

YOUNG INVESTIGATOR PERSPECTIVES

Phytoestrogen Action in the Adult and Developing Brain

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Key words: soy, phytoestrogens, coumestrol, isoflavones, genistein, daidzein, endocrine disruptors, oestrogen, sex behaviour, ER α , ER β , brain, hypothalamus.

Abstract

Soy isoflavonoids are plant phytoestrogens available as dietary supplements and are increasingly advocated as a natural alternative to oestrogen replacement therapy. As weak oestrogen agonists/antagonists with a range of other enzymatic activities, the isoflavonoids provide a useful model to investigate the actions of endocrine disruptors. Here, the activational and organisational effects of these compounds on the brain are reviewed. In spite of their preferential affinity for oestrogen receptor (ER) β *in vitro*, isoflavonoids act *in vivo* through both ER α and ER β . Their neurobehavioural actions are largely anti-oestrogenic, either antagonising or producing an action in opposition to that of oestradiol. Small, physiologically relevant exposure levels can alter oestrogen-dependent gene expression in the brain and affect complex behaviour in a wide range of species. The implications for these findings in humans, and particularly in infants, largely remain uninvestigated but are a subject of increasing public interest.

Compounds classified as endocrine disruptors (EDCs) are regarded as toxic substances and are presumed to have deleterious effects on mammalian physiology. However, although most endocrine disruptors are associated with dire predictions with respect to declining reproductive function and increased cancer risk, one group of these compounds, the phytoestrogens, is touted as providing an array of beneficial effects including preventative or therapeutic actions in carcinogenesis, atherosclerosis and osteoporosis. Phytoestrogens have gained considerable notoriety in the popular literature and, in October 1999, the US Food and Drug Administration (FDA) approved the health claim that daily consumption of soy phytoestrogens is effective in reducing the risk of coronary artery disease (1).

Phytoestrogens are a class of naturally occurring plant compounds with oestrogen-like structure and actions (Fig. 1) (2). There are several classes of phytoestrogens (Table 1), of which the most intensely studied are the isoflavones. Soy, soy-based foods and other legumes are rich in isoflavones, the two most significant of which are genistein and daidzein (Table 2) (3). Numerous dietary supplements are also available containing a wide range of isoflavone phytoestrogens (4). Although they behave similarly to other EDCs on molecular and cellular targets, the attitude regarding their phytoestrogen effects is generally positive, whereas the similar effects of

their synthetic counterparts are of increasing public concern. To a large degree, this paradoxical attitude is based on the presumption that, because phytoestrogens are natural, and are consumed in large quantities by Asian populations with low cancer rates (5), they must be beneficial, whereas synthetic compounds, by virtue of their industrial origins, must be detrimental. However, the rapidly expanding literature on phytoestrogens may suggest that the highly celebrated health benefits of soy are not entirely merited (6, 7). For the moment, the effects of these compounds on the brain and behaviour remain largely unexplored. Although phytoestrogen effects in the central nervous system have received much less attention than their effects in the periphery, the low doses reported for isoflavonoid actions in the brain suggest that the brain and behaviour may be important targets of isoflavonoid action, particularly during development.

Mechanisms of action

The primary mechanism by which the phytoestrogens are thought to influence oestrogen-responsive systems is through binding with oestrogen receptors (ER). The oestrogen receptor (ER) subtypes ER α and ER β are differentially distributed in both the adult (8, 9) and neonatal brain (10). This suggests that the two ER subtypes may regulate different aspects of

