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Assessing estrogenic activity of phytochemicals using transcriptional activation and immature mouse uterotrophic responses

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Abstract

The estrogenic responses of several phytoestrogens including genistein, daidzein, coumestrol, α-zearalanol, zearalenone, naringenin, taxifolin and biochanin A were compared over a wide dose range using an in vitro assay that measures transcriptional activation of the estrogen receptor (ER) and an in vivo immature mouse uterotrophic assay consisting of measuring uterine wet weight increase plus sensitive morphological and biochemical endpoints in the uterus. The transcriptional activation assay showed activation of the ER by all compounds tested except taxifolin with varying magnitudes of response as compared to estradiol or diethylstilbestrol. Results from the uterotropic bioassay showed that genistein, coumestrol, zearalanol, and zearalenone caused an increase in uterine wet weight, while naringenin, taxifolin, daidzein and biochanin A failed to do so over the dose range tested. However, sensitive morphological and biochemical parameters such as uterine epithelial cell height increase, uterine gland number increase, and induction of the estrogen-responsive protein lactoferrin demonstrated that all compounds tested in this study gave some measure of estrogenicity although a wide range of estrogenic responses across compounds was shown. Use of multiple in vitro and in vivo estrogenic endpoints as described in this paper will be useful in developing estrogenic profiles for individual compounds and ultimately mixtures of compounds. Furthermore, having an estrogenic ''fingerprint'' for each phytochemical is an essential first step in determining potential adverse effects of exposure to phytoestrogens.

Keywords: Endocrine disrupter testing; Phytoestrogens; Diethylstilbestrol; Estrogen receptor; Lactoferrin

1. Introduction

Permanently altered reproductive tract structure and function have been attributed to exposure to synthetic and naturally occurring estrogenic compounds if exposure occurs at critical stages of differentiation. For example, treatment of pregnant women with estrogenic compounds such as diethylstilbestrol (DES) was associated with genital tract abnormalities including cancer in their male and female offspring [1]. Experimental animal studies have since replicated and, in some cases, predicted, many of these DES-associated abnormalities [2]. Recently other experimental animal studies have shown that similar adverse effects occur following developmental exposure to the phytoestrogen, genis-

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tein [3]. Together, these data suggest the developing fetus is highly susceptible to the toxic and tumorigenic effects of estrogenic substances including phytoestrogens. Therefore, knowledge of the estrogenic potency of various phytochemicals, to which pregnant women and infants may be exposed, is extremely important.

Although phytoestrogens occur naturally in the diet, the nutritional and pharmaceutical use of these compounds has dramatically increased over the last decade due to a number of reports suggesting that phytoestrogens might lower the risk of severe chronic diseases, ease the symptoms of menopause, and protect against various cancers. In fact, protective effects of genistein are assumed from epidemiological findings showing a higher incidence of some common types of cancer (i.e. breast, prostate, and colon) and of coronary heart diseases in Western populations exposed to low levels of genistein in their diet [4]. Despite these possible beneficial effects, adverse effects have also been reported [5]. Concern exists in feeding infants soy-based formulas that contain several dietary estrogens including genistein [6]. While much attention has been given to the beneficial effects of dietary estrogens, it is important to balance these against the potential for deleterious effects especially if exposures to the substances occur during critical stages of development [7,8]. In fact, a recent report from our laboratory has shown that female CD-1 mice treated neonatally with genistein develop uterine adenocarcinoma later in life [3]; the incidence of this lesion is similar in mice treated with an equally estrogenic dose of diethylstilbestrol (DES), 35% and 31%, respectively.

In light of the adverse effects of exposures to estrogens during critical periods of development, several naturally occurring compounds were tested in our laboratory to compare their estrogenic activity using an in vitro assay that measures the transcriptional activation of the estrogen receptor (ER) and an in vivo immature mouse uterotrophic bioassay that includes sensitive morphological and biochemical endpoints in addition to the traditional uterine wet weight marker [9]. In the current study, estrogenic activity was determined for genistein, daidzein, and coumestrol (found in soy beans and soy-based products), naringenin (found in grapefruit), taxifolin (found in green tea) and biochanin A (found in bourbon) and compared to estradiol and DES.

Also of interest are the mycotoxins or mycoestrogens, zearalanol and zearalenone that are produced by mold and frequently found to infect pasture grasses, legumes, and corn. In veterinary medicine, zearalanol is used in animal feed as a growth promoter, anabolic agent, and an estrogenic agent [10]. Zearalenone has been shown to cause persistent estrus in laboratory animals as demonstrated by cornification of vaginal epithelial cells [11]. The association of ''moldy corn'' syndrome, and farm animals grazing in fields of clover rich in phytoestogens with reproductive problems including infertility has been well established [12].

In addition, numerous studies with experimental animals have shown deleterious long-term effects of developmental exposure to some of these phytoestrogens. For example, animals exposed to coumestrol exhibited ovary-independent persistent vaginal cornification as well as cervico-vaginal pegs and downgrowths, uterine squamous metaplasia, and hemorrhagic follicles [13]. A more recent study from our laboratory has shown preliminary results from a large multi-generational study following exposure to genistein showing effects on the reproductive tract in both female and male rats [14].

