Phytoestrogens in Soy-Based Infant Foods: Concentrations, Daily Intake and Possible Biological Effects

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ABSTRACT

Exposure to estrogenic compounds may pose a developmental hazard to infants. Soy products, which contain the phytoestrogens, genistein and daidzein, are becoming increasingly popular as infant foods. To begin to evaluate the potential of the phytoestrogens in these products to affect infants, we measured total genistein and daidzein contents of commercially-available soy-based infant formulae, infant cereals, dinners and rusks. We also assayed phytoestrogens in dairy-based formulae and in breast milk from omnivorous or vegetarian mothers. In most cases, the glucoside forms of the phytoestrogens were hydrolyzed before separation by HPLC. Mean (+ SEM) total genistein and daidzein contents in four soy infant formulae were 87 + 3 and 49 + 2 μg/g, respectively. The phytoestrogen content of cereals varied with brand, with genistein ranging from 3 to 287 μg/g and daidzein from 2 to 276 μg/g. By contrast, no phytoestrogens were detected in dairy-based infant formulae or in human breast milk, irrespective of the mother's diet (detection limit = 0.05 μg/ml). When fed according to the manufacturer's instruction, soy formulae provide the infant with a daily dose rate of total isoflavones (i.e. genistein + daidzein) of approximately 3 mg per kg body weight, which is maintained at a fairly constant level between 0 and 4 months of age. Supplementing the diet of 4-month old infants with a single daily serving of cereal can increase their isoflavone intake by over 25%, depending on the brand chosen. This rate of isoflavone intake is much greater than that shown in adult humans to alter reproductive hormones. Since the available evidence suggests that infants can digest and absorb dietary phytoestrogens in active forms and since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu, we suggest that it would be highly desirable to study the effects of soy isoflavones on steroid-dependent developmental processes in human babies.

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INTRODUCTION

The use of soy-containing infant foods is increasing as the public has been made aware of the health promoting properties of soy. Even in 1986, approximately 25%

of the liquid infant formulae sold in North America were soy based (1). Although soy also contains antinutritive factors (2), these are largely eliminated by processing or counteracted by supplementation for infant feeding (3). By contrast, the isoflavone phytoestrogens, genistein and daidzein, present in raw beans primarily as the glucosides, genistin and daidzin (4), are heat stable and show substantial carry-over through regular processing methods (5). Recently, concern has been expressed that exposure to soy isoflavones may pose a developmental hazard to infants, particularly to the reproductive system (6- 9). This is because the isoflavones may cause perturbations of the sex steroid milieu, which are poorly tolerated by neonates (7). In vitro experiments have shown that the soy isoflavones can bind to estrogen receptors and act as competitive agonists or antagonists to endogenous estrogens depending on relative concentrations and affinities (10-12). Moreover, they can influence endogenous steroid metabolism by inhibiting 17β - hydroxysteroid oxidoreductase Type 1, which is the enzyme responsible for converting relatively impotent estrone to the more potent estradiol and, to a lesser extent, androstenedione to testosterone (13). Genistein can also inhibit protein tyrosine kinases, which phosphorylate intracellular proteins and are necessary for the action of insulin-like and epidermal growth factors (14,15). For example, genistein blocks transforming growth factor-a induction of aromatase by inhibiting protein tyrosine kinase (16) and thus decreases the aromatization of androgens to estrogens. There are therefore several pathways by which soy isoflavones might affect sex steroid synthesis and activity in vivo.

The first step in evaluating the potential of the phytoestrogens in soy-based infant foods to affect the reproductive system of infants is to determine the isoflavone intake of infants. Because there are very few data available on the genistein, genistin, daidzein and daidzin contents of typical infant foods (17), we measured their concentrations in: 1) several brands of soy- or dairy-based infant formulae commonly used in New Zealand and U.S.A., 2) breast milk from omnivorous or vegetarian women, and 3) several brands of infant cereal, dinners and rusks. We were then able to calculate the daily phytoestrogen intake of infants fed breast milk or the commercial foods given according to the manufacturer's instructions.

