights of dry samples, feces were ground to a fine powder in a coffee mill (Braun Company Inc., Lynnfield, MA). Ten g of each dry fecal sample was stored in a -20°C freezer until analysis.

Soy Milk Powder and Soy Foods Analysis.

The concentrations of twelve isoflavone isomers in soymilk powder were measured according to the method described by Wang and Murphy (1994).

Plasma, Urine and Fecal Sample Analysis.

Sample preparation for plasma and urine isoflavone analysis was performed according to the methods described by Lundh et al. (1988). Plasma and urine samples were treated with glucuronidase/sulfatase (Sigma Chemicals Company, St. Louis, MO) to produce the parent isoflavones. Chromatographic analysis followed the conditions described by Xu et al. (1994). Samples were automatically injected with a Spectra-Physics Autosampler Model 8780XR (Spectra-Physics, Fremont, CA). Isoflavones were separated in a Waters 3.9 mm i.d. × 30

cm length μ-Bondapak C_{18} reverse phase column with gradient elution at ambient temperature. Two Beckman Model 110B pumps,and one Beckman Model 420 Microprocessor solvent flow controller were used. Beckman Model 163 variable wavelength detector set at 254 nm was connected with Beckman Model 427 Integrator by which chromatographic data were calculated and plotted.

Daidzein (4',7-dihydroxyisoflavone) was obtained from Life Science Group, ICN Pharmaceuticals, Inc., Plainview, NY. Genistein (4',5,7-trihydroxyisoflavone) was obtained from Calbiochem Corporation, La Jolla, CA. Equol (4',7-dihydroxyisoflavondiol) was a generous gift from Dr. H. Adlercreutz (Department of Clinical Chemistry, University of Helsinki, Finland).

A series of standards of 0.25, 0.5, 1, 2, 3, 4, 6 μg/mL, were run to quantify isoflavones. For each subject, plasma and urine samples were spiked randomly with daidzein, genistein and equol standards (0.15 μg isoflavone/100 μL added to each sample) to measure recovery.

Fecal sample preparation and analysis were performed as described by Xu et al.(1994). To ensure the quality of analysis, a series of standards (0.25, 0.5, 1, 2, 3, 4, 6 μg/mL) and fecal samples spiked with 500 μL of 8 μg/mL daidzein, genistein and equol standards were run to measure recovery.

Natural Killer Activity Assay

The blood was diluted 1:1 with HBSS (GIBCO) and 4 ml layered over 3 ml of Ficol-hypaque (Pharmacia), and centrifuged for 20 min at 400 g. The interface were washed 3 times with HBSS, enumerated using the NOVA Cell-Track (NOVA Biochemical), and diluted to 10^6 cells/ml in complete media (2 mM L-glutamine, 5 mM HEPES, 50 μg/mL glutamine and 10 % FBS, BIBCO-BRL). The natural killer activity of isolated cell was

measured using a cell mediated cytotoxicity assay and ^{51}Cr-release from labeled targets (Cunnick et al., 1988). The target cells (K562) were labeled for 70 min by incubating 7.5 x 10^6 targets with 200 µCi of ^{51}Cr (as sterile aqueous sodium chromate, Dupont-NEN). The targets were washed 3 times to remove exogenous ^{51}Cr and incubated (37°C) for 30 min prior to the last wash to reduce spontaneous release during the assay.

The effectors and targets were plated in triplicate at 4 ratios of 50:1, 25:1, 12.5:1, and 6.25:1 in a total volume of 250 µl in 96 well plates. Controls consisted of wells containing 250 µl if media with targets alone (Spontaneous release) and wells containing 100 µl of targets (For Maximum release). The cultures were incubated for 4.5 h at 37°C in a humidified CO_2 (5%) incubator.

At the end of the incubation period 150 µl of 10% Trichloroacetic acid (TCA) was added to the maximum release control wells to lyse all targets. The plates were centrifuged for 5 min at 500 rpm, 100 µl of the supernate were removed from each well. The radioactivity of the samples were determined on a gamma-scintillation counter (Gamma-Track).

