Effects of Neonatal Administration of Zearalenone on the Reproductive Physiology of Female Mice

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(Received 14 March 1994/Accepted 18 September 1994)

ABSTRACT. The effects of neonatal (1–10 days) administration of various doses (5–30 μg/animal) of zearalenone, a mycotoxin, on the sexual maturation and reproductive physiology of female ICR mice were investigated. The drug was found to have mimic estrogen actions, causing delayed vaginal opening, persistent estrus (60–80% incidence) and sterility. Daily doses of zearalenone (10–30 μg/animal) for 3–5 neonatal days resulted in infertility accompanied by thickening of the vaginal epithelium, even after ovariectomy and adrenalectomy, indicating mucosal independence of endogenous estrogen. Thus, it may be possible that zearalenone acts by binding irreversibly to estrogen receptors in the target tissues.

KEY WORDS: mouse, persistent estrus, sterility, zearalenone.


Zearalenone, a mycotoxin produced by Fusarium spp., is important in the field of food and feed hygiene as it occurs ubiquitously. Its natural occurrence has been reported in many countries [3, 4], including Japan [10], China [27] and Korea [16]. The estrogenic activity of zearalenone has been well known [18], especially in swine, and the toxin has caused vulvovaginitis [24], abortion [11] and infertility [19]. Transmission of zearalenone into the milk of the mother has been proven in the cow and ewe [8, 20]. Since only a few experimental studies have been conducted on the estrogenic actions of zearalenone on the reproductive organ system of rodents [15, 23], we examined the effect of the neonatal administration of this toxin on the reproductive physiology of mice.

MATERIALS AND METHODS

Zearalenone was kindly provided by Dr. Bachman, IMC Chemical Group Inc. (Indiana, U.S.A.). Purity of the sample was 99.8%. Zearalenone was suspended in olive oil and injected 10 μl/animal intraperitoneally with a microsyringe into female newborn mice. During the first five days after birth, each mouse was given a single or multiple dosage of 0, 5, 10, 20 and 30 μg/animal.

ICR mice of both sexes were purchased from Japan SLC, Inc., Hamamatsu, Japan. The mice were housed in plastic cages (5 per cage) with sawdust beddings with the room temperature of 23±1°C, humidity 55±5% and 12/12 hr lighting cycle. They were given pellet diet MF (Oriental Yeast, Co., Tokyo, Japan) and water ad libitum. Mice were mated at 10 weeks of age. Two hundred and six female newborns were used for this study. They were suckled 8 per litter, weaned at 3 weeks, and then fed as their mothers. The weaned mice were checked for vaginal opening, and the vaginal smear was examined daily until 8 weeks of age. Vaginal smears were stained with Giemsa and evaluated for 4 stages (proestrus, estrus, metestrus and diestrus). The mice were considered to have developed persistent estrus when the definite estrus phase was found in more than 50% of the experimental period. All mice were weighed weekly after weaning.

Ovariectomy and/or adrenalectomy was performed 5 weeks of age. The mice were anesthetized by intraperitoneal injection of 0.1 ml/10 g b.w. of diluted pentobarbital sodium (5 mg/ml) supplemented by ether inhalation during the operation. Through a dorsal medial incision, bilateral ovaries were extirpated after suturing the paraovarian tissue. The adrenals were extirpated with a ring forceps without suture. After adrenalectomy the mice were given physiologic saline instead of water.

For determination of reproductive function, female mice received the toxin in neonatal period and were mated at 8 weeks of age with non-treated males of the same age. Mating rate, fertilization rate and sexual behavior were recorded. The pregnant mice were killed on day 18 of pregnancy by cervical dislocation. The number of implantations and status of the fetuses (live, dead, macerated and absorbed) were examined from the removed uterus. The live fetuses were weighed. The ovaries, uterus and pituitary of dams were weighed and fixed in 10% neutral buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. The mice surviving at 11 weeks of age were killed and examined similarly.

For determination of the uterotropic action of zearalenone, female mice treated with 30 μg/animal of the toxin during the first five days after birth were orally given 10 mg/kg of the toxin at 3 weeks old. They were killed after 24 hrs and the uteri were weighed.

From each mouse receiving 30 μg of zearalenone during the first five days after birth and those of the corresponding controls in the same stage of estrus cycle, the blood was taken from the abdominal aorta at 3 and 4 weeks of age. The serum was separated and stored at −60°C until analyzed. Serum estrogen levels were measured in duplicate using radioimmunoassay method (lower detectable
concentration: 1 µg/ml of hormone). Estrone (E1) levels were kindly measured by Otsuka Assay Laboratory (Tokushima, Japan). Estradiol (E2) levels were measured by RIA using the E2 Kit Daichi II (Daichi Radioisotope Laboratory, Tokyo, Japan). The pituitaries of these mice were fixed in formalin, embedded in paraffin. Paraffin sections were then stained with Azan and the numbers of acidophilic, basophilic, and chromophobic cells were counted on about 1,000 cells per animal.

Statistical evaluations were performed using Student’s t-test following F-test for equality of variance or chi square test.