Because of the multiple biological effects of estrogens and the influence of absorption, metabolism, distribution, and excretion on the manifestation of their estrogenic activities, it is important to look at biochemical and cellular endpoints following exposure to potential estrogenic compounds in vivo. We previously reported the use of several bioassays including ER transcriptional activation and the immature mouse uterotrophic bioassay for testing compounds for estrogenicity [15]. As expected, the inclusion of multiple tests was more sensitive than any one assay alone. Further, a recent study from our laboratory describes sensitive morphological and biochemical endpoints of estrogenic activity that can be easily incorporated into the immature mouse uterotrophic assay that traditionally measures only uterine wet weight increase [16]. Again, these estrogenic endpoints make the uterotrophic assay very sensitive in detecting compounds with estrogenic activity. Thus, we incorporated these endpoints into the study of phytochemicals. In addition to uterine

wet weight gain, uterine epithelial cell height, uterine gland formation, and the production of the estrogen-inducible protein, lactoferrin (LF) in the uterine epithelial cells were determined. Using the data generated from both the in vitro transcriptional and in vivo uterotrophic assays for estrogenicity, estrogenic responses were determined for each compound and compared to estradiol and DES.

2. Materials and methods

2.1. Chemicals

Details of the chemicals used in this study are listed in Table 1.

2.2. ER transcriptional activation assay

The BG1Luc4E2 cell line has been stably transfected with a luciferase reporter gene that is responsive to the exposure of the cells to estrogen or chemicals that are estrogen agonists as previously described [17]. The cell line was grown in estrogen free DMEM for 4 days containing 5% FCS (fetal calf serum) that was carbon/dextran filtered to remove endogenous estrogen activity. Just prior to dosing the BG1Luc4E2 cells, the test chemicals dissolved in DMSO (<0.1%) were suspended in cell culture medium. These solutions were then used to expose monolayers of our cells grown in 96 well culture plates. In addition to the dilutions of test samples, a standard curve of 17β-estradiol was assayed. The plates were incubated to induce optimal expression of luciferase activity in a humidified CO_2 incubator. Following incubation, the media was removed and the cells were microscopically observed for viability. The luciferase assay was run to quantify the induction of luciferase activity. Microscopic examination of the cells following exposure to the sample extracts did not reveal any indication of toxicity to the cells at any dose tested.

Table 1
Chemicals used in this study

Compound	Cas #	Source
Estradiol-17β	[50-28-2]	Sigma, St. Louis, MO
DES	[56-53-1]	Sigma, St. Louis, MO
Genistein	[446-72-0]	Sigma, St. Louis, MO
Coumestrol	[479-13-0]	Acros Organics, NJ
Daidzein	[486-66-8]	Research Triangle Institute, Durham, NC
α-Zearalanol	[26538-44-3]	Sigma, St. Louis, MO
Zearalenone	[17924-92-4]	Sigma, St. Louis, MO
Biochanin A	[491-80-5]	Sigma, St. Louis, MO
Naringenin	[480-41-1]	Sigma, St. Louis, MO
Taxifolin	[480-18-2]	Sigma, St. Louis, MO

2.3. Uterotrophic bioassay

Female CD-1 mice [Crl: CD-1 (ICR)] (Charles River Breeding Laboratories, Raleigh, NC) were bred to males of the same strain at NIEHS. All animals were housed in a temperature-controlled room (21–22 °C) with a 12-h light and 12-h dark cycle. Mice were provided with fresh water and fed NIH-31 lab chow ad libitum. This chow was chosen because it contained moderately low levels of phytoestrogens that are necessary in studies conducted for estrogenicity [18]. All animal procedures complied with NIH/NIEHS animal care guidelines. Pregnant females delivered their young on day 19 of gestation. At delivery, pups from all litters were pooled, and then each dam was randomly assigned 10 female pups (male pups were used for another study). As previously described [19], female pups were weaned on day 17 and housed five per cage. Starting on day 17, immature mice were subcutaneously injected with varying doses of diethylstilbestrol (DES) or 17β-estradiol as positive controls or the test phytochemical dissolved in corn oil at doses ranging from 0.01 to 1 000 000 μg/kg per day. The amount of corn oil given to each mouse was 0.01 ml/g body weight. Control mice received corn oil injections. Mice were injected for 3 consecutive days and sacrificed on the morning of the 4th day (20 days old). Body weights and uterine wet weights were determined. Care was taken to not lose uterine luminal fluid since fluid imbibition is an important estrogenic response. Uterine tissues were fixed in Bouin's fixative and processed for histological examination. A minimum of five mice per compound per dose was used. Statistical analysis was performed by ANOVA (StatView SE+Graphics, Abacus Concepts, Inc., Berkeley, CA); values were determined to be significant at 95%.

