Bioavailability and biological effects of the isoflavone glycitein and isoflavone glucuronides: role of glucuronide in human natural killer cell modulation in vitro

Yan Zhang
Iowa State University

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Bioavailability and biological effects of the isoflavone glycine and isoflavone glucuronides: role of glucuronide in human natural killer cell modulation in vitro

by

Yan Zhang

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

DOCTOR OF PHILOSOPHY

Major: Toxicology
Major Professor: Suzanne Hendrich

Iowa State university
Ames, Iowa
2000

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ABSTRACT

Lower incidence of breast cancer in Asia has been connected to the higher intake of isoflavone-containing soy foods. The effort to study soy isoflavones as health protective compounds has come to some positive results, like their effects in cancer prevention, lowering blood cholesterol level and osteoporosis prevention. In order to characterize the bioavailability of the third major isoflavone glycitein and to assess its potential health-protective effects, a comparison study with other isoflavones was conducted in humans with moderate fecal isoflavone degradation activity. The average 48 h urinary excretions of daidzein, glycitein and genistein were 50, 45 and 35% of the total dose ingested, respectively, at which daidzein and glycitein were not different from each other but they were significantly greater than genistein \( (p = 0.01) \). Plasma isoflavone concentrations paralleled relative amounts of isoflavones in soymilk at 6 and 24 h but not in soygerm in women at 6 h.

To characterize soy isoflavone metabolism, three feeding protocols were conducted in women. By using selective hydrolysis enzymes, the distribution of glucuronide and aglycone isoflavones in both urine and plasma were obtained. At 3 h after soymilk intake, 59.4% of total daidzein and 50.9% of total genistein appeared in plasma as glucuronides. About 76.8% of total daidzein and 70.9% of total genistein presented in urine (24 h) were glucuronides. Significantly higher percentages of aglycone appeared in plasma than in urine. A steady state was observed with six consecutive days feeding. As the major metabolite of isoflavone, the glucuronide forms might contribute to observed effects of soy isoflavones.

The major metabolites of daidzein and genistein glucuronides were assayed for their ability to modulate human natural killer (NK) cells and NK3.3 cell line alone or in
combination with aglycone daidzein and genistein in nutritionally relevant concentrations. Daidzein and genistein glucuronides were as active as their aglycone counterparts but activated human NK cells in a wider range (up to 10 \( \mu \text{mol/L} \)) than aglycone isoflavones (below 5 \( \mu \text{mol/L} \)). No activation was observed with NK3.3 cell line for both daidzein and genistein. These data suggested that soy isoflavone metabolites indirectly activated human NK cells.
GENERAL INTRODUCTION

A. Introduction

Expanding scientific knowledge has supported the idea that foods play a vital role in overall health and well being. Phytochemicals are nonnutritive secondary plant metabolites present in relatively small amounts. To date, most research related to phytochemicals has focused on cancer prevention. At least a dozen distinct classes of nonnutrient phytochemicals in fruits and vegetables with demonstrated anticancer activity have been identified (Stemmetz and Potter, 1991). Soybeans contain several classes of phytochemicals, such as saponins, protease inhibitors, isoflavones and inositol hexaphosphate (phytates). The main forms of isoflavones found in soybean are daidzin, genistin, glycitin, malonyl- and acetyl-glucosides and their corresponding aglycones daidzein, genistein and glycitein. Consumption of soybean isoflavones has been associated with several health-enhancing properties such as lowering the rates of breast, colon and prostate cancers (Barnes et al., 1994); reducing blood cholesterol level and decreasing the risk of cardiovascular disease (Potter et al., 1998); prevention of osteoporosis and reduction of post-menopausal symptoms (Messina and Messina, 2000).

It was found that the content of the total isoflavones and different forms of isoflavone differ in soybeans based on source, variety and year (Wang and Murphy, 1994a). The bioavailability and biological activities of different forms of isoflavone also differ to some extent. Bioavailability refers to the portion of a substance absorbed that reaches its active sites. Bioavailability studies have been performed for daidzein and genistein. But the bioavailability of the third major isoflavone, glycine, has not been studied at all. To compare
glycitein bioavailability and metabolism with other isoflavones to begin to understand this compound is one of the objectives of my research.

After absorption, over 95% of the isoflavones appear as conjugated forms (mainly as glucuronides and less as sulfates) in the circulation in cow and sheep (Lundh, 1995). The percentage of conjugates in human urine and plasma was estimated to be over 90%. But no definite number is known because most of the studies used hydrolysis enzymes to obtain aglycones and all the results were reported as aglycones. Classically, conjugation has been considered as a detoxification step. But in some cases, after conjugation, the biological activities of the conjugates have been enhanced. The formation of aromatic amine glucuronides can cause bladder cancer (Thorgeirsson et al., 1983). Retinoyl β-glucuronide (RAG) and retinyl β-glucuronide (ROG) were more active than their parent compounds in inhibiting prolactin-induced DNA synthesis in organ cultures of mouse mammary glands (Olson et al., 1992). Daidzein and genistein glucuronides showed some biological activities in in vitro assays including weak estrogenicity and natural killer (NK) cell modulation activity (Zhang et al., 1999a). To our knowledge, research about isoflavones has focused on the biological activities of daidzein, genistein and equol. However, the concentrations that have been applied in many studies were very high, such as 0.15 mmol/L genistein used as a MCF-7 cell cycle arrest agent (Constantinou et al., 1998b). It will be crucial for us to know the amount and percentages of both aglycones and conjugated isoflavones in the circulation in order to understand isoflavone biological activities, especially their anticancer effects. To measure the amount of glucuronide as well as aglycone isoflavones in circulation after different feeding protocols is another objective of my research.
Natural killer (NK) cells are the third major class of lymphocytes after T and B cells and comprise 10-15% of the lymphocyte population in blood, liver, and especially in the spleen in humans. NK cells have nonspecific cytotoxicity and represent one of the first lines of immune defense against tumor cells and virally infected cells in the body. Previously we reported that genistein at concentrations of 0.1-5 μM, daidzein and genistein glucuronides at concentrations of 0.1-10 μM were able to activate NK cells (Zhang et al., 1999a). Based on our human feeding study results, daidzein, genistein and their glucuronides can all be detected in circulation. In order to best understand NK activation activity of isoflavones, it is our goal to investigate the effect of these four major isoflavone metabolites in combination on NK activity. Since previous studies of NK cells used were separated from peripheral blood (i.e. not pure NK cell populations), by using a NK cell line we can clarify the direct effects of isoflavones on NK cell function as well.

B. Dissertation organization

This dissertation consists of a literature review and three papers. The first paper, “Urinary disposition of the soybean isoflavones daidzein, genistein and glycitein differs among humans with moderate fecal isoflavone degradation activity” has been published in Journal of Nutrition. 129: 957-962. The second paper, “Glucuronides are the main isoflavone metabolites in women fed soy milk powder” and the third paper, “The combination of daidzein, genistein and their glucuronides activated human natural killer cells in nutritionally relevant concentrations” will be submitted to Journal of Nutrition. A general conclusion follows the three papers.
LITERATURE REVIEW

A. Plant and commercial food product sources of isoflavones

The flavonoids are a major class of phenylpropanoid-derived plant products. Their fifteen carbon (C_6-C_3-C_6) backbone can be arranged as a 1,3-diphenylpropane skeleton (flavonoid nucleus) or as a 1,2-diphenylpropane skeleton (isoflavonoid nucleus). The 1,3-diphenylpropane flavonoid derivatives are mainly found in terrestrial plants. The isoflavonoids are primarily restricted to the Leguminosae, rarely they can be found in other families such as Apocynaceae, Pinaceae, Compositae, and Moraceae (Dixon et al., 1999).

There are nearly 900 natural isoflavonoid aglycones, which can be divided into nine major classes based on differences in their basic carbon skeletons. The isoflavones and pterocarpans are the most abundant isoflavonoids, with 334 and 152 different structures, respectively. These diversities are caused by various substituents that occur on many different positions of the A and B rings (Fig. 1), such as methoxyl, prenyl and methylenedioxy.

Clover is one of the legume plants, consumed by animals, especially sheep, that contains relatively high amount of isoflavones. Isoflavones are high in subterranean clover, for example, the Yaloop cultivars contained 1.5, 2.8 and 0.5% of dry matter as formononetin, genistein and biochanin A, respectively. In red clover, and relatively less in white clover, such as the red clover Ladino cultivar contained about 0.02, 0.01 and 0.02% of dry matter as formononetin, genistein and biochanin A, respectively (Cheeke, 1985). The amount that is present in clover depends upon many factors. Rossiter and colleagues in a series of studies from 1966 to 1973 have thoroughly examined the environmental
Common isoflavone aglycones

![Chemical structure of isoflavone and common isoflavone aglycones](image)

<table>
<thead>
<tr>
<th>Compounds</th>
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<th>R2</th>
<th>R3</th>
<th>R4</th>
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<tr>
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<td>H</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Genistein</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Glycitein</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Formononetin</td>
<td>OH</td>
<td>H</td>
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<td>OCH₃</td>
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Figure 1: Chemical structure of isoflavone and common isoflavone aglycones
influences on the isoflavone levels in subterranean clover. The concentrations of isoflavones, especially formononetin, in the fully expanded leaf were increased markedly by mineral deficiencies of nitrogen, sulfur, and especially phosphorus. The effects of potassium, zinc and copper on isoflavone concentrations were negligible. Both high and low temperatures and very low light intensities usually lowered isoflavone levels. The concentrations of isoflavones vary within the plant, being highest in the leaves, intermediate in flowers, and lowest in petioles, stems and roots. Also, genetic background (Morley and Foaucis, 1968) and growth stages (Hanson et al., 1965) play an important role in isoflavone concentration variations. For soybeans, the location and crop year also influence the content of isoflavone present (Wang and Murphy, 1994a).

Soybean is the major plant seed consumed by humans which contains high amounts of isoflavones. The amount of isoflavones present in soybeans is about 1-3 mg/g (Wang and Murphy, 1994b). The major isoflavones found in soybeans are the polar glycosylated forms: daidzin, genistin, glycitin, malonyl- and acetyl-glucosides of these forms and their respective aglycone forms: daidzein, genistein and glycitein. Most of the soy isoflavones are concentrated in the hypocotyl and are four times more concentrated than in the cotyledons, as reported by Kudou et al. (1991). Glycitein and its glucoside derivatives are mainly found in the hypocotyl.

The distributions of the isoflavone isomers in commercial soybean foods are quite different. In soy germ, glycitein is the predominant form with daidzein second highest and genistein the minor form with a ratio of 50:40:10. Soy ethanol extract (from Novasoy) has an isoflavone distribution with genistein:daidzein:glycitein in a ratio of 50:40:10. which is
comparable to soybean isoflavone distribution (Hendrich and Murphy, 2000). The total amount and/or the forms of isoflavones may be altered during food processing. The isoflavonoid moieties of isoflavone glucosides are not destroyed by most conventional food processing operations. The concentrations of the acetyl glucosides are increased in toasting soy flakes (Farmakalidis and Murphy, 1985). Malonylglucoside is converted to the β-glucosides in an equimolar distribution with a minimal heat processing (Wang and Murphy, 1996). Microbial enzyme can hydrolyze the glucosides to produce aglycones in fermented products like tempeh, miso and natto (Buseman, 1996; Wang and Murphy, 1996).

Soy ingredients, which include raw (or unprocessed) soybeans, soy flours (defatted and full fat), soy concentrates, soy isolates, texturized soy protein (TVP) and hydrolyzed soy protein, contain the highest concentrations of isoflavones with a total normalized isoflavone content ranging from 466 µg/g to 1640 µg/g. The isoflavones tend to associate with the protein fraction in soybeans. The higher the fat content, the less isoflavones will be detected. Soybean oil contains no detectable isoflavones, while the defatted soy flour contain the highest concentrations of isoflavones. The extraction solvents also have a big influence on isoflavone concentrations. Isoflavones have a high solubility in alcohols. Alcohol washed soy concentrate contains little isoflavone (Hendrich and Murphy, 2000).

Traditional soy foods, which have been widely used in Eastern Asia, including tofu, soymilk, tempeh, miso, natto, soy sauce, soynuts (roasted or fried soybean) and kinako also have significant amounts of isoflavones with a range of 100 µg/g to 1600 µg/g (Hendrich and Murphy, 2000). The level of isoflavones in these products depends on the isoflavone
concentrations of the starting soybean. Kinako, which comes from toasted soy flour, has the highest isoflavone content (2438 μg/g wet weight). The fried or roasted soynuts are whole soybeans and are concentrated with the loss of water, so they have the highest isoflavone concentrations among available food products (Hendrich and Murphy, 2000).

The second generation of soy foods are foods where soy protein is used to replace muscle or dairy proteins. The most widely available products are soy hot dogs, soy burgers, soy bacon, soy/beef burger blends, soy cheese and other soy meat analogues. These products are low in isoflavone content with less that 100 μg isoflavones per g of product due to the small amount or low isoflavone level in the soy protein ingredients being used. Interestingly, the soy infant formulas, which use soy isolate as the replacement for dairy proteins, contain relatively high amounts of isoflavones ranging from 214 μg/g to 285 μg/g of dry formula among the six major brands marketed in the United States (Murphy et al., 1997).

Reduced fat soy foods have been a great attraction to consumers due to their low- or no-fat content. These products like soymilk and tofu can be produced by different processing methods, such as skimming the soymilk to reduce fat or adding soy isolate/soy concentrate to increase protein concentration and dilute fat concentration. The total amount of isoflavones varies in each product, with a range of 700 to 1800 μg/g product (Hendrich and Murphy, 2000).

Dietary supplements can also be found in the market and are available to consumers. These supplements (about 100 options in U.S.) are coming from different sources with different concentrations and distributions of isoflavones. The major sources are soy germ or
soy hypocotyl produced by Schouten Company, soy ethanol extract marketed as "Novasoy", red clover and Kudzu (an Asian plant) extract. Compared with any other food sources of isoflavones, these isoflavone supplements are tenfold or more concentrated (Hendrich and Murphy, 2000).

The role of isoflavones in plants is not obvious. They frequently form a substantial proportion of the dry matter of the plant, which has suggested that isoflavones are simply storage compounds. However, isoflavones are synthesized primarily during active growth when demand for carbon is greatest and not at maturity when carbon is available for storage, suggesting that they may play important roles in the life of plants. The isoflavones from red clover may inhibit the germination of its own seed (Change et al., 1969). Isoflavones can act as stimulatory as well as inhibitory factors in legume and fungi interactions (Dixon et al., 1999). Daidzein and genistein, released in soybean root exudates, act at nanomolar concentrations as chemoattractants for zoospores of *Phytophthora sojae*, and also induce their encystment and germination (Morris and Ward, 1992). Biochanin A and several pterocarpan phytoalexins, including medicarpin and pisatin, stimulate germination of *Fusarium solani* spores at micromolar concentrations (Ruan et al., 1995). Isoflavones may also inhibit the action of microorganism invasion and function as phytoalexins (Barz and Welle, 1992). Daidzein and genistein are also reported to inhibit the ability of *Rhizobium* bacteria to induce root nodules ((Firmin et al., 1986). On the other hand, *Bradyrhizobium sp.* has been reported to be attracted to the plant to form root nodules when specific flavonoids and isoflavonoids are secreted from the plant roots. Isoflavones in soybean function as inducing compounds of nod genes, a common gene required for the early stages of nodule formation, in
Bradyrhizobium japonicum (Kossak et al., 1990) and as precursors of inducible pterocarpan phytoalexin, glyceollin I-III (Ebel and Grisebach, 1988). Reduced synthesis of daidzein in soybean roots at suboptimal temperatures limits rhizobial colonization (Zhang and Smith, 1996).

Isoflavone biosynthesis has been extensively studied in chickpea and soybeans. A phenyl-propanoid pathway has been proposed and chalcone synthase (CHS), chalcone reductase (CHR) as well as isoflavone synthase have been identified as the key enzymes (Braz et al., 1990; Ebel et al., 1986). CHS catalyzes the formation of chalcone by a head-to-tail condensation of 4-coumaroyl CoA and three molecules of malonyl CoA. CHS has been purified, characterized and its gene cloned from many plant species (Dixon et al., 1998). CHS is encoded by multigene families, consisting of at least seven in soybeans. The multiple forms of CHS in legumes may have evolved to serve particular specializations of the flavonoid/isoflavonoid pathway, but there is no direct evidence currently to support this hypothesis. In addition to the formation of naringenin chalcone, in the presence of high concentration of NADPH and the enzyme of CHR, 2',4,4'-trihydroxychalcone and its corresponding flavanone liquiritigenin are also produced. CHR is encoded by a small gene family in soybean and alfalfa. This enzyme co-acts with CHS and exhibits approximately 90% maximal activity at a molar ratio (CHS : CHR) of 2 : 1 (Dixon et al., 1999). The third important enzyme in isoflavone synthesis is isoflavone synthase. It catalyzes the conversion of naringenin to genistein or 2(S)-liquiritigenin to daidzein, in the presence of NADPH (Hagmann and Grisebach, 1984). To date, there is no report on the purification to homogeneity or the molecular cloning of the gene for this enzyme. These aglycones can
undergo glucosylation in the 7'- O-position to form the predominant glucoside forms of isoflavones in soybean. Further methylation at the 4'-position of daidzein and genistein, catalyzed by 4'-isoflavone methyltransferase, yield formononetin and biochanin A, respectively (Wengenmayer et al., 1974). 4'-O-methylation appears necessary prior to further modification of the isoflavonoid nucleus by 2'-hydroxylation and subsequent reduction to isoflavanone, the substrate for formation of pterocarpans (Hinderer et al., 1987) (Fig 2).

B. Metabolism of isoflavones

Most of the isoflavones occur in soybean as glycosides and aglycones, as stated above. In ruminants, the glycosides are hydrolyzed by gut microfloral β-glucosidases in the rumen and further demethylated and reduced by the microorganisms. A very minor portion of these hydrolyzed isoflavones is absorbed very quickly from the rumen, and reaches the circulation unconjugated. The bulk is conjugated in gastrointestinal epithelium by phase II biotransformation enzymes, namely UDP-glucuronyltransferases (UGT) and sulfotransferases (SULT) (Fig 3). The remaining unconjugated isoflavones that enter the circulation are mainly conjugated by the liver and perhaps also by other extrahepatic tissues such as kidney. Lundh (1995) reported that in cattle and sheep, the conjugation of isoflavones mainly happened in gastrointestinal epithelium, which indicated that the liver was not the predominant detoxifying organ for isoflavones in ruminants. When rats were infused intestinally with 14C labeled genistein, 70% of the labeled genistein was found in its glucuronidated form from the collected portal blood (Sfakianos et al., 1997). Thus, the intestinal mucosa was the major site of isoflavone biotransformation in rats.
Figure 2: Biosynthesis pathway of isoflavone daidzein and genistein.
Figure 3. Metabolic pathways of dietary isoflavones (adapted from Setchell and Adlercreutz, 1988).
Doerge et al. (2000) evaluated substrate activities of daidzein and genistein with recombinant human UGT and SULT. The human colon UGT 1A10 isoform was found to be specific for genistein, although several other UGTs (1A1, 1A4, 1A6, 1A7, 1A9 and 1A10) catalyzed 7 and 4'-glucuronidation of both daidzein and genistein. The SULTs 1A1*2, 1E and 2A1 catalyzed formation of a single genistein sulfate; the 1A2*1 and 1A3 isozymes had no observed activity. None of the SULTs listed above showed any activity with daidzein. Clearly, there were tissue and enzyme isoform specificities towards the conjugation of daidzein and genistein.