MATERIALS AND METHODS

Samples: Infant formulae and other food items were commercially available and were purchased from a local supermarket. Four commonly-used brands of powdered soy-based infant formulae, and one liquid 'ready to feed' variety were used. All dairy-based formulae were powdered; three were cow's milk, while the fourth was goat's milk. The dinners were two brands of chicken and vegetable puree.

Milk samples from each of 11 breast-feeding mothers were frozen immediately following their collection. Two of these women were vegetarians. Although the diets of the mothers were not controlled, we monitored their intake of soy products for 48 hr before the breast milk samples were collected. The soy consumption by the women over this 48-hr period was classed as follows: 1) No known soy consumed (n=6), 2) soy consumption < 10 g (n=3), 3) soy consumption between 10 and 50 g (n=1), and 4) soy consumption > 50 g (n=1).

Chemicals: All solvents (Mallinkrodt, ChromAR grade), hydrochloric acid and acetic

acid (BDH, AnalaR grade) were purchased from Lab Supply Pierce (Auckland, New Zealand). Genistein (Sigma Chemical Co., St Louis, MO) and daidzein (ICN Biomedicals, Aurora, OH) were used without further purification.

Extraction and hydrolysis of isoflavones: Genistein and daidzein were extracted from food items according to the method of Franke et al. (18). Samples (approximately 2.5 g for solid samples and 5 ml for liquid samples) were refluxed in 50 ml ethanol:hydrochloric acid (4:1) for 2 hr. The extracts were cooled and immediately passed through a 0.45 u filter before 20 ul was injected onto the HPLC system.

Instrumentation and chromatographic conditions: HPLC determinations were carried out on a Waters WISP 710B equipped with a 20 ul injection loop and an ICI LC1200 UV/Vis detector. A 3.9 x 300 mm uBondapak C18 reversed-phase column (Waters, Milford, MA) connected to a uBondapak C18 GuardPak (Waters, Milford, MA) pre-column insert was used. Elution was carried out as described by Franke & Custer (19): i.e. mobile phase 20:80 acetonitrile:10% acetic acid for 15 min and then 70:30 acetonitrile:10% acetic acid for 15 min at a flow rate of 0.8 ml/min. Analytes were monitored at 260 nm during each run.

The mean spike recovery of isoflavones was 93% from solid samples and 85% from liquid samples. The detection limits were 0.1 μg/g for solid samples and 0.05 μg/ml for liquid samples.

Most commercial infant formulae and cereals were analyzed in triplicate; however occasionally the batch analyzed differed between runs. The mean percentage coefficient of variation (i.e. sd/mean x 100) of concentration estimates was 12.5%, which takes into account variation due to batch and to measurement error.

Isoflavone analysis without prior hydrolysis: To determine the distribution of isoflavones into glucosylated and aglucone forms, selected formula and cereal samples were extracted in 80:20 methanol:water for 4 hr (20) and submitted to HPLC analysis as described by Murphy & Wang (21).

RESULTS

Glucosylated isoflavones were the predominant form in both soy-based infant formulae and cereals (Fig. 1). Isoflavone concentrations in commercially available infant formulae and food are shown in Table I. In this case, glucosylated isoflavones were hydrolyzed before the HPLC separation and therefore results are expressed as total genistein and total daidzein concentrations. The soy-based formulae were 13.8% + 0.2 protein according to the information on each product's label, with soy being the major protein source. The isoflavone concentrations in the soy formulae were 17.7% + 0.5 mean values in soy protein isolates (20), which is consistent with the formulae being simple dilutions of soy isolates.

The total genistein and daidzein concentrations in dairy- based infant formulae were less than the detection limit of the analytical method (i.e. < 0.1 μg/g). Similarly, genistein and daidzein concentrations in all breast milk samples were also less than the method's detection limit (i.e. < 0.05 μg/ml). By contrast, the ready-to-feed soy formula contained total genistein and daidzein concentrations of 18 and 15 μg/ml, respectively.

We used the isoflavone concentrations measured in infant formulae and cereals to estimate the mean daily intake (i.e. total genistein plus total daidzein consumed) and the daily isoflavone dose per kg body weight basis received by infants when the products were fed as recommended by the manufacturer (Table II). For soy formulae, the dose rate received by infants remained fairly constant between <1 and 4 months, with a mean (+ SEM) value of 3.2 + 0.2 mg/kg per day. There was little variability in intake due to the brand of formula chosen (Table I; also see the small SEM in Table II). By contrast, the isoflavone intake provided by cereals differed markedly with brand. For example, feeding 4-month old infants one serving of Cereal A each day would increase the daily isoflavone dose by 28% (Table II).

