proestrus and diestrus, and especially the former, the luminal and glandular epithelial cells along with stromal cells beneath the luminal epithelium are strongly positive for ER-alpha mRNA expression. At estrus, the expression is slightly diminished in luminal cells, but is almost completely lacking in glandular cells. At metestrus, positive signals appear again in the latter. In the myometrium, expression is constant in all estrous stages. Thus cell-type specific patterns of ER-mRNA expression characterize the uteri of normal estrous cycling rats[26].

In general, the adult stage in rats is from minimum breeding age to maximum age (about 360–450 days), and thereafter the aged stage starts[25]. In Donryu rats, however, estrous cycle abnormalities increase age-dependently after 26 weeks of age, and almost all animals show persistent estrus at 52 weeks of age. In contrast, vaginal smears of F344 rats indicate a normal estrous cycle until 52 weeks of age[27]. In our studies, various histological changes such as follicular cysts and atrophic changes such as absence of corpora lutea in the ovary and cornification of epithelium in the vagina in Donryu rats were observed to be linked with persistent estrus, and increased with time, especially after 10 months of age. In F344 rats, in contrast, atrophy of the ovary is observed in only a few animals at 15 months of age. As a result of ovarian atrophy, in Donryu rats, the plasma values of E2 and P, and especially the latter, decrease with age, the E2:P ratio becoming elevated, and the bromodeoxyuridine (BrdU)-labeling indices of uterine endometrial cells are age-dependently increased[28]. This age-related hormonal imbalance and the constant high level of proliferating activity of epithelial cells are considered to play important roles in high yield development of spontaneous uterine endometrial adenocarcinomas in this rat strain[1,28].

Toxicologic and/or Carcinogenic Effects of EDCs on the Female Genital Organs of Rodents

Rodents in the first 2 weeks of postnatal life, termed "a critical point" or "a window of vulnerability", are very sensitive to exogenous estrogens and androgens including EDCs, because the reproductive tract undergoes rapid growth and differentiation within this period, as mentioned above. Thus, the OECD (Organization for Economic Cooperation and Development) recently proposed the immature rat uterotropic assay as one of the screening test methods for the detection of estrogenic or anti-estrogenic properties of chemicals[29]. In studies using adult animals, the ovariectomized (OVX) animal model is also effective for the detection of estrogen agonists, because the effects of endogenous estrogen can be minimized[30]. In various toxicity studies using adult animals, oral administration has generally been used to assess the toxicity of chemicals, and the OECD has also proposed a new 28-day repeated oral-dosing toxicity test protocol using adult rats, the enhanced OECD TG407 protocol, for the assessment of the toxic effects of EDCs. For the detection of endocrine disrupting activity of direct-acting chemicals, however, other administration routes such as subcutaneous injection may provide greater sensitivity than oral administration, because this eliminates the direct effects of metabolism of the chemicals during first passage through the liver.

As mechanisms for the biological effects of EDCs on their target organs, their binding to growth factor receptors and arylhydrocarbon (Ah) receptors as well as steroid receptors has been considered to be very important. Furthermore, some chemicals have been shown to have effects on endogenous estrogen metabolism, resulting in disturbance of the hormonal milieu.

Effects of High-doses of EDCs
Effects on Growth and Development of Female Reproductive Organs
Prenatal and/or Neonatal Exposure

Inappropriate exposure to estrogens and also EDCs in the prenatal and/or neonatal period has been well established to exert irreversible influence directly and indirectly on the female reproductive system[31,32]. "Androgenization" is characterized by direct modulation of the hypothalamo-pituitary-gonadal control system, resulting in lowering of gonadotropin levels and persistent estrus as an indirect effect, and abnormal uterine/vaginal development and/or growth as direct influences.

Alkylphenolic compounds are derived from biodegradation of nonionic surfactants, alkylphenol ethoxylates, which are widely used as detergents in many industries. Alkylphenol ethoxylates are also broken down in the process of sewage-treatment or in rivers into alkylphenols, such as nonyl or octylphenol (NP or OP), which are well known representative EDCs with weak estrogenic activity, acting via binding to ER. In vitvo data indicate that OP has the most potent estrogenic activity of the alkylphenols (approximately 1000 times less estrogenic than E2), although NP is detected with higher levels than OP in the environment.

