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

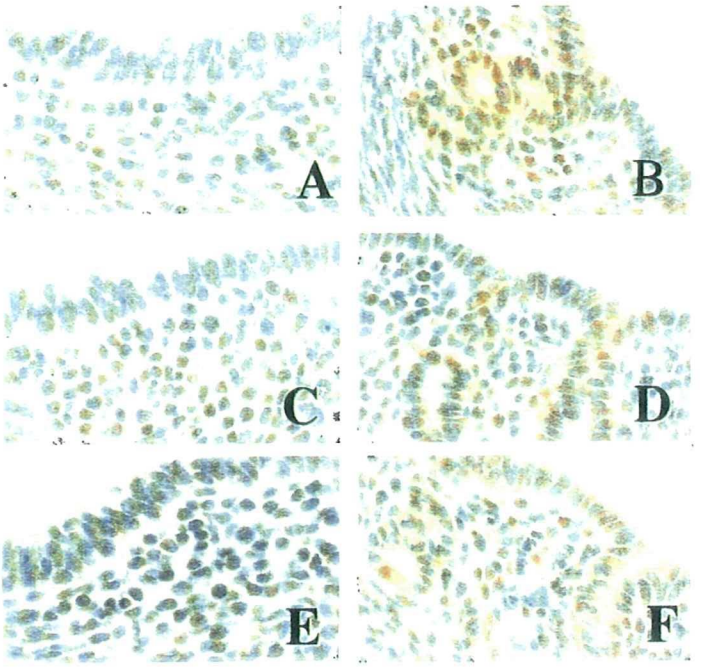

Fig.1. Immunohistochemistry of estrogen receptor α in the uteri of female offspring at post natal days (PNDs) 10 (A, C, E) and 14 (B, D, F) in 0 mg/kg (A, B) and 0.006 mg/kg (C, D) and 6 mg/kg (E, F) bisphenol A(BPA)-treated groups. At PND 10 ERα was expressed in stromal cells of the endometrium but 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.

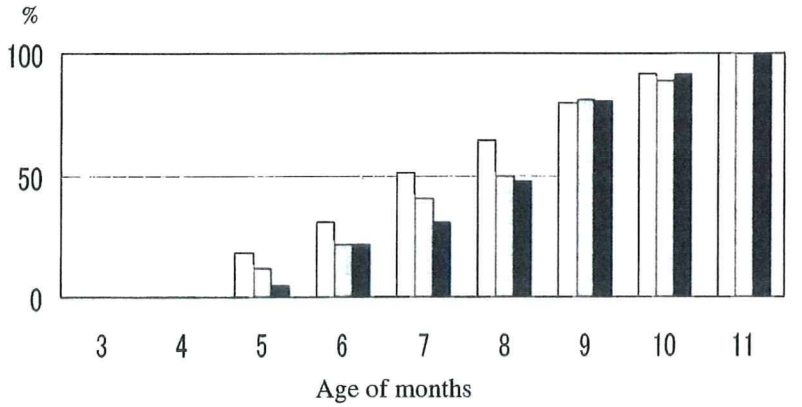

Fig. 2. Sequential development of persistent estrus in female offspring perinatally exposed to 0, 0.006 or 6 mg/kg bisphenol A (BPA). White, grey or black column indicates offspring treated with BPA at doses 0 mg/kg/day, 0.006 mg/kg/day and 6 mg/kg/day, respectively.

