

Urinary isoflavonoid excretion is similar after consuming soya milk and miso soup in Japanese-American women

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Based on the hypothesis that isoflavones are absorbed more efficiently from fermented than from non-fermented soya foods, we compared the urinary isoflavonoid excretion (UIE) after intake of miso soup or soya milk. We recruited twenty-one women with Japanese ancestry who consumed standardized soya portions containing 48 mg isoflavones. On day 1, half the women consumed soya milk, the other half started with miso soup. On day 3, the subjects ate the other soya food and on day 5, they repeated the first food. Each participant collected a spot urine sample before and an overnight urine sample after soya food intake. All urine samples were analysed for daidzein, genistein and equol using LC-MS and were expressed as nmol/mg creatinine. We applied mixed models to evaluate the difference in UIE by food while including the baseline values and covariates. Relative to baseline, both groups experienced significantly higher UIE after consuming any of the soya foods. We observed no significant difference in UIE when soya milk was compared to miso soup ($P=0.87$) among all women or in the seven equol producers ($P=0.88$). Repeated intake of the same food on different days showed high reproducibility within subjects. These preliminary results indicate similar UIE after consuming a fermented soya food (miso) as compared to a non-fermented soya food (soya milk). Therefore, recommendations favouring fermented soya foods are not justified as long as the intestinal microflora is capable of hydrolysing the isoflavone glucosides from non-fermented soya foods.

Soya: Isoflavones: Urinary isoflavonoid excretion: Equol

Soya foods may protect against chronic diseases^(1,2). Isoflavones in soybeans, i.e. daidzein, genistein and glycitein, are active substances that have chemical structures similar to mammalian oestrogens⁽³⁾. In food, they are primarily found as β -glucosides with and without additional malonates and acetate esters⁽⁴⁾. Once ingested, these conjugated isoflavones undergo hydrolysis by β -glucosidases mainly from intestinal bacteria, releasing the principal bioactive aglucone (glucose-free) form that is absorbed, whereas the highly water-soluble glucosides are not absorbed^(5–8). Therefore, intestinal bacteria are crucial for the absorption and bioavailability of isoflavones^(5,8,9). It is the aglycones (sugar-free forms) that show an affinity for oestrogen receptors and have other non-hormonal effects on the cell machinery⁽⁶⁾. The isoflavone daidzein is metabolized to equol and *O*-desmethylangolensin by gut bacteria and excreted predominantly through the urine⁽¹⁰⁾. The ability to produce equol is limited to 30–50% of the population but whether this metabolic feature results in more beneficial health effects from soya consumption remains uncertain^(9,11). The extent of isoflavone metabolism varies among individuals and may be influenced by additional dietary factors^(12,13).

Dietary isoflavonoids are specific to soya foods and urinary isoflavonoid excretion (UIE) serves as an excellent marker for the bioavailability of isoflavones^(14,15). As a result of their rapid metabolism, urinary appearance of isoflavonoids reflects

circulating levels when the timing of specimen collection is accurately considered^(8,16,17). Due to micro-organism-induced hydrolysis in fermented soya products, e.g. tempeh and miso, the predominant form of isoflavones in these foods are aglucones. Consuming fermented soya foods was reported to lead to higher levels of absorption and urinary isoflavone recovery in some^(18–20) but not in other studies^(21–23). We hypothesized that women will excrete more isoflavonoids and produce more equol after consuming equivalent isoflavone amounts in one serving of miso soup than in one serving of soya milk because the aglucone form present in fermented soya products is more bioavailable than the conjugated isoflavones in the non-fermented soya milk.

Methods

Participants

We recruited a convenience sample through employees, families and friends of the Cancer Research Center of Hawaii. Eligible participants were at least 18 years of age, at least 50% Japanese ancestry, current residents of Hawaii and free of any known soya allergies. Women with Japanese ancestry were chosen because they were expected to have had previous soya food exposure and a more comparable gut flora than subjects with different ancestries⁽²⁴⁾. During the

Abbreviation: UIE, urinary isoflavonoid excretion.