RESULTS

Sexual Maturity: Repeated administration of zearalenone in the neonatal period (day 1 to 5) showed a tendency to delay the vaginal opening of mice, while single administration (30 µg/animal) on day 10 after birth accelerated it (Table 1).

Vaginal Estrus Cycle: Zearalenone, given on day 1 to 5, caused disturbance of estrus cycle following vaginal opening. Persistent estrus developed in a considerable number of animals and a single or multiple dosage of 10, 20 or 30 µg/animal was more effective (Table 1). In the animals without persistent estrus, the interestrus period lasted up to 11 days. The control mice and each animal administered 30 µg of the toxin 8 or 10 days postnatally showed a regular 3- to 4-day cycle.

Ability of Mating, Pregnancy and Maturation: Reduced mating rate and infertility were noted in the mice after 3 or 5 injections during the first five days after birth. Infertility was also induced by single injection of the highest dose on days 1, 3 and 5 after birth (Table 1). Most of the 16 mice given the toxin continuously were unsuccessful in mating for 3 weeks of coupling, showing unusual sexual behavior. They showed no lordosis when mounted by proven male mice, but acted like a male attacking a male or trying to mount another female.

In fertilized mice, the pregnant course was uneventful without abortion. At autopsy on day 18 of pregnancy, the proportion of live fetuses and mean body weight of fetuses were not significantly different in the zearalenone-treated mice with the exception of the group injected with 10 µg/animal in the neonatal period. In the zearalenone-treated group, a significantly high mortality (25.3% vs. 8.2% in control, p<0.01) and increased fetal weight (1.57±0.13 g vs. 1.38±0.21 g in control, p<0.01) were recorded. The litter size varied but tended to decrease in the treated groups. No anomalies were observed in the fetuses of any group.

Effect of Ovariectomy and/or Adrenalectomy on Sexual Maturation of Female Mice Neonatally Treated with Zearalenone: The mice were each given 5 neonatal injections of 30 µg of zearalenone and underwent ovariectomy, with or without adrenalectomy, at 5 weeks of age. Bilateral ovariectomy immediately interrupted persistent estrus, and the vaginal pattern showed metestrus (Fig. 1). After 3 to 14 days the vaginal cycle reappeared in 6 of 7 mice but was irregular. In the bilaterally ovariectomized controls, the vaginal cycle entered diestrus and remained to the end of the experiment.

In 4 of 5 animals receiving bilateral adrenalectomy, the estrus phase reappeared after 2 to 11 days of the interphase period, but with an irregular cycle. In the adrenalectomized controls, the vaginal cycle changed a little with slight elongation of the interestrus period. By combined bilateral ovariectomy and adrenalectomy, the results were similar to that of the animals which had undergone bilateral ovariectomy.

Modification of Uterotrophic Action of Zearalenone by Neonatal Treatment with Toxin: Oral administration of 10 mg/kg zearalenone to 3-week-old female mice induced uterine enlargement in 24 hrs (51.8±9.3 mg vs. 31.2±6.0 mg in control, p<0.001). In each mouse neonatally treated with 30 µg of zearalenone for 5 days, the uterus

<table>
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<th>Dose (µg)</th>
<th>No. of times injected</th>
<th>Postnatal day of injection</th>
<th>No. of mice</th>
<th>Day of vaginal opening Mean±SD</th>
<th>No. of mice showing Persistent Estrus/ Treated</th>
<th>No. of mice Fertilized/Mated/Used</th>
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* * *: Significantly different (*P<0.05, **P<0.01, ***P<0.001) from control. a) All control data were combined as there was no difference between groups given the vehicle alone on day 1, 1-3 and 1-5 days after birth, respectively. Only the statistically significant results are tabulated.
was also enlarged significantly by oral administration (39.0±7.4 mg vs. 29.3±7.4 mg in the neonatally treated, p<0.01), but compared with the effect on the neonatally non-treated animals, the degree of enlargement was less marked (51.8±9.3 mg vs. 39.0±7.4 mg, p<0.001).

Serum E1 and E2 Levels in Mice Treated with Toxin during the Neonatal Period: Serum E1 levels of the toxin-treated animals were not significantly different from those of the control animals at 3 weeks of age (254.3±50.0 pg/ml vs. 299.3±12.6 pg/ml, respectively) and at 4 weeks of age (298.7±104.7 pg/ml vs. 269.7±4.0 pg/ml, respectively). E2 levels tended to be higher in the treated than in the control animals but with no significant differences (52.2±43.5 pg/ml vs. 10.4±10.6 pg/ml, respectively, at 3 weeks of age, and 122.5±99.3 pg/ml vs. 46.5±19.8 pg/ml, respectively, at 4 weeks of age).

Quantitative study of the pituitary gland revealed an increase in the number of acidophilic cells from 88.9 to 95.9% (p<0.01) and a decrease in the basophilic cells from 4.3 to 1.1% (p<0.01) in the mice neonatally treated with the toxin, compared with the control.

