Influence of inulin on plasma isoflavone concentrations in healthy postmenopausal women

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ABSTRACT

Background: Bacterial intestinal glucosidases exert an important role in isoflavone absorption. Insoluble dietary fibers such as inulin may stimulate the growth of these bacteria in the colon and, hence, stimulate the absorption of these substances in subjects who may need isoflavone supplementation.

Objective: The objective was to assess the influence of inulin on plasma isoflavone concentrations after intake of soybean isoflavones in healthy postmenopausal women.

Design: Twelve healthy postmenopausal women participated in a randomized, double-blind, crossover study. They consumed 40 mg of a conjugated form of soybean isoflavones (6 mg daidzein and 18 mg genistein as free form) with or without 3.66 g inulin twice daily in two 21-d experimental phases. Blood samples were collected 0, 1, 2, 3, 4, 6, 10, 12, and 24 h after intake of isoflavones with breakfast and dinner at the end of each 21-d experimental phase. Plasma concentrations of isoflavones were assessed by HPLC with an electrochemical detector.

Results: Plasma 24-h areas under the curve indicated that the intake of soybean isoflavones with inulin for 21 d was followed by higher plasma concentrations of daidzein and genistein (38% and 91%, respectively) compared with the formulation without inulin. Furthermore, the time for the maximum concentration of daidzein and genistein appeared to be lower after the 21-d intake of soybean isoflavones, with or without inulin. However, the time for the maximum concentration of daidzein and genistein after supplementation with the inulin-containing formulation on day 21 was not significantly different from that after supplementation with the formulation without inulin.

Conclusions: Inulin may increase the apparent plasma concentrations of the soybean isoflavones daidzein and genistein in postmenopausal women. The higher plasma concentrations of the 2 isoflavones suggest that the absorption of each was facilitated by the presence of inulin. Am J Clin Nutr 2007;86:775–80.

KEY WORDS Isoflavones, plasma inulin concentrations, postmenopausal women

INTRODUCTION

The role of dietary flavonoids in the prevention of several chronic diseases is the subject of intense research, and soybean isoflavones have been the focus of particular attention (1–3). Assessment of the potential importance of flavonoids in human health, however, should be supported by the best knowledge of the absorption, distribution, metabolism, and excretion of these substances after ingestion.

Estrogen replacement therapy is effective in prevention as well as in the treatment and reversal of postmenopausal bone loss. However, this therapy is associated with serious side effects, such as breast or uterine cancer (4). Recent reports have concluded that the combined use of estrogen and progesterone as part of hormone replacement therapy is partially effective in the prevention of coronary heart disease and cognitive decline (5, 6). Consequently, there is considerable interest in the identification of alternative estrogens for the prevention of these chronic diseases. Soybean contains large quantities of isoflavones, the so-called phytoestrogens. These substances include genistein, daidzein, and glycitein, which have structural similarities to 17β-estradiol and exhibit estrogenic-like effects by binding to estrogen receptors (5). Epidemiologic studies indicate a lower incidence of osteoporosis in populations who consume more soybean and soy products (7).

In postmenopausal women, soybean isoflavones are an alternative to conventional hormone replacement therapy because of their cholesterol-lowering action and hot flush prevention (8–10) and because of their effect on bone (11–13). Furthermore, daily consumption of soy food may induce endocrine effects in postmenopausal women (14, 15).

Bacterial intestinal glucosidases (ie, from lactobacilli, bacteroides, and bifidobacteria) have an important role in the absorption of isoflavones (16). Insoluble dietary fibers such as fructooligosaccharides and inulin may stimulate the growth of these bacteria in the colon (17). Thus, it is possible that supplementation with inulin may influence plasma concentrations of soybean isoflavones, possibly by increasing their absorption. Because of the relevance of isoflavones on alimentation after menopause, the present study investigated the influence of inulin on plasma concentrations of soybean isoflavones in healthy postmenopausal women.

SUBJECTS AND METHODS

Isoflavone formulations

We compared 2 drinkable formulations (prepared by Pharbenia-Bayer, Milan, Italy) containing 40 mg of a conjugated...
form of soybean isoflavones (25 mg free form): 6 mg daidzein, 18 mg genistein, and 1 mg glycin. Both formulations also contained 500 mg Ca and 7.5 μg (equivalent to 300 units) vitamin D₃. The formulations identified as Iso contained no inulin, whereas the Iso+ Inu formulations also contained 3.66 g inulin.

