Figure 4. *Representative thoracic mammary whole-mounts of 28-day-old female Sprague-Dawley rats. a) Untreated control; b) 1.5 mg/kg genistein prenatally; c) 30 mg/kg genistein prenatally; d) 1.5 mg/kg genistein prepubertally; e) 30 mg/kg genistein prepubertally. All glands have TEBs at the periphery, with lateral bud formation. No developmental changes were observed at this time-point.*

Table III. *Effect of prenatal and prepubertal genistein exposure on percentages of ER-, PgR-, p63- and PCNA-positive mammary terminal end bud (TEB) cells in 28-day-old female Sprague-Dawley rats.*

Group	Genistein treatment	ER-positive (%)	PgR-positive (%)	p63-positive (%)	PCNA-labeling (%)
1	Untreated	75.2 ± 1.8	70.3 ± 0.5	39.0 ± 1.7	84.3 ± 2.0
2	1.5 mg/kg/day prenatally	65.4 ± 2.0**	61.6 ± 1.0**	32.5 ± 1.3**	82.8 ± 1.4
3	30 mg/kg/day prenatally	54.4 ± 2.2**	50.1 ± 0.5**	33.1 ± 1.7**	79.0 ± 2.4*
4	1.5 mg/kg/day prepubertally	52.3 ± 2.1**	51.6 ± 0.4**	34.4 ± 1.0**	75.2 ± 1.5**
5	30 mg/kg/day prepubertally	56.2 ± 2.8**	51.6 ± 1.4**	32.4 ± 1.1**	72.3 ± 0.8**

Values represent mean ± SEM. Each group consists of 6 rats. *$p<0.05$ and **$p<0.01$ *versus* group 1.

Table IV. *Effect of genistein exposure on MNU-induced mammary carcinogenesis in female Sprague-Dawley rats.*

Group	Treatment	No. of rats	No. of rats with carcinomas ≥1 cm (%)	Total no. of carcinomas	Tumor multiplicity	Tumor latency (days) Range	Mean ± SEM
1	Untreated	20	14 (70)	31	1.6 ± 0.2	85-186	120 ± 8
2	1.5 mg/kg prenatally	24	13 (54)	38	1.6 ± 0.3	85-186	136 ± 9
3	30 mg/kg prenatally	23	13 (57)	40	1.7 ± 0.4	93-186	133 ± 8
4	1.5 mg/kg prepubertally	24	10 (42)*	29	1.2 ± 0.2	98-186	141 ± 10
5	30 mg/kg prepubertally	23	11 (48)	31	1.3 ± 0.3	98-186	128 ± 10

Values represent mean ± SEM. * $p<0.05$ *versus* group 1.

of genistein used in the present study produced no clinical signs of toxicity. At 28 days of age, although there were no noticeable histological changes in the ovaries or uterus, relative uterine-ovarian weights of genistein-treated rats were lower than those of the control group (32). Prepubertal genistein treatment accelerates puberty onset (21). In the present study, vaginal opening was accelerated in rats that received 30 mg/kg/day genistein prepubertally. In addition, all genistein-untreated control rats had a normal (4- or 5-day) estrous cycle, and the cycle was disturbed in <20% rats in each group. In previous studies, prepubertal genistein treatment prolonged the estrous phase (21), whereas perinatal (prenatal and postnatal) genistein did not alter the percent of time spent in each phase of the estrous cycle (33). Similarly, in the present study, prepubertal genistein treatment prolonged the estrous phase, whereas the percent time spent in each phase was not changed by prenatal treatment. The estrous cycle was observed in all genistein-treated animals. Functional changes in the estrogen target organ indicate endocrine disruption.

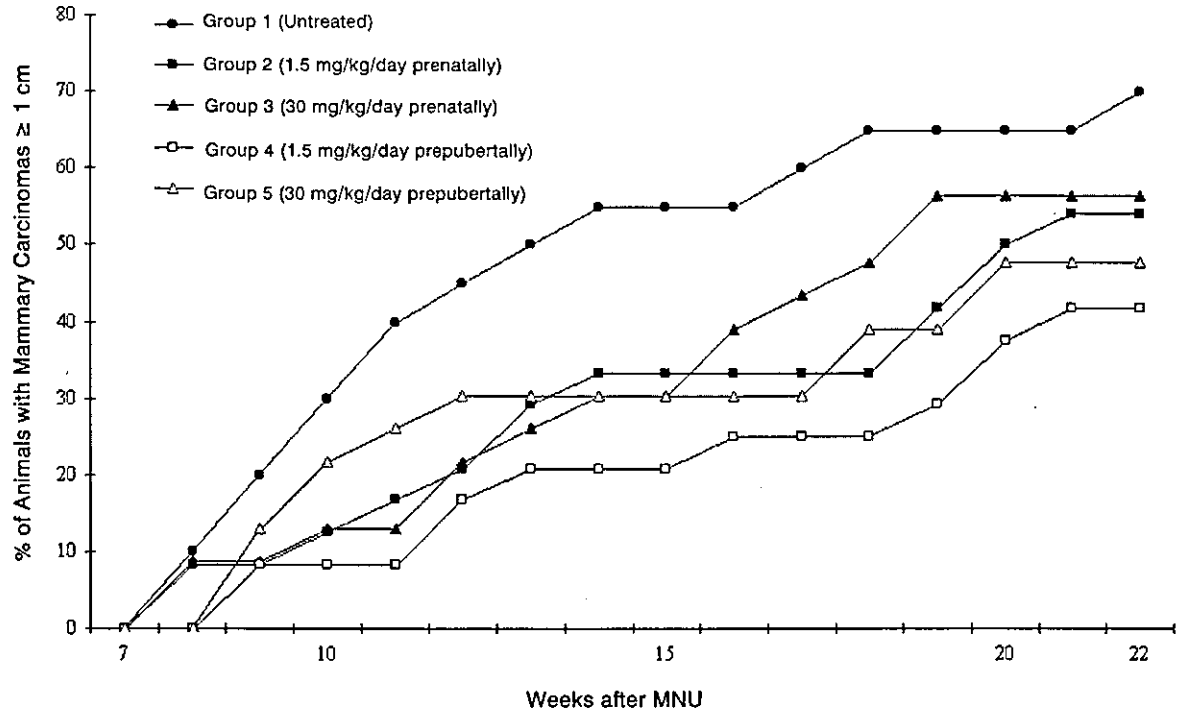

Figure 5. *Effect of genistein exposure on cumulative incidence of MNU-induced mammary carcinomas ≥1 cm in female Sprague-Dawley rats.*

In the present study, at 28 days of age (time at MNU inoculation), no alterations in mammary structure were detected in genistein-treated rats; structural development of the mammary glandular tree was comparable to that of genistein-untreated controls. The mammary epithelium was concentrated near the nipple and TEBs were observed at the margin. Thus, structurally similar mammary glands received the same carcinogenic stimuli before puberty onset; vaginal opening was not observed in any of the rats at 28 days of age.

In the present study, mammary cancer was significantly suppressed in rats treated prepubertally with 1.5 mg/kg genistein, and non-significant mammary cancer suppression was observed in rats treated prepubertally with 30 mg/kg genistein. Exposure to estrogen or estrogen-like chemicals early in life reduces the incidence of mammary cancer in animals (34, 35). Neonatal (20) and prepubertal (21) genistein exposure reduces rat mammary tumorigenesis. In contrast, maternal exposure to genistein during pregnancy increases mammary cancer in female rat offspring (16, 17). The low incidence of mammary cancer in rats exposed to genistein early in life is attributed to accelerated mammary maturation at the time of carcinogen administration (23). Accelerated mammary carcinogenesis in female offspring of pregnant rats exposed to genistein is attributed to persistent TEBs at the time of carcinogen administration (36). In the present study, MNU was administered before puberty (at 28 days of age); genistein did not act as a mammary morphogen at this time (there were no morphological differences among groups). Thus, both prenatal and prepubertal genistein treatment with a physiological or pharmacological dose causing decreased incidence of MNU-induced mammary cancer must be explained differently.

Table V. *Incidence of hormone-dependency in largest mammary carcinomas taken from female Sprague-Dawley rats bearing mammary carcinomas ≥1 cm, as determined immunohistochemically.*

Group	Genistein treatment	No. of carcinomas	No. of hormone-dependent carcinomas (%)
1	Untreated	14	13/14 (93)
2	1.5 mg/kg prenatally	13	13/13 (100)
3	30 mg/kg prenatally	13	12/13 (92)
4	1.5 mg/kg prepubertally	10	10/10 (100)
5	30 mg/kg prepubertally	11	10/11 (91)

No significant differences among groups.