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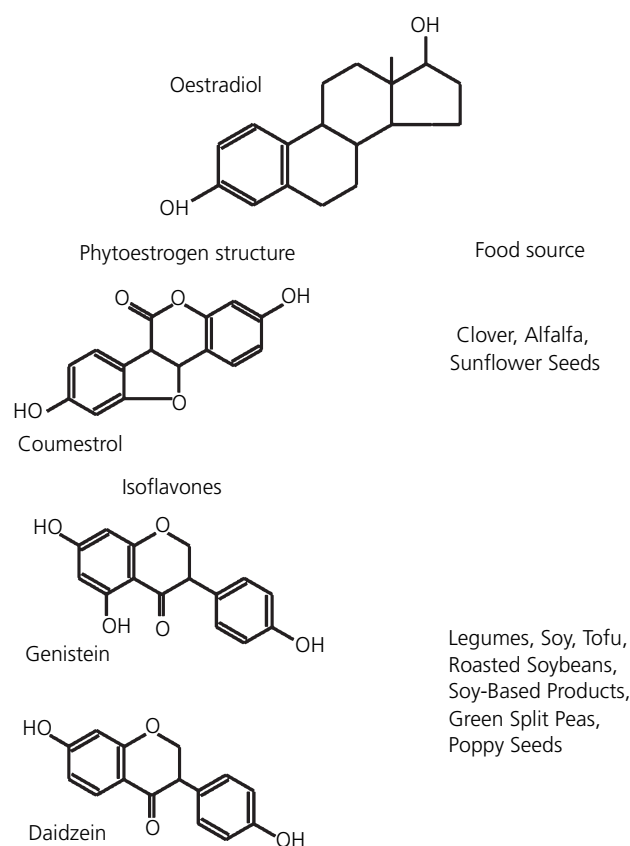


FIG. 1. Structure and dietary source of notable phytoestrogens.

behaviour and neuroendocrine function, and have differential roles across the lifespan. For example, $ER\beta$ has a higher level of expression than that of $ER\alpha$ in the basal forebrain, hippocampus and cerebral cortex in the adult (8, 9), which are all brain regions critical to memory function and vulnerable to Alzheimer's disease. In some hypothalamic nuclei that are crucial for the regulation of reproductive behaviour, neurones express primarily one ER subtype. For example, in rodents, the paraventricular nucleus of the hypothalamus (PVN) contains high concentrations of $ER\beta$ but virtually no $ER\alpha$, whereas the opposite is true of the ventromedial nucleus of the hypothalamus (VMN) (11, 12). Assays performed *in vitro* have found that isoflavonoids bind to both ER isoforms, with a generally higher affinity for $ER\beta$ than for $ER\alpha$, and that they activate ER-dependent gene transcription (13). The coumestan phytoestrogen coumestrol (Fig. 1) has a relative binding affinity for $ER\beta$ approximately equivalent to 17β -oestradiol, whereas the affinity of genistein for $ER\beta$ is approximately four-fold lower than 17β -oestradiol. In general, the isoflavonoid phytoestrogens have higher binding affinities for both $ER\alpha$ and $ER\beta$ than most organochlorides and other synthetic EDCs (13) (Table 3).

In assays performed *in vitro*, phytoestrogens have many ER-independent actions. Enterolactone, genistein and daidzein have been shown to stimulate hormone-binding globulin (SHBG) synthesis *in vitro* in liver cells (14), and several phytoestrogens are capable of competitively displacing both 17β -oestradiol and testosterone from SHBG in plasma (15).

TABLE 1. Classification of Notable Phytoestrogens.

Flavonoids
Flavanones
4',7-Dihydroxyflavanone
Naringenin
Flavones
Apigenin
4',5-Dihydroxyflavone
4',6-Dihydroxyflavone
Flavonols
Kaempferol
Hydroxychalcones
Phloretin
Isoliquiritigenin
4,4'-Dihydroxychalcone
Isoflavonoids
Isoflavones
Daidzein
Formononetin
Genistein
Biochanin A
Isoflavanones
O-Desmethylangolensin
Isoflavans
Equol
Coumestans
Coumestrol
Lignans
Enterolactone
Enterodiol

TABLE 2. Isoflavone Content of Soy-Based Foods and Infant Formulas.

Soy product	Daidzein (µg/g)	Genistein (µg/g)	Method	Reference
Soy flour	523.5	854.1	HPLC, GC-MS	(5, 89, 90)
Soy nuts	575	935	HPLC	(89)
Soy hot dogs	49	139	HPLC	(89)
Tofu	76	166	HPLC	(89)
Prosobee soy formula	17	22	HPLC	(78)
Isomil soy formula	19	23	HPLC	(78)

HPLC, High-performance liquid chromatography.

TABLE 3. Relative Binding Affinities for Oestrogen Receptor ($ER\alpha$ and $ER\beta$) of Endocrine Disrupting Compounds, Including the Phytoestrogens.