Subjects
Twelve healthy postmenopausal women aged 51–64 y participated in this study. They were derived from a group of 32 volunteers initially prescreened and selected by phone interview from 76 volunteers who responded to an announcement posted in the local media. Screening of recruited volunteers concerned general health status, body mass index (BMI; in kg/m²), gastrointestinal function, and dietary habits (with the use of a dietary questionnaire). Volunteers were excluded if they had known or suspected hypersensitivity toward any of the constituents of the study medication or an allergy, had given blood within the past 3 mo, had any condition likely to change the absorption of the study medication, or had a BMI < 18 or > 30. The subjects were asked not to change their dietary habits, not to consume food containing soybean products or isoflavones, and not to change their physical activity patterns during the period of the study. In particular, they were instructed to consume no soy products from the time of screening until the end of the trial. The study was conducted at the University of Catania Medical Center (UCMC) in accordance with the protocol, good clinical practice requirements, and the principles of the Declaration of Helsinki and the applicable regulatory requirements. All subjects provided written informed consent. The study design and protocol, the informed consent text, and the fee amount for the subjects were approved by the Ethical Committee of the UCMC.

Experimental design
The crossover study was double-blind and randomized and consisted of 2 experimental phases. On day 1 of the first experimental phase, the subjects who had fasted overnight (14 h) were admitted to the UCMC. A venous blood sample (0 time) was collected into heparinized tubes. Within 30 min, the subjects consumed a standard breakfast and thereafter 1 of the 2 experimental formulations according to the randomization assignment for this phase. Breakfast consisted of milk and 6 biscuits and optional coffee or tea with sugar. Additional blood samples were withdrawn 1, 2, 3, 4, 6, 10, and 12 h after intake of the study formulation. The subjects remained at the UCMC during the 12-h period. They consumed a standard lunch at noon after blood was drawn at 4 h and consumed dinner at 2100 after blood was drawn at 12 h. Lunch consisted of lettuce, sliced tomato, shredded carrot, 2 pieces of white bread, tuna, mayonnaise, a fresh apple, a small package of butter cookies, and regular ginger ale. Italian dressing, salt, and pepper were optional. Dinner consisted of tomato soup, herbed chicken prepared with garlic powder, corn oil, oregano, cooking sherry, broccoli, a dinner roll, butter, sparkling water, and salt and pepper (optional). The subjects spent the night at the UCMC to provide a blood sample 24 h after intake of the study formulation. They then consumed the formulation twice daily, always at 0800 and 2000, and returned to the UCMC 20 d later. Blood samples were drawn according to the same procedure as described for day 1 of the first experimental phase. After a 7-d washout period, the subjects were readmitted to the UCMC, and the same procedure used during the first phase was repeated in the second experimental phase according to the randomization assignment applied for this phase. Blood samples (8 mL) were obtained by venipuncture via an indwelling catheter for the more frequent samplings or were collected into evacuated tubes for the later sample times, depending on the choice of the individual. Blood samples were centrifuged at 3000 × g for 10 min at 4 °C within 30 min of blood withdrawal. Plasma was separated and stored at −20 °C until analyzed.

Adverse events experienced throughout the study were recorded for each subject. The Common Toxicity Criteria suggested by the National Cancer Institute (Bethesda, MD) were used to assign a severity grade to each event.

Measurement of plasma isoflavones
Concentrations of isoflavones (daidzein and genistein) were measured in plasma samples by HPLC with electrochemical detection (HPLC/ECD). Estriol 3-(β-glucuronide) was used as internal standard. Daidzein (product no. D7802), genistein (product no. G6776), and estriol 3-(β-glucuronide) sodium salt (product no. E2002) were obtained from Sigma-Aldrich (St Louis, MO). Plasma preparations were derived according to a previously described HPLC-ECD procedure (18). One milliliter of plasma was mixed with 15 μL of 60 μg/mL estriol 3-(β-glucuronide) solution, 0.25 mL of 1.0 mol ammonium acetate buffer/L (pH 5.0), and 1000 units β-glucuronidase from Helix pomatia (Sigma, St Louis, MO) and incubated overnight at 37 °C to release the free forms of isoflavones from the glucuronide. After the addition of 0.1 mL glacial acetic acid, the hydrolysates were washed with 5.0 mL hexane.