Effects on Organ Weights: No changes in body weight were observed in any experimental groups compared with the corresponding control groups. The weight of ovaries (9.2±0.6 mg, p<0.01) was reduced, while those of the pituitary gland (3.3±0.5 mg, p<0.01), and uterus (212±69 mg, p<0.01) were increased in the treated mice compared to the non-treated controls (14.2±4.1, 2.5±0.4, 124±50 mg, respectively). Similar changes in organ weights were observed in the ovarietomized or adrenerectomized mice. Combined ovariectomy and adrenalectomy caused a remarkable decrease in pituitary

Fig. 1. Effects of bilateral ovariectomy on persistent estrus induced by 5 injections of 30 μg/animal of zearalenone on the first 5 days after birth. Open circles show the day of vaginal opening; periods shown by the upper thick lines stand for the estrus phase, those shown by the lower thin lines for the proestrus, metestrus and diestrus phase, and the arrow above indicates the day of ovariectomy.

Fig. 2. Ovary of a control mouse sacrificed at 8 weeks old. Many mature follicles and corpora lutea are present (Hematoxylin and Eosin, bar = 100 μm).

Fig. 3. Ovary of an 8-week-old mouse treated with 30 μg/animal of zearalenone on days 1-5 after birth. The ovarian cortex contains many immature follicles but no corpora lutea. The medulla was occupied by hypertrophied interstitial tissues (Hematoxylin and Eosin, bar = 100 μm).
weight (1.6±0.5 mg), which was partly inhibited by neonatal treatment with zearalenone (2.3±0.2 mg, p<0.05).

**Histopathological Changes:** The histological changes observed in the mice neonatally treated with zearalenone were basically identical, unrelated to the doses, injection period, number of injections, the time of sacrifice and the extirpation of ovaries and adrenal glands. The ovaries were atrophic with immature vesicular follicles and proliferation of the interstitium. No mature corpora lutea were found in the infertile mice (Figs. 2 and 3). The endometrium was unremarkable. The vaginal mucosa was thickened with hyperkeratosis, occasional parakeratosis and basal cell hyperplasia. The adrenal glands showed remarkable lipid deposition in the reticular zone.

**DISCUSSION**

Neonatal treatment with zearalenone showed profound effects on the sexual maturity and reproductive functions of the female mouse, apparently similar to those of estrogen: 1) delayed vaginal opening; 2) persistent estrus; 3) sterility and 4) decreased serum level of estrogens and decreased number of basophilic cells of the pituitary gland.

Vaginal opening of the female newborn was delayed when zearalenone was administered 3 or 5 times within the first 5 postnatal days and seemed to be accelerated when given after the 5th day (Table 1). According to Forsberg [5], mitosis of the uterine and vaginal epithelial cells is inhibited by estrogen administered 1 to 4 times within 4 days after birth, possibly causing delayed vaginal maturation. Meanwhile, administration of estrogen after 5 postnatal days resulted in early appearance of vaginal opening for more than 1 week [22]. These observations indicate that differentiating vaginal epithelium has a critical period, 5 days after birth, for the response to estrogenic components. Thus, zearalenone seems to affect this critical stage of development as estrogen does.

Persistent estrus was induced by administration of estrogen within 5 postnatal days [13, 25]. Administration of zearalenone within 5 days after birth caused persistent estrus following vaginal opening (Table 1). The rate of induction of persistent estrus was dose-dependent when injected on day 1. This persistent estrus was not influenced by ovariectomy and/or adrenalectomy, indicating that the vaginal mucosa became independent of endogenous estrogen.

Sterility may result from both neuroendocrine and local disturbance caused by zearalenone. Infertility was induced after 3 or 5 zearalenone administrations of 10, 20 and 30 µg/animal. The ovaries of these mice showed no corpora lutea indicating no ovulation (Fig. 3). Similar results have been observed by neonatal injection of estrogen in rats with anovulation. Hypertrophic reaction of the uterus by zearalenone treatment was partially suppressed by a large oral dose of zearalenone in the neonatal period, just as with estrogen following neonatal estrogen injection [26].

Zearalenone binds with estrogen receptors of the rat [14], possibly binding in a competitive manner [2, 7]. It is conjectured that saturation of uterine estrogen receptors with zearalenone occurs by neonatal administration. The demonstrated quantitative decrease in basophilic cells and the higher level of circulating estrogen in the zearalenone-treated mice with persistent estrus in the present study runs parallel to the decrease in pituitary basophiles [1, 12] and higher serum estrogen than controls at diestrus [21] observed in rats neonatally given estrogen as reported elsewhere. It seems possible that higher serum estrogen levels may lead to low levels of FSH and LH released from the pituitary gland. In the rat treated neonatally with estrogen a decrease in estrogen receptors in the hypothalamus and pituitary has been shown [17]. Thus, the mechanisms of action of these two drugs may well be the same. Destruction of the mediobasal part of the preoptic area of the neonatal rats induced persistent estrus after vaginal opening [9].

In summary, injection of zearalenone in the neonatal period induced persistent anovulatory estrus of female mice, independent of estrogen from the ovary and adrenal gland. Judging from the lesions and functional disturbances of the varied organs, such as uterus, vagina, adrenals, pituitary and ovary, zearalenone seems to affect not only the sexual organs but also the hypothalamic-pituitary system.

**REFERENCES**