Based on the observation by Sfakianos et al. (1997) that 75% of a dose of $^{14}$C-genistein was recovered from bile within 4 h after duodenal infusion of rats, biliary excretion may be considerable. The biliary isoflavone metabolite of genistein was the 7-O-glucuronide. However, human feeding studies (Xu et al., 1994, 1995a & b) showed that the recovery of isoflavones from feces is usually nearly nil. With about 10-50% of ingested isoflavones recovered from urine, this suggests that biliary excretion of isoflavones is followed by the breakdown of isoflavones by some microorganism, namely *Clostridium, Butyrivibrio* and *Eubacterium* (Hawksworth et al., 1971).

Glucuronidation is the major detoxification system for several potentially toxic endogenous and exogenous substances, including phytoestrogens. Formononetin, daidzein and equol are mainly found as glucuronide conjugates in blood. Not more than about 5% of the total amount was found as unconjugated substances in plasma at any time (Lundh, 1995). However, different species may differ in their ability to metabolize isoflavones. Studies performed by Cimino et al. (1999) showed that man and rat excrete different amounts of
genistein, daidzein and their conjugates from urine. Twenty-six female rats fed isolated soy protein, which contains 1.36 and 0.65 mg of genistein and daidzein equivalent per g protein, excreted genistein and daidzein: 52±4 and 42±3% as free aglycones, 28±3 and 18±4% as glucuronides, 26±4 and 40±3% as sulfates, respectively. The male human subject excreted genistein and daidzein: 0.36 and < 1% as free aglycones, 86 and 75% as glucuronides, 4 and 11% as sulfates. Peterson et al. (1998) reported that they could detect genistein 7-sulfate after incubating MCF-7 and T47D human cancer cell lines with genistein; but the hydroxylated and methylated metabolites were suggested as the active forms of genistein in inhibiting cell growth.

Basically, the mode of formation of isoflavone metabolites in humans seems to be similar to that in rats. Administration of soy protein resulted in a marked increase in the excretion of equol (a metabolite of daidzein formed by intestinal bacterial action) (Axelson et al., 1984). Some subjects may not be able to form equol (Axelson et al., 1984) and germ-free rats did not excrete equol when given commercial pelletted food (Axelson and Setchell, 1981). O-desmethylangolensin (ODMA) seems to be a minor metabolite in man (Adlercreutz et al., 1986).

Different isoflavone components in food may produce different metabolites. When Yasuda et al. (1994) orally administered male Sprague-Dawley rats (120-160 g) with daidzein (60 mg/kg), the urine contained daidzein 7-O-β-D glucuronide, daidzein 4'-O-sulfate and daidzein. The urine of rats treated with daidzin (100 mg/kg) contained daidzein 7, 4'-di-sulfate and the other three compounds mentioned above.
Rat intestinal microflora culture study has confirmed some of the metabolites of isoflavones. p-Ethylphenol, a metabolite of genistein was identified by paper chromatography and thin layer chromatography (TLC) and confirmed by gas chromatography (GC). Unfortunately, the presence of this metabolite in human urine has not been established (Heinonen et al., 1999). Equol was also detected (Griffith and Smith, 1972) in rats and human (Heinonen et al., 1999). The tentative identification of trans-4-OH-equol in human urine were reported by Kelly et al. (1993) and Joannou et al. (1995) based on their GC-MS spectrometric data and microscale synthesis of possible reference compounds. In 1998, Wåhälä et al. synthesized cis- and trans-4-OH-equol and characterized them by 2D$^1$H and $^{13}$NMR and MM calculation. Heinonen et al. (1999) fed six healthy volunteers with three soy bars (39 g) per day for 2 weeks and analyzed their two consecutive days urine samples. They isolated and characterized the urine metabolites with absorption chromatography on Sephadex LH-20 and gas chromatography-electron ionization mass spectrometry (GC-EIMS), and found that the known and previously authentically identified isoflavones daidzein, genistein, glycitein, equol, ODMA, dihydrodaidzein (4', 7-dihydroxyisoflavanone) and mammalian lignans enterolactone and enterodiol. They also isolated and characterized cis-4-OH-equol, dihydrogenistein (4', 5' 7-trihydroxyisoflavanone), and 6'-OH-ODMA from these urines. Dihydrodaidzein and dihydrogenistein were identified in all the urine samples and were found to be the main metabolites of daidzein and genistein, respectively. However, the levels of genistein, dihydrogenistein and 6'-ODMA were especially low when compared to the levels of daidzein and its metabolites, although almost equal amounts of daidzein and genistein were ingested (41.4 and 34.4 mg per 100 g bar, respectively). Kelly et al. (1993) reported in their
study that dihydrogenistein was only a minor metabolite and they only identified this metabolite in the urine of one volunteer. This result was in disagreement with the result of Heinonen et al. (1999). It seems that feeding methods and chemical extraction and quantification methods greatly influence the identification and characterization of isoflavone metabolites. Standardizing analysis methods will facilitate isoflavone research.

C. Isoflavone bioavailability

Bioavailability is the proportion of a substance having access to its sites of action. Isoflavone bioavailability is a major determinant of the safety and efficacy of these compounds. Many factors might influence isoflavone bioavailability, such as structure, dose, background diet, soy food type, and gut microflora.

In most soy foods, more than 90% isoflavones are in glycosidic form. These glycosides are probably poor substrates for mammalian β-glycosidases. They must be cleaved by gut microflora β-glycosidases before the isoflavone aglycone can be absorbed. Farmakalidis et al. (1985) showed that daidzin and genistin, when fed in equivalent molar amount, had the same estrogenicity in mice as did their respective aglycones. This suggested that cleavage of glycosides to aglycones by gut microfloral β-glycosidases was efficient. King et al. (1996) observed nearly identical total urinary excretions when they fed 20 mg/kg body weight genistein or equivalent dose of genistein glucoside forms to 10-wk old Wistar rats. Yasuda et al. (1994) had similar observation about daidzein and daidzin with their male SD rats (120-160 g) which were fed either 100 mg/kg daidzin or 60 mg/kg daidzein. Neither
The study reported urinary excretion of isoflavone glycosides. In general, the glycosides would be considered to have less biological activity due to their greater hydrophilicity and molecular weight, and have been shown to have less antioxidant activity than did the aglycones (Nairn et al., 1976). The direct absorption of glycosides has not been reported so far. On the other hand, aglycones may be absorbed more rapidly than isoflavone glycosides. King et al. (1996) saw a higher initial plasma concentration at 2 h in genistein-treated rats (11.0 μmol/L) than in soy extract-treated rats (4.93 μmol/L). But similar long-term concentration existed for both forms of isoflavones, indicating that their overall bioavailability may be the same.

Isoflavone aglycones are slightly different in their structures (Fig. 1). Genistein has three hydroxyl groups and daidzein has two. The 5-OH group on genistein makes it more susceptible to microbial breakdown (Griffiths and Smith, 1972). More degradation of genistein makes it less available in the system. Based on urinary excretion, daidzein is more bioavailable than genistein.

Many feeding studies have been performed to look at the influence of dose on isoflavone excretion and plasma concentrations. Two review papers have summarized the results (Hendrich et al., 1999a and 1999b). Basically, at nearly equal doses, urinary excretion of daidzein was more than twofold greater than genistein. Plasma concentrations and urinary isoflavone levels were linearly related to isoflavone doses. Frequent intake will prolong isoflavone retention in the body.

The low recovery of isoflavones from feces and the recovery of only 10-50% of ingested isoflavones from urine in human feeding studies indicated that these compounds may be degraded or significantly altered by gut microflora. Flavonoids and isoflavonoids may be...
susceptible to degradation by strains of *Clostridium*, *Butyrivibrio* and *Eubacterium* (Hawksworth et al., 1971). In vitro anaerobic incubation of human feces with brain/heart infusion (BHI) media showed that daidzein completely disappeared after 72 h and genistein after 18 hrs. The disappearance of isoflavone fits first-order kinetics, with half-lives of daidzein and genistein estimated to be 7.5 and 3.3 h, respectively (Xu et al., 1995). Samples taken from 15-20 subjects over a ten-month period showed that people could be grouped into three different phenotypes according to their isoflavone degradation rate as assessed by in vitro fecal incubation (Hendrich et al., 1998). The rate constant, k, was calculated as the negative slope of the regression line plotted for isoflavone content of each sample over time. At day 0, daidzein degradation rates were: $k_{\text{low}} = 0.012$, $k_{\text{moderate}} = 0.055$ and $k_{\text{high}} = 0.299$; for genistein, they were: $k_{\text{low}} = 0.023$, $k_{\text{moderate}} = 0.163$ and $k_{\text{high}} = 0.299$. Within each compound, each phenotype differed significantly ($p < 0.05$) from other phenotypes. At day 300, similar patterns were seen. These data suggested that human gut microflora differ in ability to degrade isoflavones in a relatively stable way. The degrees and strains of human gut microflora vary from individual to individual, which highly influence the metabolism and bioavailability of isoflavones. However, which microorganism(s) are the major ones responsible for isoflavone degradation remains to be determined.

Based on this phenotype identification method, Zhang et al. (1999b) selected 15 subjects (7 male and 7 female) who are all moderate excretor phenotypes to participate in a human feeding study. In this study, except for a controlled amount of soyfoods, diet intake was ad libitum. Individual isoflavone urinary excretion varied about 8-fold for all three major isoflavones, daidzein, genistein and glycinein. In a diet-controlled study, Karr et al. (1997) fed
different levels of soy protein to 14 subjects. They found that urinary excretion of genistein varied by as much as 12-fold, daidzein by 15-fold within diet treatment. Compared to Karr’s study, screening subjects to control for isoflavone degradation phenotype seemed to decreased isoflavone excretion variability to some extent. Pre-screening for degradation phenotype could be a useful tool in studying isoflavone bioavailability.

Gastrointestinal (GI) bacteria play an important role in determining the magnitude and degradation pattern of isoflavone bioavailability. Extensive metabolism and degradation of isoflavones by intestinal microflora, as occurs in the low excretor phenotype in vitro, prohibits isoflavone absorption. In this case, low fecal isoflavone and lesser total urinary recovery may be observed. Xu et al. (1995a) examined seven women fed a dose of 10, 20 and 30 μmol isoflavones/kg one day. They found that two subjects showed about two- to three-fold greater urinary isoflavone excretion of both daidzein and genistein than the other five subjects. This was accompanied by 10-fold greater recovery of isoflavones in their feces compared with other subjects.

King et al. (1998) investigated the plasma and urinary kinetics of daidzein and genistein in humans by feeding them a single soy meal. Isoflavone concentrations in plasma rose slowly and reached maximal values of 3.14 μmol/L at 7.42 h for daidzein and 4.09 μmol/L at 8.42 h for genistein. Urinary excretion rate of daidzein was greater than that of genistein throughout of postmeal period, with mean recovery of 62% and 22% for daidzein and genistein, respectively. The ratio of the areas under curve for plasma concentrations vs time for daidzein and genistein was equal to the ratio of the concentrations of the respective
isoflavones in the soy meal. They concluded that daidzein and genistein bioavailability were
similar even though daidzein had greater urinary excretion.

Biliary excretion of isoflavones occurred in rats as well as humans. The first-pass
effect, the fraction of ingested isoflavones entering hepatic portal circulation from the intestine
that is immediately passed back out into the intestine by biliary excretion, may be
considerable. Sfakianos et al. (1997) studied disposition of $4^{-14}$C-genistein infused into the
duodenum of rats. This $14$C-genistein was rapidly absorbed from the intestine, taken up by the
liver and excreted into the bile as 7-O-$\beta$-glucuronide conjugate. The total recovery of $14$C-
radioactivity within 4 h in bile was 70-80% of the administered dose. The total urinary and
fecal $14$C-genistein excreted from rats in metabolic cages within 120 h after dosing were 21%
and 2%, respectively. Considering the recovery observed in human studies, a total of less than
50% of ingested isoflavones in urine and nearly nil from feces (Xu et al., 1994, 1995a, 1995b)
suggests that biliary excretion of isoflavones is followed by microbial breakdown.

D. Anticancer effects and mechanism of isoflavones

Diet has been considered to play an important role in cancer prevention. 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 soy-rich diets can decrease the
incidence of hormone-dependent cancers, such as breast, colon and prostate cancers. British
intake of isoflavones has been estimated to be < 1 mg/day (Jones et al., 1989), while the
average consumption of these compounds in Asian countries is 50-100 mg/day (Barnes et al.,
1990; Griffiths et al., 1999). Urinary equol excretion in Japanese was up to twenty times
greater than in Western populations, and the circulating levels of isoflavones in plasma were also much greater. In 1978, Nomura et al. found an association between greater intake of miso soup or tofu and subsequent decreased risk of breast cancer. Hirayama (1986) also showed that a high intake of soybean paste soup was associated with a reduced cancer risk in Japanese women. Lee et al. (1991) in their case-control study found a significant (p<0.01) association in premenopausal women between decreased breast cancer risk and greater soybean protein intake. These epidemiological observations have been verified by animal experiments.

N-methylnitrosourea (NMU)-induced mammary tumor load in female rats was reduced from 8 to 3 by feeding 20% powdered soybean chips compared with a diet containing casein (Barnes et al., 1990). However, when the isoflavones were chemically extracted from the soybean product, no reduction in mammary carcinogenesis was observed (Barnes et al., 1990). Soybean protein isolate decreased mammary tumors by 50% in Sprague-Dawley female rats dosed with 40 mg NMU/kg body weight for 7 weeks compared to rats fed 20% casein (Hawrylewicz et al., 1991). When rats consumed a powdered soybean diet (15%), there was a 50% reduction in X-ray radiation induced mammary tumors (Troll et al., 1980). Lamartiniere et al. (1995) also showed that administration of genistein (5 mg subcutaneous injection at day 2, 4 and 6 postpartum) to female Sprague-Dawley CD rats suppressed mammary cancer induced by dimethylbenz[α]anthracene (DMBA) (80 μg/g body weight at day 50), compared to vehicle-(dimethylsulfoxide) treated rats.

In explaining the anticarcinogenic functions of isoflavones, several mechanisms have been proposed. Initially, isoflavones, especially genistein were considered to have estrogen
agonist/antagonist activity due to their structural similarity to the physiological estrogens such as 17β-estradiol, and to their estrogenic effects (mouse uterine weight increase) when 1 or 2 mg genistein, or 2 mg genistin was administered to immature female mice (Cheng et al., 1953). However, other mechanisms have also been postulated, including protein tyrosine kinase (PTK) inhibition, topoisomerase II inhibition, induction of differentiation and antiproliferation, and inhibition of oxidation events.

1. **Antioxidant functions of isoflavones**

Hepatic neoplastic nodules from rats initiated with a single injection of diethylnitrosamine (DEN), 0.15 μmol/kg body wt, intraperitoneal) and promoted by 0.05% phenobarbital showed increased levels of reactive oxygen species (ROS), predominantly superoxide anion radicals (O$_2^-$) and hydroxyl peroxide (H$_2$O$_2$), which was measured by the emission of lucigenin-chemiluminescent signals (Scholz et al., 1990). Certain ROS, such as hydroxyl radicals (•OH), O$_2^-$ and H$_2$O$_2$, and their subsequent modification of macromolecules (such as protein, RNA and DNA) may be involved in development of multistage carcinogenesis (Fisher et al., 1988; Frenkel, 1992; Cerutti, 1985; Sun, 1990). Antioxidants may protect cells from the oxidative damage that occurs during carcinogenesis (Sun, 1990). Antioxidant effects of isoflavones may inhibit tumor promoter-induced oxidant formation and inflammatory responses which could lead to anticarcinogenic activities. Naim et al. (1976) reported that isoflavones inhibited lipoxygenase action and prevented peroxidative hemolysis of sheep erythrocytes in vitro. In female CD-1 mice (6-7 weeks old) fed 250 ppm genistein
for 30 days, the activities of antioxidant enzymes including catalase, superoxide dismutase (SOD), glutathione peroxidase and glutathione reductase in skin and small intestine were enhanced about 10-30%. When comparing the effect of isoflavones on H2O2 production by 12-O-tetradecanoylphorbol-13-acetate (TPA)-activated HL-60 cells and O2·− generation by xanthine/xanthine oxidase, Wei et al. (1995) showed that genistein (IC50 = 25 μmol/L) and daidzein (IC50 = 150 μmol/L) were among the most potent inhibitors of hydrogen peroxide formation. Biochanin A and apigenin had little effect. They also showed that dietary administration of 250 ppm genistein for 30 days significantly enhanced the activities of antioxidant enzymes in the skin and small intestine of mice.

In 1996, Cai and Wei fed SENCAR mice with 50 or 250 ppm genistein for 30 days. The activities of antioxidant enzymes, such as catalase, SOD, and glutathione peroxidase in liver, kidney, lung and intestine were significantly increased compared to vehicle treated groups. The 4’-position hydroxyl group was proposed to be crucial for isoflavone antioxidant activity.

Flavonoids and isoflavonoids may also have some effects on antioxidant protein expression. Kameoka et al. (1999) treated human intestinal Caco-2 cells with 100 μmol/L genistein, daidzein, biochanin A or kaempferol and measured the mRNA level of metallothionein (MT), catalase and SOD. MT mRNA level was increased up to 15-fold by these compounds, but catalase and SOD mRNA levels were not affected.

The antioxidant activities of isoflavones may also modulate the effects of glucose. Soy phytochemical extract (SPE) containing isoflavone daidzein and genistein was found to
protect against glucose-induced oxidation of human low-density lipoprotein in vitro. In this system, equol was as effective as dietary flavonols quercetin and kaempferol; it also had a similar antioxidant potency as endogenous 17β-estradiol, and was more effective than daidzein and genistein (Vedavanam et al., 1999). SPE was a weak inhibitor of glucose uptake into rabbit intestinal brush border membrane vesicles in vitro. The properties of isoflavone-containing SPE as an estrogenic agent, an inhibitor of intestinal glucose uptake and a preventive agent for glucose-induced lipid peroxidation may make it a good candidate as antidiabetic agent (Vedavanam et al., 1999).