Daidzein and genistein were extracted twice with 3 mL ethyl ether, and the extracts were evaporated with a vacuum system (Buchi multiple evaporator); the resulting residue was dissolved in 100 μL methanol followed by the addition of 100 μL mobile phase and an aliquot of 100 μL was injected into the HPLC column. The HPLC system was composed of a ProStar 410 AutoSampler VARIAN (Palo Alto, CA) series, ProStar 230 Solvent Delivery Module, and an 8-channel CouArray detector ESA (Chelmsford, MA) model 5600A. Applied potentials were 460, 530, and 560 mV. The separation was done on a polar column (LiChrospher 60RP-select B, 125 mm × 4 mm, 5 μm; Merck, Darmstadt, Germany) and precolumn (LiChroCart, 4 mm × 4 mm, 5 μm; Merck, Darmstadt, Germany). The mobile phase was composed of 2 mixtures: mixture A consisted of water-methanol-acetonitrile (55:40:4, by vol) containing 0.2 mol ammonium acetate buffer/L (pH 4.8); mixture B consisted of water-acetonitrile (20:75:5, by vol) containing 0.2 mol ammonium acetate buffer/L (pH 4.8). The flow rate was set at 0.7 mL/min. A gradient in 10 min, from 0% (by vol) to 70% of mobile phase B, was introduced 10 min after injection. At 25 min, the system returned to 100% A in 5 min and was kept under this condition for 3 min to reequilibrate. The eluent of isoflavones was quantified by determining the peak areas in the chromatograms calibrated against known amounts of standards with high purity (>99%). All plasma samples collected from each subject during the 2 phases were analyzed in the same batch. The postprandial absorption and metabolism curves for genistein and daidzein were constructed by plotting plasma concentrations over time. The maximum concentration in plasma (Cmax) postprandially, the time for the maximum concentration to be reached (tmax), and the area under the curve (AUC₀–tmax) were determined.
However, the AUC0–24 values on day 21 for both isoflavones, Iso and Iso, were higher than those at baseline (day 1) after the consumption of both the Iso and Iso+Inu formulations. Because there was considerable variation in the rate of absorption and appearance of isoflavones in plasma between the subjects, the AUC0–24 values for daidzein and genistein from the study formulations are shown in Tables 1 and 2. Because all of the subjects consumed the same defined and controlled diet during the first 24 h on days 1 and 21 of each experimental phase while they stayed at the UCMC, we compared the AUC0–24 values for plasma daidzein and genistein.

After 21 d of supplementation with soybean isoflavones, the AUC0–24 values for daidzein and genistein appeared to be higher than those at baseline (day 1) after the consumption of both the Iso and Iso+Inu formulations (Table 1 and 2, respectively). However, the AUC0–24 values on day 21 for both isoflavones were higher after the Iso+Inu formulation than after the Iso formulation. This increase was 38% (95% CI: 5.6) for daidzein and 91% (95% CI: 12.2) for genistein.

Plasma $C_{\text{max}}$ values of daidzein appeared to be higher after 21 d of supplementation with both the Iso and Iso+Inu formulations (Table 1). In contrast, plasma $C_{\text{max}}$ values of genistein appeared to be higher only after supplementation with the Iso+Inu formulation.

As shown in Tables 1 and 2, we observed a decrease in $t_{\text{max}}$ values for daidzein and genistein after 21 d of supplementation with both the Iso and Iso+Inu formulations. However, the $t_{\text{max}}$ value after supplementation with the Iso+Inu formulation was not significantly different from that after the Iso formulation on day 21 for either isoflavone. No adverse events were recorded for any of the subjects participating in the trial.

**RESULTS**

The postprandial plasma concentrations and time curves for daidzein and genistein in postmenopausal women after intake of the Iso and Iso+Inu formulations are depicted in Figure 1 and Figure 2, and the pharmacokinetic parameters of these concentrations and time curves are summarized in Table 1 and Table 2.

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**DISCUSSION**

The aim of the present study was to investigate whether inulin enhances soybean isoflavone absorption in postmenopausal women. The results indicated a significant difference in plasma isoflavones concentrations as a result of the presence of inulin. In fact, daidzein and genistein absorption appeared to be relatively faster, and $C_{\text{max}}$ values for plasma isoflavones were found at times ranging from 2 to 8 h after ingestion. Furthermore, after a 21-d supplementation period, plasma concentrations of both daidzein and genistein were significantly higher in subjects who took isoflavones with inulin. This difference was particularly evident for genistein, the AUC0–24 of which increased by ~91% in the subjects who consumed isoflavones with inulin for 21 d compared with the increase in the daidzein AUC0–24 of only ~38%.

