Isoflavones in breastfed infants after mothers consume soy1–3

Adrian A Franke, Brunhild M Halm, Laurie J Custer, Yvonne Tatsumura, and Sandra Hebshi

ABSTRACT

Background: The bioavailability of isoflavones in children after soy exposure is uncertain.

Objective: We aimed to compare isoflavone patterns in infants exposed to isoflavone-containing breast milk (BF), in tofu-fed (TF) infants, and in mothers consuming a soy beverage.

Design: Eighteen nursing mothers who were not feeding soy foods to their infants consumed one daily serving of a soy protein beverage for 2–4 d and collected their own milk and urine and infant urine. Plasma was collected from infants if venous blood draws were ordered by pediatricians. Blood and urine were collected from additional children after they consumed tofu. Isoflavones were measured by liquid chromatography–mass spectrometry.

Results: In 7 subjects, isoflavone values increased significantly from baseline after mothers ate soy: in maternal urine (± SEM) from 18.4 ± 13.0 to 135.1 ± 26.0 nmol/mg creatinine, in breast milk from 5.1 ± 2.2 to 70.7 ± 19.2 nmol/L, and in infant urine from 29.8 ± 11.6 to 111.6 ± 18.9 nmol/mg creatinine. The mean isoflavone concentration in plasma obtained from 11 BF infants was 19.7 ± 13.2 nmol/L. TF infants had much higher mean isoflavone values (urine, 229 ± 129 nmol/mg creatinine; plasma, 1049 ± 403 nmol/L). Statistically significant correlations were observed between the types of fluids investigated within mothers, between mothers and infants, and within infants. Urinary isoflavone excretion per hour adjusted for dose per body weight was 81% lower for BF infants and 24% higher for TF infants than for their mothers after eating soy.


KEY WORDS Isoflavones, soy foods, infants, breast milk, urine, biological markers, creatinine, plasma

INTRODUCTION

Soy foods contain high concentrations of the isoflavones genistein, daidzein, and glycitein (~0.01–0.3% as consumed) and account for most dietary isoflavone exposure (1), particularly in soy-consuming populations (1–4). Isoflavones are efficiently absorbed by adults after oral administration (5–10), and urinary or plasma isoflavones are used as reliable biomarkers of soy consumption in adults (11–18). According to numerous animal and human studies, isoflavones are suggested to protect against many chronic diseases, including breast, prostate, and colorectal cancer; osteoporosis; menopausal symptoms; and cardiovascular disorders (19–25). Isolated isoflavones as present in nutritional supplements are mostly ineffective (26, 27), except for improving endothelial function (28). Some studies find health benefits from soy foods that are low in or devoid of isoflavones (29, 30), whereas others find that isoflavones from soy alone or in combination with other soy components result in health benefits (27, 28, 31–34).

Although well researched in adults (6–8, 35), the bioavailability of isoflavones after soy exposure has been minimally investigated in infants and children (6–8). In one study, mean plasma isoflavone concentrations in seven 4-mo-old boys fed soy-based formula, cow milk–based formula, or human milk were 3.7, 20, and 16 μmol/L, respectively (36). Urinary isoflavone concentrations were reported to be lower in up to 4-mo-old boys than in adults when both were exposed to comparable isoflavone doses (37). Comparisons in isoflavone bioavailability between children or infants and their mothers in dietary intervention studies have so far not been performed. Details about the absorption, metabolism, and excretion of milk components in infants often remain uncertain, including the fate and quantitative pattern of dietary agents, even vitamins (38, 39). Thus far, no information is available about isoflavone concentrations in infants after they consume isoflavone-containing breast milk. We and others previously showed that lactating women secrete isoflavone into breast milk after soy intake (40, 41) and that this happens in a dose-dependent fashion (42).

We report here on isoflavone values in urine (IU) and plasma (IP) of infants who breastfed from soy-consuming mothers (BF) or who consumed tofu (TF) to find out whether isoflavones consumed by breastfed infants as glucuronate and sulfate conjugates appear in these infants differently than when consumed as β-glucosides as present in soy foods. In addition, we compared these findings with the isoflavone patterns in urine and milk of the soy-consuming mothers (MU and MM, respectively).

