Table 1. Mean Food Consumption of the Experimental Groups and Mean Daily Intakes of Hydroxymatairesinol (HMR) in the Dosed Groups[a]

Group	Consumption (g/kg/day)	HMR intake (mg/kg/day)
Control—conventional diet (CRF-1)	45.6±1.1	—
Control—basal diet (1324 diet alone)	53.1±1.9**	—
200 ppm HMR in the basal diet	55.2±1.4**	11.0±0.3
600 ppm HMR in the basal diet	54.5±1.9**	32.7±1.1***

[a] Values represent the mean ± SEM. Mean food consumption in Group 1 was significantly lower than that in Groups 2, 3, and 4 (**, $P < 0.01$). Mean HMR-intakes are significantly different (***, $P < 0.001$) between Groups 3 and 4.

were stored at –70°C until analysis of lignans such as HMR, secoisolariciresinol (SECO), mateiresinol (MR), enterodiol (END), hydroxyenterolactone (HEL), and ENL was made. Aliquots of 0.5 ml of thawed urine samples were mixed with 1.0 ml of 0.15 *M* sodium acetate buffer (pH 4.0) and 15 μl of *Helix pomatia* enzyme mixture. For hydrolysis of lignan conjugates, the samples were incubated at 37°C overnight. The hydrolyzed samples were extracted using Sep-Pak tC18 columns (Waters Associates, Milford, MA) conditioned with 2.0 ml of 0.15 *M* sodium acetate buffer. The urine samples to which 2.5 μg of the internal standard flavone had been added were loaded into columns and washed with 0.15 *M* acetate buffer, then the polyphenolic fraction was eluted with 2.0 ml methanol. The samples were gently evaporated to dryness under nitrogen flow in a water bath at 45°C, dissolved in 5.0 ml methanol, and then an aliquot of 0.1 ml was diluted with 0.9 ml of 0.1% acetic acid. The final flavone concentration was 50 ng/ml. A variety of lignans were analyzed by HPLC-MS-MS using a PE Sciex API3000 triple quadrupole mass spectrometer equipped with a Turbo ion spray ionization source (electrospray ionization). Detailed methods have been described in a previous paper (26).

Statistical Analysis. Data on tumor incidence were statistically analyzed using the cumulative chi-square test (27). If significance was detected, differences between groups were confirmed by chi-square test. Other data were analyzed using the Student's *t* test for comparison between two groups and one-way analysis of variance (ANOVA) for multiple groups. *Post hoc* multiple comparisons were performed by Tukey's test when numbers of data were equal or Scheffe's test in other cases. A *P* value less than 0.05 was considered to be statistically significant.

Results

Food Consumption and Daily Intake of HMR in the Experimental Groups. Data for food consumption and intake of HMR in the experimental groups are summarized in Table 1. In the control group fed conventional diet (Group 1), food consumption was lower than those in Groups 2, 3, or 4 ($P < 0.01$). There were no significant differences in food consumption among the other 3 experimental groups. Mean daily intakes of HMR were 11.0 and 32.7 mg/kg/day in the 200- and 600-ppm dosed groups, respectively, and they differed statistically ($P < 0.001$).

Table 2. Delay of Persistent Estrus by Hydroxymatairesinol (HMR) Dosing[a]

Group	Mean week of age of persistent estrus start
Control—conventional diet (CRF-1)	30.3±1.2
Control—basal diet (1324 diet alone)	32.1±1.3
200 ppm HMR in basal diet	35.4±1.6*
600 ppm HMR in basal diet	35.3±1.6*

[a] Values represent the mean ±SEM, n = 25, 27, 27, and 26 rats of each group, respectively. Means of Group 3 and 4 are significantly different (*$P < 0.05$) from Groups 1 and 2.

Effects of HMR on Start of Persistent Estrus. At 4 months of age (about 1 month after dosing started), the estrous cycle stage could be easily identified using vaginal smears, a precise 4-day cycle being evident in all groups. In the control groups supplied with the conventional (Group 1) or basal (Group 2) diet, no significant difference of the beginning of persistent estrus, characterized by vaginal smears exhibiting nucleated epithelial or cornified cells, was detected. Mean ages of persistent estrus start were 30.3 and 32.1 weeks in Groups 1 and 2, respectively. However, in HMR-dosed groups (Groups 3 and 4), persistent estrus was significantly lengthened for 3 to 5 weeks ($P < 0.05$), as shown in Table 2. Almost all animals in the experimental groups were in persistent estrus until 12 months of age.

Uterine Proliferative Lesions and Other Histopathologic Findings. During the experimental period, no animals in any of the groups died. No significant changes in relative weights of the uterus and ovaries were detected at termination at 15 months of age (data not shown). A comparison of development of uterine proliferative lesions in controls and HMR-dosed rats is given in Table 3. Almost all animals had endometrial hyperplasia or adenocarcinoma. Total incidences of endometrial hyperplasia were 64%, 63%, 78%, and 80% in Groups 1–4, respectively, no shifting the burden from late stage lesions to early stage being obvious. Most hyperplasias were focal proliferations of uterine glands with apparent duct structures in the stroma of the endometrium. The characteristics and incidences of endometrial hyperplasia did not differ among the groups. Endometrial adenocarcinoma was significantly decreased by the HMR treatments ($P < 0.05$). Incidences of adenocarcinoma in 200- and 600-ppm, HMR-dosed groups were reduced to 11% and 15%, respectively, compared with those

Table 3. Numbers and Incidences of Uterine Endometrial Proliferative Lesions at 15 Months of Age in the Four Experimental Groups[a]

Group	N	Hyperplasia				Adenocarcinoma
		–	+	++	+++	
Control—conventional diet (CRF-1)	25	0	2	8	6	9
		(0)	(8)	(32)	(24)	(36)
Control—basal diet (1324 diet alone)	27	2	2	8	7	8
		(7)	(7)	(30)	(26)	(30)
200 ppm HMR in the basal diet	27	3	5	9	7	3*
		(11)	(19)	(33)	(26)	(11)
600 ppm HMR in the basal diet	26	1	4	12	5	4*
		(4)	(15)	(46)	(19)	(15)

[a] Values in parentheses are incidences (%). Significantly different from Groups 1 and 2 (* $P < 0.05$). These data show that hydroxymatairesinol (HMR) significantly reduced the number of endometrial adenocarcinomas versus controls in Groups 1 and 2 given no HMR.

of the two control groups (conventional and basal diet), being 36% and 30%, respectively. The adenocarcinomas were well-differentiated, invading the serosa of the corpora uteri, glandular structures being obvious.

Ovarian atrophy or cyst formation and lack of any corpora lutea were observed in almost all animals. Proliferation of ovarian interstitial cells was also evident. Various neoplastic and nonneoplastic lesions were also found in other organs and tissues, but no differences were apparent among the groups.

Urinary Lignans. Urinary concentrations of lignans such as HMR, SECO, MR, END, HEL, and ENL at 8 months of age are shown in Figure 3. Those in the control group supplied with conventional diet (Group 1) were comparable to those with the basal diet (Group 2), and HMR was undetectable in either control group. In the 200- and 600-ppm groups, however, urinary concentrations of HMR were dose-dependently increased at 25 and 88 μg/ml, respectively, and similar elevation was evident for ENL and HEL concentrations. Urinary concentrations of SECO, MR, and END were low or undetectable.

