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ISOFLAVONES IN SOY INFANT FORMULA: A Review of Evidence for Endocrine and Other Activity in Infants*

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■ **Abstract** Soy infant formulas are widely used, but few studies have evaluated long-term safety or examined specific forms of toxicity, such as to the endocrine or immune systems. This review focuses on newer experimental studies of the effects on estrogen activity, immune function, and thyroid economy of genistein and daidzein, two isoflavones in soy infant formula, and existing human studies of soy formula use. In order to judge the likelihood that an endpoint seen in laboratory studies might occur in soy-fed infants, we examined the doses and the resulting serum or plasma concentrations from the laboratory studies and compared them with doses and concentrations seen in soy-fed infants. We also summarized the estimates of the potency of the isoflavone compounds relative to estradiol. Given the scarcity and inconsistency of existing human data and the substantial laboratory evidence of hormonal and other activity at doses relevant to the soy-fed infant, we conclude that more clinical and epidemiological study is warranted.

CONTENTS

INTRODUCTION

Soy infant formulas have been used in the United States for five decades, and at present approximately 25% of all formula sold in the United States is soy-protein based (4). In developed countries, reports of morbidity in infants from formula of any sort are rare, and soy formula in particular is commonly used uneventfully in children with a history of feeding problems. Soy formulas, however, contain significant amounts of genistein and daidzein, two phytoestrogens of the isoflavone class. Genistein is structurally similar to 17β-estradiol, binds to estrogen receptors (ER) α and β and sex hormone–binding globulins (SHBGs), and has both estrogen agonist and antagonist activity (24). Genistein can also inhibit tyrosine kinase and topoisomerase (3), and both genistein and daidzein have antiandrogen activities (80). Irvine et al. (41) reported that infants as young as 4 weeks old can digest, absorb, and excrete genistein and daidzein from soy-based infant formulas as effectively as do adults consuming soy products. Although there is some support for the use of soy isoflavones in preventing hormone-related conditions in adults (2, 65, 67), whether benefits accrue to children or, indeed, whether soy formula is safe for infant use has been extensively debated (7, 9, 26, 29, 32, 36, 39, 44, 58, 68, 72, 75, 83). The United Kingdom (19), Australia (6), and New Zealand (59) have issued position statements cautioning against indiscriminate use of soy infant formula. The concern is based largely on studies in laboratory animals and on in vitro experiments. In that spirit, the American Academy of Pediatrics Committee on Nutrition did not recommend restriction in its 1998 review, pointing out that soy phytoestrogens had low affinity for human postnatal ERα and low estrogenic potency in bioassays (4). Thus, in the United States, those who make recommendations concerning infant nutrition face a conundrum—soy products are widely promoted as a natural, safe way to achieve at least some of the benefits of hormone replacement therapy in adults, and there is substantial laboratory evidence supporting their potential for hormonal activity. Infants on soy formula, however, achieve doses of the active compounds that are much higher than can be achieved by an adult eating soy foods, but do not appear to be affected.

Since 1998, when the Academy of Pediatrics published its statement, new experimental evidence on the estrogenic potency of these compounds has become available, including preferential binding to ERβ; in addition, there are new data on adverse effects on development of the female genital tract and differentiation of immune cells. Here we review selected newer laboratory work as well as clinical and epidemiologic studies, focusing on the quantitative relationship between administered dose, achieved circulating concentration, and effects. We compare the serum concentrations seen in the experimental studies with those achieved

by soy-fed infants, and compare the infant's exposure from soy formula with the dose-response data from the experimental literature. This allows a semiquantitative prediction of the effects likely to be seen in soy-fed infants. We use the newer estimates of estrogenic potency to express the infant's administered dose of genistein in units of oral contraceptives. We find that the serum/plasma concentration of genistein achieved by the infant fed soy formula exceeds the concentration seen in laboratory studies using doses that were pharmacologically or toxicologically active, but the lack of data from human infants and the lack of obvious toxicity observed in the large number of infants fed soy formula over the past 50 years make a unified interpretation of the current literature impossible. Although current recommendations regarding soy formula use in the United States are likely still valid, we make suggestions for relevant research.

EXPERIMENTAL DATA

Reproductive System

UTERINE ADENOCARCINOMA Diethylstilbestrol (DES) is a well-established carcinogen in humans and in experimental animals, and bioassays for carcinogenicity of hormones are available that have been validated using DES as a positive control. Using such a bioassay, Newbold et al. (61) reported that outbred neonatal CD-1 mice treated by subcutaneous (sc) injections with genistein (50 mg/kg/day) or an approximately equal estrogenic dose of DES (0.001mg/kg) on postnatal days 1–5 had prevalences of 35% and 31%, respectively, of uterine adenocarcinoma at 18 months (Table 1). At these doses, genistein and DES induced a 202% and 190% gain in uterine weights by day 5, demonstrating equal estrogenic potency at the time of exposure. The maximum serum concentration (mean $\pm$ SD) of total [conjugated plus aglycone (the active form)] genistein was 6.8 $\pm$ 1.4 μM in females and 3.8 $\pm$ 1.1 μM in males (27). The carcinogenic potential of lower doses of genistein, as well as the effects of genistein treatment through the diet instead of sc injection, is now under investigation.