2.4. Cell height and gland number determinations

Uterine tissues, collected and fixed in Bouin's from the uterotropic bioassay, were used for cell height and gland number determinations. Tissues were processed and embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin (H&E). Uterine tissue sections were evaluated for epithelial cell height with an Olympus BH-2 Microscope (Olympus Corp., New Hyde Park, NY) interfaced with an image analysis system (Southern Micro Instruments, Atlanta, GA) as previously described [16]. Cell height measurements were determined by obtaining three measurements per uterine tissue from a minimum of three uterine horns. Gland number was determined from two consecutive uterine cross-sections of a minimum four mice for each treatment. These values were used to determine the mean±standard error (SE) for each group. Statistical analysis was performed by ANOVA (StatView SE+ Graphics, Abacus Concepts, Inc., Berkeley, CA); values were determined to be significant at 95%.

2.5. LF immunohistochemistry

Uterine tissues collected from the uterotropic bioassay were used for the determination of LF protein. Bouin's fixed tissues were embedded and cut in 4-micron sections. Uterine tissue sections were immunostained using a horseradish peroxidase immunocytochemistry technique [20]. Briefly, tissue sections were deparaffinized, washed in Tris buffered saline, pH 7.6 (TBS) containing 0.6% Tween-20 (TBS-T; Bio-Rad Laboratories), and then treated with 3% H_2O_2 in deionized water to block endogenous peroxidase. After washing in TBS-T, tissue sections were covered with 10% bovine serum albumin (BSA) in TBS-T for 1 h. After washing in TBS-T, excess buffer was removed and tissue sections were incubated for 2 h at room temperature with rabbit anti-mouse LF IgG antibody diluted 1:200 in TBS-T. Normal rabbit IgG (Accurate Chemical Company, Westbury, NY) diluted 1:200 was used as a negative control. After washing in TBS-T, tissues were then incubated for 1 h at room temperature with donkey anti-rabbit Ig horseradish peroxidase-linked $F(ab')_2$ (Amersham International, Arlington Heights, IL) diluted 1:1000 in TBS-T. Tissue sections were washed in TBS-T and incubated 20 min in 0.05 *M* Tris–HCl buffer containing 0.01 *M* imidazole (Sigma, St. Louis, MO), DAB and 0.015% H_2O_2. Sections were washed in TBS, vapor-fixed for 10 min with 2% osmium tetroxide (Sigma), washed again in TBS, dehydrated and coverslipped. LF intensity was scored on the image analysis system described for cell height measurements. Three measurements of intensity were taken from a minimum of three areas for each of three uteri. The intensity scale ranged from 0 (no stain) to 150 (very intense stain). Statistical analysis was performed as described for cell height calculations.

3. Results

3.1. ER transcriptional activation assay

The results of the transcriptional activation assay can be seen in Fig. 1. Of these compounds genistein, daidzein, coumestrol, zearalanol, zearalenone, biochanin A, and naringenin were found to induce transcriptional activation of luciferase through the ER. The only compound that did not induce transcriptional activation at any of the doses tested was taxifolin. We also found various magnitudes of activation based on the compound tested. For example, genistein, daidzein, and biochanin A had a higher magnitude of response than estradiol or DES although the peak response occurred at a much higher dose, while zearalanol, zearalenone, coumestrol, and naringenin demonstrated a lower magnitude of response than estradiol or DES.

3.2. Uterotrophic bioassay

The results of the uterotropic bioassay are shown in Fig. 2. Uterine wet weight to body weight ratios were plotted for all doses of all compounds tested. The positive controls in this experiment were DES, which showed an increase in uterine wet ratio with the maximum response at 10 μg/kg and estradiol with a maximum response at 500 μg/kg. Coumestrol showed an increase in uterine wet weight with a peak estrogenic response at 10 000 μg/kg, which was equal in magnitude to the positive controls. Since zearalanol and zearalenone were difficult to dissolve,

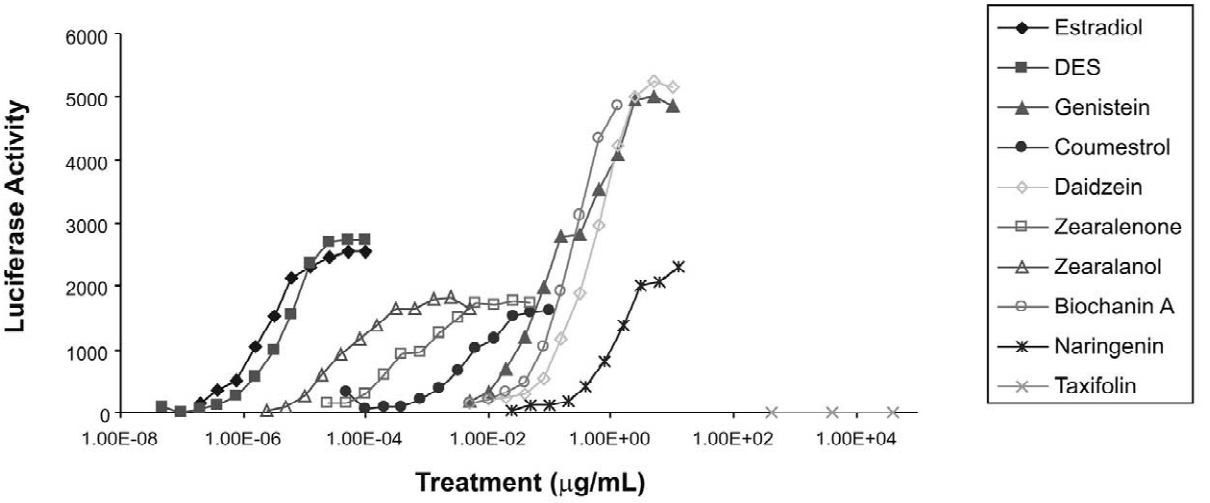

Fig. 1. Results of transcriptional activation of ER following treatment with various phytochemicals. The estimated EC50 for each compound can be seen in Table 3.