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

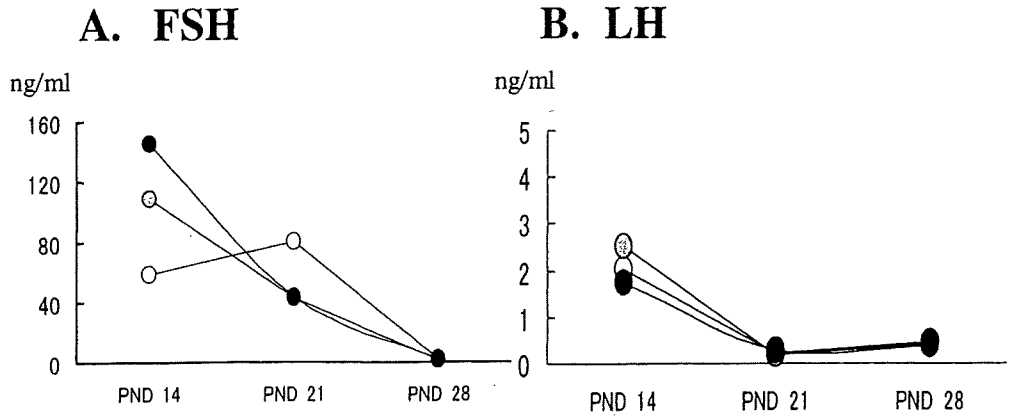

Fig. 3. Sequential change in gonadotropin levels (A, FSH; B, LH) of female offspring up to puberty in 0 mg/kg (white circle), 0.006 mg/kg bisphenol A (BPA) (grey circle) and 6 mg/kg/day BPA (black circle) groups.

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.

Acknowledgements

This study was supported by a Grant-in Aid from the Ministry of Health, Labor and Welfare of Japan. We sincerely thank Mr. Murakami K and Mr. Ochiai K for their expert animal technical assistance and Ms. Ichihara H for the histopathology.

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J Toxicol Pathol 2003; 16: 259–266

Original

Effects of Maternal Exposure to Nonylphenol on Growth and Development of the Female Reproductive System and Uterine Carcinogenesis in Rats

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Abstract: Effects of maternal exposure to nonylphenol (NP) on growth and development of the female reproductive system and uterine carcinogenesis in Donryu rats were investigated. Dams were administered 0, 0.1, 10 or 100 mg/kg NP daily by gavage from gestation day 2 up to the day before weaning. The treatment with NP did not influence the reproductive ability of the dams. In their female offspring, there were no significant effects on the reproductive system such as uterine growth and development, vaginal opening, and hormonal secretion until puberty. Moreover, NP had no apparent influence on estrous cyclicity after maturation, morphology of the reproductive organs, and uterine carcinogenesis initiated by N-ethyl-N'-nitro-N-nitrosoguanidine. Regarding biotransfer of NP, the chemical was detected at low levels in the milk of dams given NP at 10 and 100 mg/kg/day in a dose-dependent manner, but not in the serum. In the offspring also, NP was not detected in the liver in any of the treated groups. Taken together, maternal exposure of rats to 0.1 – 100 mg/kg NP did not have any effects on the female reproductive system of offspring from puberty up to 15 months of age. NP at 10 mg/kg and 100 mg/kg doses was transferred from dams to their offspring via the milk, but with these doses no accumulate in the liver of offspring was evident.
(J Toxicol Pathol 2003; **16**: 259–266)

Key words: nonylphenol, endocrine disrupting chemicals, female reproductive system, maternal exposure

Introduction

Alkylphenolic compounds are derived from biodegradation of nonionic surfactants, alkylphenol ethoxylates, which are widely used as lubricating oil additives, plasticizers, resins, detergents, and surface-active agents. The nonylphenol group of ethoxylates is broken down into nonyphenol (NP), mainly found in rivers[1]. NP exerts weak estrogenic activity *in vivo* and *in vitro*[2,3], binding to both estrogen receptors (ER)α and ERβ with low affinity[4]. Uterotropic effects of NP at high doses have been reported in immature or ovariectomized female rats[2,5,6], as well as with octylphenol[7–9].

The most serious issue with endocrine disrupting chemicals (EDCs) is potential effects of prenatal and/or neonatal exposure on offspring. Inappropriate exposure to endogenous and/or exogenous estrogens is known to induce irreversible change in the reproductive system, with an influence on uterine carcinogenesis[10–15]. However, the perinatal effects of EDCs on the reproductive organs in rodents are very complex and the underlying mechanisms remain to be determined in detail. In reproductive toxicity studies, high-dose NP exposure resulted in estrogenic-effects on pubertal development in male and female rats[16–18]. However, relatively low dose maternal or perinatal exposure to NP demonstrated no adverse effects on the reproductive tract in rodents[17], although perinatal treatment with estrogenic EDCs at doses comparable to human exposure levels has been reported to exert an influence[19–22]. For human risk assessment, it is very important to determine the effects of actual exposure to EDCs on reproductive organs, but the studies so far conducted in accordance to the test guidelines for safety evaluation did not demonstrate adverse effects or the results were controversial. One reason for the latter is the lack of established endpoint markers to detect maternal or perinatal effects of EDCs in rodents, especially females. It has been reported that a 'delayed' influence of EDCs on the reproductive system of rodents exposed