2. Antiproliferation and differentiation induction properties of isoflavone

Another main mechanism proposed for the anticarcinogenic function of isoflavones is antiproliferation and inhibition of cell growth. As the main transduction signal, increased activity of protein tyrosine kinases (PTKs) will give cells a proliferation advantage. As a specific inhibitor of tyrosine protein kinase (Akiyama et al., 1986), genistein could act as an antitumor agent.

Genistein is a more potent inhibitor of PTK than daidzein, so the investigation of antiproliferation activities of isoflavones has mainly focused on genistein, even though there are some reports about daidzein. Genistein inhibited the PTK activity of growth factor receptors, such as epidermal growth factor (EGF) receptor and pp60^c-src PTK (IC$_{50}$ of 22-26 μmol/L), and inhibited intact A431 PTK with an IC$_{50}$ of 148 μmol/L (Akiyama et al., 1987 and 1991). Aluminum tetrafluoride stimulated inositol phosphates in 3T3 cells inhibited by genistein and daidzein (Higashi and Oganara, 1992). Genistein (IC$_{50}$ = 24-45 μmol/L)
inhibited both estrogen-and growth factor-stimulated proliferation of human breast cancer cell lines, including MCF-7, T47D ER\textsuperscript{−}, T47D ER\textsuperscript{+}, BT-20 and ZR-75-1 (Peterson and Barnes, 1991 and 1996). However, the requirement for the presence of functional estrogen receptor or inhibition of epithelial growth factor PTK activity is not the only mechanism for the growth inhibition effect of genistein. Interfering with signal transduction events stimulated directly by estradiol or growth factors may also account for the mammary cell growth blockage function of genistein. Like the blockage of human colon cancer cell (Caco-2) proliferation stimulated by estradiol (Domenico et al., 1996). Estradiol triggered a rapid and transient activation of the mitogen-activated protein (MAP) kinases, erk-1 and erk-2. This activation was mediated by the estradiol receptor since it was inhibited by the pure antiestrogen ICI 181.780. Both erk-1 and erk-2 are activated by receptor and non-receptor tyrosine kinase. It appeared that estradiol activated MAP kinases in Caco-2 cells was through stimulation of c-src kinase family members. Genistein (40 \text{ \mu mol/L}), a src family kinase inhibitor, abolished the erk-2 stimulation induced by estradiol in Caco-2 cells (Domenico et al., 1996).

Meng et al. (1999) synthesized fat-soluble isoflavone esters and incorporated these compounds via artificial transfer system into low density lipoprotein (LDL) and delivered them into U937 cells. LDL containing 7-oleates or 4′, 7-dioleates of genistein and daidzein significantly reduced U937 cell proliferation by 36-43%. The strongest inhibitory effect was shown by daidzein 4′, 7-dilinoleate with 93% reduction of cell proliferation. This in vitro study suggested that isoflavone fatty acid esters may transfer into cells via LDL-receptor pathway and exhibit their effects inside the cells. Whether this could occur physiologically remains to be seen.
The antiproliferative activity of isoflavones and flavonoids was probably not related to their structures either on basis of the classes or with respect to position of substitutions within a class (Kuntz et al., 1999) on cancer cell lines of Caco-2, MCF-7, LLC-PK1 and HT-29. About 30 compounds they screened showed antiproliferation activities with $EC_{50}$ values between 39.7 to 203.6 $\mu$mol/L. In different cancer cell lines, flavonoids of the flavone, flavonol, flavanone, and isoflavone classes possess different antiproliferative effects. Based on this study, the capacity of flavonoids for growth inhibition and induction of apoptosis also cannot be predicted on the basis of their chemical composition and structure.

The other major cellular effect of isoflavones is to induce cell differentiation. Decreased activity of either topoisomerases, important enzymes in the process of replication, transcription, and other DNA processing, or tyrosine kinases have been implicated in the differentiation of a number of cell types (Markovits et al., 1989). Genistein (18.5-74 $\mu$mol/L) induced erythroid differentiation of erythroleukemia cells (Watanabe et al., 1991). Myeloblastic ML-1 cells were induced to differentiate into promyelocytes, and promyelocytic HL-60 cells were induced to differentiate into mature granulocytes by genistein (35 and 50 $\mu$mol/L, respectively) (Makishima et al., 1991; Constantinou et al., 1990). Mouse C1 line cells which are megakaryoblastic cells established by coinfection of Abelson murine leukemia virus and recombinant simian virus were induced to differentiate and the $v$-abl tyrosine kinase activity was inhibited by genistein (4.6 $\mu$mol/L) (Honma et al., 1991). Constantinou and Huberman (1995) investigated the differentiation induction by genistein in human promyelocytic HL-60, erythroid K-562 leukemia cell and SK-MEL-131 melanoma cells. The
optimal concentrations of genistein for each cell line were 37, 37 and 55.6 μmol/L, respectively. They suggested that genistein triggers the pathway that leads to cellular differentiation by stabilizing protein-linked DNA strand breakage.

The cellular level of p53 may be an important determinant of differentiation. Normal cells contain certain amount of p53. Melanoma cells either completely lack or contain different levels of wild-type p53. When treated with genistein, these melanoma cell growths were significantly inhibited, and melanin content, tyrosinase activity were increased. A dendrite-like structure was caused to form in these cells. Transfection study showed that p53 transfectants expressed high levels of wild-type p53 became resistant to genistein (Rauth et al., 1997).

Isoflavones bearing different structures have been associated with their different differentiation induction effects. Jing and Waxman (1995) studied the differentiation effects of isoflavones on mouse erythroleukemia (MEL) cells based on benzidine staining. Genistein, daidzein and genistin induced MEL differentiation, but biochanin A and apigenin had no effects. The potency of these chemicals on cell growth inhibition was apigenin > genistein > genistin > biochanin A > daidzein at assay doses from 11 to 100 μmol/L. They concluded that the 4′-hydroxyl groups are essential for the differentiation induction effect, while trihydroxy derivatives are good growth inhibitors. Daidzein was considered to be a potent differentiation inducer with the least cytotoxic effect. Unfortunately, daidzein showed no differentiation effect on breast cancer cell lines MCF-7 (estrogen-receptor positive) and MDA-MB-468 (estrogen-receptor negative) (Constantinou et al., 1998b). However, both estrogen-positive
and negative cells became differentiated in response to genistein treatments, suggesting that the estrogen-like function of genistein was unrelated to the mechanism of cell differentiation.

3. **Cell cycle arrest and induction of apoptosis by isoflavones**

   In most cells, the events in the nuclear cycle (spindle pole duplication and separation, DNA replication, spindle formation, chromosome segregation) are organized into a dependent sequence of steps. Any perturbation may arrest cells at a certain stage or may delay the transition from one stage to another. There are two important checkpoints in the cell cycle. One is at the G1/S boundary. Any damage to DNA may delay the transition. Another one is at the G2/M boundary. DNA damage, unreplicated DNA or unassembled microtubules may delay progression from G2 into mitosis (Hutchison and Glover, 1995).

   Cells die primarily by one of two major mechanisms: necrosis or apoptosis. Cell death by necrosis occurs as a result of a marked toxic or physical insult. Programmed cell death, apoptosis, is a process in which individual cells die in a controlled manner in response to specific stimuli following an intrinsic program. Apoptosis characteristically affects single cells and is not accompanied by an inflammatory reaction. Apoptosis plays a key role in the control of cellular populations in development, in the immune system, and in carcinogenesis (Cohen et al., 1995).

   Normal tissue homeostasis is maintained by a balance of cell input, due to cell proliferation and renewal, and cell death. Cancer represents an imbalance, which may be due to an increase in cell proliferation and/or a decrease in cell death. The induction of cancer is a multistep process involving tumor initiation, promotion, and progression. Apoptosis may
interfere with tumor formation at one or more of the steps in carcinogenic process. A defect in the ability of a cell with damaged DNA to undergo apoptosis may therefore lead to an increase in cancer incidence. Certain oncogenes and tumor suppressor genes such as \textit{c-myc}, \textit{bcl-2} and \textit{p53} may play important roles in determining a cell's fate. Any reagent that can cause increased expression of \textit{c-myc} and \textit{p53} and decreased \textit{bcl-2} expression in cancer cells will increase the possibility of cell apoptosis (Cohen et al., 1995).

Three DNA damage-responsive cell cycle checkpoints were shown to operate in diploid human fibroblasts (Kaufmann, 1998). Progression from G2 to M is controlled in part by cyclin-dependent kinase (cyclin B/Cdk1) that is regulated by tyrosine phosphorylation. Genistein (45\textmu mol/L) inhibits tyrosine kinase, including the one that phosphorylates Cdk1 on Tyr15 (Akiyama et al., 1987). This kinase is rapidly induced by treatments that trigger cell cycle checkpoints and may contribute to the delay of mitosis onset. Down regulation of cyclin B, up regulation of the p21WAF1 growth-inhibitory protein by genistein (30 \mu mol/L) were also seen in prostate cancer cells (Davis et al., 1998), in non-small cell cancer cell line H460 (Lian et al., 1998). Genistein (starting at 20 \mu mol/L, complete inhibition at over 80\mu mol/L) also inhibits type II DNA topoisomerase to produce a form of DNA-damage that triggers all of the DNA damage responsive cell cycle checkpoints (Markovits et al., 1989). Topoisomerase II poisons also are powerful clastogens inducing lethal and carcinogenic chromosomal aberrations. Type II topoisomerase can break DNA in a region of chromosome 11q23 that contains the ataxia telangiectasia gene (ATM). ATM controls all the DNA damage-responsive cell cycle checkpoints (Kaufmann, 1998). Thus topoisomerase poisons
such as genistein may trigger chromatid breakage to inactivate AT gene function, disable cell cycle control and induce gene instability.

Induction of \( p53 \) level may also account for an important mechanism of phytochemicals inducing apoptosis, as mentioned above. Non-cancerous human mammary epithelial 184-B5 cells were pretreated with benzopyrene (\( \alpha \)), the altered cell proliferation was inhibited and apoptosis was induced by applying cruciferous glucosinolate indole-3-carbinol, tea polyphenol(-)epigallocatechin gallate or genistein (39 \( \mu \text{mol/L} \)) (Katdarc et al., 1998). Treating the cells with these phytochemicals resulted in a 1.8-6.9 fold increase in apoptosis. The induction of apoptosis was accompanied by enhanced \( p53 \) immunoreactivity within the cells.

Over-expression of the \( bcl-2 \) gene makes cells resistant to apoptosis without affecting proliferation. At a concentration of 0.15 mM, after 24 hrs treatment, genistein inhibited the phosphorylation of \( bcl-2 \). The \( p53 \) level in MCF-7 cells reached a steady-state (Constantinou et al., 1998b).

At different concentrations, genistein may interrupt different stages of the cell cycle. Spinozzi et al. (1994) added different concentrations of genistein to Jurkat T-leukemia cells. They found that at low concentrations (18.5-37 \( \mu \text{mol/L} \)), genistein arrested cell cycle at G2/M phase; at higher concentrations (74-111 \( \mu \text{mol/L} \)), it arrested cells in S-phase. Apoptosis followed in genistein-treated cells.

Daidzein can also inhibit cell growth at cell cycle checkpoint. After stimulation by bombesin plus insulin for 4.6 h, Swiss 3T3 cell progression was blocked at G1 phase by
daidzein at concentrations over 37 μmol/L. After removal of daidzein, insulin or insulin-like growth factors (IGFs) reinitiated daidzein-blocked cell cycle progression without further addition of bombesin. In vitro study showed that daidzein inhibited the activity of casein kinase II which is required for the commitment of mitogenic signal by insulin or IGF in G1 phase (Higashi and Ogawara, 1994).

4. Estrogenic and antiestrogenic properties of isoflavones

Isoflavones have structural similarities to estrogen, suggesting that they may act as phytoestrogens. These phytoestrogens may cause beneficial or adverse hormonal effects, depending on the effectiveness and amount of the ingested hormone agonist, synergistic and antagonistic effects with other dietary or endogenous hormones, interactions with other dietary compounds (e.g. fiber and fat intake), and the hormone status of the individual. Certain cancers (breast, prostate and colon) may be hormone-dependent, such as breast cancer which is associated with estrogen and prolactin activity (Welsch, 1976). Estrogens bind to cytoplasmic receptors, and elicit their translocation to the nuclear estrogen receptor complex for a short time. These events initiate DNA transcription, and RNA synthesis, which in turn leads to protein synthesis and cell growth (Anderson, 1977). So, increasing the levels, intensity and duration of exposure of estrogen might increase the risk of breast cancer as well as other sex-hormone dependent cancers.

The phenolic ring of isoflavones, which is a prerequisite for binding to the estrogen receptor, is similar in structure to mammalian estrogen 17β-estradiol. However, the estrogenicity of daidzein and genistein were about $10^3$-$10^7$ times less than estradiol or the
synthetic estrogen diethylstilbestrol (DES) (Farmakalidis et al., 1985; Kitte et al., 1980; Newsome and Kitte, 1980). The sheep uterine estrogen receptor binding of daidzein and genistein were only 0.1% and 0.9% that of 17β-estradiol, respectively (Shutt and Cox, 1972). Glycitein had similar estrogen receptor binding affinity to daidzein, but it increased female B6D2F1 mice uterine weight by 150% compared to control, which was higher than that of daidzein (50% increase over control) (Song et al., 1999). Equol and O-desmethylangolensin (ODMA), metabolites of daidzein, and daidzein glucuronides (CB50 = 14.7 μmol/L) and genistein glucuronides (CB50 = 7.27 μmol/L) also bind to estrogen receptors and have weak estrogen activity (Shutt and Cox, 1972; Adlercreutz et al., 1992; Zhang et al., 1999). Certain soybean foods, such as tofu and soymilk, contain comparatively large amount of isoflavones. After consumption, the plasma concentration of glucuronide isoflavones, isoflavone aglycones and other metabolites may exceed endogenous estrogens by several orders of magnitude, which could effectively compete with estrogen by competitive binding to estrogen receptor, suppressing estrogen-stimulated cell growth (Adlercreutz et al., 1995).

Estrogen receptor (ER) is a ligand-activated transcription factor that after activation binds to specific DNA elements and modulates the transcription levels of target genes. Two Ers, ERα and ERβ, have been identified. ERβ was identified in 1996 (Mosselman et al., 1996). The two receptors have some similar structures and certain properties, but also differ in many ways. Both receptors contain five different domains in their sequences. The N-terminal A/B region is a transactivation domain possessing constitutive and hormone-dependent transcription-activation function. In ER also as in many glucocorticoids receptors,
it is a ligand-independent transactivation domain, usually called AF1 region. The C region is the most conserved region between different members of the steroid receptor family, which is the DNA-binding domain (DBD). The D domain is regarded as a flexible hinge region between DBD and the E domain. E is the ligand binding domain (LBD) also called AF2 region. F is a domain of unknown function. It does not appear in all nuclear receptors, but does exist in ERs. Ligand binding induces a conformational change in ER and subsequent DNA binding to specific estrogen response elements (ERE) present in target genes. ERα has 599 amino acids and ERβ has 485 amino acids. There are about 97% and 60% amino acid homology in DBD and LBD respectively between these two receptors. A/B domains and activation function (AF1) are quite different. The AF2 core sequences are identical. ERβ has a slightly low affinity to the ERE than ERα. Tissue distribution was slightly different in rats (Kuiper et al., 1997). Kidney has only ERα. Uterus, pituitary and epididymis dominated by ERα. In ovary, prostate and brain, equal or greater levels of ERβ have been found. As a weak estrogen, isoflavones may bind to these two receptors with different affinity. Makela et al. (1999) found that genistein bound to ERβ of carotids with 20-fold higher affinity than to ERα in ovariectomized female rats. Different ligand binding affinity to these two receptors may contribute to the selective action of estrogens in different tissues (Kuiper et al., 1997).

In addition to the competitive estrogen receptor binding ability, isoflavones may also exert their antiestrogenic effects by directly or indirectly increasing the synthesis of sex-hormone binding globulin (SHBG), a protein that binds dihydrotestosterone, testosterone and estradiol. Mousavi and Adlercreutz (1993) found that at concentrations of 5-40 μM, genistein
significantly increased hepatocarcinoma (Hep-G2) cell SHBG production, but suppressed the proliferation of these cancer cells at a stage when SHBG production continues to be high. The urinary excretion of the phytoestrogen equol had a positive relationship with plasma SHBG and negative association with plasma free estradiol, found by Adlercreutz (1987). The high SHBG levels in plasma will decrease hormone exposure by reducing the metabolic clearance rate or lowering the uptake of sex hormone in many tissues (Adlercreutz et al., 1987; Monsavi and Adlercreutz, 1993), which in turn will decrease the risk of sex-hormone dependent cancers.

Decreasing estrogen levels by isoflavones can also be achieved by inhibition of aromatase activity, a cytochrome P-450 enzyme that catalyzes the conversion of androgens to estrogens (Kellis et al., 1984; Adlercreutz et al., 1993). The competitive binding of lignans to aromatase with a binding affinity of 1/75-1/300 of natural substrates testosterone and androstenedione will result in a decreased substrate binding affinity (Adlercreutz et al., 1993). Urinary excretion of estradiol, estrone, estriol and total estrogens, as well as excretion of hypothesized genotoxic estrogen metabolites 16α-hydroxyestrone, 4-hydroxyestrone and 4-hydroxyestradiol were decreased compared to control diet with isoflavone consumption in 12 healthy premenopausal women (Xu et al., 1998). This result indicated that estrogen synthesis can be decreased and metabolism of estrogen can be altered by isoflavone intake. However, isoflavones are significantly poorer inhibitors of aromatase than flavones (Kao et al., 1998).

Several studies have examined the biological effects of isoflavones in women. Consumption of 45 mg/day glycosylated isoflavones in 60 g textured vegetable protein over a 1-month period significantly (p<0.01) prolonged the length of menstrual cycle of
premenopausal women, and suppressed luteinizing hormone (LH) \( (p<0.05) \), and follicle-stimulating hormone (FSH) \( (p<0.01) \) (Cassidy et al., 1994, 1995). These findings suggested that soybean isoflavones may exert weak estrogenic effects on the hypothalamic-pituitary-gonadal axis, and may be beneficial with respect to factors stimulating breast cancer. Dietary soy supplementation of 84 premenopausal women with 45 mg isoflavone showed that nipple aspirate levels of apolipoprotein D were significantly lowered and pS2 levels raised, indicative of estrogenic stimulus (Hargreaves et al., 1999). Isoflavones exert weak estrogenic activities on postmenopausal women. Eighteen postmenopausal women consumed low- or high-content isoflavone diet for 93 days, isoflavone significantly decreased estrogen sulfate but significantly increased SHBG. No estrogenic effects were observed on vaginal epithelium or endometrium (Duncan et al., 1999).