OVARIAN FOLLICLES In a report from the same lab (43), Jefferson et al. treated transgenic ERα or ERβ knockout mice by sc injections on postnatal days 1–5 with 0 (vehicle), 1, 10, or 100 μg/pup/day ($\sim$0.5, 5, and 50 mg/kg/day) of genistein and examined the ovaries histologically at day 19. Wild-type CD-1, wild-type C57BL/6, and ERα knockout mice had multi-oocyte follicles in both the 10-μg/pup dose group (prevalences of 2/8, 9/11, 4/6) and the 100-μg/pup dose group (prevalences of 6/8, 11/11, not determined); mice with no ERβ receptor had no statistically significant response in multi-oocyte follicles to genistein treatment (1/2 in vehicle; 0/4 in genistein 1-μg/pup group, 0/5 in genistein 10-μg/pup group, and 1/3 in genistein 100-μg/pup group). Wild-type CD-1 mice treated on a similar protocol with lavendustin, an inhibitor of tyrosine kinase without estrogenic activity, showed no multi-oocyte follicles. These data imply that genistein induces

TABLE 1 Doses and serum/plasma concentration of isoflavones in the studies and their effects

Route	Authors	Subjects	Dose and time period	Effects	Serum/plasma concentration
Feeding soy formula	Setchell et al. (68)	4-month-old infant	Total isoflavones 6–9 mg/kg/day		Genistein: 2.5 ± 1.6 μM Daidzein: 1.2 ± 0.2 μM
	Sharpe et al. (70)	4- to 5-day-old marmoset	~8 h each day (2 h at weekends) until 35–45 days of age	Decrease in plasma testosterone concentration	
Ingestion	Delclos et al. (22)	Rat in utero and postnatal	Genistein 0, 5, 25, 100, 250, 625, or 1250 ppm from gestation day 7 through lactation for dams and after weaning until PND 50 for pups	Higher doses: hyperplasia in mammary glands; abnormal vaginal cellar maturation and ovarian antral follicles in females; abnormal spermatogenesis in males	
	Wisniewski et al. (77)	Rat in utero and lactational	Genistein 0, 5, 300 ppm from gestational day 1 until PND 21	Smaller testis size, fewer preputial separation at PND 40; lower plasma testosterone concentration at PND 70	
	Yellayi et al. (78)	32- to 34-day-old ovariectomized mice	Genistein 1000 or 1500 ppm for 12 days	10% or 25% decrease in thymic weight	0.97 ± 0.04 μM or 1.12 ± 0.18 μM
	Yellayi et al. (79)	32- to 34-day-old ovariectomized mice	Genistein 1000 or 1500 ppm for 28 days	50% decrease in DTH response; 50% decrease in $CD4^+$ and $CD8^+$ cells in popliteal lymph node	
	Guo et al. (38)	Rat in utero and postneonatal	Genistein 0, 25, 250, 1250 ppm from gestational day 7 to PND 22 for dams and PND 22–64 for pups	Increased splenic weight, decreased NK cell percentage in spleen	

	Chang et al. (17)	Rat in utero and postnatal	Genistein 0, 5, 100, and 500 ppm for dams continuously and for pups after weaning	Dose-response decrease up to 80% in rTPO activity, more pronounced in females	ppm Male (μM) Female (μM) 5 0.06 ± 0.01 0.10 ± 0.01 100 0.59 ± 0.03 0.94 ± 0.21 500 6.00 ± 0.65 7.94 ± 2.47
			NIH 31 (genistein and daidzein 30 ppm each) from weaning until 6 months	50% decrease in rTPO	Genistein: 0.35 ± 0.03 0.62 ± 0.05 Daidzein: 0.20 ± 0.02 0.25 ± 0.02
Ingestion/ injection	Lewis et al. (55)	Neonatal rat	Genistein 0.2 or 2 mg/kg/day injection for PND 1–6; 4 or 40 mg/kg/day gavage for PND 7–21	High dose: doubled uterine weight; four-day advancement of vaginal opening; majority of female vaginal smear pattern of persistent cornification	
Injection	Newbold et al. (61)	Neonatal CD-1 mice	Genistein 50 mg/kg/day on days 1–5	35% uterine adenocarcinoma at 18 months	
	Jefferson et al. (43)	Neonatal CD-1 mice	Genistein 0.5, 5, or 50 mg/kg/day on days 1–5	Dose-related increase in incidence of multi-oocyte follicles	
	Yellayi et al. (78)	100-day-old ovariectomized mice	Genistein 2, 8, 20, 80, or 200 mg/kg/day for 21 days	Dose-related decrease in thymic weight	
	Yellayi et al. (79)	32- to 34-day-old ovariectomized mice	Genistein 8, 20, or 80 mg/kg/day for 28 days	46%–67% decrease in DTH response	

Abbreviations: DTH, delayed-type hypersensitivity response; PND, postnatal day; TPO, thyroid peroxidase.

multi-oocyte follicles through an ERβ-receptor mediated mechanism that does not depend on tyrosine kinase inactivation.

VAGINAL OPENING A U.K. study reported the effect on neonatal rats of sc injection of genistein on postnatal days 1–6 (0.2 or 2.0 mg/kg/day) and gavage for days 7–21 (4 or 40 mg/kg/day, respectively) (55). The higher dose increased the uterine weight at postnatal day 22, advanced the mean day of vaginal opening by 4 days, and induced permanent estrus in the developing female pups, though no such effects were seen in the lower dose group.

HISTOLOGICAL CHANGES Delclos et al. (22) administered genistein in a soy- and alfalfa-free diet to pregnant Sprague-Dawley rats from gestation day 7 through gestation, lactation, and then to the pups from postnatal day 21 until sacrifice at postnatal day 50. The doses were 0, 5, 25, 100, 250, 625, or 1250 ppm. The highest dose caused a decrease in maternal and pup weights. Histopathologic changes in the female pups included ductal/alveolar hyperplasia of the mammary glands (250–1250 ppm), abnormal cellular maturation in the vagina (625- and 1250-ppm groups), and abnormal ovarian folliculogenesis (1250-ppm group). In male pups, the changes observed were mammary gland ductal/alveolar hyperplasia and hypertrophy (25–1250 ppm), aberrant or delayed spermatogenesis in the seminiferous tubules (1250 ppm) and hypospermia in the head of epididymis (625 and 1250 ppm). The results of this short-term study confirm the potential of dietary genistein to affect multiple estrogen-sensitive organs in both male and female experimental animals.