Statistical Methods

Analysis of variance (General Linear Model) were used. Subjects and feeding times were treated as blocks. Effects of gender, plasma concentration and type of isoflavones as well as subject and feeding time upon NK cell activity were determined. Linear regression model relating NK activity and effector to target ratio was estimated. Meanwhile, effects of gender, dosage and type of isoflavones as well as subject and feeding time upon plasma, urine and fecal isoflavone levels were also evaluated. If the effect from any one of these factors other than gender and type of isoflavones was significant, Tukey's test was used for comparison within the factor. The statistical analysis was performed with the

Statistical Analysis System (SAS Institute, Inc., Cary, NC) version 6.06 on the Iowa State University mainframe computer. A *P* value of 0.05 or less was considered to be significant.

Results

Men had greater amounts of total energy intake as well as protein, total fat, carbohydrate and insoluble fiber intake than those of women in each of last two days of feeding (**Table 1**).

Isoflavone concentrations in soymilk powder were presented in **Table 2**. Plasma concentrations of isoflavones were dose-dependent after soymilk feeding (**Table 3**). Plasma daidzein concentration in males was significantly greater than that in females after soymilk dosing ($P < 0.01$).

In both men and women blood natural killer cell activities after one week soy milk ingestion were not significantly affected by the isoflavone dosages, nor were plasma isoflavone concentrations ($P > 0.1$). Although average NK cell activities of male subjects were 20-25% greater than those of females after soy milk ingestion (**Table 4**), the difference between genders was not significant ($P > 0.1$) due to great overall variation among subjects. Natural killer cell activity of both genders at each dosage was decreased by the reducing of effector to target ratio in a approximate linear relationship ($r^2=0.78$).

Urinary excretion of isoflavones on day six of feeding was very close to that on day seven (**Table 5**). Male subjects excreted significantly greater amount of daidzein than female subjects did after soy milk dosing ($P < 0.01$). For both male and female subjects, urinary daidzein was about two fold greater than genistein in each of last two feeding days ($P < 0.01$). Only a small amount of equol, about one tenth to one thirteenth of the amount of daidzein, was found in urine on each day.

The amounts of fecal isoflavone excretion were not affected by sex or type of isoflavones ($P > 0.1$) (**Table 6**). Fecal isoflavone excretion was significantly increased with the isoflavone dosages ($P < 0.05$). There were great variations among subjects. One woman excreted about 7-8 times more isoflavones than other women. Two men had about two to three fold greater fecal isoflavone excretion than did other men at 1.6 mg /kg body wt dosage.

Discussion

This study extends our knowledge of soy isoflavone absorption and excretion to continuous soy milk feeding rather than single dose. As reported in our previous studies (Xu et al., 1994 and 1995), daidzein had much greater bioavailability than genistein, and fecal excretion of soy isoflavones was very low in most of our subjects. These are still true in this study (**Tables 3, 5** and **6**). Furthermore, similar to pattern we saw in previous study (Xu et al., 1995), one female subject who excreted 7-8 times more fecal isoflavones did have greater over urinary excretion of isoflavones, especially, genistein compared to subjects with close body weight in the same study.

At 6.5 h after seventh soy milk dosing in this study, average plasma concentrations of daidzein and genistein after 0.8 mg/ kg dosage were about 1.93 and 1.73 μM, respectively, and 2.96 and 2.71 μM, respectively, after 1.6 mg/ kg dosage (**Table 3**). Whereas, at 6.5 h after single soy milk dosing in previous study (Xu et al., 1994), average plasma concentrations of daidzein and genistein were 1.22 and 1.07 μM, respectively, after 1.3 mg/ kg dosage, and 2.24 and 2.15 μM, respectively, after 2.0 mg/ kg dosage. These differences suggested that continuous soy milk dosing in 24 h interval may still prolong and increase the presence of isoflavones in plasma compared to single soy milk dosing. This certainly

enhance the chance for isoflavones to exert their health protective effects through physiological amounts of soy food consumption.

Urinary excretion of isoflavones at day 6 and day 7 of continuous soy milk feeding were very close at each of two isoflavone dosages (**Table 5**). This suggested that absorption and excretion of isoflavones may already enter a steady state after six days of continuous soy milk dosing. In addition, equol was found in plasma, urine and feces at day 6 and day 7 of continuous soy milk feeding although it was only present in a small amount (**Tables 3, 5** and **6**). In previous single day soy milk feeding studies no equol was detected in plasma, urine and fecal samples (Xu et al., 1994 and 1995). This difference suggested that human subjects in our study may need several days of soy ingestion to achieve the induction of equol production by gut microflora.