SUBJECTS AND METHODS

Subjects and study design

Breastfeeding mothers and their infants were recruited from the local children’s hospital and through word of mouth. All were

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A total of 18 mothers and their BF infants (9 males, 9 females) aged 2–45 wk participated in this study. All infants consumed breast milk in frequency and volume ad libitum. Some participants repeated collections after several soy interventions, which were ≥2 wk apart.

To compare the effects of isoflavone exposure via breast milk with that via solid foods, we collected blood and urine additionally from 3 infants (aged 9, 10, and 24 mo; 2 females, one male) 2 to 4 h after they consumed on average 44 g tofu (equivalent to 7.4 mg isoflavones) (43). No samples were collected from their mothers, and values from TF infants were included in the IP-IU comparisons.

The protocol of this dietary intervention study was approved by the Institutional Review Board of Kapiolani Medical Center for Women and Children/Honolulu and the University of Hawaii Committee on Human Subjects. All participants signed an informed consent form for themselves and their children before entry into the study.

Blood was collected in lithium-heparin–containing evacuated tubes, and urine was collected from infants in special sex-specific collection bags in 2 sizes depending on age (First-Time; Hollister Inc, Libertyville, IL). Sterile screw-cap containers were used to collect MM and MU. Immediately after collection, blood was transported at 5–8 °C in the dark to the laboratory via courier, followed by immediate processing and final storage of plasma at −20 °C. Urine and milk samples were allowed to be stored in refrigerators until transported to the laboratory on wet ice via courier. These samples were always kept at temperatures <8 °C until final storage at −20 °C, no later than 24 h after collection. These conditions were shown not to degrade any of the measured isoflavones (data not shown).

**Specimen analysis**

The soy protein beverage contained 55 mg isoflavones, composed of daidzein, genistein, glycitein, and their glycoconjugates as determined by HPLC with photodiode array detection (PDA) (5), as shown in Table 1. Daidzein, genistein, glycitein, equol, dihydrodaidzein, dihydrogenistein, and O-desmethylangolensin were analyzed from urine, plasma, and human milk by HPLC with PDA followed online by electrospray ionization (negative mode) ion trap mass spectrometry (ESI-MS) (44, 45). In brief, triply 13C-labeled internal standards of daidzein, genistein, equol, and O-desmethylangolensin (University of St Andrews, St Andrews, United Kingdom) were added to each specimen hydrolyzed with glucuronidase and sulfatase (Sigma Chemical Co, St Louis, MO, or Roche Applied Sciences, Indianapolis, IN) followed by repeated phase separation with diethyl ether (5). Milk was additionally defatted with hexane before this procedure (40). The combined ether fractions were dried under nitrogen and redissolved in a 1:1 mixture of acetonitrile–sodium acetate buffer (0.2 mol/L, pH 5). Five to 20 μL of this extract was analyzed by liquid chromatography/PDA/ESI-MS with an LCQ Surveyor-Advantage ion trap mass spectrometry system (ThermoElectron Corp, San Jose, CA) equipped with a HydroBond PS C18 (100 × 3.0 mm; 5 μm) reversed-phase column coupled to a HydroBond PS C18 (25 × 3.2 mm; 5 μm) direct-connect guard column (MacMod Analytic Inc, Chadds Ford, PA). The elution, absorbance detection, and mass spectrometric measurements were performed as applied previously (34, 44, 46, 47). Limits of quantitation for all analytes were 0.3 mL plasma, 1.8 mL milk, or 1.2–1.8 mL urine were 2.5 nmol/L except for dihydrodaidzein and dihydrogenistein (1.5 nmol/L) and O-desmethylangolensin (5.0 nmol/L). Between-day CVs for plasma, milk, and urine ranged from 4% to

**TABLE 1**

Composition of the soy protein beverages consumed by mothers

<table>
<thead>
<tr>
<th>Amount per dose (mg)</th>
<th>With aspartame</th>
<th>Without aspartame</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzin</td>
<td>7.5</td>
<td>7.2</td>
</tr>
<tr>
<td>Glycitin</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Genistin</td>
<td>10.3</td>
<td>10.2</td>
</tr>
<tr>
<td>Daidzin malonate</td>
<td>11.8</td>
<td>11.7</td>
</tr>
<tr>
<td>Glycitin malonate</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Genistin malonate</td>
<td>13.8</td>
<td>14.0</td>
</tr>
<tr>
<td>Daidzin acetate</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Glycitin acetate</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Genistin acetate</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Daidzin</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Glycitein</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Genistein</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Total daidzein</td>
<td>24.3</td>
<td>24.2</td>
</tr>
<tr>
<td>Total glycitein</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Total genistein</td>
<td>28.5</td>
<td>28.5</td>
</tr>
<tr>
<td>Total isoflavones</td>
<td>55.1</td>
<td>54.5</td>
</tr>
</tbody>
</table>