TESTOSTERONE CONCENTRATION AND FERTILITY A feeding study in twin marmoset monkeys showed that the twin fed with soy formula for ~8 h each weekday and 2 h on weekends from postnatal days 4–5 until postnatal days 35–45 had low plasma concentrations of testosterone (1.3 $\pm$ 2.1 ng/ml) compared with the twin fed with cow-milk formula (2.8 $\pm$ 3.9 ng/ml) (70). The body weight, testis weight, and formula intake were similar in the two groups. The Sertoli and germ cell counts did not differ between the twins; Leydig cell counts were higher in those fed the soy formula. Based on the measured intake of soy formula per day and the average concentration of isoflavones measured in the brand of soy formula used (25.5 mg aglycone/L), intake of isoflavone per day in the study was in the range of 1.6–3.5 mg/kg/day over the 5- to 6-week study period.

A recent in vitro study indicated estradiol and genistein had effects in promoting mice sperm capacitation, acrosome reaction, and fertilizing ability, but, unexpectedly, genistein was much more potent than E_2. Hydroxytamoxifen did not block these responses (1). An in vivo study in mice, however, suggested that exposing the dam by gavage during gestation and lactation (gestational day 12 to postnatal day 20 except parturition day) to genistein (0, 0.1, 0.5, 2.5, and 10 mg/kg/day) did not produce significant effects on sperm count, percent of motile sperm, or the number of motile sperm at postnatal day 105 and 315 (31). But in rats, Wisniewski

et al. (77) observed demasculinization of the reproductive system in pups after gestational and lactational exposure to dams (0, 5, 300 ppm from gestational day 1 until postnatal day 21), including smaller testis size, fewer pups with preputial separation at postnatal day 40, lower plasma testosterone concentration (3.72 $\pm$ 0.55, 1.76 $\pm$ 0.31, and 2.23 $\pm$ 0.42 ng/ml in 0, 5, and 300 ppm, respectively), and fewer males capable of ejaculation at postnatal day 70. Sperm counts, however, were not statistically different in the groups of this study.

Immune System

In vitro, genistein can inhibit bovine mononuclear cell proliferation, interleukin-2 synthesis, protein tyrosine kinase, and leukotriene B4 production (5). Yellayi et al. (78) conducted an in vivo study and found that genistein could reduce thymic weight and decrease the number of $CD4^+CD8^-$ and $CD4^+CD8^+$ thymocytes. The 100-day-old ovariectomized adult mice receiving 21 daily injections of 8, 20, 80, or 200 mg/kg/day genistein showed 17%, 40%, 73%, or 78% decrease in thymic weights compared with controls receiving dimethyl sulfoxide. The antiestrogen ICI 182,780 partially blocked the genistein effects. The highest dose of genistein, 200 mg/kg/day, resulted in near total elimination of $CD4^+CD8^-$ and $CD4^+CD8^+$ thymocytes.

A shorter 7-day course of genistein injections at the submaximal dose (80 mg/kg/day) also decreased thymic weight by 62%, and increased thymocyte apoptosis as indicated by the percentages of thymocytes in both a relatively early stage (genistein 2.4 $\pm$ 0.4% versus control 0.8 $\pm$ 0.2%) and a late stage (genistein 5.5 $\pm$ 0.8% versus control 2.6 $\pm$ 0.3%) of apoptosis.

A similar effect of genistein on thymic weights was found when younger mice, 25–27 days old when treatment started, were treated for 5 weeks with the same doses. Genistein treatment in young mice also decreased splenic $CD4^+CD8^-$ cells, and thus might affect both humoral and cell-mediated components of immunity. In mice dosed with 8 mg/kg genistein, serum concentrations at 0.5, 1, and 2 h post injection were comparable to those in soy-fed infants (68). Concentrations then declined to 0.4 $\pm$ 0.4 μM by 6 h and were not measurable after 24 h.

Yellayi et al. (78) also reported feeding studies. Feeding 32- to 34-day-old ovariectomized mice with phytoestrogen-free AIN-93G diet supplemented with 1000 or 1500 ppm of genistein for 12 days reduced thymic weights approximately 10% and 25%. These diets produced serum genistein concentrations of 0.97 $\pm$ 0.04 and 1.12 $\pm$ 0.18 μM, respectively (78), which were only 37% and 45% of the average genistein concentration in a 4-month-old soy-fed human infant (2.5 μM) (68).

Recently, Yellayi et al. (79) reported reversible changes in the cell-mediated immunity after administration of genistein. Mice (32- to 34-days-old) given genistein at 8, 20, 80 mg/kg/day for 28 days showed 46%–67% decrease in delayed-type hypersensitivity response to a hapten, 4-hydroxy-3-nitrophenyl acetyl succinimide (NP-O-SU), as compared with controls. Dietary genistein (1000 or 1500 ppm) also

decreased the delayed-type hypersensitivity response by almost 50%. The number of both $CD4^+$ and $CD8^+$ T cells in the popliteal lymph nodes of mice were reduced approximately 50% in two dietary genistein groups or 75% in an sc injection group with 80 mg/kg/day (79). Previously, Salem et al. (66) reported that estradiol had a more pronounced effect on the delayed-type hypersensitivity response rather than the immediate-type response, which suggests that the genistein effect could have been through an estrogenic mechanism.

