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Inulin Effects on Bioavailability of Soy Isoflavones and Their Calcium Absorption Enhancing Ability

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The effect of inulin on isoflavone absorption and the effect of isoflavones and synergy with inulin on calcium absorption in rats was investigated. Rats ($n = 48$) were divided into three groups and fed inulin (50 mg/g), isoflavone (8 mg/g) or inulin + isoflavone (50 mg/g + 8 mg/g) diets for 21 days. After a 2-h fast, rats were given ^{45}Ca orally or intraperitoneally, together with 25 mg of calcium as calcium acetate. Blood and femurs were collected 4 days later. Sera were analyzed for isoflavones using HPLC-MS, femurs for ^{45}Ca by β-scintillation counting, and total femoral calcium by atomic absorption spectroscopy. Both groups fed isoflavones had similar and significantly higher weight-adjusted total femoral calcium content compared to the inulin-fed group ($p < 0.0001$). ^{45}Ca absorption was significantly higher ($p < 0.01$) when isoflavones were added to the diet, and serum equol was significantly lower ($p < 0.01$) when inulin was added to the diet containing isoflavones. We conclude that isoflavones enhance calcium absorption, without synergy from inulin, and that inulin decreases equol production.

KEYWORDS: Soy isoflavones; inulin; equol; calcium absorption; rats

INTRODUCTION

Estrogen replacement therapy (ERT) is effective in the prevention as well as treatment and reversal of menopausal bone loss. However, it is accompanied by side effects including breast and uterine cancers (*1*). Recent reports have concluded that the combined use of estrogen and progesterone (in the form of the therapeutic agents premarin and medroxyprogesterone acetate (Pempro, Wyeth labs)) as part of hormone replacement therapy (HRT) is ineffective in the prevention of coronary heart disease and cognitive decline (*2*, *3*). As a consequence, there is a considerable interest in the identification of alternative estrogens for the prevention of each of these chronic diseases. Soy contains large quantities of isoflavones, so-called *phytoestrogens*. These isoflavones (i.e, genistein, daidzein, and glycitein, usually as their glycosidic conjugates) have structural similarities to 17β-estradiol, exhibiting similar estrogenic action by binding to the estrogen receptors (*2*). Although no benefits of feeding isoflavones on preventing bone loss were seen in some animal studies (*3*−*6*), there were favorable effects demonstrated on bone in other studies in ovariectomized (OVX) rats and mice and in vitro studies (*7*−*12*). Epidemiological studies indicate a lower incidence of osteoporosis in populations who consume more soybeans and soy products (*13*).

After ingestion, soy isoflavone glycosides are hydrolyzed to their aglycones by phlorizin lactose hydrolase in the apical membrane of the lumen of the small intestine, as well as by bacterial intestinal glucosidases (i.e., from lactobacilli, bacteroides, and bifidobacteria) (*14*, *15*). Conversion of daidzein into its bacterial metabolites, dihydrodaidzein, *O*-desmethylangolensin, and equol also precedes absorption from the colon. Following hepatic uptake and excretion into bile of the β-glucuronides of genistein, glycitein, and daidzein and its metabolites, a second round of hydrolysis occurs (*16*). Insoluble dietary fibers such as fructooligosaccharides (FOS) and inulin are known to stimulate the growth of these bacteria in the colon (*17*). These same dietary fibers have also been shown to increase calcium absorption both in rats (*18*−*29*), and humans (*30*−*33*) by acting as prebiotics. The mechanism of enhanced calcium absorption by insoluble dietary fiber is debated. It is hypothesized that (1) colonic bacteria ferment the fiber into short chain fatty acids, which lower the lumenal pH and therefore increase calcium absorption and (2) proliferation of the mucosal cells occur that leads to an enlargement of the absorptive surface area and thus to increased calcium absorption. Our recent study of kinetic modeling of calcium metabolism in ovariectomized

