British Journal of Nutrition (2010), **104**, 709–715
doi:10.1017/S0007114510000978

Overnight urinary excretion of isoflavones as an indicator for dietary isoflavone intake in Korean girls of pubertal age

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(Received 9 October 2009 – Revised 17 February 2010 – Accepted 18 February 2010 – First published online 13 April 2010)

Little is known about the bioavailability of isoflavones in children. Previous studies have shown that children excrete more isoflavone in urine compared with adults. Thus we examined the relationship between usual dietary isoflavone intake and the urinary excretion of isoflavonoids in Korean girls of pubertal age. Twelve girls each were selected from the lowest and the highest quartiles of isoflavone intake among 252 Korean girls aged 8–11 years. Age, BMI and sexual maturation stage were matched between the two groups. Dietary intakes for 3 d by diet record and overnight urine samples were collected at baseline and at 6 and 12 months. Total and individual isoflavone (daidzein, genistein and glycitein) intakes were calculated from diet records. The parent isoflavone compounds (daidzein, genistein and glycitein) and their metabolites (equol, *O*-desmethylangolensin (*O*-DMA), dihydrodaidzein and dihydrogenistein) present in the urine samples were analysed using liquid chromatography–MS. Intake levels of total and individual isoflavone compounds were significantly higher in the high isoflavone (HI) group than the levels in the low isoflavone (LI) group ($P<0·05$). Urinary excretion of all isoflavone parent compounds was significantly higher in the HI group than in the LI group ($P<0·0001$). Among isoflavone metabolites, only *O*-DMA and total metabolites were significantly different ($P<0·05$). Total isoflavone intake was highly correlated with the urinary excretion of total parent compounds (r 0·68; $P<0·01$), parent compounds plus their metabolites (r 0·66–0·69; $P<0·01$) and total isoflavonoids (r 0·72; $P<0·0001$). In conclusion, overnight urinary excretion of total isoflavonoids is a reliable biomarker of usual isoflavone intake in Korean girls of pubertal age.

Young Korean girls: Isoflavone intake: Isoflavone metabolites: Overnight urinary excretion

Isoflavones have been associated with a lower risk of chronic diseases, including prostate, breast and colorectal cancer, CHD, osteoporosis and menopausal symptoms[1–3]. Isoflavones are present in soya foods, mainly as glycosides. The major dietary isoflavones are daidzein, genistein and glycitein. Isoflavone glucosides are hydrolysed by intestinal β-glucosidases to produce aglycones (daidzein, genistein and glycitein) in the small intestine[4]. These aglycones are then absorbed by diffusion through the enterocyte and across the intestinal wall[5] and are further metabolised by the intestinal microflora in the large intestine. Daidzein is converted into dihydrodaidzein (DHDE), equol and *O*-desmethylangolensin (*O*-DMA), and genistein is converted into dihydrogenistein (DHGE) and *p*-ethylphenol, which are less predominant metabolites[6]. These compounds and metabolites circulate through the blood and are excreted in urine usually within 48 h after ingestion[7,8].

In several studies, urinary isoflavones were found to be reliable biomarkers for isoflavone consumption[9–14], and the urinary appearance of isoflavonoids accurately reflected circulating levels when the timing of specimen collection was considered[15]. Urinary isoflavone excretion has been well researched in adults as a suitable index of isoflavone intake. This is especially true in middle-aged women because of the possible role of isoflavones in women's health after the menopause. In several large epidemiological studies, strong preventive effects against breast cancer later in life were observed in women who had consumed soya at a young age[16,17]. Higher isoflavone intake during adolescence significantly reduced the risk of adult breast cancer by 20 % in Canadian women[18]. A recent study showed that the relative risks for breast cancer were reduced to 40 and 80 % among children and adolescents with high soya intake (1·5–8·8 times/week) compared with those with low soya intake (0–0·75 times/week)[19].

Abbreviations: DHDE, dihydrodaidzein; DHGE, dihydrogenistein; *O*-DMA, *O*-desmethylangolensin.

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Some studies have suggested that the bioavailability of isoflavones after soya consumption may be different in infants and children compared with adults[20,21]. One study showed that children aged 3–12 years had a higher urinary isoflavone excretion rate of both parent compounds and total isoflavones, as compared with their biological parents[21]. Little is known, however, about urinary excretion rates in children after isoflavone consumption. The relationship between urinary excretion of isoflavonoids and usual isoflavone intake among pubertal children requires further study.