Sex-related differences have been reported in the activity of liver sulfotransferases, but not UDP-glucuronosyltransferases, toward phenolic compounds in rats, with the activity of phenol sulfotransferase being two- to threefold higher in cytosol prepared from liver of adult male rats compared to females (Matsui and Watanabe, 1982; Singer et al., 1982). Consistent with these in vitro data, Meerman et al. (1987) reported that male rats excreted a significantly greater amount of phenol with higher percentage of them as phenolic sulfates and no difference in phenolic glucuronides compared to female rats following iv administration of 133 μmol phenol/ kg body wt. In our study males excreted about 80 % more isoflavones than females did on day 6 or 7 (**Table 5**). Plasma concentration of daidzein in male subjects was significantly greater than that of females (**Table 3**). This difference may be due to greater sulfotransferation of isoflavones in men, which promote their urinary isoflavone excretion, prohibit rapid distribution of absorbed isoflavones

into the tissues and maintain greater plasma concentration, especially, daidzein since daidzein is more hydrophilic than genistein. Study to compare urinary excretion of isoflavone sulfates and glucuronides between male and female subjects as well as sulfotransferation of daidzein and genistein will help to examine our hypothesis.

Isoflavones can have a profound impact on natural killer cell activity. NK activity is regulated in a negative manner by prostaglandins (Herberman, 1989) which are produced by many cells of the body including macrophages and neutrophils (Kunkle et al., 1984). Of particular interest is PGE_2 which inhibits T-cell production of IL-2 as well as NK cell activity (Baxevanis et al., 1993; Ohnishi et al., 1991). Several tumors have been shown to produce PGE_2 and are then able to down regulate the immune system and escape lysis (Earnest et al., 1992). Inhibitors of the cyclo-oxygenase pathway such as indomethacin are known to decrease PGE_2 synthesis (Earnest et al., 1992). This decrease in PGE_2 is associated with upregulation of IL-s production and NK activity (Baxevanis et al., 1993). Isoflavones as antioxidants can increase glutathione peroxidase activity (Hendrich et al., 1994) which inhibits prostaglandin production (Marshall et al., 1988), and may also enhance IL-s production and NK activity. Data from our glutathione peroxidase activity and prostaglandin assays will clarify the role of these factors in mediating isoflavone effects.

On the other hand, genistein can inhibit activation of NK cell by specifically blocking tyrosine kinase activity. NK cell activation is dependent upon the stimulation of cell surface receptors and proper regulation of intracellular second messengers. The activation of protein tyrosine kinase after the binding of NK receptors with IL-2, IFN-α, CD16, or tumor cell receptors is crucial step for NK cell activation (Borrego et al., 1993). Inhibitors of tyrosine kinase such as

genistein (at 20-110 μM) can inhibit NK cell activity (Gerosa et al., 1993; O'Shea et al., 1992). However, genistein plasma concentrations from physiologically achievable amounts of soy food consumption does not reach the inhibitory level based on extensive human bioavailability data collected by our laboratory. Our data from this study further indicated that genistein from physiological amount of soy food intake will not cause the suppression of NK activity in humans.

In addition to exogenous factors, some endogenous hormones such as glucocorticoids have many immunosuppressant effects, which can reduce the release of various interleukins and suppress NK activity (Hadley 1993). Because plasma glucocorticoid concentration varies diurnally, its level at 1400-1500 h when subjects had their post-feeding blood draw is about 100 % higher than that in early morning pre-feeding blood draw. This difference of glucocorticoid concentrations may be one of the reasons why there was no significant difference in NK activity before and after one week soy milk feedings. In addition, Nilsson and Carlsten (1994) reported that estradiol reduced NK cell cytotoxicity at ^{51}Cr- release assay with YAC-1 cells as target cells in a dose-dependent manner. Among males and females of six mouse strains, estrogen suppressed NK activities by 25-80% compared with sham-treated controls after subcutaneous implantation of 5-mm long siliac tube packed with 2.5 mg 17β-estradiol benzoate for 4 weeks. These may explain why female subjects had lower average pre- and post-feeding NK activities than male subjects although the difference was not statistically significant. Furthermore, although isoflavones may be antiestrogenic, they do not seem to be able to relieve the suppression of NK cell activity by endogenous estrogens in female subjects after seven days of soy milk consumption. Neither do isoflavones in the doses given down-regulate NK activity, even though they may inhibit tyrosine kinase activity. Further study is