Equol is one of the daidzein metabolites produced by the intestinal microflora and absorbed in plasma, where it remains for a relatively longer time than genistein and daidzein (21). Because equol concentrations were not measured in our samples, we do not know whether inulin in the Iso+Inu formulation caused modifications in plasma equol concentrations. However, it should be considered that other studies did not consider plasma equol concentrations as an important measure of soybean isoflavone pharmacokinetics (22).

It is not easy to explain the mechanism by which isoflavone absorption was facilitated by inulin intake under the present experimental conditions. The chemical forms in which isoflavones appear in food or supplements have been considered to be important to their plasma concentrations and, thus, for their biological activity. Isoflavones in soybean exist in 4 chemical...
forms: aglucone, glucoside, acetylg glucoside, and malonylglucoside. However, the glucoside forms are predominant (23) and represent the major naturally occurring isoflavones in soybean and soybean-based food products (24, 25), although variation in food-processing techniques may alter the relative content of acylglucoside, malonylglucoside, and simple glucosides (26). After ingestion, isoflavone glucosides are hydrolyzed to their aglucones by phlorizin lactose hydrolase in the apical membrane of the lumen of the small intestine and by bacterial intestinal glucosidases (ie, from lactobacilli, bacteroides, and bifidobacteria) (16, 27). Conversion of daidzein into its metabolites dihydridaidzein, O-desmethylyangolensin, and equol also precedes absorption from the colon. After hepatic uptake and excretion into the bile of the β-glycuronides genistein, glycitein, and daidzein, and its metabolites, a second round of hydrolysis occurs (28). Insoluble dietary fibers, such as fructooligosaccharides and inulin, may stimulate the growth of these bacteria in the colon (17). Thus, it is possible that inulin-stimulated bacterial growth induced soybean glucoside conversion and, hence, facilitated their absorption.

It is possible that inulin altered the pharmaceutical properties of the formulations that were prepared for this study. This is a well-known problem in the pharmaceutical industry dealing with food products, which puts considerable effort into the preparation of drugs to overcome this difficulty. Concern for this issue comes from the study by Busby et al (21), which suggests that significant amounts of isoflavones are never absorbed or excreted in the feces or may be metabolized to other compounds that were not measured.

The intestinal absorption of isoflavones requires a release of free forms from glucosidic conjugates. Thus, intestinal absorption of glucoside forms of isoflavones can slow down until they enter the large intestine, where colonic microflora may release aglucones (29). However, glucoside forms are subjected to hydrolysis by β-glycosidases in the small intestine and appear in plasma within a short period of time (30, 31). Other factors, including dietary habits, the food matrix, the extent of intestinal bacterial fermentation, intestinal transit time, and age may influence the intestinal metabolism and plasma concentrations of isoflavones in humans (32, 33).

Whether a 7-d washout period was sufficient to avoid the possible enterohepatic recirculation of isoflavones is not certain. In fact, it is possible that a pool of isoflavones in the gut undergoes enterohepatic recirculation, which allows a postprandial reentry into the blood and alters the AUC calculations for the new preparation. However, preclinical studies in rats suggest that genistein is absorbed very well from the intestines and is excreted into the bile in only small proportions. An important factor that may alter the initial intestinal absorption and the subsequent enterohepatic recirculation of genistein is bacterial metabolism.

### TABLE 1
Pharmacokinetics parameters of daidzein

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<tr>
<th>Daidzein</th>
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<td>Iso formulation</td>
<td>Iso+Inu formulatation</td>
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<tr>
<td></td>
<td>Day 1</td>
<td>Day 21</td>
</tr>
<tr>
<td>AUC_{0–24}</td>
<td>4083.0 ± 181.0</td>
<td>4614.3 ± 188.8</td>
</tr>
<tr>
<td>C_{max}</td>
<td>240.4 ± 10.3</td>
<td>408.7 ± 39.5</td>
</tr>
<tr>
<td>t_{max}</td>
<td>7.9 ± 0.3</td>
<td>5.5 ± 0.2</td>
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</table>

All values are x ± SD; n = 12 healthy postmenopausal women. The SAS statistical software package (SAS Institute, Cary, NC) was used to analyze the data. AUC_{0–24}, area under the curve over 24 h calculated by using the Westlake and Schuirmann test; t_{max}, time for the maximum concentration (C_{max}) to be reached determined by using a nonparametric test (Wilcoxon matched-pair ranks test). The Iso formulation contained soybean isoflavones without inulin; the Iso+Inu formulation contained soybean isoflavones with inulin. Each of the 2 formulations was consumed for 21 d. There were no significant differences between the day 1 values for any of the variables. The final AUC_{0–24} for daidzein calculated for the Iso+Inu formulation was 38% higher than that for the Iso formulation.