In our studies of the toxicologic/carcinogenic effects of EDCs on the female genital organs, OP was selected as a representative compound. It has already been reported that neonatal treatment with OP disrupts estrous cyclicity after weaning in female rats[33]. We also examined the effects of neonatal exposure to a high dose of p-tert octylphenol (t-OP) on the female genital organs of Donryu rats[34], and the results were in line with those of other papers: long-term persistent irreversible effects such as lower gonadotropin levels at prepuberty, inhibition of uterine gland genesis, persistent estrus shown by vaginal cytology, and polycystic ovaries. In our recent study, newborn female pups were injected with 100 mg/kg t-OP subcutaneously within 24 h of birth. Administration was repeated every other day until PND 15 (PNDs 1–15), and animals were observed till PND 77. Histologically, inhibition of uterine gland genesis was apparent during the immature period before weaning. The day of vaginal opening was about 4 days earlier in OP-treated animals than in controls, and after vaginal opening,

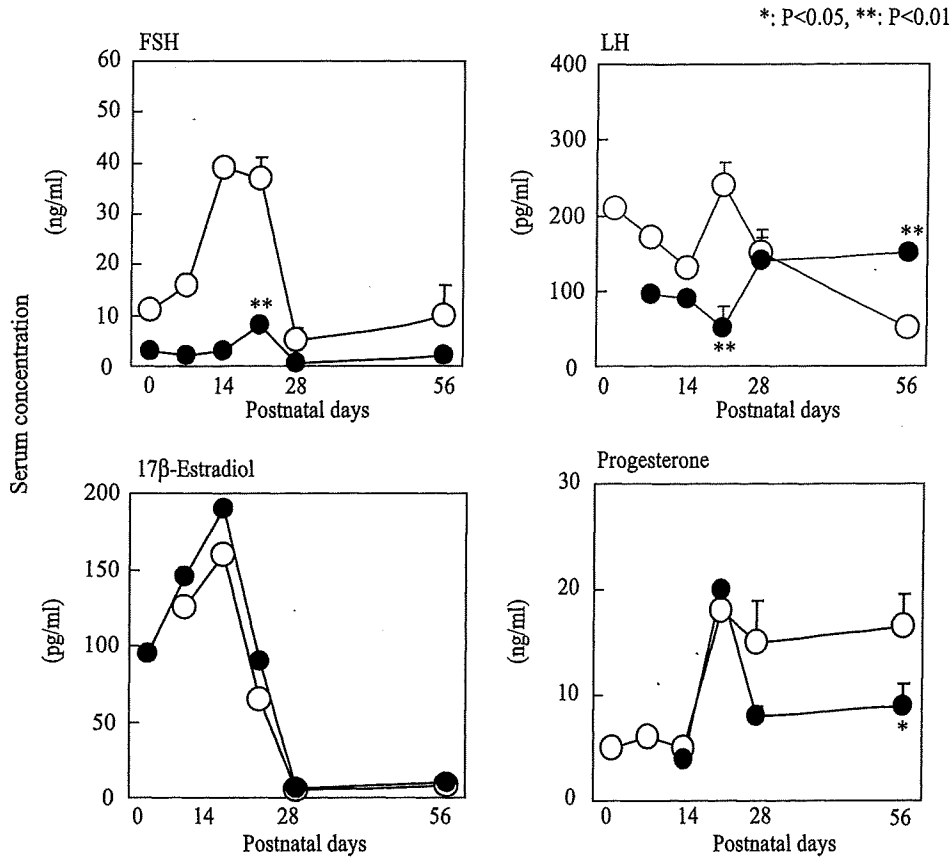

Fig. 2. Serem gonadotropins and sex steroid hormones in control and PNDs 1–15 OP-treated rats. Open circles (○), controls; black circles (●), PNDs 1–15 OP-treated. Katsuda *et al.*, Toxicol Appl Pharmacol 2000; 165: 217–226.