Discussion

In the present study, HMR, obtained from the heartwood of spruce (*Picea abies*), demonstrated an inhibitory effect on the development of uterine adenocarcinoma in Donryu rats initiated by ENNG, in line with the experimental evidence of inhibition of the growth of DMBA-induced rat mammary tumors published earlier (8, 9). Secoisolariciresinol diglycoside (SDG), isolated from flaxseed, is metabolized to both END and ENL, and has shown chemopreventive properties in the DMBA-induced mammary-tumor model (28). ENL potently inhibits the growth of DMBA-induced mammary carcinoma in the rat (29). Until now, however, there has been no report of anticarcinogenic effects of lignans on female genital tracts, including rodent uteri. The present results provide the first support for the hypothesis that long-term exposure to HMR might similarly result in a chemopreventive effect on rat uterine carcinogenesis.

Plant lignans are metabolized by the mammalian gut microflora mainly to ENL and END, called mammalian lignans (30). The urinary lignans of HMR-dosed animals were mostly HMR, HEL, and ENL in the present study; the increase was 63- and 210-fold for HEL and 5- and 10-fold for ENL in urine 24 hours after feeding 200- and 600-ppm HMR-containing diets, respectively. Previously, it was reported that ENL excretion in urine was elevated 1.5- to 9-fold after single oral dosing of HMR at 3–50 mg/kg (8). Oral administration of HMR to Sprague-Dawley rats resulted in doubled excretion of ENL, with a single gavage at 25 mg/kg (26). Daily dosage of HMR in the present study was 11.0 and 32.7 mg/kg, respectively; thus urinary ENL concentrations after a single administration in the present study were comparable to those in the previous reports. In a recent *in vitro* study, metabolites of HMR generated by human intestinal microflora were characterized as ENL and HEL (31), strongly suggesting that HMR might be transformed into these two forms in the mammalian gut (Fig. 1).

Estrogens are well established as important etiological agents for uterine carcinogenesis in humans (32–35). Although exact roles remain to be detailed, tumor-promoting effects involving up-regulation of cell proliferation have long been suggested. Recently, natural compounds having antiestrogenic activity were proven to have chemopreventive effects against estrogen-dependent carcinoma development (1). Competition for estrogen receptor-binding (36) and inhibition of aromatase activity (29) are plausible explanations for chemopreventive effects of compounds such as flavonoids and lignans. Lignans and endogenous estrogens have structural similarities, suggesting possible estrogen-like or antiestrogen-like activity. Secoisolariciresinol diglycoside feeding to rats during pregnancy and lactation has been found to increase the uterine weights of offspring at weaning, but not at later stages (37). It causes irregular estrous cycling and/or persistent estrus in adult, normal-cycling rats (38). HMR, however, exerted no significant estrogen-like or antiestrogenic effects on the immature rat uterine growth test (8). There were also no

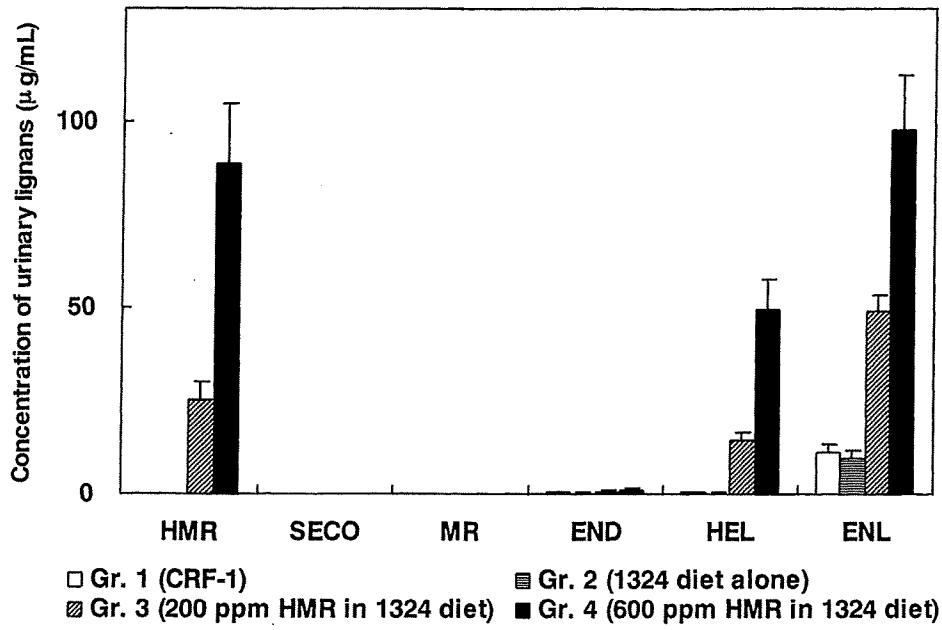

Figure 3. Urinary lignans at 8 months of age. Hydroxymatairesinol (HMR), secoisolariciresinol (SECO), mateiresinol (MR), enterodiol (END), hydroxyenterolactone (HEL), and enterolactone (ENL) were analyzed by HPLC-MS-MS. Values are mean ± SEM (n = 6).

effects on uterine weights, ovarian weights, or estrous cycle-period in the present experiment.

We have documented that the Donryu rat is a high-incidence strain for spontaneous development of endometrial adenocarcinoma and is associated with a hormonal imbalance characterized by early development of persistent estrus (15–17). Previously, it was reported that disorders or suppression of the estrous cycle, appearing very early in rats exposed prenatally to DES, might be associated with neoplastic development (39). It has been shown that *p-tert*-octylphenol, known as an EDC and having estrogenic activity, causes early occurring, persistent estrus with exposure for the first 5 days after puberty, although no abnormalities in growth and development of the reproductive organs could be found up to maturation; finally, development of uterine adenocarcinoma was accelerated (22). Although the pathogenesis of uterine tumor development by these compounds remains to be elucidated, a hormonal disorder characterized by early development of persistent estrus and increase of the E2/P ratio is exclusively involved (21). In the present study, delay in starting persistent estrus because of HMR dosing was significant. Persistent estrus results from anovulation, which is effected by change in action of various hormones such as LH-RH, LH, and estrogen. Recently, ENL was demonstrated to act as a weak aromatase inhibitor *in vitro* and to reduce the relative uterine weights of DMBA-treated, nonovariectomized rats (29). Aromatase inhibitors and antiestrogenic pharmaceuticals can reduce estrogen levels, followed by elevation of FSH and growth of ovarian follicles. The mechanisms underlying the delay of persistent estrus with HMR dosing is unclear, but ENL, a major metabolite, could be responsible through its action on aromatase.

In the present experiment, the 1324 diet was selected as a basal diet, instead of the conventional CRF-1 diet for Donryu rats (21), but both were included as controls, the CRF-1 group for historical background data in the rat strain and the 1324 diet group for HMR dosing. No significant differences in tumor development, start of persistent estrus, or other parameters were evident between the two control groups. There is evidence that subcutaneous injections of genistein and daidzein have an inhibitory effect on endometrial carcinogenesis in *N*-methyl-*N*-nitrosourea and E2-treated mice (40). The fact that the tumor incidences in both controls were comparable despite different contents of isoflavones suggests that these isoflavones are unlikely to have an inhibitory effect on cancer development in the present study. Difference in route of exposure, dose and/or inhibitory effect on the aromatase activity of these isoflavones (41) might be responsible for the discrepancy. Tumor incidence in the conventional-diet group was relatively low compared with that of the previous data (21). Although the reason is unclear, the design of the experiment appears appropriate for investigation of the chemopreventive effects on uterine carcinogenesis, given the positive influence detected.

In conclusion, long-term administration of HMR can reduce the development of uterine adenocarcinoma in Donryu rats, suggesting that its indirect modulation of hormonal regulation and its effect on estrogen production create an unfavorable milieu for tumor growth. To test this hypothesis, further examination of the detailed mechanisms of HMR's cancer-chemopreventive activity will be required.