The estrogenic/antiestrogenic activities of isoflavones may also have some effects on other parts of the human body, such as prevention of bone loss and cardiovascular disease. Ishimi et al (1999) administered genistein \((0.1-0.7 \text{ mg/day})\) or \(17\beta\)-estradiol \((0.01-0.1 \mu\text{g/day})\) to sham-operated or ovariectomized (OVX) female mice for 2-4 weeks, and found that genistein did not reverse OVX-induced uterine atrophy, but restored B-lymphopoiesis and trabecular bone volume. These results indicated that genistein exhibited estrogenic action in bone and bone marrow to regulate B-lymphopoiesis and prevent bone loss without exhibiting estrogenic action in the uterus. In another study done by Draper et al. (1997), oral intake of 131 mg isoflavone per week had no preventive effect on bone loss of oophorectomized rats.

Estrogen-based drug therapy in cardiovascular disease has been difficult because the wanted vasculoprotective effect cannot be separated from the unwanted effects of the
hormone on the reproductive system. Isoflavones may act as drug candidates for cardiovascular disease prevention. Makela et al. (1999) used endothelial denudation (at carotid artery) in rats as their study model, and found that genistein (0.0025-2.5 mg/kg body weight) had a similar dose-dependent vasculoprotective effects as 17β-estradiol. Both genistein and 17β-estradiol were equally inhibitory to the replication and migration of smooth muscle cells at concentrations < 10 μM in vitro. Treatment with 17β-estradiol but not with genistein caused a uterotrophic effect, which indicated that genistein was a vasculoprotective estrogen analog devoid of effects on the reproductive system.

E. Immune system modulation effects of isoflavones

In malignancy, natural killer (NK) cells appear to represent a first line of defense against the metastatic spread of blood-borne tumor cells, and normal NK activity may be important in immune surveillance against cancer. The development and progression of cancer has been found to be associated with decreased natural immunity, as measured in vitro by NK activity and/or absolute numbers of circulating NK cells (Whiteside and Herberman, 1989). Many cells of the body including macrophages and neutrophils can produce prostaglandins (PG) (Kunkle et al., 1984), which suppress the activation of all of the immune system: B cells (Thompson et al., 1984), T cells (Parhar et al., 1988), NK cells (Herman and Rabson, 1984), lymphokine-activated killer (LAK) cells (Parhar and Lala, 1988), as well as macrophages (Parhar and Lala, 1988). Tumors which produce PGE₂ are able to down-regulate the immune system and escape lysis (Earnest et al., 1992). For the T cells, PGE₂ inactivation is mediated
by blockage of interleukin-2 (IL-2) production (Lala et al., 1988; Chouaib et al., 1985), down-regulation of IL-2 receptors production (Lala et al., 1988) and transferrin receptors (Chouaib et al., 1985). Due to the dependence of NK cells on interferons as well as IL-2 for their activation, PGE$_2$ action on both of these pathways remains a strong possibility. Isoflavones as antioxidants can increase glutathione peroxidase activity (Hendrich et al., 1994) which inhibits prostaglandin production by removing the hydroperoxides required for prostaglandin synthetic activity (Marshall et al., 1988). So, it is possible that isoflavones may also enhance IL-2 production and NK activity.

NK cells kill malignant and virus-infected cells either by Fc receptor (FcR)-dependent or direct cytotoxic mechanisms (Trinchieri, 1989). The FcR complex expressed on NK cells (FcyRIII) is a low-affinity receptor for the Fc fragment of IgG and is composed of a 55- to 70-kDa transmembrane protein (CD16) complexed with at least two other polypeptide chains (Einspahr et al. 1991). Antibody-dependent cellular cytotoxicity (ADCC) is initiated by the stimulation of this FcR complex. Direct cell-mediated cytotoxicity is thought to be triggered by the interaction of an unidentified NK cell surface receptor with a putative target structure on malignant or virus-infected cells. In order for NK cells to become active cytolytic effectors, these membrane receptor-ligand interactions must be translated into biochemical signals that stimulate intracellular response systems. The suggested signals involved in the activation of NK cell activity including tyrosine phosphorylation, serine/threonine phosphorylation, phospholipase C, phosphatidylinositol and Ca$^{2+}$ (Chow et al., 1988; Einspahr et al., 1991; Shenoy et al., 1993; Whalen et al., 1993). As the most potent activator of NK cells, IL-2 activation of NK cells is via the induction of tyrosine phosphorylation of multiple
proteins (Einspahr et al., 1990 and 1991). The IL-2 receptor itself lacks intrinsic tyrosine kinase activity (Waldman, 1991). Other non-receptor, membrane-associated tyrosine kinases, such as src-family tyrosine kinases, may associate with the IL-2 receptor and thereby relay information from the receptor to the cell interior. Genistein, a PTK inhibitor, like herbimycin A, demonstrated concentration-dependent (0.3-9 μmol/L) suppression of direct and FcR-mediated NK cellular cytotoxicity (Einspahr et al., 1991). Therefore, the effect of isoflavones on NK cell activity may depend on the balance between their inhibition of tyrosine kinase activity and activation of cellular cytokine production.

An experiment performed by Drs. Cunnick and Hendrich (unpublished data) showed that after feeding 240 mg isoflavones/kg diet for 10 days, rat liver-associated NK cell activity was two-fold greater than that in the control group. However, rats receiving a dose of 480 mg isoflavone/kg diet did not differ in NK cell activity compared with the control group. This study indicated that soybean isoflavone extracts can affect NK cell activity in a dose-dependent manner.

Exon et al. (1997) studied the effect of biochanin A on NK cell activities in vivo as well as in vitro. They either gavaged 8 mg/kg or intraperitoneally injected 4 mg/kg biochanin A into rats. Similar to control, NK cell activity was observed 29 h after treatment. When administered three times a week for three weeks, biochanin A did not affect NK cell response, antibody production or delayed-type hypersensitivity reactions. In vitro, NK cell cytotoxicity was significantly enhanced in rat splenocytes exposed to 10 μmol/L of biochanin A or genistein.
Zhang et al. (1999a) co-cultured genistein, daidzein and genistein glucuronides with human peripheral blood lymphocytes at concentrations of 0.1-10 μmol/L. Genistein at concentrations < 0.5 μmol/L, and daidzein and genistein glucuronides at concentration of 0.1-10 μmol/L significantly (p < 0.05) enhanced NK cell-mediated K562 cancer cell killing. When used together with IL-2, genistein and the glucuronides additively increased NK cytotoxicity, which indicated that isoflavones may function at a site different from IL-2 when they activate NK cells.

Other potent activators of NK cell activity are IL-12, IL-15 and interferon gamma (IFN-γ). IL-12 is known as an NK-stimulating factor and is a protective cytokine in infection involving intracellular pathogens. It works best in synergy with IL-2 to activate NK cells (Peakman and Vergani, 1997). The role of IL-12 in NK cells was demonstrated by culturing NKR-P1⁺CD56⁺ populations with IL-12-induced differentiation into the mature NKR-P1⁺CD56⁺ stage. These cells lysed K562 tumor targets (Sivakumar et al., 1998). This suggested a possible role for IL-12 in differentiation of NK cells in later stages.

IL-15 is a differentiation factor. It shares many features with IL-2 such as the functional similarity of activating T and NK cells (Grabstein et al., 1994). However, these two cytokines differ in certain ways, like cells secreting these cytokines, sites of production, regulation of secretion. IL-2 is produced primarily by activated T cells, while IL-15 message is most abundant in placenta, skeletal muscle, kidney, lung and heart. IL-2 secretion seems to be regulated at the level of transcription and message stabilization, whereas IL-15 secretion is controlled at different levels including translation and entry into the secretory pathway. IL-
15 may play a major role as a differentiation factor for NK cells in certain cell line, like NK1.1<sup>+</sup>, or inducing a more immature (CD34<sup>-</sup>CD7<sup>-</sup>) population of human cord blood cells into CD56<sup>-</sup>CD3<sup>-</sup> cells (Bennett, 1996).

IFN-γ is a major activating factor for macrophages. It can increase the metabolism and phagocytic and killing activities of macrophages. IFN-γ can also induce differentiation of B cells and NK cells and affect T cell development. It can increase NK cell adherence to target cells and activate and increase the rate of target lysis. Other factors like IL-1, type I interferons (α and β) may play a minor but important role in enhancing NK cell cytotoxicity. Are isoflavones enhancing any of these cytokine secretions from other lymphocytes and thus affecting function of NK cells or do the isoflavones directly affect NK cells? This remains unknown.

In addition to the possible effect on NK cells, isoflavones, especially genistein, may have some influence on B cells and T cells. In human B-cell lineage lymphoid cells, genistein inhibited multiple effects of CD-40 (in B cells function as signal transduction), namely tyrosine phosphorylation of several proteins, phosphoinositide turnover, and activation of serine/threonine protein kinase (Uckun et al., 1991). In 1995, Uckun et al. reported that genistein (1.8 x 10<sup>5</sup> cpm/nmol of <sup>125</sup>I-genistein), conjugated to the B-cell specific CD-19 receptor with monoclonal antibody B43 (one molecule of genistein conjugated to one molecule of mAb B43 through SANPAH cross-linker), was highly effective in treating leukemia in a nude mouse in a model of pre-B cell human leukemia. At a concentration of 111 μmol/L genistein completely blocked mitogen induced T-cell receptor (TCR)
phosphorylation. In the presence of the same concentration of genistein (111 μmol/L), T-cells stimulated with phytohemagglutinin (PHA) or with OKT3, a monoclonal antibody to CD3, failed to undergo blast transformation (Mustelin et al., 1990).

When Swiss mice were fed 20 or 40 mg daidzein/kg (Zhang et al., 1997), their peritoneal macrophages and thymus weights were increased. Both humoral and cell-mediated immunity were enhanced. In an in vitro study, at concentrations of 0.01-10.0 μM, daidzein significantly inhibited proliferation of mixed mice splenocyte cultures activated with concanavalin A or lipopolysaccharide in a dose-dependent manner, whereas genistein had no influence on the response (Wang et al., 1997).

There are few reports on effects of isoflavones on the immune system. Clearly, more work needs to be done on isoflavones' immunoregulatory function.

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URINARY DISPOSITION OF SOYBEAN ISOFAVONES DAIDZEIN, GENISTEIN AND GLYCITEIN DIFFERS AMONG HUMANS WITH MODERATE FECAL ISOFAVONE DEGRADATION ACTIVITY\(^1,2\)


(Erratum to be submitted)

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Abstract

Glycitein metabolism was compared with other isoflavones, to begin to understand the effect of this compound. Total isoflavones of 4.5 \(\mu\)mol/kg body weight from soymilk (high in genistein and daidzein) and soygerm (high in daidzein and glycitein) were fed to 7 women and 7 men. Only subjects with moderate fecal isoflavone degradation rates (half-

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lives of daidzein and genistein were 15.7 and 8.9 h, respectively) were included to minimize interindividual variation. The average 48 h urinary excretion of glycine, daidzein and genistein were approximately 55%, 46% and 29% of the dose ingested, respectively, which were significantly different from each other in men and women ($p < 0.001$). Plasma isoflavone contents at 6 and 24 h after soymilk feeding paralleled relative amounts of isoflavones in soymilk (genistein > daidzein > glycine) ($p < 0.05$), but plasma isoflavone contents after soygerm feeding did not parallel soygerm isoflavone contents in women, because genistein and glycine were not different from each other at 6 h after feeding. Six hours after soygerm dosing, plasma isoflavone contents paralleled soygerm isoflavone contents in men. Based on plasma isoflavone concentrations at 6 h after dosing, the bioavailabilities of daidzein and genistein were similar in men and women. But at the high glycine dose, plasma concentration at 24 h after dosing suggested a modest gender difference in glycine bioavailability.

Key Words: • Fecal isoflavone degradation • Glycine • Humans • Isoflavones • Urinary disposition

Introduction

Isoflavones are one of the principal classes of plant-derived diphenols in the human food supply. Because the presence of isoflavones in human urine was related to lower mortality from sex hormone-dependent cancers, isoflavones have excited scientific researchers (Barnes et al., 1991). Isoflavones might be partly responsible for soy's effect to lower the risk of cardiovascular diseases (Anderson et al., 1995) and to prevent bone mineral loss in
ovariectomized rats (Arjmandi et al., 1996). Daidzein, genistein and their corresponding glucosides account for the major portion of soy isoflavones and have been the focus of numerous studies. But a third soy isoflavone, glycinein, may also contribute to soy's health effects.

Systemic studies of the absorption, metabolism and excretion of isoflavones are needed to determine their bioavailabilities and biological effects. Among the soy isoflavones, a greater proportion of the dose of daidzein was excreted in urine than that of genistein (Xu et al., 1994; King and Bursill, 1998; Watanabe et al., 1998). Watanabe et al. (1998) reported maximum plasma concentrations of $2.44 \pm 0.65 \mu\text{mol/L}$ at 6 h for genistein and $1.56 \pm 0.34 \mu\text{mol/L}$ at the same time for daidzein after ingestion of 103 $\mu$mol daidzein and 112 $\mu$mol genistein. King and Bursill (1998) reported peak of $4.09 \pm 0.94 \mu\text{mol/L}$ at 8.42 $\pm$ 0.69 h for genistein and $3.14 \pm 0.36 \mu\text{mol/L}$ at 7.42 $\pm$ 0.74 h for daidzein after 3.6 $\mu$mol genistein and 2.7 $\mu$mol daidzein/kg body weight soy meal ingestion. The plasma concentration/time curves of both daidzein and genistein were the same. Daidzein and genistein may have similar bioavailabilities but the longer elimination half-life of genistein may contribute to its potential for greater efficacy than daidzein.

Glycitein and its corresponding glucosides account for 5-10% of the total isoflavones in most soy foods. In soygerm, glycinein accounts for at least 40% of total isoflavones. No biological activity or bioavailability studies of glycinein have been reported to date. Therefore, it is of great interest to study the bioavailability of glycinein which may be an important determinant of glycinein's biological potency.
Gut microflora may play an important role in isoflavone degradation and may be a critical factor in determining isoflavone bioavailability. Xu et al. (1995) showed that two women who excreted greater amounts of fecal isoflavones had greater urinary and plasma isoflavone levels than five other women who excreted small amounts of isoflavones in feces, and these high excretors experienced more prolonged daidzein and genistein bioavailability. Gut motility and gut microflora differ among individuals. A study of gut microfloral metabolism of isoflavones in vitro in 15 subjects over a ten-month period showed that the subjects sorted into three distinguishable groups with respect to the ability of their feces to degrade daidzein and genistein. Degradation rate constants for daidzein and genistein ($k_D$ and $k_G$, respectively) (calculated as the negative slope of the regression line plotted for isoflavone content of each sample over time) were significantly different at day 0 of the study among the three excretor phenotypes: Low excretors ($n = 5$), average $k_D = 0.012$ and $k_G = 0.023$; Moderate excretors ($n = 10$), average $k_D = 0.055$ and $k_G = 0.163$; High excretors ($n = 5$), average $k_D = 0.299$ and $k_G = 0.299$. These phenotypes proved to be stable when reexamined after ten months (Hendrich et al., 1998). These excretor phenotypes might be responsible in large part for the observed great interindividual variation in human bioavailability and likely variability in health effects of soy isoflavones.

To study glycitein absorption and excretion patterns, male and female subjects possessing the moderate isoflavone degradation phenotype were chosen to minimize interindividual variation. A single dose of isoflavones from soymilk (which contained predominantly equal amounts of daidzein and genistein) and soygerm (containing
approximately equal amounts of daidzein and glycine) were provided in a well-controlled liquid diet. Plasma and urine isoflavone contents were measured after feeding.

**Materials and Methods**

**Experimental procedures**

*Subject screening.* The participants in this experiment were selected from 25 volunteers according to their gut microfloral ability to degrade isoflavones. Freshly voided fecal samples from volunteers were diluted and homogenized with sterilized brain-heart infusion culture medium (Difco laboratories, Detroit, MI), containing 0.5 mg cysteine hydrochloride (Aldrich Chemical Company, Milwaukee, WI) as reducing agent and 1 mg resazurin (Aldrich Chemical Company, Milwaukee, WI) as O₂ indicator, under anaerobic conditions. After centrifugation, supernatant was added to duplicate sterilized culture tubes containing sterilized daidzein and genistein (final concentration of each compounds: 590 μmol/L) and incubated anaerobically at 37°C. At time 0, 6, 12, 24 and 48 h intervals, 3 mL of culture mixture was obtained and mixed with 0.6 mL of 100% methanol and 0.9 mL of 0.4 mol/L trichloroacetic acid (Sigma, St. Louis, MO) containing 0.6 mol/L glycine (pH 2.0). Centrifuged and filtered daidzein and genistein were analyzed by HPLC, as described below.

The logarithm of isoflavone concentration (C) was plotted against incubation time intervals. The equation used was: \( \ln(C_0/C) = kt \), with \( C_0 \) represented the initial isoflavone concentration and \( k \) represented the rate constant, \( t \) was the reaction time. The time needed for half of the isoflavone to disappear (\( t_{1/2} \)) from the culture medium was derived from the
equation: \( t_{1/2} = \frac{\ln(2)}{k} \). Half-lives of daidzein and genistein for participants were calculated (Table 2).

**Participants.** Fourteen healthy subjects (seven male and seven female), with age between 19 and 35 years, body weight of 66.0 ± 11.2 kg, body mass index of 22.4 ± 2.3 kg/m², participated in this study (Table 2). The Human Subject Committee of Iowa State University (ISU) approved the procedures for this feeding study. Informed consent of subjects was obtained in writing.

**Diet.** The study consisted of two breakfast feedings, which were separated by a one-week washout period. Subjects were randomly assigned to consume either soymilk (Feain Natural Foods, Mequon, WI) or soygerm powders (Soylife, Schouten USA, Inc. Minneapolis, MN) mixed into cranberry or orange juice with an ad libitum breakfast chosen from wheat toast, cereals, skim milk, apple, banana or orange in a crossover design. The total amount of soy isoflavones fed from either soy source was 4.5 μmol/kg body weight. All subjects were instructed to avoid any soy food and products containing texturized vegetable protein (TVP) and hydrolyzed vegetable. A list of soy-containing foods was given to subjects.

**Blood sample collection.** Venous blood samples (10 mL) were collected into EDTA containing vacuum containers by a licensed medical technologist under stringent aseptic conditions. A blood sample was collected 18 h before dosing (baseline), and at 6 and 24 h after soy containing breakfast. Samples were centrifuged within 1 h after collection at 3000 g for 25 min at 4°C (Model 4D, International Equipment Co.; Needham Hts., MA). Plasma was separated and stored in a -20°C freezer before analysis.
**Urine sample collection.** Each subject provided a urine sample immediately before dosing (time 0). After dosing, urine from each subject was pooled over the following time periods: 0-6, 6-12, 12-24 and 24-48 h. The total sample volume was recorded and 50 mL aliquots of each pooled sample were stored in a -20°C freezer until analysis.