Compound	$ER\alpha$	$ER\beta$
17β -oestradiol	100	100
17α -oestradiol	7	7
Diethylstilbestrol (DES)	236	221
4-OH-tamoxifen	257	232
Coumestrol	20	40
Daidzein	0.1	0.5
Genistein	4	87
Bisphenol A	0.01	0.01
Methoxychlor	0.01	0.01
Testosterone	< 0.01	< 0.01
Progesterone	< 0.01	< 0.01

Listed values are percent affinity relative to 17β -oestradiol and taken from Kupier *et al.* (13).

By altering either the total amount or availability of SHBG, phytoestrogens may affect the free fraction of endogenous hormones in circulation either systemically or locally. Circulating endogenous oestrogen levels may also be affected by disruption of aromatase. Isoflavone and lignan phytoestrogens, including equol, genistein and biochanin A, are potent inhibitors of ovarian aromatase activity *in vitro* (16–18). This effect has not yet been replicated in the mammalian brain *in vivo* (19). Coumestrol has been shown to reduce the conversion of [³H]-estrone to [³H]-oestradiol *in vitro* by inhibiting the enzyme 17 β -hydroxysteroid oxidoreductase type 1 in a dose-dependant fashion (20). Genistein, although weaker, demonstrated a similar dose-dependant inhibitory effect.

Genistein is notable among the isoflavones because it is also a potent inhibitor of tyrosine protein kinases (PTKs) (21, 22). PTKs catalyse phosphorylation of their own tyrosine residues and those of other proteins, including growth factors involved in tumour cell proliferation. By inhibiting PTKs, genistein can potentially slow tumorigenesis, an effect that has driven investigators to evaluate its use as a therapeutic agent for breast and prostate cancer. Genistein can also inhibit DNA topoisomerases I and II, which are enzymes essential for DNA replication (23, 24). These observations suggest that genistein (and perhaps other phytoestrogens) has the potential to affect the nongenomic effects of oestrogen mediated through the activation of a wide array of intracellular signalling mechanisms (25, 26).

Effects in the adult brain

Phytoestrogens have widespread effects on oestrogen-sensitive gene expression in the adult brain and generally act as oestrogen antagonists. Both the PVN and the VMN are critical nuclei for the generation and regulation of sex behaviour (27) and each contains primarily one ER subtype. Only ER β is expressed in the PVN, whereas the VMN contains primarily ER α (28). Studies using ER α KO mice have conclusively demonstrated that ER α is required for the normal expression of both male and female sexual behaviour (29–31) and the regulation of oxytocin receptor (OTR) expression in the VMN by oestradiol is ER α -dependent (32, 33). Oxytocin is critical for the facilitation of sexual behaviour (34, 35) and the oestrogen-dependent regulation of oxytocin production in the PVN is regulated through ER β (33).

A number of phytoestrogens, including coumestrol and genistein, stimulate ER β mRNA expression in the PVN, an effect opposite to that of 17 β -oestradiol (Fig. 2A) (36, 37). In rats, consumption of a commercially prepared phytoestrogen supplement attenuated the oestrogen-dependent up-regulation of OTR in the rat VMN (Fig. 2B) (38), a nucleus critical for the generation of the lordosis response in females (27). Consistent with these findings, this phytoestrogen supplement significantly impaired female sexual behaviour (39). Intriguingly, genistein alone did not produce similar effects on sexual behaviour even though it also has the opposite effect to oestradiol on ER β mRNA expression in the PVN. Even at a relatively high concentration of 1000 p.p.m., female sexual behaviour and OTR expression in the VMN was unaltered compared to control animals on a genistein-free diet (37).

Thus, genistein may only be active through ER β *in vivo* and the disruption of sexual behaviour observed with the phytoestrogen supplement appears to be through an ER α -dependent mechanism.