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perinatally may be manifested after puberty or sexual maturation[15,23,24]. Thus, relatively long-term comprehensive studies of endocrinological and morphological aspects might be necessary for determination of the impact of perinatal treatment with EDCs. At the same time, with maternal exposure it is crucial to examine biotransfer of chemicals from dams to offspring, because the effects of EDCs on the target organs are fundamentally related to serum levels[8]. Little information is available regarding test compound transfer from dams to offspring via the placenta or milk.

The purpose of the present study was to investigate the effects of maternal exposure to NP at low to high exposure-doses, with reference to growth and development of the female reproductive tract and also uterine carcinogenesis in rats. For these purposes a relatively long-term period from prepuberty up to 15 months of age was employed with many parameters to detect effects of EDCs reported previously. In addition, we assessed transfer of NP to offspring via the placenta and milk.

Materials and Methods

Animals, NP treatment, and housing conditions

Thirty seven pregnant female Crj:Donryu rats at gestation day (GD) 2, checked by plugs and sperm in the vagina and judged to be pregnant by the breeder, were purchased from Charles River Japan (Kanagawa, Japan) and allocated into four groups: 0 mg/kg/day (vehicle controls, 10 dams), 0.1 mg/kg/day NP (Tokyo Kasei Kagaku, Tokyo, Japan, 10 dams), 10 mg/kg/day NP (10 dams), and 100 mg/kg/day NP (7 dams). The highest dose is known to have adverse effects, with uterotropic- and cell-proliferative activity observed in the uterus and mammary glands in rats when given by gavage[25]. The middle-dose was selected as near the no observed effect level in multiple generation reproductive study using rats[16] and the lowest dose as relevant to human daily intake of isoflavones in Germany (1 mg/kg), because uterotrpopic activity of NP was reported to be 10 times stronger than that of daidzein, a major isoflavone[26]. NP was suspended in 0.05% carboxymethylcellulose (CMS) solution (Wako Pure Chemicals, Osaka, Japan) for this purpose. The females were orally administered NP or vehicle solution (0.05% CMC), every morning from GD 2 to the day before weaning (21 days after delivery) by gavage. The treatment period was selected to observe effects of maternal exposure to NP as long as possible. Commercial pellet diet (CRF-1, Oriental Yeast, Co., Japan) and drinking water stored in plastic containers were available *ad libitum* throughout the study. 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 PNDs 4 or 6. All pups were weaned at PND 21 and female pups in the same treatment group were housed together in cages (3 or 4 pups per cage). Animals were maintained in air-conditioned animal rooms under constant conditions of 24 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

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. Dams were euthanized at weaning (PND21 of their offspring) 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 and examined histopathologically.

Examination of offspring

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

Uterine growth and development at prepuberty and ovulation: To investigate uterine growth and development of female offspring up to puberty, 3 or 4 animals per group being different littermate were euthanized by decapitation at PNDs 10, 14, 21, and 28, the individual animals at each time-point being derived from different dams. After the uteri were weighed, the numbers of uterine glands were histopathologically quantified. Briefly, the uteri were fixed in 10% neutral buffered formalin solution, 21 cross sections per uterine horn were taken from upper, middle and lower parts of the bilateral uterine horn, and examined histopathologically. To assess ERα expression and cell proliferative activity in the developing uteri, serial uterine sections from slices used for measurement of uterine gland-genesis were incubated with anti-ERα and anti-proliferating cell nuclear antigen antibodies (Dako, Kyoto, Japan), for immunohistochemical comparison with control animals. In the morning of the estrus stage at 8 weeks of age, 4 animals per each group were euthanized and the numbers of ova in the oviduct were counted. The ovaries, vagina and other representative organs were fixed in 10% neutral buffered formaldehyde solution at PNDs 10, 14, 21, and 28 and 8 weeks of age and processed routinely for histopathological examination.