Guo et al. (38) also found that genistein dietary exposure (0, 25, 250, 1250 ppm) could modulate the immune response in Sprague-Dawley rats. Gestational and lactational exposure (F_0 dams ingested genistein from gestational day 7 to postnatal day 22) and postnatal ingestion for F_1 rats (pups, postnatal days 22 to 64) increased splenic weight relative to body weight (25, 1250 ppm in males and all doses in females). In males, the number of T cells, T-helper cells, and cytotoxic T cells increased at all genistein doses and splenic B cells increased at the highest dose (1250 ppm), although the percentage of these cells remained unaffected. The percentage but not the number of natural killer (NK) cells in males decreased, but, at the highest dose in females (1250 ppm), T cells, T-helper cells, and cytotoxic T cells showed relative increases, with relative decreases in B cells and NK cells. Another study with gestational and lactational exposure to genistein also showed changes in the percentages of $CD4^+CD8^+$ thymocytes, $CD8^+$ splenocytes, and splenic total T cells (51). Together, these studies suggest immune dysfunction occurs following developmental exposure to genistein.

Thyroid Peroxidase

In vitro incubation of purified bovine thyroid peroxidase (TPO), porcine TPO, rat microsomal TPO, and human TPO with genistein (10 μM) and H_2O_2 (100 μM) for 5 min produced activity losses of 53 $\pm$ 3, 40 $\pm$ 6, 66 $\pm$ 7, and 62 $\pm$ 3%, respectively (means $\pm$ SD) (17). In vivo, rat pups born to dams fed with 5, 100, or 500 ppm genistein and continued on genistein-supplemented chow to postnatal day 140 showed dose-related decreases in TPO activity. On the 500-ppm diet, genistein concentrations in rat serum were similar to those in infants on soy formulas. Rats on the 100-ppm diet had genistein concentrations similar to those found in adults who consume typical Asian diets or soy isoflavone dietary supplements. Rats consuming 5 ppm and control diets had the low genistein concentrations typically found with a Western diet. Despite the observed adverse effects on TPO, there was no obvious effect on gland weight, histopathology, or thyroid function (17).

HUMAN DATA

Human studies on soy formula have mainly focused on nutritional status, with only one on long-term (i.e., two to three decades) safety. In that retrospective cohort study, Strom et al. (72) followed 811 subjects (85% of the initial study cohort) in

their twenties or early thirties who, as infants, had been given soy formula (120 males and 128 females) or cow-milk formula (295 males and 268 females) in a clinical trial. Interviews were conducted over the telephone. Those given soy formula did not differ from those given cow-milk-based formula on their answers to general questions about health and reproduction. However, women who had been fed soy formula as infants reported longer duration of menstrual bleeding (about 8 hours) and greater discomfort with menstruation; they also reported more use of asthma or allergy drugs and a greater tendency for sedentary activities. This study found little or no evidence of excess morbidity among the women given soy as infants, nor did it find large differences in measures plausibly related to reproductive function, such as menstrual cycle length. In addition, the authors reported multiple comparisons and did not claim strong prior hypotheses, and thus the differences they did find may be due to chance. This study also had the most appropriate and sensitive measure of exposure possible—a record of the group to which the child had been assigned—and thus whatever it shows is of interest. Nonetheless, studies that persuasively demonstrate safety are notoriously difficult to do. Because so many children are exposed to soy formula, events occurring at background frequencies of 1% or even 0.1% that are doubled in frequency among soy-fed girls would be of interest, but such differences would not likely be detectable with only 128 subjects. In addition, interviews might not be the most sensitive method of ascertaining endocrine function, and physical examinations and laboratory tests are not possible with the study design used. Finally, excesses of conditions that occur later in life cannot be detected among any-sized group of women who are only in their thirties. The study is most appropriately regarded as placing an upper bound on morbidity differences, rather than as demonstrating the safety or hazard of soy formula.

Beginning in 1978, pediatric endocrinologists in Puerto Rico reported clinical evidence of an increase in isolated early breast development in girls (precocious thelarche), which was originally attributed to consumption of estrogen-contaminated foodstuffs, especially meat. Freni-Titulaer et al. (35) conducted a case control study of 120 girls in Puerto Rico with precocious thelarche diagnosed between 1978 and 1982 and 120 matched controls. Cases had onset of breast development before 2 years of age. Extensive interviews of the mothers produced differences in three potential risk factors: maternal history of ovarian cysts [odds ratio (OR) 3.8, multivariate 95% confidence interval (CI): 1.1–6.8], consumption of fresh chicken (OR 4.9, 95% CI: 1.1–21.9), and consumption of soy-based formula (OR 2.7, 95% CI: 1.1–6.8) (35). Corn consumption appeared to be protective (OR 0.2, 95% CI 0.0–0.9). Because many of the case children had none of the risk factors, the authors did not consider the associations causal. Further, the authors suggested that the association with ovarian cysts in the cases' mothers might be due to increased diagnostic scrutiny, such as ultrasound examinations, done because of the child's condition, and that the association with chicken might have arisen because publicity preceding the interviews had linked chicken consumption with the condition. There was, however, no advance belief that soy formula was

involved, and thus, although the association might still be due to chance, it was not the result of recall or interview bias. The reports of early thelarche in Puerto Rico have so far led to multiple studies, none of which have clearly resolved the magnitude, duration, or cause of the outbreak. Although soy formula cannot be the sole cause, some role for soy estrogen in a multifactorial condition is possible. However, in a review that included consideration of breast bud and gynecomastia in infants but not specifically the situation in Puerto Rico, Klein (50) proposed that there was no convincing evidence of endocrine effects from infant consumption of modern soy-based formulas.

