A Case of a Laboratory Animal Feed with High Estrogenic Activity and Its Impact on *in Vivo* Responses to Exogenously Administered Estrogens

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We recently noted that immature rats failed to exhibit a normal uterine response to exogenously administered estradiol as assessed by both biochemical (induction of gene expression) and morphological (altered uterine and vaginal histology and size) end points. An initial analysis suggested that this was due to a high degree of estrogenization from a dietary source which was producing a near maximal uterotrophic response prior to hormone treatment. Subsequent chemical analysis indicated that the feed in question contained high amounts of two well-known phytoestrogens, genistein (210 mg/kg) and daidzen (14 mg/kg), and the lot of feed in question produced a large uterotrophic effect when fed to immature ovariectomized rats. These findings illustrate that, despite increased awareness of phytoestrogens, some batches of animal feed contain very high amounts of estrogenic components which have marked effects on *in vivo* end points of hormone action. These observations have important implications for both basic research and screening methods that utilize *in vivo* approaches. *Key words*: animal feed, estrogen, mycoestrogens, phytoestrogens. *Environ Health Perspect* 106:369–373 (1998). [Online 4 June 1998] *http://ehpnet1.niehs.nih.gov/docs/1998/106p369-373boettger-tong/abstract.html*

It is well known that many edible plants produce nonsteroidal compounds that can either mimic or antagonize the effects of endogenous steroid hormones, including estradiol and related compounds. These compounds may either be produced by plants themselves (referred to as plant estrogens or phytoestrogens) or by fungi that infect plants or plant-derived materials such as grain during storage (referred to as mycoestrogens). Based on their chemical structures, the most potent phytoestrogens are coumestans, such as coumestrol, and isoflavones, such as genistein and daidzein (*1,2*). Alfalfa is one of the richest sources of coumestans, and isoflavone levels are high in soybean products. The most potent mycoestrogens are resorcylic acid lactones such as zearalenone (*3,4*) produced by the species *Fusarium* (see Fig. 1), which grows on corn, oats, and other grains. It is unequivocally established that all of these compounds can bind to the estrogen receptor and can produce estrogenlike effects in animals and cultured cells (*1*).

Historically, there has been concern that such compounds might be present in the human diet and in grain and forage eaten by domestic animals or wildlife. For example, it has been widely recognized since mid-century that the ingestion of certain plants can cause infertility in domestic animals, e.g., the clover disease described by Bennets et al. (*5*). Such concerns recently led the U.S. Congress to pass the Safe Drinking Water Act and the Food Quality Protection Act in 1996. This legislation mandates that the U.S EPA develop screening and testing guidelines for endocrine disruptors, including environmental estrogens. Environmental estrogens include naturally occurring plant and fungal compounds such as those noted above and a variety of man-made chemicals used in agriculture, e.g., pesticides such as DDT, and manufacturing, e.g., phenolic compounds used to produce plastics (*6,7*).

While firm guidelines have not yet been developed, the general consensus seems to be that some combination of *in vivo* and *in vitro* testing procedures is likely to be employed for this purpose. This raises potential concerns about factors likely to affect screening tests as well as basic research that utilizes *in vivo* experimental models. One such factor is the presence of compounds with estrogenic or antiestrogenic activities in the feed of laboratory animals.

It was clearly documented in NIEHS laboratories over a decade ago that common bioassays for estrogenic activity, e.g., rodent uterotropic assays, are highly susceptible to influence by compounds with estrogenic activity present in laboratory animal diets fed to test animals (*8,9*). This is a particular concern because the richest natural sources of isoflavones and coumestans are soy and alfalfa, respectively, which are common ingredients of laboratory animal feeds. In addition, materials from these plant sources are susceptible to infection by *Fusarium*, which can lead to feed contamination by mycotoxins such as zearalenone, with potent estrogenic activity. Two years ago we experienced a case of extreme estrogenization of immature female rats. We were able to identify the commercial rodent feed being used in our animal facility as the source of this hormonelike activity. This episode, the fact that this diet has not been previously documented to contain estrogenic contaminants, the resultant interactions with the manufacturer, and the current interest in the study of environmental estrogens prompted us to share our experience.