Reproduction is not the only behavioural system vulnerable to disruption by phytoestrogens. The stress response and social behaviours are also affected through a complex, but largely undescribed, mechanism that likely involves the regulation of vasopressin and its receptors. The soy-rich 5001 rodent diet from Purina Mills Inc. (St Louis, MO, USA) increased aggression and significantly altered vasopressin receptor expression (V1a) in the lateral septum and the lateral hypothalamus of male Syrian hamsters compared to control animals fed a soy-free diet (40). A phytoestrogen diet containing substantially lower levels of phytoestrogens than the 5001 diet increased anxiety and elevated stress-induced plasma vasopressin and corticosterone levels in adult male rats (41). Hypothalamic vasopressin was also increased in rats fed a diet containing 1250 p.p.m. genistein over their entire lifetimes (42). Phytoestrogen-rich diets have also been found to increase aggressive behaviours in nonhuman primates. Male cynomolgus monkeys fed soy protein isolate containing 1.88 mg isoflavones/g protein over 18 months demonstrated higher frequencies of intense aggressive (67% higher) and submissive (203% higher) behaviours, as well as a decreased proportion of time spent in physical contact with other monkeys (68% reduction) (43). Timing and duration of exposure may mediate these effects. Anxiolytic effects of phytoestrogen-rich diets were observed in intact male and female rats exposed over their entire lifetimes but not when administered briefly in adulthood (19).

Hypothalamic vasopressin plays an important role in the regulation of stress (44). Vasopressin is colocalised with ER β but not ER α in the supraoptic nucleus (SON) (45) suggesting that oestrogen-dependent vasopressin secretion in the SON is regulated through ER β . Although vasopressin expression has been shown to be regulated by an ER β -dependent mechanism in the PVN (46), a role for ER β in the regulation of vasopressin secretion in the SON remains to be demonstrated definitively.

Human studies

Results from studies of phytoestrogen action in humans have been controversial and inconsistent. Dose and treatment diet vary considerably across studies, with most experiments reporting very small sample sizes, and several studies being funded largely by the manufacturers of soy and soy-based supplements, leading to some mistrust. Nonetheless, it is becoming clear that soy-rich diets can affect hormone balance and thyroid function in human adults, and the health implications of these effects remain the subject of intense debate.

Consumption of isoflavones-rich soy foods by humans has been shown to marginally suppress circulating oestrogen and progesterone levels and, in some cases, attenuate the ovulatory surge of luteinising hormone and follicle-stimulating hormone (47–49). The observed neuroendocrine effects may be due, in part, to increased clearance and metabolism of endogenous oestrogens. A soymilk diet delivering approxi-

