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Phytoestrogen-Low Diet for Endocrine Disruptor Studies

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Hormonally active chemicals (HACs) that are capable of inducing adverse effects on wildlife as well as human beings are featured as "endocrine disruptors". Various animal studies conducted to clarify the characteristics of HACs, including the uterotrophic assay, are sufficiently sensitive to detect the effect of 17-β-estradiol in micrograms per kilogram of body weight or lower. In such systems, a trace amount of HACs in the dietary pellets may interfere with the test results and thus can be a serious problem for the low-dose issue, which is now a major topic in the field of endocrine disruptor research. Here, the significance of the hormonal effects of phytoestrogen components in the NIH-07 diet is confirmed and a NIH-07-based open formula "phytoestrogen-low diet" (PLD) is proposed, which effectively reduces uterine weight as well as the uterine luminal epithelial labeling index in ovariectomized rats.

KEYWORDS: Phytoestrogen; genistein; genistin; daidzein; daidzin; rodent diet; uterotrophic response

INTRODUCTION

Hormonally active chemicals (HACs) that are capable of inducing adverse effects on reproduction and/or carcinogenesis in wildlife as well as human beings are featured as "endocrine disruptors" (ED). The National Academy of Science (*1*) and the U.S. Environmental Protection Agency (*2*) recommend various animal studies to clarify the characteristics of those HACs. Some methods, including the uterotrophic assay, are sensitive enough to detect the effect of 17-β-estradiol in micrograms per kilogram of body weight or lower. In such systems, a trace amount of HACs, if any, in the dietary pellets may interfere with the test substance. This dietary hormonal effect can even be a serious problem in assays especially aimed at the low-dose issue, which is now a major topic in the field of ED research (*3*). Odum et al. (*4*) recently reported that five different rodent diets containing different amounts of soy/alfalfa-derived isoflavones (β-glucuronidase-treated genistein and daidzein ranging from barely detectable levels to approximately 18 and 11 mg/100 g of diet, respectively) showed different effects on sexual development in rats, indicating that dietary phytoestrogens can be a modifier of certain hormone-sensitive endpoints.

Indeed, NIH standard dietary pellets (NIH-07 open formula) for experimental animals contain a certain amount of phytoestrogens known to originate from soy products as well as alfalfa (*5*−*8*) (**Table 1**). Here we report the significance of the hormonal effects of the NIH-07 diet and, at the same time, propose an open formula "phytoestrogen-low diet" (PLD), a modified NIH-07 diet. Additionally, a uterotrophic assay was performed to test the effect of dietary phytoestrogens on its response.

Table 1. Contents[a] of Genistein/Genistin and Daidzein/Daidzin in Diets and Components

	genistein	genistin	daidzein	daidzin
NIH-07(OY)[b]	1.6	10	1.4	6.4
PLD	nd[d]	nd	nd	nd
PLD-S[c]	2.8	8.8	2.2	5.1
dried skim milk	nd	nd	nd	nd
fish meal (65% protein)	nd	nd	nd	nd
soybean meal (45% protein)	9	91	7.1	60
alfalfa meal	nd	nd	nd	nd
ground shelled corn	nd	nd	nd	nd
corn gluten meal	nd	nd	nd	nd
wheat (flour)	nd	nd	nd	nd
casein	nd	nd	nd	nd
dried skim milk	nd	nd	nd	nd
soy oil	nd	nd	nd	nd
corn oil	nd	nd	nd	nd

[a] Expressed as mg/100 g. Measured by LC; detection level > 0.5 mg/100 g. [b] NIH-07 slightly modified by Oriental Yeast Co., solely due to material availability. [c] For reference, PLD-S was prepared; genistein, genistin, daidzein, and daidzin in the amounts found in NIH-07(OY) were added back to PLD. Slight change in free/conjugated ratio may be due to heating during pellet formation. [d] nd, not detected.

MATERIALS AND METHODS

Measurement of Phytoestrogens in Diet. Geinstein, genistin, daidzein, and daidzin were measured by a liquid chromatography (LC) technique (*9*) at the Japan Food Research Laboratories, Tokyo, Japan. Approximately 2 g of sample diet was mixed with 80% ethanol and