1 Significantly different from the day 1 value (P < 0.05, ANOVA) and from the day 21 value (P < 0.01, ANOVA) for the Iso formulation.

2 Significantly different from the day 1 value (P < 0.05, Wilcoxon matched-pair ranks test).

### TABLE 2
Pharmacokinetics parameters of genistein

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<tr>
<th>Genistein</th>
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<tr>
<td></td>
<td>Iso formulation</td>
<td>Iso+Inu formulatation</td>
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<tr>
<td></td>
<td>Day 1</td>
<td>Day 21</td>
</tr>
<tr>
<td>AUC_{0–24}</td>
<td>4772.9 ± 206.6</td>
<td>4397.0 ± 236.9</td>
</tr>
<tr>
<td>C_{max}</td>
<td>294.6 ± 12.1</td>
<td>288.2 ± 18.5</td>
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<tr>
<td>t_{max}</td>
<td>20.2 ± 0.7</td>
<td>8.0 ± 0.6</td>
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All values are x ± SD; n = 12 healthy postmenopausal women. The SAS statistical software package (SAS Institute Inc, Cary, NC) was used to analyze the data. AUC_{0–24}, area under the curve over 24 h calculated by using the Westlake and Schuirmann test; t_{max}, time for the maximum concentration (C_{max}) to be reached determined by using a nonparametric test (Wilcoxon matched-pair ranks test). The Iso formulation contained soybean isoflavones without inulin; the Iso+Inu formulation contained soybean isoflavones with inulin. Each of the 2 formulations was consumed for 21 d. There were no significant differences between the day 1 values for any of the variables. The final AUC_{0–24} for genistein calculated for the Iso+Inu formulation was 91% higher than that for the Iso formulation.

1 Significantly different from the day 1 value (P < 0.01, ANOVA) and from the day 21 value (P < 0.01, ANOVA) for the Iso formulation.

2 Significantly different from the day 1 value (P < 0.05, ANOVA).

3 Significantly different from the day 1 value (P < 0.05, Wilcoxon matched-pair ranks test).
replacement therapy. A daily intake of hot flushes (8–10) as an alternative to conventional hormone therapy in women (19). Menopausal subjects are primarily interested in treatments. However, it should be noted that the pharmacokinetic parameters measured in the postmenopausal women in the present study were lower than those described by others in premenopausal subjects (17, 19). Thus, it seems likely that postmenopausal women may show decreased absorption of isoflavones and need higher doses to achieve plasma concentrations similar to those of younger subjects. Indeed, gut microflora is important for plasma soybean isoflavone concentrations in women (19). Menopausal subjects are primarily interested in isoflavone supplementation because these substances are chemically and functionally similar to 17β-estradiol and seem to be effective during menopause at preventing osteoporosis (7, 11–13), lowering plasma cholesterol concentrations, and preventing hot flushes (8–10) as an alternative to conventional hormone replacement therapy. A daily intake of ~50 mg isoflavones, which is equal to the dose adopted in the present study (6 mg daidzein, 18 mg genistein, and 1 mg glycitein twice daily), seems to have been based largely on the early observation that the daily consumption of soy foods providing 45 mg isoflavones resulted in hormonal changes in menopausal women (14, 15).

The excellent tolerability of soybean preparations used in the present trial does not deserve comment. Indeed, in another study, dietary supplementation with purified unconjugated isoflavones administered in single doses greatly exceeding normal dietary intake resulted in minimal clinical toxicity (22).

More research is required to establish precisely the health benefits of soybean isoflavones, the site and the mechanism of their absorption, and their interaction with inulin. Interestingly, a randomized, double-blind, placebo-controlled study recently found that dietary supplementation of functional foods with isoflavones may cause changes in the diversity and composition of dominant intestinal bacterial communities in postmenopausal women (36). Another unanswered question refers to the effects of total isoflavones compared with those of their individual metabolites on calcium absorption and bone health. However, the present study clearly showed that supplementation with inulin increases plasma concentrations of soybean isoflavones and suggests that this substance should be consumed with isoflavones by subjects who may need isoflavone supplementation.

The authors’ responsibilities were as follows—CP, BM, and GML: analytic measurements; MGP: quality insurance; TI, MRM, and MAR: clinical portion of the study; and FD: study coordinator. No conflicts of interest were disclosed by any of the authors.

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