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intervention, none of the participants were taking antibiotics, food supplements or probiotics that could alter the intestinal flora. The study protocol was approved by the Committee on Human Subjects at the University of Hawaii and all subjects gave written informed consent.

Data collection

Demographic, familial and medical information was collected by questionnaire. Regular soya intake was assessed with a twelve-item questionnaire which elicited the frequency and average serving size of soya food consumed during the last 12 months⁽²⁵⁾. To obtain a summary score, we multiplied the frequencies of intake for each of the twelve soya foods by the estimated isoflavone content from the food composition database maintained by the Nutrition Support Shared Resource at the Cancer Research Center of Hawaii. Isoflavone concentrations for soya foods in this database were primarily derived from an analysis of representative local foods⁽⁴⁾. An additional lifetime questionnaire estimated soya exposure since birth using the following stages: infancy (1 year), childhood (1–9 years), adolescence (10–19 years), early adulthood (20–29 years) and late adulthood (30+ years)⁽²⁶⁾. Participants indicated the annual frequency of four categories of soya foods (tofu; soyabeans and sprouts; soyabean drink or milk; and other soya products) for each period. To obtain a summary score, we multiplied the frequency of intake for each of the four foods by the estimated isoflavone amount and added the total amounts of the four foods in each stage of life to determine an annual intake for each stage.

Intervention and urine collection

The 6 d study protocol included three spot urine collections after a soya-free day and three overnight urine collections after consumption of a standardized portion of miso soup or soya milk. According to the HPLC analyses, one serving of soya milk (250 ml Edensoy[®] Original Organic soya milk) contained 48.3 mg isoflavones as aglycone equivalents which was matched with 31 g Haccho miso (again determined by HPLC) to be dissolved in one cup of water; 98% of the isoflavones in miso were aglycones but only 2% in the soya milk. During and 1 d before starting the intervention, participants were instructed to abstain from all soya foods other than those provided to them for the study. Half of the participants started with soya milk (group A) as follows. On day 1, at around 18.00 hours, the women collected a spot urine sample, consumed one container of soya milk, and collected all urine thereafter until they got up the next morning (overnight urine). On day 2, participants proceeded with their normal diets. On day 3, subjects followed the same protocol as day 1, but drank one serving of miso soup. On day 4, participants again returned to their normal diets. On day 5, subjects followed the same protocol as day 1 exactly, collected a spot urine sample at around 18.00 hours, consumed one container of soya milk, and collected all overnight urine. The other half of the participants (group B) followed the same protocol except starting with miso soup (day 1) followed by soya milk (day 3) and finally miso soup again (day 5). The urine collection containers contained ascorbic and boric acid to prevent bacterial contamination and degradation of analytes.

The samples were stored in refrigerators and transported in chilled coolers. The urine samples were aliquoted into 2 ml vials and stored at -80°C until analysed. Based on the collection times recorded by each subject, we calculated the total number of overnight collection hours.