Although early studies had shown that soy infant formula decreased the incidence of major allergic diseases in infants with a strong family history of allergy compared with cow-milk formula (48, 57), further studies failed to document any advantage of soy formula in the prophylaxis of allergic disease (11, 49). A recent five-year follow-up of high-risk infants with a family history of allergy demonstrated that exclusive breast-feeding is associated with lower incidence of atopic diseases and food allergy as compared with soy or cow-milk formula; cumulative incidence of asthma and eczema did not differ over five years from birth between soy and cow formula (16).

Zoppi et al. (81) showed that the best response to vaccinations (polio, tetanus, diphtheria, and pertussis) was obtained in healthy breast-fed infants and the worst in healthy infants fed (now outmoded) soy flour–based formulas. Also, Zoppi et al. (82, 84) found evidence of abnormal gamma globulin, immunoglobulin, transferrin, complement fractions, and T-lymphocyte markers in healthy infants fed soy formulas. However, a later study in babies of atopic families showed no negative influence of feeding type on the concentration of antibody response or seroconversion rates after oral poliovirus vaccination (13). Recent studies examining whether nucleotide supplementation of soy formula might affect immune status included three arms—soy (N = 92), soy supplemented with nucleotides (N = 94), and cow-milk formula/human milk (N = 81). Infants fed nucleotide-supplemented soy had higher levels of antibody to H. influenza type B at 7 months (7 versus 3 μg/dl) and 12 months (0.78 versus 0.52 μg/ml) than the cow-milk/human-milk group; however, the soy formula group had a lower polio viral neutralization antibody at 12 months (276 versus 620 viral neutralization units) than the cow-milk/human-milk group (62). This study also found infants fed soy formula demonstrated immune cell status similar to infants fed human milk or cow-milk formula, consistent with normal immune system development. Among 32 immune cell populations analyzed, only the percentage of $CD57^+$ NK T cells at 12 months was higher in the infants fed human milk or cow-milk formula than in the soy-fed ones (20).

There are old case reports of goiter occurring in infants fed soy formula, which resolved after their diets were changed to a cow-milk formula. After 1959, when manufacturers began supplementing soy formulas with iodine, no further cases of soy formula–induced goiters were reported (40, 63, 71). However, Chorazy et al. (18) reported that an infant with congenital hypothyroidism was insensitive to L-T4 treatment while consuming soy infant formula, and after he switched to cow-milk

formula, his thyroid functions normalized even though he was receiving the same or a smaller dose of L-T4 in microgram per kg/day. Both soy formula and congenital hypothyroidism are relatively common, so it is reassuring that no further case reports have appeared. Fort et al. (33) conducted a retrospective case-control study of 59 teenage children diagnosed with autoimmune thyroid diseases (Hashimoto's thyroiditis or Grave's disease) using telephone interviews of their parents, their 76 healthy siblings, and 54 healthy nonrelated control children. The frequencies of use of soy formula in early life were 31%, 12%, and 13%, respectively.

None of the studies noted have documented the degree of exposure to the isoflavones or other components of soy formula, which vary from cultivar to cultivar and presumably from batch to batch. In addition, feeding a complex mixture of compounds, including lignans and phytate that might change absorption or metabolism of other components, cannot reasonably be expected to produce the same findings as administering pure compound to an experimental animal. Thus, the human data remain unclear.

ISOFLAVONES IN SOY INFANT FORMULA

In soybeans and in soy formula, genistein and daidzein occur mostly as the β-glycoside conjugates, genistin and daidzin, and as malonyl conjugates, 6″-O-malonyl genistin and 6″-O-malonyl daidzin (69). Although "genistein" strictly refers to the aglycone, or unconjugated, moiety, the terms "genistein aglycone" and "unconjugated genistein" are used, usually to distinguish the contribution of the aglycone to the total amount of isoflavone present, which includes the conjugates (37).

Conjugation blocks the site that would occupy estrogen receptor, and the unconjugated forms should be the ones most biologically active. Although the glycoside is stripped in the gut by structural and microbial glucosidases, it is replaced by a glucoronide and the compounds circulate in mostly conjugated form. Although serum concentrations of the unconjugated compounds are low, target organs, such as ovary, uterus, and mammary gland, can store the aglycones at concentrations higher than those in serum (25). It can thus be difficult to estimate dose effects on target organs either from administered dose or from serum concentration of the aglycones.

Although the amounts of each vary by batch, the unconjugated genistein and daidzein (aglycones) typically account for 3.2%–5.8% of the total isoflavones in soy-based formulas. In one report, the total isoflavone concentrations per gram soy protein isolate were similar among five commercially available soy-based infant formulas (69). When prepared for consumption according to the manufacturer's directions, the isoflavone concentration in powdered formulas was 32–46 μg/ml. Considering the age and body weight of infants and volume of their daily milk intake, the estimated average dose of isoflavone intake is 6–9 mg/kg/day for infants at 4 months of age (69). Setchell et al. (68) determined that infants on soy formula

received a daily exposure to isoflavones that was 6 to 11 times higher on a body weight basis than the dose that appeared to affect hormonal regulation of the menstrual cycle in women consuming soy proteins (15). However, Irvine et al. (41) suggested that soy-based formulas provided a mean ± SEM daily dose of isoflavones (genistein plus daidzein) of 3.2 ± 0.2 mg/kg, which remained fairly constant regardless of age of infants ≤16 wk. Franke et al. (34) also determined the isoflavone concentration in four different soy-based infant formulas and found that the average concentration of total isoflavone was 0.21 mg/g. After adjustment for body weight, the estimation of intake was ≈7 mg/kg, which was four to six times greater than that of adults consuming soy foods regularly (30 g soy protein/day).