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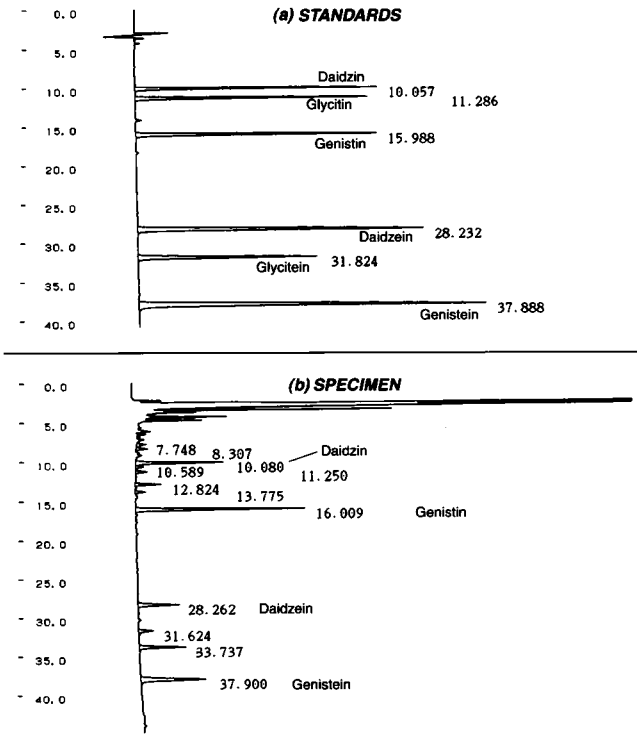

Figure 1. HPLC chromatography of (a) standards and (b) specimen. Elution times for genistein, genistin, daidzein, and daidzin were about 38, 16, 28, and 10 min, respectively.

Table 2. Ingredients of the Diets

ingredient	NIH-07 (original)	NIH-07(OY)[a] (used in this study)	PLD (used in this study)
dried skim milk, %	5.0	5.0	
fish meal (60% protein), %	10.0		
fish meal (65% protein), %		10.0	14.0
soybean meal (49% protein), %	12.0		
soybean meal (45% protein), %		11.75	
alfalfa meal, %	4.0	4.0	
corn gluten meal, %	3.0	3.0	
ground shelled corn, %	24.5	24.5	8.0
ground hard winter wheat, %	23.0		28.5
wheat middlings, %	10.0		
wheat (flour), %		32.87	40.62
casein, %			
brewer's dried yeast, %	2.0	2.0	2.0
dried molasses, %	1.5		
molasses, %		0.75	0.75
soy oil, %	2.5	2.5	
corn oil, %			2.5
salt, %	0.5	0.33	0.33
dicalcium phosphate, %	1.25		
ground limestone, %	0.5		
premixes, %	0.25		
mineral premixes,[b] %		1.05	1.05
vitamin premixes,[b] %		1.0	1.0
total	100.0	100.0	100.0

[a] NIH-07 slightly modified by Oriental Yeast Co., solely due to material availability. [b] Adjusted to original NIH-07.

extracted twice for 1 h. The extract was filtered, concentrated, and measured by an LC-10ATvp/SPD-10AVvp liquid chromatograph (Shimadzu Corp., Kyoto, Japan), using a YMP-Pack ODS-A A-312 f 6 mm × 15 cm column (YMC Co., Ltd., Kyoto, Japan). Standard phytoestrogens were purchased from Funakoshi Co. Ltd., Tokyo, Japan. Standards were dissolved in 80% ethanol (v/v) at 0.25−20 μg/mL, and standard curves were drawn. The conditions for LC were as follows: mobile phase A, 2.5% acetic acid/acetonitrile/methanol, 85+10+5 (v/v); mobile phase B, 2.5% acetic acid/acetonitrile/methanol, 58+19+23 (v/v/v); a gradient program of (A) to (B) in 45 min, linear gradient; flow rate, 1.0 mL/min; column oven, 50 °C; detection, 260 nm; injection volume, 10 μL; measurement interval, 66 min.