none of the OP-treated rats showed a regular estrous cycle, and persistent estrus was ultimately observed in these animals. Atrophic and polycystic ovaries without corpora lutea were anovular. In the endometrium, cell-proliferative activity and cell-death were increased and decreased, respectively, and expression of estrogen receptor alpha mRNA was apparent on in situ hybridization. At 8 weeks of age, treated animals exhibited luminal epithelial hyperplasia with overexpression of ER-mRNA. During the immature period, serum FSH and LH levels were consistently lower in OP-treated rats than in controls. In particular, serum FSH levels remained uniformly low. Serum E2 levels demonstrated essentially the same pattern as in controls, being elevated at PND 14, and then falling to low levels. After weaning before sexual maturation, FSH values in treated rats remained low, while those of control animals decreased rapidly and were maintained at the same levels as in the OP-treated case. In contrast, LH levels of treated animals increased after weaning and remained high until the end of the experiment (PND 77). Serum P levels of both OP-treated and control rats were constant, but the level in the former was only half of the latter value (Fig. 2). Serum inhibin levels of OP-treated rats were nearly the same as in controls at PND 28. The results resembled those of male or androgenized female rats in the secretary pattern of gonadotropins at this age[10,11], indicating that neonatal treatment with high-dose t-OP affects gonadotropin secretion during the developmental period of sexual maturation with direct masculinization of hypothalamic function.

In another of our studies, neonatal exposure for the first 2 weeks (PNDs 1–15) to 100 mg/kg t-OP induced an early and enhanced ER expression in the luminal epithelium compared with age-matched controls, and increased proliferating cell nuclear antigen (PCNA) positive cells, though expression in the glandular epithelium was suppressed in relation to inhibited gland-genesis. Therefore neonatal exposure to high doses of EDCs with estrogenic activity can induce abnormal differentiation in the developing rat uteri via abnormal ER expression and subsequent alteration of cell proliferating activity[35].

Recently, however, it has been reported that prenatal and/or neonatal exposure to high doses of estrogens or EDCs with estrogenic activity also exerts a "delayed" influence, different from that of typical androgenization. The delayed influence is probably caused by delayed modulation of the hypothalamo-pituitary-ovarian control system[36]. A number of investigators have described effects of neonatal exposure

Table 2. Uterine Gland Genesis before Puberty in Control and PNDs 1–5 or PNDs 1–15 OP-treated Rats*

	No. of uterine gland / section (Mean ± SD)		
	Control	PNDs 1–5	PNDs 1–15
PND 10	0	0	0
PND 14	3.94 ± 0.5	4.05 ± 1.5	0.1 ± 0.13**
PND 21	4.58 ± 0.6	5.57 ± 1.7	2.55 ± 1.5**
PND 28	6.42 ± 1.5	7.83 ± 1.3	3.14 ± 2.2**

*: Yoshida *et al.*, Carcinogenesis 2002; 23: 1745–1750.
**: Significantly different from the control value ($P<0.05$).

Table 3. Sequential Changes in Incidences of Persistent Estrus in Control and PNDs 1–5 or PNDs 1–15 OP-treated Rats*

	Incidence of persistent estrus (%)									
Group	1.5	2	3	4	5	6	8	10	11	(Months of age)
Control	0	0	0	2.6	17.9	30.8	64.1	85.7	100	
OP-treated (PNDs 1–5)	4.9	12.2	53.7**	70.1**	87.8**	100**	100**	100	100	
OP-treated (PNDs 1–15)	100	100	100	100	100	100	100	100	100	

*: Yoshida *et al.*, Carcinogenesis 2002; 23: 1745–1750.
**: Significantly different from the control value ($P<0.05$).

to EDCs including estrogens or androgens, but information on such delayed effects is limited. In our recent study, exposure after birth to 100 mg/kg t-OP for the first 5 days (PNDs 1–5) caused a "delayed" influence which was characterized by accelerated appearance of atrophic ovary, manifested by early-occurring and long-term continuing persistent estrus, whereas no abnormalities could be found with regard to growth and development of the reproductive organs and the hypothalamo-pituitary-gonadal control system up to maturation[37], thus differing from the case of exposure for PNDs 1–15 to the same dose of t-OP[34] (Tables 2 and 3). Previously, we confirmed neonatal OP-treatment of 50 mg/kg/day every other day for PNDs 1–15 did not affect estrous cyclicity[34], the total administration-dose (400 mg/kg) being higher than that (300 mg/kg) in the PNDs 1–5 study. This result indicates that the differences were due to the treatment period, rather than the total dosing volume.