DISCUSSION

Our results show that considerable amounts of isoflavones remain after processing and formulation of most soy infant foods. By contrast, the isoflavone contents of cow- or goat- based formulae were less than the method's detection limit of 0.05 μg/ml. Likewise, isoflavones were not detectable in human breast milk regardless of the mother's soy consumption.

Although endogenous steroids are present in human breast milk, total concentrations (i.e. conjugated plus free steroids) in most women are only 1-5% those in plasma (22). Similarly, steroids in oral contraceptive pills are transferred into breast milk in small quantities, but the amounts are usually very low or insufficient to allow detection in the infants (23,24). Moreover, in cattle, ovarian steroids used to induce lactation are not excreted through the milk in detectable concentrations (25). It seems likely therefore that the transfer of plasma phytoestrogens into milk may also be inefficient. In young vegetarian women, plasma total genistein and daidzein concentrations have been reported to be 118.5 nmol/L (i.e. approximately 0.04 μg/ml) (26). Even if plasma phytoestrogens passed into milk with 100% efficiency, the resulting milk concentrations would be less than we could detect. On the other hand, the total isoflavone concentrations in soy infant formula diluted as used were at least 660 times higher than the maximum level possible in breast milk (i.e. our detection limit). This indicates that for infants exposure to phytoestrogens via milk is much less significant than exposure via soy formulae.

As in other soy products (4,20,21), the isoflavones in soy- based infant foods exist predominantly as glucosides, which are biologically inert (5). However, in adult humans, these glucosides are readily hydrolyzed in the acidic environment of the stomach and by intestinal bacteria (27,28). Consequently, isoflavones are rapidly and efficiently absorbed and digested (29), with plasma concentrations of genistein and daidzein rising significantly after ingestion of soy products (27).

Elimination of isoflavones occurs largely via the urine, mainly as glucuronide conjugates (28,30). Fecal excretion is only 1-2% of intake (30).

Although gut microflora appear during the first week of life (31), the ability of infants to digest ingested isoflavones has not been investigated before four months of age (32). An earlier abstract in which efficient digestion was reported at two months of age has not yet been confirmed (33). In a recent study, male babies, fed from birth on soy formulae, were found to excrete significantly more daidzein and genistein in the urine at 4 months of age than did infants drinking cow's or mother's milk (32). This shows that 4-month old infants can hydrolyze the soy isoflavones into active

forms and absorb these (32). Furthermore, the small amount of phytoestrogen excretion by babies fed on cow's or mother's milk is consistent with our finding of undetectably low phytoestrogen concentrations in breast milks. Interestingly, urinary concentrations of equol, a more potent estrogenic metabolite of daidzein (10,32), are similar and low in infants fed soy, cow's or mother's milk (32). The absence of equol in the urine of soy fed infants may be because the gut microflora needed to produce it from daidzein have not yet developed (32). Alternatively, it may simply reflect the variability in its production observed in adult humans (34 cf 26; 35; 30 cf 31; 36).

Our data show that as the infant's weight increases from 3 to 7 kg (32) the daily rate of intake of soy isoflavones provided by infant formulae is held fairly constant at 3.2 + 0.2 mg/kg when formulae are fed as recommended by the manufacturers. Supplementing the diet of 4-month old infants with a single serving of cereal each day can increase their daily intake by over 25%, depending on the brand chosen.