Urine analysis

Daidzein, genistein and equol were analysed from urine by LC–MS using a triple quadrupole TSQ Ultra system (ThermoFisher, San Jose, CA, USA) with electrospray ionization in negative mode and multiple reaction monitoring^(27,28). In brief, triply ^{13}C -labelled internal standards of daidzein, genistein and equol (University of St Andrews, UK) were added to 100 μl urine and hydrolysed for 1 h at 37°C with glucuronidase and sulphatase (Roche Applied Sciences, Indianapolis, IN, USA) followed by phase separation with diethyl ether⁽²⁹⁾. The diethyl ether fractions were dried under nitrogen and re-dissolved in a 1:1 mixture of methanol–sodium acetate buffer (0.2 M, pH 5). The extract (25 μl) was analysed by LC–MS/MS after separation on a BetaBasic C8-column (100 mm \times 2.1 mm internal diameter, 3 μm) coupled to a BetaBasic C8-precursor (10 mm \times 2.1 mm internal diameter, 3 μm ; both from ThermoFisher)^(27,28,30). The elution was performed with methanol–acetonitrile–water (10:10:80 to 33:33:34) in 6 min, holding there for 1 min before changing in 0.1 min to the starting mixture for equilibration. Ammonium hydroxide (aqueous 2.5% at 10 $\mu\text{l}/\text{min}$) was infused to enhance the signal. Daidzein was monitored using the transitions (m/z) 253.020 to 222.988, 207.980 and 131.949; for genistein the transitions were 269.090 to 159.050, 133.035 and 132.032, and for equol they were 241.130 to 134.950, 121.000 and 118.960. Limits of quantitation for all analytes were 1.5 nmol/l for daidzein and genistein and 3.0 nmol/l for equol for post-intervention samples and half of those values for pre-intervention samples due to differences in concentration steps prior to analysis. Mean intra- and inter-day CV of LC–MS/MS quantitation for daidzein (422 nmol/l), genistein (35.9 nmol/l) and equol (9.9 nmol/l) were 8.3, 13.6 and 2.9%, and 7.3, 17.9 and 3.5%, respectively. Recoveries were 72–88%. Urinary creatinine concentrations were measured with a Roche-Cobas MiraPlus chemistry analyser using a kit from Randox Laboratory (Crumlin, UK) that is based on a kinetic modification of the Jaffe reaction. Limits of quantitation were $<15 \mu\text{mol}/\text{l}$ and the mean inter-assay CV was 0.8% at 187 $\mu\text{mol}/\text{l}$. The sum of daidzein, genistein and equol was expressed in nmol isoflavonoid/mg creatinine. We also calculated the isoflavonoid excretion as nmol/h based on urine weight, hours of urine collection and isoflavonoid concentration in urine.

Statistical analysis

All data management and statistical analyses were performed using SAS release 9.1 (SAS Institute, Cary, NC, USA). To define equol producer status, we used 0.05 nmol/mg creatinine as a cut-off value. Two-sample *t* tests were used to compare study characteristics by group at baseline. Due to their non-normal distribution, we log transformed the UIE. We examined overall mean differences of UIE after soya milk and miso soup intake in one model that included all six UIE

values for each woman. This examination was carried out using maximum likelihood estimation of a mixed general linear model that takes into account the covariance structure of the repeated measures within subjects⁽³¹⁾. The order (group A or B), day, age, weight and equol producer status were included into the model as covariates. In addition, we computed adjusted least square means of UIE for the three food categories (no soya, soya milk, miso soup).

Results

We enrolled twenty-one participants with a mean age of 49.4 (range 20–83) years (Table 1). The mean body weight was 58.0 (SD 11.7) kg. All women reported at least 50% Japanese ancestry. Self-reported isoflavone intake during the previous 12 months varied among the participants with a mean of 20.1 (range 0.1–85.6) mg/d. While self-reported early life soya intake was only 0.98 (range 0–7.1) servings/d, adult soya intake was 1.7 (range 0.2–10.2) servings/d. Although group B reported higher soya and isoflavone intake than group A during their previous life, none of the differences was statistically significant. The time during which overnight urine was collected covered a mean of 10.8 (SD 1.5) h and did not differ by group (11.0 *v.* 10.5 h; *P*=0.67).

The unadjusted mean UIE was 135 nmol/mg creatinine after first soya milk as compared to 148 nmol/mg creatinine after

first miso soup intake (Fig. 1). When we expressed UIE as an hourly rate⁽³²⁾, the results remained unchanged; the respective medians for soya milk and miso soup were 6.39 and 6.57 $\mu\text{mol/h}$. The correlation between the two UIE values expressed in different units was 0.95. Based on all 6 d, the mean UIE on days after soya food intake were significantly higher than on the days without soya intake (*P*<0.001; Fig. 2). In a mixed model that included the baseline UIE values, the difference in UIE after soya milk *v.* miso soup consumption was not significant (*P*=0.87). The respective adjusted mean UIE for no soya, soya milk and miso soup were 5.7, 116.9 and 111.7 nmol/mg creatinine, respectively. Order (group A or B) was not significant (*P*=0.98). Inclusion of body weight and age did not change the effect estimate for UIE (*P*=0.19 and *P*=0.38, respectively).