**Analysis methods**

**Soy products analysis.** Two soy products, soymilk and soy germ powders were chosen as glycinein sources. The sample extraction and concentration determination were modified from the method described by Wang and Murphy (1994). Two grams of each soy product was extracted with 10 ml of acetonitrile and 2 mL of 0.1 N HCl for 2 h at room temperature. The filtered and vaporized sample was dissolved in 10 mL of 80% methanol in water and analyzed by HPLC. The total isoflavone content was the sum of total daidzein, total genistein and total glycinein (Table 1).

**Plasma and urine sample analysis.** Sample preparation and analysis for plasma and urine isoflavones were modified from Lundh et al. (1988). One mL plasma or 5 mL urine was incubated with β-glucuronidase-sulfatase (Sigma chemical, St. Louis, MO) at 37°C for 20 h to release aglycones of isoflavones. Fifty μL of 2,4,4'-trihydroxydeoxybenzoin (THB) (Murphy et al., 1997) used as internal standard was incubated together with urine samples. Incubation mixtures were loaded onto Extrelut® QE columns (EM Science, Gibbstown, NJ) and extracted with ethyl acetate. The eluent was collected and dried. The extracted plasma and urine isoflavones were dissolved in 2 mL of 80% methanol in water for HPLC analysis.
The HPLC analysis was carried out on a Hewlett Packard 1050 series system (Hewlett Packard Company, Scientific Instruments Division, Palo Alto, CA). THB and the three aglycone isoflavones were separated and quantified on a YMC-Pack ODS-AM C18 reverse phase column (5 μm, 25 cm x 4.6 mm i.d.) (YMC Inc., Wilmington, NC) attached to a precolumn in-line filter (0.45 μm, Alltech, Deerfield, IL). A linear HPLC gradient was composed of (A) 0.1% (g/L) glacial acetic acid in water and (B) methanol. Following 20 μL sample injection, solvent B was increased from 30% to 50% over 45 min, and then maintained at 50% for 5 min. The solvent flow rate was 1 mL/min. Analyses were monitored with PDA from 200 to 350 nm. Ultraviolet spectra were recorded and area responses were integrated by Chem Station® software (Hewlett Packard Company, Scientific Instruments Division, Palo Alto, CA). Standard curves were established with a series of concentrations of each standard to quantify extracted daidzein, genistein and glyciten.

**Recovery study.** Daidzein, genistein and THB were chemically synthesized and glyciten was purified in Dr. Murphy's lab at ISU. One woman's urine was collected and used in this recovery study. Fifty μL of THB and different amounts of external standards (daidzein 10.83 to 173.20 μmol/L; genistein 10.4 to 166.46 μmol/L; glyciten 5.38 to 86.08 μmol/L) were added to the urine samples. The extraction and analysis methods were the same as described above. Duplicates were run at each standard concentration. Recoveries for each compound were reported as the combined results of three repeated assays. Plasma samples were randomly spiked with daidzein, genistein and glyciten standards to measure recoveries.
(0.1 g isoflavone/100 L plasma was added to each sample). Plasma and urine isoflavones obtained from feeding study were calculated with adjustment for recoveries.

**Statistical methods**

Analysis of variance was performed on the data obtained from this experiment with the SAS program (version 6.03, SAS Institute Inc. Box 8000, 1995, Cary, NC). Plasma concentrations of daidzein, genistein and glycitein at certain time points were analyzed separately and by repeated measurement analysis. Urinary excretions of the three isoflavones during 0-12 h after feeding were compared with the urinary excretions from 12-48 h by paired t-test. Data from male and female subjects were analyzed separately for both plasma and urine. Tukey's test was used for comparison within factors if there was significant effect. A \( p \) value of 0.05 or less was considered to be significant. All values are reported as means ± SD.

**Results**

Soy milk powder contained 9.0 \( \mu \)mol isoflavones/g; 43.3% daidzein, 48.9% genistein and 7.8% glycitein, whereas soy germ powder contained 71.4 \( \mu \)mol isoflavones/g; 48.5% daidzein, 12.6% genistein and 38.9% glycitein (Table 1). Among the selected fourteen subjects, the average fecal incubation half-lives of daidzein and genistein were 15.7±5.3 and 8.9±4.3 hrs, respectively (Table 2).

Linear regression was found in urinary recovery studies over the concentration range used for daidzein, genistein and glycitein (\( R^2 = 0.9984, 0.9979 \) and 0.9928 respectively). The urinary recoveries of daidzein, genistein and glycitein were: 76.4±3.5%, 85.6±3.1% and
61.6±4.2%, respectively. Recoveries of plasma isoflavones were: daidzein; 76.5±5.2%, genistein; 72.3±4.8%, and glycitein; 63.6±4.6%.

After soymilk feeding, the plasma glycitein concentrations were significantly lower than daidzein and genistein, and daidzein was significantly lower than genistein (p < 0.001) at both 6 and 24h. At 6 h after soygerm feeding, the plasma concentration of daidzein was significantly higher (p < 0.001) than genistein or glycitein (Table 3). Glycitein concentration was significantly (p < 0.001) higher than that of genistein in male subjects but not in female subjects. At 24 h after soygerm feeding significantly different plasma concentrations of daidzein and glycitein were only found in female subjects, whereas plasma genistein concentration differed from neither daidzein nor glycitein.

The two soy treatments had no significant influence on the urinary isoflavone excretion as a percentage of ingested doses (Table 4). Men and women had similar urinary recoveries after consuming these two soy products, so pooled results are presented in the table.

Urinary excretion (as a percentage of ingested isoflavone) (Table 4) was significantly less (p < 0.001) for genistein than for daidzein and glycitein, and there were no differences in urinary excretion as percentage dose between daidzein and glycitein after 6 h. After 12 h, all three compounds differed significantly in their urinary excretions in the order of glycitein > daidzein > genistein (p < 0.001). Total excretions of the three compounds after 48 h were significantly different from one another (p < 0.001), just as at 12 h. Interindividual variation in isoflavone excretion was high. Genistein excretion varied by as much as eight-fold (8.5-69.6%), daidzein by five-fold (17.4-87.7%) and glycitein by four and half-fold (19.7-91.3%), among the soy product treatments. Urinary excretion of daidzein and glycitein from both soy
products and genistein from soymilk product were significantly greater during the first 12 h than at later times (p < 0.05).

Discussion

Gut microflora play important roles in isoflavone metabolism and bioavailability (Xu et al., 1995). It is possible that the interindividual variation of isoflavone bioavailability may be due partly to the action of gut microflora. In order to better characterize the bioavailability of isoflavones, in this study, subjects were chosen from volunteers according to the ability of their fecal microflora to degrade isoflavones. The moderate fecal isoflavone metabolism rate phenotype was distinguished by the character that their fecal bacterial could rapidly degrade genistein with average half-lives (G½) of 5.0 h and daidzein (D½) of 17 h (Wang, 1997). In this study, the mean fecal degradation half-lives of genistein and daidzein were 8.9 and 15.7 h respectively. Less fecal degradation would result in appearance of greater amount of isoflavones in the circulation, and greater urinary isoflavone excretion. The average urinary excretion of daidzein (46.4%) was nearly two-fold that of genistein (28.7%) in this study, which agreed with the average fecal metabolism results. However, with relatively few volunteers participating in the prescreening, this study included a few subjects with relatively long or short fecal incubation half-lives (e.g. D½ = 21.2 h and G½ = 16.3 h for one subject; D½ = 7.1 h and G½ = 4.1 h for another subject, Table 1). Individual urinary excretions of isoflavones varied widely (4.5-8 fold) even with selection of subjects by fecal degradation phenotype. Dietary patterns may be able to alter gut motility and fecal isoflavone degradation. For example, increasing insoluble dietary fibers would increase fecal bulk and decrease gut
transit time (Jenkins et al., 1986) and microorganism populations, which would influence isoflavone metabolism and absorption. In a diet controlled study, Karr et al. (1997) fed different levels of soy protein which contained 2.52 μmol daidzein/g protein and 4.07 μmol genistein/g protein to 14 subjects in four 9-day diet treatment periods. Urinary excretion of genistein varied by as much as 12-fold and daidzein by as much as 15-fold within diet treatments. In our study, except for a controlled amount of soyfoods, diet was ad libitum. Compared with Karr’s study, the individual isoflavone urinary excretion varied within a lesser range. Overall, the subject screening method we used seemed to minimize the individual isoflavone excretion variability to some extent. Screening methods similar to ours may be important in understanding the role of gut microflora in phytochemical metabolism and for validation of the health effects of soy isoflavones and related phytochemicals. Further characterization of this screening technique is needed.

Glycitein has an -OCH₃ group at the 6-position and neither glycitein nor daidzein have a 5-OH. This structural difference from genistein may result in less microfloral degradation than for genistein. Griffiths and Smith (1972) reported that isoflavones and flavonoids that possess a hydroxyl group in the 5 position of the A-ring, such as genistein, are much more susceptible to C-ring cleavage by rat gut bacteria. The more isoflavones are broken down by bacteria, the less isoflavones would be detected in urine. This may explain why less genistein was recovered from urine and could also explain why genistein had a shorter half-life than daidzein when anaerobically incubated with fecal samples in vitro (Xu et al., 1995). Gut microfloral isoflavone metabolites, such as equol (Adlercreutz et al., 19820; Axelson et al., 1982), and O-desmethylangolensin (ODMA) (Adlercreutz et al., 1981; Bannwart et al., 1984),
metabolites of daidzein; and p-ethylphenol (Griffith and Smith, 1972), a metabolite of
genistein, have been identified. The metabolites of glycitein are unknown. It will be of great
interest to develop methods to measure glycitein metabolites and to study the potential
biological activity of both glycitein and its metabolites.

When the molar ratio of isoflavones fed was 1.1 genistein : 1 daidzein : 0.2 glycitein
(from soymilk, Table 1), plasma concentrations of genistein were significantly higher than that
of daidzein, and plasma glycitein was nearly negligible. These patterns were similar to the
soymilk feeding study of Xu et al. (1994). When soygerm (isoflavone molar ratios of 0.26
genistein : 1 daidzein : 0.8 glycitein, Table 1) was fed, plasma glycitein concentrations were
significantly lower than daidzein concentrations in women. But plasma glycitein and genistein
did not differ. In men, only at 6 h after feeding soygerm did plasma isoflavones corresponded
with soygerm isoflavone contents (daidzein > glycitein > genistein). The two plasma time
points partially reflected dietary contents of isoflavones as well as individual metabolic
differences. For example, subjects #10 and #11 had the highest fecal degradation half-life for
daidzein (Table 2), and their plasma concentrations and total urinary excretions of daidzein
after soygerm ingestion were also the largest, with 3.10 μmol/L, 60.3% (subject #10) and
3.06 μmol/L, 65.1% (subject #11), respectively. However, the reasons for the observed
gender and time differences (Table 3) are unclear. Plasma pharmacokinetic studies after an
oral dose of nearly equal amounts of genistein and daidzein fed male subjects (Watanabe et al.,
1998) showed that more genistein appeared in the circulation than did daidzein. Our soymilk
feeding study, which had similar proportions of daidzein : genistein to Watanabe et al. (1998),
had similar results to theirs in both plasma and urinary excretion (Table 3 & 4). After soygerm feeding, genistein and glycitein plasma concentrations at 6 h (in women) and 24 h (both genders) did not differ, although the intake of glycitein was about 4 times that of genistein. If glycitein pharmacokinetics followed the same pattern as genistein and daidzein (Watanabe et al., 1988, King and Bursill, 1998), lower plasma concentrations of glycitein than daidzein would be expected because a greater proportion of glycitein dose was found in urine than that of daidzein. According to retention times of the isoflavones on our reverse phase HPLC system, daidzein is more water-soluble than is glycitein, and glycitein is more water-soluble than is genistein. Accordingly, daidzein would be expected to be excreted in urine more readily than genistein, but glycitein ought to be less readily excreted in urine than daidzein. Perhaps glycitein's major metabolite(s) are more water-soluble than are daidzein and genistein's major (glucuronide) metabolites.

Urinary excretion of daidzein within the first 12 h was significantly greater than during the latter 36 h (p < 0.05). This observation agreed with Xu et al. (1994) and Tew et al. (1996). Glycitein showed the same excretion pattern as did daidzein. The proportion of total ingested daidzein, genistein and glycitein excreted in urine were the same after soygerm and soymilk feeding, indicating that isoflavone bioavailability was not affected by soy food type. This result agreed with Tew et al. (1996), who showed that after women consumed a single dose of 3.4 μmol isoflavone/kg body weight as either tofu or TVP, the percentage of ingested genistein or daidzein excreted in urine did not differ between the isoflavone sources.

The present study showed that the urinary disposition of three main isoflavones was different, with more glycitein excreted than daidzein and more daidzein excreted than
genistein. A detectable amount of glycitein appeared in plasma even after soymilk feeding. Although glycitein is a minor isoflavone, these results suggest that determining biological effects of glycitein would be worthwhile.

References


Table 1

*Isoflavone content of soy isoflavone sources*¹

<table>
<thead>
<tr>
<th></th>
<th>Daidzein (µmol/g)</th>
<th>Genistein (µmol/g)</th>
<th>Glycitein (µmol/g)</th>
<th>Total (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soymilk (µmol/g)</td>
<td>3.9</td>
<td>4.4</td>
<td>0.7</td>
<td>9.0</td>
</tr>
<tr>
<td>(%)</td>
<td>43.3</td>
<td>48.9</td>
<td>7.8</td>
<td>100.0</td>
</tr>
<tr>
<td>Soygerm (µmol/g)</td>
<td>34.6</td>
<td>9.0</td>
<td>27.8</td>
<td>71.4</td>
</tr>
<tr>
<td>(%)</td>
<td>48.5</td>
<td>12.6</td>
<td>38.9</td>
<td>100.0</td>
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</table>

¹Sample analysis was performed in duplicate.
Table 2

Subject characteristics and fecal degradation half-lives

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>Weight</th>
<th>Body mass index (kg/m²)</th>
<th>Feeding</th>
<th>D₁₂</th>
<th>G₁₂</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>81.7</td>
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<td>2</td>
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<td>68.1</td>
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<td>22</td>
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<td>4.0</td>
</tr>
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<td>17.7</td>
<td>8.3</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
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<td>81.7</td>
<td>25.22</td>
<td>A</td>
<td>17.3</td>
<td>7.1</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
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<td>74.9</td>
<td>22.37</td>
<td>B</td>
<td>13.5</td>
<td>6.8</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>21</td>
<td>56.8</td>
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<td>5.4</td>
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<td>9</td>
<td>F</td>
<td>35</td>
<td>65.8</td>
<td>25.70</td>
<td>B</td>
<td>5.7</td>
<td>5.6</td>
</tr>
<tr>
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<td>F</td>
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<td>12.3</td>
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<td>56.8</td>
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<tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>2.3</td>
<td>8.9</td>
</tr>
</tbody>
</table>
Table 2 (continued)

1Feeding sequence means which soy product was taken first. A = soymilk intake first; B = soygerm intake first. There was a one-week washout period between each feeding.

2This person did not finish her soymilk. Sample was not collected.

3Daidzein and genistein degradation half-life by gut microflora.
Table 3

Plasma concentration of isoflavones in humans after a single dose of 4.5 μmol total isoflavones/kg body weight from soymilk or soygerm

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0h 6h 24h</td>
<td>0h 6h 24h</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>μmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soymilk powder</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daidzein</td>
<td>ND 1.04±0.61b</td>
<td>0.20±0.11b</td>
<td>ND 1.29±0.50b</td>
<td>0.20±0.05b</td>
</tr>
<tr>
<td>Genistein</td>
<td>ND 1.70±1.01a</td>
<td>0.39±0.28a</td>
<td>ND 1.78±0.83a</td>
<td>0.54±0.35a</td>
</tr>
<tr>
<td>Glycitein</td>
<td>ND 0.20±0.08c</td>
<td>NDf</td>
<td>ND 0.22±0.08c</td>
<td>NDf</td>
</tr>
<tr>
<td>Soygerm powder</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daidzein</td>
<td>ND 1.63±1.03X</td>
<td>0.22±0.15X</td>
<td>ND 1.16±0.44X</td>
<td>0.24±0.04X</td>
</tr>
<tr>
<td>Genistein</td>
<td>ND 0.51±0.19Y</td>
<td>0.16±0.12XY</td>
<td>ND 0.47±0.29Z</td>
<td>0.19±0.07X</td>
</tr>
<tr>
<td>Glycitein</td>
<td>ND 0.73±0.22Y</td>
<td>0.08±0.07Y</td>
<td>ND 0.85±0.25Y</td>
<td>0.29±0.08X</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. n = 6 for female soymilk feeding and n = 7 for male soymilk and both male and female soygerm feeding. ND = not detectable, at a detection limit of 0.05 μmol/L injected.

2 Plasma samples from soymilk and soygerm were statistically analyzed separately. Values in a column with different superscripts are significantly different (p < 0.05).
Table 4

*Human urinary isoflavone excretion as a percentage of dose ingested after a single dose of 4.5 μmol total isoflavones/kg body weight from soymilk vs soygerm*¹

<table>
<thead>
<tr>
<th>Time interval after dosing</th>
<th>Soymilk</th>
<th>Soygerm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% ingested dose</td>
<td>% ingested dose</td>
</tr>
<tr>
<td></td>
<td>Daidzein</td>
<td>Genistein</td>
</tr>
<tr>
<td>0-6 h</td>
<td>14.2±11.5³</td>
<td>6.3±5.4³</td>
</tr>
<tr>
<td>6-12 h</td>
<td>20.5±10.4³</td>
<td>9.5±6.1³</td>
</tr>
<tr>
<td>12-24 h</td>
<td>10.7±7.9</td>
<td>9.0±8.0</td>
</tr>
<tr>
<td>24-48 h</td>
<td>3.2±3.5</td>
<td>2.8±3.6</td>
</tr>
<tr>
<td>Total</td>
<td>48.6±23.1³</td>
<td>27.6±20.7³</td>
</tr>
</tbody>
</table>

¹Values are means ± SD. For soygerm intake results, n = 14; for soymilk intake results, n = 13.

²The amount of each isoflavone compound intake was considered as 100%.

³Values in a column with different superscripts are significantly different (p < 0.05).
Erratum

The soybean isoflavone content was calculated according to their extinction coefficients and normalized to the total daidzein, genistein and glycitein calculated as aglycone isoflavones. Due to re-purification or recent commercial availability, the extinction coefficients of acetylgendistin and acetyldaidzein were re-studied. The new extinction coefficients for these two compounds were 38946 and 29007, respectively.

According to these new data, the isoflavone contents of the soygerm and soymilk used in this feeding study were over-estimated previously. The recalculated isoflavone contents in both soy milk and soygerm powders are shown in Table 1.

