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Effects of Soy Isoflavone Intake on Urinary and Fecal Isoflavone Excretion in Rats*

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This study was undertaken to determine the bioavailability of isoflavones in weanling Sprague-Dawley rats by providing diets containing different levels of soy isoflavones for 6 weeks: 0.025% (low isoflavone intake; LI), 0.125% (medium isoflavone intake; MI), and 0.25% (high isoflavone intake; HI). The subsequent fecal and urinary excretion of daidzein and genistein was then measured. As the levels of dietary isoflavones increased, the amount of food intakes significantly decreased, and weight gain was slower in female rats. In male rats, there was no significant difference in weight gains related to dietary intakes. Urinary excretion of daidzein and genistein was significantly higher in the MI and HI groups in both male and female rats than the control and LI groups. The recovery % of daidzein and genistein in the urine was significantly lower in the MI and HI groups. Fecal daidzein increased as dietary isoflavone intakes increased in female rats; however, in male rats the increase was significant only in the HI group. The recovery % of daidzein and genistein in the feces of female rats was not significantly different among the four groups. When dietary isoflavones were increased from 0.025% to 0.25%, the amounts of daidzein and genistein excreted in the urine and feces increased; however, the low recovery rate of both daidzein and genistein in the urine implies an increased bioavailability of isoflavones. We also observed sex-related differences in the urinary and fecal recovery of isoflavone intakes.

Key words : Isoflavones, Daidzein, Genistein, Urinary excretion, Feces, Recovery

INTRODUCTION

Recent epidemiological studies have found that the incidence of hormone-dependent diseases such as breast cancer, prostate cancer and osteoporosis, and of cardiovascular diseases in westerners is much higher than in Asians who consume more soybean products than their western counterparts. This has heightened interest in searching for physiologically active substances in soybeans.[1,2] Soybeans contain 0.1 to 0.4% of isoflavones, which are considered to be the main active substance in soybeans.[3] Isoflavones are known to have many pharmacological properties including anti-cancer effects, reductions in low density lipoprotein (LDL) cholesterol, and the prevention and suppression of osteoporosis.[4,5]

Isoflavones are diphenol substances which are widely distributed in the plant kingdom, and have both structures and molecular weights which are similar to those of the human reproductive hormone, estrogen isoflavones are therefore also called phytoestrogens.[6,7] Isoflavonoid has structural similarity to estrogen and binds to estrogen receptor, suggesting that it exhibits estrogenic action in various tissues. It has demonstrated that soy isoflavones prevent bone loss under estrogen-deficient conditions in ovariectomized animals.[8,9] Hence, it is critical to distinguish the doses of isoflavones that affect bone and uterus to establish the dose that promotes beneficial effects on bone in osteopoorosis with promoting uterine hypertrophy.

The isoflavones include such substances as genistein, daidzein, and glycitein. Genistein and daidzein are present as glycosides in soybeans.[10] These glycosides are not physiologically active in humans or animals however, they are activated when they are hydrolyzed to aglycon substances by peptic acid and micro-organisms in the intestines.[11] The ingested isoflavones are either excreted in feces or are excreted in the urine after being absorbed and utilized by the body through enterohepatic circulation.[12] Genistein, dadizein, the metabolites of daidzein (equol and *O*-desmethylangoensin (*O*-DMA), and the metabolites of genistein (6′hydroxy-*O*-DMA), are substances found in the urine of humans following consumption of foods containing isoflavones.[13,14]

The absorption and excretion of isoflavones vary according to the level of isoflavone intake as well as to intestinal microflora conditions. Slavin et al.[15] reported that when 0, 5, 10, or 20 g of soybean protein (0~36 mg of isoflavones) were given to adults over a 9 day period, the excreted levels of daidzein, genistein, equol, and total isoflavonoids