Serum/Plasma Concentration

Mean (±SD) plasma concentrations of total genistein and daidzein in the seven 4-month-old infants fed soy-based formulas were 2.53 ± 1.64 μM and 1.16 ± 0.23 μM, respectively, which was significantly greater than in the infants fed either cow-milk formulas (11.6 ± 2.5 nM and 8.1 ± 1.1 nM) or human breast milk (10.2 ± 2.7 nM and 5.86 ± 0.51 nM) (68). Equol, the metabolite of daidzein, was lower in infants on soy milk than in those on cow milk, partly because of the reduced intestinal biotransformation resulting from the lack of fully developed microflora in early life or inactivity of the enzymes essential for the further metabolism of isoflavones (68).

Genistein and daidzein excretion rates in urine samples after soy-milk consumption were only available in Irvine et al.'s study (41), showing 0.15 ± 0.03 to 0.32 ± 0.04 mg/kg/day (roughly 13 ± 3% of daily intake) for genistein and 0.37 ± 0.03 to 0.58 ± 0.06 mg/kg/day (roughly 38 ± 4% of daily intake) for daidzein.

Estrogenicity

The estrogenicity of isoflavones, especially genistein, has been estimated based on estrogen receptor binding, transcriptional activation activity, and estrogen-regulated gene expression in vitro. Genistein showed much higher affinity for ERβ than for ERα (52, 53), although the estimation of estrogenicity varied broadly (Table 2). Human ERα and ERβ show different tissue distributions: ERα concentrations were much higher than ERβ in osteoblasts (23), mammary gland, and endometrium (28), whereas ERβ but not ERα was expressed in isolated ovarian granulosa cells and developing spermatids and umbilical vein endothelial cells (28). While in ovary and in lung, the amounts of ERα and ERβ mRNA were approximately equal. There are likely to be species-specific aspects of the tissue distribution of these receptor subtypes, thus requiring caution in extrapolating the animal study results to human beings (28).

Different studies on ERα or ERβ transactivation activity also provided different estimates of the estrogenicity of genistein, from 10^{-5} to 0.4 times that of estradiol (14, 45, 52, 53). However, expression levels of endogenous estrogen-regulated

genes (e.g., PS2) only suggested a mild estrogenicity (about 10^{-5}) of genistein (54). A combined in vivo and in vitro study from Jefferson et al. (45) indicated that the estrogenicity of genistein was 10^{-5} that of estradiol except for the most sensitive indicator, which was an increase in the number of uterine glands (Table 2).

In addition to the binding of ER, genistein showed binding activity to human sex hormone–binding globulin (hSHBG) with IC50 of 6.8×10^{-4} relative to estradiol, although its glycoside derivative genistin did not (21, 56). The binding with hSHBG may transport genistein into plasma and increase the concentrations of unbound estradiol and testosterone.

Administered Dose in Birth Control Pill Units

One way to fit these data into the more general picture of estrogen effects in humans is to estimate the dose to the child in units of birth control pills (BCPs). These are designed to have the lowest estrogen dose consistent with reliable contraception, and thus they provide a benchmark for dose comparison. Estrogen intake from modern oral contraceptive pills ranges from 20 μg/day to 50 μg/day. If we assume the average weight of women taking such pills is 50 kg, the daily estrogen intake is 0.4–1 μg/kg/day. As for infants fed with soy formula exclusively, the total daily genistein intake is about 5 mg/kg/day (70% of total isoflavones) (69). Because the estrogenicity of genistein relative to estradiol varies widely depending on the method used, the relative quantitative estimation of bioactive dose of genistein is also variable. A 10^{-3} or 10^{-5} relative estrogenicity of genistein to estradiol would yield a relative intake of 5 μg/kg/day or 0.05 μg/kg/day estradiol for these infants. The health effects based on these estimations are not readily predictable, but if the relevant exposure is more like five oral contraceptives/day, then observable responses to the estrogen signal are more likely to be seen. Note that Klein (50), using data on potency available up to 1998, gives estimates of birth control pill equivalents that are all at the low end of our estimates. The variability in the estimation of the estrogenicity of genistein makes investigation of estrogen effects in newborns using direct clinical and laboratory observation more reliable.

DISCUSSION

The evidence from laboratory studies showing biological activities at doses or tissue concentrations relevant to soy-fed infants is difficult to reconcile with the long record of uneventful use of these formulas. The laboratory studies do use pure compounds, sometimes by injection, in species with different rates of metabolism. These practices affect the relation between administered dose and tissue concentration, and so should not be important if serum concentrations are known in the experimental animal and in humans. On the basis of the serum measurement achieved by soy formula–fed infants, it is not possible to dismiss the laboratory evidence because the doses studied in the laboratory are too high. However, even when the