To assess the repeatability of UIE after consuming the same food, we compared the first and second soya milk intake for group A (Fig. 3) and the first and second miso soup intake for group B (Fig. 4). There was no significant difference in UIE between first and second intake for group A or for group B (*P*=0.65 and *P*=0.81, respectively).

Equol excretion was observed in seven of our twenty-one (33%) participants. Three of the seven women showed equol excretion on all feeding days, three only on the initial feeding day, and one on feeding days 2 and 4. We observed no significant difference in UIE between soya milk and miso

Table 1. Characteristics of the study population

Group	Age (years)	Body weight (kg)	Soya intake (servings/d)		Mean isoflavone intake (mg/d)*
			Early life	Adult	
A	83	39.5	1.42	2.07	26.3
A	54	49.5	0.41	1.20	4.4
A	20	50.0	1.71	2.28	21.9
A	48	52.3	0.16	0.20	3.7
A	59	70.5	0.41	0.74	3.9
A	54	70.9	0.71	1.38	8.7
A	52	52.3	0.10	0.23	1.4
A	45	61.4	0.20	0.33	8.8
A	50	44.1	0.56	1.17	24.5
A	50	47.3	0.16	0.20	5.5
B	32	47.7	0.48	0.96	13.5
B	53	76.8	0.03	0.21	0.1
B	29	54.1	7.14	10.20	46.3
B	43	47.7	2.53	4.09	11.4
B	53	50.9	0.03	0.49	22.5
B	56	76.4	0.00	2.14	85.6
B	58	60.5	0.71	0.95	45.8
B	47	72.7	0.41	0.74	42.2
B	58	76.4	2.92	4.92	34.2
B	41	58.2	0.28	0.37	7.9
B	52	59.5	0.21	0.49	3.4
A + B					
Mean	49.4	58.0	0.98	1.7	20.1
SD	21.9	11.6	1.6	2.3	21.1
A					
Mean	51.5	53.8	0.58	0.98	10.9
SD	14.5	10.0	0.53	0.73	9.0
B					
Mean	47.5	61.9	1.3	2.32	28.4
SD	9.6	11.1	2.1	1.9	24.3
<i>P</i> value†	0.68	0.11	0.82	0.27	0.38

* Self-reported intake during the previous 12 months according to a FFQ.

† Two-sample *t* tests using log-transformed values for difference between groups A and B.

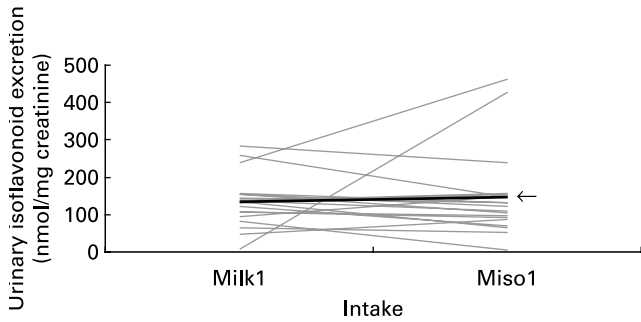


Fig. 1. Urinary isoflavonoid excretion on days 2 and 4 comparing first soya milk (Milk1) and miso soup (Miso1) intake. Each line represents one of the twenty-one Japanese-American women. ←, Mean.

soup for the seven equol producers ($P=0.88$). Based on the mixed model, there was no difference in overall UIE between equol producers and non-producers ($P=0.87$).

Discussion

In this small intervention study among twenty-one women with Japanese ancestry, we observed no significant difference in overnight UIE after intake of one serving of soya milk or miso soup with equivalent isoflavone content. Expressing UIE as nmol/mg creatinine or as nmol/h gave similar P values⁽³²⁾ and adjustment for potential confounders did not change the result. For the majority of subjects, the UIE did not vary by food (Fig. 1). The results are contrary to our expectation of a higher UIE for miso soup that contains readily available aglucones, whereas the isoflavones in soya milk require hydrolysis prior to uptake. Repeated intake of the same food on different days showed high reproducibility within subjects. There was also no difference in UIE among the subgroup of seven equol producers or between them and the non-equol producers. The fact that one-third of the participants excreted equol agreed with previous reports for Caucasians⁽¹¹⁾ but is low for persons of Asian ancestry⁽³³⁾.

