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Effects of Prepubertal Zeranol Exposure on Estrogen Target Organs and *N*-Methyl-*N*-nitrosourea-induced Mammary Tumorigenesis in Female Sprague-Dawley Rats

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Abstract. *Background: There are no previous reports of the effects of prepubertal exposure to zeranol, an estrogenic substance, on estrogen-responsive reproductive organs and mammary glands in rats, or its effects on N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Materials and Methods: Prepubertal female Sprague-Dawley rats were treated daily with either 0, 0.1 or 10 mg/kg body weight of zeranol between 15 and 19 days of age. They were given 50 mg/kg body weight MNU at 28 days of age, and were monitored for occurrence of mammary tumors ≥1 cm in diameter. Body weight gain, structures and functions of estrogen target tissues, and mammary carcinogenesis were compared between dosage groups. Results: Zeranol did not affect body weight gain. At 28 days of age, zeranol-treated and -untreated rats showed similar development of reproductive organs and mammary glands. However, both low- and high-dose zeranol treatment caused significantly earlier vaginal opening, irregularity of estrous cycle (high frequency of prolonged estrous or prolonged diestrous) at 8 to 11 weeks of age, and anovulatory ovary (ovaries without newly formed corpora lutea). At 37 weeks of age, the high-dose zeranol-treated group exhibited increased relative uterine-ovarian weight, but mammary gland development was comparable to that of untreated rats. Mammary carcinogenesis was not affected by low- or high-dose zeranol treatment. Conclusion: Short-duration zeranol treatment in the prepubertal period severely damaged ovarian functions and structure, but mammary carcinogenesis was not affected. The present results suggest that ingestion of foods containing zeranol in the infantile period can cause dramatic endocrine disruption in later life.*

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The β-resorcylic lactones zeranol (α-zearalanol) [6-6,10-dihydroxyundecyl-β-resorcylic acid lactone] and zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid-lactone] are naturally occurring substances that have estrogenic activity (1,2). Zearalenone is a mycotoxin synthesized by *Fusarium* molds, and is present as a natural contaminant in food as a result of infection of grain (3). Zeranol is a natural metabolic product of zearalenone (4). Zearalenone and zeranol are classified as mycoestrogens (5), and have been widely used to promote growth of livestock in the United States due to their potent anabolic effects (3). Zeranol has greater estrogenic potency than zearalenone (6), and has been commercially produced (7). Zeranol can enter the human food chain directly *via* ingestion of contaminated grain (8), or indirectly *via* consumption of meat products from animals fed a mold-infected grain or animals injected with zeranol for growth stimulation. Thus, it is difficult to avoid exposure to zeranol *via* consumption of human food products. Although no marked toxic, mutagenic or carcinogenic effects of zeranol have been observed (8), studies indicate that the timing and dosage of exposure to estrogenic chemicals can greatly influence their effects.

There has been increasing concern about the impact of exposure to dietary compounds with hormone-like action during critical periods on human development and reproductive health. Estrogens play important roles in the maintenance and function of the reproductive tract and mammary glands. Zearalenone disrupts reproductive function when given to prenatal mice (9) or prepubertal rats (10). Risk assessment of endocrine disruption by xenoestrogens and their metabolites requires accurate evaluations of their estrogenic potency. However, the effects of zeranol exposure during development on estrogen target tissues have not previously been precisely analyzed.

In addition, estrogens have long been recognized as important mitogens in the breast, and are associated with increased risk of breast cancer. Estrogens and estrogenic compounds can have considerable effects on breast carcinogenesis in humans and animals during critical

periods of development. Epidemiological evidence suggests that high intake of soy (rich in phytoestrogens) during adolescence affects the risk of breast cancer later in life (11), and experimental results indicate that prepubertal exposure to estrogenic chemicals such as genistein (12), resveratrol (13) and zearalenone (10) modifies mammary cancer risk in female rats. There have been no studies of the effects of consumption of zeranol in the early period of development on mammary cancer risk. Thus, the object of the present study was to examine the effects of prepubertal administration of zeranol on estrogen target tissues and the occurrence of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumors in female Sprague-Dawley rats.