Early pregnancy reduces the incidence of mammary cancer in women (19) and in animals (30, 34). Pregnancy suppresses the number of ERα- and PgR-positive cells and lowers the cell proliferation rate in non-tumoral mammary glands (31). In the present study, both prenatal

and prepubertal genistein treatment lowered the number of ERα- and/or PgR-positive cells and decreased PCNA-labeling index in TEB cells. In addition, the number of p63-positive cells (mammary basal cells), which are presumed to be mammary stem cells (37, 38), was decreased. The finding that the majority of the mammary carcinomas were hormone-dependent suggests that the mechanism of suppression of mammary cancer in rats by prenatal/prepubertal genistein is reduction of ERα- and/or PgR-positive cells (presumed to be progenitors of hormone-dependent carcinomas) and reduction of the cell renewal and cell turnover necessary for tumor promotion, resulting in a lower number of mammary carcinomas ≥1 cm. ERα is known to be associated with increased cell proliferation and breast cancer risk. Asian women who consume high levels of soy-based foods have a low incidence of breast cancer, even if they migrate to the West as adults (39). This demonstrates that exposure to genistein or other phytoestrogens in the early period of a woman's life is critical for protection against breast cancer. In the present study, pregnant and prepubertal rats were administered genistein at a concentration similar to Asian dietary levels (29) and a concentration 20 times greater. Prepubertal administration of a physiological dose of genistein suppressed mammary cancer significantly.

In conclusion, prenatal and prepubertal genistein exposure at a physiological dose or a dose 20 times greater is safe. A physiological dose of genistein administered prepubertally can protect the mammary glands of female Sprague-Dawley rats from chemically-induced carcinogenesis.

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Prepubertal resveratrol exposure accelerates *N*-methyl-*N*-nitrosourea-induced mammary carcinoma in female Sprague–Dawley rats

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Abstract

The major object of this study was to characterize the effect of prepubertal trans-3,4′,5-trihydroxystilbene (resveratrol) exposure on *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis in female Sprague–Dawley rats. Prepubertal rats (15 to 19 days of age) were treated daily with either 10 or 100 mg/kg resveratrol for 5 days, and were compared with resveratrol-untreated animals (30 rats in each group). Six rats in each group were autopsied at 49 days of age, and their growth was evaluated. All remaining rats were given 50 mg/kg MNU, followed by monitoring for occurrence of mammary carcinoma. A dose of 100 mg/kg (but not 10 mg/kg) resveratrol significantly increased incidence of rat with mammary carcinomas ≥ 1 cm and multiplicity (all histologically detected mammary carcinomas per rat), but did not affect latency, compared with untreated controls. Resveratrol did not affect body weight increase, but 100 mg/kg resveratrol caused slightly earlier vaginal opening. Although all rats cycled, resveratrol-treated animals exhibited significantly increased irregularity of estrous cycle, spending more time in the estrus phase. Thus, short resveratrol treatment of prepubertal female rats affected endocrine function, and accelerated development of MNU-induced mammary carcinomas.

Keywords: Resveratrol; *N*-Methyl-*N*-nitrosourea; Mammary carcinoma; Sprague–Dawley rat

1. Introduction

Geographical differences in the incidence of cancer indicate that diet and nutrition play a critical role in carcinogenesis [1,2]. Epidemiological studies indicate that dietary factors influence the development of breast cancer, and experimental analysis suggests that natural products in the diet can act as modifying factors against breast cancer. Certain nonnutritive phytochemicals, particularly those in daily diets, have marked chemopreventive properties. For example, Asian women who consume a soy-rich diet have about 6-fold lower risk of developing breast cancer than their Western counterparts [3,4]. Genistein, a flavonoid abundant in soy,

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shows anticancer activity [5]. One benefit of plant-derived bioactive agents is that they are less toxic.

Resveratrol is a naturally occurring phytochemical that is found in approximately 72 plant species, including grapes, peanuts and various herbs [6]. Resveratrol, a phytoalexin, may have roles in protecting plants against fungal infections and in conferring disease resistance. Resveratrol is highly concentrated in grape skin, and is also abundant in red wine [7]. The French have a lower incidence of coronary heart disease, despite having a diet relativity high in fat. This phenomenon, called the 'French paradox' [8], may be due to their high consumption of red wine. Resveratrol exerts anticarcinogenic activity in animal models of skin [6], esophageal [9] and mammary carcinogenesis [10,11], when given to adults. In cell culture studies, resveratrol reportedly inhibits estrogen receptor (ER)-positive and -negative breast cancer cell growth [12–14]. Phytoestrogens are plant chemicals that resemble endogenous estrogens of humans and animals [15]. Resveratrol's stilbene structure shows structural similarity to estradiol and diethylstilbestrol [7]. Resveratrol is a phytoestrogen that binds to ER and induces various estrogenic effects [14,16].

Estrogens have long been known to be important mitogens in the breast, and thus are associated with increased breast cancer risk. Estrogens and estrogenic compounds are a matter of considerable concern for human and animals during critical periods of development. The perinatal period of development is most sensitive to estrogenic chemicals, and human data indicate that high maternal exposure to estrogen during pregnancy increases the risk of breast cancer among daughters [17,18]. Genistein, a phytoestrogen, accelerates mammary tumor development when administered to prenatal rats, and suppresses mammary tumor development when given to prepubertal rats [19–22]. Zearalenone, a mycoestrogen used as an anabolic agent to enhance growth in cattle and lambs, reduces incidence of mammary tumors when a physiological dose is administered in the prepubertal period [19], and accelerates mammary tumor when a pharmacological dose is used [23]. The available experimental evidence regarding breast cancer risk and consumption of estrogenic chemicals during critical periods of development is inconsistent. The timing and level of exposure to estrogenic chemicals are apparently important. However, most resveratrol studies have been conducted using established cancer cell lines in culture, and few in vivo studies have been performed using adult animals [10,11]. There has been no report of the effect of prepubertal administration of resveratrol on mammary carcinogenesis. In the present study, we examined the effects of prepubertal resveratrol exposure on *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas in female Sprague–Dawley rats.

2. Materials and methods

2.1. Animals

Ninety 14-day-old female Sprague–Dawley rats (10 pups per nursing mother) were obtained from Charles River Japan (Atsugi). To avoid exposure to endocrine-disrupting chemicals, rats were housed in standard polyisopentene rat cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River Japan, Yokohama) as bedding. To avoid phytoestrogens in the diet, NIH-07 PLD (phytoestrogen low diet; Oriental Yeast, Chiba, Japan), which effectively reduces adverse endocrine-disrupting activity [24], was fed, and water was supplied in polycarbonate bottles with rubber stoppers, throughout the experiment. Thus, known endocrine-disrupting agents were eliminated from the environment.

2.2. Chemicals

Resveratrol (trans-3,4′,5-trihydroxystilbene) was purchased from Sigma (St Louis, MO). The purity was 99%, as determined by gas chromatography. The compound was supplied in powder form (trans isomer), and was kept at 0 °C in the dark. Immediately before use, resveratrol was dissolved in dimethylsulfoxide (DMSO) (Nacalai Tesque, Kyoto), and stored at 4 °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.

2.3. Experimental procedures

Animals 15 to 19 days of age were randomized into 3 groups of 30 animals each, and were given daily

subcutaneous injections of resveratrol at 10 or 100 mg/kg body weight, or an equal volume of the vehicle DMSO for 5 days. Resveratrol doses were selected on the basis of previous studies [6,10]. Rats were weaned at 21 days of age, and were then observed daily for vaginal opening (puberty onset). Body weight was recorded weekly, and growth rate was compared among groups. At 49 days of age, 6 rats per group were randomly selected and autopsied to assess the effect of resveratrol. The remaining rats (24 rats per group) received a single intraperitoneal injection of freshly prepared 50 mg/kg body weight MNU, and were observed for mammary tumor development. All animals were weighed weekly until the end of the experiment. The Animal Experimentation Committee at Kansai Medical University approved all procedures involving animals.