A recent study showed that usual isoflavone intake has a favourable effect on bone metabolism in young Korean women[22]; however, few data on isoflavone dietary intake and metabolism are available for younger populations. Studies on isoflavones are important among Koreans, because their usual diet includes high consumption rates of isoflavones, mainly as soyabeans, compared with diets in Western countries. Among studies conducted in Koreans, isoflavone intakes were 7·8 mg/d for Korean children, 17 mg/d for college women and 24 mg/d for middle-aged women[23]. The level of isoflavone intake is lower than that of other Asian populations with a high intake of soyabeans, such as Chinese or Japanese women reported to eat 15–40 mg isoflavones daily[24–26]. However, the isoflavone intake is higher than that of Caucasian middle-aged women reported to consume between 0·2 and 5 mg isoflavones daily[27–30].

In the present study, we examined the relationship between usual dietary isoflavone intake and urinary excretion of isoflavonoids in Korean girls of pubertal age, who consumed high or low levels of isoflavones in their habitual diet.

Experimental methods

Subjects

The subjects in the present study were selected from among 252 girls, aged 8–11 years, who had participated in two dietary surveys conducted 10 months apart. The surveys were conducted with the permission of the principal and teachers at an elementary school in Seoul, Korea, after explaining the study purpose and procedures. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional Review Board of the Graduate School of Public Health, Seoul National University. Written informed consent was obtained from all subjects.

At the initial pre-survey, all participants completed a 3 d dietary record that included two weekdays and one weekend day, while they consumed their usual diets. At the first meeting, detailed instructions for the 3 d dietary record were given to the subjects by trained dietitians. These instructions included pictures of food and detailed instructions on how to record the exact amounts of food intake. An explanation of the procedure was also given to the students to take home and show their parents, who could then provide any necessary help with the survey. During the following week, the students brought the records to the survey team and were interviewed to complete any missing information. Height and body weight were measured while the subjects were wearing light clothes without shoes. Body weight was measured by Inbody 3.0 (Biospace Co. Ltd, Seoul, Korea). To identify pubertal maturation stage, Tanner's puberty standard assessment was completed using a self-assessment sheet showing pictures of pubic hair and breasts[31]. Subjects were grouped into quartiles according to their daily mean intake of total isoflavones during two survey periods (pre-survey and first survey). Twenty-five girls were included in the lowest quartile from both survey periods and eighteen girls were included in the highest quartile from both survey periods. Of these, some of the girls did not agree to the urine collection. Finally, twelve girls each from the lowest and highest quartiles were selected as the 'low intake group' (between 0·06 and 3·67 mg/d) and the 'high intake group,' (between 9·29 and 51·59 mg/d), respectively. Mean daily intakes of major nutrients and isoflavones, i.e. daidzein, genistein and glycitein, from three survey periods were calculated using the nutrient database of the Korean Nutrition Society[32] and the isoflavone compound database developed by our group[23]. An isoflavone database was established with a systematic evaluation system. Among 2932 food items in the Korean Nutrient Database, 142 food items were assumed to have isoflavones. Among these, twenty-five food items were evaluated from analytical values and nineteen food items were used from the United States Department of Agriculture database. The remaining ninety-eight items were replaced with adaptations or calculations from similar items. Subjects in the two groups were matched as closely as possible for age and other physical characteristics, including BMI and Tanner stage.

Data collection

After the pre-survey, the first study survey was conducted and follow-up surveys were conducted at 6 months and 12 months after the first survey on the subjects selected for the present study. At each survey, dietary intakes of 3 d were collected as in the previous surveys. At the first survey, the subjects were asked to collect one overnight urine sample during the week for which they recorded their dietary intake. Overnight urine samples replaced 24 h urine collection because of difficulties in completing the urine collection from children during the daytime. Overnight urine samples were collected in urine bags prepared with 0·2 g ascorbic acid and 0·3 g boric acid as preservatives to prevent bacterial growth leading to isoflavone degradation. The bags were distributed to the subjects at the first meeting for each survey. The subjects were instructed to record the time of the last urination before going to bed, collect all urine including the one after getting up in the morning, and record the time of the last urine collection. Urine samples were retrieved on the morning of sample collection and transported on ice to the laboratory. Upon arrival, the urine samples were mixed thoroughly, urine volume was measured, and samples were stored at −20°C until further analysis. The average intake calculated from three dietary records of a total of 9 d over 12 months, and the average of a total of three urinary excretion samples over 12 months were used for data analysis.