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were positively correlated with the amount of dietary intake of isoflavones. Another study of Japanese subjects who had high isoflavone consumption showed a positive relationship between levels of soy protein intakes and of urinary excretion of isoflavonoids.[16] In a study where three groups of adult female subjects were given soy milk containing different concentrations of isoflavones, the group who had the highest fecal excretion of isoflavones also had a 2.5 times higher excretion of genistein and daidzein in the urine, compared to the group with lowest fecal excretion the half life of genistein and of daidzein in the large intestine were only 7.5 hours and 3.5 hours, respectively.[17] Thus, the bioavailability of isoflavones in the body depends on the relative ability of intestinal microorganisms to break down isoflavones. Also, fermentation of soybeans could affect the bioavailability of isoflavones.[18] This effect could be due to changes in the structure of the constituents of soybeans caused by the fermentation process using Rhizopus oligosporus, which improved the rates of digestion and utilization of isoflavones.[19-21]

Isoflavones may act as estrogen agonists or antigonists.[22] A study of the metabolism and disposition of soy isoflavones in humans reported that initial urinary excretion, long-term excretion pattern, and recovery in urine of daidzein and genistein in men is different those in women during one month of daily soy ingestion.[23] Thus, isoflavone metabolism and disposition are affected by the duration of soy ingestion and by sex.

Therefore, this research was undertaken to study the bioactivity of isoflavones by giving diets containing three different concentrations of soy isoflavones to male and female rats and then measuring the amounts of daidzein and genistein excreted in the urine and feces.

MATERIALS & METHODS

Experimental Animals

Weanling male and female Sprague-Dawley rats were obtained from Daihan Biolink (Umsung, Korea). The Rats were individually caged in an environmentally controlled animal laboratory at a temperature of 23±1℃, a relative humidity of 65±57%, and a 12-h light/dark cycle maintained. After a one-week acclimation period, female rats weighing 100~114 g and male rats weighing 105~115g were devided by initial body weight into 4 blocks of nine rats each, using a randomized complete block design. Water and food were given ad libitum for 6 weeks.

Experimental Diets

The American Institute of Nutrition diet (AIN-76) was fed to all rats; in addition, except for the control group where no isoflavones were fed, 0.025% of isoflavones (low isoflavone intake; LI)), 0.125% of isoflavones (medium isoflavone intake; MI), and 0.25% of isoflavones (high isoflavone intake; HI), were added to the basic diet (Table 1). The amount of isoflavones added to the LI group was derived from the daily human consumption of 25 g of soybean protein recommended by the FDA (Food and Drug Administration) which equates to approximately 50 mg of isoflavones, and from 30 mg of average consumption of isoflavones by Koreans. Aglycone-type isoflavone powder manufactured from soybean germs and containing 32.18% of isoflavone glycoside (genistein 3.76%, daidzein 19.33%, and glycitein 9.09%) was purchased from Shindongbang (Ansan, Korea). The vitamin and mineral mixtures were purchased from Harlan Teklad (Madison, USA). Cellulose, DL-methionine, and choline chloride were purchased from Sigma (St. Louis, USA). The source of casein was the Junsei Chemical (Tokyo, Japan). Corn starch, sucrose and corn oil were purchased in the local market.

Table 1. Composition of experimental diets

Ingredients (g/kg diet)	Control	LI	MI	HI
Corn starch	450	449.75	448.75	447.5
Sucrose	200	200	200	200
Cellulose	50	50	50	50
Casein	180	180	180	180
DL-methionine	3	3	3	3
Corn oil	70	70	70	70
Mineral mixture[1] (AIN-76)	35	35	35	35
Vitamin mixture[2] (AIN-76)	10	10	10	10
Choline chloride	2	2	2	2
Isoflavone powder	0	0.25	1.25	2.5
Total	1000	1000	1000	1000

1) Mineral mixture provides calcium phosphate, 17.5g: sodium chloride, 2.59g: potassium citrate, 7.7g potassium sulfate, 1.82g: magnesium oxide, 0.84g: manganous carbonate 0.1225: ferric citrate, 0.21g: zinc carbonate, 0.056g: cupric carbonate, 0.0105g: potassium iodate, 0.00035g: sodium selenite, 0.00035g: chromium potassium sulfate, 0.01925g: sucrose finely powdered, 4.13105g