2.4. Growth evaluation

Effect of prepubertal resveratrol exposure was evaluated in rats killed and autopsied at 49 days of age. Animals were killed by an overdose of ether, followed by cervical dislocation. Uterus and ovaries were weighed, and formalin-fixed samples of uterus, ovaries and mammary gland were sectioned at a thickness of 4 μm, followed by staining with hematoxylin and eosin (HE). Mammary whole-mounts were prepared using the thoracic mammary gland (gland pair number 3) [22]. Also, the formalin-fixed, paraffin-embedded slides from inguinal mammary glands (gland pair number 4) were processed for immunohistochemical evaluation of the number of ERα- and progesterone receptor (PgR)-positive cells. Antibodies to ERα (6F11, Novocastra, Newcastle upon Tyne, UK) and PgR (PR10A9, Immunotech, Marseille, France) were used. The assay was performed using the labeled streptavidin–biotin (LSAB) method and an LSAB staining kit (DAKO, Carpinteria, CA), in accordance with the manufacturer's instructions. Antigenicity was retrieved by treatment in a microwave oven. Approximately 1000 mammary glandular cells were counted in ducts and acini from more than five different areas per tissue section, and the resulting data was used to calculate numbers of ERα- and PgR-positive cells.

2.5. Vaginal smears

Estrous cyclicity was monitored by examination of vaginal smears [25], performed at the same time daily from 8 to 11 weeks of age. Estrous cycles were classified as follows [26]: (i) 4- or 5-day cycle (normal duration) consisting of full estrus, diestrus I and II, and proestrus period, or including an additional 24 h of diestrus (diestrus III); (ii) 3-day cycle (irregular shortened); (iii) 6-day cycle (irregular elongated). Percent time spent in estrus phase was calculated.

2.6. Mammary tumor detection

To determine the effect of prepubertal resveratrol exposure on mammary tumorigenesis, MNU-treated animals were examined. All animals were palpated weekly for mammary tumors, and tumor location was recorded. Rats were autopsied when the largest tumor grew to ≥ 1 cm in diameter. All surviving animals were killed 32 weeks after MNU treatment, and the experiment was terminated. At autopsy, all visible mammary tumors were dissected, fixed in 10% neutral buffered formalin, and stained with HE. In addition, 'normal' mammary glands were dissected and processed to produce routine histologic 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. [27]. Mammary tumors diagnosed as adenocarcinoma were analyzed. Data analysis included the number of animals with mammary carcinomas ≥ 1 cm (carcinoma incidence), the number of carcinomas (all sizes) per animal (carcinoma multiplicity), and latency (time from MNU administration to point when largest mammary tumor grew to ≥ 1 cm in diameter).

2.7. Statistical analysis

All data were expressed as mean ± SD. Vaginal opening and carcinoma incidence were analyzed by Mantel–Cox Logrank test. Patterns of estrous cycle were analyzed by chi-squared 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 pretests 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.

3. Results

3.1. Body weight gain

Prepubertal exposure of resveratrol did not affect body weight gain (Fig. 1). Between the first day of resveratrol exposure (day 15) and the day of MNU administration (day 49), the weight increase was 5.5 ± 0.4-fold in the untreated control, low-dose treated (10 mg/kg) and high-dose treated (100 mg/kg) rats. Thereafter, body weight increases of all groups were comparable.

3.2. Endocrine status

Vaginal opening (puberty onset) occurred from 33 to 40 days of age. In rats treated with 100 mg/kg resveratrol, vaginal opening occurred slightly earlier, but there was no significant difference in timing of vaginal opening among the groups (Fig. 2). At 49 days of age, although all groups had comparable body weight, relative uterine and ovarian wet weights were heavier in the 100 mg/kg resveratrol group (Table 1), while histological sections of uterus and ovaries showed no detectable difference. At this time point, in all groups, whole-mount preparations of the thoracic mammary gland showed terminal end buds (TEBs) at the periphery, with lateral bud formation (Fig. 3a–c), and all groups had comparable HE-stained sections. Resveratrol treatment tend to increase percentages of ERα- and PgR-positive cells, respectively, but this trend did not achieve statistical significance (Table 2). Vaginal smears taken from 8 to 11 weeks of age indicated that all animals were cycling. However, incidence of irregular cycle was significantly increased in resveratrol-treated rats (Fig. 4a), and this irregularity consisted primarily of prolongation of time spent in estrus phase (Fig. 4b). Prepubertal resveratrol treatment disrupted endocrine function in a dose-dependent manner.

3.3. Mammary carcinogenesis

As shown in Fig. 5, tumor incidence (rats with mammary tumor ≥ 1 cm) was significantly higher in the high-dose resveratrol-treated group than in the untreated controls ($P < 0.05$), whereas there was no significant difference between the low-dose resveratrol-treated rats and the untreated controls. Histopathological examination revealed that all mammary tumors ≥ 1 cm were adenocarcinomas. We compared

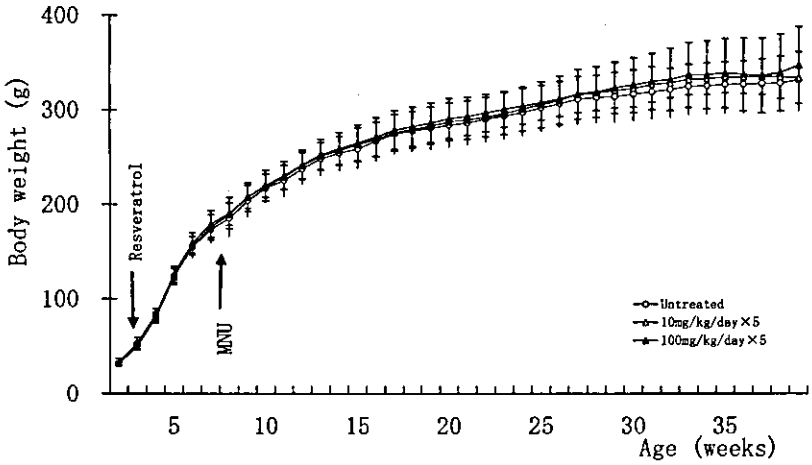

Fig. 1. Body weight changes in prepubertal (15 to 19 days of age) resveratrol-treated or untreated, and at day 49 MNU administered female Sprague–Dawley rats.

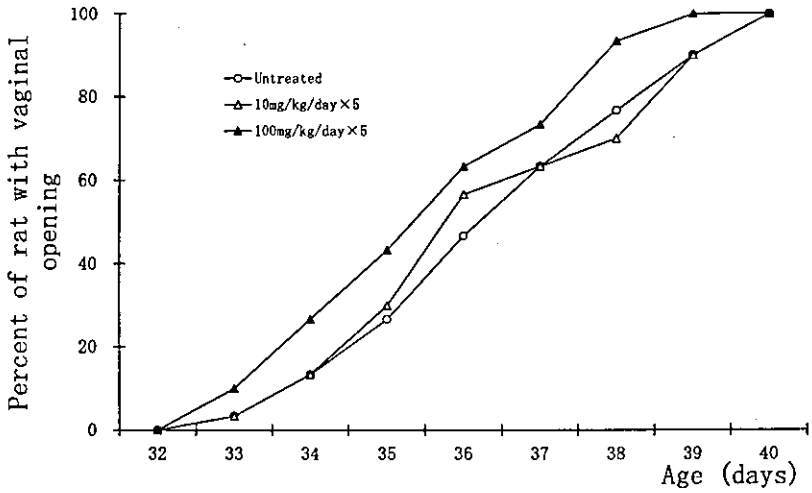

Fig. 2. Relation between vaginal opening and prepubertal resveratrol treatment.

the incidence, numbers and multiplicity of histologically confirmed mammary carcinomas. The mammary carcinogenesis data are summarized in Table 3. In the 100 mg/kg resveratrol-treated group, there was a significantly higher incidence of mammary carcinoma ≥ 1 cm (carcinoma incidence), and there was a high yield of histologically detected mammary carcinomas and significantly higher cancer multiplicity than in the untreated control rats. Low-dose resveratrol treatment had no adverse effects on mammary carcinogenesis. There was no difference in cancer latency among the groups. In MNU-treated rats, we observed one leukemia in the resveratrol-untreated group; one mammary fibroadenoma, one leukemia and two ear duct tumors in the low-dose resveratrol-treated group; and one mammary fibroadenoma in the high-dose resveratrol-treated group. There were no differences in occurrence of these tumors among the groups.