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—Original—

Maternal Exposure to Low Doses of Bisphenol A Has No Effects on Development of Female Reproductive Tract and Uterine Carcinogenesis in Donryu Rats

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Abstract. Effects of maternal exposure to low doses of bisphenol A (BPA), including those comparable with human exposure levels, on growth and development of the female reproductive system and uterine carcinogenesis in Donryu rats were investigated. Dams were administered BPA (0, 0.006 and 6 mg/kg/day) daily by gavage from gestation day 2 up to the day before weaning (postnatal day 21 at offspring). The serum levels of BPA were significantly elevated in the dams receiving 6 mg/kg/day, however, BPA levels in the milk of dams, and those in the serum and liver of offspring were similar between control and treated groups. The treatment did not exert any influences on uterine development including weight, gland genesis and estrogen receptor α expression, vaginal opening and gonadotropin secretion in the female offspring up to puberty. After maturation, no effects were evident with regard to estrous cyclicity in female offspring treated with BPA. In addition, the treatment had no effects on age-related morphological changes of the reproductive and endocrine organs and uterine carcinogenesis until 15 months of age. The results demonstrate that maternal exposure to BPA at levels comparable to human exposure did not have any effects on the female reproductive system of offspring in rats. In addition, BPA was also found in the serum, milk and liver of control dams and pups, and low levels of BPA were detected in drinking water and pellet diet. The present study showed that the experimental animals were also exposed to environmental BPA in the animal room.

Key words: Bisphenol A, Low doses, Female reproductive system, Toxicity, Uterine carcinogenesis

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Bisphenol A (BPA), volume chemical used in the manufacture of polycarbonate plastics, has been found in canned foods packed in lacquer subjects, containers and composite dental sealants [1]. BPA is reported to be an endocrine disrupting chemical (EDC) with weak estrogenic activity in both *in vitro* and *in vivo* systems [2], binding to both estrogen receptor (ER)α and ERβ with low affinity and causing reporter gene transactivation *in vitro* [3, 4]. Uterotropic effects of BPA at high doses (3 daily oral applications of 400, 600 or 800 mg/kg/day) were reported in immature female rats [5], and significant increases in the luminal epithelial height and the thickness of both stromal and myometrial layers of the uterus were also observed in ovariectomized mice injected 0.8–8 mg/day of BPA for 4 days [6]. A study conducted by the National Toxicology Program (NTP) in the USA demonstrated that maternal exposure to high doses of BPA, at 0.5 or 1.0% in feed (approximately daily intakes of 875 and 1750 mg/kg/day), reduced the number of live pups per litter and litters per pair in

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first generation mice [7]. However, pre- and/or postnatal high dose BPA exposure did not have any apparent adverse effects on pubertal development in female rats or reproductive functions in rats and mice [8–10].

On the other hand, perinatal treatment with BPA at much lower doses has been reported to influence growth and male reproductive organ parameters such as weights of the testis, prostate, preputial glands and epididymis, and the efficiency of sperm production in rodents [11–15]. However, other investigators have reported no treatment-related effects of low doses of BPA given to pregnant mice [16–18] and to rats in a three generation reproductive toxicity study [19].

In female rodents, inappropriate perinatal exposure to endogenous and/or exogenous estrogens is known to induce serious and irreversible effects on the reproductive system [20–24]. Perinatal and/or postnatal effects of EDCs on the reproductive organs in rodents are very complex and the underlying mechanisms remain to be fully determined. Recently, some investigators have reported 'delayed' influences of perinatal exposure to estrogens or EDCs on the female reproductive system which are manifested after puberty or sexual maturation [25–27]. In fish, the ovo-testis can serve as a good indicator of estrogenic effects of EDCs [28]. For assessment to human health, it is very important to investigate the effects of low doses of EDCs, including BPA, on the reproductive organs at levels comparable to human exposure. Although there is as yet no consensus regarding the endpoint markers for detecting perinatal effects of EDCs in rodents, the following phenomena have been pointed out as perinatal effects of estrogens or EDCs with estrogenic activities: lowering of gonadotropin levels at prepuberty, anovulation, polycystic ovary, persistent estrus, early vaginal opening, abnormal development of uterus such as inhibition of uterine gland-genesis and abnormal expression of ERα, and increased uterine or vaginal carcinogenicity [10, 20–25, 27, 29, 30]. In particular, induction of uterine cancers by perinatal exposure to estrogens or EDCs with estrogenic activity is the most striking event, since natural occurrence of uterine cancer is generally rare in rats. Our co-workers found that uterine endometrial adenocarcinomas spontaneously developed in aged Donryu rats with a high incidence, and that the tumors showed a number of morphological and biological similarities to humans, such as ovarian hormonal imbalance leading to elevation of the serum estrogen/progesterone ratio [31–33]. Therefore, we selected this rat strain in the present study for the experimental animal.

In the investigations of maternal exposure to EDCs, especially with low dose exposure, it is very important to examine the biotransfer of chemicals from dams to offspring, because the effects of EDCs on the target organs are fundamentally related to serum EDCs level [34]. Although there has been much speculation about the potential adverse effects of low dose exposure to estrogenic EDCs including BPA [15–17], little information is available regarding test compound transfer from dams to offspring via the placenta or milk.

The purpose of the study was to investigate the effects of maternal exposure to low doses of BPA, at levels comparable to human exposure, on the growth and development of the female reproductive tracts, and also uterine carcinogenesis in rats observed from prepuberty up to 15 months of age, using the many endpoint markers reported previously. In addition, we monitored the transfer of BPA to offspring via the placenta and milk.

Materials and Methods

Animals

Forty-six pregnant female Crj:Donryu rats at gestation day (GD) 2, verified by plugs and sperm in the vagina and judged pregnant by the breeder, were purchased from Charles River Japan (Kanagawa, Japan).

Treatment of BPA

Animals were allocated into three groups: 0 mg/kg/day (control group, 12 dams), 0.006 mg/kg/day BPA (Tokyo Kasei Kagaku, Tokyo, Japan)(15 dams) group and 6 mg/kg/day BPA (19 dams). The concentration of 0.006 mg/kg was selected as relevant to provide the 63 ppb that is defined as the average daily intake from canned food in human beings [35]. The 6 mg/kg was selected as appropriate to simulate the maximum dose level (80 ppm) detected in plastic plates for children [35]. BPA was suspended in 0.05% carboxymethylcellulose solution (CMC; Wako Pure Chemicals, Osaka, Japan) for dosing. The dams

were orally administered BPA or the vehicle, 0.05% CMC (2 ml/kg body weight), every morning from GD 2 to the day before weaning (21 days after delivery) by gavage. The treatment period was selected to observe the effects of maternal treatment with BPA for as long as possible.

Examination of dams

Body weights of dams were checked once a week during the pregnancy and lactating periods. All dams were observed at least twice a day for morbidity, mortality and treatment-related clinical signs. The day of birth was designated postnatal day (PND) 0. After delivery, dams with offspring were housed in plastic cages containing wooden chips, and litter sizes were adjusted to 8–10 pups/dam at PND 4 or 6. All dams were euthanatized at weaning (PND 21) and the numbers of implantation sites in the uterus were recorded after complete necropsy. The uterus, vagina, ovaries, pituitary, adrenals, liver and kidneys were fixed in 10% neutral buffered formaldehyde solution, routinely processed and examined histopathologically.

Examination of offspring

Body weights, sex, external abnormalities and the number of offspring: The number and sex of offspring were checked at PNDs 1. At PNDs 1, 7, 14 and 21, body weights and external abnormalities were examined.