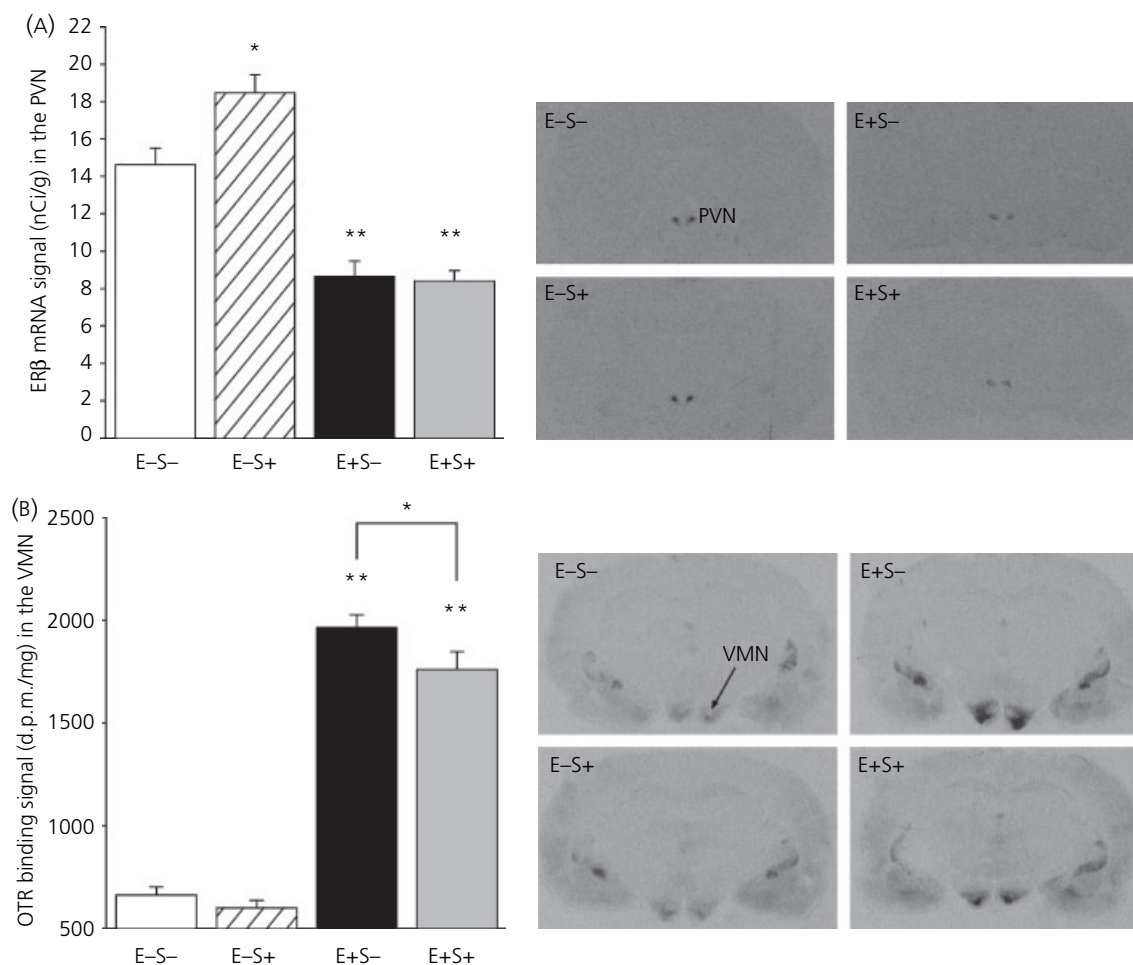


Fig. 2. A commercially available isoflavone supplement can disrupt both $ER\alpha$ and $ER\beta$ - dependent gene expression in the adult female rodent brain [38]. (A) $ER\beta$ mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) is decreased by 17β -oestradiol (E+S-) but increased by the isoflavone supplement (E-S+). When 17β -oestradiol and the isoflavones are given together (E+S+), the supplement has no effect on the oestrogen-dependent decrease in $ER\beta$ mRNA expression. This effect is mediated through $ER\beta$. (B) The isoflavones supplement attenuates the effect of oestrogen on oxytocin receptor (OTR) binding in the ventromedial nucleus of the hypothalamus (VMN) but has no effect in the absence of oestrogen. This effect is mediated through $ER\alpha$. (E = 17β -oestradiol, S = isoflavone supplement, * $P < 0.01$, ** $P < 0.001$)

mately 158 mg of isoflavones per day increased the ratio of 2-hydroxyestrone to 16- α -hydroxyestrone in urine, suggesting that soy isoflavones may increase the metabolism of endogenous oestrogens to the protective 2-hydroxylated oestrogens in women (50). It does not appear that phytoestrogens affect circulating oestradiol levels through decreased SHBG levels. Studies evaluating SHBG levels in women after a dietary soy challenge providing between 32 and 68 mg of isoflavones per day showed no significant changes (47, 48, 51). Interestingly, consumption of an isoflavone-free soymilk has also been shown to influence hormonal profiles (52). Thus, the possibility that the neuroendocrine effects of soy foods may be due to characteristics other than isoflavones content warrants further investigation.

In humans, soy consumption has also been shown to have a depressive effect on thyroid function. A Japanese study group found that consumption of 30 g of soybeans per day significantly increased thyroid-stimulating hormone (TSH) levels and many of the subjects experienced goiters and symptoms consistent with hypothyroidism (53). Other studies

have found more modest changes in thyroid function (54, 55), suggesting that soy may have a dose-dependent effect on thyroid function. At low doses, soy may increase T4 concentrations, perhaps by displacing T4 from its binding proteins (56), but decreases T4 concentrations at higher doses by stimulating TSH secretion (57). Because oestradiol increases the sensitivity of the thyroid gland to feedback mechanisms mediated through the pituitary (58, 59), phytoestrogens may in part be influencing thyroid function by disrupting the effects of oestradiol.