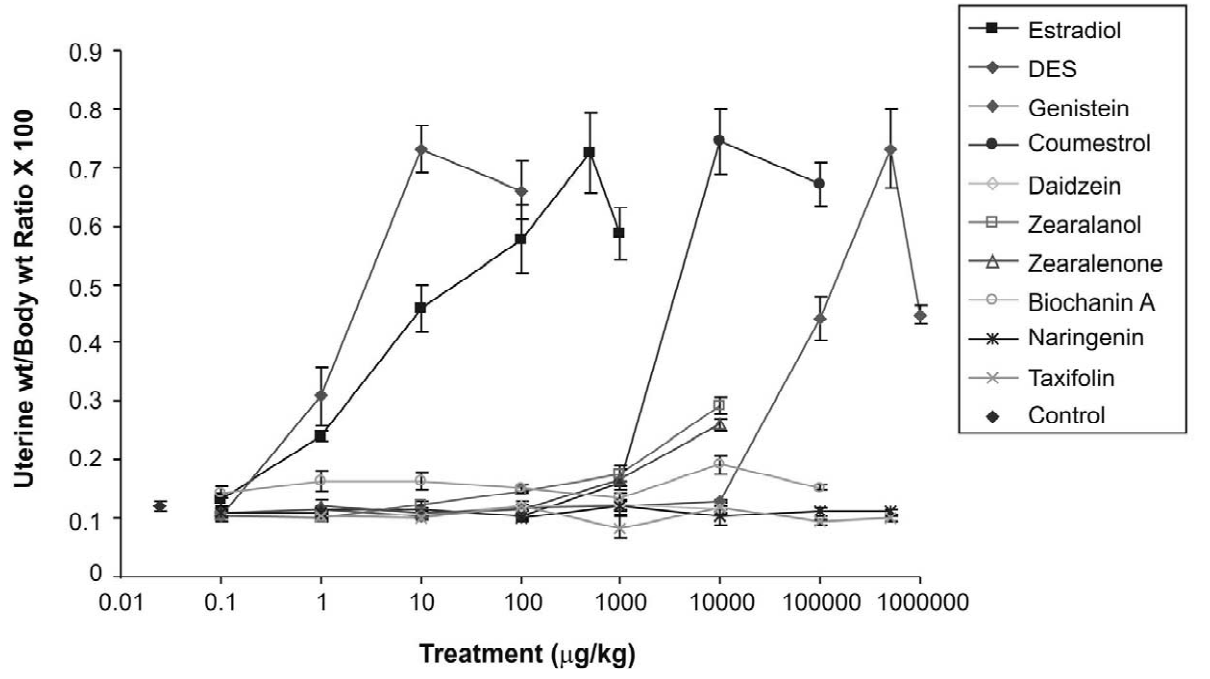

Fig. 2. Results of the immature mouse uterotrophic bioassay of various phytochemicals. The following treatments showed statistically significant increases over control at $P<0.05$. DES and estradiol at doses ≥ 1 μg/kg; coumestrol at doses $\geq 10\,000$ μg/kg; genistein at doses $\geq 100\,000$ μg/kg; zearalanol and zearalenone at doses $\geq 10\,000$ μg/kg. All other compounds did not significantly increase uterine wet weight.

doses higher than 10 000 μg/kg could not be tested, but similar increases in uterine wet weight as that of DES at a dose of 1 μg/kg suggested that it would take approximately 10 000 times as much of these compounds as DES to elicit a similar response. Genistein also demonstrated a positive response in the uterotropic bioassay with the uterine wet weight increase equal in magnitude to DES at a dose of 500 000 μg/kg, which is 50 000 times the amount needed for the same response by DES. Naringenin, taxifolin and daidzein did not demonstrate an increase in uterine wet weight at the doses tested in this study, however, other estrogenic endpoints were positive as further described.

3.3. Uterine epithelial cell height

The results of the uterine epithelial cell height measurements can be seen in Fig. 3. The compounds tested in this study showed an increase in uterine epithelial cell height that closely resembles the increase in uterine wet weight. However, the increase in cell height appears to be a more sensitive indication of estrogenicity with an increase in cell height occurring at a lower dose than the increase in uterine wet weight for most compounds. Of the compounds that demonstrated an increase in uterine wet weight, all of them showed an increase in cell height over untreated controls. Naringenin and biochanin A, which did not increase uterine wet weight, showed an increase in uterine epithelial cell height at the highest doses tested although they were less effective in increasing this endpoint as compared to estradiol or DES; biochanin A was significantly increased only at the highest dose tested. One compound, taxifolin demonstrated an unusual pattern of increased cell height with the increase occurring at the lowest doses tested instead of the highest dose tested. Daidzein was the only compound that did not demonstrate any increase in cell height at any dose tested.

