Reproductive Toxicology 47 (2014) 81–88

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Multigenerational exposure to dietary zearalenone (ZEA), an estrogenic mycotoxin, affects puberty and reproduction in female mice

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ARTICLE INFO

Article history:
Received 16 February 2014
Received in revised form 13 June 2014
Accepted 17 June 2014
Available online 24 June 2014

Keywords:
Zearalenone
Multipregnancy
Multigeneration
Vaginal opening
Embryo implantation
Litter size
Female reproduction

ABSTRACT

This study investigated potential cumulative effects of multiple pregnancy and multigenerational exposure to dietary ZEA (0, 0.8, 4, or 20 ppm) on female puberty and reproduction in C57BL/6J mice. Multiple pregnancies did not significantly affect litter size or offspring puberty. Significant effects were observed in 20 ppm ZEA-treated females: advanced puberty onset in F0, F1, and F2 generations; decreased implantation rate, pregnancy rate, and litter size, and increased pregnancy gap and gestation period in F1 and F2 generations; and reduced fertility index in F2 generation. F3 females from 0 and 20 ppm groups were split into 0 or 20 ppm ZEA diets at weaning, with advanced puberty onset seen in 0-20 and 20-20 groups and decreased implantation rate observed in 20-20 group. In summary, 20 ppm dietary ZEA advanced puberty onset without obvious cumulative effect and impaired fertility with multigenerational cumulative effect, which could be partially alleviated upon exposure cessation.

1. Introduction

Mycotoxin zearalenone (ZEA) is commonly found in livestock feed and in human food. Its contamination levels are in the range of ppb to low ppm with the highest reported at 600 ppm [1,2]. Plant foods (e.g., corn and wheat) can contain ZEA through fungal contamination. Animal foods (e.g., meat and dairy products) can be contaminated with ZEA via intake of fungus contaminated feedstuff by livestock or can contain zeranol (α-zearalanol), a derivative of ZEA used as a growth promoter in livestock [3,4]. Contaminated food is the main source of human exposure to ZEA [1–3]. The median and 95th percentile daily dietary ZEA exposure in European population were estimated to be <0.1 μg/kg body weight and <0.3 μg/kg body weight, respectively [2]. The tolerable daily intake (TDI) for ZEA established by the Panel on Contaminants in the Food Chain in Europe is 0.25 μg/kg body weight [2].

A study on 76 girls/2 boys with idiopathic precocious puberty (IPP) and 99 girls/1 boy in the control indicated positive correlation between ZEA and IPP, with an odds ratio of 8.833 (95% confidence interval: 2.281–34.208) [5]. Epidemiological studies support ZEA and its derivative mycotoxins as a triggering factor for precocious pubertal development in prepubertal exposed girls (reviewed in [4]). Although human data on definitive causative effects of ZEA and its metabolites on puberty and female reproductive system are unavailable, the estrogenicity of ZEA and its metabolites renders them the potential to influence puberty and functions of the female reproductive system [1].

Female puberty and reproduction are regulated by estrogen [6–8], therefore, these processes could be affected by ZEA. Our previous study showed that postweaning exposure to 10 ppm or 40 ppm dietary ZEA promoted premature onset of puberty and 40 ppm dietary ZEA also disrupted early pregnancy events in female mice [9]. ZEA is quickly absorbed and the unconjugated ZEA has an elimination half-life of 16.8 h after oral administration in male rats [2]. Placental transfer of ZEA and its metabolites in rats and pigs, as

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http://dx.doi.org/10.1016/j.reprotox.2014.06.005
0890-6238/

well as lactational transfer of ZEA and its metabolites in the bovine milk, have been demonstrated (reviewed in [2]). These observations suggest that placental and lactational transfer of ZEA and its metabolites may also occur in other species, such as human and mouse. Given that ZEA is a common contaminant in the human diet [2], which is consumed daily across generations, it is natural to ask if there are any cumulative effects of ZEA on female puberty and reproduction during multigenerational exposure.

Multigenerational studies on reproduction usually cover the F0 (parental), F1 and F2 generations, with exposure commencing from the F0 generation prior to mating and continuing through the F2 generation until the F2 offspring are weaned [10], allowing the investigation of reproduction upon exposure for one or two generations. A survey of multigenerational studies of 316 chemicals in rats indicates that more chemicals have reproductive effects on the F1 generation than on the F0 generation and more adult reproductive effects are seen in the F1 generation than in the F0 generation [11]. Therefore, it is necessary to assess the effects of ZEA on female puberty and reproduction in a multigenerational setting. It was hypothesized that ZEA in the diet could have cumulative adverse effects on female puberty and reproduction. This hypothesis was tested in C57BL/6J mice. Vaginal opening, pregnancy rate, and litter size were among the parameters used to determine effects of ZEA treatments on female puberty and reproduction.