essential to characterize the mechanism for modulation of NK cell activity by isoflavones.

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Table 1. Nutritional Analysis of Day 6 and 7 in Men and Women

	Day 6	Day 7
Female Control (n=8)		
Kilocalories	1887 ± 15.6	2115 ± 20.1
Protein (g)	84.4 ± 6.7	94.9 ± 7.9
Carbohydrate (g)	258.2 ± 5.0	292.3 ± 6.1
Total Fat (g)	53.8 ± 5.6	60.4 ± 4.9
Saturated Fat (g)	20.5 ± 2.8	23.0 ± 2.2
Monounsaturated Fat (g)	21.2 ± 3.1	25.5 ± 3.5
Polyunsaturated Fat (g)	10.4 ± 1.3	10.2 ± 1.2
Cholesterol (mg)	125.1 ± 8.5	123.0 ± 10.1
Dietary Fiber (g)	15.5 ± 2.0	17.3 ± 2.4
Insoluble Fiber (g)	10.0 ± 2.1	11.9 ± 3.0
Female 0.8 mg/kg (n=8)		
Kilocalories	2092 ± 18.9	2034 ± 15.1
Protein (g)	90.4 ± 7.2	87.9 ± 6.7
Carbohydrate (g)	310.1 ± 6.4	282.3 ± 5.3
Total Fat (g)	52.3 ± 5.1	60.1 ± 4.5
Saturated Fat (g)	19.5 ± 2.3	25.2 ± 2.4
Monounsaturated Fat (g)	20.2 ± 2.6	23.1 ± 3.1
Polyunsaturated Fat (g)	9.0 ± 0.9	10.0 ± 1.1
Cholesterol (mg)	130.9 ± 8.1	134.7 ± 8.9
Dietary Fiber (g)	16.2 ± 2.7	16.9 ± 2.1
Insoluble Fiber (g)	11.3 ± 2.5	10.8 ± 2.6
Female 1.6 mg/kg (n=8)		
Kilocalories	2007 ± 19.3	2022 ± 12.1
Protein (g)	90.0 ± 4.2	89.5 ± 3.7
Carbohydrate (g)	274.2 ± 5.3	285.7 ± 5.6
Total Fat (g)	60.7 ± 5.9	57.4 ± 4.0
Saturated Fat (g)	24.5 ± 3.0	21.7 ± 2.0
Monounsaturated Fat (g)	25.2 ± 3.4	23.5 ± 3.3
Polyunsaturated Fat (g)	9.4 ± 1.1	11.2 ± 1.5
Cholesterol (mg)	129.3 ± 8.9	119.2 ± 8.1
Dietary Fiber (g)	17.0 ± 2.9	18.5 ± 3.1
Insoluble Fiber (g)	10.4 ± 2.3	12.7 ± 3.3
Male Control (n=8)		
Kilocalories	2612 ± 27.0	2227 ± 18.4
Protein (g)	119.3 ± 10.3	100.5 ± 7.5
Carbohydrate (g)	337.1 ± 13.6	304.6 ± 8.9
Total Fat (g)	85.7 ± 10.3	65.1 ± 7.0