2) Vitamin mixture provides thiamin HCl, 0.006g: riboflavin, 0.006g: pyridoxine HCl, 0.007g: niacin, 0.03g: calcium pantothenate, 0.016g: folic acid, 0.002g:biotin, 0.0002g: vitamin B_{12}, 0.00001g: dry vitamin A palmitate, 4.000U: dry vitamin E acetate, 50U:vitamin D_3 trituration, 1.000U: menadione sodium bisulfite complex, 0.00005g: sucrose finely powdered, 9.81g

LI; low isoflavone intake, MI; medium isoflavone intake, HI; high isoflavone intake

Collection of Feces and Urine

After 5 weeks on their respective diets, the rats were moved to individual metabolic cages where their feces and urine were collected for 24 hours. The total volume of urine was measured and frozen until analysis. The feces were weighed after removing hair and other non-fecal substances, and were frozen-dried until further analysis.

Determination of Urinary Isoflavones

The genistein and daidzein excreted in the urine were measured by a modified method of Record et al.,[24] using reversed-phase high performance liquid chromatography (HPLC). 40㎕ of 0.17M ammonium acetate (pH 4.6), containing β-glucuronidase (1,670 unit/ml), were added to 20㎕ of the urine sample, and the mixture was incubated at 37℃ for 16 hours in a constant temperature water bath in order to completely hydrolyze the isoflavones. 100㎕ of ethyl ether were added and centrifuged at 5,000 rpm for 15 minutes, following which the supernatant was collected; this process was repeated once more to ensure the complete extraction of isoflavones. The resulting supernatant was evaporated with N_2 gas then, 30㎕ methanol was added to dissolve extracted isoflavones and was analyzed using HPLC. The conditions of the HPLC analysis are shown in Table 2.

Table 2. HPLC conditions for the determination of isoflavones

Parameter	Conditions
Instrument	Waters 600
Detector	Waters 486, UV/VIS
Integrator	Waters 746 data module
Column	Nova-Pak C_{18} 4㎛ 3.9×300mm
Mobile phase	Water : Acetonitrile = 85:15
Wavelength	UV 254 nm
Flow rate	1.0 ml/min
Injection volume	10㎕

Determination of Fecal Isoflavones

Fecal genistein and daidzein were analyzed by using the HPLC method as described for urine. The samples were prepared for analysis by first adding 0.3 g of feces (freeze-dried and powdered) to 5 ml of ethanol. After completely sealing, the samples were incubated for 30 minutes in a shaking incubator at 55℃. After the complete extraction of isoflavone, the samples were centrifuged at 3000 rpm for 15 minutes. 1 ml of supernatant was added to 1 ml of HCl solution (4 mol/L), and the sample mixture was put in a constantly boiling incubator for 30 minutes in order to hydrolyze isoflavone conjugates. 2 ml of methanol and 2 ml of water were added to C_{18} solid-phase extraction cartridges (sep-pak plus, Waters, U.S.A.) for conditioning; the hydrolyzed samples were loaded after rinsing with 2 ml water, and were eluted with 2 ml of 80% methanol. 10㎕ of the extracts were used for subsequent analysis.

Statistical Analysis

The experimental data were expressed as means and standard errors of means, and were analyzed by one-way ANOVA. The differences in mean values between the group were tested using Duncan's multiple range test and were considered significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

Food intakes and weight gains

The levels of food intake of the experimental female and male animals are presented in Tables 3. In female rats, food intake decreased as the level of dietary isoflavones increased, but in the case of male rats there were no significant differences among the groups. Food intake of the female HI group was significantly lower than the female control group. According to Tables 4, the amount of weight gain decreased as the amount of isoflavones increased in the diet in female rats however, there were no such differences among the male rats. The amount of weight gain in the male rats was slightly higher than that in the females.