TABLE 2 The estimated estrogenicity of isoflavones in vitro and in vivo

				Relative estrogenicity				
Route	**Authors**	**Animal/cell line/system**	**Endpoints**	**E_2**	**DES**	**Genistein**	**Genistein:E_2**	**Daidzein**
In vitro	Fang et al. (30)	Rat uterine cytosol	Relative ER binding activity (IC50)	100	400	0.45	0.0045	0.023
	Kuiper et al. (52)	Synthesized human ERα (β) used in ligand competition experiments	Relative ERα binding activity	100		5	0.05	
			Relative ERβ binding activity	100		36	0.36	
	Kuiper et al. (53)	Human ER protein expressed in the baculovirus-Sf9 insect cell system	Relative ERα binding activity	100		0.7	0.007	
			Relative ERβ binding activity	100		13	0.13	
		Human embryonal kidney 293 cells transfected with ERE-TATA-Luc reporter plasmid, and pSG5-hERα (β) expression plasmid	Relative ERα transactivation activity (EC50)	100		0.025	0.00,025	
			Relative ERβ transactivation activity (EC50)	100		0.8	0.008	
	Jefferson et al. (45)	BG1Luc4E2 cell line	ER transactivation assay EC50	100	200	0.001	0.00,001	0.0004
	Casanova et al. (14)	HepG2 human hepatoma cells transfected with rat ERα (β) plus an estrogen-responsive luciferase reporter gene	ERα transactivation EC50	100		1	0.02	0.08
			ERβ transactivation EC 50	100		30	0.3	1.7
	Leffers et al. (54)	Expression levels of endogenous estrogen regulated genes in MCF7 breast cancer cells	PS2 significant induction	100	100	0.001	0.00,001	
			ATB0+	E2> DES>>>>genistein				
			TGFβ3	E2 = DES>>>>genistein				
			Monoamine oxidase A	E2>DES>>>> genistein				
			MRG1/p35srj	DES>>E2>>>>genistein				
In vivo	Jefferson et al. (45)	CD-1 mice weaned on PND 17, injected for 3 consecutive days and sacrificed at PND 20	Uterine wet weight to body weight ratio increase	100	100	0.001	0.00,001	
			Uterine epithelial cell height increase	100	100	0.001	0.00,001	
			Uterine gland number increase	100	1000	0.2	0.002	0.1
			Uterine lactoferrin intensity	100	1000	0.001	0.00,001	

Abbreviations: DES, diethylstilbestrol; EC50, half-maximal activation; ER, estrogen receptor; IC50, inhibitory concentration 50%; PND, postnatal day.

circulating concentration of genistein inter alia is measured in the experimental system and is comparable to that in soy-fed infants, metabolic differences may matter. Huggett et al. (39) argued that free genistein or daidzein (aglycone) in the plasma should be the active moieties, rather than the total isoflavone that Setchell et al. (68) measured in infants fed soy formula. Deorge et al. (27) had quantified both conjugated and unconjugated genistein in mice used in Newbold et al.'s study of neonatally treated mice (61), and found 31% of total serum isoflavone were as the aglycone (27). In contrast, Huggett et al. (39) found no aglycones in plasma samples from four soy milk–fed babies. Soy formula is also a complex mixture that might include compounds whose activity blocks or moderates any effect of the isoflavones. Thus far no compounds of sufficient potency and concentration have been identified, although they have been sought (12).

The explanation for the absence of documented effects in soy-fed infants that seems most likely, however, is that relatively subtle effects on thyroid hormone metabolism, estrogen metabolism and regulation, and immune function have not been sought in studies of sufficient size using sufficiently sensitive measures. In addition, soy formula use is often an alternative chosen for children past the newborn period who are having feeding or gastrointestinal problems with cow milk–based formula, rather than as the formula that a newborn starts with and stays with. Such use makes opportunistic study difficult because special measures are required to assemble a group of healthy infants who have been fed only soy formula.

Assuming we want to go to the trouble and expense of special studies, the possibility that soy formula has endocrine activity in children still presents the researcher with a dilemma. If such effects are present, they are mild enough to escape clinical notice. This does not rule out effects detectable with epidemiologic study, as has been done with pollutants in breast milk, low-level lead exposure, and passive smoking by infants. But epidemiologic study is time-consuming and expensive, and thus the interested researcher, however intrepid, must consider whether the question is sufficiently important and addressable with sufficient sensitivity and validity that an answer, positive or negative, is valuable. Elements of importance include the number of children exposed, which is very large for soy formula, as well as the strength of the laboratory evidence, the plausibility of the mechanisms of action, and the dose, which are all arguably applicable enough to the soy-fed infant that careful study is worthwhile. Another element is whether study of this question might shed light on other, larger questions. One such larger question is whether, in general, exogenous chemicals, such as phytoestrogens (like genistein), pesticides (like DDT), plasticizers (like phthalates), and other compounds that have endocrine activity in laboratory tests might disrupt human endocrine function at levels below those that produce acute toxicity and that might occur in the environment. This possibility, usually referred to as the endocrine disrupter hypothesis, has gained sufficient currency that Congress included specific language on endocrine disruption in the Food Quality Protection Act and amended the Safe Drinking Water Act in 1996. The former mandated that Environmental Protection Agency

(EPA) develop an endocrine disruptor screening program, whereas the latter authorizes EPA to screen endocrine disruptors found in drinking water sources (76). The National Toxicology Program has considered the use of short-term, receptor-based assays for endocrine disrupter detection and the existence of "low-dose" endocrine disrupting effects in laboratory studies (60). The purpose of this regulatory activity, the results of which have large social and economic consequences, is to predict whether endocrine effects will occur in human beings exposed to a tested agent. There is, however, relatively little evidence that endocrine disruption actually occurs in children exposed to these substances (64), and thus the validity of the process has been questioned. It might be, for example, that estrogen receptor occupancy or production of murine endometrial thickening, although indeed properties of estradiol, do not imply that any chemical that also has such activity will produce an estrogenic effect when a human being is exposed to it.