Uterine development: Three to 5 animals from different dams per group were euthanatized at PNDs 10, 14, 21, 28 and 8 weeks of age to investigate uterine development in female offspring except uterine weights at PND 10 and uterine gland genesis at 8 weeks. After the uteri were weighed, uterine gland-genesis in the uterine horn was histopathologically quantified as follows. The uteri were fixed in 10% neutral buffered formalin solution, 4 to 7 cross sections per uterine horn were dissected from the upper, middle and lower parts of the uterine horn, and routinely processed for histopathology. The number of uterine glands in 4 to 7 cross sections per animal was measured and the average number of glands was calculated for each group. To observe ERα expression and cell proliferating activity in the developing uteri, the same uteri as those measured for uterine gland genesis were incubated with anti-ERα (DakoCytomation, Kyoto, Japan) and anti-proliferating cell nuclear antigens (Dako Japan) at PNDs 10, 14, 21 and 28, and the expression against their antibodies was examined immunohistochemically.

Ovulation: On the morning of estrus stage at 8 weeks of age, 4 animals from different dams per each group were euthanatized and the number of ova in their oviducts were counted.

Age of vaginal opening and estrous cyclicity: All female offspring were checked daily for vaginal opening. After the opening, estrous cyclicity was examined by vaginal cytology throughout the study.

Hormone profiles: Blood from the animals used for examination of uterine development was collected by decapitation and the serum was stored at –80C until assay. Up to PND 14, pooled serum samples from the animals examined for uterine growth and gland genesis were used. Serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels at PNDs 10, 14, 21 or 28 were measured using NIDDK-rat-FSH and -LH radioimmunoassay (RIA) kits (NIAMDD; NIH, Bethesda, MD, USA), and compared among three groups, according to the method reported previously [36,37].

Uterine carcinogenicity study: For initiation of carcinogenesis, female pups (35, 36 or 35 animals in 0, 0.006 or 6 mg/kg group, respectively) at 11 weeks of age were administered a single dose of 20 mg/kg N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG; Nacalai Tesque Inc., Tokyo, Japan) into a uterine horn using a stainless steel catheter via the vagina, as reported previously [38]. The ENNG-treatment is reported to exert no toxic or carcinogenic effect on tissues or organs other than the uteri in rats [38]. At the termination of the experiment, all surviving animals (15 months of age) (24, 30 and 30 animals in 0, 0.006 and 6 mg/kg groups, respectively) underwent histopathological examination. Animals found dead and sacrificed when moribund were also examined similarly. After complete necropsy, the reproductive and representative organs were fixed in 10% neutral buffered formalin, and then routinely processed. Each uterus was cut into about 12 slices in cross-section for hematoxylin and eosin staining. Endometrial proliferative lesions were classified into three degrees of hyperplasia (slight, moderate or severe) and adenocarcinomas, according to our categories described previously [39]. In addition,

adenocarcinomas were subdivided into well, moderately and poorly differentiated types, and also classified as to the degree of invasion: limited to the uterus, invading into the serosa and/or surrounding adnexae, and with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers [40].

Histopathological examination: The ovaries, vagina and other representative organs including liver, kidneys, brain and endocrine organs were fixed in 10% neutral buffered formaldehyde solution at PNDs 10, 14 21, 28 and 8 weeks of age for histopathological examination.

Serum and tissue concentrations of BPA in dams and their offspring

In dams, BPA concentrations in the serum at weaning (PNDs 21) of their offspring and in milk collected in the stomachs of their pups at PNDs 10 and 14 were analyzed by gas chromatography mass spectrometry (QT-5050; Shimadzu, Kyoto, Japan) using the modified method reported previously [34]. Samples of milk were pooled from each litter for analysis.

In offspring, BPA concentrations in the serum and the liver were sequentially measured at PNDs 10, 14, and 21 in the same manner as their dams.

Housing conditions including measurement of environmental BPA

Animals were maintained in an air-conditioned animal room under constant conditions of 24 ± 2C and 55 ± 10% humidity with a 12 h light/dark cycle (light, 0800–2000 h; dark, 2000–0800 h). All pups were weaned at PND 21 and female offspring in the same treatment group were housed 3 or 4 pups per cage. Commercial pellet diet and drinking water were available *ad libitum*, and animals drank tap water stored in plastic containers throughout the study. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

To examine environmental BPA, concentrations of environmental BPA in the animal room, samples of fresh tap water, drinking water stocked in plastic containers used for water supply to the animals and in fresh pellet diet were determined using high performance liquid chromatography (HPLC). For tissue preparation, an internal standard (dimethylbuthylidene-bisphenol) was added to tap water and drinking water samples and evaporated to dryness for sample preparation. Pellet diet samples were grained, added to distilled water and 2 N sodium hydroxide and sharken for 1 hr. The samples were centrifuged and the aqueous layer was added to an internal standard and 2 N hydrogen chloride. The mixture was extracted twice with ethyl acetate and the organic solvent layers were evaporated to dryness. Both the residue of water and pellet diet samples were dissolved in 60% acetonitrile solution and subjected to HPLC analysis. HPLC was carried out using a M-600 pump (Waters, USA), Mightysil RP-18GP (Kanto Kagaku Co. Ltd, Tokyo, Japan) and a F-1080 fluorescence detector (Hitachi Co., Tokyo, Japan) [41].

Statistical analysis

Values for incidences were statistically analyzed using Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between BPA-treated and control groups were made with the Dunnett's t-test. P values less than 0.05 were considered to be statistically significant.

Results

The body weights were similar in the control and treated groups during the BPA-treatment period (GD2 to PND 21), and no treatment-related clinical signs were observed in dams (data not shown). Table 1 summarizes data for reproductive ability of dams. There were no significant differences among the groups in all parameters: gestation period, the number of implantation sites, the average number of offspring per litter, and the body weights of offspring at birth. No external abnormalities were detected in any offspring. The body weights of female offspring were similar among control and treated groups from puberty up to 15 months of age.

The days of vaginal opening of offspring are shown in Table 1. No significant inter-group differences were found. After vaginal opening, precise 4-day cyclicity was observed in all animals. Table 2 shows uterine weights and uterine gland genesis from PNDs 10 up to 8 weeks of age. At PNDs 14, 21, 28 and 8 weeks of age, uterine weights did not differ among three groups. Sequential changes in the number of uterine glands in the

Table 1. Dams and offspring data during gestation and lactation period

Dose	0 mg/kg/day	0.006 mg/kg/day	6 mg/kg/day
Number of dams examined	12	15	19
Gestation period (days)	22.0 ± 0	21.9 ± 0.5	22.1 ± 0.2
Number of pups/dam at birth (A)			
Total	12.8 ± 2.0	14.1 ± 1.3	13.7 ± 2.0
Female	6.1 ± 2.2	7.1 ± 1.3	6.9 ± 2.0
Male	6.7 ± 2.6	7.0 ± 1.1	6.9 ± 2.5
Number of implantations (B)	13.4 ± 2.7	14.8 ± 1.0	14.9 ± 1.4
A/B	0.96 ± 0.05	0.95 ± 0.07	0.92 ± 0.13
Body weights of pups			
Females			
PND 1	5.89 ± 0.35	5.87 ± 0.34	5.52 ± 0.78
PND 7	13.90 ± 1.43	14.04 ± 1.25	14.12 ± 1.03
PND 14	28.32 ± 1.78	29.06 ± 1.97	29.67 ± 1.86
PND 21	44.13 ± 2.44	45.18 ± 2.42	45.58 ± 2.37
Males			
PND 1	6.24 ± 0.35	6.21 ± 0.33	6.10 ± 0.66
PND 7	14.90 ± 1.43	15.11 ± 1.20	14.86 ± 0.69
PND 14	29.61 ± 1.92	30.72 ± 1.56	29.77 ± 1.68
PND 21	46.13 ± 2.66	47.97 ± 2.45	47.69 ± 2.51
Age of vaginal opening of female offspring (days)			
	29.4 ± 1.9	29.5 ± 1.4	30.0 ± 1.4

PND, post natal day. Values are means ± SD.