Postnatal Exposure

Chronic administration of OP to adult male rats causes alteration in hormonal secretions[38], and also induces atrophies of the testis and other genital organs[39]. We therefore tested estrogenic effects of t-OP using adult OVX Donryu rats given daily subcutaneous injections of 6.25, 12.5, 25, 50 or 100 mg/kg for 2 or 14 days. t-OP was detected in serum at doses of 25 mg/kg and above for 2 days and of 12.5 mg/kg and above for 14 days, and uterine weights and luminal epithelial heights were increased dose-dependently. OP-treatment for 2 days caused a dose-related increase in proliferation of uterine luminal, glandular and stromal cells and vaginal epithelial cells, and the effects were fundamentally related to the serum OP levels[40].

Effects of t-OP on the female reproductive tract of normal cycling rats were also investigated. F344 and Donryu rats were used, and t-OP was subcutaneously injected for 28 days at similar concentrations to those applied to OVX rats. The most notable changes were disappearance of normal cyclicity in 50 mg/kg or more OP-treated rats and appearance of persistent estrus in the 100 mg/kg group. In rats showing abnormal cyclicity, the uterine morphology deviated from the normal at each estrous stage of cycling rats, and cell proliferation in the endometrium was slightly increased. However, the data for uterine weights, luminal epithelial cell heights and/or numbers of epithelial cells in the endometrium demonstrated only equivocal alteration. In treated rats, the serum E2 levels were decreased with 50 mg/kg of OP or more. Donryu and F344 rats showed similar sensitivity to estrogenic effects of OP, no strain difference being evident. The results indicate that vaginal cytology may be the most sensitive endpoint for the detection of estrogenic activity of potential EDCs in studies using adult female rats[41]. It was also demonstrated that vaginal cytology or its morphological features might be very useful in animal toxicity studies for assessment of the individual hormonal milieu including dysfunction of the hypothalamo-pituitary-gonadal control system[42].

The suitability of the 28-day repeated oral-dosing study for risk assessment of EDCs or strain differences was investigated in adult SD, F344 and Donryu female rats given 60 or 250 (150) mg/kg/day of NP, or 5 or 50 mg/kg/day of atrazine by stomach tube for 28 days. No morphological changes were noted in any reproductive organs of the treated animals, although abnormal estrous cycles were detected in high-dose groups of all strains, without any strain differences[43]. The results also indicate that vaginal smear is the most sensitive parameter for detection of effects of estrogenic or anti-estrogenic chemicals, when normal

cycling animals are used. Although atrazine is an agrochemical having weak estrogen-antagonistic activity, an anti-estrogenic property was not clear in the study. However, effects were detected in the immature rat uterotropic assay, in which atrazine alone was not associated with any changes in uterine weight, but co-treatment with atrazine and E2 reduced E2-induced increase of uterine weight[44].

Effects on Uterine Carcinogenesis

While the etiology of uterine adenocarcinomas in women is still inconclusive, hormones such as estrogens are considered to be of essential importance[2,3]. The carcinogenic effects on the female genital tract in mammals, including humans, are considered to be one of the most important adverse consequences of EDCs with estrogenic activity. However, there have been only a few reports of unequivocal induction of carcinomas in experimental animals by EDCs, except with diethylstilbestrol (DES), as reviewed previously[1]. In humans, the causation of vaginal and uterine cancers by prenatal exposure to DES is a striking example of environmental carcinogenesis[45]. In experimental animals also, the effects of prenatal DES exposure have been studied in rats and mice, as reviewed by Marselos and Tomatis[46]. Vaginal and uterine adenocarcinomas were induced in mice exposed prenatally to DES[47,48]. In rats following in utero DES exposure, however, mammary and vaginal tumors, rather than uterine tumors, were observed[46]. Thereafter, uterine carcinomas were also induced in Donryu rats by transplacental administration of DES[49]. In the study, interestingly, data for persistent estrus incidence indicate a "delayed" influence in offspring exposed prenatally, similar to our recent report[37].