Assuming efficient absorption as suggested by the work discussed above, would this isoflavone intake be sufficient to exert biological effects? Since species may differ in responsiveness to phytoestrogens, this question is best answered by considering data from humans.<nothing> Unfortunately, few controlled studies have been reported on the effect of dietary soy on the human reproductive system. Two groups have used postmenopausal women to search for estrogenic effects of soy-supplemented diets (estimated intake = 1.2-2.5 mg total isoflavones/kg (27) assuming an average body weight of 65 kg) on the reproductive system. These have yielded contradictory results, with one group observing increased vaginal cell proliferation (37) and the other finding no significant changes in the vaginal epithelium, plasma gonadotropin or sex hormone binding globulin concentrations (27). Baird et al. (27) speculated that the soy isoflavones may act primarily as antiestrogens in the reproductive system. Their effects would therefore be easier to detect in premenopausal women than in postmenopausal women whose endogenous estrogens are already very low (27). Supporting this deduction, Cassidy et al. found that in premenopausal women a high soy intake significantly alters reproductive hormones (38). That is: when young women consume 45 mg total genistein and daidzein (i.e. 0.73 mg/kg body weight) daily, the peri-ovulatory follicle-stimulating hormone (FSH) and luteinizing hormone (LH) surges fall to one-third and one-half, respectively, of control values, while follicular phase estradiol levels are raised significantly (38). These hormonal changes are accompanied by a lengthened follicular phase and/or delayed menstruation (38). Although treatment lasted one month, the effects persisted for up to three months (38).

The isoflavone dose rate given infants fed on soy-based formulae is more than four times that shown to alter reproductive hormone secretion in cyclic women. Since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu (7), what consequences might result from such a high isoflavone intake by infants?

It has long been known that modification of the sex steroid milieu in neonatal rodents alters reproductive axis function and sexual behavior, and leads to structural changes in specific areas of the brain. The effects of neonatal steroid treatment, although irreversible, are often not manifested until the reproductive system is activated at puberty (39,40).

Moreover, there is only a limited window during development, the 'critical period', when sex steroids can markedly influence neuronal structure and function. In humans unlike rodents, this critical period was thought to occur before birth (41,42). However, recent theories on human sexual differentiation propose that there are several critical periods for development which occur not only prenatally but also during the early postnatal period (43). The timing of these critical periods seems to vary from tissue to tissue so that a temporary perturbation of the sex steroid environment may affect the development of only the one tissue that was passing through its critical period at that time (43). In extreme cases, this can lead to the development of sexual mosaics in which masculinized and feminized tissues coexist within the same body (43).

Because the cerebral cortex develops late relative to other neural regions, the postnatal critical periods may be particularly important for cognitive development and other aspects of behavior that are mediated cortically (43).

In both humans and monkeys, the reproductive axis is active soon after birth. In males (44-47), plasma testosterone concentrations increase post-partum and are maintained at levels similar to those in adults for 20-90 days. In females, plasma estradiol concentrations approximately double during the first month of life (47). After this, gonadal steroid levels decline and remain at low basal levels until puberty (44-47). Observations in males strongly suggest that the postnatal period of raised testosterone secretion is a critical period for normal sexual development. For example, blocking the testosterone surge in male monkey infants significantly delays puberty (46). Once pubescent, treated monkeys have lower plasma LH and testosterone concentrations, and reduced testicular volumes and sperm counts compared with normal controls (46). Moreover, there appears to be permanent impairment of the central nervous system pathway regulating gonadotropin-releasing hormone (GnRH) secretion (48), sexual behavior is compromised (49), and bone density is reduced (48). Interestingly, no adverse effects of blocking the postnatal testosterone surge were noted before puberty.

Although ethical considerations prevent intentional blocking of the postnatal testosterone surge in human infants, studies of boys with congenital hypogonadotropic hypogonadism suggest that early gonadal steroid deficiency may subsequently contribute to impaired testicular descent and maturation leading to oligospermia in the adult (46,50). The postnatal androgen surge may also prime the urogenital tract by promoting early growth and by potentiating the maturational effects of testosterone at puberty (51). For example, boys born with microphallus related to androgen lack have inadequate androgen-mediated growth of the external genitalia if therapy is not commenced until puberty. Responses are normal if androgens are replaced during infancy (51). With regard to cognitive development: prepubertal androgen deficiency in boys results in impaired spatial perception which normally is more acute in men than women (52). Likewise, in monkeys gender differences in maturation rate of learning ability (53) and performance of delayed visual discrimination tasks (54) can be manipulated by altering the postnatal sex steroid milieu.

The effect of modifying the sex steroid milieu in female primates has been little studied. This could be because the female's postnatal rise in plasma estradiol concentrations is much more subtle than the male's postnatal testosterone surge

(47). However it is still possible that these low levels of estrogen are needed for normal sexual development as has been clearly shown in female rats (55,56).