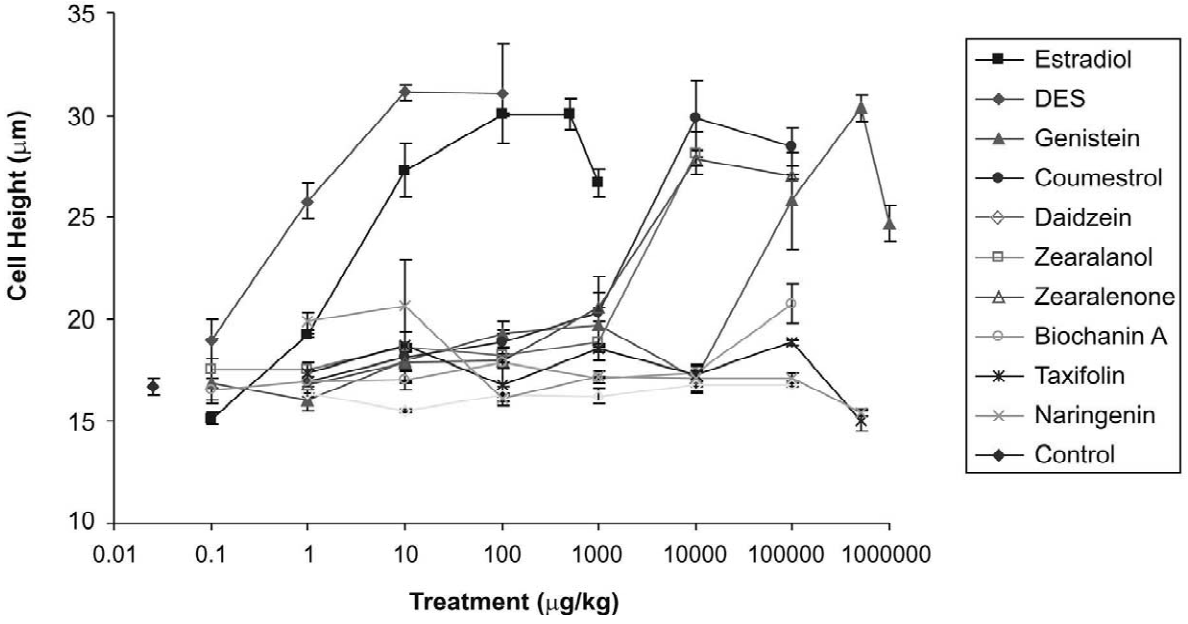

Fig. 3. Results of uterine epithelial cell height following exposure to various phytochemicals. The following treatments showed statistically significant increases over control at $P<0.05$. DES and estradiol at doses ≥1 μg/kg; coumestrol at doses ≥10 000 μg/kg; genistein at doses ≥100 000 μg/kg; zearalanol and zearalenone at doses ≥10 000 μg/kg; biochanin A at a dose of 100 000 μg/kg. Taxifolin exhibited an unusual pattern with a statistically significant increase at the 1 μg/kg dose but not at the higher doses. All other compounds did not significantly increase uterine epithelial cell height.

Table 2
Uterine gland number following exposure to various phytoestrogens

Treatment (μg/kg)[a]	Dose[b]	Gland number[c]
Control	0	11.1±1.4[d]
DES	10	21.6±3.9
Estradiol-17β	100	20.9±1.7
Genistein	50 000	19.8±1.4
Coumestrol	100 000	20.8±3.0
Daidzein	100 000	19.8±1.2
Zearalanol	10 000	14.5±1.6
Zearalenone	1000	22.3±3.8
Biochanin A	100 000	16.0±3.3
Naringenin	10 000	15.8±3.6
Taxifolin	500 000	15.3±1.3

[a] Mice were treated on days 17, 18, and 19 with test chemical; uterine tissues were collected on day 20.

[b] The dose for each compound represents the maximum uterine gland response.

[c] Uterine cross-sections were examined and the number of glands per section (two sections per mouse) were determined for a minimum of four mice per treatment group.

[d] Numbers are the mean±SEM.

3.4. Uterine gland number

The increase in gland number following 3 days of treatment by each compound is shown in Table 2. An increase in gland number was seen in all of the test compounds. DES and zearalenone gave the largest increase in gland number with lower varying responses from the other compounds. Although naringenin, daidzein, biochanin A and taxifolin did not have significant increases in uterine wet weight, gland numbers were significantly increased over control.