Materials and Methods

Animals. Ninety 14-day-old female Sprague-Dawley rats (10 pups per 1 nursing mother) were obtained from Charles River Japan (Hino). To avoid exposure to endocrine-disrupting chemicals, rats were housed in standard rat polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River Japan, Yokohama) as bedding. To avoid phytoestrogens in the diet, rats were fed the low-phytoestrogen diet NIH-07 PLD (Oriental Yeast, Chiba, Japan), which effectively reduces adverse endocrine-disrupting activity (14), and water was supplied in polycarbonate bottles with rubber stoppers, throughout the experiment. Thus, known endocrine-disrupting agents were eliminated from the environment of the rats. The animal facility was maintained at 22±2˚C with 60±10% humidity and a 12-h dark/light cycle.

Chemicals. Zeranol was purchased from Wako Pure Chemical Industries (Osaka, Japan). The purity was 99%, as analyzed by high-performance liquid chromatography. It arrived in powder form, and was kept at 0˚C in the dark. Immediately before use, zeranol was dissolved in 100% dimethylsulfoxide (DMSO) (purity ≥ 99%, Nacalai Tesque, Kyoto, Japan), and stored at -80˚C.

MNU was obtained from Nacalai Tesque. Upon arrival, it was kept at -20˚C in the dark. MNU was dissolved in physiological saline containing 0.05% acetic acid immediately before use.

Experimental procedures. Animals were randomly divided into 3 groups of 30 animals each. From 15 to 19 days of age, 2 groups received a daily subcutaneous injection of zeranol at 0.1 (low-dose group) or 10 (high-dose group) mg/kg body weight, and the third group (untreated controls) received an equal volume of the vehicle (100% DMSO). There have been no reports of measurements of serum concentrations of zeranol in humans, but human exposure to zearalenone in the United State is 1 to 5 mg/day (0.02-0.1 mg/kg body weight per day) (15). Thus, zeranol doses of 0.1 and 10 mg/kg per day were selected for the present study. Rats were weaned at 21 days of age, and animals were then observed daily for vaginal opening (puberty onset). At 28 days of age, 6 randomly selected rats per group were autopsied to assess the effects of zeranol. Rats were killed by an overdose of ether followed by cervical dislocation. On the same day, the remaining rats (24 rats per group) received an intraperitoneal injection of freshly prepared MNU at 50 mg/kg body weight. They were then housed in groups of 3 to 4 animals and palpated weekly for mammary tumors. Rats were autopsied when their largest tumor was ≥ 1 cm in diameter. At 33 weeks after MNU administration, all remaining animals were killed and the experiment was terminated. Body weight was recorded weekly from 2 weeks of age to the end of the experiment (37 weeks of age), and growth rate was compared among groups. The Animal Experimentation Committee at Kansai Medical University, Japan approved all procedures involving animals.

Evaluation of estrogen target organs. At 28 days of age (the time of MNU exposure; 6 animals in each group) and at 37 weeks of age (all remaining rats in each group), animals were weighed and killed and the acute and chronic effects of prepubertal zeranol exposure, respectively, were assessed. Uterus and ovaries were excised and weighed, and formalin-fixed samples of uterus, vagina, ovaries and mammary glands were cut into sections (thickness, 4 μm), which were stained with hematoxylin and eosin (HE). Mammary gland growth and differentiation were also evaluated on whole-mount sections. Vaginal opening was checked, and estrous cyclicity was monitored by examination of vaginal smears (16), performed at the same time daily from 8 weeks of age for 4 consecutive weeks. Estrous cycles were classified as follows: i) normal length, 4 or 5 days; ii) prolonged estrous, irregular cycle in which estrous phase constitutes more than 50% of the observation period; iii) prolonged diestrous, irregular cycle in which the diestrous period constitutes more than 30% of the observation period. When rats were sacrificed during the experimental period, reproductive organs were routinely fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with HE.