4. Discussion

Phytoestrogens alter endocrine status when administered to immature animals, in a manner similar to that of prepubertal estrogen treatment [28]. Prepubertal treatment with estradiol, diethylstilbestrol or testosterone not only advances vaginal opening but also precipitates the age-related decline in estrus cyclicity [28]. Immature female rats fed the phytoestrogen coumestrol, a major coumestan, exhibit similar acceleration of the age of vaginal opening and the decline in vaginal cyclicity [29]. Coumestrol treatment of neonatal mice results in prolonged estrus phase in the estrous cycle [30]. Genistein treatment of prenatal, neonatal and prepubertal rats results in irregularity in the length of the estrous cycle due to prolonged estrus phase [31,32].

There is limited information available on endocrine disruption by resveratrol in growing rats. In studies, daily three administrations of 120 mg/kg resveratrol [33], or even 575 mg/kg resveratrol [34], produce no detectable activity in immature rat uterotrophic assay. Resveratrol apparently exerts little or no estrogen activity in reproductive and non-reproductive estrogen

Table 1
Effect of prepubertal resveratrol treatment on body weight and relative uterine–ovarian weight in female Sprague–Dawley rats at 49 days of age

Resveratrol treatment	Body weight (g)	Relative uterine–ovarian weight
Untreated	184 ± 8	0.29 ± 0.03
10 mg/kg/day × 5	176 ± 19	0.32 ± 0.05
100 mg/kg/day × 5	178 ± 20	0.37 ± 0.08*

Each group consists of 6 rats. Values represents mean ± SD.
*$P < 0.05$ compared with untreated control.

Fig. 3. Representative thoracic mammary whole-mounts of 49-day-old female Sprague–Dawley rats. (a) Untreated control; (b) 10 mg/kg/day × 5 resveratrol; (c) 100 mg/kg/day × 5 resveratrol. All glands have terminal end buds (TEBs) at the periphery, with lateral bud formation. No developmental changes were observed at this time point.

target tissues in growing rats [35]. Effects of resveratrol on immature rats must be evaluated under restricted conditions. In the present study, the rats were fed the NIH-07 PLD diet, which contains no 'phytoestrogens', and were kept in an estrogen-free environment throughout the observation period, to minimize the effects of exogenous estrogens. The present high-dose prepubertal regimen (100 mg/kg × 5 resveratrol) slightly accelerated vaginal opening, and significantly increased the incidence of irregular estrous cycles with prolonged estrus phase. Nevertheless, all resveratrol-treated animals were cycling. In contrast to these functional alterations, structural alteration such as estrogen-like proliferative change were not observed in the uterus and ovary at 49 days of age (time at MNU administration). Thus, prepubertal resveratrol treatment altered endocrine function, whereas all groups had comparable structural changes (uterine–ovarian histology). However, as in two previous studies [27,35], uterine–ovarian weight was greater in the 100 mg/kg resveratrol group; the mechanisms by which this occurs are presently unknown.

In adult rats, resveratrol administered by gavage or in the diet has been found to significantly suppress mammary tumorigenesis, in MNU and DMBA models, respectively [10,11]. However, caution must be taken when phytoestrogens are administered during critical periods of development (e.g. prepubertal stage). In the present study, a dose of 100 mg/kg resveratrol accelerated occurrence of MNU-induced mammary cancer, but a dose of 10 mg/kg did not. Cancer incidence and multiplicity were significantly higher in the high-dose group, whereas latency was unchanged. Perhaps a given phytoestrogen can have opposing effects on mammary cancer risk, depending on the timing of exposure. Increase in the number of TEBs and reduction of their differentiation into alveolar buds play a critical role in increasing breast cancer risk [36]. However, in the present study, number of TEBs among groups at 49 days of age (time at MNU exposure) was not compared. Resveratrol treatment tend to increase ERα- and PgR-positive cells in normal mammary gland at this time point (this trend did not achieve statistical significance). As the majority (>80%) of MNU-induced mammary carcinomas are hormone-dependent [37,38], resveratrol appears to increase the ER- and/or PgR-positive cells presumed to be the progenitors of hormone-dependent carcinomas resulting in a higher mammary carcinoma yield. Doses of 10 and 100 mg/kg/day resveratrol are 500 and 5000 times the amount that is consumed by a person drinking one glass of red wine a day, respectively [39]. However, in the present study, all groups had comparable body weight gain and growth rate; i.e. there was no growth toxicity. Daily oral administration of 20 mg/kg resveratrol for 28 days in adult Sprague–Dawley rats does not affect final body weight or mean growth rate, and does not exhibit any adverse toxicity [39].

Table 2
Effect of prepubertal resveratrol exposure on percentages of ER- and PgR-positive mammary glandular cells in 49-day-old female Sprague–Dawley rats

Resveratrol treatment	ER-positive (%)	PgR-positive (%)
Untreated	30.8 ± 4.0	37.9 ± 3.8
10 mg/kg/day × 5	33.2 ± 3.8	38.7 ± 3.7
100 mg/kg/day × 5	34.7 ± 3.8	40.6 ± 1.7

Values represent mean ± SD. Each group consists of 6 rats.

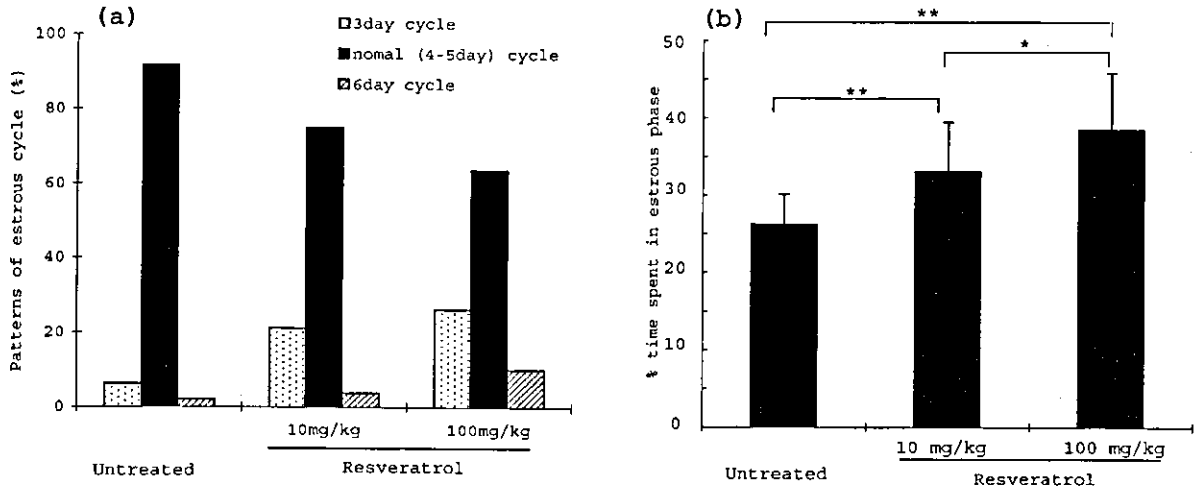

Fig. 4. Patterns of estrous cycle (a) and percent time spent in estrus phase (b) in relation to prepubertal resveratrol treatment. (a) $P < 0.01$ among groups; (b) $^{*}P < 0.05$, $^{**}P < 0.01$.

In summary, short prepubertal exposure to resveratrol resulted in endocrine disruption and increased risk of MNU-induced mammary carcinogenesis in female Sprague–Dawley rats. Although the precise mechanism by which resveratrol accelerates mammary cancer occurrence requires further clarification, the present results may have important implications for public health. If the present findings are applicable to development of breast cancer in humans, new dietary guidelines for prepubertal infants, such as prohibition of foods containing resveratrol, may be useful for prevention of breast cancer.

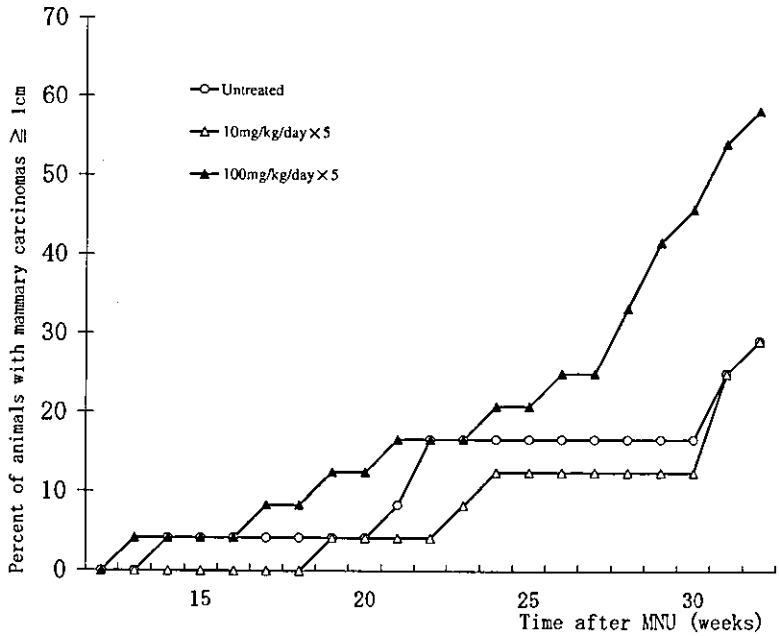

Fig. 5. Effect of prepubertal resveratrol exposure on cumulative incidence of MNU-induced mammary tumor ≥ 1 cm in female Sprague–Dawley rats.