3.5. Uterine lactoferrin intensity

The results of the LF immunohistochemistry image analysis can be seen in Fig. 4. DES and estradiol as positive controls showed an increase in LF production in the uterine epithelial cells. Coumestrol, genistein, zearalanol, and zearalenone

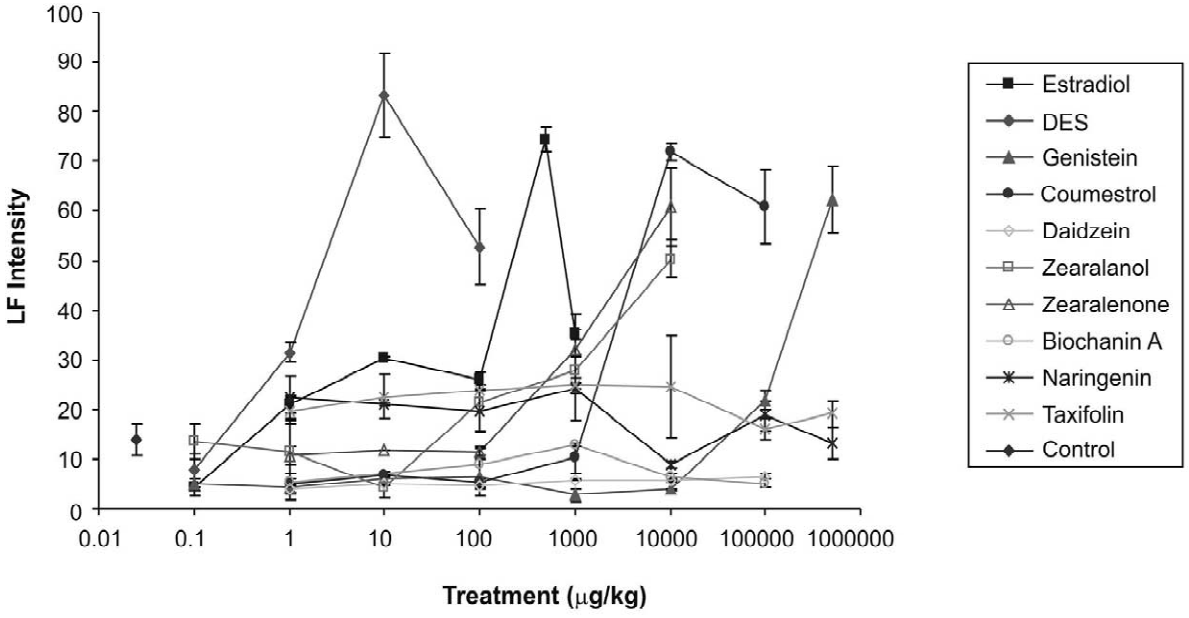

Fig. 4. Image analysis of LF immunohistochemistry of various phytochemicals. The following treatments showed statistically significant increases over control at $P<0.05$. DES at doses ≥1 μg/kg; estradiol at doses ≥10 μg/kg; coumestrol at doses ≥10 000 μg/kg; genistein at a dose of 1 000 000 μg/kg; zearalanol and zearalenone at doses ≥1000 μg/kg; biochanin A at a dose of 100 000 μg/kg. All other compounds did not significantly increase lactoferrin expression.

also showed an increase in the uterine epithelial cells with similar levels of intensity at the highest doses tested for each respective compound. The immunolocalization of LF can be seen in Fig. 5 showing representative sections from several compounds. The dose response curves for these compounds also followed the increase in uterine wet weight. In addition, as with uterine wet weight, naringenin, daidzein, biochanin A, and taxifolin did not demonstrate any LF immunostaining at any of the doses tested in this study.

A summary of the estrogenic responses for each chemical is shown in Table 3. This data demonstrates the wide array of estrogenic responses based on the chemical exposure. Some compounds like DES, estradiol, genistein, and coumestrol are highly positive in all of the endpoints but others like daidzein are only positive in one endpoint (gland ≠) and a few compounds are highly positive in some parameters but negative in others like naringenin.

4. Discussion and conclusions

Human and wildlife exposures to phytoestrogens and synthetic estrogens form the basis for the emerging field of environmental endocrine disrupters. The presence of phytoestrogens, including genistein, in soy-based infant formulas is of concern among public health practitioners. Previous work in