Evaluation of mammary tumorigenesis. To determine the effect of prepubertal zeranol exposure on mammary tumorigenesis, all visible mammary tumors were dissected, fixed in 10% neutral buffered formalin, and cut into sections (thickness, 4 μm) that were stained with HE. In addition, normal-appearing mammary glands were dissected and processed to produce routine histological preparations, to detect microscopic tumors. Histopathology of mammary tumors of all sizes was evaluated from HE-stained sections. The histological criteria for identification of mammary tumors were based on those of Russo *et al.* (17). Data analysis included the number of animals with gross mammary carcinomas (≥1 cm) and latency (from MNU administration until largest mammary tumor reached ≥1 cm in diameter). Also, numbers of histologically detected mammary carcinomas (total number of carcinomas of all sizes) and the number of carcinomas per animal (multiplicity) were analyzed.

Statistical analysis. All data were expressed as mean±SE. Vaginal opening and cumulative incidence of gross mammary tumors (≥1 cm) was analyzed by Mantel-Cox Log-rank test. For all other data, after assurance of homogeneity of variance, analysis was performed using non-repeated measure ANOVA parametric test or Kruskal-Wallis non-parametric test. If the p value of these pre-tests was < 0.05, *post-hoc* analysis was performed using Fisher's protected least significant difference test. Differences between groups were considered significant if the p value was < 0.05.

Results

Body weight gain and reproductive organ function and structure. Prepubertal exposure to zeranol did not affect

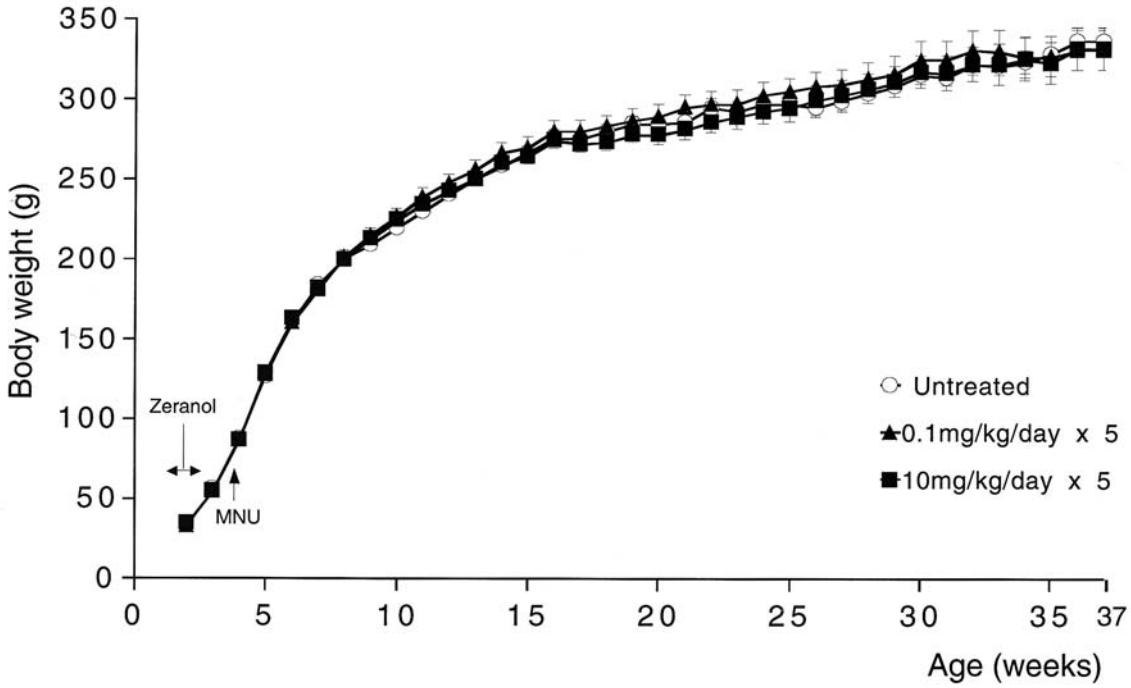

Figure 1. *Body weight gain in female Sprague-Dawley rats treated daily with 0.1 or 10 mg/kg zeranol or vehicle from 15 to 19 days of age and administered 50 mg/kg MNU at 28 days of age. Differences between zeranol-untreated and zeranol-treated groups were not significant.*