Table 3
Effect of prepubertal resveratrol treatment on MNU-induced mammary carcinogenesis in female Sprague–Dawley rats

Resveratrol treatment	No. of rats	No. of rats with carcinomas ≥ 1 cm (%)	Total no. of carcinomas	Cancer multiplicity[a]	Cancer latency[b] (weeks)
Untreated	24	7(29.2%)	22	0.92 ± 0.19	31.7 ± 2.6
10 mg/kg/day × 5	24	7(29.2%)	24	1.00 ± 0.27	34.3 ± 2.0
100 mg/kg/day × 5	24	14(58.3%)*	41	1.71 ± 0.28*	32.6 ± 1.6

Values represents mean ± SD. *$P < 0.05$ compared with untreated control.
[a] Number of carcinomas (all sizes) per rat.
[b] Interval between MNU administration to largest mammary tumor reached ≥ 1 cm in diameter.

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Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring

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Abstract

The objective of this study was to examine the effects of maternal exposure to xenoestrogen, at levels comparable to or greater than human exposure, on development of the reproductive tract and mammary glands in female CD-1 mouse offspring. Effects of genistein (GEN), resveratrol (RES), zearalenone (ZEA), bisphenol A (BPA) and diethylstilbestrol (DES) were examined. Beginning on gestational day 15, pregnant CD-1 mice were administered four daily subcutaneous injections with 0.5 or 10 mg/kg/day of GEN, RES, ZEA or BPA, 0.5 or 10 μg/kg/day of DES dissolved in dimethylsulfoxide (DMSO), or DMSO vehicle ($n = 6$). Vaginal opening was monitored, 6 animals per group were autopsied at 4, 8, 12 and 16 weeks of age and estrous cyclicity was monitored from 9 to 11 weeks of age. Maternal exposure to xenoestrogen accelerated puberty onset (vaginal opening) and increased the length of the estrous cycle; mice treated with GEN, RES, BPA or DES spent more time in diestrus, and ZEA-treated mice spent more time in estrus. Lack of corpora lutea and vaginal cornification were observed at 4 weeks of age in the high-dose GEN (33%) and RES (17%) groups, and in the high- and low-dose BPA groups (33 and 50%, respectively) and DES groups (83 and 100%, respectively). Lack of corpora lutea and vaginal cornification was observed in the high-dose ZEA group at 4, 8, 12 and 16 weeks of age (83, 100, 83 and 33%, respectively). Mammary gland differentiation was accelerated in ZEA- and BPA-treated mice with corpora lutea at 4 weeks of age. ZEA-treated mice without corpora lutea showed mammary growth arrest at 8, 12 and 16 weeks of age; their mammary glands consisted only of a dilatated duct filled with secreted fluid. Mammary gland growth was similar with xenoestrogens other than ZEA or BPA to that of the controls at all time points. High-dose GEN and RES and high- and low-dose BPA and DES exerted transient effects on the reproductive tract and mammary glands, whereas ZEA exerted prolonged effects.

Keywords: Genistein; Resveratrol; Zearalenone; Bisphenol A; Diethylstilbestrol; CD-1 mouse; Prenatal; Maternal

1. Introduction

Endocrine-disrupting chemicals such as xenoestrogens are naturally occurring substances (i.e., phytoestrogens and mycoestrogens) or synthetic chemicals that are released into the environment and can interfere with the endocrine system and exert various effects in vertebrates [1]. These effects can be severe, particularly in prepubertal children where endogenous estrogen concentration is low [2]. In utero exposure to diethylstilbestrol (DES; (*E*)-3,4-bis(4-hydroxyphenyl)-3-hexene) as an antiabortive induces clear cell adenocarcinoma of the vagina in daughters after puberty [3]. Although the risk among the DES exposed daughters for the development of clear cell adenocarcinoma is small (<1%), DES is linked to more frequent benign reproductive tract dysfunction and structural abnormality. Development of estrogen target tissues appears to be particularly vulnerable to effects of xenoestrogens during the prenatal period [4]. Mice are especially sensitive to estrogens in utero; exposure to natural or synthetic estrogens in utero can produce various postnatal effects [5].

Naturally occurring and synthetic chemicals that exhibit estrogenic activity are widely distributed in the environment [1]. Among the chemicals that exhibit such activities are genistein (GEN), resveratrol (RES), zearalenone (ZEA) and bisphenol A (BPA). GEN (4′,5,7-trihydroxyisoflavone) is a major component in soy-based foods and reports indicate that the level of GEN exposure in Asian populations consuming a soy-rich diet ranges from ~1 to 30 mg/day (~0.02–0.55 mg/kg/day) [6]. Infants consuming a diet of soy-based formula may be exposed to ~20–40 mg/day

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(4–6 mg/kg/day) of soy isoflavones, of which GEN accounts for >65% [7]. RES (*trans*-3,4′,5-trihydroxystilbene) is found in grapes and red wine [8]. Red wine is believed to be the main source of RES, and a person drinking one glass of red wine a day consumes ~0.02 mg/kg/day of RES [9]. ZEA (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resocylic acid-lactone) is a mycotoxin synthesized by *Fusarium* mold, and is present as a natural contaminant in food as a result of infection of grain by *Fusarium* species. Human exposure to ZEA in the United States is 1–5 mg/day (0.02–0.1 mg/kg/day) [10]. BPA (4,4′-isopropylidenediphenol), an industrial chemical that exhibits estrogenic action, is a monomer used in the manufacture of many chemical products including the interior lining of food and beverage cans, dental sealants and polycarbonate plastic products including baby bottles. Typical human exposure to environmental BPA ranges from 0.025 to 0.25 mg/kg/day [11]. To date, the estrogenic potency of these chemicals has not been compared.

The present study has compared the effects of early exposure to xenoestrogens in pregnant mice treated subcutaneously (s.c.) with each xenoestrogen at 0.5 mg/kg/day to mimic human-relevant doses, and also treated with a 20-fold higher dose (10 mg/kg/day). DES was included as a positive estrogenic control at doses approximately 1000 times lower than those of the other xenoestrogens (0.5 μm/kg/day or 10 μg/kg/day). Xenoestrogens interact with the estrogen receptor (ER) and evoke estrogenic activity [5]. The present dose of DES was based on the finding that DES has a 1000-fold higher affinity for ERα than GEN [12]. We examined the effects of these xenoestrogens on the reproductive tract and mammary glands.

2. Materials and methods

2.1. Test chemicals

GEN was purchased from Fujicco (Kobe, Japan), and RES, ZEA, BPA and DES were obtained from Sigma (St. Louis, MO). The purity of all tested chemicals was ≥99%. All chemicals arrived in powder form and were kept at 0 °C in the dark. Immediately before use, each chemical was dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto), and stored at 4 °C.

2.2. Animals

Outbred Crj:CD-1 (ICR) timed pregnant mice were purchased from Charles River Japan (Atsugi), and arrived in our laboratory on day 14 of gestation.

2.3. Experimental environment

The animals were kept at 22 ± 2 °C and 60 ± 10% humidity, with a 12 h/12 h light/dark cycle. To avoid exposure to endocrine-disrupting chemicals, mice were housed in standard mouse polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River, Yokohama) as bedding. To avoid exposure to dietary phytoestrogens, mice were fed a low-phytoestrogen diet (NIH-07 PLD; Oriental Yeast, Chiba, Japan); NIH standard dietary pellets (NIH-07 open formula) contain phytoestrogens from soy products and alfalfa [13]. Water was supplied in polycarbonate bottles with rubber stoppers. Thus, exposure to known environmental endocrine-disrupting agents were minimized.