Table 2. Sequential changes of uterine weight and gland genesis of female offspring

	Doses of BPA		
	0 mg/kg	0.006 mg/kg	6 mg/kg
Number of female offspring examined			
PND 14	5	3	4
PND 21	4	4	4
PND 28	3	3	3
8 weeks of age	3	3	3
Uterine weight (mg)			
PND 14	36.8 ± 4.6	39.7 ± 5.5	32.0 ± 3.4
PND 21	51.0 ± 4.2	44.0 ± 6.2	48.3 ± 9.7
PND 28	218.5 ± 23.9	384.7 ± 177.1	318.3 ± 114.8
8 weeks of age[a)]	488.3 ± 33.5	540.7 ± 107.0	533.3 ± 55.8
Uterine gland genesis (number of glands in 3–7 cross sections)			
PND 10	0 ± 0	0 ± 0	0 ± 0
PND 14	5.14 ± 1.64	4.90 ± 0.99	4.13 ± 1.36
PND 21	4.76 ± 1.35	5.85 ± 1.68	4.76 ± 1.73
PND 28	7.63 ± 2.19	7.23 ± 2.89	8.38 ± 1.85

PND, post natal day. Values are means ± SD. a) The animals were euthanatized in the morning at estrus.

treated groups at PNDs 10, 14, 21 and 28 were similar to those in the control group. No obvious morphological changes, including expression of ERα and the labeling index for cell proliferation activity in the uterus were observed in either of the BPA-treated groups before puberty (Fig. 1). In all of the 3 groups, persistent estrus, characterized by vaginal smears exhibiting nucleated epithelial and/or cornified cells, began to appear after 5 months of age and then gradually increased with age, so that

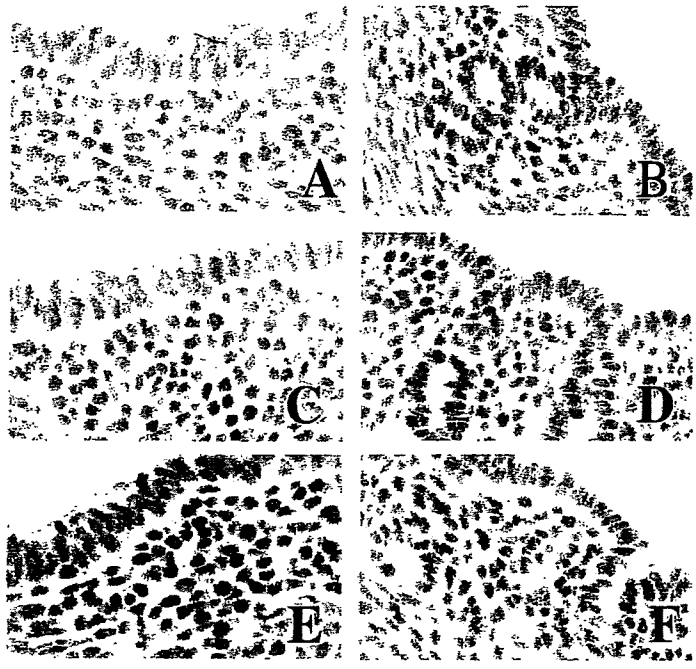

Fig.1. Immunohistochemistry of estrogen receptor α in the uteri of female offspring at post natal days (PNDs) 10 (A, C, E) and 14 (B, D, F) in 0 mg/kg (A, B) and 0.006 mg/kg (C, D) and 6 mg/kg (E, F) bisphenol A(BPA)-treated groups. At PND 10 ERα was expressed in stromal cells of the endometrium but not luminal epithelial cells. At PND 14, uterine glands developed into the endometrium, and ERα was expressed in both luminal and glandular epithelium. There were no differences in ERα expression of the uterus and gland genesis at PNDs 10 and 14 among the control and BPA-treated groups.

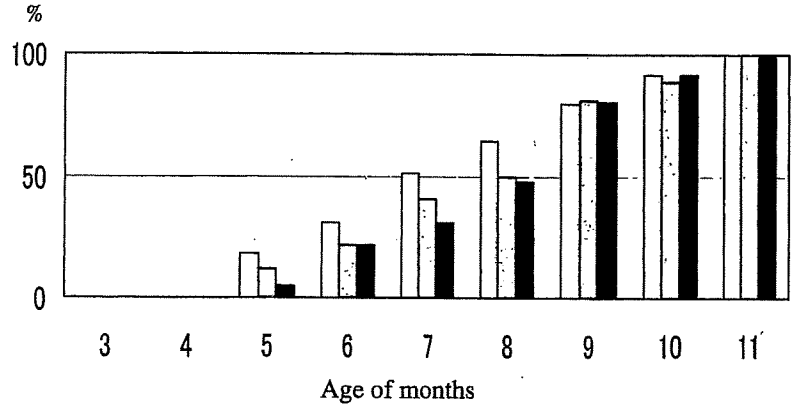

Fig. 2. Sequential development of persistent estrus in female offspring perinatally exposed to 0, 0.006 or 6 mg/kg bisphenol A (BPA). White, grey or black column indicates offspring treated with BPA at doses 0 mg/kg/day, 0.006 mg/kg/day and 6 mg/kg/day, respectively.

all animals were affected by persistent estrus at 11 months of age (Fig. 2). In endocrine tissues and representative organs such as those of the alimentary, urinary, respiratory and nervous systems, treatment-related lesions were not morphologically detected.

The average numbers and SD values of ova at 8 weeks of age were 13.3 ± 1.0, 13.0 ± 2.8 and 12.7 ± 1.2 in control, 0.006 and 6 mg/kg BPA-treated groups, respectively, with no significant differences.

Serum gonadotropin levels for BPA-treated and control rats in the immature period are shown in Fig. 3. During the immature period, serum FSH and LH levels were comparable among the BPA and control groups, the differences at each PND being not significant.

The incidences of uterine preneoplastic and

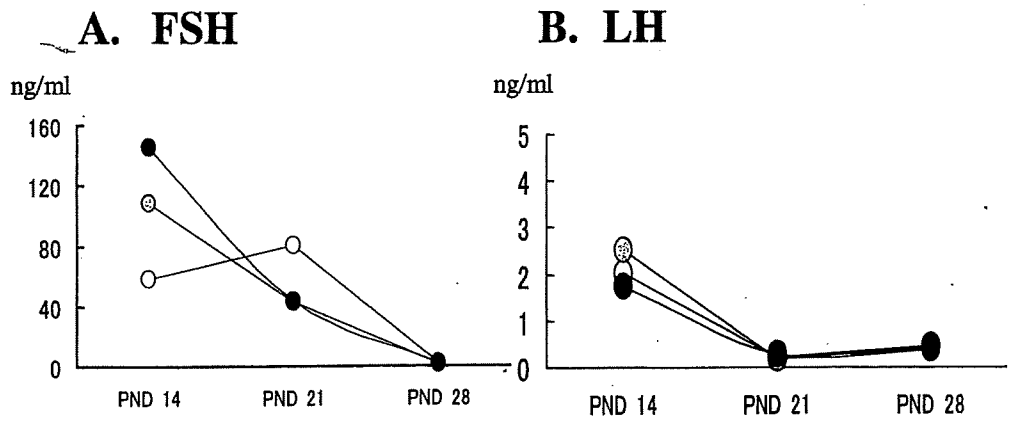

Fig. 3. Sequential change in gonadotropin levels (A, FSH; B, LH) of female offspring up to puberty in 0 mg/kg (white circle), 0.006 mg/kg bisphenol A (BPA) (grey circle) and 6 mg/kg/day BPA (black circle) groups.