Hormonal profiles at prepuberty: Blood from the same animals used for histopathological examination was collected after decapitation and serum was stored at –80°C until assayed. Up to PND 14, pooled serum samples were used, since volume of serum per single animal was too small to allow analysis. Serum follicle stimulating hormone (FSH) and inhibin (INH) levels at PNDs 10, 14, 21, or 28 were measured using NIDDK radioimmunoassay kits for rat FSH[27–29].

Vaginal opening and estrous cyclicity: After weaning, female pups were checked daily for vaginal opening. After this was confirmed, estrous cyclicity in all animals was examined by vaginal cytology throughout the study.

Uterine carcinogenicity study: All female pups at 11

Table 1. Reproductive Ability and Body Weights of Offspring

	Group			
	0 mg/kg	0.1 mg/kg	10 mg/kg	100 mg/kg
No. of dams	10	10	10	7
Pregnant	10	10	10	7
At the termination of PND21	10	10	10	7
Pregnant period	21 ± 0.0 (#)	21.11 ± 0.33	21 ± 0.0	21 ± 0.0
No. of pups at birth (g)				
Female	5.6 ± 1.84	6.5 ± 1.84	6.3 ± 1.06	5.2 ± 2.66
Male	5.5 ± 1.96	5.9 ± 2.64	6.8 ± 1.75	6.5 ± 2.42
Total (a)	11.1 ± 2.13	12.4 ± 1.71	13.1 ± 1.20	12.0 ± 2.71
No. of dead pups during PNDs1–5				
Female	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Male	0 ± 0.00	0 ± 0.00	0.7 ± 1.89	0.3 ± 1.41
No. of implantation (b)	12.5 ± 1.35	13 ± 1.41	13.9 ± 0.99	13.0 ± 1.63
a/b	0.89 ± 0.15	0.95 ± 0.09	0.94 ± 0.07	0.9 ± 0.17

A ± B (#), Mean ± SD. PNDs, post natal days.

weeks of age were administered a single dose of 20 mg/kg N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG, Nacarai Tesque Inc., Tokyo Japan) into a uterine horn using a stainless steel catheter via the vagina, as reported previously[30]. At 12 months of age, 5 or 6 animals per group were euthanized and examined histologically to evaluate development of uterine proliferative lesions. At the termination (15 months of age), all surviving animals underwent a histopathological examination. After complete necropsy, the reproductive and representative organs were fixed in 10% neutral buffered formalin, and then routinely processed. Animals found dead and killed when moribund were also examined similarly. Each uterus was cut into about 12–16 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[31]. 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 tumors with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers[32].

Serum and tissue concentration of NP in dams and their offspring

Serum and milk of dams were collected at weaning from offspring and at PNDs 10, respectively. The milk in the stomachs of the male and female pups was collected and pooled for each litter. In offspring, NP levels in the liver were sequentially measured at PNDs 21 and 28. The analysis was accomplished by gas chromatography mass spectrometry (QT-5050, Shimadzu, Kyoto, Japan) using the modified method reported previously[8].

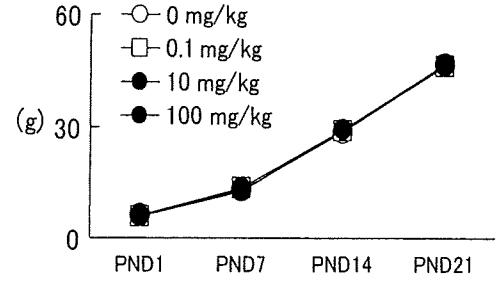

Fig. 1. Growth curve of female offspring up to the weaning. The body weights are comparable among the groups.

Statistical analysis

Values for incidences were statistically analyzed using the Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between NP-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 of dams were comparable in the control and treated groups during the NP-treatment period (GD2 to PND21) and no treatment-related clinical signs were observed in any treated groups. Table 1 shows data of reproductive ability of dams. Examinations at birth and necropsy revealed no significant differences among the groups in the gestation period, the number of implantation sites, the average number of offspring per litter, and the body weights of offspring.

