In utero Exposure of Fisher 344 Rats to Low Doses of Zeranol via the Maternal Diet Produces Dose-Dependent Effects on Sexual Development and Reproduction

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Abstract. Zeranol is a semi-synthetic derivative of zearalenone, a mycotoxin produced by Fusarium fungi that contaminate grain. Zeranol is a potent mycoestrogen with activity comparable to that of diethylstilbestrol, a synthetic estrogen previously used to prevent miscarriages in at risk women. Diethylstilbestrol was also used in livestock for many years to enhance meat production. Zeranol was developed as a replacement for diethylstilbestrol after human studies demonstrated that children and grandchildren of pregnant mothers who took the drug had an increased risk of cancer. Although zeranol has been used for more than 40 years in the USA and many other countries to enhance meat production and quality, its use is banned in the European Union and many Asian countries. Most human exposures to zeranol occurs result from consumption of contaminated meat and grain. Previous studies showed that both zearalenone and zeranol are efficiently absorbed from the diet and are readily transported cross the placenta, resulting in significant exposure of the fetus. However, the effects of in utero exposures at low-doses have not been studied in offspring or in subsequent generations. The long-term goal of our research is to determine if in utero exposure to zeranol at doses that approximate the Allowable Daily Intake (ADI) of 1.25 g/kg/day set by the US Food and Drug Administration has adverse, transgenerational effects on reproduction, sexual development and risk of cancer. In the present study, we treated pregnant Fisher F-344 with doses of zeranol (0, 1.25, 3.75, 7.5, 15 or 25 µg/kg/day) that ranged from the approximated human ADI to doses exceeding the ADI by 20-fold. Dams were exposed daily starting on gestational day 7 through weaning. Dose-dependent effects of zeranol on dam body weight, litter sizes, gender ratio and progeny body weights were assessed in at least three independent litters. The results indicated that developmental exposure to low-levels of zeranol induced significant effects on reproduction and sexual development.

Keywords: developmental exposure, endocrine disruptors, mycoestrogens.

1. Introduction

Over the last few decades there has been increasing concern over the human health effects of environmental toxicants with endocrine activity. These compounds can interfere with or disrupt normal endocrine function, leading to adverse effects on sexual development, fertility, obesity and the risk of developing tumors in hormone-responsive tissues [1], [2]. The unfortunate link between the use of Diethylstilbestrol (DES) to prevent miscarriages and the increased risk of clear cell cancer of the vagina and breast cancer in the daughters of women who took the drug, provided the clear evidence for fetal origins of adult disease in humans [3]-[9]. Recognition that exposure to endocrine disruptors during critical developmental windows increases the risk of cancers and other diseases later in life (F1) and across...
subsequent generations (Fn) [10]-[12] led increased scrutiny and regulation of compounds with relatively weak estrogenic activities, including such as Bisphenol A (BPA), genistein, and phthalates. By contrast, two potent environmental estrogens, zeranol and zearalenone, with significant potential for human exposure, have escaped critical reevaluation.

Zearalenone is a potent mycoestrogen with estrogenic activity that is orders of magnitude higher than most other environmental xenoestrogens [13]. The growth promoting effects of zearalenone were first noted in cattle fed with grain contaminated by *Fusarium* [14], [15]. Zeranol, a semi-synthetic derivative of zearalenone, was subsequently developed for use in livestock to enhance meat production. Zeranol pellets are implanted subcutaneously in cattle to stimulate weight gain and increase meat quality [16]-[18]. Zeranol cannot be used in breeders or dairy cows due to the documented adverse effects on reproduction [19]-[21] and transmission into milk [22]. There is currently no requirement to remove implants prior to slaughter. Zeranol can also be detected in meat [17], [23], prompting the FDA to set allowable limits food limits of 2 μg/kg (2 ppb) in muscle and 10 μg/kg (10 ppb) in liver (FAO/WHO, 1995). Based on its potent estrogenic activity, zeranol was banned by the European Union and in much of Asia, although random spot checks of meat sold to consumers indicate continued illegal use in European countries [17], [24]-[26].

The known health effects of zearalenone and zeranol mycoestrogens in humans are largely the result of high-dose accidental or occupational exposures. Dietary exposure to high levels of zeranol was implicated in early thelarche, the triggering of central precocious puberty (CPP) and high growth rates in young girls in Puerto Rico [27], [28]. Occupational exposure of workers at a plant producing zeranol resulted in gynecomastia in both the workers and their sons under the age of five [29]. In an Italian study, increased serum levels of zearalenone and its metabolites were observed in girls exhibiting precocious puberty [30]. Although most human exposures are at lower levels, even low-level oral zeranol and zearalenone exposures can be significant as they result in higher estrogenic potency than other route of exposure. These mycoestrogens are extremely stable (surviving most cooking conditions) and are poorly metabolized in humans, with a half-life of 22 hours [13], [31], [32].