Percentage of total (%)

| Glucosides           | 34             | 34                 |
| Malonyl-glucosides   | 48             | 48                 |
| Acetyl-glucosides    | 10             | 10                 |
| Aglucons             | 7              | 8                  |
| Total daidzein       | 44             | 44                 |
| Total glycitein      | 4              | 4                  |
| Total genistein      | 52             | 52                 |
12% for daidzein, 5–18% for genistein, and 3–14% for glycitein. Isoflavone metabolites (equol, dihydrodaidzein, dihydrogenistein, and O-desmethylangolensin) were not considered here because of amounts at detection limits. Urinary creatinine was measured by using a Roche Cobas MiraPlus clinical autoanalyzer (Roche Diagnostics, Indianapolis, IN) with a kit from Ran- dox Laboratories (Crumlin, United Kingdom) that is based on a kinetic modification of the Jaffe reaction.

**RESULTS**

Sixteen mothers correctly complied with the protocol and collected at least one specimen (milk, urine, or plasma). Reasons for missing samples were lack of sufficient volume for analysis (MF, IU) or forgetting to collect (MU). One of the noncompliers forgot to consume the soy beverage; samples for that subject pair were used for baseline measurements only. No data from the other noncomplier were included in the analysis. Three mothers gave their infants soy-based infant formula; therefore, only the mothers’ specimens could be included. Mean values were used from each individual when repeated collections were available. Sixteen mothers correctly completed the collection of MU and MM, and 13 and 11 infant samples were collected for IU and IP, respectively. For specimen pair comparisons, 12 participants completed the protocol correctly for the donation of MM-IU correlation being nonzero. Means were calculated by using one data point per subject before or after the intervention.

**Statistical procedures**

Student’s paired t test was applied to test the significance of differences in isoflavones before and after the soy intervention in MU, IU, and MM with the use of EXCEL software 1998 for Macintosh (Microsoft Inc, Redmond, WA). Fisher’s z-transformation was used to test the statistical significance of the correlation coefficients by using EXCEL software 1998. This transformation allowed us to test any null hypothesis, ie, not just to test whether the true correlation was zero. It is more flexible and appropriate than the t test for correlation when the distribution of sample values of the correlation is skewed under the true

![Graph](image-url)
pairs, 13 for MU-IU pairs, 14 for IP-IU pairs (11 BF, 3 TF), and 16 for MU-MM pairs (Table 2).

The protocol was challenging for most participants because it required the collection of multiple samples and the timed collection of IU with the use of clinical urine bags and because of the general stress connected with a new baby, particularly for primiparas. Therefore, baseline MU, MM, and IU was obtained from only 7 mother-infant pairs with 3 female and 4 male infants. The mean (±SEM) values of these 7 subjects before and after the intervention were 18.4 ± 13.0 and 70.7 ± 26.0 nmol/mg creatinine (Cr) for MU, 5.1 ± 2.2 and 70.7 ± 19.2 nmol/L for MM, and 29.8 ± 11.6 and 111.6 ± 18.9 nmol/mg Cr for IU (Figure 1). Isoflavone values increased significantly after the intervention in these 7 participants for all the body fluids provided (MU, MM, IU) according to paired Student’s t tests. When the data from additional participants who donated specimens exclusively after the intervention were included, the results were similar, ie, mean overall values for MU after the intervention (9 additional mothers consuming soy) were 157.1 ± 482 nmol/mg Cr and 1048.6 nmol/L in plasma (median: 145 nmol/mg Cr; range: 61–482 nmol/mg Cr) and 1048.6 nmol/L in plasma (median: 663.1 nmol/L; range: 629.1–1853.6 nmol/L). The unexpected and extremely high plasma value of 1.86 ± 0.2 ng/mL was obtained from a child (aged 11 mo) who consumed 90 g tofu for this study. In addition to passing the exclusion criteria during the recruitment phase, we confirmed with the parents after study completion that this child was not fed any soy formula or soy supplement during or before this study.