In female offspring, the growth curves were comparable among control and treated groups from prepuberty (Fig. 1) up to 15 months of age and no external abnormalities were detected in any offspring. Data of

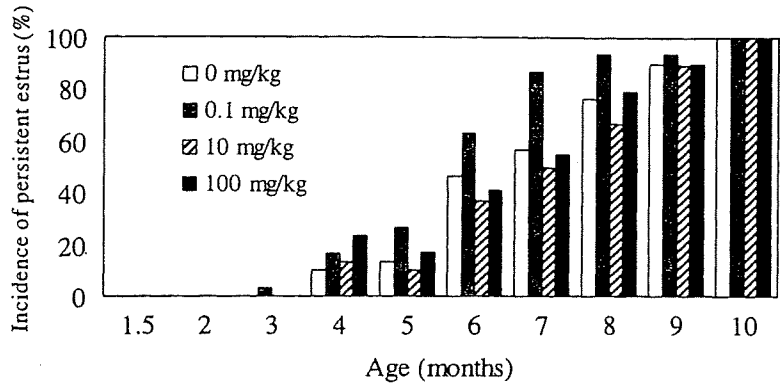

Fig. 2. The incidence (%) of animals showed persistent estrus (PE) at their vaginal cytology. The number of the animals with PE is increased with advanced age and their incidence is not significantly different among the groups.

Table 2. Growth and Development of the Female Reproductive Organs in Offspring

Time of examined	Group			
	0 mg/kg	0.1 mg/kg	10 mg/kg	100 mg/kg
Number of rats examined				
PNDs10	4	3	4	2
PNDs14	4	3	4	3
PNDs21	4	4	4	4
PNDs28	4	4	4	4
17wks*	4	4	4	4
Uterine weights (mg)				
PNDs14	26.8 ± 6.0	28.3 ± 5.0	32.3 ± 4.3	21.0 ± 1.0
PNDs21	42.3 ± 5.6	41.0 ± 7.2	38.3 ± 7.7	46.3 ± 3.8
PNDs28	141.6 ± 34.2	187.3 ± 137.4	242.3 ± 145.2	290.8 ± 134.6
17wks	712.3 ± 117.7	653.3 ± 76.8	653.3 ± 76.8	720.8 ± 113.1
Uterine gland-genesis (Number of gland per cross section)				
PNDs10	0	0	0	0
PNDs14	4.07 ± 1.49	4.27 ± 1.74	4.29 ± 1.59	3.00 ± 0.87
PNDs21	4.57 ± 1.60	4.76 ± 1.15	5.31 ± 1.49	4.78 ± 2.46
PNDs28	6.00 ± 2.62	5.92 ± 1.26	5.73 ± 2.34	5.07 ± 1.77
The time of vaginal opening (days)				
	29.6 ± 1.8	30.1 ± 1.3	29.8 ± 1.1	29.0 ± 1.4
Number of rats examined				
	34	34	34	34
Ovulation (Number of ova in the oviduct in the morning of estrus at 17wks of age)				
	11.3 ± 1.5	11.3 ± 1.5	11.5 ± 1.0	12.3 ± 0.6

*; Examined in the morning at estrus stage.

females are shown in Table 2; the days of vaginal opening of offspring demonstrated no significant intergroup differences. Thereafter, precise 4-day cycles of estrous stages started in all animals. Persistent estrus, characterized by vaginal smears exhibiting nucleated epithelial and/or cornified cells, began to appear after 5 months of age in control animals and gradually increased with age in this group, all animals showing persistent estrus at 10 months of age, as shown in Fig. 2. In all NP-treated groups also, occurrence of persistent estrus demonstrated a similar profile. At PNDs 14, 21, 28, and 8 weeks of age, uterine weights did not differ among the groups, and sequential changes in number of uterine glands in both treated and control animals were comparable (Table 2). No obvious changes in morphology, expression of estrogen receptor α or proliferative activity in the uterus were observed in any of the treated groups before puberty, compared to those in controls. Other endocrine tissues and representative organs showed no abnormalities in NP-treated and control groups. The numbers of ova at 8 weeks of age were not significantly different among the groups (Table 2).