2. Materials and methods

2.1. Animals

The C57BL/6J mice were initially derived from animals at Jackson Laboratories (Bar Harbor, ME) [9]. All mice were housed in polypropylene cages with free access to a casein-based phytoestrogen-free AIN-93G diet (Bio-Serv, Frenchtown, NJ) or a ZEA in AIN-93G diet and to water in polypropylene water bottles. Polypropylene containers were not significant sources for endocrine disruption [12]. Generally, ZEA levels in rodent diets were undetectable or very low (<30 ppb) [13,14]. The animal facility was maintained on a 12-h light/dark cycle (0600–1800 h) at $23 \pm 1\,^{\circ}C$ with 30–50% relative humidity. All methods used were approved by the Animal Subjects Programs of the University of Georgia and conform to National Institutes of Health guidelines and public law.

2.2. ZEA treatment, mating, and data collection

Homemade diets containing 0, 0.8, 4, and 20 ppm ZEA (Fermentek, Israel) were prepared as previously described [9]. These ZEA levels were within the range of levels in the highly contaminated food [1,2]. The treatment regimen is outlined in Fig. 1. F0 females were treated from weaning (3 weeks old) to dissection; F1 and F2 females were exposed to ZEA diets during their entire lives, from gestation to dissection (Fig. 1A).

Newly weaned F0 generation females were randomly assigned into 0, 0.8, 4 and 20 ppm ZEA-treated groups with littermates assigned into different groups. At 8 weeks old, the F0 females in each group were mated with fresh stud males, which were exposed to the same ZEA diets as the females only during mating, to produce three consecutive litters, F1a, F1b and F1c (Fig. 1B). Another set of F0 females were mated and dissected on gestation day 4.5 (D4.5) to determine embryo implantation in the F0 generation. At 8 weeks old, F1a and F1b females were mated with fresh stud males. The F1a females would eventually produce three consecutive F2 litters, F2a, F2b, and F2c. The F1b females were dissected on D4.5 to determine embryo implantation in the F1 generation (Fig. 1B).

At 8 weeks old, F2a and F2b females were mated with fresh stud males. Since no obvious adverse effects on puberty and fertility were observed in the 0.8 and 4 ppm ZEA-treated F0 and F1 females, F2a and F2b females in the 0.8 and 4 ppm ZEA-treated groups were dissected on D4.5 to determine implantation without producing an F3 generation for these two dose groups. F1c and F2c pups were sacrificed before or at weaning. In the 0 ppm ZEA group, 11 F2a and 2 F2b females were mated to produce the F3a and F3b litters; in the 20 ppm ZEA groups, 3 F2a and 8 F2b females were mated to produce the F3a and F3b litters (Fig. 1B). The rest of the F2b females in the 0 and 20 ppm ZEA were dissected on D4.5 to determine embryo implantation in the F2 generation.

At weaning, F3 female littermates from each dam in the 0 and 20 ppm ZEA groups were randomly split into 0 or 20 ppm ZEA-treated groups, resulting in a total of four groups: 0-0, 0-20, 20-0, and 20-20 (Fig. 1A). At 8 weeks old, all F3 females were mated with fresh stud males and dissected on D4.5 to determine embryo implantation in the F3 generation.

During mating in each generation, each female was checked every morning for the presence of a vaginal plug to determine mating in the previous night. They were separated from the mating males ~D15.5 and housed individually when an enlarging belly (to indicate pregnancy) was clearly observed. Some females had demonstrated more than one mating plug before a pregnancy was evident. Dams that lost all pups before weaning or had pups weaned at three weeks old were back to mating after one week of rest if they were scheduled to produce more litters.

Water consumption and food consumption were recorded weekly during postweaning (F0 and F1) and lactation (F0, F1, and F2). Postnatal body weights were monitored at 7, 14, 21, 22, 29, 36, 43, 50, and 57 days of age. Body weights during pregnancy were recorded every 5 days from D2.5 to D17.5 for F0, F1, and F2 dams and their body weights during lactation were measured weekly. Litter size was recorded at birth. Gender ratio of the pups was determined at weaning (3 weeks old).

Female pups were monitored daily after weaning for signs of vaginal opening; the age at vaginal opening was recorded as an indication of puberty onset [9,15,16]. As previously described [9,17], embryo implantation and pregnancy status on D4.5 were determined by using Evans blue dye reaction, or for those without implantation sites, by uterine flushing to detect the presence of healthy-looking embryos. All male pups were sacrificed at weaning without further study.

The number of animals in each group was indicated in the figure legends. All groups began with at least 6 mice. However, since not all mated mice were pregnant, not all F0 and F1 pregnant mice produced three litters as planned, and there was further impaired fertility in the F1, F2, and F3 20 ppm ZEA-treated groups, the number of pregnant females per group analyzed for food and water consumption during lactation, body weight during gestation and lactation, length of the gap between mating and delivery (pregnancy gap), age of dams at delivery, and number of implantation sites could be less than 6.

2.3. Statistical analysis

ANOVA analyses were done using SigmaPlot 12.0. ANOVA on ranks followed by Dunnett's test was used for analyzing the age at vaginal opening and the number of implantation sites. One way ANOVA followed by Dunnett's test was used for analyzing food and water consumption, body weight during gestation and lactation, mating duration, and pregnancy gap. Two-way repeated measures ANOVA followed by Dunnett's method was used for analyzing gestational period and litter size from the females that gave birth two (F2) or three (F0 & F1) times and postweaning body weight for each mouse that was repeatedly measured weekly. Two-way