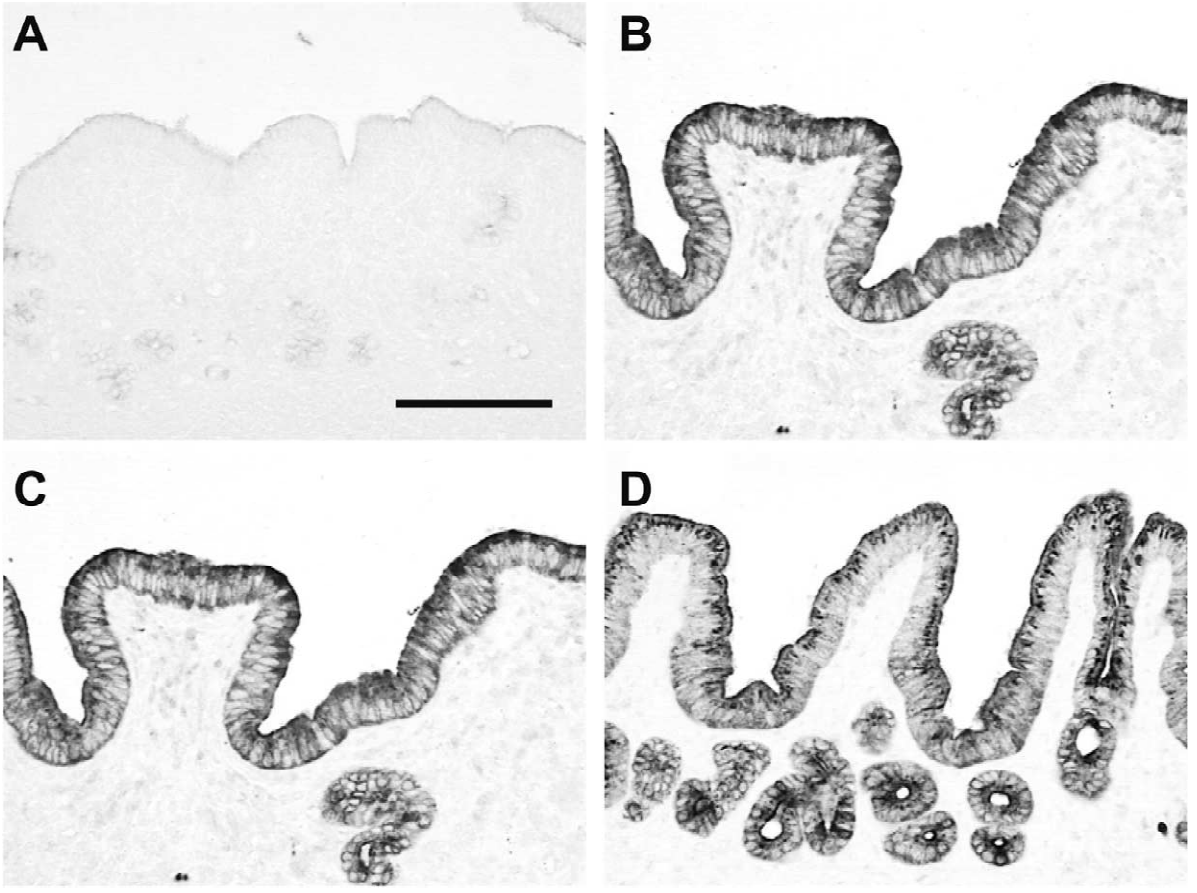

Fig. 5. Immunohistochemical localization of LF in the uterus following exposure to various phytochemicals. Panel A: control uterus showing no LF immunostain; Panel B: DES (10 μg/kg) treated uterus showing strong LF immunostain in the epithelial cells; Panel C: genistein (500 000 μg/kg) treated uterus showing strong immunostain in the epithelial cells; Panel D: zearalenone (10 000 μg/kg) treated uterus showing strong immunostain in the epithelial cells. Bar=100 μm.

Table 3
Summary of estrogenicity test results

Chemical	Transcription assay EC50 (μg/ml)[a]	Uterotrophic assay[b]	Cell height[b]	Gland number[b]	LF[b]
Estradiol-17β	0.0000023	++++	++++	++++	++++
DES	0.0000049	++++	++++	++++	++++
Zearalanol	0.000039	++	+++	++	+++
Zearalenone	0.00053	++	+++	+++	+++
Coumestrol	0.0052	+++	++	+++	+++
Genistein	0.19	+++	++	+++	+++
Biochanin A	0.21	±	+	++	−
Daidzein	0.52	−	−	+++	−
Naringenin	1.22	−	±	+	±
Taxifolin	N/A[c]	−	±	+	−

[a] The estimate of the EC50 (dose that demonstrates 50% efficacy) for each compound was determined by using the Hill equation.
[b] The assignment of ± to +++ was determined by comparing all of the compounds to each other and taking into account the magnitude of response as well as the dose that it took to achieve that response ranging from no response (−) to the strongest response (++++).
[c] N/A=not applicable; taxifolin did not demonstrate a response in this assay so the EC50 could not be determined.

our laboratory as well as others with the synthetic estrogen DES has demonstrated that perinatal exposure induces reproductive tract abnormalities as well as subfertility [21,22]. Although there have been a few studies of beneficial effects from phytoestrogen treatment including a possible reduction in breast cancer risk in rats [23] and cholesterol synthesis rates in humans [24], little data exists on long-term effects on reproductive tract function and cancer. We recently reported an increase in uterine adenocarcinoma at 18 months of age in mice following neonatal exposure to genistein [3]. The incidence of uterine adenocarcinoma was 35% in the genistein 50 000 μg/kg group and 31% of mice treated with DES at a dose of 1 μg/kg which are equal in estrogenicity [3]. In addition, serum levels of genistein were measured in these neonatal mice and the circulating levels were comparable to the amounts found circulating in human infants fed soy-based infant formula [25]. Although the injection dose was slightly higher, and the routes of administration different, the actual circulating levels are similar in neonatal mice and human infants. Another recent study showed an increase in carcinogen-induced mammary tumorigenesis in female rats following prenatal exposure to genistein [26]. Further, two epidemiology reports have shown an association of a vegetarian diet, which usually contains high levels of soy, consumed during human pregnancy, with an increased incidence of hypospadias in the male offspring [27], and an increase in autoimmune disease and the use of allergy medicines in children fed soy-based infant formulas [28]. Together these studies indicate the need to more fully assess the effects of exposures to phytoestrogens during development.