Tamoxifen (TAM) is a non-steroidal anti-estrogen which competes with estrogen for binding to ER. However, its pharmacology is very complex, and both estrogen agonistic and antagonistic properties have been found, depending on the species, age, exposure duration, dose, route and organs in experimental studies[50]. It has been pointed out that the risk of endometrial cancer may be increased in postmenopausal women exposed to TAM for mammary cancer therapy, the agent acting on the uterus as a weak estrogen agonist[51,52]. In experimental studies using adult rats and mice, however, it has been impossible to cause endometrial cancers by TAM treatment, although endometrial carcinomas were induced in mice treated neonatally[53]. Also the incidences of uterine and cervical/vaginal cancers increased in rats, in the absence of any estrogen agonistic effect, when tamoxifen was administered orally on days 2–5 after birth[54]. Previously we reported that TAM showed potent anti-estrogenic effects on the adult rat uterus and inhibited the development of endometrial adenocarcinomas in our two-stage uterine carcinogenesis model[55]. In that study, however, the dose levels used might have been high. Quite recently, we also reported that TAM showed promotion, but not progression, effects on mouse uterine carcinogenesis, so that the influence in the progression stage appears to be different from the estrogen agonism reported for human beings, although TAM did show estrogen agonistic effects in the promotion stage[56].

In one study, atrazine slightly increased the incidence of endometrial adenocarcinomas in female F344 rats, when given in the diet[57]. Quite recently, however, it was reported that atrazine administered in diet has no modifying effects on uterine carcinogenesis in ICR mice initiated with N-ethyl-N-nitrosourea[58]. Vinclozoline, a pesticide also showing an anti-estrogenic effect, induced uterine adenocarcinomas in female Wistar rats, as well as ovarian sex cord-stromal tumors, when given orally[59]. The carcinogenic mechanisms of these chemicals with anti-estrogenic activity are not clear and further studies are needed to elucidate them.

Dioxin (2,3,7,8-TCDD) is known to exert its modulatory actions through the Ah receptor, and there is experimental evidence suggesting that it can also act in both estrogenic and anti-estrogenic manners, depending on the dose, species, and organ system involved. In rodents, TCDD induces mainly hepatocellular tumors. In addition, in an initiation-promotion study, morphological changes were also noted in both the uterus and the ovary. Although there is no evidence that TCDD can induce tumors in the female genital tract of rodents, it was reported to cause endometriosis in monkeys[60].

Another interesting example is ethylenethiourea (ETU), a metabolic product of ethylenebisthiocarbamate fungicides such as maneb and zineb, which are also listed as EDCs. ETU itself is a well established carcinogen, inducing thyroid tumors in rats and hepatic and lymphoid tumors in mice. In addition, it reacts with nitrite under acidic conditions in vitro and in vivo to form a mutagenic and carcinogenic compound, N-nitroso ETU[61]. Concurrent oral administration of ETU and sodium nitrite is reported to induce uterine endometrial adenocarcinomas in mice[62]. In our two-stage uterine carcinogenesis model using Donryu rats, concurrent oral administration of ETU (80 mg/kg) and sodium nitrite (56 mg/kg) resulted in uterine endometrial carcinomas without initiation by intrauterine administration of ENNG, and also promoted development of the tumors in animals initiated by ENNG, presumably by influencing the hormonal balance[63]. Both ETU and nitrite are known environmental chemicals which are included in foods. Our confirmation that endometrial adenocarcinomas can be induced in this way in rats as well as mice, thus points to an importance of the oral route of exposure to these chemicals, although the doses used in the study were much higher than those in the diet.

The effects of high-dose t-OP on uterine carcinogenesis were investigated using adult Donryu rats initiated with a single intrauterine treatment of ENNG at 11 weeks of age and exposed thereafter to 100 mg/kg/day t-OP by s.c. injections until 15 months of age. Adult OVX rats were also treated in the same way. t-OP had no effect on the occurrence of persistent estrus in non-OVX rats, although uterotropic effects were obvious in the OVX case. At the

Table 4. Uterine Adenocarcinomas in ENNG-initiated Rats with Exposure to High-dose OP*