Serum FSH and inhibin levels for NP-treated and

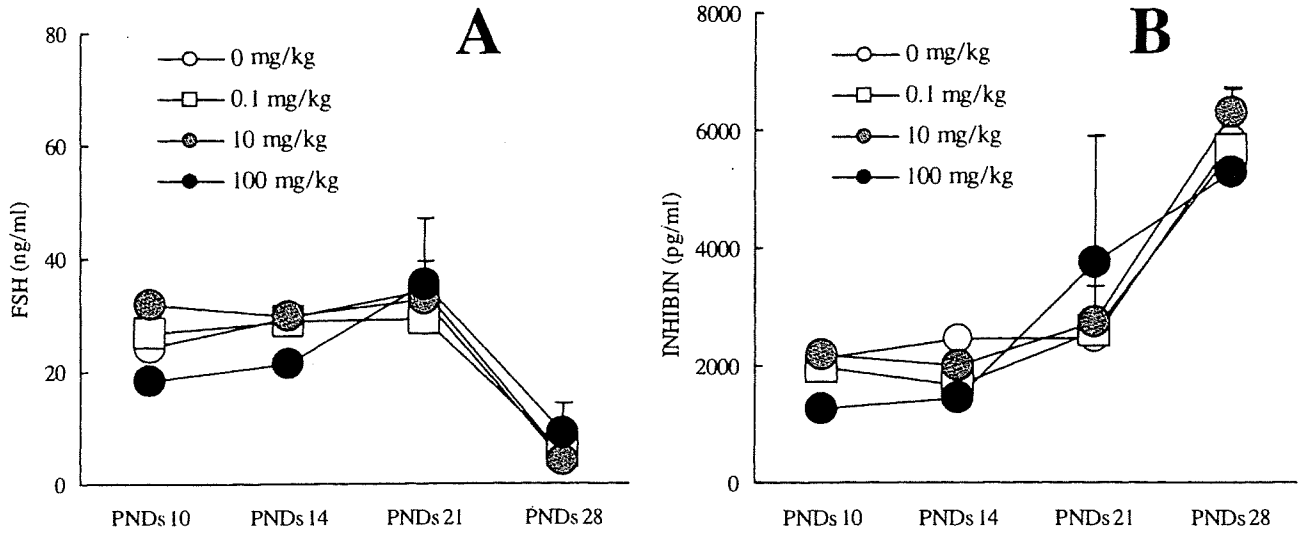

Fig. 3. Serum hormone profiles of follicle stimulating hormone, gonadotropin (A), and inhibin, a hormone secreted in the ovary (B) up to puberty. In all treated groups, FSH levels are comparable to that in controls. Inhibin levels were anti-related with levels of FSH.

Table 3. Incidence of Uterine Proliferative Lesions

Group	No. of rats examined	No. of rats without lesions	No. of rats with lesions			
			Hyperplasia			Adenocarcinoma
			Slight	Moderate	Severe	
0 mg/kg	24	3 (13)*	3 (13)	8 (33)	4 (17)	6 (25)
0.1 mg/kg	24	2 (8)	3 (13)	9 (38)	1 (4)	9 (38)
10 mg/kg	23	1 (4)	1 (4)	10 (43)	3 (13)	8 (35)
100 mg/kg	22	1 (5)	2 (9)	5 (23)	4 (18)	10 (45)

*( ); Percentage.

control rats at prepuberty are shown in Fig. 3. Up to PND 28, serum FSH and its linked inhibin levels were comparable among NP-treated and control groups, and the gonadotropins demonstrated no tendency for lowering with the treatment.

The incidences of uterine preneoplastic and neoplastic lesions are shown in Table 3. There were no significant differences and/or treatment-related tendencies among the groups. Sub-classification of adenocarcinomas by differentiation and invasion also demonstrated no variation. Most of the ovaries in all groups were atrophic with small cystic follicles and lacking corpus lutea. Various non-neoplastic and neoplastic lesions were observed in the representative organs and other endocrine tissues such as the liver, kidneys, adrenals, pituitary, and thyroids in all groups, although there were no significant differences among the NP-treated and control groups. Necropsy of animals found dead or euthanized when moribund did not reveal any treatment-related changes.