Urinary isoflavone excretion as a percentage of ingested dose were modified accordingly. Because there was difference in urinary excretion as percentage of ingested dose according to the soy food administered, combined data are shown in Table 2. Total urinary excretion of daidzein (52.4%) was slightly greater than that of glycitein (46.7%), but there was no significant \( p = 0.08 \) difference between them. Both daidzein and glycitein excretion were significantly greater than that of genistein (37.0%) \( p = 0.0004 \) and \( p = 0.01 \) respectively.

Female subjects excreted a slight greater amount of all three compounds at each time points than did male subjects \( p = 0.03 \). The overall urinary excretion as percentage of ingested dose (total over 48 h) of daidzein, genistein and glycitein were 57.4, 42.3 and 50.2% in female, and 48.8, 36.4, 42.1% in male, respectively.
Based on these new data, the urinary disposition of the three main isoflavones were more daidzein and glycitein excreted than genistein, and daidzein and glycitein had similar excretion. This disposition pattern was different from that reported previously (glycitein greater than daidzein, Zhang et al., 1999). On reverse phase HPLC analysis, daidzein and glycitein appeared earlier than genistein by more than 5 min, and glycitein was eluted about 1 min later than was daidzein. These HPLC results suggested that daidzein and glycitein had similar hydrobicity and they were more hydrophilic than was genistein. The absence of 5-OH group makes both daidzein and glycitein more hydrophilic. The 5-OH forms part of an internal hydrogen bond that makes genistein more hydrophobic although it contains more free hydroxyl than glycitein or daidzein. The absence of the 5-OH probably makes daidzein and glycitein less susceptible to microbial breakdown than is genistein (Griffiths and Smith, 1972). The new calculated data urinary disposition of isoflavones fit well with their observation structural properties.

Little study has been done comparing isoflavone bioavailability between genders. Lampe et al. (1998) fed people with soy protein containing 22 mg daidzein and 8 mg genistein for 4 days. They did not find any differences in daily excretion of daidzein, genistein and O-desmethylangolensin between equol excreters and nonexcreters and between men and women. The slight gender differences in isoflavone urinary excretion observed in this study need further study.

Overall, re-calculation showed that daidzein and glycitein had similar bioavailability and they were more bioavailable than genistein, based on urinary disposition.
Table 1

*Isoflavone content of soy isoflavone sources*

<table>
<thead>
<tr>
<th></th>
<th>Daidzein (μmol/g)</th>
<th>Genistein (μmol/g)</th>
<th>Glycitein (μmol/g)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soymilk</td>
<td>3.3</td>
<td>3.7</td>
<td>0.7</td>
<td>9.0</td>
</tr>
<tr>
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<td>48.1</td>
<td>9.0</td>
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</tr>
<tr>
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<td>27.8</td>
<td>71.4</td>
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<td>41.7</td>
<td>8.1</td>
<td>50.2</td>
<td>100.0</td>
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</tbody>
</table>

1Sample analysis was performed in duplicate.


<table>
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<tr>
<th>Time interval after dosing</th>
<th>0-6 h</th>
<th>6-12 h</th>
<th>12-24 h</th>
<th>24-48 h</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>% ingested dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daidzein(^1,^3)</td>
<td>15.9±11.6(^a)</td>
<td>20.0±9.5(^a)</td>
<td>12.1±9.8</td>
<td>4.3±4.2</td>
<td>52.4±19.6(^a)</td>
</tr>
<tr>
<td>Genistein(^2,^3)</td>
<td>7.9±6.0(^b)</td>
<td>10.8±6.8(^b)</td>
<td>13.0±16.9</td>
<td>5.3±5.4</td>
<td>37.0±25.9(^b)</td>
</tr>
<tr>
<td>Glycitein(^2,^3)</td>
<td>13.5±10.5(^a)</td>
<td>18.7±12.0(^a)</td>
<td>10.7±8.7</td>
<td>3.7±2.0</td>
<td>46.7±18.1(^a)</td>
</tr>
</tbody>
</table>

\(^1\)Values are means ± SD. There was no soy type differences in urinary excretion. A combined data from soymilk and soy germ is presented in this table.

\(^2\)The amount of each isoflavone compound intake was considered as 100%.

\(^3\)Values in a column with different superscripts are significantly different (p < 0.05).
GLUCURONIDES ARE THE MAIN ISOFLAVONE METABOLITES IN WOMEN FED SOY MILK POWDER

A paper to be submitted to the Journal of Nutrition

Yan Zhang\textsuperscript{1}, Suzanne Hendrich\textsuperscript{1,2} and Patricia A. Murphy\textsuperscript{1}

Abstract

Three experiments were conducted to characterize metabolism of isoflavone from soymilk in women: two meals in two weeks separated by a one-week washout, one meal feeding, and six consecutive days of feeding. Urine and plasma samples were extracted directly or pre-digested before extraction with either \textbeta-\textendash glucuronidase/sulfatase (H-2 type) to obtain total isoflavones or \textbeta-\textendash glucuronidase (B-3 type) to eliminate isoflavones that came from sulfate forms. The percentages of glucuronide and aglycone isoflavones were calculated. Among the three experiments, no significant differences were found in the proportion of glucuronide and aglycone isoflavones recovered from plasma or urine. The percentages of daidzein and genistein glucuronides were 76.8$\pm$7.5 and 70.9$\pm$8.4\% of total daidzein and genistein excreted from urine, and 59.4$\pm$9.5 and 50.9$\pm$9.5\% of total daidzein and genistein presented in plasma, respectively. Percentages of aglycone daidzein and genistein were 4.8$\pm$2.9 and 5.7$\pm$3.6\% of total daidzein and genistein in urine, 19.6$\pm$4.9 and 21.2$\pm$7.6\% of total daidzein and genistein present in plasma, respectively. With six-day

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continuous feeding, total urinary excretion at day 5 and 6 respectively were $54.3 \pm 11.9$ and $48.3 \pm 7.6$% of daidzein ingested, and $23.7 \pm 17.0$ and $20.1 \pm 9.7$% of genistein ingested. The $p$ value between these two days was 0.12. These studies show that significant amounts of aglycone isoflavones circulate in the human body. But because the glucuronide isoflavones predominate in vivo, these metabolites might contribute to observed effects of isoflavones.

Key words: glucuronide • aglycone • isoflavone • proportion • feeding protocol

**Introduction**

Since the detection and identification of isoflavonoids in animal and human diets and body fluids, many studies on their biological roles in health and disease have been carried out. The isoflavones daidzein, genistein, glycitein, formononetin, and biochanin A possess estrogenic/anti-estrogenic activities, which may affect hormone production and metabolism as well as cancer cell growth by a number of mechanisms that make them candidates for a protective role with special regard to sex hormone dependent cancers.

Upon ingestion, glycosides of isoflavones may undergo acidic and enzymatic hydrolysis in the intestine. Free aglycone isoflavones undergo further reactions by gut microflora. The metabolism of isoflavones has been of interest, because their metabolites may contribute to observed effects of ingested isoflavones. In the 1950s, the metabolites, equol from daidzein, and daidzein from formononetin have been attributed as the major causes of infertility syndrome among sheep grazing on subterranean clover. The metabolite of genistein, p-ethylphenol, has been considered as a biologically less active compound than is genistein (Shutt, 1976). Both daidzein and genistein are active compounds. Daidzein was
reportedly able to enhance mitogen-stimulated activation of murine lymphocytes in physiologically relevant concentrations of 0.01-10.00 μM, and to enhance mouse immune functions at a dose of 20 or 40 mg/kg body weight, but genistein failed to do that (Zhang et al., 1997 and Wang et al., 1997). Genistein is a specific inhibitor of protein tyrosine kinases, topoisomerase II and histidine protein kinase. Tyrosine kinases appear to play an important role in cell proliferation and transformation. They are correlated with the ability of retroviruses to transform cells, and are also associated with expression of breast cancer oncogenes (Herman et al., 1995). The protein kinase inhibition ability of genistein may explain its cancer prevention effects. Arora et al. (1998) reported that the possible metabolites of daidzein and genistein, 4 and 5-hydroxy derivatives of equol (4'-7-dihydroxyisoflavan), were more potent antioxidants than daidzein and genistein in inhibition of lipid peroxidation in a liposomal system. It is possible that in biological systems, many beneficial effects exerted by soy isoflavone consumption may come from the combined effects of isoflavones and their metabolites. It is worthwhile to study all the metabolites to help us fully understand isoflavone activities and mechanisms of effects.

After absorption, the predominant forms of isoflavones in human urine and plasma are their glucuronide, sulfate, and sulfoglucuronide conjugates (Adlercreutz et al., 1993 & 1995, Yasuda et al., 1994). The aglycone forms are biologically active, as reported by many studies. The activity of both sulfate and glucuronide conjugates are not well studied. The glucuronides may possess biological activity. Weak estrogenic and human natural killer cell activation activities of daidzein and genistein glucuronides at range of 0.1 -10 μmol/L were noted in vitro (Zhang et al., 1999a). Peterson et al. (1998) detected genistein 7-sulfate in
human breast cancer cell lines after incubation with [4-\(^{14}\)C]genistein. But the hydroxylated and methylated metabolites, not the sulfate form, were considered as the active forms of genistein in these cancer cell lines. The conjugates might be deconjugated by the target organ to the active free forms, as with deconjugation of estradiol- and estrone-3\(\beta\)-D-glucuronides demonstrated in Syrian hamster kidney and liver lysosomes and microsomes (Zhu et al., 1996).

Conjugations are considered to be the major detoxification system and glucuronides are the major portion of isoflavones in plasma and urine (Lundh, 1995). Doerge et al (2000) found that several UDP-glucuronyltransferase isoforms catalyze 7- and 4'-glucuronides of isoflavone formation, but the 1A10 isoform from human colon microsomes was specific for genistein, and equal activity was found for daidzein in human liver and kidney microsomes. Even with this detailed study, the distribution of glucuronide, sulfate and free isoflavones in biological systems has not been well characterized. By labeling with \(^{14}\)C, Sfakianos et al. (1997) infused genistein into duodenum and recovered 70-75\% of the dose from bile as 7-\(\beta\) glucuronide conjugate. Recently, by feeding one man with a single dose of soy protein isolate. Cimino et al. (1999) reported that about 86\% of genistein and 75\% of daidzein appearing in urine were glucuronides. In comparison, only 28\% of genistein as glucuronide and 18\% of daidzein as glucuronide were found in Sprague Dawley rats. The aglycones daidzein and genistein were less than 1\% of total isoflavones recovered in urine, but over 20\% of both compounds recovered from rat urine. On the other hand, in their feeding study, Holder et al. (1999) reported that over 90\% of the isoflavones appearing in the blood after Sprague Dawley rats received 0.5-1250 \(\mu\)g genistein/g of chow were genistein glucuronides.
Lundh (1995) reported that the total amount of unconjugated isoflavones in plasma were less than 5% at any time in cows and sheep, with free equol constituting about 5% in cows and 1% in sheep, and daidzein concentrations at around detection limit. No studies have been reported regarding glucuronide distributions in women. In order to probe mechanisms of isoflavone action in vivo, isoflavone metabolism, pharmacokinetics and bioavailability should be understood.

To investigate the proportion of glucuronide and aglycone isoflavones in both plasma and urine after soy consumption, healthy women were fed 25 g soy milk powder at breakfast. Also, three different feeding protocols were used to study isoflavone absorption and excretion patterns.

**Materials and methods**

Three feeding studies were performed. Six healthy female subjects were recruited for the first study. This study consisted of two breakfast meals, which were separated by a 1-wk washout period. Subjects were instructed not to take any foods except the soymilk for breakfast, and to eat nothing before blood was drawn 3 hrs after breakfast. In the second (single meal) and third (six meals) studies, five healthy female subjects participated in each study. The same amount of soymilk (25 g/person) was provided, but bread, milk, bananas, apples and raspberry jelly were also available to them in the latter two studies. Subjects ate whatever they wanted except for avoiding soy foods or foods containing textured vegetable protein and hydrolyzed vegetables protein for at least two days prior to the study. The
Human Subjects Committee of Iowa State University (ISU) approved the procedures for these feeding studies. Informed consent of subjects was obtained in writing.

Soymilk powder was purchased from a local grocery store (Now Foods, Glendale, IL). Isoflavone content was extracted and analyzed by HPLC in Dr. Patricia A. Murphy's laboratory at ISU, with total of 0.82 mg/g soy of daidzein and 0.97 mg/g of genistein on aglycone basis (Wang and Murphy, 1994).

Venous blood samples were collected into EDTA-containing vacuum containers by a licensed medical technologist under stringent aseptic conditions. Blood samples of 10, 25 and 25 mL were collected at 3 h for the first, second and third study, respectively. After dosing, urine was collected over 24 hrs from each subject for the first and second study. For the third study, urine was collected for 24 hrs over each of days 5, 6, and 7. The treatment of blood and urine samples was the same as described by Zhang et al. (1999b).

Total daidzein and genistein concentrations in plasma and urine samples were analyzed by using β-glucuronidase/sulfatase H-2 type (EC 3. 2. 1. 31. from Helix pomatia, Sigma Chemicals) as the deconjugation enzyme. To investigate the amount of isoflavone glucuronide alone, β-glucuronidase B-3 type (EC 3. 2. 1. 31. from bovine liver, Sigma Chemicals), which does not contain any detectable sulfatase activity, was used. The units from B-3 type were adjusted to match the units of glucuronidase in H-2 type. Enzyme of 5,000 units per sample was used for urine analysis and 2,000 units per sample for plasma analysis. A preliminary analysis with greater amount of enzyme at 7,000 or 100,000 units per sample were tested on urine samples and 5,000 units for plasma samples in order to make sure the reaction was complete with the amount we selected. The preparation, extraction and
HPLC methods were the same for both enzyme treatments and were the same as stated in Zhang et al. (1999b).

For the study of free isoflavone concentration in plasma and urine without any deconjugation, 3 mL samples were mixed with 1.5 mL 1.5 mol/L sodium acetate buffer (pH = 5.5), internal standard 2, 4, 4'-trihydroxydeoxybenzoïn (THB) and injected into pre-wet C_{18} Sep-Pak (Minipore Co., Bedford, MA). After washing with 2 mL 0.15 mol/L sodium acetate buffer (pH = 3), plasma free isoflavones were eluted with 4 mL methanol, dried under N\textsubscript{2} and further dissolved with 0.25 mL 80% methanol in water, while urine free isoflavones were washed out with 2 mL 80% methanol in water.

The percentages of daidzein and genistein that come from glucuronidation alone were calculated by the following formula: \((IB_3 - IFr)/IH_2 \times 100\). \(IB_3\) represented isoflavone came from B_{3} type enzyme hydrolysis. \(IFr\) represented free isoflavone without hydrolysis. \(IH_2\) represented isoflavone came from H_{2} type enzyme hydrolysis. The percentages of sulfate isoflavones were calculated as: \((IH_2 - IB_3)/IH_2 \times 100\).

Statistical analysis was applied with the SAS program (version 6.03, SAS institute, 1995 Cary, NC). Total isoflavones and aglycones in both plasma and urine were analyzed separately. When three studies were analyzed together, each study was treated as a block. The differences between urine and plasma glucuronide percentage were compared by paired t-test. A \(P\) value of 0.05 or less was considered to be significant.
Results

In the two-meal feeding study, results were collapsed across feeding, so urinary and plasma results from both feedings were combined among subjects. The urinary excretion of isoflavones over 24 h after soy milk ingestion is shown in Table 1. There were no significant differences (p = 0.13) in total isoflavone excretion between 24 h urine samples analyzed from two-meal and one-meal studies and on days 5 and 6 in the six-meal study. All of these 24 h samples contained significantly (p < 0.05) more isoflavones than did the sample collected on day 7 in the six-meal study. No significant differences of urinary isoflavone excretion (p = 0.12) were found between day 5 and day 6 in the six-meal study. With soymilk intake at breakfast, the average total excretion of daidzein (Daid-H$_2$) and genistein (Gein-H$_2$) was 58.2±11.5% and 22.3±11.6% of ingested doses, respectively, which were significantly (p < 0.001) different between these two compounds. Aglycone isoflavones were also detected in the samples. No significant differences in urinary aglycone excretion were found among the three studies. Total aglycone daidzein and genistein excreted in 24 h urine samples when soymilk was fed at breakfast were 2.5±0.9 and 1.2±0.6% of ingested dose, respectively.

The plasma concentrations of total daidzein (Daid-H$_2$) and genistein (Gein-H$_2$) were significantly (p < 0.05) greater in the two-meal study than in the other two studies (Table 2). No significant differences in plasma concentrations were found between day 5 and day 6 in the six-meal study and between the one-meal and six-meal studies. In all three studies, significant (p < 0.001) differences were found between daidzein and genistein. In the two-meal study, the total amount of plasma obtained was not enough for aglycone isoflavone
analysis by our method. In the one-meal and six-meal studies, we were able to analyze aglycone isoflavone content with increased blood volume. Again, no significant differences were found between plasma taken on day 5 and day 6 in the six-meal study and between the one-meal and six-meal studies, and the amounts of daidzein and genistein found were significantly different ($p < 0.001$). The average plasma aglycone daidzein and genistein concentrations were $0.47 \pm 0.09$ and $0.11 \pm 0.05$ $\mu$M, respectively.

The percentage of isoflavone glucuronide was calculated by deducting isoflavone measured without enzyme digestion from that obtained after B3 enzyme digestion. The amount obtained from this calculation was divided by that seen after H2 digestion and then multiplied by 100. With no significant differences among the three studies, percentages of urine glucuronide or aglycone isoflavone were combined (Fig. 1). For plasma, only the one-meal and six-meal study results were combined. In both plasma and urine, percentage of daidzein glucuronides (DGs, 64.2 and 73.4% respectively) were significantly ($p < 0.01$) greater than that of genistein glucuronides (GGs, 53.4 and 69.1% respectively). The percentage of aglycone genistein (25.6%) was significantly ($p < 0.05$) greater than that of aglycone daidzein (19.4%) in plasma, but not in urine (4.1 and 4.5%, respectively).

Comparing plasma to urine results, the percentages of both DG and GG in plasma were significantly ($p < 0.05$) less than in urine, but the percentages of aglycone daidzein and genistein were the counterpart.