Group	No. of rats examined	Incidence of endometrial lesions						
		Hyperplasia			Adenocarcinoma			
		+	++	+++	total	differentiation**		
						G1	G2	G3
1. Control	23	2	8	7	4	4	0	0
2. OP-treated (Adulthood)	26	1	8	5	12***	9 (G1 and/or G2)		3
3. Control	23	3	7	5	6	6	0	0
4. OP-treated (PNDs 1–5)	28	1	3	5	18***	17	1	0
5. OP-treated (PNDs 1–15)	22	2	2	1	8	1***	3	4***

*: Groups 1–2: Katsuda *et al.*, Jpn J Cancer Res 2002; 93: 117–124.
Groups 3–5: Yoshida *et al.*, Carcinogenesis 2002; 23: 1745–1750.
**: Histological grades of uterine adenocarcinomas by tumor differentiation.
G1: well differentiated; G2: moderately differentiated; G3: poorly differentiated.
***: Significantly different from the control value ($p<0.05$).

end of the experiment, however, development of uterine adenocarcinomas was significantly increased in animals exposed to t-OP during adulthood, but no tumors developed in OVX rats. This finding suggests that high-dose t-OP has tumor-promoting effects on the ENNG-treated endometrium of rats, possibly due to direct action on the uterus, as indicated by the uterotropic effect of OP[64] (Table 4).

Uterine carcinogenesis in Donryu rats treated neonatally with a high-dose of t-OP has also been investigated. Female pups were subcutaneously administered 100 mg/kg/day t-OP every other day for the first 5 days after birth (PNDs 1–5), or the first 2 weeks (PNDs 1–15). Thereafter, they received a single intrauterine injection of 20 mg/kg ENNG at 11 weeks of age and were observed until 15 months of age. PNDs 1–5 OP-treated animals showed normal development of the female reproductive system, including uterine gland genesis before weaning and normal estrous cycling immediately after vaginal opening. However, the treatment accelerated the occurrence of persistent estrus after 6 weeks of age, and increased the number of well-differentiated uterine adenocarcinomas at the end of the experiment (15 months of age), as compared with controls. This indicates that PNDs 1–5 OP-treatment resulted in delayed modulation of the hypothalamus-pituitary-ovarian hormonal control system, and thus increased the serum E2:P ratio, leading to promotion of uterine carcinoma development. On the other hand, PNDs 1–15 OP-treatment demonstrated immediate and irreversible influences on the control system, called "androgenization", and induced suppression of uterine gland genesis as well as abnormal uterine development manifested by prolonged persistent estrus immediately after vaginal opening, similar to our previous report[34]. In addition, at the end of the experiment, uterine tumor malignancy as assessed by morphological and biological properties was clearly increased, although there was no significant alteration in the total incidence of adenocarcinomas. The total incidence of hyperplasias was significantly lowered, probably related to suppression of uterine gland genesis (Table 4). That study provided evidence that neonatal exposure during PNDs 1–5 or 1–15 to high-dose t-OP enhances uterine carcinogenesis in ENNG-initiated rats, and that the type of uterine tumor is changed by the period of neonatal treatment[37].

Concerning the histogenesis of endometrial adenocarcinomas in Donryu rats, the tumors are considered to arise from hyperplasias of the luminal or glandular epithelium, especially the latter[28]. In humans, it has been pointed out that the presence or absence of hyperplasia as the background is important for the biological behavior of endometrial adenocarcinomas. High-dose OP treatment at PNDs 1–15 induced luminal epithelial hyperplasia in the uteri of rats at 8 weeks of age[34], and finally increased development of undifferentiated adenocarcinomas, although the incidence of hyperplasias was decreased[37]. Carthew *et al.* also reported that tamoxifen induced uterine adenocarcinomas, including biologically malignant examples, in rats in the absence of endometrial hyperplasia, when given on days 2–5 after birth[54]. These results are very interesting in consideration of the histogenesis of uterine adenocarcinomas.