Serum and tissue concentrations of NP are summarized in Table 4. In dams, NP was detected in milk at PND 14 with dose dependence in the 10 and 100 mg/kg groups, but not serum. NP was not found to have accumulated in the livers of offspring in any of the treated groups at PNDs 21 and 28, the latter being 7 days after the final treatment.

Discussion

Inappropriate exposure to estrogens or EDCs with estrogenic activity in the fetal and/or newborn period is well known to exert irreversible influence on the female reproductive system due to disruption of the hypothalamic-pituitary-ovary controlled system. Typically 'androgenized' influences appear in prepuberty, characterized by lowering of gonadotropin levels, anovulation, hypoplastic ovary, persistent estrus status immediately after early vaginal opening, and abnormal uterine development, with inhibition of gland-genesis and anomalous ERα expression[10,14,15,33]. In addition to the typical 'androgenized' effects described above, perinatal exposure may also cause 'delayed' effects including the 'anovulatory syndrome'[15,23,24]. For example, high-dose exposure to *p-tert*-octylphenol, an alkylphenolic compound with weak estrogenic activity, during the first 5 days after birth in female Donryu rats exerted a delayed influence, detected as ovarian atrophy with polycystic

Table 4. Tissue and Serum Concentration of Nonylphenol

	Group			
	0 mg/kg	0.1 mg/kg	10 mg/kg	100 mg/kg
Dam				
Serum level at PNDs21	<0.1 ppm*	<0.1 ppm	<0.1 ppm	<0.1 ppm
Number of dam pooled	5	5	5	5
Milk at PNDs14	<0.1 ppm	<0.1 ppm	0.4 ppm	1.6 ppm
Number of pup stomach collected	8	8	8	6
Offspring				
Liver at PNDs21	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm
Number of pup pooled	4	4	4	6
Liver at PNDs28	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm
Number of pup pooled	4	4	4	4

*; Under the detectable limit.

Table 5. Comparison of Effects on the Female Reproductive System in the Present Study with Previous Studies Showing Typical Androgenized- or Delayed Androgenized-effects

	Nonyphenol by gavage (present study)			Octhylphsenol (previous studies)	
	0.1 mg/kg	10 mg/kg	100 mg/kg	Typical androgenization (a) 100 mg/kg subcutanrous	Delayed-androgenization (b) 100 mg/kg subcutaneous
Examinations at prepuberty					
Growth	N (c)	N	N	N	N
Uterine weight	N	N	N	N	N
Uterine gland-genesis	N	N	N	↓(d)	N
Expression of estrogen receptor in the uteri	N	N	N	Abnormal	N
Time of vaginal opening	N	N	N	Early opening	N
Gonadtoropin secretion	N	N	N	↓	N
Examinations after maturation					
Ovulation	N	N	N	Anovulation	N
Estrous cyclicity	N	N	N	PE (e)	Normal after vaginal opening but earlier occurrence of PE
Uterine carcinogenesis	N	N	N	Enhanced	Enhanced

(a); Exposure of octylphenol during first 15 days after the birth referred by Katsuda *et al.*, 2000A(14) and Yoshida *et al.*, 2002A(33).
(b); Exposure of octylphenol during first 5 days after the birth Referred by Yoshida *et al.*, 2002B(15).
(c); Normal or comparable data compared with those in the control animals.
(d); Decreased.
(e); PE, Persistent estrus at vaginal cytology.

follicles resulting in early persistent estrus compared to aged-matched control animals, although no apparent abnormalities were found up to maturation[15]. In the present study, however, NP-treated animals showed no abnormalities in gonadotropin and associated ovarian hormone secretion, or in uterine growth and development. Thus uterine gland-genesis and ERα expression as well as the time of vaginal opening and subsequent sexual maturation were comparable to those in controls. The treatment also did not exert any effects on ovulation and estrous cyclicity throughout the study, as compared to the age-matched control animals or our control data for the Donryu strain rat. The results clearly demonstrated that maternal treatment with 0.1 – 100 mg/kg NP did not exert any influence on the female reproductive system of offspring at prepuberty, and delayed modulation of the system after sexual maturation appeared lacking.