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Table 1. Composition of the Control and Modified AIN 93G Diets Fed to Rats in the Pilot Study and the Main Study

ingredients (g/kg diet)	AIN 93G[c]	inulin[d] (1%)	inulin[e] (5%)	isoflavone[f] (0.8%)	isoflavone (0.8%) + inulin (5%)[f]
corn starch	397.5	386.36	341.8	294.75	239.05
casein	200	200	200	200	200
choline-bitartrate	2.5	2.5	2.5	2.5	2.5
cystein	3	3	3	3	3
dextrinized corn starch	132	132	132	0	0
fiber	50	50	50	50	50
oligo fiber[a]	0	11.14	55.7	0	55.7
Advanta soy clear[b]	0	0	0	20	20
mineral mix	35	35	35	35	35
soybean oil	70	70	70	70	70
sucrose	100	100	100	314.75	314.75
vitamin mix	10	10	10	10	10

[a] 90% inulin, degree of polymerization >8. [b] Soy isoflavones content 40–50 wt %. [c] Control diet fed to the rats in the pilot study for 10 days. [d] 1% inulin diet fed to the rats in the pilot study for 10 days. [e] 5% inulin diet fed to the rats in the pilot study for 10 days and in the main study for 21 days. [f] Modification of the diets is explained under Materials and Methods.

rats showed that inulin had to be in the same meal with calcium to enhance calcium absorption (*29*). This observation supports the mechanism of fiber fermentation in the lower gut. Therefore, the present study was designed to investigate (1) the effect of inulin on isoflavone absorption and (2) whether inulin could further enhance the effect of dietary isoflavones on calcium absorption in a rat model.

MATERIALS AND METHODS

Pilot Studies. A series of pilot studies were conducted to determine an effective level of inulin and length of prefeeding for enhancing calcium absorption as well as appropriate length of time for harvesting of femurs post dose. The successful pilot study will be described. Male 10 week old Sprague−Dawley rats (340 g) were randomized into three groups. Group 1 ($n = 16$) was the control group that received AIN-93G nutritionally adequate semi-purified diet (*34*), without inulin, group 2 ($n = 8$) was fed 1% inulin, and group 3 ($n = 16$) was fed 5% inulin by weight in the AIN-93G diet. Diets were adjusted so as to be both isocaloric and isonitrogenous (**Table 1**). After feeding the experimental diets for 10 days, rats were fasted overnight. Eight rats in each group were administered 10 μCi of ^{45}Ca and 25 mg of calcium as calcium acetate by gavage prepared in water. Eight rats in groups 1 and 3 were given 10 μCi ^{45}Ca by intraperitoneal (IP) injection in 0.5 mL saline. Because no difference was found earlier in rats when fed control or 1% inulin supplemented diets (data not shown), the eight rats in the control group served as IP group for the 1% inulin group as well. They were given the food back an hour after dosing until 96 h when they were sacrificed. Femurs were collected from them and analyzed for ^{45}Ca. Percent calcium (mean ± SD) for rats fed 5% inulin for 10 days (44 ± 2) was significantly higher compared to both the control group (37 ± 4) and the 1% inulin group (41 ± 2) ($p < 0.05$). Despite the significantly higher calcium absorption of the group fed the 5% inulin supplemented diet, the difference among the groups was small. Therefore, rather than increase the inulin or calcium concentration in the diets to less practical levels, we decided for the main study to (1) increase the feeding period up to 21 days, (2) harvest the femurs 4 days later after the isotope (^{45}Ca) administration to ensure colonic absorption adaptation, (3) reduce the fasting time from 14 h to a minimum of 2 h so as not to minimize the mucosal cell proliferation effect of inulin, and (4) give a higher dose of 20 μCi ^{45}Ca.

Animals and Diets. Adult male (6 weeks old) Sprague−Dawley rats ($n = 48$) were purchased from Harlan, Indianapolis, IN. Female rats were avoided to prevent the effect of menstrual cycles on calcium absorption). After a week of adaptation, they were divided into three groups ($n = 16$/group). Group 1 was fed a 5% inulin diet, group 2 was fed a 0.8% isoflavone diet, and group 3 was fed a 0.8% isoflavones plus 5% inulin diet. Diets were prepared by replacing cornstarch in the AIN 93G diet with inulin and isoflavones. After feeding for 6 days, it was decided to substitute dextrinized cornstarch and part of the cornstarch with sugar to curb weight loss due to the bitter tasting isoflavone diets. Dietary composition is given in **Table 1**. Rats were then continued with their respective diets for 21 days.