The Lack of Response to Exogenously Administered Estrogens

Our laboratories have been studying estrogen-induced changes in the rodent female tract at both the ultrastructural and molecular levels for approximately 25 years; thus, we have extensive experience with this experimental system. We had been receiving animals from the same vendor, a major national supplier, during the entire 25-year period and had not encountered any problems using these animals in many types of studies. In our institution animals are received, maintained, and treated in our Center for Laboratory Animal Medicine and Care, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. It should also be noted at the outset that the animal supplier also owns a subsidiary company that produces laboratory animal feed. The same rodent diet is thus used in their breeding facility as well as in our animal quarters.

Two years ago in a series of experiments, the uteri of immature female rats seemed to be exhibiting an abnormally low response to injected estradiol based upon the induction of several marker genes such as c-*fos*, which we and others have shown are induced by ovarian (*10,11*) and environmental estrogens (*12,13*). For these experiments we were using immature Sprague-Dawley female rats that had arrived at our institution at 20–21 days of age; animals underwent ovariectomy 1–2 days later

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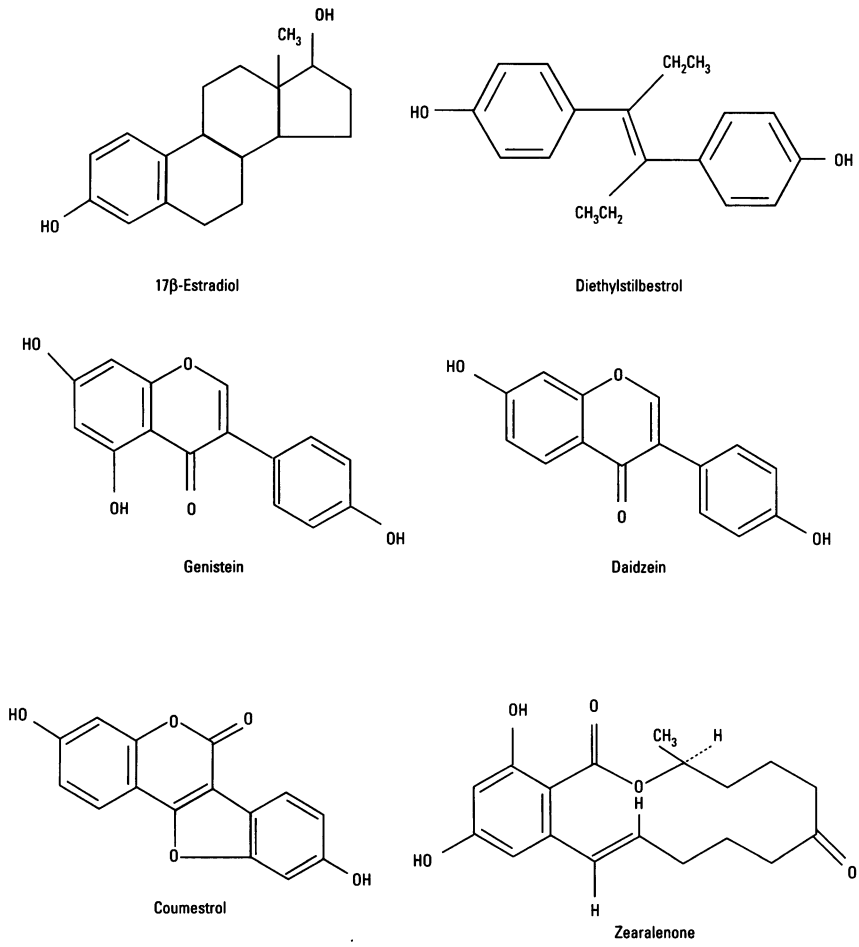