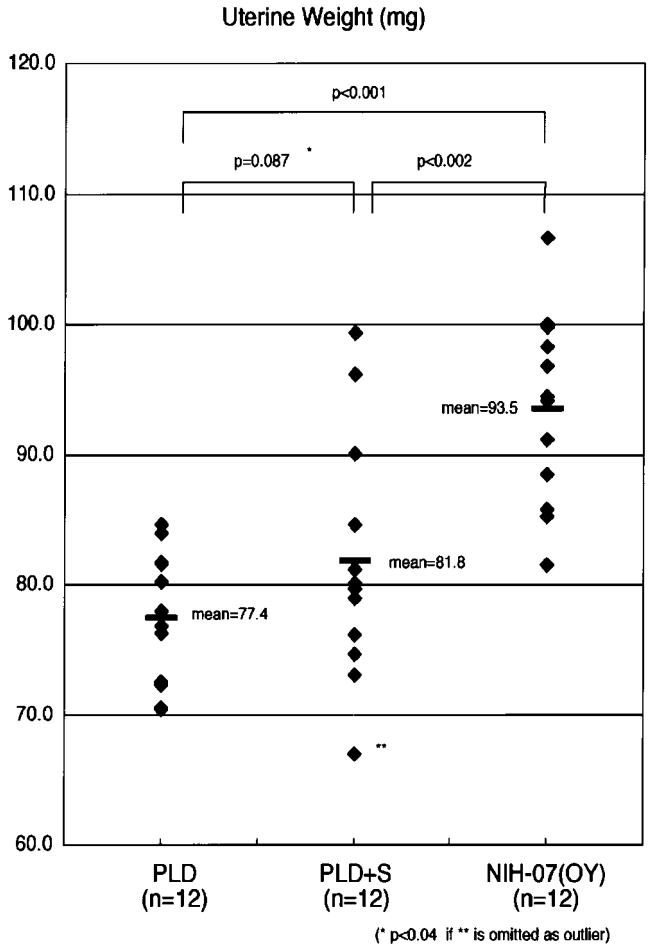

Figure 2. Uterine weight of ovariectomized rats maintained with NIH-07 and PLD: ovariectomized Crj:CD(SD) IGS female rats were used. In the PLD group, animals were maintained on PLD for 4 weeks; in the PLD-S group, animals were given PLD for 2 weeks and then given PLD-S for another 2 weeks; in the NIH-07(OY) group, animals were given PLD for 2 weeks and then given NIH-07(OY) for another 2 weeks.

Diet. The NIH-07 open formula diet (slightly modified by the Oriental Yeast Co., cf. **Table 1**), the PLD, and the PLD supplemented with geinstein, genistin, daidzein, and daidzin (PLD-S) were obtained from the Oriental Yeast Co., Ltd.

Animals. Female Crj:CD(SD)IGS rats (Charles River Japan, Atsugi, Japan) were used. Rats were kept in SPF condition (23 ± 3 °C, 55 ± 15% humidity).

Uterotrophic Assay. Thirty-six 6-week-old female rats were ovariectomized, fed on PLD for 2 weeks, and divided randomly into three groups of 12 rats each. The first group was given PLD for 2 additional weeks. The second and third groups were fed PLD-S or NIH-07 diet for an additional 2 weeks, respectively. BrdU (4 mg/kg) dissolved in DMSO was injected intraperitoneally 2 h prior to necropsy. Aortic blood was collected under ether anesthesia. Blotted uterine weights were measured. Uteri were fixed with buffered formalin and subjected to histology preparation and immunohistochemical staining for BrdU.

RESULTS

The chromatograms of standards and one sample as a representative are shown in **Figure 1**. The elution times for genistein, genistin, daidzein, and daidzin were approximately 38, 16, 28, and 10 min, respectively. The calculated amounts

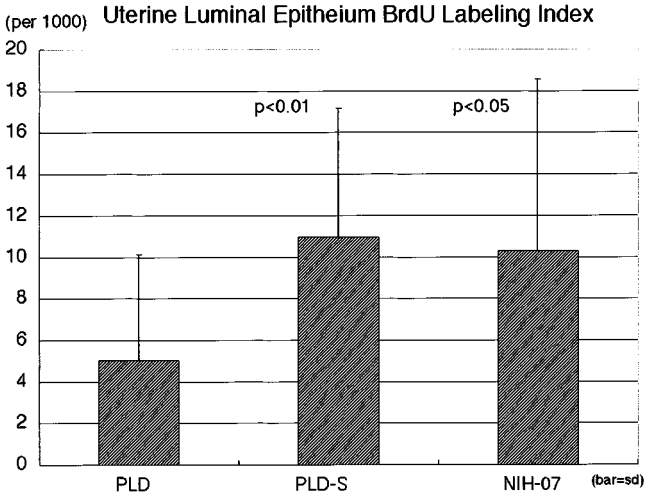

Figure 3. BrdU labeling index of uterine luminal epithelium (number of positive cells per 1000, BrdU 4 mg/kg ip injection 2 h prior to sampling). Both NIH-07 and PLD-S showed significantly high indices when compared to PLD.

of genistein, genistin, daidzein, and daidzin in the NIH-07 diet and its components are shown in **Table 1**. PLD was composed of components free of these four phytoestrogens by current measurements. Although negative for these four phytoestrogens, alfalfa has been reported to include phytoestrogens, and daily products such as dried skim milk and casein, which can contain estrogens of bovine origin, were also removed from the PLD component list. The protein content was adjusted by increasing the percentage of fish meal (**Table 2**). PLD-S, PLD supplemented with genistein, genistin, daidzein, and daidzin at concentrations measured in the NIH-07 diet, was prepared by adding standard phytoestrogen powder to PLD. PLD and PLD-S were also measured for the four phytoestrogens (**Table 1**).