We have sought to devise several epidemiologic tests of the endocrine disruption hypothesis as it relates to maternal and child health, with mixed results. That search, however, has yielded the conclusion that, of all nonpharmacological sources of exogenous compounds that have laboratory evidence of estrogen activity to which the general population is exposed, an exclusively soy-fed infant arguably has the highest exposure. Thus, study of these infants might be thought of as a test case of the larger question of laboratory detection and prediction of human endocrine activity, or at least short-term activity.

Given that endocrine effects of soy formula in human infants might be worth studying, the question remains whether they can be studied validly. Valid study would require that the outcomes reflect plausible effects of estrogen exposure in infants, and that the exposure can be measured and is not assigned in some way that is also related to the outcome.

That fetuses and newborns respond to estrogens is well known. That response is, in fact, so well known and predictable that it forms part of the Ballard evaluation for gestational age (8). During pregnancy, circulating estrogen increases exponentially until an abrupt drop at term. Both male and female fetuses have estrogen-responsive breast and genital tissue. The degree of estrogenization is maximal in the term newborn, with palpable breast tissue in both sexes, coverage of the labia minora by the majora in the female, and a rugose pendulous scrotum in the male. Examination of an infant of unknown gestational age for these findings and neurological maturity constitute the Ballard examination, a well-validated and familiar clinical scale. These findings may be accompanied by ultrasonographic findings, such as small ovarian cysts, an endometrial stripe (74), and increased prostate volume, which are also plausible effects of estrogen but are not part of the Ballard score. Although the appearance of these findings on the Ballard scale by gestational age less than 40 weeks is well worked out, the time over which they disappear once the child is born and there is no more influence of maternal estrogen is not well described. The presence of estrogenic compounds in human milk and cow-milk formula has been looked for and found to be much lower than in soy formula (10). This leads to the hypothesis that, in the term infant, signs of estrogen effect should wane

more slowly in infants fed soy formula than in those fed human milk or cow-milk formula. This system should be sensitive enough to distinguish between the extremes of the laboratory-based potencies of the phytoestrogens because a dose of 1 to 5 BCP units should be detectable, whereas a dose of 0.01 BCP units should not.

With clinical collaborators at the Children's Hospital in Philadelphia and in Boston, and laboratory collaborators at the Centers for Disease Control and Prevention, the Food and Drug Administration, and the National Institute of Child Health and Human Development, our sister organization at the National Institutes of Health, we have begun pilot studies of the natural history of the physical and ultrasonographic findings associated with estrogen exposure in term newborns, and will examine children up to one year old (http://www.codares.com/index2.html). Although we are noting the way in which the children are fed, we do not expect that these studies will allow us to test hypotheses, but rather will allow us to perform defensible sample size calculations for a later study. We are also collecting samples of blood, saliva, and urine for testing of endogenous hormone levels and phytoestrogen exposure.

Ideally, we would use data from these pilot, cross-sectional studies to design a prospective, longitudinal study with a randomized-trial component. At its most complex, such a trial would have four arms, with infants who were breast fed and bottle fed serving as nonrandomized controls, and soy-fed infants randomized to standard soy formula and soy formula with reduced or absent phytoestrogens. The feasibility of manufacturing such an isoflavone-depleted formula is unclear. A patented method that produces isoflavone-depleted soy protein isolate exists (73). A small trial evaluating very short-term measures of tolerance for an isoflavone-depleted formula has appeared (42). There is up to 50% variability in isoflavone content in soy protein isolate prepared from beans harvested at different times of the year (47), and this property might be exploited. Thus far, however, we have not identified a source for such a formula. If we cannot find such a formula, we could do either a longitudinal study of children on different feeding regimens or attempt to randomize children to soy-based or cow milk–based formulas. We believe that relatively few mothers would be willing to be randomized in such a study, and that it would be very difficult to preserve any kind of blinding, because soy-fed infants smell different from cow-milk or formula-fed infants (46), and the formula looks and smells different. A longitudinal study without randomization would be subject to the criticism that the examiners were aware of which formula the child was taking. In addition, certain indications for switching formula group might be associated with the outcomes we were interested in, such as time of disappearance of breast buds, and milk allergy or intolerance.

Even though these studies are a complex undertaking, they will not answer all short-term questions and will not contribute to the long-term questions at all. If these studies, however, show some evidence of estrogen effect in infants, then researchers, funding agencies, and the formula companies may find the area more attractive for study.

Other relevant work might include studies of conjugated and unconjugated genistein circulating in infants, studies of thyroid economy, and further studies of immune response, especially response to immunizations. Further studies of the tissue distribution of ERα and ERβ in infants might guide interpretation of which of the several methods of judging genistein's potency relative to estradiol is most appropriate for estimating effects in newborns. In addition, although the lack of severe effects seen in the 128 young women who were given soy as infants is reassuring, more and longer follow-up data are necessary in order to exclude even large effects on infrequent outcomes.

CONCLUSIONS AND PERSPECTIVE

We reviewed new evidence on possible effects of isoflavones in soy infant formula from both experimental and epidemiological studies and compared the dose administered, serum concentration, and biological effects. Both ingestion and injection of genistein can affect development of the reproductive system, decrease thymic weight and delayed-type hypersensitivity response, modulate immune response, or reduce thyroid peroxidase. Results from epidemiological studies are inconclusive. Limited data did not indicate major developmental or functional disorders related to soy infant formula use, though possible perturbation of the menstrual cycle, sexual maturity, immune response, and thyroid function merit more investigation. Because the soy-fed infant appears to be exposed to enough compounds to be pharmacologically active and yet there is no indication of such action in the 50 years the formulas have been used, a unified interpretation of the current literature is not possible. This highlights the urgent need to evaluate the effects of isoflavones in soy infant formula clinically, prospectively, and longitudinally.

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