Analysis of urinary isoflavones and their metabolites

The urinary analysis of isoflavonoids was performed as described previously[33–35]. The urine samples were completely

thawed at room temperature, and the isoflavonoids were extracted and then quantified by liquid chromatography–MS. Isoflavones (daidzein, genistein and glycitein) and their major metabolites (equol, *O*-DMA, DHDE and DHGE), in addition to creatinine, were analysed from each urine sample. Creatinine was determined in 0·01 ml urine using a commercial test kit using a Cobas MiraPlus chemical autoanalyser (Kit 555; Sigma, St Louis, MO, USA). The isoflavonoid concentrations in urine were adjusted for the creatinine level, and the final isoflavonoid excretion values are reported in nmol/mg creatinine. Total isoflavonoid excretion was calculated as the sum of all excreted isoflavonoids measured. The mean value of three urine samples from each subject was used to analyse the relationship between dietary intake and urinary excretion of each isoflavonoid.

Statistical analysis

The results are expressed as mean values and standard deviations, or CV. Statistical analyses were performed using SAS software (version 9.1; SAS Institute, Inc., Cary, NC, USA). Differences in the baseline characteristics of the subjects by isoflavone intake or differences in urinary excretion of isoflavones and their metabolites between the two groups (by isoflavone intake) were evaluated using Student's *t* tests. Urinary excretion of isoflavones and their metabolites were expressed as mean and CV to compare the values considering the difference because the mean values among isoflavone metabolites were very different from each other. To investigate the relationship between dietary intake and urinary excretion of isoflavonoids, the mean value from three surveys was used. Dietary and urinary values of isoflavonoids were log-transformed to compensate for a skewed distribution. Owing to the small number of subjects in the study, Spearman's correlation coefficients were used in correlation analyses. Statistical significance was defined as $P<0·05$.

Results

The girls in the low isoflavone intake and high isoflavone intake groups were comparable in age, anthropometry and energy intake. Girls in the low intake group had higher fat intake and lower protein intake as a percentage of total energy intake, compared with the girls in the high intake group (Table 1). The mean isoflavone intake was significantly different between the low and high intake groups for all survey periods (Fig. 1). The mean daily intake of total isoflavones was 3·0 mg in the low intake group and 13·3 mg in the high intake group ($P<0·0001$). Although the intakes of individual isoflavones (daidzein, genistein and glycitein) were higher in the high intake group compared with the low intake group, the contributions of the three individual isoflavones relative to total isoflavones were similar between the groups. Black beans, soyabean paste and tofu (soyabean curd) were the primary foods that contributed to isoflavone intake in the high intake group, whereas seasonings such as soyabean paste and Tcha Jang were the main contributors in the low intake group.

Table 2 shows the amounts of isoflavones and their metabolites excreted in the urine. The most abundant isoflavone excreted in urine was daidzein (47 % of total excretion), followed by genistein (17 %). Approximately 63 % of the excreted urinary isoflavonoids were the unmetabolised parent compounds. On average, urinary excretion of the parent compounds (daidzein, genistein and glycitein; $P<0·01$) and total isoflavonoids ($P<0·0001$) was significantly higher in the high intake group than in the low intake group; however, urinary excretion of isoflavone metabolites except for *O*-DMA did not differ between the two groups. The urinary excretion of *O*-DMA was higher in the high intake group compared with the low intake group ($P<0·05$).

The correlation coefficients between isoflavone intake and urinary excretion of the isoflavone compounds are shown in Table 3. Dietary intakes of daidzein, genistein and glycitein were highly correlated with their respective urinary excretion

Table 1. Subject characteristics and dietary intake of isoflavones in pubertal Korean girls
(Mean values and standard deviations)

	Low intake group (*n* 12)			High intake group (*n* 12)			
	Mean	SD	Percentage of total	Mean	SD	Percentage of total	*P**
Characteristic							
Age (years)	10·3	0·5		10·2	0·7		0·748
Height (cm)	140·3	6·1		139·0	5·9		0·589
Weight (kg)	35·9	8·4		33·1	4·4		0·329
BMI (kg/m^2)	18·1	3·3		17·1	1·6		0·375
Energy intake (kJ)	6486	1149		6576	1280		0·857
Percentage of total energy intake from each macronutrient							
Carbohydrate	55·2	4·3		56·5	3·8		0·435
Protein	15·2	1·2		16·5	1·05		0·016
Fat	29·5	3·2		26·5	3·5		0·039
Isoflavone intake (mg/d)							
Daidzein	1·2	0·3	40·9	5·3	1·9	39·8	<0·0001
Genistein	1·4	0·5	48·2	6·2	1·6	47·1	<0·0001
Glycitein	0·3	0·1	11·8	1·8	1·2	13·7	0·002
Total isoflavones	3·0	0·9		13·3	3·4		<0·0001

* *P* values by Student's *t* test were used to evaluate significant differences between the mean values of the two groups.