Isotope Administration. The rats were fasted for 2 h 4 days before they were sacrificed. Each rat was given 20 μCi of ^{45}Ca either orally or IP together with 25 mg of calcium as calcium acetate dissolved in double deionized water. The oral dose was given by gavage prepared by dissolving calcium acetate in water and labeled with ^{45}Ca. For the IP dose, ^{45}Ca was injected in the IP cavity in a 0.5-mL saline carrier, while calcium acetate was given by gavage as for the oral group. An hour after dosing, food cups were returned until 4 days later, when they were sacrificed by exposure to CO_2. Blood was collected immediately by heart puncture and then centrifuged to separate sera. Sera were stored at −80 °C until analyzed for isoflavone concentration. Right femurs were harvested from the rats for ^{45}Ca analysis and total calcium measurements. All protocols were approved by the institutional animal care and use committee at Purdue University, West Lafayette, IN.

Analysis. Diets and sera from group 2 (isoflavone alone diet) and group 3 (isoflavone plus inulin diet)were analyzed for isoflavone concentrations by high performance liquid chromatography−mass spectrometry (HPLC-MS). Diet samples were extracted with 10 volumes of 80% aqueous methanol at 4 °C for 2 h. To quantify the extraction, internal standard, 50 μL of 20 mg/mL fluoroscein in 80% MeHO, was added prior to extraction. Extracts were centrifuged at 14000*g* for 10 min prior to HPLC analysis. Aliquots (10−20 μL) were injected onto a 22-cm × 4.6-mm i.d. 300-Å pore size, C_8 reversed-phase HPLC column (Rainin Instruments, Woburn, MA) with a 3-cm × 4.6-mm i.d. precolumn cartridge. The mobile phase solvents were 0.1% (v/v) trifluoroacetic acid in water (A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (B). The solvent gradient used to separate the isoflavones was 0−1 min of 10% solvent B in solvent A, 1−31 min of a linear gradient 10−40% solvent B in solvent A increasing at 1% solvent B/min, and 100% solvent B for 5 min, followed by reequilibration in solvent A for 5 min. Isoflavones were detected in the eluate using a UV-diode array. Quantitative measurements were obtained by the absorbance at 260 nm. All samples were measured in duplicate. Acceptable replicate errors were 1−2%.

Sera were analyzed as previously described (*35*). In brief, internal standards to monitor the hydrolysis of the β-glucuonides and sulfates and the recovery of the aglycones (phenolphthalein β-glucuronide, 4-methylumbelliferone sulfate and apigenin, respectively) were added to each serum diluted into 150 mM ammonium acetate, pH 5 buffer. β-glucuronidase/sulfatase from *Helix pomatia* was added, and the samples were incubated at 37 °C overnight. Glacial acetic acid was added, and the samples were extracted three times with 2 mL diethyl ether. The ether extracts were combined and evaporated to dryness under N_2. The dried residues were reconstituted in 100 μL of 80% aqueous methanol. Aliquots (20 μL) were analyzed by HPLC-electrospray ionization multiple reaction ion monitoring using a 10-cm × 4.6-mm i.d. 300-Å pore size, C8 reverse-phase HPLC column (Rainin Instruments, Woburn, MA) under isocratic conditions with 30% acetonitrile in 10 mM ammonium acetate at a flow rate of 1.0 mL/min on a Shimadzu VP HPLC System (Kyoto, Japan). The eluate was split so that 10 μL/min was passed into the IonSpray interface of a PE Sciex API III triple quadrapole mass spectrometer (Toronto, CA) operating in the negative ion mode with an orifice potential of −60 V. Individual isoflavones and their metabolites and the internal standards were measured using the following parent/daughter ion pairs: daidzein *m*/*z* 253/223; genistein *m*/*z* 269/133; dihydrodaidzein *m*/*z* 255/149; *O*-desmethylangolensin *m*/*z* 257/108; equol *m*/*z* 241/119; phenolphthalein *m*/*z* 317/93; 4-methylumbelliferone *m*/*z* 175/119, and apigenin *m*/*z* 269/149. Areas of each peak were determined using MacQuant software supplied by the manufacturer. Isoflavanoid peak areas were corrected for using the area of the apigenin internal standard and compared to