The Amounts of Isoflavones Excreted in the Urine

Tables 5 and 6 present the levels of daidzein and genistein excreted in the urine of the female rats over a 24-hour period. As the level of dietary isoflavones increased, the urinary excretion of daidzein and genistein significantly increased in the MI and HI groups compared to the LI group. The difference between the MI and the HI groups was not significant. The amounts

Table 3. Food intake in female and male rats[1)]

(g/week)

Sex	Group	Experimental period, week						
		1	2	3	4	5	6	Average
Female	Control	99.9±2.6^{a}	107.7±2.5^{a}	102.5±3.2^{a}	104.2±4.2	77.6±4.3^{a}	111.1±5.2^{a}	100.5±3.7^{a}
	LI	95.7±1.8^{ab}	105.9±3.5^{a}	96.3±1.2^{a}	97.5±3.9	76.8±3.6^{ab}	101.1±5.0^{ab}	95.6±3.2^{ab}
	MI	92.0±2.7^{bc}	92.0±1.9^{b}	85.7±1.7^{b}	97.6±4.6	68.7±2.2^{ab}	94.1±2.3^{bc}	88.4±2.6^{ab}
	HI	87.0±1.4^{c}	93.5±8.7^{b}	84.4±2.0^{b}	92.1±4.0	67.5±2.3^{b}	84.2±1.8^{c}	84.8±3.4^{b}
Male	Control	111.3±4.2	131.7±2.3^{a}	131.4±4.6^{ab}	139.9±3.8	152.7±3.0^{a}	146.8±5.3	135.6±3.9
	LI	107.8±3.2	134.9±3.6^{a}	134.6±2.8^{a}	140.5±2.3	152.1±3.9^{a}	150.5±3.8	136.7±3.3
	MI	111.2±1.1	133.8±2.9^{a}	124.3±1.7^{b}	149.4±3.5	146.4±4.4^{ab}	148.4±2.3	135.6±2.7
	HI	104.6±3.8	123.4±2.4^{b}	128.7±1.9^{ab}	142.7±3.6	139.1±3.7^{b}	141.3±2.4	129.7±3.0

1) Values are means±SEM (n=9). Values in a column with different superscripts in the same sex are significantly different ($P < 0.05$) as assessed by Duncan's multiple range test.

LI; low isoflavone intake, MI; medium isoflavone intake, HI; high isoflavone intake

Table 4. Weight gain in female and male rats[1)]

(g/week)

Sex	Group	Experimental period, week						
		1	2	3	4	5	6	Average
Female	Control	34.4±1.5^{a}	23.7±1.7	27.4±2.0^{a}	14.0±3.4	23.5±3.3^{a}	11.7±2.1	22.5±2.3
	LI	32.9±0.7^{a}	22.3±2.1	24.3±2.5^{a}	18.5±2.5	20.0±3.6^{ab}	8.7±3.0	21.1±2.4
	MI	28.0±1.7^{b}	19.3±1.8	16.4±1.3^{b}	20.0±2.0	13.7±1.3^{b}	6.1±1.3	17.3±1.6
	HI	24.4±1.4^{b}	18.5±1.8	17.6±1.5^{b}	13.1±5.0	13.9±2.4^{b}	5.9±1.1	15.6±2.2
Male	Control	49.9±1.7	48.0±1.4^{a}	48.3±2.1^{ab}	33.4±2.4	42.2±2.9^{ab}	28.2±2.9	41.7±2.2
	LI	49.8±1.9	47.7±1.9^{a}	50.7±2.0^{a}	36.8±1.8	43.4±2.2^{a}	28.5±2.9	42.8±2.1
	MI	49.8±1.0	46.9±1.1^{a}	43.5±2.0^{b}	42.5±3.5	37.1±1.3^{ab}	26.9±2.6	41.1±1.9
	HI	45.5±3.2	41.4±2.7^{b}	45.3±2.2^{ab}	37.5±3.8	33.9±3.9^{b}	23.4±2.4	37.8±3.0

1) Values are means±SEM (n=9). Values in a column with different superscripts in the same sex are significantly different ($P < 0.05$) as assessed by Duncan's multiple range test.
LI; low isoflavone intake, MI; medium isoflavone intake, HI; high isoflavone intake

Table 5. Dietary intake and the urinary and fecal excretion of daidzein in female and male rats[1)]