continued

continued, Table 1

Saturated Fat (g)	29.5 ± 4.4	22.1 ± 3.1
Monounsaturated Fat (g)	28.1 ± 5.1	25.9 ± 4.0
Polyunsaturated Fat (g)	23.5 ± 2.8	15.4 ± 1.5
Cholesterol (mg)	191.2 ± 9.1	182.4 ± 11.7
Dietary Fiber (g)	23.3 ± 4.0	20.5 ± 3.0
Insoluble Fiber (g)	17.3 ± 3.6	15.2 ± 2.9
Male 0.8 mg/kg (n=8)		
Kilocalories	2481 ± 22.6	2384 ± 20.6
Protein (g)	116.2 ± 9.1	102.4 ± 8.0
Carbohydrate (g)	327.5 ± 12.3	314.0 ± 9.4
Total Fat (g)	75.6 ± 8.0	72.0 ± 8.4
Saturated Fat (g)	26.5 ± 3.7	25.8 ± 3.3
Monounsaturated Fat (g)	25.2 ± 4.3	26.0 ± 4.2
Polyunsaturated Fat (g)	21.0 ± 3.2	18.3 ± 2.2
Cholesterol (mg)	188.5 ± 10.3	176.2 ± 9.6
Dietary Fiber (g)	22.7 ± 4.2	21.9 ± 3.5
Insoluble Fiber (g)	16.1 ± 3.3	14.8 ± 2.1
Male 1.6 mg/kg (n=8)		
Kilocalories	2504 ± 24.5	2352 ± 19.3
Protein (g)	110.0 ± 8.5	107.4 ± 8.1
Carbohydrate (g)	366.9 ± 15.2	324.2 ± 10.6
Total Fat (g)	66.1 ± 7.1	69.3 ± 7.0
Saturated Fat (g)	24.9 ± 2.8	23.2 ± 3.0
Monounsaturated Fat (g)	23.3 ± 3.5	25.8 ± 3.8
Polyunsaturated Fat (g)	18.0 ± 2.8	17.4 ± 2.0
Cholesterol (mg)	174.6 ± 8.5	180.8 ± 9.9
Dietary Fiber (g)	25.4 ± 6.0	22.1 ± 3.8
Insoluble Fiber (g)	18.2 ± 5.2	15.9 ± 2.5

Table 2. Isoflavone content in soymilk powder[1]

	µg/g
Daidzin	559
Genistin	1000
Glycitin	59
Mal Daidzin	176
Mal Genistin	625
Mal Glycitin	62
Ac Daidzin	247
Ac Genistin	497
Ac Glycitin	55
Daidzein	5
Genistein	16
Glycitein	ND
Total Daidzein	572
Total Genistein	1250
Total Glycitein	102

[1]Values were the means (n=3). ND = Not Detectable.

Table 3. Plasma concentrations of isoflavones 6.5 h after 7th day soy milk feeding

Dosages	0 mg/kg	0.8 mg/kg	1.6 mg/kg
		μM	
Female (n=8)			
Daidzein	0.08±0.04	1.33±0.44	2.36±1.15
Genistein	0.04±0.04	1.48±0.49	2.47±0.91
Equol	ND[2]	0.16±0.12	0.27±0.12
Male (n=8)			
Daidzein	0.04±0.02	2.52±0.69[1]	3.56±0.79[1]
Genistein	ND	1.98±0.74	2.94±0.80
Equol	ND	0.16±0.11	0.33±0.21

[1]Significantly greater than that in females ($p<0.01$) by using ANOVA (GLM) with mean square for subjects within the same gender as the error term.

[2]ND= Not detectable.

Table 4. Natural killer cell activity at pre- and post- feeding

	Pre-feeding	Post-feeding
Effector to target ratio = 50:1		
0 mg/kg		
Female (n=8)	37.6 ± 5.4	44.8 ± 7.3
Male (n=8)	44.0 ± 5.3	53.1 ± 7.5
0.8 mg/kg		
Female (n=8)	41.0 ± 7.2	41.8 ± 7.4
Male (n=8)	47.1 ± 4.0	52.3 ± 7.2
1.6 mg/kg		
Female (n=8)	40.8 ± 7.5	41.9 ± 7.4
Male (n=8)	41.7 ± 6.7	53.4 ± 7.8
Effector to target ratio = 25:1		
0 mg/kg		
Female (n=8)	23.0 ± 5.1	29.6 ± 6.7
Male (n=8)	29.2 ± 6.7	38.5 ± 7.8
0.8 mg/kg		
Female (n=8)	26.9 ± 9.4	30.2 ± 8.4
Male (n=8)	29.5 ± 5.1	36.1 ± 7.0
1.6 mg/kg		
Female (n=8)	28.3 ± 7.0	32.5 ±10.0
Male (n=8)	26.4 ± 7.1	38.1 ± 9.2
Effector to target ratio = 12.5:1		
0 mg/kg		
Female (n=8)	12.2 ± 9.9	16.9 ±12.0
Male (n=8)	18.4 ±15.4	21.1 ±12.0
0.8 mg/kg		
Female (n=8)	17.6 ±15.2	18.5 ±15.7
Male (n=8)	16.5 ±11.8	17.9 ± 9.2
1.6 mg/kg		
Female (n=8)	15.2 ±13.8	18.6 ±17.8
Male (n=8)	16.2 ±14.4	20.8 ±12.2