body weight gain (Figure 1). The doses of zeranol used were not toxic. In the untreated and 0.1 and 10 mg/kg zeranol-treated groups, there were 2, 2 and 1 moribund animals, respectively. These rats were excluded from the calculation. At 28 days of age (time at carcinogen administration), differences in body weight among groups were not significant, and relative uterine-ovarian weights were comparable among groups (Table I); histological sections of uterus, vagina and ovaries showed no detectable difference. At 28 days of age, whole-mount preparations of mammary gland showed similar differentiation; mammary ducts ended in club-shaped terminal end buds, and alveolar differentiation was seen proximal to the nipple.

Table I. *Effect of prepubertal zeranol treatment on body weight and relative uterine-ovarian weight in female Sprague-Dawley rats at 28 days of age.*

Zeranol treatment	No. of rats	Body weight (g)	Relative uterine-ovarian weight (mg/100g B.W.)
Untreated	6	88.1±1.9	267.5±29.9
0.1 mg/kg/day x5	6	84.2±2.0	258.2±23.7
10 mg/kg/day x5	6	84.9±2.1	232.6±26.1

Values are mean±S.E; **p* value <0.05 compared with untreated group.

Vaginal opening (puberty onset) occurred from 27 to 42 days of age. Compared with zeranol-untreated controls, 0.1 and 10 mg/kg zeranol-treated rats had significantly earlier vaginal opening (Figure 2): untreated controls, 36.4±0.6 days of age; 0.1 mg/kg zeranol, 31.2±0.6 days; 10 mg/kg zeranol, 32.2±0.7 days. Daily vaginal smears taken from 8 weeks of age for 4 weeks indicated that, whereas zeranol-untreated animals had an average cycle length of 4.6 days, both low- and high-dose zeranol-treated groups had a significant increase in cycle length due to prolonged estrous (Table II). In zeranol-untreated and 0.1% and 10% zeranol-treated groups, prolonged estrous was seen in 0% (0/22), 59% (13/22) and 78% (18/23) of animals, respectively, and prolonged diestrous was seen in 5% (1/22), 9% (2/22) and 9% (2/23) of animals, respectively. In rats sacrificed at the termination of the experiment (Table III), body weight was comparable among groups, but 10 mg/kg zeranol significantly increased the relative uterine-ovarian weight. In addition, the frequency of rats with no newly formed corpora lutea in the ovaries (indicating anovulation) increased in the zeranol-treated groups. Prepubertal zeranol treatment (both high- and low-dose) disrupted endocrine function and ovarian structure in adulthood. However, at 28