Table 2. Dietary Analysis of Isoflavones Fed to Rats

diets (mg/g)	daidzein	glycitin	genistin	daidzein	glycitein	genistein	total
isoflavone	2.91	1.42	0.50	0.10	0.11	0.24	5.44
isoflavones + inulin	2.99	1.47	0.51	0.10	0.11	0.23	5.41

Table 3. Effect of Feeding 5% Inulin, 0.8% Isoflavone, and Isoflavone plus Inulin Diets for 21 Days on Ca and Body Weight in Rats (Mean ± SD)[a]

groups	% ^{45}Ca absorption in femurs (n = 8/group)	total Ca (mg)/ femur (n = 16/group)	total femoral Ca (mg)/ 100 g body weight (n = 16/group)	final body weight (g) (n = 16/group)	weight gain (g) (n = 16/group)
inulin	42 ± 4a*	175 ± 20a	58 ± 6a**	301 ± 19a	103 ± 15a
isoflavone	49 ± 5b	167 ± 12a	75 ± 5b	226 ± 2b	23 ± 11b
isoflavone plus inulin	46 ± 9ab	168 ± 20a	74 ± 10b	225 ± 10b	24 ± 10b

[a] Means with different letters within columns are significantly different at *, $p < 0.01$, and **, $p < 0.0001$.

Table 4. Analysis of Isoflavones in Rats' Sera Fed either Isoflavone Alone or a Combination of Isoflavone plus Inulin in the Diet for 21 Days (Mean ± SD) (n = 16/Group)[a]

groups	equol (μM)	daidzein (μM)	DHD (μM)	*O*-DMA (μM)	genistein (μM)	total isoflavones (μM)
2. isoflavone	26.0 ± 10a	3.5 ± 2	5.5 ± 3	10.6 ± 5	0.9 ± 0.5	46.5 ± 14
3. isoflavone plus inulin	16.7 ± 9b	2.9 ± 2	7.2 ± 8.5	15.6 ± 16	0.6 ± 0.5	43.0 ± 15.6

[a] Means with different letters within columns are significantly different at $p < 0.01$.

the areas of known isoflavone standards. All samples were measured in duplicate. Acceptable duplicate error ranged from 3 to 10%.

Femurs were digested overnight in concentrated nitric acid and counted for ^{45}Ca by β-scintillation counting. Percent ^{45}Ca absorption was calculated by the ratio of percent oral dose in femur versus percent IP dose in femur by the following formula:

$$\%\ ^{45}\text{Ca absorption} = \%\ ^{45}\text{Ca oral dose}/\%\ ^{45}\text{Ca IP dose} \times 100$$

Total calcium in the femurs was measured by atomic absorption spectroscopy. It was corrected with body weight and reported as mg calcium per 100 g of body weight. Weight gain was calculated by subtracting the initial weight (the weight at the time when they were put on the experimental diet) from the final weight when they were sacrificed.

Statistical Analysis. Data are presented as means ± SD. One way analysis of variance along with Tukey testing was used. Statistics were performed using Statistical Analysis System software (SAS, Carey, NC) and a p value of $p < 0.05$ was considered significant.

RESULTS

Calcium Absorption and Femoral Calcium. Calcium absorption as measured by ^{45}Ca femur uptake was higher in rats fed isoflavones alone than for those fed inulin alone. Calcium absorption in rats fed isoflavones plus inulin did not further enhance calcium absorption. After correcting for body weight, there was significantly higher total femoral calcium content in both isoflavone groups with or without inulin supplementation compared with the inulin alone group ($p < 0.0001$) (**Table 3**).