Table 3. Proliferative lesions in the uteri and histopathology of the ovary at 15 months of age

Dose	0 mg/kg/day	0.006 mg/kg/day	6 mg/kg/day
Number of female offspring examined	24	30	30
Uterus:			
Hyperplasia			
Slight	4 (21)[a)]	6 (20)	4 (13)
Moderate	5 (21)	6 (20)	14 (47)
Severe	3 (13)	5 (17)	5 (17)
Endometrial adenocarcinoma	8 (33)	10 (33)	6 (20)
Sub-classification of adenocarcinoma			
Differentiation			
Well-differentiated	8	10	6
Moderately- or poorly-differentiated	0	0	0
Invasion			
Limited to the uterus	8	10	6
Invading into the serosa	0	0	0
Distant metastasis	0	0	0
Ovary:			
Atrophy with cystic follicles and absence of corpus luteum	24	30	29

a) Values in parentheses indicate percentage of incidence.

neoplastic lesions at the termination of the experiment are shown in Table 3. There were no significant differences or treatment-related tendencies among the groups. Sub-classification of adenocarcinomas with regard to their differentiation and invasion status also revealed no inter-group variation. Most of the ovaries in all groups showed atrophy with small cystic atretic follicles and an absence of any corpus luteum (Table 3). Various non-neoplastic and neoplastic lesions including mammary or pituitary tumors were observed of animals in all groups, but again the incidences were not significant among the three groups. In addition, the animals found dead or euthanatized when moribund showed no treatment related changes histopathologically.

Serum and tissue concentrations of BPA are shown in Table 4. BPA was detected in all serum and tissues examined in all groups of the present study. In dams, serum BPA was significantly

Table 4. BPA concentration (ppb) in the serum, milk and liver of dams and pups

Sample	Age of examination		Doses of BPA (ppb) 0 mg/kg		0.006 mg/kg		6 mg/kg	
Dam:								
Serum	PND 21 of offspring		3 ± 0	(5)	4 ± 0	(5)	11 ± 4*	(5)
Milk	PND 10 of offspring		28 ± 9	(3)	8 ± 21	(3)	8 ± 3	(3)
	PND 14 of offspring		255 ± 78	(4)	205 ± 7	(3)	185 ± 50	(4)
Offspring:								
Serum	PND 10	Females	4	(6)	10	(3)	23	(4)
		Males	15	(3)	5	(3)	7	(3)
	PND 14	Females	5	(2)	4	(3)	3	(4)
		Males	4	(2)	5	(6)	4	(6)
	PND 21	Females	9	(4)	3	(4)	9	(4)
		Males	14	(2)	9	(4)	20	(5)
Liver	PND 10	Females	13	(6)	12	(3)	17	(4)
		Males	9	(3)	9	(3)	14	(3)
	PND 14	Females	22	(2)	100	(3)	18	(4)
		Males	45	(2)	14	(6)	16	(6)
	PND 21	Females	60	(4)	70	(4)	37	(4)
		Males	69	(2)	9	(4)	60	(5)

PND, post natal day. Values are means ± SD. Values in parentheses are numbers of animals examined. The serum and liver tissues obtained from offspring were pooled for analysis. *$p<0.05$.

Table 5. Environmental BPA

Instruments or diet	Concentration of BPA	
Fresh tap water	0 ± 0 ng/ml	(3)
Drinking water stored in plastic containers	2.56 ± 2.51 ng/ml	(3)
Pellet diet (on opening)	40.06 ± 2.59 ng/g	(3)
Pellet diet (several days after opening)	39.70 ± 0.56 ng/g	(3)

Values are means ± SD. Values in parentheses indicate numbers of samples.

elevated in the 6 mg/kg group as compared to the controls. BPA levels in the milk, however, did not significantly vary among the BPA-treated and control groups. In the offspring, there were no differences in BPA levels in the serum and liver among BPA-treated and control animals from PND 10 up to PND 21.

The concentrations of environmental BPA in the animal room specimens are shown in Table 5. BPA was not detected in fresh tap water, but was identified in drinking water stored in the plastic containers. BPA was also detected in fresh pellet diet at levels several times higher than in the stocked water.

Discussion

The present study was performed to investigate the effects of maternal exposure to low doses of BPA, at levels comparable with human exposure, on growth and development of the female reproductive system and uterine carcinogenesis in Donryu rats. Unappropriate maternal and/or neonatal exposure to estrogens has been well known to exert irreversible influence directly and indirectly on the reproductive system. The typical influences called 'androgenization' are characterized by lowering of the gonadotropin levels in prepuberty, anovulation, polycystic ovary and persistent estrus immediately after vaginal opening, resulting from direct modulation of the hypothalamic-pituitary-gonadal axis [21, 23]. Androgenized uteri showed abnormal development such as inhibition of uterine gland-genesis or abnormal expression of ERα, and these effects were detectable until maturation [20, 29]. In addition, perinatal exposure to high-doses

estrogens or EDCs with estrogenic activity induced a 'delayed' influence with a different phenotype from that of typical androgenization and is probably caused by delayed modulation of the hypothalamic-pituitary-ovarian control system [25–27]. For instance, first 5 days exposure after birth to 100 mg/kg *p-tert*-octylphenol, this dose is estrogenic [42] and extremely higher (about $\times 10^6$) than waste water, caused a 'delayed' influence which was characterized by accelerated appearance of atrophic ovary compared to controls. This was manifested by an early occurring and a long-term continuing persistent estrus status, whereas no abnormalities could be found with regard to growth and development of the reproductive organs and the hypothalamic-pituitary-gonadal control system up to maturation [27]. In the present study, the treatment did not exert any influences on the reproductive ability of the dams and also on the uterine growth and development of BPA-treated offspring with reference to ERα expression, cell proliferating activity and gland genesis in the uterus, estrous cyclicity, vaginal opening and hormonal secretion up to sexual maturation. These results demonstrate no influence of low doses of BPA on the hypothalamic-pituitary-gonadal control system and the reproductive system up to puberty. After maturation, no disruption of ovarian function reflected by vaginal cytology was also noted, indicating no 'delayed' modulation effects on the reproductive system under the present experimental conditions. Vaginal cytology or its morphological feature in the vagina might be useful for assessment of the individual hormone milieu including dysfunction of the hypothalamic-pituitary-ovarian axis, as previously reported [43].

The most striking examples of changes caused by prenatal exposure to EDCs in the reproductive system of humans and rodents are uterine or vaginal cancers [29, 30, 44, 45]. Many studies have also demonstrated the induction of uterine endometrial adenocarcinomas in rats by perinatal treatment with estrogenic compounds [27, 46]. Uterine endometrial adenocarcinoma is one of the most common malignant tumors in women and has increased in number in recent years, although some epidemiological aspects remain unclear [47, 48]. The Donryu strain rat is a high-incidence strain for spontaneous endometrial adenocarcinoma development with aging, and the tumors have morphological and biological similarities to those found in humans [30, 31]. In this strain, earlier occurrence of ovarian atrophy with cystic atretic follicles but without corpus luteum leads to ovarian hormonal imbalance, resulting in prolonged elevation of the serum estrogen/progesterone ratio and then early onset of persistent estrus [33]. Under such characteristics, spontaneous uterine cancer development is ascribed to the age-dependent modulation of the ovarian hormonal control, as similarly evidenced in humans [49]. We also reported that neonatal exposure of Donryu rats to high-dose *p-tert* octylphenol enhanced uterine carcinogenesis with prolonged persistent estrus status [27]. The present study clearly demonstrated that maternal treatment with low doses BPA did not affect ovarian function manifesting as vaginal cytology throughout the experiment or susceptibility to uterine carcinogenesis. It might be recommended that relatively long-term comprehensive studies of endocrinological and morphological aspects are necessary for determination of perinatal effects of EDCs.