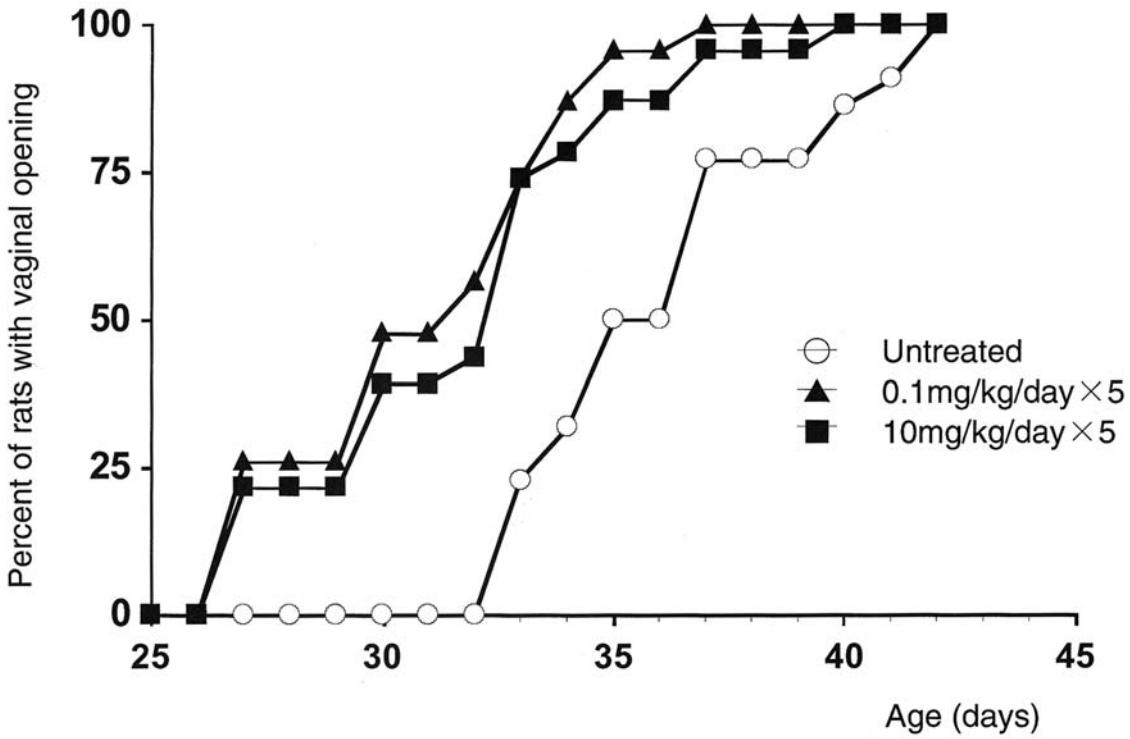

Figure 2. *Vaginal opening in female Sprague-Dawley rats after prepubertal zeranol treatment. Zeranol-treated groups showed earlier vaginal opening than untreated controls ($p<0.01$, respectively).*

Table II. *Estrous cycle in prepubertal zeranol-treated and -untreated female Sprague-Dawley rats administered MNU at 28 days of age.*

Zeranol treatment	One cycle length (days)	% of time spent in each phase of cycle			
		Proestrus	Estrous	Metestrous	Diestrous
Untreated	4.6±0.2	25.6±1.6	31.2±1.3	25.6±2.4	17.0±1.9
0.1 mg/kg/day x5	5.3±0.2	10.6±1.5*	56.6±2.5**	18.4±2.2*	14.0±2.0
10 mg/kg/day x5	5.9±0.4**	11.4±1.5**	56.2±2.4**	18.1±2.1*	13.8±2.1

Examined from 8 weeks of age for 4 weeks. Values are mean±S.E. *p value<0.05 and **<0.01 compared with untreated group.

days of age, zeranol treatment caused no apparent histological changes in the vagina or uterus, and mammary gland development was comparable among groups.

Mammary tumorigenesis. Gross mammary tumors (≥1 cm) were all histologically confirmed to be carcinomas. Development of gross mammary carcinomas (≥1 cm) tended to be delayed in zeranol-treated groups, especially in the 0.1 mg/kg group, compared with the untreated group (Figure 3). However, log-rank test analysis indicated that differences in cumulative incidence of gross mammary carcinoma among groups were not significant. Also, incidence of gross mammary

Table III. *Effect of prepubertal zeranol treatment on body weight and relative uterine-ovarian weight in female Sprague-Dawley rats at 37 weeks of age.*

Zeranol treatment	No. of rats	Body weight (g)	Relative uterine-ovarian weight (mg/100 g B.W.)
Untreated	8	336.0±8.2	291.7±27.7
0.1 mg/kg/day x5	12	330.3±13.6	294.9±24.4
10 mg/kg/day x5	10	328.5±14.5	340.7±27.8*

Values are mean±S.E; *p value <0.05 compared with untreated group.