Figure 1. Structures of estrogens including a steroidal estrogen (estradiol), a stilbene (diethylstilbestrol), isoflavones (genistein and daidzen), a coumestan (coumestrol), and a resorcylic acid lactone (zearalenone).

and were then allowed to recover for another week before estradiol administration and euthanasia at approximately 30 days of age. Upon analysis of our data, the major problem appeared to be that the levels of these transcripts were abnormally high in control animals that had not received exogenous estrogen treatment. In other words, we were not observing the typical robust response to exogenously administered estrogens because the response in question was already stimulated to near maximal levels in the untreated control animals. This suggested that the animals were being estrogenized via the diet or another route of environmental exposure.

Evidence of Exposure to Environmental Estrogens

To explore this possibility, we prepared histological sections of the uteri and vaginae from 30-day old ovariectomized animals we were using at this time (i.e., 1995) that had not received hormone treatment. This was done because transcripts such as c-*fos* can be induced by nonestrogenic as well as estrogenic stimuli (*14*) so that expression of this gene cannot be taken as unequivocal evidence of exposure to estrogens. Representative uterine and vaginal sections from these animals are are shown in Figure 2 (OV-95-30d). Because we suspected that our untreated animals were somehow estrogenized, we retrieved sections we had prepared 2 years earlier (in 1993) from similar animals (i.e., ovariectomized without subsequent estrogen administration) obtained from the same vendor to examine as historical controls. At that time, basal levels of gene expression and other parameters in our laboratory were very low and hormonal responses were very robust. These sections are also shown in Figure 2 (OV-93-30d). The sections prepared in 1995 and 1993 showed strikings differences. For example, in the 1995 sections relative to the 1993 samples, the vaginal epithelium is much thicker, the height of the uterine luminal epithelial cells is markedly increased, the uterine stroma is much more edematous, etc.

The Location and Nature of Estrogenic Exposure

The histological changes seen in Figure 2 are virtually diagnostic for exposure to estrogens and thus confirmed our suspicions of an environmental exposure to estrogens. Because these sections were prepared from 30-day-old animals that had been in our institution for approximately 10 days, the suspected "estrogenization" could have occurred at the vendor's breeding facility, in our animal care center, or both. To explore these possibilities, we obtained a new batch of 20-day-old animals from the vendor and sacrificed them immediately upon arrival in our facility. Representative sections of vagina and uterus from these animals are shown in Figure 2 (OV-95-20d). These animals show clear signs of estrogen exposure, albeit to a lesser degree than those held in our facility for an additional 10 days. For example, the uterine stroma shows clear signs of edema (compare to the OV-93-30d) and the vaginal epithelium is slightly thickened, but the luminal epithelial cells are not markedly increased in height. This suggested that the animals were already estrogenized when they arrived from the vendor, but that they were receiving further hormonal stimulation in our animal quarters.

As an additional comparison, we obtained similar 20-day-old rats of the same strain from an alternative vendor and had them delivered to our neighboring institution Baylor College of Medicine, which is located within two blocks of our facility. These animals were thus from a totally different vendor and were never exposed to the environment of our animal care quarters, but experienced a similar geographical environment. Representative sections of these animals are also shown in Figure 2 (AV-95-20d). These sections showed no apparent signs of estrogenization that we could detect, and are comparable to those from animals obtained from our original vendor in 1993. This further indicated that the problem was limited to animals from our original vendor.