Sex	Group	Dietary Intake (μg/d)	Urinary excretion (μg/d)	Recovery in urine (%)	Fecal excretion (μg/d)	Recovery in feces(%)
Female	Control	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	LI	630.71± 41.56^{b}	47.34± 5.36^{a}	8.12±1.41^{c}	4.56± 4.56^{a}	1.13±1.13^{a}
	MI	3105.90±141.54^{c}	123.04±18.61^{b}	3.96±0.59^{b}	26.09±18.16^{b}	0.83±0.57^{a}
	HI	5544.53±293.51^{d}	155.40±28.86^{b}	2.85±0.56^{b}	74.23±22.37^{b}	1.38±0.41^{a}
Male	Control	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	LI	1004.19± 31.75^{b}	33.77± 6.36^{a}	3.46±0.70^{c}	0^{a}	0^{a}
	MI	5236.01±153.29^{c}	126.31±30.51^{b}	2.50±0.62^{bc}	109.07±29.07^{a}	2.04±0.53^{b}
	HI	9480.74±287.51^{d}	131.45±13.69^{b}	1.39±0.14^{b}	419.56±93.19^{b}	4.49±1.63^{c}

1) Values are means±SEM (n=9). Values in a column with different superscripts in the same sex are significantly different ($P < 0.05$) as assessed by Duncan's multiple range test.
LI; low isoflavone intake, MI; medium isoflavone intake, HI; high isoflavone intake

Table 6. Dietary intake and the urinary and fecal excretion of genistein in female and male rats[1)]

Sex	Group	Dietary Intake (μg/d)	Urinary excretion (μg/d)	Recovery in urine (%)	Fecal excretion (μg/d)	Recovery in feces(%)
Female	Control	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	LI	122.67± 8.08^{b}	9.85±1.59^{b}	8.79±1.78^{c}	4.27±4.27^{a}	2.95±2.95^{a}
	MI	604.21±27.54^{c}	18.92±2.51^{c}	3.13±0.42^{b}	5.73±5.73^{a}	1.05±1.05^{a}
	HI	1078.39±57.09^{d}	23.64±4.13^{c}	2.20±0.35^{ab}	7.79±7.79^{a}	0.82±0.82^{a}
Male	Control	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	LI	195.31± 6.14^{b}	9.40±1.91^{b}	4.97±1.08^{c}	0^{a}	0^{a}
	MI	1018.59±29.82^{c}	17.88±4.16^{c}	1.83±0.44^{b}	0^{a}	0^{a}
	HI	1843.97±55.92^{d}	13.26±0.65^{bc}	0.73±0.04^{ab}	45.93±16.12^{b}	2.54±0.86^{b}

1) Values are means±SEM (n=9). Values in a column with different superscripts in the same sex are significantly different ($P < 0.05$) as assessed by Duncan's multiple range test.
LI; low isoflavone intake, MI; medium isoflavone intake, HI; high isoflavone intake

of urinary excretion of daidzein and genistein in male rats belonging to the MI and HI groups were significantly higher than in the LI group, which is a similar observation in female rats. Our results agree with a study conducted on women given 0.7 mg, 1.3 mg, or 2.0 mg of isoflavones provided in soy milk per kg of body weight and who were found to excrete in the urine 4.5, 11.5, and 14.2 μmol of diadzein, and 0.9, 3.9, and 6.3 μmol of genistein, respectively[25)]; thus, it appears that urinary excretion tended to increase as intakes of isoflavones increased. Most of the daidzein and genistein in soybean exist as conjugates which are hydrolyzed into desmethylangolensin, eguol, 4-ethyl phenol, etc., before being absorbed in the intestines.[26,27)] Once they are absorbed, they are not further metabolized except into glucuronide and sulfate conjugates.[28)] Thus, the differences in absorption and metabolism of isoflavones are due to factors such as the extent of deconjugation and degradation by intestinal bacteria, the ratio and amounts of isoflavones transported through intestinal walls, and the levels of oxidation and reduction of isoflavones.[6,29)]