Diet and Serum Isoflavones. Dietary isoflavone content of the isoflavone-containing diets is given in **Table 2**. There were no differences found in the total isoflavones, daidzein, geinstein, DHD or O-DMA serum concentrations between the two groups fed isoflavone alone and isoflavone plus inulin in the diet (**Table 4**). Rats receiving both inulin and isoflavones had significantly lower equol concentrations ($p < 0.01$) when fed isoflavone plus inulin diet compared to rats fed an isoflavone diet without inulin supplementation.

Body Weight. Despite increasing sugar levels in the isoflavone diets, rats did not eat as well as rats on the inulin alone diet. This was reflected in their final weight and weight gain (**Table 3**).

DISCUSSION

We have previously shown that inulin enhances calcium absorption in rats (*29*). Here we show that ^{45}Ca absorption was enhanced more by the presence of isoflavones in the diets than by inulin at the levels used (**Table 3**). Furthermore, adding inulin to the diet containing isoflavones did not further increase ^{45}Ca absorption over isoflavones alone. Total femoral calcium after correcting for body weight was significantly higher in isoflavone-groups with or without inulin supplementation over the inulin alone group. Thus, we did not find a synergy between inulin and isoflavones in contrast to Ohta et al. (*36*) who found an additive effect of inulin (5%) and isoflavone (0.2%) on the femoral bone mineral density in OVX mice fed for 6 weeks. The difference between our study and theirs could be due to many factors, including a different level of isoflavone (0.8 vs 0.2%) or type of isoflavones, a different animal model (young adult male rats, 6 weeks old, vs OVX female mice, 8 weeks old), or a different length of feeding time (21 vs 42 days), respectively.

Our other aim was to investigate whether inulin enhances isoflavone absorption in rats. We did not see any significant difference in serum isoflavone concentration due to the presence of inulin. However, in both isoflavone-fed groups, daidzein and genistein were low compared to their metabolites such as equol, DHD, and O-DMA. This may suggest the highly bioavailable nature of the isoflavone product. Equol was significantly lower in rats fed isoflavones plus inulin. This suggests an interaction between inulin and isoflavones when fed together. Urinary or bile excretion of isoflavones were not measured. It is possible that equol was excreted earlier by the isoflavone plus inulin fed rats resulting in the lower blood equol concentration or that inulin suppressed the bacteria responsible for the formation of equol. No enhancing effect of inulin on isoflavone absorption

was observed in the present study, unlike another study (*37*), which reported that an enhancing effect of FOS was found on the intestinal bioavailability of genistein and daidzein when administered as a single dose to rats by gavage. The rationale presented by these investigators for the increased absorption of isoflavones was that prefeeding inulin stimulates the growth of bifidobacteria in the large intestine that produce β-glucosidases, which then help cleave the glycosidic bonds of the isoflavone conjugates, and therefore, improve the absorption of isoflavone metabolites.

There is yet a great deal to learn about the metabolism of isoflavones and their interaction with inulin. Isoflavone bioavailability may increase with the hydrolysis of the glycosidic bonds of isoflavones. Glucosidase-producing bacteria flourish in the large intestine, yet the isoflavone absorption takes place much earlier than the large intestine. In a human study (*38*), genistein and daidzein was detected in blood within 15 min after the consumption of textured soy protein, although this may have come from small amounts of isoflavone aglycones in the product. In a rat study, Ioku et al. (*39*) measured β-glucosidase activity in the small intestine, suggesting that hydrolysis of isoflavones can occur in the jejunum. The bacterial β-glucosidase activity is now thought to be minor when compared to the intestinal membrane protein lactase phlorizin hydrolase (*40*). Not surprisingly then, in the present study, we saw little effect of inulin on overall isoflavone absorption. However, serum equol concentrations were significantly lower in the group co-fed with inulin compared to the group fed isoflavone without inulin ($p < 0.01$) **(Table 4)**. This indicates that inulin may alter the colonic bacterial flora in such a way that equol production is lowered.

More research is needed to establish the health benefits of isoflavones, the site and the mechanism of their absorption, isoflavone and inulin interaction, the effects of total isoflavones versus their individual metabolites on calcium absorption, and bone health. In this study, we did not see evidence that inulin enhances isoflavone absorption. Although inulin and isoflavones separately enhance calcium absorption, there was no apparent benefit to feeding these functional food ingredients together.

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