When considering about effects of maternal exposure to low doses of EDCs on the offspring, the subject of most concern is transfer of EDCs from dams to pups through the placenta and/or milk and subsequent modification of toxicokinetics [50], however, the data about transfer of test compounds are quite limited. BPA is known to form its major metabolite, bisphenol A glucuronide in the liver and is excreted very quickly via feces and urine [51–53]. In the present study, serum BPA levels were elevated in the dams given 6 mg/kg BPA, but BPA was not elevated in the milk, serum or liver of offspring. Surprisingly, however, BPA was detected in the serum and tissues of all the animals examined, including the controls. Furthermore, environmental BPA was found in the drinking water and more prominently in the diet. The increase in the serum of dams at 6 mg/kg group might be related to the BPA-treatment; however the influences of biotransfer of 0.006 mg/kg BPA could not be decided in the present study due to the environmental BPA. In the animal room, there were a number of instruments made with BPA such as plastic cages and water containers, and pellet diet and wood chips are packed in plastic wrapping. Therefore, the presence of environmental BPA in the present study is not considered to have been an incidental contamination, and indicates the possibility that

animal studies using rats or mice are always exposed to environmental BPA. While the influence of long-term exposure to environmental BPA on experimental animals remains to be determined, the possibility that major effects on the female reproductive system occur is unlikely, since the present data were similar to those of relevant studies previously reported in rats [10, 54] and our background data [55, 56]. In addition, any abnormalities, which are defined as effects of perinatal exposure to estrogens or EDCs with estrogenic activity on the female reproductive system, or suggested disruption of the hypothalamic-pituitary-gonadal axis were not detected in the present study [10, 20–27, 29].

In conclusion, transplacental and lactational exposure to BPA at levels comparable to human exposure did not exert any adverse influence on the female reproductive system such as uterine growth and development, and uterine carcinogenesis. Maternal exposure to BPA did not result in appreciable transfer to the offspring, although serum BPA levels in dams treated with 6 mg/kg BPA group were significantly elevated. The situation, however, is complicated, because low doses of environmental BPA were detected in the drinking water and diet.

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Review

Toxicologic/carcinogenic Effects of Endocrine Disrupting Chemicals on the Female Genital Organs of Rodents

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Abstract: Toxicologic/carcinogenic effects of some representative endocrine disrupting chemicals (EDCs) having estrogenic activity, such as alkylphenols, on the female genital organs of rodents, especially rats, are reviewed and discussed, focusing on our recent research. Neonatal treatment of high-dose p-tert octylphenol (t-OP, 100 mg/kg s.c. injection every other day from postnatal day 1 (PND 1) to PND 15) induced various long-term persistent irreversible effects on the female reproductive system of Donryu rats, such as lower gonadotropin levels at prepuberty, inhibition of uterine gland genesis, persistent estrus and polycystic ovaries. The result indicates that neonatal high-dose treatment of estrogenic EDCs can affect gonadotropin secretion during the developmental period of sexual maturation with direct masculinization of the hypothalamic function. Exposure limited to the first 5 days after birth (PNDs 1–5) to 100 mg/kg t-OP, however, caused delayed influence which was characterized by accerelated appearance of atrophic ovary, manifested by early-occurring and long-term continuing persistent estrus after puberty, whereas no abnormalities could be found with regard to growth and differentiation of the reproductive organs and the hypothalamo-pituitary-gonadal control system up to maturation, the influence being caused by delayed modulation of the hypothalamo-pituitary-gonadal control system. The most notable effect on the female reproductive system when normal cycling rats were exposed to high-doses t-OP for 28 days, was disappearance of the estrous cycle, but no clear changes were detected in other parameters such as uterine weight and morphology. These results indicate that the most serious issue with EDCs is the potential effects of prenatal and/or neonatal exposure on rodents. Well or moderately differentiated adenocarcinomas increased in Donryu rats initiated with N-ethyl-N'-nitro-N-nitrosoguanidine, when high-dose t-OP was given subcutaneously during adulthood. Neonatal exposure for PNDs 1–5 to high-dose t-OP also showed promoting effects on uterine adenocarcinoma development. However, in rats given t-OP for PNDs 1–15, uterine tumor malignancy was clearly increased, although there was no significant alteration in the total incidence of adenocarcinomas. The results are very interesting in consideration of the histogenesis of uterine adenocarcinomas. However, maternal exposure to low doses of EDCs such as nonylphenol and bisphenol A at actual human exposure levels by the oral route showed no effects on growth and development of the female reproductive system or uterine carcinogenesis. These results indicate that dietary exposure to low doses of EDCs might not induce any adverse effects on the female genital system in mammals including humans. (J Toxicol Pathol 2004; **17**: 69–83)

Key words: endocrine disrupting chemicals (EDCs), toxicity/carcinogenicity, female genital organs, rodents

Introduction

Recently, the possible adverse consequences arising from the release of man-made substances with estrogenic, anti-estrogenic or androgenic properties, so called endocrin disrupting chemicals (EDCs), into the environment have become an important environmental problem. There is much concern that these EDCs may have the potential to disturb normal sexual differentiation and development in wild life and mammals, including humans, and exert various deleterious effects on many organs, with carcinogenic effects being particularly important in mammals. The genital organs are the obvious target organs of various EDCs, and various toxicologic changes have been reported to be induced in both male and female genital organs of rodents. Unfortunately, however, there is less information

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on females than males, although many EDCs have estrogenic properties.

In the present report, toxicologic/carcinogenic effects of some representative EDCs, such as alkylphenols, on the female genital organs of rodents, especially rats, are briefly reviewed and discussed from the point of view of extrapolation to humans, mainly focusing on our recent research. In our studies of the effects of EDCs, Donryu rats were mainly used. The Donryu rat is a unique domestic strain having a regular 4-day estrous cycle at the juvenile stage. After 5 months of age, however, persistent estrus appears and increases age-dependently, and a high occurrence of spontaneous uterine adenocarcinomas is observed at about 2 years of age or thereafter (Table 1)[1]. In this rat strain, the early appearance of persistent estrus results in an increase in the estrogen (E2):progesterone(P) ratio (E2:P ratio). In humans, it has been reported that relatively high E2:P values increase the endometrial cancer risk[2,3]. Using this strain, we recently demonstrated effects of reproduction on uterine carcinogenesis, in line with the known lower risk of uterine adenocarcinoma in multiparous as compared to nulliparous or infertile women. The incidence of spontaneous endometrial adenocarcinomas showed a tendency to decrease in animals having three reproductive experiences, compared to the nulliparous case, although the incidence was not influenced by a single pregnancy[4]. Thus, this rat strain is a good animal model for endometrial adenocarcinoma development due to the imbalance of endogenous steroid hormones, as found in humans. We also succeeded in obtaining high induction of tumors in this strain by single intra-uterine administration of N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), thereby establishing a two-stage uterine carcinogenesis model (Fig. 1)[1,5]. Quite recently, Vollmer[6] reviewed experimental endometrial cancer models, including Donryu rats, and considered them useful for studies on molecular aspects of endometrial cancer and carcinogenesis.

Table 1. Persistent Estrus and Spontaneous Uterine Tumors in Donryu and F344 Rats*

Sequential Changes in Persistent Estrus Incidences in Female Donryu and F344 Rats

	Incidence (%)						
Strain	4	5	6	8	10	12	15 (Months of age)
Donryu	0	17	32	64	87	88	85
F344	0	0	2	0	6	11	4

Main Spontaneous Uterine Tumors in Donryu and F344 Rats

Uterine tumors	Incidences (%)	
	Donryu	F344
Mean survival time (weeks)	108.8 (62–120)	114.1 (60–131)
Endometrial adenocarcinoma	35	1
Endometrial stromal polyp	1	21

*: Maekawa *et al.*, J Toxicol Pathol 1999; 12: 1–11.