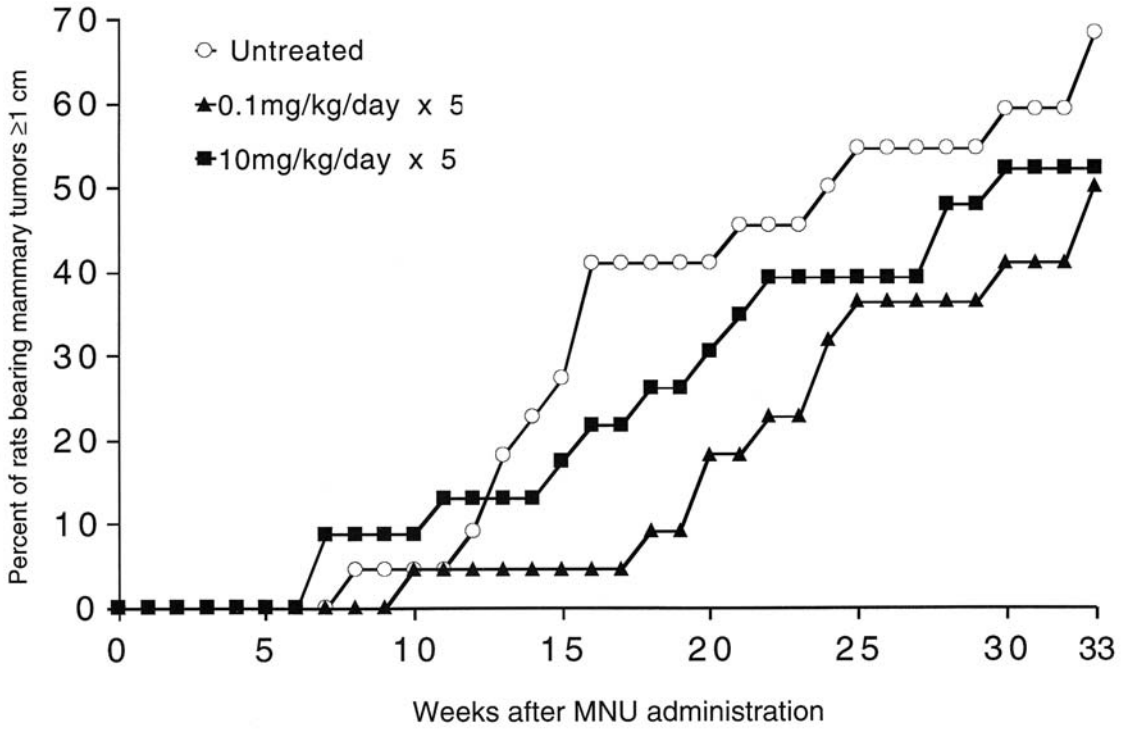

Figure 3. *Effects of prepubertal zeranol exposure on cumulative incidence of MNU-induced mammary tumors ≥1 cm in female Sprague-Dawley rats.*

carcinoma at the final time point did not significantly differ among groups (Table IV), and latency was also not significantly affected by zeranol treatment. When all sizes of histologically detected mammary carcinomas were analyzed, the number of mammary carcinomas per rat (multiplicity) did not significantly differ among groups, although the 0.1 mg/kg group tended to have a higher number of carcinomas per rat. Zeranol did not significantly affect mammary carcinoma yield, as indicated by cumulative incidence of gross mammary carcinomas, number of carcinomas per rat and latency. Zeranol tended to increase the incidence of fibroadenomas dose-dependently, and the reasons for this are unclear. Ovarian thecoma and adenoma, squamous cell carcinoma of the skin and renal mesenchymal tumors were observed after MNU administration, but the incidence of these tumors did not correlate with zeranol treatment.