Zeranol binds to estrogen receptors with an affinity similar to that of estradiol [33]. Although most human exposures to zeranol are thought to be at levels that will not have significant deleterious effects in adults, the potential health effects of exposures occurring during developmental windows of susceptibility has not been investigated in a systematic way. Recently, our group reported that zeranol, zearalenone and their metabolites are detectable in the urine of prepubescent girls, and that the levels were correlated with the consumption of beef and popcorn [34]. The levels of zeranol and its metabolites were associated with changes in height, body mass index and Tanner stage, suggesting effects on growth and onset of puberty [34]. Despite the limitations of a cross-sectional study design, our study indicated that the human exposure to zeranol is occurring during vulnerable developmental stages and is having an effect on normal endocrine function and sexual development. We therefore initiated studies in rats to look at effects on development and understand the underlying mechanisms.

Previous studies in animals demonstrated that zearalenone, zeranol, and their metabolites can cross the placenta and are not bound by serum binding proteins [13], suggesting the possibility of significant fetal exposure, even at low-dose exposures. At high maternal doses, both mycoestrogens disrupt fetal development and induce spontaneous abortions in many mammalian species [13], [35], [36]. However, few studies have examined the effects of exposures at doses relevant to the human Acceptable Daily Intake (ADI) of 1.25 μg per kg body weight per day established by the USFDA. More importantly, no previous studies have examined the potential transgenerational effects of zeranol when exposure at these low doses occurs during critical developmental windows.

2. Materials and Methods

2.1. Test Chemicals

All test chemicals were purchased from Sigma Aldrich St. Louis, MO, USA. Zeranol (alpha-zearalanol, ~97% in powdered form) was protected from light and stored at -20°C. Safflower oil was protected from light and stored at room temperature.
2.2. Animals

All research protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee. Animals were housed in the AAALAC accredited Rutgers School of Public Health vivarium. Pregnant Fisher 344 rats were obtained from Charles River Laboratories International Inc. (Kingston, NY). Rats were fed ad libitum with AIN-76A non-endocrine disrupting diet (Research Diet New Brunswick, NJ). The animal facility was maintained at 22±3°C, with 55-65% humidity and a 12-h dark/light cycle. Body weights of control and exposed dams were determined every week. Litter size and mean weights of pups we determined as on postnatal day 6.

2.3. Zeranol Exposure

Zeranol was dissolved in acetone, evaporated and reconstituted in safflower oil and loaded on wafers at doses of 0.0, 1.25, 3.75, 15.0, and 25.0 µg/kg/day. Rats were fed either vehicle or zeranol treated wafers seven days a week, beginning at gestational day 7 (GD-7) until weaning [37]. Dams were observed to ensure consumption the entire wafer each day.

2.4. Necropsy/Histopathology

For necropsy, animals were weighed then euthanized by asphyxiation with CO₂. Liver, spleen, kidney, mammary glands, uterus, testes, and ovaries were collected for histopathology. Tissues were fixed in 10% neutral buffered formalin and processed for routine hematoxylin and eosin staining. Organs collected for RNA and DNA extraction were wrapped in aluminium foil, snap-frozen in liquid N₂ and stored at -80°C.

2.5. Statistical Analysis

All data were analyzed and graphs generated by GraphPad Prism version 5.01 for Windows, GraphPad software, San Diego, CA (www.graphpad.com). One-way ANOVA with Dunnett's posthoc test was performed for all dose response studies.

3. Results

3.1. Low Doses of Zeranol Cause Adverse Reproductive Effects

The overall goal of this study was to evaluate the effect of developmental exposure to dietary zeranol at doses that close to the human ADI exposure level established by the USFDA. Beginning on gestational day seven (GD7) pregnant and continuing until weaning, Fischer 344 dams were exposed to dietary zeranol at daily doses of (0, 1.25, 3.75, 7.5, 15 or 25 µg/kg/day). These doses ranged from the approximated human ADI (1.25 µg/kg/day) and up to 20-fold above the ADI. At least three F1 litters were examined for each of the phenotypes which included dam body weight, average number of pups per litter and mean body weight of F1 progeny. The results presented in Fig. 1 indicated that developmental exposure had significant effects on the F1 progeny, even at maternal doses that approximated the human ADI for dietary zeranol.

Exposure of pregnant dams did not have significant effects on the weight of pregnant dams, except at the highest dose (p<0.04) (Fig. 1B). The data further suggested a non-monotonic dose response, with strong effects on both litter size (p<0.05) (Fig. 1A) and mean weight (P<0.0001) (Fig. 1C) of exposed pups at twice the human ADI (1.25 µg/kg/day). Gender comparisons yielded no significant differences in male to female ratios in F1 litters of vehicle or zeranol-treated rats (data not shown).