The present results agree with some previous reports^(21–23,34,35), but they are in conflict with others^(18–20,36,37). Urinary isoflavonoid recovery did not differ significantly after a meal of cooked soybeans, tofu, texturized vegetable protein or tempeh⁽³⁴⁾ between subjects who ingested soyabean isoflavone glycosides or red clover isoflavone aglycones⁽³⁵⁾, and in a

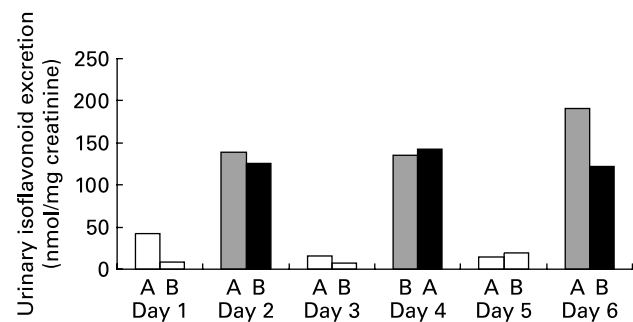


Fig. 2. Urinary isoflavonoid excretion before (days 1, 3 and 5; □) and after soya food intake (days 2, 4 and 6; ■, soya milk; ■, miso soup) in twenty-one Japanese-American women in two groups (A and B). Values are medians.

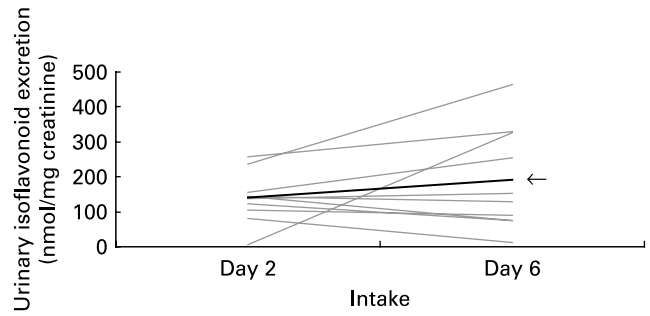


Fig. 3. Urinary isoflavonoid excretion after soya milk intake on days 2 and 6. Each line represents one of the ten group A Japanese-American woman. ←, Mean.

cross-over trial that provided regular soya milk containing mostly glucosides and soya milk treated by probiotic bifidobacteria to produce aglucones⁽²¹⁾. Similarly, equivalent bioavailability was observed when isoflavones were given as glucosides as naturally present in soya drinks and after enzymatically hydrolysing these drinks to produce aglucones⁽²³⁾, and bioavailability of isoflavones was similar among American women after consumption of aglucone or glucoside tablets⁽²²⁾. A comparison of isolated aglucones and conjugated isoflavones also showed identical uptakes for both⁽⁸⁾. On the other hand, a randomized cross-over trial described higher urinary isoflavonoid recovery after eating tempeh than soyabean pieces⁽¹⁸⁾, plasma concentrations over 24 h were higher after the administration of aglucone tablets than an equivalent glucoside preparation⁽¹⁹⁾, isoflavone aglycones were absorbed in greater amounts than their glycosides in a comparison of regular soya milk, fermented soya milk and β -glucosidase-treated soya milk⁽²⁰⁾, and tempeh resulted in larger areas under the curve than textured vegetable protein⁽³⁷⁾. Still another report demonstrated higher bioavailability of glucosides v. aglucones when the area under the plasma curve after oral dosage of 50 mg β -glucosides (daidzin, genistin) was compared to that after intake of the equivalent amount of aglycones (daidzein, genistein)⁽³⁶⁾.