Classification of EDCs

Various chemicals have been shown to have endocrine disrupting effects not only on wildlife but also mammals including humans. Major representative EDCs are as follows, according to their use, chemical structures and/or chemical characteristics.

1. DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane), DDE (1,1'-(dichloro-ethenylidene)bis(4-chlorobenzene)), dieldrin: agricultural chemicals (insecticides) with properties of high-accumulation and resistance to degradation.

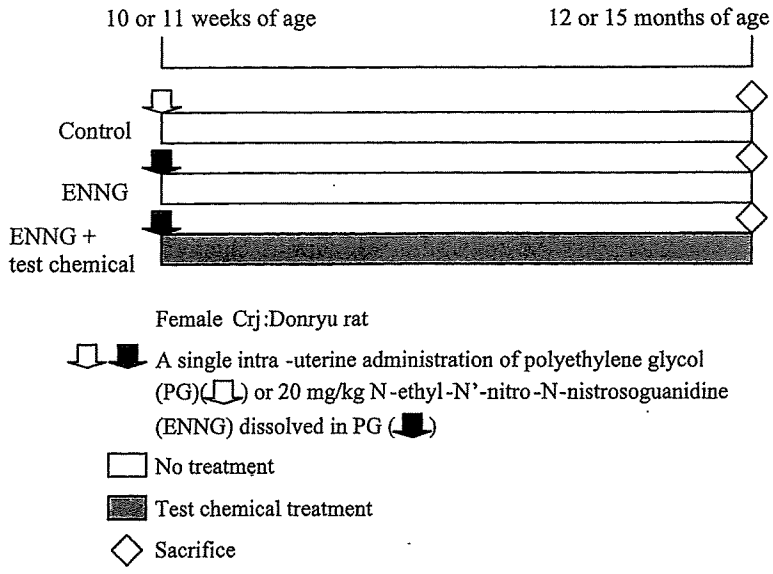

Fig. 1. Two-stage rat uterine carcinogenesis model

2. PCBs (polychlorinated biphenyl), PBB (polybrominated biphenyl): industrial chemicals (insulators etc.) which accumulate and are difficult to degrade.
3. DEHP (di(2-ethylhexyl)phthalate), alkylphenols such as nonylphenol and octylphenol, bisphenol A: industrial chemicals widely used as plasticizers or surfactants.
4. Dioxins such as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), dibenzofuran: chemicals naturally produced by dust-incineration.
5. TBT (tributyltin), TBTO (tributyltin oxide): industrial chemicals used for coating of ships' bottom.
6. DBCP (1,2-dibromo-3-chloropropane), atrazine, vinclozolin: agricultural chemicals.
7. DES (diethylstilbestrol), tamoxifen, oral contraceptives: medical drugs.

Development of the Female Genital Organs and Profile of Hormonal Secretions in Rodents

In general, development of the female genital organs in rats is roughly classified into 3 stages, prenatal (embryonic), neonatal/juvenile and adult/aged. The prenatal stage is from the day of fertilization, i.e., day 1 post-coitum till birth (about 20–22 days in rats). The embryonic bipotential gonad develops from mesoderm in the gonadal ridge located on the dorsal coelomic walls. The primordial germ cells and the gonadal ridge are visualized as condensations of cells localized ventral to the mesonephrons by gestational day 12 in rodents. In the embryonic developmental stage, two sets of paired tubular organs develop: the Wolffian ducts and the Mullerian ducts. It is well established that the presence or absence of functioning embryonic testes plays a major role in determining which duct system undergoes further development. In the rat, the critical time period for Mullerian duct development covers days 14–18 of gestation. On day 18, in the absence of testicular hormones including anti-Mullerian hormone (AMH) from Sertoli cells, the Mullerian ducts undergo further development and the Wolffian ducts degenerate.

The female reproductive tract of rodents is immature at birth and the developing uterus undergoes a period of rapid growth and differentiation during the first 2 weeks of postnatal life. In rats, the uterus at birth corresponds developmentally to the fetal uterus at gestation day 100 in human beings[7]. During this period, luminal epithelial cells invaginate into the underlying stroma to form uterine glands[8]. The uterine growth phase in this period coincides with an elevation of serum estradiol levels beginning on postnatal day (PND) 9. Thus, the role of endogenous estrogen (17β-estradiol, E2) and its receptor (ER) are very important for uterine growth and differentiation. In normal rats, ER expression in the uterine epithelium appears at various days from PND 7 to PND 15[9].

In female rats, serum FSH (follicle-stimulating hormone) levels rise to a peak at PNDs 15–16 followed by an abrupt nadir, while LH (luteinizing hormone) concentrations are high at PNDs 2–10 followed by gradual decline during sexual maturation; E2 levels also rise to a peak during the first 2 weeks of age[10,11]. On the other hand, α-fetoprotein, the estrogen-binding protein produced in the liver, is found in very high concentrations for several weeks after birth[12]. It is well known that the increase of FSH before puberty is caused by nullification of the negative feedback of estrogen because of the high concentrations of serum α-fetoprotein[13].

A striking sexual dimorphism in gross morphology of the medial preoptic area (sexually dimorphic nucleus of the preoptic area: SDN-POA) has been recognized in the rat brain[14]. The development of this nucleus starts during late fetal life and depends on the hormonal environment at the critical period of sexual differentiation[14–16]. In genetic males, the relatively high levels of perinatal testosterone are aromatized to estradiol in the nervous cells of SDN-POA and the estrogenic signals may be directly responsible for the increase of SDN-POA volume. In genetic females, in contrast, estrogenic effects on SDN-POA are prevented because estrogen is bound to the serum binding protein, α-fetoprotein, during the late embryonic and early neonatal periods. The female SDN-POA is smaller than that of males as a result of an orchestrated pattern of decreased cell proliferation and/or increased programmed cell death[17,18]. It is well known that the SDN-POA volume of genetic females becomes larger than normal on perinatal exposure to testosterone or high amounts of some estrogenic compounds[19]. Analogues of SDN-POA have also been identified in various animal species such as the gerbil, Guinea pig, ferret, quail and human[20]. Recently, another sexual dimorphism had been demonstrated in the anteroventral periventricular nucleus of the preoptic area (AVPvN-POA) and the locus coeruleus[21,22]. The volumes of these are larger in females than males, but a direct correlation with the hormonal environment has not yet been clarified.

In the rat brain, ERα expression is found primarily in ventral midline structures such as bed nucleus of the stria terminals, hypothalamic medial preoptic area, hypothalamic ventromedial nucleus, hypothalamic arcuate nucleus, septohypothalamic nucleus, septum and central gray area of the midbrain. ERβ is similarly distributed in the brain and is additionally detected in the paraventricular nucleus of the hypothalamus and the hippocampus[23,24].

After weaning, the first estrous cycle starts at about 36 days of age, and the minimum breeding age is about 84 days of age[25]. The estrous cycle is characterized by cyclic changes of the epithelial surface in the vagina and the uterus. The estrous cycle in the rat lasts 4–5 days and is divided into proestrus, estrus, metestrus and diestrus. Uterine weights increase with luminal excretion from diestrus to proestrus, showing a peak at proestrus, but decrease at estrus and metestrus.

In normal cycling rats, the E2 and P levels are highest at proestrus, corresponding with the increased uterine weights. Thereafter, the E2 level drops toward estrus and slightly increases again at diestrus. The P value increases slightly at metestrus, although it is low at estrus and diestrus. At