Chemical Analysis of Animal Feed for Mycoestrogens and Phytoestrogens

Using animals from the alternative vendor, we next conducted a series of studies to determine if the source of the apparent estrogenization in our facility was primarily the feed, bedding, or other factors (e.g., air in certain rooms). For example, we placed some animals from the same shipment in a given room in wire bottom cages and some in polycarbonate cages with bedding material, we fed both groups the identical diet to determine if the bedding was the source of the estrogenization. These studies eliminated the bedding, water, or specific holding rooms as the source of the apparent contamination and thus strongly suggested that the diet was the most likely source of any estrogenic substances. To examine this possibility directly, we tested lots of the feed being used in our

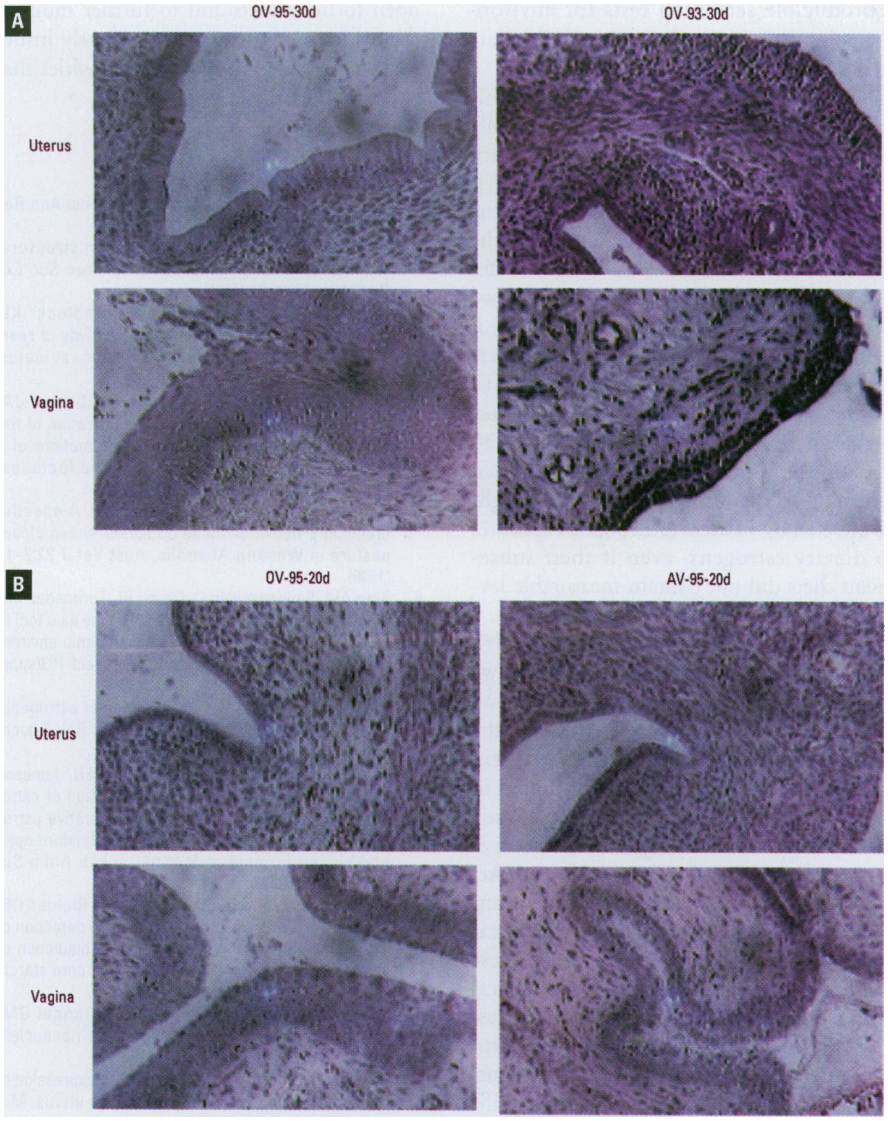

Figure 2. Histological evidence of estrogenization. (A) Sections of uteri and vaginae from 30-day-old rats received from the original vendor in either 1995 (OV-95-30d) or 1993 (OV-93-30d). (B) Sections from 20-day-old rats received in 1995 from either the original vendor (OV-95-20d) or an alternate vendor (AV-95-20d).