2.4. Experimental procedures

Beginning on gestational day 15, mice were given four daily s.c. injections of 0.5 or 10 mg/kg/day of GEN, RES, ZEA or BPA, 0.5 or 10 μg/kg/day of DES, or vehicle DMSO alone (untreated control). Doses were adjusted daily according to body weight, to provide constant dose levels.

Female offspring were weaned at 21 days of age. Timing of vaginal opening was recorded. In 12 mice from each group, vaginal smears were taken from 9 to 11 weeks of age, and estrous cycle was monitored. Six randomly selected mice in each group at 4, 8, 12 and 16 weeks of age were weighed, anesthetized, euthanized by cervical dislocation, and autopsied. Ovaries, uterus, vagina and inguinal mammary glands were fixed in 10% neutral buffered formalin. Mid-uterine transverse segments, vaginal transverse segments, the center of each ovary, and inguinal mammary glands were sectioned (thickness, 4 μm) and stained with hematoxylin and eosin (HE). Thoracic mammary glands were processed for whole-mount preparation and the degree of differentiation was arbitrarily scored from 1 to 4 using the following criteria: Score 1, low degree of differentiation, terminal end buds (TEBs) in the periphery with lateral buds but no alveolar development; Score 2, small number of alveoli in poorly developed ductal tree; Score 3, intermediate development of ductal and alveolar structure; Score 4, high degree of development, and lobulo-alveolar formation in the gland. Our experimental protocol was approved by the Animal Experimentation Committee, Kansai Medical University.

2.5. Statistical analysis

All data were expressed as mean ± S.E. 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.

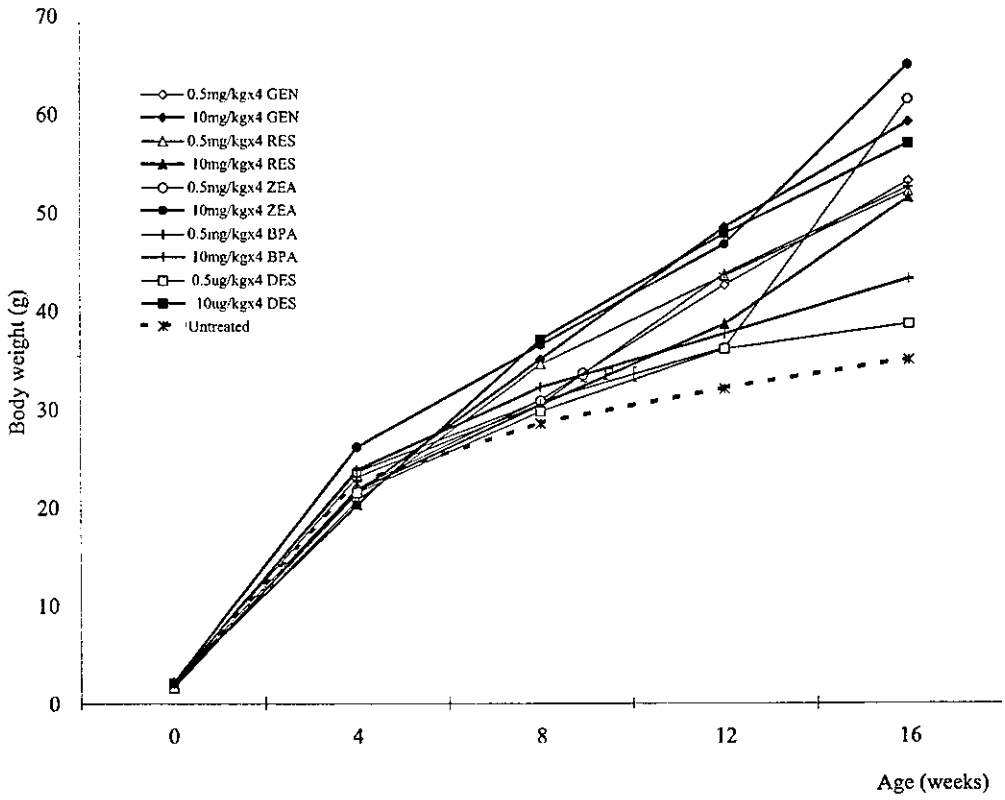

Fig. 1. Body weight gain in female CD-1 offspring of mothers administered four daily low- or high-dose injections of xenoestrogen, beginning on gestational day 15.

3. Results

3.1. Body weight gain in female offspring

Maternal exposure to test chemicals accelerated body weight gain in female offspring compared with untreated controls (Fig. 1). At 16 weeks of age, all mice other than the low-dose DES group were significantly heavier ($P < 0.01$, respectively) than untreated controls.

3.2. Vaginal opening

Xenoestrogens accelerated timing of vaginal opening, compared with untreated controls (Table 1). In the GEN, ZEA, DES and high-dose BPA groups, vaginal opening was significantly earlier. In the RES and low-dose BPA groups, there was no significant difference.

3.3. Estrous cycle

All untreated control mice exhibited a regular cycle of 5.2 ± 0.1 days, with 18.7 and 24.2% of time spent in estrus and diestrus phase, respectively (Table 2). Although all xenoestrogen-treated mice were cycling, cycle length was significantly elongated in all groups other than low-dose DES. In the GEN, RES, BPA and DES groups, the percentage of time spent in the diestrus phase was significantly longer than in untreated controls. In the ZEA groups, the percentage of time spent in the estrus phase was significantly longer than in untreated controls.

Table 1
Age at vaginal opening in female offspring of mothers exposed to xenoestrogen (four daily injections, beginning on gestational day 15)

Test chemical	Dose	Vaginal opening (days)
Untreated	–	26.0 ± 0.2
Genistein	0.5 mg/kg × 4	25.0 ± 0.2**
	10 mg/kg × 4	25.5 ± 0.2*
Resveratrol	0.5 mg/kg × 4	25.7 ± 0.1
	10 mg/kg × 4	26.0 ± 0.1
Zearalenone	0.5 mg/kg × 4	24.8 ± 0.1**
	10 mg/kg × 4	24.4 ± 0.2**
Bisphenol A	0.5 mg/kg × 4	25.8 ± 0.2
	10 mg/kg × 4	24.8 ± 0.2**
Diethylstilbestrol	0.5 μg/kg × 4	24.5 ± 0.1**
	10 μg/kg × 4	24.1 ± 0.2**

Values represent mean ± S.E. Each group consists of 24 mice.
* $P < 0.05$, compared with untreated controls.
** $P < 0.01$, compared with untreated controls.

Table 2
Estrous cycle alteration in female offspring of mothers exposed to xenoestrogen (four daily injections, beginning on gestational day 15)

Test chemical	Dose	One cycle length	Percent of time spent in	
			Estrus	Diestrus
Untreated	–	5.2 ± 0.1	18.7 ± 0.4	24.2 ± 2.1
Genistein	0.5 mg/kg × 4	6.4 ± 0.3**	15.9 ± 0.7	31.0 ± 1.7**
	10 mg/kg × 4	7.2 ± 0.4**	14.3 ± 0.8	34.5 ± 1.8**
Resveratrol	0.5 mg/kg × 4	6.5 ± 0.2**	14.7 ± 0.9	33.7 ± 2.7**
	10 mg/kg × 4	6.1 ± 0.2**	15.1 ± 0.5	29.8 ± 1.7
Zearalenone	0.5 mg/kg × 4	7.7 ± 0.3**	29.8 ± 4.7**	29.8 ± 3.0
	10 mg/kg × 4	10.9 ± 0.5**	48.4 ± 4.0**	23.4 ± 2.2
Bisphenol A	0.5 mg/kg × 4	8.0 ± 0.4**	13.1 ± 0.6	38.9 ± 2.0**
	10 mg/kg × 4	8.2 ± 0.3**	13.9 ± 0.4	40.5 ± 1.2**
Diethylstilbestrol	0.5 μg/kg × 4	5.5 ± 0.1	17.5 ± 0.7	30.2 ± 2.1*
	10 μg/kg × 4	5.8 ± 0.1*	17.5 ± 0.9	37.3 ± 1.7**

Values represent mean ± S.E. (days).
* $P < 0.05$, compared with untreated controls.
** $P < 0.01$, compared with untreated controls.