Glycitein values were negligible in MM and IP, with a contribution of ≈2% relative to the total isoflavone concentrations. In MU and IU, however, glycitein contributed 9% and 17%, respectively. The daidzein-to-genistein ratio was on average 0.6, 2.4, 1.3, and 0.4 in MM, MU, IU, and IP, respectively. However, in IP of TF infants, this ratio was 1.1 and therefore almost 3-fold that in BF infants.

As shown in Figure 2, high correlations were observed between the types of fluids investigated and all were statistically significant according to a two-tailed z test whether within mothers (MM versus MU: r = 0.661), between mothers and infants (MM versus IU: r = 0.775; MU versus IU: r = 0.863), or within infants (IP versus IU: r = 0.975). Correlation coefficients and P values did not change considerably when applying median or logged values (data not shown).
To compare urinary isoflavone excretion (UIE) between infants and their mothers relative to dose (Table 3), we used the known isoflavone dose in mothers (55 mg/d), the established assumption of daily breast milk intake of 100–150 mL/kg body wt of infants (48), and the measured isoflavone dose of 7.37 mg in tofu (43) based on the amount fed (=44 g) according to the mothers. This resulted in a dose per body weight of 1.01 mg/kg in mothers on the basis of their mean body weight of 55 kg, 0.003 mg/kg in BF infants on the basis of the isoflavone concentration in breast milk of 95 nmol/L (Figure 1), and 0.69 mg/kg for the 3 TF infants on the basis of their individual weights. Relative to their mothers, this body weight–adjusted dose is 69% in TF infants but only 0.3% in BF infants.

UIE adjusted for creatinine concentration was found to be 157, 0.186, and 229 nmol/mg Cr, and when adjusted to dose per kg body wt, these values changed to 156, 60, and 345 (nmol/mg Cr)/(mg · kg⁻¹ · d⁻¹) in mothers, their BF infants, and TF infants, respectively. Relative to their mothers, this creatinine-based UIE-dose ratio was less than one-half in BF infants but >2-fold higher in TF infants.

The daily creatinine excretion in urine can vary greatly between individuals but is mainly determined by sex, age, and body weight (49,50). Under consideration of these factors, an accurate conversion of creatinine-based urinary excretion rates to hourly urinary excretion rates was performed by using established values for daily creatinine excretion per kg body wt, ie, 21.0, 10.2, and 11.8 mg in mothers aged 28–36 y, BF infants at a mean age of 2 mo, and TF infants at a mean age of 15 mo, respectively (49–51). Our measured creatinine-based UIE values converted in this process to hourly UIE of 138, 0.08, and 113 nmol · h⁻¹ · kg⁻¹, respectively (Table 3). When these body weight–adjusted hourly UIE values were additionally adjusted to dose per kg body wt (UIE/dose; Table 3) they changed to 137, 26, and 169 (nmol · h⁻¹ · kg⁻¹)/(mg · kg⁻¹ · d⁻¹) for mothers, their BF infants, and TF infants, respectively. Relative to their mothers, this body weight– and time-adjusted UIE/dose ratio was found to be ≈81% lower in BF infants but 24% higher in TF infants, with the former but not the latter value reaching statistical significance.

DISCUSSION

We found low circulating concentrations and urinary excretion rates of isoflavones in breastfed infants of mothers who consumed usual servings of a soy protein drink despite the presence of these isoflavones as glucuronide and sulfate conjugates, which are easily hydrolyzed to bioavailable aglycons. This finding was probably due to the very low isoflavone dose but also to a lower ability of isoflavone uptake relative to adults when adjusted to dose. In contrast, TF infants showed high isoflavone amounts in plasma and urine, which exceeded those found in adults eating soy. This indicates that the isoflavone conjugation pattern alone does not determine isoflavone uptake capacities in children.