animal care quarters for genistein and daidzein by chemical analysis using an isotope dilution gas chromatographic-mass spectrometric method (*15*). We analyzed the feed chemically for these two compounds because they are the isoflavones present in highest concentration in most soy products (*1*), because soybean meal is a component of many laboratory animal feeds, and because these two chemicals had previously been detected in commercial rodent feeds (*16*). Our chemical analyses detected high levels of genistein (21 mg/100 g feed) and daidzein (14 mg/100g feed), but the feed could also contain other phytoestrogens. Lower levels of genistein (6.8 mg/100 g feed) and daidzein (0.6 mg/100 g feed) had been found in commercial rodent feeds in an earlier study (*16*).

These compounds are well-known phytoestrogens and would be expected to produce a clear estrogenic response at these levels, given normal consumption of feed by ovariectomized rats. Recently, Santell et al. (*13*) showed that diet supplemented with 375 µg genistein per 1 g diet (i.e., 37.5 mg/100 g) induced uterine weight gain in rats, and a twofold higher dose elicited induction of uterine *c-fos* and estrogenic effects in mammary gland and pituitary, in addition to uterine weight gain. Furthermore, it is possible that the diet contains other estrogens because we only performed the chemical analysis for a limited number of preselected compounds. Other analyses performed by outside contract laboratories did not, however, detect measurable levels of the mycoestrogens zearalenone or zearalenol in this lot of feed.

Interactions with the Animal/Feed Vendor

At this point, we informed the vendor of our findings and provided the company

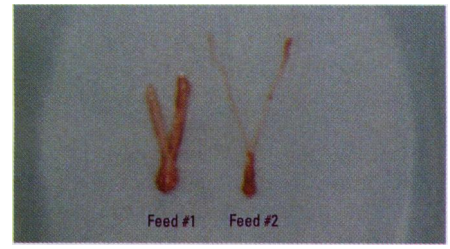

Figure 3. Uterotrophic effect of feeding rat diet. Rats were obtained from the alternate vendor at 20 days of age, ovariectomized, and fed either the rat diet from our original vendor, which we suspected contained estrogenic components (Feed #1), or from an alternate supplier (Feed #2) for 10 days. Euthanasia was then performed at 30 days of age and uteri were removed.

with a sample of the batch of the lab feed being used at this time in our facilitiy. The vendor performed an independent bioassay of this feed using the growth of estrogen-responsive cultured cells as an end point (*17*). While these bioassays did not indentify specific estrogenic chemicals or their levels in the feed, the vendor voluntarily acknowledged that "It appears that a component of the ... rat/mouse diet which was being used at your facility in the summer of 1995 had a high level of estrogen."

The feed in question was prepared primarily from soy and alfalfa, but the vendor had not retained samples of the raw materials used to prepare the specific lot of feed in question. However, by analyzing other lots of feed prepared with the same or different batches of raw materials, the vendor believed that the source of estrogenic activity in this particular batch of feed was due to the alfalfa. However, this is not consistent with our chemical analysis, as alfalfa is not known to contain significant amounts of isoflavones such as genistein and daidzein that we identified chemically (*18*); thus, the exact source of the phytoestrogen contamination in the rodent diet remains an open question.

To further confirm or refute the diet as a source of exposure to estrogen, we obtained 20-day-old animals from the alternate vendor (see Fig. 2) and had them delivered to our facility. We ovariectomized the animals within a day of arrival at our facility and divided them into two groups. One group received the standard diet we had been using throughout these studies, and the second group received a rat diet of the same general composition that we purchased from another supplier. Neither group of animals received any exogenous estrogen treatment. The animals were then sacrificed at 30 days of age (i.e., after 10 days on either of the two different diets). Representative uteri from the two groups are illustrated in Figure 3; the

uterus from an animal fed our vendor's diet is dramatically increased in size and vascularity compared to the uterus from an animal that received the diet obtained from an alternate source.