Table 5. Comparison of Urinary Excretion of Isoflavones on the Last Two Days of Feedings

	Day Six			Day Seven		
Dosages	0 mg/kg	0.8mg/kg	1.6 mg/kg	0 mg/kg	0.8 mg/kg	1.6 mg/kg
			mg			
Female (n=8)[2]						
Daidzein	0.30±0.38	7.92±2.34	15.36±3.85	0.16±0.15	6.87±2.42	14.91±5.39
Genistein	0.04±0.05	3.22±1.55	6.99±2.38	0.11±0.10	3.11±1.61	7.53±2.77
Equol	ND	0.54±0.49	1.01±0.75	ND	0.65±0.56	1.13±0.80
Male (n=8)[2]						
Daidzein	0.16±0.19	13.19±4.57[1]	24.04±4.22[1]	0.12±0.10	12.95±3.51[1]	24.18±7.57[1]
Genistein	0.02±0.06	5.30±2.95	8.98±3.66	0.14±0.15	5.43±2.87	8.79±4.11
Equol	ND	0.71±0.50	1.25±0.63	ND	0.76±0.42	1.20±0.74

[1]Significantly greater than that in females ($p<0.01$) by using ANOVA (GLM) with mean square for subjects within the same gender as the error term.

[2]For both male and female subjects, urinary daidzein was significantly greater than genistein and equol in each of last two soy feeding days ($p<0.01$).

Table 6. Total fecal isoflavone excretion at last two days of one week soy milk feeding[1]

Dosages	0 mg/kg	0.8 mg/kg	1.6 mg/kg
		mg	
Female (n=8)			
Daidzein	0.03±0.04	1.44±1.00	3.02±1.80
Genistein	0.03±0.07	1.28±0.89	3.03±2.76
Equol	ND	0.34±0.20	0.49±0.31
Male (n=8)			
Daidzein	0.03±0.04	1.45±0.43	3.12±1.28
Genistein	0.03±0.04	1.18±0.36	3.17±1.35
Equol	ND	0.29±0.19	0.51±0.28

[1]The amounts of fecal isoflavone excretion were not affected by sex or type of isoflavones ($p>0.1$) but the dosage ($p<0.05$) in ANOVA (GLM).

SUMMARY

Our studies indicated that human absorption and excretion of isoflavones from soybean and soy foods are dose-dependent. Daidzein is a more bioavailable soy isoflavone than is genistein as reflected in urinary recovery of ingested isoflavone.

Gut bacteria play a important role in determining the pattern and magnitude of soy isoflavone absorption and degradation. Modulation of gut bacteria may provide additional means to optimize soy isoflavone bioavailability and potential health protective effects.

Variation of protein and fat content in background diets had little effect on isoflavone bioavailability reflected in their urinary recovery. Different soybean foods containing various amounts of isoflavone conjugates and aglycones did not change human bioavailability pattern and magnitude. These results suggested that ad libitum diet may be appropriate in the future short-term human soy isoflavone feeding studies.

Isoflavones can reach steady state in shorter than five days of continuous soy milk feeding. Disposition pattern of isoflavones in this one-week feeding study showed sex-related difference with greater amounts of urinary excretion and higher plasma concentration of daidzein in male subjects than in female subjects. There was no significant difference in NK activities between different sex, dosages as well as pre- and post-soy milk feeding. Further well-controlled experiments are essential to prove the hypothesis that isoflavones can increase NK activity by inhibition of prostaglandin synthesis in monocytes.

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