Interventions that measured isoflavones in blood agree that the peak plasma level is achieved faster when aglucones as opposed to glucosides are consumed^(19,20,36,37). In the present study, it was not possible to assess the speed of isoflavone absorption during the first hours of consumption as described in studies that collected repeated blood samples^(8,19,20,37). However, the parallel pattern of plasma isoflavone levels

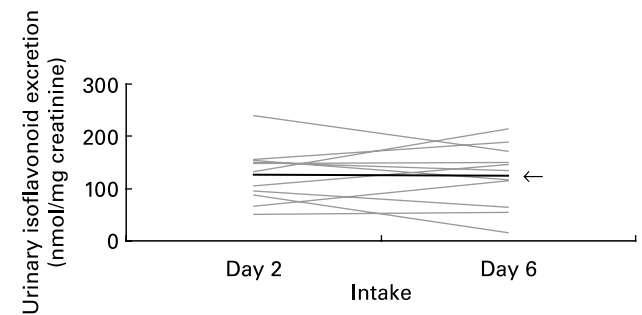


Fig. 4. Urinary isoflavonoid excretion after miso soup intake on days 2 and 6. Each line represents one of the eleven group B Japanese-American woman. ←, Mean.

and UIE when plotted as a function of time has been shown⁽⁸⁾. Published reports are consistent in that isoflavones in liquids rather than solids are absorbed faster, but cumulative uptake may be higher for solids^(8,37,38). Therefore, fermentation of soya foods leads to faster absorption and higher peak levels, but it appears likely that similar amounts of isoflavonoids from non-fermented products will become available over time.

The results of the present study have to be considered in light of several limitations, foremost the small sample size. Given the strong variation across subjects, the minimum difference by food that could have been detected with the present study, given a power of 0.80, would have been 49 nmol/mg creatinine. Because all subjects were free-living, we were not able to verify the food intake and the exact times of soya consumption. In fact, it appears that at least one subject may not have consumed the soya milk at all (Fig. 1). Although our choice of two liquid soya foods eliminated bias due to the differential uptake according to the texture of the soya foods^(37,38), the women may have consumed the study foods as part of a regular meal whose composition may have affected the uptake of the isoflavones⁽³⁷⁾. On the other hand, previous studies do not indicate a major effect of regular diet on the excretion of isoflavones^(34,35). Another issue was the lack of 24 h urine collections. Overnight urine samples represent the UIE that occurred over 10–12 h. Therefore, we have no information on isoflavonoid excretion during the next day. However, the time frame in the present study covered approximately 70% of the total UIE⁽⁸⁾. Total excretion levels could have also been determined in 24 h urine samples but extensive periods of urine collection tend to result in low compliance.

The homogeneous ethnic background of the women with regular soya intake in the past was a strength because it reduced possible variation in UIE due to the development of specialized intestinal flora adapted to the breakdown of soya foods⁽²⁴⁾. Individual differences in this cross-over study were minimized since the subjects served as their own controls and baseline UIE values were included in the statistical models. Results could be different in other ethnic groups who were not exposed to soya foods since childhood⁽²⁴⁾. The wide age range in the present study, as well as unmeasured genetic and lifestyle factors, e.g. alcohol intake and exercise, may have also affected isoflavone uptake. Although UIE does not directly assess isoflavone absorption, the use of urine seemed adequate to estimate isoflavone bioavailability due to the high correlation of isoflavone appearance patterns in plasma and urine⁽⁸⁾, the general high correlation between plasma and urine values when samples are collected correctly^(32,39), and the strong correlation between plasma and urine values as determined in different experimental settings^(16,17).

The interest in isoflavone uptake from fermented soya foods is based on the idea that the aglucones may be more readily bioavailable and more beneficial to human health⁽²⁰⁾. Due to the continued ambiguity, the present study examined this question using an ethnically homogeneous population, a cross-over design, two liquid soya foods and a timed urine collection. The present results do not support the idea that the consumption of fermented soya foods results in higher isoflavonoid exposure than the intake of unfermented soya foods. Future studies with repeated blood and/or urine collections

over more than 24 h among a larger study population are necessary to assess the speed of isoflavonoid uptake. At this time, the overall evidence does not justify nutritional recommendations that favour fermented soya foods as long as the intestinal microflora is capable of hydrolysing the isoflavone glucosides from unfermented soya foods.

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