The results presented in this study demonstrate various estrogenic responses of naturally occurring compounds in the environment. The ability of these compounds to bind to the estrogen receptor and elicit transcriptional activation has been demonstrated for the phytochemicals tested in this study except taxifolin. Although the dose required to give a response was higher than DES or estradiol for all of these compounds, the magnitude of response was similar for most of them. This would indicate that there is a potential for endocrine disrupting activity.

In addition to transcriptional activation, the in vivo estrogenic potential of some of these phytoestrogens appears to be relatively high, like genistein, zearalanol, zearalenone and coumestrol. A recent paper by Mehmood et al. using uterine wet weight gain in immature mice and several other endpoints showed that zearalanol was approximately 5000 times less potent than DES [29] similar to our findings. Others have shown a range of potency for zearalanol as well as other phytoestrogens compared to DES in different assays [30–34]. The magnitude of response is often equal to DES or estradiol although the dose required to achieve this response is much greater. Naringenin, taxifolin, biochanin A,

and daidzein appeared to be much less potent estrogens but positive results in several of the cellular response endpoints would suggest that these compounds could also cause deleterious effects if exposure occurs during sensitive windows of development.

Of particular significance is the positive results seen in the uterine response endpoints like gland number and cell height when a compound did not show an increase in uterine wet weight. Although several compounds followed the same pattern in each test as that of uterine wet weight gain, some compounds showed little uterine wet weight gain but a strong positive response in another sensitive endpoint. For example, daidzein appeared to be negative in all of the in vivo tests with the exception of an increase in uterine gland number, and biochanin A gave varying results for each endpoint. These compounds may activate different pathways of response depending on their interaction with the estrogen receptor. This provides an interesting area of research. The regulation of cell growth may be separate from that of cellular differentiation and the results could be specific for each pathway. For example, some compounds may interact with ER (α and/or β) to elicit a cell growth response such as an increase in uterine wet weight whereas another compound may interact with the ER subtypes to stimulate cellular differentiation such as an increase in uterine glands. While an increase in uterine wet weight has been the ''gold standard'' for detecting the estrogenic activity of a compound, these other endpoints are also considered markers of estrogen action and therefore are important in the determination of the overall estrogenic potential of a particular compound. It is interesting to note that these other endpoints could occur independently and without an accompanying increase in uterine wet weight. It is possible that some of these compounds are very specific in their mode of action and may cause particular effects especially if studied for long-term effects.

Another important issue concerning phytoestrogens is the relative content of each of them in commercial diets for laboratory animals and the effects they might have on endocrine disrupting research [18,35,36]. In this study, we specifically chose a diet that was low in phytoestrogen activity. In a recent report, we examined the phytoestrogen content of several rodent diets and determined that the relative amounts of these compounds is highly variable from diet to diet [18]. Therefore, research conducted using different diets may give different results based on the amount of these phytoestrogens present in the diet. In fact, it may be particularly difficult to interpret the results of sensitive endpoints in any system where phytoestrogens may have their own effect. This should be carefully considered when designing experiments that are specifically addressing endocrine disrupting endpoints. While it is not necessary to use phytoestrogen free diets, and at times, it may be contraindicated [37], it is important to know the levels of phytoestrogen activity in the diet.

In light of recent findings of low dose exposures to estrogenic compounds during development causing effects on the developing reproductive tract [38], even the phytochemicals that have low estrogenic activity may have adverse effects. There have been reports demonstrating effects on reproductive tract tissues with doses of estrogenic chemicals that were previously thought to have no effect. For example, DES has been shown to increase prostate weight following prenatal exposure to very low doses of DES (0.02 μg/kg per day) [39]. Another paper has also shown a similar effect on the prostate following prenatal exposure to the weaker environmental estrogen, bisphenol A [40]. Testing of these weaker phytoestrogens for low dose effects is of great importance and would offer support to the idea that low estrogenic activity can cause alterations in the reproductive tract if given during critical periods of development. In addition to the low dose issue, it is possible to envision that the additivity or synergistic potential of these compounds should also be considered since these compounds are rarely found alone in the environment. Since these compounds may influence estrogen-mediated processes in development, they should be thoughtfully considered when determining risks to the developing organism.

In conclusion, determining the estrogenic potential of a substance is very important. As previously reported, estrogenic compounds can significantly alter the reproductive and endocrine system of both animals and humans [1]. It is well known that estrogens, synthetic or otherwise, introduced into an organism during critical periods of development can cause many deleterious effects including cancer and

infertility [41]. For this reason, the determination of the estrogenic potential of phytochemicals is of great importance to human health, particularly since these chemicals can be readily found in the diets of young children. Long-term studies with some of the less potent estrogenic substances would help to determine their potential adverse effects on the developing reproductive tract; this is essential before dismissing them as non-problematic because of their weak estrogenicity.

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