Discussion

Zeranol does not exhibit toxicity when properly used as an anabolic in animal production (18). However, because zeranol has strong estrogenicity, as indicated by *in vivo* bioassays (19), detailed analysis of the effects of zeranol exposure during development on estrogen-responsive reproductive system functions and structure is of clinical importance. In rodents, neonatal and prepubertal zearalenone treatment has been shown to cause accelerated vaginal opening, persistent estrous and structural changes in ovaries indicating sterility (9,10,20-22). In the present study, although we did not see structural changes in female genital organs at 28 days of age, even low (0.1 mg/kg)-dose prepubertal zeranol exposure induced early puberty onset and profound disruption of estrous cyclicity in a considerable number of animals examined at 8 to 11 weeks of age. The ovaries of some zeranol-treated rats sacrificed during the experiment lacked newly formed corpora lutea, indicating anovulation and sterility. Taken together, the present data indicate that zeranol causes profound endocrine disruption, leading to anovulation (sterility) in female rats. Neonatal exposure to DES or genistein (a phytoestrogen) induces development of uterine adenocarcinoma in mice (23). In the present study, zeranol did not induce abnormalities in the uterus or vagina. However, longer observation is necessary to exclude the possibility that zeranol can induce tumorigenesis in female reproductive organs. Prenatal zeranol treatment (0.15 mg/kg x 2) causes abnormal testicular differentiation in male mice (24). There is a need for further studies of the effects of zeranol on the male reproductive tract, including its tumor-producing effects.

Table IV. *Effects of prepubertal zeranol exposure on mammary tumorigenesis in female Sprague-Dawley rats administered 50 mg/kg MNU at 28 days of age.*

Zeranol treatment	No. of rats	No. of rats with carcinoma >1 cm (%)	Total no. of carcinoma	No. of carcinoma per rat	Latency (weeks)	Total no. of fibroadenoma	Other tumors
Untreated	22	18 (82)	33	1.5±0.3	19.3±2.1	1	2 ovarian thecomas, 1 skin squamous cell carcinoma
0.1 mg/kg/day x5	22	14 (64)	50	2.3±0.4	23.7±2.0	6	1 ovarian adenoma, 2 renal mesenchymal tumors
10 mg/kg/day x5	23	16 (70)	34	1.5±0.3	18.6±2.3	13	1 ovarian thecoma

Values are mean±S.E

In addition to its effects on genital organs, zeranol exhibits acute hepatotoxicity and induces hepatic carcinogenesis in American hamsters, a rodent that is especially sensitive to the hepatotoxic effects of exogeneous estrogens (25). Mammary glands are also sensitive to estrogenic chemicals, and timing and dosage of exposure of immature animals to xenoestrogens influence the development of mammary tumors. The phytoestrogens genistein (12) and resveratrol (13) and the mycoestrogen zearalenone (10) affect the occurrence of MNU-induced mammary tumors (carcinomas) in female Sprague-Dawley rats when administered during the prepubertal period. Exposure of prepubertal children to zeranol may pose a risk of increased mammary tumorigenic potential when the endogenenous estrogen level is low (26). Zeranol, which is a more potent estrogen than zearalenone, stimulates developmental growth of mammary glands in ovariectomized mice (27). The degree of differentiation of mammary glandular structure at the time of carcinogen exposure appears to play a critical role in mammary cancer risk (28). Human exposure to carcinogenic stimuli can occur at any time of a woman's life. MNU can induce mammary tumors in sexually immature rats (29). In the present study, MNU was administered at 28 days of age and, at this time point, results of qualitative analysis of mammary gland differentiation did not significantly differ between females not exposed to zeranol and those exposed daily to zeranol from 15 to 19 days of age. Zeranol administered in the prepubertal period did not affect mammary tumorigenic potential, but had irreversible effects on the female reproductive system.

In conclusion, prepubertal exposure to zeranol did not affect occurrence of mammary carcinoma in female rats later in life, but it had profound effects on ovarian function and structure, indicating that consumption of zeranol-containing foods by humans in the prepubertal period should be avoided.

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