As expected, there was a significant increase in isoflavone amounts between baseline and the soy intervention in mothers (8-fold in MU and 14-fold in MM) but also in their BF children (4-fold in IU). When we included the 5 to 9 participants without baseline samples, the combined mean values after soy intervention were similar, and the differences were most likely due to the known interindividual variability in isoflavone uptake (35, 52). Isoflavone values in TF infants’ plasma and urine were on average 53-fold and >1200 times the values in BF infants, respectively. Concentrations of this magnitude in children were previously thought to exist only in soy formula–fed infants, who are exposed to very high isoflavone doses (6–8 mg/kg body wt) (36, 42). Plasma isoflavone concentrations in adults were found to peak at 1.5 μmol/L after 27 mg isoflavone (6) or 10–50 nmol/L per mg isoflavone consumed (reviewed in reference 10), with less efficient uptake at greater doses (53). Our findings in TF infants were much higher, 135 nmol · L⁻¹ · mg⁻¹, and may have even been greater because our collections might not have coincided with peak plasma isoflavone concentrations. These findings suggest a higher isoflavone exposure in children than in adults, particularly when considering that children eat more per kg body wt. This might shed a different light on isoflavone effects. High soy consumption in some Asian countries may lead to high systemic isoflavone exposures in Asian children.
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The low isoflavone values in urine and plasma of BF infants, even after consideration of the low dose, could be due to the impaired ability of the nonmatured gut flora to cleave glucuronide and sulfate conjugates for the production of the aglycons required for isoflavone uptake. However, the constant exposure to isoflavone in infants breastfed by mothers regularly consuming soy will result in more uniform systemic concentrations, and bioavailability as measured by area under the curve might be higher than single isoflavone plasma concentrations indicate. Nevertheless, although detectable isoflavone concentrations were measured, the urine and plasma values in infants exposed to isoflavone-containing breast milk were low but might still be in the pharmacologically relevant range. Conversely, the higher isoflavone bioavailability after soy intake in weaning infants relative to adults could be due to the maturing gut flora that has attained the ability to hydrolyze beta glucosides efficiently (6) for the production of aglycons but has not yet developed fully to degrade unconjugated isoflavones (71). This would result in an overall higher survival of ready-for-uptake isoflavone aglycons in the gut (52). Despite similar isoflavone doses per kg body wt through adjustment of soy doses, we found higher creatinine-based UIE in 8–14-y-old girls than in adults (72), and this difference was little changed after conversion to hourly UIE (G Maskarinec, unpublished observations, 2005). This agrees with the present findings and supports our hypothesis of higher isoflavone bioavailability in children than in adults. Collection of the entire urine amount in a timed fashion bypasses the need for conversion of creatinine-based UIE; although 24-h (or longer) urine collections result in very precise data, a protocol of this nature is often difficult or impossible to perform in human studies for various reasons. A good compromise is the collection of overnight urine, which resulted in very high compliance previously (2, 73) and which is particularly recommended for research in children once bladder control is achieved.

Weaknesses of this study are the small sample size and the lack of control over infants’ sample collections. In addition, all specimens were collected in a 1–2-h time period in the afternoon after the mothers had consumed their soy dose in the morning. This time frame could lead to major differences in isoflavone appearance in plasma, urine, or breast milk as a result of the fast isoflavone pharmacokinetics (7–9, 40). Correlations between the various types of fluids analyzed could probably be improved with a more uniform and defined schedule for soy consumption, breastfeeding, and sample collections. With this in place, changes in uptake in infants as a function of age could be evaluated. Despite the relatively small number of samples in this study, we obtained robust results considering the insignificant change in all correlation coefficients and $P$ values when applying median or logged values. To our knowledge, this is the first report on isoflavone values in urine and plasma of infants who were breastfed by soy-consuming mothers or who ate tofu. It adds critical information to the previous animal and recent epidemiologic studies showing beneficial effects of soy exposure by elucidating isoflavone appearance in humans at an early age. The current study shows that, independent of relative bioavailability, isoflavone exposure in soy-consuming populations is probably higher in children than in adults, particularly as a result of larger intake when adjusted for body weight.

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LJC performed the chemical analyses. YT and SH were the study coordinators. SH also assisted in manuscript preparations. BMH assisted in study design, recruited the study participants, performed sample collections, and was available for medical advice. She also assisted in matters related to the institutional review board and in data interpretations. AAF, the principal investigator of this project, was responsible for all parts of the study, designed and directed the study, performed data interpretation, and prepared the manuscript. None of the authors had a conflict of interest.

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