The feeding study using ovariectomized adult female rats showed that the uterine weight, an indicator of estrogenic potency, was significantly lower in rats given PLD than in those given NIH-07 (**Figure 2**). Additionally, the standard deviation of the uterine weight was smallest in the PLD-fed group. The BrdU-labeling index of the luminal epithelium of the uterus was significantly higher in the NIH-07 and PLD-S groups than in the PLD group (**Figure 3**).

DISCUSSION

We have long been aware of HACs such as diethylstilbesterol, a synthetic estrogen, bisphenol A, an industrial chemical, and methoxychlor, a pesticide. Endocrine-disrupting chemicals can be defined as HACs that induce adverse effects in intact organisms, including humans and rodents as our surrogate. Because HACs affect organisms by binding to the hormone receptors, endocrine disruption can be considered a receptor-mediated adverse effect or toxicity.

A characteristic effect of the receptor-mediated toxicity that differs from the traditional toxicity is the effective dose range. Because the endocrine system functions at low concentrations of ligands in the body, subtle hormonal insult by xenoestrogens can induce a cascade of events including altered gene expression. The presence of estrogenic components in the diet, therefore, can be a concern for sensitivity as well as specificity in highly sensitive ED experiments. Our "phytoestrogen-low" diet enhances the sensitivity and reduces the interlaboratory/interexperimental variations. The latter has often been a focus of discussion in the interpretation of the results generated by low-dose HAC experiments (*4*, *10*).

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LITERATURE CITED

(1) Committee on hormonally active agents in the environment, Board on environmental studies and toxicology, Commission on life sciences. *Hormonally Active Agents in the Environment*; National Research Council, National Academy Press: Washington, DC, 1999.

(2) Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). Final Report, 1998; http://www.epa.gov/oscpmont/oscpendo/history/finalrpt.htm.

(3) Solicitation of Comments on Proposed Peer Review of Low-Dose issues for Endocrine Disruptors, 2000; http://ntp-server.niehs.nih.gov/htdocs/liason/LowDoseEndocrineFR.html, http://ntp-server.niehs.nih.gov/htdocs/liason/EndocrineMtgDelayFR.html.

(4) Odum, J.; Tinwell, H.; Jones, K.; Van Miller, J. P.; Joiner, R. L.; Tobin, G.; Kawasaki, H.; Deghenghi, R.; Ashby, J. Effect of rodent diets on the sexual development of the rat. *Toxicol. Sci.* **2001**, *61*, 115−127.

(5) Thigpen, J. E.; Li, L. A.; Richter, C. B.; Lebetkin, E. H.; Jameson, C. W. The mouse bioassay for the detection of estrogenic activity in rodent diets: II. Comparative estrogenic activity of purified, certified and standard open and closed formula rodent diets. *Lab. Anim. Sci.* **1987**, *37*, 602−605.

(6) Santell, R. C.; Chang, Y. C.; Nair, M. G.; Helferich, W. G. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. *J. Nutr.* **1997**, *127*, 263−269.

(7) Boettger-Tong, H.; Murthy, L.; Chiappetta, C.; Kirkland, J. L.; Goodwin, B.; Adlercreutz, H.; Stancel, G. M.; Makela, S. A case of a laboratory animal feed with high estrogenic activity and its impact on in vivo responses to exogenously administered estrogens. *Environ. Health Perspect.* **1998**, *106*, 369−373.

(8) Thigpen, J. E.; Setchell, K. D.; Ahlmark, K. B.; Locklear, J.; Spahr, T.; Caviness, G. F.; Goelz, M. F.; Haseman, J. K.; Newbold, R. R.; Forsythe, D. B. Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. *Lab. Anim. Sci.* **1999**, *49*, 530−536.

(9) Nakamura, Y.; Tsuji, S.; Tonogai, Y. Determination of the levels of isoflavonoids in soybeans and soy-derived foods and estimation of isoflavonoids in the Japanese daily intake. *J. AOAC Int.* **2000**, *83*, 635−650.

(10) Ashby, J.; Tinwell, H.; Odum, J. Uterotrophic activity of a "phytoestrogen-free" rat diet. *Environ. Health Perspect.* **2000**, *108*, A12−13.

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