The most striking examples of the effects caused by EDCs on the female reproductive system are induction of vaginal or uterine cancers in humans and rodents[34–36]. Recently many studies of induction of uterine endometrial adenocarcinomas in rodents by perinatal treatment with estrogenic compounds or EDCs have been conducted[12,15,35,37]. The 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[38,39]. The Donryu strain rat is a high-incidence strain for spontaneous endometrial adenocarcinomas and the tumors have morphological and biological similarities to those found in

women[40–42]. Similar to the human case, ovarian hormonal imbalance is a crucial factor. In particular, the model features early occurrence of ovarian atrophy with cystic atresia follicles and lack of corpus lutea, associated with prolonged elevation of the serum estrogen/progesterone ratio and persistent estrus in vaginal cytology[43]. In the present study, maternal treatment of NP did not exert any influence on estrous cyclicity and uterine carcinogenesis. To determine ovarian function in rats, examination of estrous cyclicity might be the most useful indicator, as previously reported[44].

For assessment of effects of EDCs on offspring with maternal treatment, biotransfer from dams is crucial because the effects on the target organs are fundamentally related to serum EDCs levels[8]. However, data for transfer of test compound via the placenta or milk to offspring and toxicokinetics of low-dose EDCs were very limited[45]. In the present study, NP levels in the milk of the 10 and 100 mg/kg groups were elevated, but the compound was not detected in the serum of dams and the liver of offspring.

A summary of the present study results in comparison with androgenized- or delayed androgenized-effects reported previously is given in Table 5. We can conclude that transplacental and lactational exposure to various doses NP does not appreciably influence the growth and development of the female reproductive system or sensitivity to uterine carcinogenesis. No accumulation of the compound was found in the offspring livers, although NP was detectable in the milk at 10 and 100 mg/kg.

Acknowledgements: This study was supported by a Grant-Aid from the Ministry of Health, Labor and Welfare of Japan. We sincerely appreciate the expert animal technical assistance of Mr. K Ohara and Mr. K Ochiai, and excellent histopathological technique of Ms. Ichihara, H. and Ms. Asako H.

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P14

Differential Enhancement by Neonatal Exposure to *p*-tert-Octylphenol of Uterine Carcinogenesis in Donryu Rats Depending on the Administrating Periods

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p-tert-Octylphenol (OP) is an environmental endocrine disrupting chemical. Despite the very weak estrogenic activity, OPs potential carcinogenicity is not necessarily ruled out, especially in the cases of fetal or neonatal exposure. The present study was conducted to investigate effects of neonatal exposure to high-dose OP on uterine carcinogenesis and the development of the female reproductive tract in rats for different treatment periods. Female Donryu rats were subcutaneously administered 100 mg/kg body weight of OP every other day for the first 5 or 15 postnatal days (PND-5 or PND-15 cases, respectively, 55 rats each). Twenty of each was serially examined until puberty, 4 weeks of age. The remaining animals received a single injection of 20 mg/kg of *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (ENNG) into the uterine horn at 11 weeks of age were euthanized at 15 months of age to assess uterine carcinogenicity. Estrous cyclicity was checked after the varginal opening thoughout a 15-month study. In PND-5, the uteri were normally developed including the gland genesis, and most of animals showed regular estrous cyclicity after the vaginal opening. At 6 weeks of age, however, persistent estrus already began to appear, the incidence increasing with age to reach 100% by 6 months of age. The incidence of well-differentiated adenocarcinomas was increased at 15 months of age. In PND-15, persistent estrus occurred immediately after the vaginal opening and lasted throughout in all animals. Gland genesis was suppressed at prepuberty, reflecting the decreased induction of endomentrial hyperplasias at 15 months of age. While the incidence of adenocarcinomas was not altered, their malignancy, assessed in terms of both histological grade and degree of invasion, was aggravated. These results indicate that neonatal OP administration enhances ENNG-induced uterine carcinogenesis and that the enhancing effects are exerted differently depending on the OPs administrating periods. Although prolonged persistent estrus status under a high estrogen:progesterone ratio is involved in either case, OP delayed influence on the hypothalamus-pituitary-ovarian hormonal control after the sexual maturation might be involved in the enhancing development of adenocarcinomas in the PND-5 case. In the PND-15 case, OP might exert immediate effects to disturb the normal uterine development and then enhance the malignant conversion of adenocarcinomas.