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Fig. 1. Daily values of total isoflavone intake for the low intake (Low) and high intake (High) groups during all survey periods. Mean values are indicated by the horizontal lines, 95% confidence intervals by the boxes and ranges by the whiskers, with ○ indicating an outlying value. Mean isoflavone intake was significantly different between the low intake and high intake groups during all survey periods ($P<0·05$).

rates (r 0·57–0·64; $P<0·01$). Total isoflavone intake (sum of daidzein, genistein and glycitein) was highly correlated with the urinary excretion of daidzein (r 0·61; $P<0·01$), genistein (r 0·64; $P<0·001$) and glycitein (r 0·59; $P<0·01$). Dietary intake of daidzein was highly correlated with the urinary excretion of *O*-DMA, which is its metabolite. Dietary intakes of daidzein and genistein were highly correlated with the urinary excretions of daidzein or genistein plus their respective metabolites (daidzein + equol + *O*-DMA + DHDE and genistein + DHGE) (r 0·72 and 0·64; $P<0·01$). Total isoflavone intake was also highly correlated with the urinary excretion of daidzein or genistein plus their respective metabolites (r 0·69 and 0·66; $P<0·01$). Average of urinary isoflavone excretion from three surveys had higher correlation with isoflavone intake than one urinary isoflavone excretion from each survey (data not shown).

Discussion

In the present study, total urinary isoflavone excretion was significantly higher in the high intake group compared with the low intake group. Total urinary isoflavonoid excretion was strongly correlated with usual dietary isoflavone intake, based on 9 d of diet records for twenty-four young girls. These results suggest that urinary isoflavonoid excretion is a reliable biomarker of usual isoflavone intake in girls of pubertal age. Specifically, the urinary excretion of total unmetabolised compounds (daidzein + genistein + glycitein) or individual parent compound plus their metabolites (daidzein + equol + *O*-DMA + DHDE and genistein + DHGE) showed a high correlation with the dietary intake of total isoflavones.

This positive relationship between dietary isoflavone intake and urinary isoflavone excretion is consistent with previous studies in adults[10–14,36]. Grace *et al.* reported significant relationships of both urinary and serum concentrations of isoflavones with dietary isoflavone intake in women aged 45–75 years who participated in the European Prospective Investigation of Cancer and Nutrition-Norfolk study[11]. Ritchie *et al.*[37] showed that isoflavone supplementation (35 mg/d)

Table 2. Urinary excretion of isoflavones and their metabolites in pubertal Korean girls
(Mean values and coefficients of variation for twelve girls per group)