3.2. Low Doses of Zeranol Cause Adverse Testicular Development

To determine if the dose zeranol used in these studies produced overt reproductive toxicity in the F1 progeny, we performed histopathological comparison of the testes of PND6 F1 male rats exposed to zeranol in utero to testes of vehicle controls. Testes of rats treated with 1.25 and 3.75 µg/kg/day demonstrated a number of effects including apoptotic germinal epithelium, hypercellularity of tubules, and hypercellularity of interstitium (Fig. 2). These findings were minimal at the lowest zeranol dose of 1.25 µg/kg/day, indicating a dose response.
A) Litter size was recorded at PND 1. Analysis 1 way ANOVA with Dunnet’s posthoc demonstrate a significance for 1.25 and 25.0 g/kg/day zeranol in comparison to the control group demonstrating that these 2 doses have a significant (p<0.05) effect on litter size. N=3.

B) Dam body weights. Dams were weighed weekly and average body weight throughout pregnancy was calculated with 1-way ANOVA. Dams that were treated with 25 g/kg/day of zeranol had increased body weight throughout pregnancy compared to control and rest of treatment group (p<0.04). N=3.

C) Mean Pup body weight. Pup weight was determined at PND 6. A one-way ANOVA analysis revealed there was significant decrease in pup body weights when dams were exposed to 1.25 and 25 g/kg/day of zeranol (p<0.001). N=3.

Figure 1. Effect of developmental zeranol exposure and reproduction.

4. Discussion

Zeranol is a potent mycoestrogen used to enhance meat production in livestock and was developed as a replacement for the DES. Zeranol is a mycoestrogenic derivative of zearalenone an endocrine disrupting chemical (EDC) that has been noted to have deleterious effects in livestock and wildlife. The long-term goal of our research is to evaluate the transgenerational effects of low dose zeranol resulting from dietary exposure of pregnant dams at levels currently permissible in humans. Although the USFDA established an ADI of 1.25 µg/kg/day for zeranol, there are no conclusive studies that detail the effects of in utero exposure in humans or rats. To date few studies have examined the effects of in utero exposure to low dose zeranol on sexual development, reproduction or obesity. Because these disease endpoints have been linked to other endocrine disruptors, such as DES, we decided to explore if similar effects would be observed in rats exposed to zeranol. Due to a lack of relevant data in the rat, the preliminary studies presented here focused on defining a dose that yields no adverse physiological or gross histopathological changes for use in future transgenerational studies. No maternal mortality was observed at any of doses used, but dams exposed to 25
μg/kg/day of zeranol had increased maternal body weight, even though they produced smaller litters. Pre-implantation loss could be determined from the current study design, as dams were not sacrificed during pregnancy to assess this parameter. We selected litter size as one of our phenotypic markers of toxicity, because a change in litter size is often indicative of reproductive toxicity. A significant decrease in litter size was observed in the 1.25 and 25.0 μg/kg/day dosing groups, suggestive of toxicity as previously demonstrated by Perez-Martinez et al 1995 [19]. Pups that were exposed in utero to concentrations of 1.25 and 25 μg/kg/day zeranol had a significantly lower body weight than the other treatment groups. These findings were similar to effects seen in NMRI mice dosed subcutaneously with 150 mg/kg/day of zeranol on gestational day 9 and 10 [19]. The pups exposed to 25 μg/kg/day of zeranol in utero had black intestines, suggesting a lack of milk in their stomachs. In later studies, Perez-Martinez et al. dosed pregnant NMRI mice subcutaneously with 150 mg/kg/day of zeranol. Mice were euthanized between gestational day 12 and 18, male fetuses were collected to microscopically examine the gonads in one study [35]. In another study, the mice were euthanized postnatal days 45, 90, 180 and 365 and results demonstrated zeranol induced more severe and earlier testicular abnormalities at PND45. The results suggested that testicular differentiation onset was earlier in estrogen-treated fetuses than observed in controls [35], [38]. We observed that in utero exposure 1.25 and 3.75 μg/kg/day zeranol caused alteration in the testes of F344 male progeny. These morphological findings suggest that prenatal exposure to zeranol induces abnormal testicular differentiation in rats at the doses used in this and previous studies. Based on these findings we selected a dose that was below the human ADI. Preliminary results of a three-generation study indicate that developmental exposure of pregnant dams at a dose of 0.625 μg/kg/day induced effects on reproduction, sexual development, onset of puberty and susceptibility to mammary carcinogenesis in exposed F1 progeny. More importantly, many of these effects are also in the F2 and F3 progeny, suggesting transgenerational effects.

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6. References