Recently, interest is growing in the potentially disruptive effects of soy-rich diets on cognitive function in ageing men and women. Postmenopausal women fed a supplement delivering 110 mg of phytoestrogens per day performed substantially better on a battery of cognitive tasks compared to their own baseline scores (60). However, a similar study administering a soy protein containing 99 mg of isoflavones to postmenopausal women did not find improved cognitive performance in the groups fed the soy-rich diet (60). To our knowledge, these cognitive studies represent the only inves-

tigations of phytoestrogen action on human behaviour. Phytoestrogen supplements are routinely advertised as an alternative to traditional hormone replacement therapy and as a way to alleviate both the physical and emotional effects of menopause. To date, there is almost no evidence to support these claims, and data from numerous animal studies suggest that soy may act to suppress sexual function and increase anxiety. The impact of soy-rich diets on human emotional and sexual health deserves to be explored in greater detail, and the numerous clinical trials now underway to examine the effects of a soy-rich diet on bone density, cancer rates and cardiovascular health, may provide a mechanism by which to do so. Asking questions of these patients regarding the impact of these compounds on their sexual and emotional health is imperative to determine whether or not soy isoflavones impact these important and relevant aspects of overall health and well-being.

Neonatal development

Phytoestrogens may have an even greater impact in the young brain. Brain development begins in embryonic life and continues through puberty in a well-orchestrated and precisely timed cascade of events controlled by genes, hormones, neurotransmitters and growth factors. Disruption of these pathways by phytoestrogens and other EDCs could result in life-long effects on both physiology and behaviour. The soy phytoestrogen genistein readily crosses the placenta and the most bioactive form of this compound is present in the fetal brain at levels comparable to the mother (61). In addition, the transfer of genistein from serum to brain appears more efficient in prenatal animals than adults (62).

The brain is sexually dimorphic, and sexual differentiation begins early in development through a process largely governed by hormones. Hormone-mediated architectural and functional changes that occur during a series of critical development periods are permanent, and ultimately affect the physiology and behaviour of the adult animal (63–65). This potentially makes development one of the most susceptible periods for phytoestrogen and EDC exposure in the life history of the animal.

The sexually dimorphic region that has undergone the most scrutiny is the aptly named sexually dimorphic nucleus of the preoptic area (SDN-POA) in the rodent brain. The volume of the SDN-POA is regulated by oestradiol aromatised from fetal, testicular androgen (63), and is five- to six-fold larger in males than in females (66). Both ER α and ER β are expressed in the SDN-POA across the lifespan (10, 28). Numerous studies have been undertaken in rats to determine whether phytoestrogens can alter SDN-POA volume, all with inconsistent results. Genistein has been shown to increase SDN-POA volume in males but not females (67) when administered across the entire lifespan, including the prenatal period. This effect was not observed in males exposed during only the postnatal period (68). By contrast, high doses of genistein had masculinising effects on SDN-POA volumes in females following postnatal exposure (69). Males fed a phytoestrogen-rich diet during development and puberty, and then switched to a phytoestrogen-free diet in adulthood, had smaller SDN-POA volumes than males fed the phytoestrogen

diet across the entire lifespan (70), but these results are difficult to interpret because unexposed, control animals were not included in the analysis.

Another sexually dimorphic nucleus sensitive to endocrine disruption is the locus coeruleus (LC). The LC is located in the brain stem and an important component of the stress axis (71). The LC is normally larger in females and contains a greater density of cells than the male LC. Pre- and postnatal exposure to moderate levels of the phytoestrogen resveratrol, a phytoestrogen found in grapes and red wine, abolished the sex difference in LC volume and cell density by demasculinising the male brain (72). In this case, no changes in SND-POA volume were found, underscoring the complexity and site-specificity of isoflavones action in the brain. Both ER α and ER β are expressed in the adult rat LC (28) but only ER α expressed in the postnatal LC (10), suggesting that resveratrol may be acting through an ER α -dependent mechanism.

Behavioural data from animals exposed to phytoestrogens during development suggest that disruption of sexually dimorphic circuits may extend beyond the SDN-POA and the LC. The lordosis response was significantly attenuated in the females exposed to resveratrol in the study described previously, and the impairment was accompanied by abnormal oestrous cycles compared to control females. Perinatal exposure to coumestrol through the lactating dam induced persistent oestrus in females and suppressed sexual behaviours in male rats once they reached adulthood (73). Plasma testosterone levels were unaffected, suggesting that these effects were not due to changes in gonadal hormone secretion. Similarly, exposure to genistein for only 5 days after birth induced a persistent oestrus accompanied by a lack of ovarian corpora lutea in female rats, and moderately suppressed lordosis behaviour compared to unexposed control females (74). These results suggest that the morphology and function of reproductive, behavioural and neuroendocrine circuits may also be impaired by phytoestrogens during development.