	1st measurement (nmol/mg creatinine)				2nd measurement (nmol/mg creatinine)				3rd measurement (nmol/mg creatinine)				Average (nmol/mg creatinine)				Percentage of total			
	Low		High		Low		High		Low		High		Low		High		Low		High	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Parent compound																				
Daidzein	7·0	69·1	16·8	98·4	6·2	207·4	21·5	123·7	2·4	147·3	10·7*	109·8	5·1	92·5	16·3**	65·4	42·9	42·2	50·8	33·3
Genistein	3·3	67·8	3·8	100·1	2·0	191·7	7·4*	90·5	0·6	76·31	5·7	153	1·9	95·1	5·7**	62·9	15·8	55·6	19·0	45·9
Glycitein	0·7	82·9	1·2	82·2	0·7	123·6	1·7	94·3	0·3	83·5	1·0*	115·9	0·5	53·8	1·3**	57·8	5·3	49·0	4·1	20·7
Subtotal	11·1	59·4	21·8	92·6	8·8	193·5	30·6	110·5	3·3	119·6	17·7*	117·9	6·6	73·6	21·7**	54·8	57·6	29·6	68·6	23·1
Metabolite																				
Equol	1·6	202·7	4·4	259·5	0·5	318·9	2·1	272·7	0·1	330·8	1·1	249·1	0·8	143·6	2·5	160·4	9·4	138·7	8·8	176·6
O-DMA	0·7	56·1	1·5*	81·4	0·6	172·6	2·2*	95·1	2·0	113·7	2·0	121·8	1·0	82·2	1·9*	62·4	10·3	85·6	6·6	49·6
DHDE	1·3	67·2	1·9	102·1	0·6	148·4	2·9	143·1	0·1	178·8	0·3	193·1	0·7	56·0	1·7	93·8	7·8	69·4	6·1	69·3
DHGE	1·3	132·0	1·0	140·3	0·7	173·8	1·8	190·7	0·3	131·9	0·5	96·8	0·8	113·2	1·1	130·9	8·4	126·7	4·7	134·4
Subtotal	4·9	74·1	8·9	138·5	2·4	140·9	9·0	134·4	2·5	84·2	3·9	88·1	4·4	60·3	8·9*	60·1	42·4	40·3	31·4	50·3
Total isoflavones	16·0	41·1	30·7	74·7	11·2	172·0	39·6*	96·0	5·8	74·1	21·4*	100·6	11·0	60·8	30·6***	48·6	100		100	

Low, low intake group; High, high intake group; *O*-DMA, *O*-desmethylangolensin; DHDE, dihydrodaidzein; DHGE, dihydrogenistein.
Mean value was significantly different from that of the low intake group at the same period: * $P<0·05$, ** $P<0·01$, *** $P<0·001$.

Table 3. Spearman's correlation coefficients between isoflavone intake and urinary isoflavonoids in pubertal Korean girls (*n* 24)

	Dietary intake			
Urinary excretion	Daidzein	Genistein	Glycitein	Total
Daidzein	0·64**	0·58**	0·63***	0·61**
Genistein	0·62***	0·62**	0·69***	0·64**
Glycitein	0·59**	0·55**	0·57**	0·59**
Subtotal	0·71***	0·63**	0·64**	0·68**
Equol	0·21	0·2	0·21	0·22
O-DMA	0·43*	0·39	0·3	0·41*
DHDE	0·36	0·38	0·17	0·35
DHGE	0·17	0·14	0·14	0·19
Daidzein + M†	0·72***	0·65**	0·63**	0·69**
Genistein + M‡	0·63**	0·64**	0·73***	0·66**
Total	0·74***	0·68***	0·71***	0·72***

O-DMA, *O*-desmethylangolensin; DHDE, dihydrodaidzein; DHGE, dihydrogenistein; M, metabolites.
* $P<0·05$, ** $P<0·01$, *** $P<0·001$.
† Metabolites of daidzein (equol + *O*-DMA + DHDE).
‡ Metabolite of genistein (DHGE).

for 3 months significantly increased urinary excretion of the isoflavones genistein and daidzein. Furthermore, there was a positive relationship between isoflavone intake and urinary excretion in fifteen premenopausal women aged 30–51 years, suggesting that 24 h urinary isoflavone levels can be used as reliable biomarkers of isoflavone intake. This trend was comparable with the results of a soya intervention study in girls in the USA. Urinary isoflavone excretion was highly correlated with total isoflavone intake (R^2 0·9506; $P<0·05$) in both Korean and US girls. In the intervention study, after soya isoflavone intake was increased from 5·4 to 32·6 mg/d ($P<0·01$) for 8 weeks in young girls aged 8–14 years, urinary isoflavone excretion increased more than 6-fold (from 23·3 to 142·1 mmol/mg creatinine; $P=0·02$)[38].

On the other hand, none of the urinary isoflavone metabolites except for *O*-DMA was significantly different between the low intake group and the high intake group. This suggests that a single metabolite as a biomarker of dietary isoflavone intake may not be adequate as much as total unmetabolised compounds or individual parent compound plus their metabolites. Lampe *et al.*[39] reported that urinary excretion of isoflavone metabolite such as *O*-DMA and equol did not differ between a high soya intake group and a low soya intake group, suggesting that the association between soya intake and DMA or equol excretion was not stronger than that between soya consumption and genistein and daidzein excretion. Moreover, only 30 % of individuals consuming Western diets are equol producers.