3.4. Reproductive tract structure

Ovarian histology in mice sacrificed at 4 weeks of age revealed that, compared with untreated control mice (Fig. 2a), corpora lutea were absent in high-dose (but not low-dose) GEN-, RES- and ZEA-treated mice (Fig. 2b) (2/6, 1/6 and 5/6, respectively), and in both low- and high-dose BPA (2/6 and 3/6, respectively) and DES-treated mice (5/6 and 6/6, respectively) (Table 3). However, corpora lutea was present in GEN-, RES-, BPA- and DES-treated mice sacrificed at 8, 12 or 16 weeks of age. In contrast, corpora lutea were absent in high-dose ZEA-treated mice sacrificed at 8, 12 or 16 weeks of age (6/6, 5/6 and 2/6, respectively); i.e., the number of mice without corpora lutea decreased with advancing age. In some ZEA-treated mice without corpora lutea, we observed ovarian interstitial cell hyperplasia composed of light-staining cells arranged in tubule-like structures, and/or squamous metaplasia of uterine glands. The other tested chemicals produced no abnormalities in the uterus. Vaginal cornification was observed in mice lacking corpora lutea, but none of the tested chemicals induced vaginal epithelial abnormality.

3.5. Mammary gland

In untreated control mice at 4 weeks of age, TEBs were observed at the periphery with lateral buds, whereas alveolar differentiation was unclear (Fig. 3a; Score 1). At ≥8 weeks of age, the alveolus and lobulus appeared. At 12 and 16 weeks of age, mammary glands showed no further differentiation. At 8, 12 and 16 weeks of age, the degree of development varied somewhat from animal to animal. In some cases mammary glands were relatively poorly differentiated, with small number of alveoli within poorly developed ducts

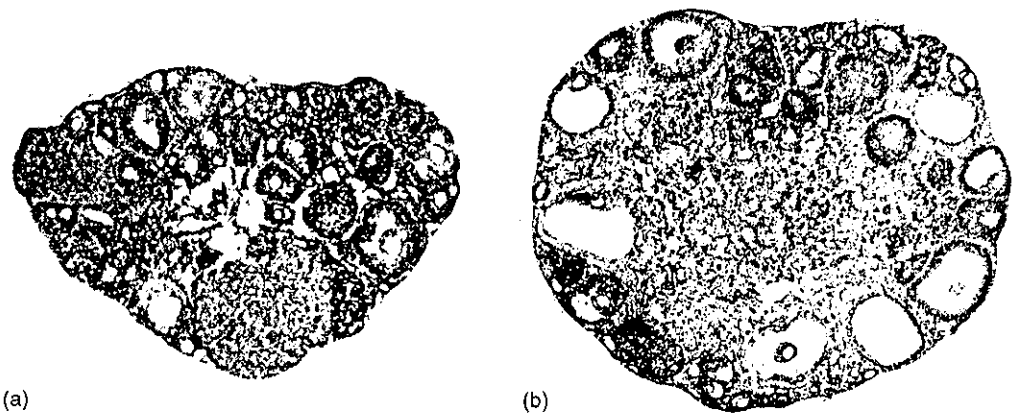

Fig. 2. Ovaries from 4-week-old CD-1 mice. (a) Mouse exposed prenatally to DMSO vehicle. Note prominent corpora lutea. (b) Mouse exposed prenatally to 10 mg/kg × 4 ZEA. Note absence of corpora lutea and conspicuous interstitial cell hyperplasia.

Table 3
Absence of corpora lutea in female mice treated with xenoestrogen prenatally

Xenoestrogens	Dose	No. of mice without corpora lutea (%)			
		4	8	12	16 (weeks)
Untreated	–	0	0	0	0
Genistein	0.5 mg/kg × 4	0	0	0	0
	10 mg/kg × 4	2 (33)	0	0	0
Resveratrol	0.5 mg/kg × 4	0	0	0	0
	10 mg/kg × 4	1 (17)	0	0	0
Zearalenone	0.5 mg/kg × 4	0	0	0	0
	10 mg/kg × 4	5 (83)	6 (100)	5 (83)	2 (33)
Bisphenol A	0.5 mg/kg × 4	2 (33)	0	0	0
	10 mg/kg × 4	3 (50)	0	0	0
Diethylstilbestrol	0.5 μg/kg × 4	5 (83)	0	0	0
	10 μg/kg × 4	6 (100)	0	0	0

(Fig. 3b; Score 2); other cases showed greater ductal and alveolar development (Fig. 3c; Score 3), and some exhibited fully developed lobulo-alveolar formation (Fig. 3d; Score 4). At 4 weeks of age, all untreated controls had Score 1 mammary glands, whereas all high- and low-dose ZEA-treated mice and 2 out of 3 high-dose BPA-treated mice with corpora lutea showed alveolar differentiation (≥Score 2) (Fig. 4a) with some alveoli showing secretory activity (Fig. 4b); however, at ≥8 weeks of age, none of the xenoestrogen-treated mice showed adverse effects on growth of mammary glands. In contrast, ZEA-treated mice lacking corpora lutea at 8, 12 and 16 weeks of age exhibited dilated beaded ducts without alveolar formation (Score 1) (Fig. 5a); these dilated ducts contained an eosinophilic substance (Fig. 5b). Scores of mammary gland differentiation after perinatal xenoestrogen exposure are summarized in Fig. 6. At 4 weeks of age,

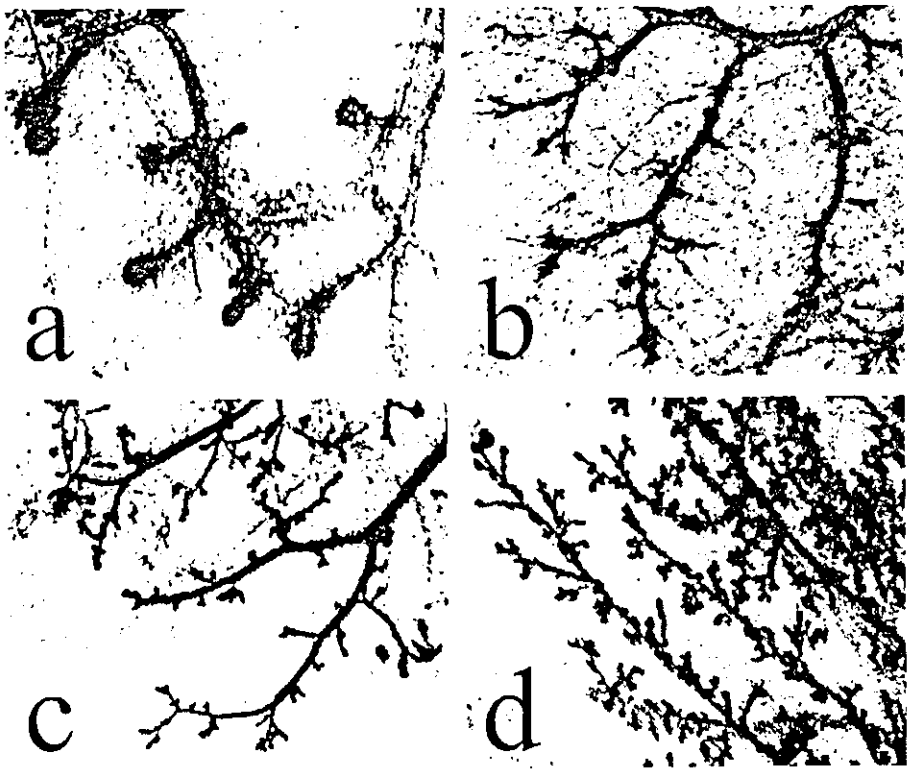

Fig. 3. Mammary glands from untreated CD-1 mice. (a) Four-week-old mouse. Note terminal end buds at the periphery with lateral bud but no alveolar differentiation (Score 1). (b) Twelve-week-old mouse. Note small number of alveoli within poorly developed duct (Score 2). (c) Twelve-week-old mouse. Note more advanced ductal and alveolar development, compared with panel (b) (Score 3). (d) Twelve-week-old mouse. Note prominent lobulo-alveolar development (Score 4).