Phytoestrogen exposure and effects in human infants

Concern has recently been raised over human infant exposure to phytoestrogens (75). Isoflavones have been found in human umbilical cord plasma and amniotic fluid at levels comparable to concentrations observed in maternal plasma. However, the greatest exposure occurs in babies raised on soy formula. Although they were initially introduced as an alternative to bovine milk formulas for babies with a milk allergy, soy infant formula has grown in popularity. The increased interest in soy formula stems largely from numerous articles in the popular media celebrating the beneficial effects of soy, which now makes up approximately 25% of the formula market in the USA (76). Total isoflavone content in soy infant formula varies widely largely due to the influence of environmental and genetic differences between batches and sources, but is consistently high, averaging near 40 μ g total isoflavones per gram of formula (77–80) (Table 2). This translates to a daily intake of approximately 6–9 mg/kg body weight per day, an amount, when adjusted for body weight, that is four- to seven-fold higher than the amounts regularly

TABLE 4. Oestradiol and Isoflavone Levels in Maternal and Neonatal Plasma.

	Plasma E ₂ (µg/l)	Plasma isoflavones (µg/l)
Adult female	0.04–0.08	40–240*
Amniotic fluid		1.5–6.5
Breast-fed Infant	< 0.02	< 1–4
Infant fed bovine formula		4–10
Infant fed soy formula		552–1755

Values listed are representative and compiled from previous studies (79, 85–88). *Values listed are for Asian women. Western women would have plasma isoflavones levels below 2 µg/l.

consumed by adults meeting the FDA guidelines for soy consumption or Asians consuming a traditional soy-based diet (0.3–1.2 mg/kg per day) (81). Infants fed soy formula have circulating phytoestrogen concentrations of approximately 1 µg/ml, which is 13 000- to 22 000-fold higher than endogenous oestrogens (82) (Table 4). These are levels high enough to produce many of the physiological effects observed in research animals and human adults.

To date, only one long-term study has examined the physiological effects of soy infant formula (83). This retrospective study telephoned adult subjects who, as infants, had been part of a clinical trial using either soy or cow milk. No significant different differences on general measures of health and reproduction were found, except longer cycles and more uncomfortable menses in women fed the soy formula as infants. These women also reported a greater use of allergy and asthma drugs and a greater tendency for sedentary activities. Although this study gained much attention when it was originally published, it has since been criticised for being underpowered and poorly executed.

Conclusions

Phytoestrogens both expand our view of environmental substances with endocrine action and demonstrate that the source of the compound in question often influences the direction of research and interpretation of available data. Although the potentially beneficial effects of soy consumption have been pursued with vigor, and often overstated, the potentially adverse effects of these compounds have not. The opposite phenomenon is often the case for synthetic endocrine disruptors, most of which have lower binding affinities for oestrogen receptors than any of the phytoestrogens. The isoflavonoids have a wide range of biological effects *in vivo* at doses and plasma concentrations observed with normal human diets. Significant responses *in vivo* have been reported for a wider range of tissues and processes than the endpoints generally used to assess other endocrine active compounds (84) and yet virtually nothing is known about how phytoestrogens affect brain function, hormone regulation and behaviour in humans, particularly children.

Despite the considerable interest, the effects of phytoestrogen-rich soy infant formulas in children remain totally unknown. Although no overtly adverse effects have been detected to date, this may simply be because no large-scale, comprehensive studies have been undertaken to address the

issue, and this may change in the near future. Considerable attention is now being given to the interaction between the environment and the neurodevelopment of children. This interest is largely sparked by a growing awareness of an apparent increase in neurobehavioural disabilities, including autism and a wide range of behavioural disorders. Whether or not soy consumption during infancy is a factor remains to be seen, but the need for a more serious, concerted effort regarding this issue is clearly indicated.

Acknowledgements

I would like to thank Larry Young of Emory University for his editorial support, Emily Rissman at the University of Virginia for suggesting the review and Eva Polston at CIIT Centers for Health Research for her many helpful comments and suggestions.

Accepted 5 January 2005

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