In the present study, daidzein had the highest rate of excretion in urine, followed by genistein and then glycitein. On average, daidzein and genistein accounted for 47 and 17 % of total isoflavone excretion. These results are consistent with previous studies[40–42]. Seow *et al.*[13] reported that urinary excretion of daidzein and genistein was 45 and 18 % of total isoflavone excretion, respectively, in Chinese women in Shanghai. Maskarinec *et al.*[12] showed that urinary daidzein was excreted at the highest rate, followed by genistein and glycitein, for all ethnic groups studied, including Japanese, Filipinos and Caucasians.

We compared urinary isoflavone excretion between young Korean girls and data gathered from adults in other studies (data not shown). Korean girls of pubertal age excreted more isoflavonoids than the adults, even though isoflavone intake was comparable. This may be explained by higher exposure per kg body weight. Higher isoflavone bioavailability in children may be a result of their gut flora, which are able to efficiently hydrolyse isoflavonoids to the bioavailable aglycone, but do not degrade the aglycones as fast as in adults[21]. A few studies have suggested that children absorb more isoflavonoids than adults[20,21,43]. Franke *et al.* reported a higher bioavailability in infants[20] and recently observed that urinary isoflavone excretion in thirty-seven healthy children was significantly higher than that in thirty-four healthy adults (39·6 *v.* 31·3 nmol/h per kg; $P<0·05$), when the subjects consumed soya nuts at a dose of 15 g per 54·4 kg body weight[43]. The authors suggested that urinary isoflavone excretion is an adequate surrogate for determining isoflavone bioavailability and for measuring soya or isoflavone intake in epidemiological studies.

The present study has some limitations. We could not collect urine over a 24 h period or longer, because of difficulties in completing the urine collection from children during the daytime; however, overnight urine collection was a good compromise, assured complete urine collections in this period, and had previously resulted in a very high compliance rate[12]. Overnight urinary isoflavone excretion has been shown to reflect reasonably well the recent or usual intake of soya foods or isoflavones[9,10]. For example, Maskarinec *et al.* showed a strong correlation between isoflavone intake (previous 24 h) and overnight urinary excretion among women in a multiethnic population (r 0·62; $P<0·0001$)[12]. Among women in the USA who consumed soya at either the two diet recalls or at the FFQ (3·5 years), isoflavone intake and urinary excretion were significantly correlated[9]. The correlation between dietary intake (previous 5 years) and overnight urinary excretion of total isoflavonoids was 0·54 in Chinese women with a high level of soya food consumption[10]. Furthermore, Franke & Custer recommended a combination of three urine samples collected every other night for each subject[34]. The isoflavone database used in the present study has not been validated. However, we believe it will be reasonable because the isoflavone database was established using the expert systematic evaluation system that the United States Department of Agriculture also used for the development of its isoflavone database[44]. Another limitation of the present study is the small number of subjects, which was due to the limited available resources. Further research on isoflavones and isoflavone metabolite excretion in urine as a useful biomarker of isoflavone intake should be conducted in a larger population. Isoflavone intervention studies are necessary to confirm the present results and to identify the beneficial effects of isoflavonoids in children or young adults.

In conclusion, our data expanded on the little-known isoflavone excretion pattern in children and showed total urinary isoflavonoid excretion was strongly associated with dietary isoflavone intake in 8- to 11-year-old Korean girls. This observation provides an important clue that overnight urinary isoflavone excretion can be used as a suitable index of usual isoflavone intake in epidemiological studies in children and adolescents. The present study also adds important data to support that dietary isoflavones may be more bioavailable in

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children than in adults. To our knowledge, there was hitherto no information available on isoflavone intake and its metabolism among children in Korea, a country with an intermediate soya exposure in adults. If confirmed, our findings have important public health implications for future research aimed at the role of soya and isoflavones in the prevention of chronic diseases.

Acknowledgements

This research was supported by the Korea Research Foundation (project number: C00079 (R04-2003-000-10199-0)) and the US National Center for Research Resources (S10RR020890).

H.-Y. P. and H. J. contributed to designing the research; H. J., H. J. K., S. L. and M. K. P. contributed in subject recruitment and data collection; Y. J. S., M. K. P. and H. J. K. contributed to dietary data analysis; J. K., H. J. K. and A. A. F. contributed to urine sample preparation and analysis; J. K., H. J. K., A. A. F. and H.-Y. P. contributed to final data analysis and manuscript preparation; H.-Y. P. had primary responsibility for the final content. All authors read and approved the final manuscript.

The authors declare that they have no competing interests.

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