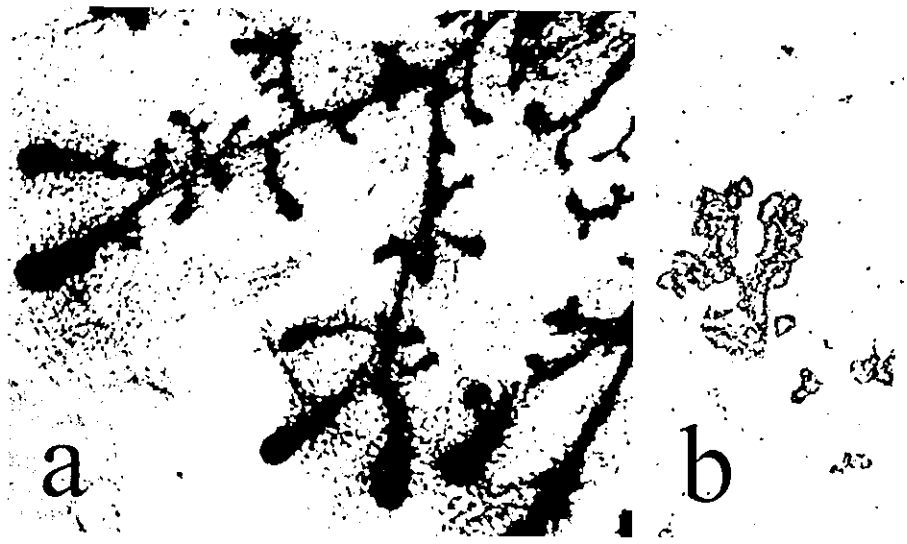

Fig. 4. Mammary gland from a 4-week-old ZEA-treated CD-1 mouse with intact ovary. (a) Note terminal end buds at the periphery with advanced alveolar differentiation (compare with Fig. 3a). (b) Alveoli exhibit secretory activity.

Fig. 5. Mammary gland from a 16-week-old ZEA-treated CD-1 mouse without corpora lutea. (a) Note dilated beaded mammary ducts without alveolus. (b) Dilated duct contains eosinophilic substance.

accelerated mammary gland differentiation was observed in ZEA- and BPA-treated mice (with corpora lutea). At 8–16 weeks of age, growth retardation was observed in high-dose ZEA-treated mice (without corpora lutea).

4. Discussion

In the present study, in utero exposure to xenoestrogens (phytoestrogens, mycoestrogens and industrial chemicals) at doses equivalent to typical human exposure and at a 20-fold higher dose produced various degrees of alteration in reproductive tract and mammary gland in female CD-1 mice. In previous studies, perinatal treatment with DES or GEN did not alter body weight [6]. ZEA and BPA have been found to increase fetal body weight, with the effect of BPA continuing into adulthood [14,15]. In the present study, at 16 weeks of age, body weight was significantly increased in all treatment groups other than low-dose DES.

Neonatal administration of estrogen or androgen causes earlier vaginal opening in rats [16]. Prenatal exposure to 2 μg/kg DES or 20 μg/kg BPA [17], and neonatal exposure to 0.4 mg/kg GEN or 0.04 mg/kg ZEA, causes earlier vaginal opening in mice [18]. In rats, prepubertal exposure to 10 mg/kg ZEA or 100 mg/kg RES causes earlier vaginal opening [19,20]. The present finding that all chemicals

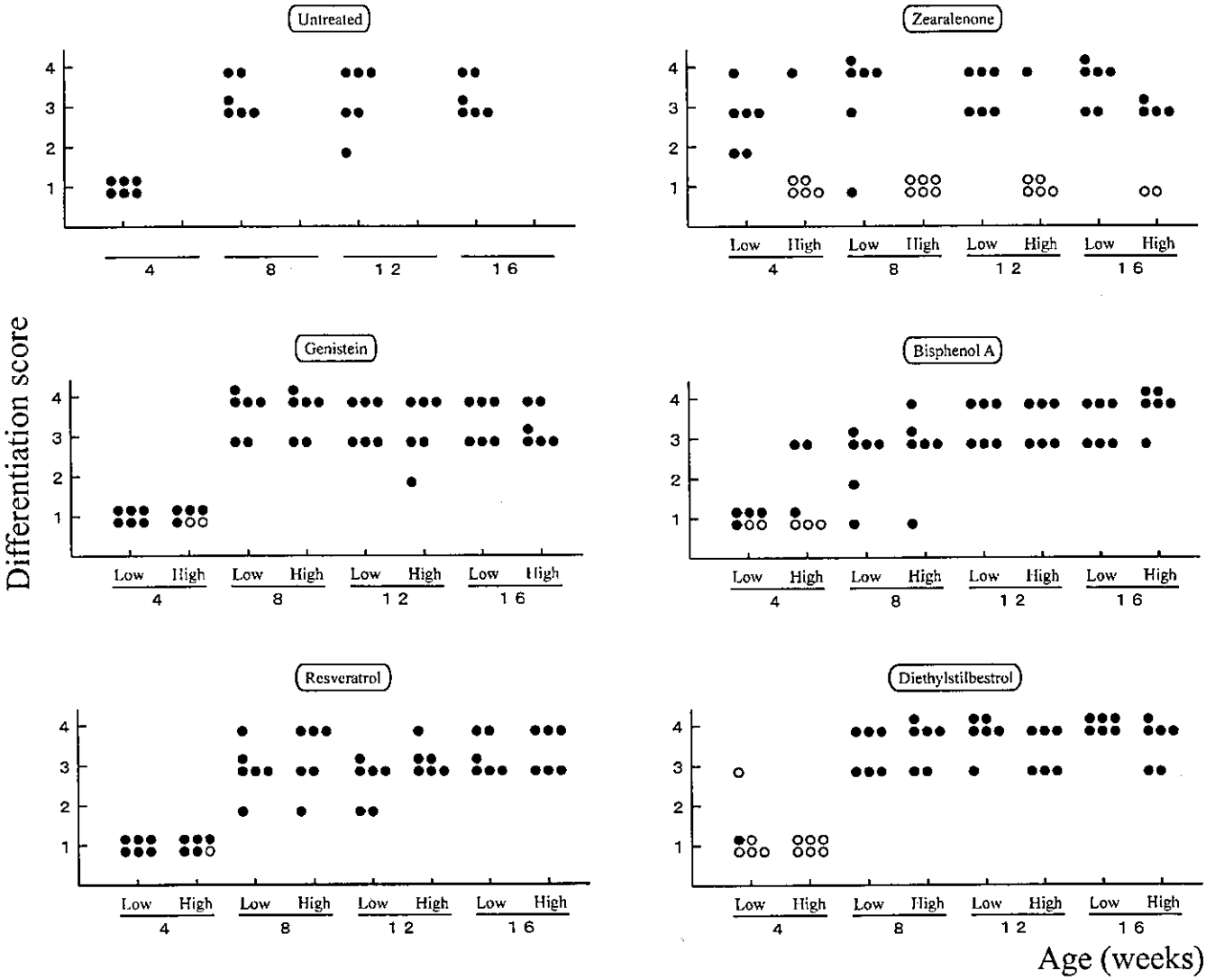

Fig. 6. Degree of mammary gland development in female CD-1 offspring of mothers administered four daily injections of xenoestrogen, beginning gestational day 15. (●) Mouse with corpora lutea; (○) mouse without corpora lutea.

tested other than RES and low-dose BPA caused significantly earlier vaginal opening in mice is consistent with previous studies.

Prenatal treatment with 10 mg/kg BPA significantly reduced the number of mice with corpora lutea at 30 days of age, but 91% of mice exhibited a normal estrous cycle at 41–70 days of age, and all were fertile at 90 days of age; suggesting transient delay of ovulation [21]. At 4 weeks of age, lack of corpora lutea was observed in the high-dose GEN and RES groups, and in the low- and high-dose BPA and DES groups. However, at ≥8 weeks of age, corpora lutea was present in all groups of mice other than the high-dose ZEA group. Rodent strains can vary dramatically in their response to estrogenic compounds, and CD-1 mice have demonstrated particularly strong resistance to the effects of estradiol [22]. In a study using BALB/c mice, a neonatal DES dose of 10 μg/kg caused lack of corpora lutea at 8 weeks of age [23]. Neonatal ZEA treatment has been shown to cause lack of corpora lutea in adult mice [24]. In the present study, although the frequency decreased with increased age, ovaries without corpora lutea were observed in high-dose ZEA-treated animals at 4, 8, 12 and 16 weeks of age, suggesting that ZEA affects ovarian structure for a longer duration than the other xenoestrogens tested. Interstitial cell hyperplasia has been observed in ovaries without corpora lutea [25]. In the present study, interstitial cell hyperplasia was seen in ZEA (16/18)- and BPA (2/5)-treated mice without corpora lutea, but not in GEN (0/2)-, RES (0/1)- or DES (0/11)-treated mice without corpora lutea; the reason for this is unclear. In humans, ovaries without corpora lutea (anovulatory ovaries) are the most frequent cause of female infertility [26].

Prenatal exposure of mice to BPA disrupts estrous cyclicity [15], and induces significantly longer estrous cycle due to