Histological sections of the vaginae and uteri from these animals indicated clear signs of estrogen exposure, but this was closer in severity to that seen in the 20-day-old group in Figure 2 and not as severe as in the OV-95-30d group. Data from the feed study and findings that 20-day-old animals displayed histological profiles of vagina and uterus characteristic of a mild estrogen exposure directly upon arrival from the vendor and 30-day-old animals displayed histological profiles characteristic of a more severe estrogenic exposure suggested to us that the animals we received in 1995 had been exposed to feed with high estrogenic activity both at the vendor's breeding facility and in our animal care quarters. This would be consistent with the fact that our animal care quarters and the animal vendor's breeding facility both used rat feed from the same supplier, which as noted previously is a subsidary of the animal supply house.

Conclusions and Implications

Throughout this entire episode we have communicated our findings and concerns to the animal vendor and their subsidiary that produces the rat feed. While they have been cooperative and have willingly undertaken an independent analysis of the estrogen content of their own feed, they have not yet incorporated routine testing of all lots of their feed or raw materials used to produce it for the presence of estrogens, antiestrogens, or other endocrine disruptors, despite the fact that we first shared our findings with them approximately 2 years ago. The supplier recently informed us, however, that they are now seriously considering the development of diets without appreciable amounts of soy- or alfalfa-based products. We have not surveyed other major vendors to determine either the frequency or rigor of testing for estrogens in their feed production processes, so we do not know the nature or extent of these practices in the industry.

This episode clearly indicated to us that not all major animal suppliers or feed vendors routinely screen for the presence of estrogenic substances in animal diets, that different lots of feed from the same supplier can vary widely in estrogen content, and that some lots of feed may contain amounts of estrogens high enough to maximally stimulate certain end points of estrogen action. Given the importance of *in vivo* studies for basic research and the intense current interest in developing accurate and reproducible screening tests for environmental estrogens, we thought it appropriate to describe our experience.

While not the primary intent of this manuscript, our findings are also potentially relevant for another reason: they confirm the published results that exposure to environmental estrogens during development and early life can have marked effects on the reproductive system (*19,20*). While we did not monitor the histological effects we observed for times longer than 30 days, it seems likely, based on the work of others (*21–23*), that these effects might well persist for the lifetime of the animals, even if they were switched to an estrogen-free diet at 30 days of age. Thus, the results of research or screening studies using adult animals might be affected by *in utero* or neonatal exposures to dietary estrogens, even if their subsequent diets did not contain measurable levels of estrogenic substances.

In terms of possible human health implications, it is also noteworthy that we measured 21 mg genistein and 14 mg daidzen per 100 g of rat feed. This is in the same general range (20–100 mg genistein and 10–70 mg daidzen per 100 g) found in typical soy products such as tofu or soy flour (*15,24*). Clearly, species differences between humans and rodents in the uptake, distribution, and elimination of these compounds could affect their potency or efficacy in humans. Even with this caveat, these measured levels and our findings in laboratory animals are consistent with the suggestions of other investigators that phytoestrogens might be present in sufficient amounts in certain human diets to produce significant health effects. We stress, however, that this is a complex issue well beyond the scope of this paper, and we do not mean to imply on the basis of our observations that the net effects of phytoestrogens on humans are necessarily deleterious. Actually, numerous epidemiological studies suggest that the overall effect of dietary phytoestrogens on human health may be quite beneficial (*25*).

In summary, we had used a particular nonpurified diet that was satisfactory for our purposes for many years, and our husbandry practices were those recommended by the National Research Council (*26*). We then received a batch of the same closed formula or proprietary feed with a level of estrogenic activity so high that it precluded our ability to further stimulate certain reproductive tract end points by administration of exogenous estrogens. Diets containing estrogenic activity have previously been reported by others (*8,9,16–18*). Collectively these observations underscore the need to use diets prepared from controlled ingredients and formulations such as open formula diets and to further monitor them for contaminants to rigorously insure that the feed does not contain activities that affect biological end points.

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