While obliteration of the postnatal testosterone surge in primate males unquestionably impairs many aspects of sexual development, there is no experimental evidence that a high level of phytoestrogen intake by primate infants does or does not alter either the sex steroid milieu or sexual differentiation. Nevertheless, in neonatal rodents isoflavone administration during the critical period can alter brain structure and the adult regulation of LH secretion in a dose-dependent manner (57). Feeding infant female pigs on soyameal causes reproductive tract abnormalities (58). Likewise, other phytoestrogens given to female neonatal mice (59) or infant pigs (60) also affect reproductive tract anatomy. When adult, phytoestrogen-treated mice develop the premature anovulatory syndrome (61), while pigs show abnormal regulation of LH secretion for weeks after phytoestrogen exposure ceases (60). Furthermore, marked effects on post-pubertal reproductive parameters have been shown in male and female rats nursed by phytoestrogen-fed mothers during early infancy (55). Early phytoestrogen exposure may also have beneficial effects. Mice injected with large doses of genistein soon after birth have reduced susceptibility to chemically-induced tumor development when adult (62). However, this treatment also impairs ovarian follicular development and cyclicity in the adult (63).

One reason for the lack of evidence linking isoflavone consumption with altered gonadal steroid-dependent developmental processes in human infants may be the probable delay in expression of the effects of early isoflavone exposure until after puberty. For example, another exogenous estrogen, diethylstilbestrol, was administered under controlled conditions to large numbers of women for over 20 years before its connection with postpubertal disorders in their offspring was observed, and several more years before the evidence of adverse effects was considered sufficiently convincing to cause withdrawal of the substance (7,8,64).

Because of these observations and the increasing use of soy products as infant foods, it would be highly desirable to study the effects of soy isoflavones on steroid-dependent developmental processes in human babies.

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Table I: Concentrations (mg/kg) of total genistein and total daidzein in commercial infant formulae and foods commonly fed in New Zealand. Shown for comparison is the isoflavone content of soy protein isolate (20)[1].

Product	Total Genistein	Total Daidzein
Soy-based Formulae		
Formula A	92	55
Formula B	81	50
Formula C	91	48
Formula D	83	44
Soy Isolate	514	248
Infant Foods		
Cereal A	287	276
Cereal B	104	95
Cereal C	3	2
Dinner A	58	45
Dinner B	32	31
Rusks	<0.1	<0.1

[1] Published weights corrected for the molar conversion of glucosides into aglucones for comparison with the present data.

[2] Most values are the mean of three analyses. The mean coefficient of variation was 12.5%, which includes variation due to measurement and to product batch.

Table II: Mean (+ SEM) daily total isoflavone (total genistein + daidzein) intake and daily isoflavone dose calculated on a body weight basis received by infants fed soy- based infant formulae as recommended by the manufacturer.

Formula means are based on the isoflavone contents of four infant formulae commonly used in New Zealand (See Table I). Also shown are the isoflavone intakes provided by three infant cereals. Because of the variability of their isoflavone contents, cereal values have not been averaged.

Age	Weight[1] (kg)	Total Isoflavones (mg/day)	Isoflavone Dose (mg/kg/day)
Soy-based Formulae			
<1 month	3	9.1 ± 0.7	3.0 ± 0.2
1 month	4	14.1 ± 0.6	3.8 ± 0.2
2 months	5	16.6 ± 1.1	3.3 ± 0.2
4 months	7	20.0 ± 2.0	2.9 ± 0.3
Cereals			
Cereal A[2]	7	5.6	0.8
Cereal B	7	2.0	0.3
Cereal C	7	0.05	0.01

[1] Weights based on data from Cruz et al., 1994 (32).

[2] Manufacturers recommend that cereal feeding should start when the infant is 4 months old. The isoflavone intake has been calculated on the basis of one 10-g cereal serving per day.

Legend to Figure

Figure 1. The percentage distribution of isoflavones into glucosides (genistin, daidzin) or aglucones (genistein, daidzein) in four soy-based infant formulae and three infant cereals. Shown for comparison is the mean isoflavone distribution in five soy protein isolates (soy isol; data from Eldridge, 1982 (20)).