Dihydrodaidzein and dihydrogenistein were detected in urine only as minor metabolites (Fig. 2). Equol was only detected in one subject’s urine in the six-meal study and also as a minor metabolite.
Discussion

In humans, the majorities of isoflavones circulate and are excreted in urine as the glucuronide conjugates, with a much lower percentage existing as other forms. Our study further confirmed this view. The percentages of DG and GG of total urinary isoflavones in our urine samples from female subjects detected by LC-UV system were slightly lower than that (75 and 86%) detected by Cimino et al. (1999) in the male subject by LC-MS system. However, in the same paper, Cimino et al. (1999) detected 52 and 26% of genistein and genistein sulfate, respectively, in their female Sprague-Dawley rat urine. Holder et al. (1999) detected over 90% of genistein glucuronide of total isoflavones in the blood in both male and female Sprague-Dawley rat blood by their LC/ES-MS system. Unfortunately, Cimino et al. (1999) did not report any blood concentrations and Holder et al. (1999) did not report any urine excretion results in their studies. However, based on our study results, blood and urine showed similar proportion of glucuronide vs aglycone isoflavones. Our glucuronidation results and calculated daidzein and genistein sulfates were in the same range as reported by Cimino et al. (1999), as stated above. Holder et al. (1999) did not report any differences in glucuronidation in both male and female rats. These results probably indicated that there was not too much gender difference in isoflavone biotransformation. However, we have to notice the significantly different results obtained by using the same rat strains (Cimino et al., 1999; Holder et al., 1999). Different extraction and analysis methods have been applied by different researchers. In order to fully understand isoflavone metabolism, standard study methods seem to be crucial.
In addition to the conjugated isoflavones and isoflavone aglycones, we also detected dihydrodaidzein and dihydrogenistein in every participant in our study (Fig. 2). Heinonen et al. (1999) detected both compounds in all the subjects when they fed them three soy bars per day for 2 weeks, and they claimed that dihydrodaidzein was the main metabolite of daidzein and dihydrogenistein was the major metabolites of genistein. Kelly et al. (1993) identified dihydrogenistein as a minor metabolite of genistein in urine in only one of 12 subjects after 40 g soy flour challenging for 2 consecutive days. In our study, both dihydrodaidzein and dihydrogenistein appeared as minor metabolites of daidzein and genistein, respectively. These metabolites appeared in the urine after a single feeding as well as during six consecutive days of feeding. The highest amount observed in all the studies was < 0.3 and 0.1 μmol/L for dihydrodaidzein and dihydrogenistein, respectively. Apparently, the formation of dihydrodaidzein and dihydrogenistein were different from equol. Equol appeared after repeated isoflavone feeding as an adaptive metabolite from gut microflora. Feeding length did not make any difference for the appearance of both dihydrodaidzein and dihydrogenistein in the current study. The difference that may explain the different results (dihydroisoflavones as minor or major metabolite) observed by three different researchers may be the population of gut microflora. As we noticed, these three studies were performed in three different countries (Australia, Kelly et al. 1993; Finland, Heinonen et al. 1999; and USA, current study, 2000). Likely isoflavone metabolism varies among people in different regions. However, no direct evidence is available at this time.

In our six-meal feeding study, we did not see any significant differences in both urine isoflavone excretion and 3 h plasma isoflavone concentrations between day 5 and day 6. Xu
(1994) also observed that urinary excretion of isoflavones at day 6 and day 7 of continuous soymilk feeding were similar. With prolonged feeding, a consistent midterm could be observed for isoflavone absorption and excretion. We observed that two out of five persons continuously excreted relatively greater proportion of isoflavones from urine in day 7 than the rest. Also we did not see any differences in urinary excretion and plasma concentrations of isoflavones from these three feeding protocols, which indicated that body isoflavones metabolism was not affected by the minor differences in the 3 protocols used.

We noticed that in our two-meal feeding study a greater plasma isoflavone concentrations was seen than in the one-meal and six-meal studies. The possible reason may be due to the feeding protocol. Subjects were limited to soymilk only at breakfast before the 3 h blood samples in the two-meal study, but had free access to breakfast foods along with soymilk in the one-meal and six-meal studies. As the only available energy source, soymilk may be digested fast and more isoflavones would enter the circulation system earlier. However, comparing 24 h urine excretion, there were no significant differences among the three studies. Either the food consumption after 3 h blood sampling in the two-meal study slowed down isoflavone excretion, or food consumption limited isoflavone conjugation and excretion. The metabolism of isoflavones by gut microflora seemingly was not influenced by meal intake patterns, which needs to be further studied.

The soy milk powder we used contained slightly higher genistein (0.97 mg/g soy) than daidzein (0.82 mg/g soy). In our previous study (Zhang et al., 1999b), at 6 h after a soy containing meal the plasma isoflavone concentrations paralleled the concentrations in the soy foods with genistein concentration higher than that of daidzein. Also, during in vitro
synthesis of isoflavone glucuronides (Zhang et al., 1999a), genistein was a better substrate for the UDP-glucuronyltransferase enzyme than was daidzein. Watanabe et al. (1998) also found that plasma concentration of daidzein was always (72 hrs continuous measurement) lower than that of genistein with their 60 g kinako (103 μmol daidzein and 112 μmol genistein) feeding. There is no evidence that biotransformation activity will dramatically change over the course of a day given normal dietary conditions. We assumed that in our feeding studies the plasma concentrations of genistein at 3 hrs should be higher than that of daidzein, paralleling food content of each isoflavone. But genistein concentrations were lower than daidzein in all three studies. This plasma result paralleled the urine excretion pattern over 24 h (the time course for near total elimination of a single isoflavones dose). Thus either urinary isoflavone excretion or 3 h plasma isoflavone concentrations may be a good reflection of overall isoflavone bioavailability. In the present study, perhaps we captured data that more accurately reflected peak isoflavone concentration in plasma by sampling at 3 h rather than the 6 h time point used in earlier isoflavone bioavailability studies.

Isoflavones can be rapidly glucuronidated in the gastrointestinal mucosa in rats, as shown by Sfakianos et al. (1997) with genistein as the model compared. The proportion of both glucuronide and isoflavone aglycones in plasma significantly differed from urine in the current studies, with relatively greater percentages of aglycone but lower glucuronides found in plasma than in urine, which indicated that other tissues are also important biotransformation sites for isoflavones.
In this study, with three different feeding protocols, we demonstrated that the body metabolizes isoflavones relatively consistently. Glucuronides are the predominant metabolites of isoflavones. Given our previous findings of biological effects of isoflavone glucuronides, these metabolites deserve further study with report to mechanisms of cholesterol lowering (Potter et al., 1998), anticancer (Barnes et al., 1994) and antiosteoporotic effects (Messina and Messina, 2000) of isoflavones. However, considerable amounts of aglycone isoflavones appeared in blood as well. In studying biological effects of isoflavones, nutritionally relevant concentrations of isoflavones and their major metabolites should be considered.

References


Table 1

Percentages (%) of isoflavones excreted from 24 h pooled human urine after ingestion of 25 g soymilk powder*

<table>
<thead>
<tr>
<th></th>
<th>Two meals</th>
<th>One meal</th>
<th>Six meals</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Measured daidzein</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>^Daid-H2</td>
<td>66.4±11.1*</td>
<td>62.0±6.8*</td>
<td>54.3±11.8*</td>
</tr>
<tr>
<td>^Daid-B3</td>
<td>46.4±7.1*</td>
<td>48.5±7.0*</td>
<td>44.5±12.2*</td>
</tr>
<tr>
<td>^Daid-free</td>
<td>2.4±0.8*</td>
<td>2.6±0.6*</td>
<td>2.5±1.4*</td>
</tr>
<tr>
<td>Calculated daidzein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daid-glucuronide</td>
<td>44.0±6.4</td>
<td>45.9±5.2</td>
<td>42.0±11.6</td>
</tr>
<tr>
<td>Daid-sulfate</td>
<td>18.3±6.3</td>
<td>13.6±4.5</td>
<td>9.8±2.6</td>
</tr>
<tr>
<td>Measured genistein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>^Gein-H2</td>
<td>27.3±10.3*</td>
<td>18.1±10.4*</td>
<td>23.7±16.9*</td>
</tr>
<tr>
<td>^Gein-B3</td>
<td>19.3±7.7*</td>
<td>13.3±8.0*</td>
<td>17.1±13.3*</td>
</tr>
<tr>
<td>^Gein-free</td>
<td>1.1±0.5*</td>
<td>0.8±0.4*</td>
<td>1.3±1.0*</td>
</tr>
<tr>
<td>Calculated genistein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gein-glucuronide</td>
<td>18.2±5.3</td>
<td>12.5±6.8</td>
<td>17.0±13.3</td>
</tr>
<tr>
<td>Gein-sulfate</td>
<td>7.7±4.6</td>
<td>4.8±2.5</td>
<td>5.4±3.9</td>
</tr>
</tbody>
</table>

* Letters with different superscripts within the same row indicate the significant differences at p = 0.05 value. The two compounds daidzein and genistein were significantly (p = 0.001) different from each other at all points. N = 6, 5 and 5 for each study, respectively.

1. H-2 type β-glucuronidase/sulfatase was used as the deconjugation enzyme.
2. B-3 type β-glucuronidase was used as the deconjugation enzyme.
3. Extracted directly without using any deconjugation enzyme.
Table 2

*Plasma isoflavone concentrations at 3 h after 25 g soymilk consumption*

<table>
<thead>
<tr>
<th></th>
<th>Two meals</th>
<th>One meal</th>
<th>Six meals</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>day 5</td>
<td>day 6</td>
</tr>
<tr>
<td><strong>Measured daidzein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¹Daid-H2</td>
<td>5.4±0.5</td>
<td>2.9±0.3</td>
<td>2.3±0.6</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>²Daid-B3</td>
<td>4.7±0.6</td>
<td>2.5±0.3</td>
<td>1.9±0.5</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>¹Daid-free</td>
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<td>0.5±0.1</td>
<td>0.5±0.1</td>
<td>0.4±0.0</td>
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<tr>
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<tr>
<td>Daid-glucuronide</td>
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<td>1.9±0.3</td>
<td>1.5±0.5</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td>Daid-sulfate</td>
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<td>0.4±0.2</td>
<td>0.6±0.3</td>
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<tr>
<td><strong>Measured genistein</strong></td>
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<tr>
<td>¹Gein-H2</td>
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<td>0.6±0.2</td>
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<tr>
<td>²Gein-B3</td>
<td>0.8±0.3</td>
<td>0.5±0.1</td>
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<tr>
<td>³Gein-free</td>
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<tr>
<td><strong>Calculated genistein</strong></td>
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<td>Gein-glucuronide</td>
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</table>

* Letters with different superscripts within the same row indicate the significant differences at p = 0.05 value. The two compounds daidzein and genistein were significantly (p = 0.001) different from each other at all points.

1. H-2 type β-glucuronidase/sulfatase was used as the deconjugation enzyme.

2. B-3 type β-glucuronidase was used as the deconjugation enzyme.

3. Extracted directly without using any deconjugation enzyme.
Figure 1: Percentages of conjugates and aglycone isoflavones in both urine and plasma. Samples were analyzed with selective enzymes of β-glucuronidase/sulfatase (H2 type) or β-glucuronidase (B3 type). Free aglycone isoflavones were analyzed without using any hydrolysis enzyme. Glucuronide was calculated by 
\[(B_3 \text{ type-Free}) \times 100 / H_2 \text{ type}\]. Sulfate was estimated by 
\[H_2 \text{ type-B}_3 \text{ type-Free}\]. The percentage of glucuronide form of both daidzein and genistein in urine were significantly \(p = 0.05\) and \(0.001\) respectively) higher than in plasma. The percentage of aglycone daidzein and genistein in plasma were significantly \(p = 0.001\) higher than in urine.
Figure 2: A representative chromatograph of urine samples with H$_2$ type $\beta$-glucuronide as the hydrolysis enzyme. Dein: daidzein; DHD: dihydro-daidzein; DHG: dihydro-genistein; Gein: genistein; THB: 2,4,4'-trihydroxydeoxybenzoin as the internal standard.
THE COMBINATION OF DAIDZEIN, GENISTEIN AND THEIR GLUCURONIDES
ACTIVATED HUMAN NATURAL KILLER CELLS AT NUTRITIONALLY
RELEVANT CONCENTRATIONS

A paper to be submitted to Journal of Nutrition

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Abstract

To characterize modulatory activity and to mimic physiological conditions, a combination of the four isoflavones daidzein, genistein and their glucuronide forms in amounts at which they are found in human blood plasma after the consumption of soybean foods were assayed with human peripheral natural killer (NK) cells and with the NK3.3 cell line. The combination of these four compounds, as well as daidzein glucuronide and genistein glucuronide alone or these two together, activated NK activity in peripheral blood leukocytes (PBLs) at concentrations ranging from 0.25 to 10 μmol/L. Daidzein alone or in combination with genistein had a relatively wider activation range than that of genistein, but a reduced activation activity was seen above 5 μmol/L. Genistein could activate NK cells at concentrations as low as 0.01 μmol/L, but not at 0.001 μmol/L. Neither daidzein nor

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genistein activated the NK3.3 cell line at any of the concentrations studied, suggesting that a
cross-talk existed among PBLs when partially purified human NK cells were used. The
activation of NK cells is an indirect effect of isoflavones.

Key words: • Combination • Isoflavone • Natural killer cell • NK3.3 cell line

Introduction

The role of host immune function has become increasingly important in the
understanding of the mechanisms underlying the body’s ability to prevent cancer. Increasing
evidence has shown that dietary alteration of host immune function is a key component of
chemoprevention (Watson, 1986; Lowell et al., 1990). Isoflavones are phytochemicals that
are mainly obtained from soybeans. Epidemiologic data suggest that consumption of
isoflavone-rich soy foods may contribute to the reduced risk of cancers at many sites
(Messina et al., 1994). As a weak estrogen and an inhibitor of protein tyrosine kinase,
isoflavones, especially daidzein (D) and genistein (G) may be able to influence the natural
immune system which consists of preexisting or rapidly inducible components. Zhang et al.
(1997) fed various doses of daidzein to Swiss mice for seven consecutive days, observing a
stimulatory effect on nonspecific immunity, humoral as well as cell-mediated immunity in
association with high doses of daidzein (20 or 40 mg/kg body weight). Wang et al. (1997)
demonstrated that daidzein (0.01-10 μmol/L) potentiated mitogen-stimulated activation of
murine lymphocytes.

Natural killer (NK) cells, an important component of the natural immune system,
provide an early host response to viral, parasitic and bacterial infections. NK cells have been
shown to provide resistance to some of these infections and to play important roles in tumor surveillance and in the regulation of hemotopoiesis (Scott and Trinchieri, 1995). Tyrosine kinase activity is crucial for the activation of NK cells and genistein is a specific inhibitor of tyrosine kinases. The effect of genistein and other isoflavones on NK cell activity may depend largely on the exposure concentrations. Zhang et al. (1999) found that genistein at concentrations < 5 μmol/L and daidzein and genistein glucuronides (DG and GG) at 0.1-10 μmol/L significantly enhanced NK cell mediated K562 cancer cell killings. At > 5 μmol/L, genistein significantly inhibited NK cytotoxicity.

After consumption, soybean isoflavones are biotransformed by phase II enzymes into conjugates, mainly glucuronides. Based on our human feeding study results and the measurements of metabolites in plasma (Zhang et al., 2000), the percentages of isoflavone glucuronides could reach 65-75% of total isoflavones in plasma. A quantifiable amount of aglycone daidzein and genistein also appeared in plasma. The ratio of DG : GG : D : G at 3 h was around 3 : 1 : 0.3 : 0.1. As stated above, a single isoflavone compound and the conjugates were able to modulate NK cytotoxicity over a wide range of concentrations. We were interested in knowing the effects when these compounds are combined together based on our observations from human feeding studies. Although low plasma concentrations were found for the aglycone isoflavones in plasma, activation of NK activity was usually seen when these concentrations were used in an NK assay. We hypothesized that the combination of D and G and their glucuronides would activate NK cells more effectively than the glucuronide forms alone at physiological concentration.
Materials and methods

Chemicals

Recombinant human interleukin 2 (IL-2) was obtained from Sigma (St. Louis, MO.). Formaldehyde (16%, Ultra pure) was obtained from Polysciences Inc.(Warrington, PA). Sterile sodium chromate ($^{51}$Cr) obtained from NEN Dupont (Boston, MA). Daidzein (D) and genistein (G) were chemically synthesized. Daidzein and genistein glucuronides (DG and GG) were prepared by the methods described in Zhang et al. (1999). FITC anti-human CD16, Biotin anti-human CD56, Biotin Mouse IgG1, κ and FITC mouse IgG1, κ and streptavindin cychrome were all purchased from Pharmingen (San Diego, CA). Gentamicin, L-glutamine and HEPES were from Gibco BRL (Grand Island, NY) and fetal bovine serum (FBS) from JRH, Biosciences (Lenexa, KS). Lymphocult-T was purchased from Biotest Diagnostics Corp (Denville, NJ).

Cell culture

A human NK cell line, NK3.3, cloned from a primary mixed lymphocyte culture, was kindly provided by Dr. Jacki Kombluth (St. Louis University, St Louis, MO). NK3.3 cell line was cultured in RPMI-1640 which contained 2 mmol/L L-glutamine, 25 mM HEPES, 50 μg/mL gentamicin, 15% FBS and 15% Lymphocult-T at 37°C and 7% CO₂. Cells were maintained at a density of $2.5-3 \times 10^5$ cells/mL. The culture medium and maintenance for NK sensitive cancer cell line K562 was the same as described in Zhang et al. (1999).
Natural killer activity assay

Lymphocult-T medium was used to maintain NK3.3 cell line. It contains IL-2 and other active components. In order to see the activation by IL-2, NK3.3 cells were activated for 18-20 h, then centrifuged and separated into two parts: one was resuspended in Lymphocult-T medium, and another was resuspended in regular NK assay medium. NK cytotoxicity was assayed in the presence or absence of IL-2. Lymphocult-T suspensions of NK3.3 cells were not assayed with IL-2 due to lack of further activation when IL-2 was present.

A total of 14 human subjects, seven females and seven males, participated in the current studies. The use of human subjects was approved by the Iowa State University Human Subjects Committee. Basically the preparation and assay methods for NK cell cytotoxicity were the same as stated in Zhang et al. (1999). The main differences were a broader range of isoflavone concentrations. In the NK3.3 cell line study, final G concentrations of 0.01, 0.1, 0.5, 1 and 5 μmol/L were used. A ratio of Effector : Target of 50 : 1 was applied. In the first human NK study, in which 6 (3 female and 3 male) human subjects participated, G concentration of 0.001 μmol/L was also included. Stock solutions of isoflavones were made using 100% DMSO due to their amphipathic/lipophilic nature. DMSO content of assay media in both studies was held constant at 0.014% for all isoflavone concentrations. In the second human NK cell study, in which 8 (4 female and 4 male) human subjects participated, D, G, DG and GG at concentrations of 0.25, 0.5, 5 and 10 μmol/L were used. A combined concentration of D+G or DG+GG at concentrations of 0.5, 1 and 10 μmol/L and a combination of DG, GG, D and G (ratio of 3 : 1 : 0.3 : 0.1) at concentrations of
0.25, 0.5, 5 and 10 μmol/L were assayed. DMSO content of assay media in this experiment was 0.028%. The Effector: Target ratio in human NK assay was 25 : 1.