The recovery rates for each of diadzein and genistein, which were calculated by dividing the amounts of isoflavone excreted in the urine over a 24 hour period

by the amounts of dietary intake, were significantly decreased in the MI and HI groups compared to the LI group in the case of female rats; the difference between the MI and HI groups was not significant. The % of recovery of daidzein in the urine of male rats was significantly lower in the MI and HI groups compared to the LI group; no significant difference was observed between the MI and HI groups. The recovery % of genistein in the urine tended to be lower in the male HI group, but this difference was not significant when compared with the male control group. The recovery % of daidzein and genistein in the male rats were slightly lower than in the females. Our results agree with those of a similar human study[19] where the urinary recovery rates of diadzein were studied in 48-hour urine samples after female subjects were given 3.4, 6.9, or 10.3 μmol of total isoflavones per kg body weight per day; in the subjects who had very low levels of fecal excretion of isoflavones, the recovery rates of daidzein were 17.2, 15.2, and 15.9%, respectively, showing that the higher intake group had a lower % of recovery of diadzein. In the subjects with high levels of fecal excretion, the recovery rates of daidzein in the urine were 33, 31.4, and 30.3% for the female subjects given 3.4, 6.9, 10.3 μmol of total isoflavones per kg body weight per day, respectively. It appears that the higher intakes of isoflavones lead to lower recovery rates of daidzein in the urine. Lesser amounts of isoflavone glycocides are absorbed in the intestine compared to aglycones, because glycosides are hydrophilic and have higher molecular weights. In animal experiments, the aglycone form of soy genistein has a higher bioavailability compared to its conjugated form.[26] Kiyosawa et al.[30] in their anti-mutation study reported that extracts from fermented food had a higher level of aglycone substances and an increased anti-mutational function thus, it appears that the consumption of fermented food compared to unfermented food improves the efficiency of utilization of isoflavones.

As for the sex difference on urinary excretion of isoflavones, women excreted more isoflavone conjugates in urine than did men during 1 month of daily soy ingestion in a metabolic unit and however, a progressive decrease in urinary excretion of genistein and daidzein was observed in women but not in men.[23] In this study, the recovery rates of genistein for LI, MI, HI groups were 8.79%, 3.13%, and 2.20%, respectively in females and 4.97%, 1.83%, 0.73%, respectively, in males. The recovery rates of genistein as well as daidzein were higher in females than in males. Also, the elimination rates for genistein and daidzein were lower in women than in men, and the excretion half-life values of them were longer in women.[23] Therefore, those results suggest the sex-related differences in the urinary recovery of isoflavone intake. Hormonal difference in males and females may be a contributing factor.

Fecal Excretion of Isoflavones

Tables 5 and 6 present the fecal excretion levels of isoflavones. As the levels of dietary isoflavones increased, the amounts of fecal excretion of diadzein and genistein increased however, there was no difference between the groups in fecal excretion of genistein in female rats. There was no difference in the recovery of fecal daidzen and genistein between the female groups. The recovery rates of daidzein and genistein in fecal excretion were 0.83-1.38% and 0.82-2.95%, and this is similar to the results obtained for human female subjects who had fecal recovery rates of 1-2% when they were given soy milk.[25]

In the male rats, there was no fecal excretion of daidzein in the LI group but there were significant levels of excretion in the MI and HI groups. The excretion of genistein was observed only in the male HI group. The recovery of daidzein in the feces was significantly higher in the HI group compared to the MI group in the male rats, with the recovery rate of isoflavones varying between 2 to 5% depending on level of dietary intake of isoflavones; these findings agree with the results of King[29] who measured a 2.3% recovery rate of daidzein and a 3.4% recovery rate for genistein in an animal experiment. In a study of Japanese males[31] reported that when 60 g of baked soy powder were given to subjects, the recovery rates of daidzein and genistein were 4.4% and 2.5%, respectively, showing as in the present study that those of the male HI group were 4.49% and 2.54%, respectively.

In summary, we showed that the amounts of daidzein and genistein excreted in the urine and feces increased and the recovery rates in the urine decreased when dietary isoflavones were increased from 0.025% to 0.25%; however, the low recovery rate of both daidzein and genistein in the urine implies an increased bioavailability of isoflavones. We also observed sex-related differences in the urinary and fecal recovery of isoflavone intakes.

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