**Fluorescent staining of lymphoid cells**

Separate aliquots (100 μL) of lymphocyte suspensions were diluted with 0.1% PBS azide (cold) and stained with either 10 μL FITC anti-human CD16 (clone 3G8) and 10 μL Biotin anti-human CD56 (clone B159), or an equivalent amount of isotype control (mouse IgG1-biotin and mouse IgG1-FITC). The solutions were incubated at 4°C in the dark for 30 min and washed with PBS 0.1% azide. The contaminated red blood cells were lysed with ammonium chloride buffer (pH = 7.4). A second step of 0.04 μg strept-Avidin Cychrome was applied. Cells were fixed with 2% PBS paraformaldehyde prior to EPICS-XL-MCI (Coulter, Miami, FL) flow cytometry analysis. Gates were set to distinguish CD16 and CD56 cells.

**Statistical analysis**

Log-transformation was applied to all the NK cytotoxicity data to achieve homogeneity of variance before ANOVA. SAS version 6.06 (SAS Institute, Cary, NC) was used for statistical analysis. A two-way ANOVA was used to compare isoflavones over three concentrations with and without IL-2. Individual treatment differences were determined using student t-test. The percentages of cells carrying CD56* alone or CD56*16* were compared between sexes using two-way ANOVA and combined with covariance for the percentage of NK cytotoxicity. Differences were considered to be significant at $p < 0.05$. 
Results

For purposes of standardization, and due to the variation of NK activity among assays and across subjects, NK cytotoxicity in the absence of IL-2 and isoflavones was set at 100% (ranging from 8.5 to 46.2% in the first six persons, and from 16.6 to 37.7% in the rest 8 persons). All the NK cytotoxicity data were transformed to percentage baseline.

**NK3.3 cell line.** Removing lymphocult-T medium from NK3.3 cells after 18-20 h activation and resuspending them in regular NK assay medium prior to cytotoxic assay was an attempt to mimic conditions used for peripheral blood NK cells. In the absence of IL-2, about 8% activation over baseline was seen at the concentrations of 0.5 or 1.0 μmol/L for D or G respectively. In the presence of IL-2, the cytotoxicity of NK3.3 cells was below baseline with the addition of D. Treatment of lymphocult-T resuspended NK3.3 cells with D or G only raised the killing ability marginally to about 5% over control. However, no significant differences were found for all the isoflavone treatments. The results presented in Fig. 1 were the average of 4 repeated measurements.

**Peripheral NK cells.** Six human subjects were recruited to study the influence of low dose genistein on NK cytotoxicity in vitro. In addition to those doses (0.1-5 μmol/L) presented in Zhang et al. (1999), two new lower doses (0.001 and 0.01 μmol/L) were included (Fig.2). The overall estimation showed that all the concentrations of isoflavones were significantly ($p < 0.01$) different from 0 μmol/L control, except for genistein at 0.001 and 0.01 μmol/L. Significant differences ($p = 0.0001$) were found between the treatment of PBL with and without IL-2. There were significant differences ($p = 0.0001$) in the cytotoxicity among different concentrations of genistein, with 5 μmol/L of genistein
significantly inhibiting the NK cytotoxicity and other genistein concentrations activating NK cells.

Four concentrations (0.25, 0.5, 5 and 10 μmol/L) were included to study the effects of the combined compounds (DG, GG, D and G at ratio of 3 : 1 : 0.3 : 0.1) on NK activity (Fig.3A&B). As a comparison, each single compound was also included and assayed at the same concentrations. Again, significantly (p = 0.0001) increased NK cytotoxicity was seen with the addition of IL-2. However, there was no interaction of IL-2 with isoflavone concentrations. G activated NK cell killing within a very narrow range. Above 0.5 μmol/L, a decrease in lytic activity was seen. At G concentrations of 5 and 10 μmol/L, a significant (p < 0.001) inhibition of NK killing activity was seen. D had a relatively wider activation range than that of G. At 10 μmol/L, we saw decreased lytic activity of NK cells, but it was not significantly (p = 0.44) different from control.

The activation of NK cell activity by DG, GG and 4 compounds combined across all the concentrations were all significantly (p < 0.01) greater than that of control. Interestingly, the combination of 4 compounds activated the NK cells less than DG, GG, the single compound D when compared at the same concentrations, but this difference was not statistically significant. In the presence of IL-2, D, G, DG, GG and 4 compounds combined additively increased NK cytotoxicity at the concentrations used (Fig. 3A).

The combination of D with G, and DG with GG were compared at concentrations of 0.5, 1 and 10 μmol/L as well (Fig. 4). Daidzein was less toxic at high concentrations (10 μmol/L) to NK cells than was G. Even though D+G together (log lsmean = 3.44) still significantly inhibited NK cytotoxicity, the actual NK cytotoxicity was greater than 10
μmol/L G (log lsmean = 3.25) used alone. At lower concentrations, D+G was as active as each compound alone. The combination of DG+GG activated NK cells at all the concentrations, and they were all significantly greater than control.

A significant gender difference was seen in the NK cytotoxicity response to IL-2 (Fig. 5) and was indicated by a significant interaction of gender and IL-2 (p < 0.001). Males had greater response to IL-2 than did females. However, no gender differences were found in the response to isoflavones.

**Fluorescent staining.** The average total population of CD56^- cells among 14 subjects was 23.0±9.1%, and the average CD56^-16^ was 18.0±6.7%. The population of CD56^-CD16^ was not great in people (ranging from 1.2-11.5%), but the NK3.3 cell line (rested) has a relatively high percentage of CD56^-CD16^ (25.3%). After activation for 18-20 h, the CD56^-CD16^ in NK3.3 cell line was increased to 28.6%. A very low percentage of CD56^-CD16^ was found in NK3.3 cell line, with 1.8% in rested cells and 4.0% in activated cells.

We used the percent total CD56^- and CD56^-16^ as covariates to examine gender differences in response to isoflavones and IL-2. However, there was no effect of gender on baseline cytotoxicity. When IL-2 effects were considered, there was significant difference (p < 0.05) between genders in their response to IL-2.

**Discussion**

NK cells have been recognized as earliest effector cells responsible for the elimination of blood-borne metastases. NK activity appears to be important in the
progression of cancer since people who have low NK activity at diagnosis tend to develop metastases more frequently than those with normal levels of NK activity (Whiteside and Herberman, 1994). Patients with congenital or acquired immunodeiciencies, including the absence or defective function of NK cells, developed at a relative high frequency of certain types of malignancies, particularly lymphomas, leukemias and Kaposi’s sarcomas (Ho et al., 1988; Rosen et al., 1995; Ullum et al., 1995). Not only in patients with hematological malignancies, but also in those with solid tissue cancers, levels of NK activity have been documented to decrease in concert with disease progression. Patients with advanced metastases often have abnormalities in NK cell function and/or NK cell numbers (Zeigler et al., 1981; Introna and Montavani, 1983). In non-cancer bearing people, increasing age is associated with decreased NK activity (Meydani et al., 1988). Increasing NK activity in both non-cancer bearing and in tumor-bearing individuals may help prevent or slow the progression of tumor metastases.

As a potential cancer preventive agent, soybean isoflavones genistein and the glucuronide forms of daidzein and genistein showed NK activation activity at low concentrations of 0.1-5 and 0.1-10 μmol/L respectively in a previous study (Zhang et al., 1999). In the present study, we further investigated NK modulation by soybean isoflavones examining the combination of the main plasma isoflavone metabolites. At total concentrations of 0.25-10 μmol/L, the combination of D, G, DG and GG activated NK cell cytotoxicity as significantly as DG, GG used alone or together, which demonstrate that isoflavones and their metabolites can be combined to be an effective NK activator in vitro. Although we had hypothesized that the natural combination of isoflavones would be more
potent as an NK activator, it is not surprising that the activating effect was similar to the individual isoflavone as they may all act through a similar mechanism.

Many investigators have suggested plausible explanations that may account for the observed cancer chemoprevention of soy isoflavones. However, the doses required to show those mechanisms in vitro are usually pharmacological (≥ 50 μmol/L). The maximum plasma level in soy consumption was reported to reach 7-8 μmol/L or lower after hydrolysis of metabolites to parent isoflavone aglycones (Barnes et al., 1995; Xu et al., 1995; Zhang et al., 2000). Based on our recent human feeding study results (Zhang et al., 2000), about 15% of total isoflavones were present in plasma as aglycones and 60% of isoflavones as glucuronides. The circulating levels of aglycone isoflavones after typical soy consumption may be unlikely to exceed 1 μmol/L. Based on these results, we generated a ratio for these major isoflavone metabolites (DG : GG : D : G at 3 : 1 : 0.3 : 0.1) and used it in our NK cytotoxicity study. The highest concentration for the combined compounds was set at 10 μmol/L, a concentration which could be observed from feeding study. Therefore, the concentrations we used in this study were well within physiologically relevant concentrations for human consumption.

As a comparison, each single compound was assayed at the same concentrations as the four compounds in combination. DG and GG were active over a broader range than previously seen with G as before (Zhang et al., 1999). D had similar NK activation activity as G at lower concentrations (below 5 μmol/L). The activation range of D may be broader than G since at 10 μmol/L, the effect of D was not significantly different from baseline, but G significantly suppressed NK activity at this concentration. When D and G were combined,
the log lsmeans for D+G at concentration of 10 μmol/L was 3.44. It was in between D and G (log lemeans 3.61 and 3.26, respectively) when they were used alone, which indicated that both isoflavones probably function at the same site to modulate NK activity.

This study also extended observations from our previous study (Zhang et al., 1999). Two lower concentrations (0.01 and 0.001 μmol/L) of G were assayed (Fig 2). NK cell cytotoxicity was marginally increased with the addition of 0.001 μmol/L G but it did not differ from the control. The significantly increased activation of NK cells with addition of isoflavones was extended from 0.1 μmol/L in the previous study to concentration as low as 0.01 μmol/L in the current study.

NK cells from male subjects had significantly greater response to exogenous IL-2 than female’s NK cells (Fig. 5). Gender differences have been shown in lymphokine production (Tollerud et al., 1989) and in immune response to exogenous compounds like cocaine (Xu et al., 1997). Brett et al. (1999) reported that male peripheral blood lymphocytes showed a significant increase in NK cell cytotoxicity when preincubated with 5 U/mL IL-2 for 70 h, while female cells failed to do so at this concentration. In our study, both males and females had a robust responses to IL-2 and males had a greater response to IL-2 (0.5 ng/well) with a 4.5 h incubation. However, no interaction was found with isoflavone and IL-2 treatments, and all the subjects responded to isoflavone treatments in a similar way. In our previous study (Zhang et al., 1999), we postulated that isoflavone activated NK cells from a site different from IL-2. Our current study further confirmed this view. Isoflavones have similar structures as the natural estrogen, estradiol and had weak estrogenic activity in some biological systems (Farmakalidis and Murphy, 1984; Song et al., 1999). In female rats, a
diminished natural killer cell activity was observed with short term exposure to elevated estradiol and low progesterone levels (Ben-Eliyahy et al., 1996). If estrogenic activity is the mechanism that could explain isoflavone effects, we would have expected a different response in males and females to isoflavones. Apparently, other mechanisms rather than estrogenicity should be considered in isoflavone's NK activation functions.

Research on NK cell biology using partially purified NK cells cannot distinguish cytotoxicity due to other immune cells, or rule out possible cross-talk between other cell types as an activation mechanism, or solve the problem of collecting an adequate quantity of pure NK cells from peripheral blood. The NK3.3 cell line was used. It was produced by harvesting peripheral blood leukocytes by density gradient centrifugation from heparinized blood from two healthy blood providers by Dr. Jacki Kornbluth (St. Louis University). This cell line has been used to provide explanations for biochemical and molecular events related to NK cell functions (Cone et al., 1993; Ortaldo et al., 1997; Umehara et al., 1997; Zhou et al., 1997). We used this cell line to help clarify the effects of isoflavones on NK cell activity. Several aspects were noticed with this cell line. The sensitivity of the K562 cancer cells to NK3.3 cell killing was not as strong as they are to the peripheral NK cells, so a higher Effector: Target ratio (50:1 instead of 25:1) was used for the cell line. This cell line was mainly of the CD56<sup>+</sup>CD16<sup>+</sup> population, but NK cells separated from human peripheral blood were mainly CD56<sup>+</sup> cells. NK3.3 cells are IL-2 dependent. After depletion of the medium and switching to regular NK assay medium, the NK cell line had a relatively lower response to IL-2 compared to the strong response by most human NK cells. The average percentage of killing after adding IL-2 over non-IL-2 treatment was 176% (ranging from 116-219%) in
NK3.3 cell line and 196% (ranging from 152-247% from 8 subjects) in human. The different response of NK3.3 cell line to IL-2 in each experiment was similar to the variability observed in humans. No additional activation was seen with IL-2 in the lymphocult-T medium, but the cytotoxicity by lymphocult-T medium alone (241%) was much greater than after the addition of IL-2 to the NK assay medium indicating that other components in the lymphocult-T medium were also important in activating the NK3.3 cell line.

No effect was seen with the addition of D or G at all the concentrations in NK3.3 cells (Fig. 1). A significant increase in cytotoxicity was seen with human peripheral blood NK cells when D or G was added at concentrations of 0.01-1 μmol/L (Fig. 2), which increased to a wider range of concentrations (up to 10 μmol/L) when DG or GG was added (Fig. 3). Our initial goal was to use the cell line to probe the molecular mechanism of the activation with isoflavones seen in human peripheral blood lymphocytes. We did not achieve this purpose. Clearly, a cross-talk existed within cells when we used partially purified peripheral NK cells. NK cells are regulated by numerous factors. Secretion of specific cytokines or regulation by specific cytokines from other cells are important effectors of NK cells. The most potent activators of NK cells are IL-2 (which we added), IL-12, IFN-γ (Sivakumar et al., 1998). To a lesser degree the following cytokines are also known to activate NK cells: IL-1, IL-15 and IFN-α and β (Kuby, 1997). It will be interesting to know which cytokine secretions are changed after co-culturing peripheral NK cells with isoflavone(s) within the assay period.

In summary, the present study provided direct in vitro evidence that isoflavones in combination of daidzein, genistein and their glucuronides activated NK cells within
nutritionally relevant concentrations. Studies with NK3.3 cell line indicated that the activation observed with isoflavones came from an indirect effect of isoflavones on NK cells. Further studies are needed in order to clarify the mechanisms involved in NK modulation activity by isoflavones and to provide more support for their cancer preventive effects.

References


Figure 1: Cytotoxicity of NK3.3 cell line against K562 target cells, expressed as percentage of baseline cytotoxicity. Each point represents the average of four different experiments from triplicate assays. In between the concentrations of 0.1-1 µmol/L, both compounds D and G marginally increased NK3.3 activity except D at the presence of IL-2. However, both compounds did not statistically significantly change NK3.3 cell's killing ability. Significant differences ($p = 0.001$) were seen among the three media treatments: NK assay media with or without IL-2, and the lymphocult-T media. D = daidzein; G = genistein; lymp represent lymphocult-T media. The regular NK assay media treatment with or without IL-2 was not specifically labeled in the graph, but are represented by the 0 µmol/L isoflavone concentrations.
Figure 2: Cytotoxicity of human peripheral blood natural killer cells against K562 target cells, expressed as percentage of baseline cytotoxicity. Genistein incubated with or without interleukin 2 (IL-2) (0.5 ng/well) was shown in this graph. Each point represents the mean cytotoxicity in 6 subjects from triplicate assays. Genistein at concentrations of 0.01 μmol/L and below marginally but not statistically significantly increased NK killing. Symbol * denotes the significant increase in NK cytotoxicity at concentrations of 0.1-1 μmol/L compared to control (p = 0.001). Symbol + represents the significant (p = 0.0001) decrease of NK activity at 5 μmol/L of genistein compared to control. G = genistein.
Figure 3: Cytotoxicity of human peripheral blood natural killer cells against K562 target cells, expressed as percentage of baseline killing. Each point in both graphs represents the mean cytotoxicity in 8 subjects. Graph A represents the individual results of 4 different isoflavone compounds (D, G, DG and GG) and the combination of these 4 compounds at concentrations of 0.25-10 μmol/L incubated with and without IL-2 (0.5 ng/well). Graph B is the overall analysis collapsing across IL-2 treatment. The four compounds in combination at all concentration were significantly different from baseline. Genistein at concentrations of 5 and 10 μmol/L were significantly different from baseline ($p = 0.001$). Symbol * represents the significant increase in NK cytotoxicity of the compound cluster. Symbol # indicates that no differences were found compared to baseline. Symbol + represents the significant decrease with the adding of isoflavone in NK cell killing ability. D = daidzein; G = genistein; DG = daidzein glucuronide; GG = genistein glucuronide; 4C = combination of D, G, DG and GG.
Figure 4: Results of two isoflavone combinations: D+G and DG+GG at three different concentrations (0.5, 1 and 10 μmol/L) incubated with and without IL-2 (0.5 ng/well). The overall estimation indicated that both combinations cross all the concentrations were significantly ($p < 0.01$) different from control. Symbols have the same meaning as in Figure 3.
Figure 5: Comparison of cytotoxicity differences in gender in response to IL-2. Each point represents the average from 4 persons. Males had significantly greater response to IL-2 than did females. Interaction of IL-2 * gender was significant at $p = 0.0001$. 
GENERAL CONCLUSIONS

Glycitein, as the third main isoflavone that is found in soy foods, has not attracted enough attention. There are few reports about glycitein’s biological activity as well as its bioavailability. Our human feeding study showed that glycitein had similar bioavailability to daidzein, and was more bioavailable than genistein. A gender difference was seen with isoflavone bioavailability which needs to be further explored.

Most studies about isoflavones are focused on their aglycone forms. Not too much research has been done with the major glucuronide metabolites. By using selective hydrolytic enzymes, our human feeding study showed that daidzien and genistein glucuronides were the dominant forms in plasma and in urine. A detectable amount of the aglycones daidzein and genistein also appeared in plasma. As the major metabolic forms appearing in circulation, the glucuronides might contribute to biological effects observed with isoflavones.

As an early line of defense, natural killer (NK) cells play an important role against cancer and some infections. It is important to keep NK cells active or to increase their activity. Daidzein and genistein glucuronides, the main metabolites in the circulation were assayed for their NK activation activity when they were in combination with the aglycone daidzein and genistein in nutritionally relevant ratios and concentrations. Our results showed that the combination of these four compounds activated human NK cells to the same level as each glucuronide used alone, and activated across a wider concentration range than the aglycones alone. No effect of isoflavone treatment was seen on the NK3.3 cell line, indicating that isoflavones activated NK activity indirectly. Our results suggested that
glucuronide forms had some biological activity, at least in in vitro. Isoflavone glucuronide activities in other biological systems should not be ignored.
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