As mentioned above, estrogen and related compounds are reported to increase the risk of endometrial adenocarcinoma development in women. Estrogens occur naturally within the normal body, and are mainly metabolized in the liver by two separate pathways, producing either catechol estrogens (2- or 4-hydroxylated products) or 16α- or 16β-hydroxylated products. 2-Hydroxylation of estradiol or estrone to a catechol is a major metabolic pathway, and the catechol estrogens 2-OHE2 and 2-OHE1 have much weaker hormonal potency than their parent hormones, and lack carcinogenic potency when given to adult animals. On the other hand, 4-hydroxyestradiol (4-OHE2) and the two 16α-hydroxylated forms, 16α-OHE1 and 16α-OHE2, retain potent hormonal activity by acting on classical estrogen receptor and also are tumorigenic[65]. In fact, induction of preneoplastic and neoplastic lesions by estrogen and its steroid metabolites (16 steroids) were studied with our two-stage mouse uterine carcinogenesis

model, and 2-OHE1 or 2-OHE2 exerted promoting, but not progressing, effects, while 16α- and 16β-OHE1 caused both promotion and progression[66].

It is known that indole-3-carbinol binds to the Ah receptor, similar to TCDD, and induces cytochrome p450 metabolic enzymes mainly in the liver. It has been reported that this chemical shows a chemopreventive effect on spontaneous endometrial adenocarcinoma development in Donryu rats when given orally, the effect being speculated to be due to enhanced 2-hydroxylation[67]. We also assessed the effect of indole-3-carbinol on uterine carcinogenesis using our two-stage rat uterine carcinogenesis model. Contrary to expectation, however, the incidences of endometrial carcinomas were increased. In rats given indole-3-carbinol, elevated liver weights and centrilobular enlargement of hepatocytes were also observed, the results indicating an effect on estrogen metabolism in the liver, and further studies are now under way, to clarify the discrepancy (Yoshida *et al.* unpublished data).

Effects of Low-doses of EDCs

The concentrations of EDCs including OP in the environment are very low, and the main exposure route is oral, rather than cutaneous, in humans. In general, the toxicokinetics of chemicals including EDCs in animals is known to be influenced by the method of administration. It has been reported that low doses of estrogens and EDCs such as OP might be removed from the blood during the first passage through the liver, when given orally[68,69]. For risk assessment of EDCs, it is very important to investigate oral dose effects at human exposure levels and thus we have also focused on relatively low doses of OP (t-OP or n-OP) by oral administration. Female Donryu rats initiated by intrauterine administration of ENNG were given diets containing 100 or 1000 ppm t-OP (about 5 or 50 mg/kg/day) or 100 ppm n-OP (about 5 mg/kg/day) from 11 weeks of age to 15 months of age. Although the concentrations are higher than those in the environment, no significant increase in the incidences of uterine adenocarcinomas was observed in any treated group at the end of the experiment, and also there was no difference in tumor malignancy among the groups (Yoshida *et al.* unpublished data).

As detailed above, exposure to high doses of estrogens or EDCs in the fetal or new born period exerts irreversible androgenization of the female reproductive organs, because of heightened sensitivity. In addition, "delayed" influences on these organs may occur after puberty or sexual maturation. Therefore, relatively long-term comprehensive studies on the endocrinological and morphological aspects may be necessary for determination of prenatal and/or neonatal effects of low doses of EDCs regarding toxicity/carcinogenicity in the female genital organs. Low doses of EDCs such as NP or bisphenol A (BPA) were given orally to pregnant rats, and offspring were observed until 15 months of age, to investigate the prenatal and neonatal effects on growth and development of the female reproductive system and uterine carcinogenesis. In the reproductive toxicity studies reported by others, high doses of NP caused estrogenic effects on pubertal development in male and female rats[70,71]. However, maternal or neonatal exposure to relatively low doses demonstrated no adverse influence on the reproductive tract[72]. In our study with NP, dams were administered 0.1, 10 and 100 mg/kg daily by gavage from gestation day 2 up to the day before weaning of their offspring. Then, all female pups at 11 weeks of age were administered a single dose of 20 mg/kg ENNG into a uterine horn, and observed until 15 months of age. The low level, 0.1 mg/kg, was selected as a dose relevant to human daily intake (1 mg/kg) of isoflavones, uterotropic activity of NP being reported to be 10 times stronger that that of daidzein, one of the major isoflavones, and the middle-dose, 10 mg/kg, was selected as near the no observed effect level in a multi-generation reproductive study using rats[71]. None of the treated groups demonstrated any alteration in reproductive ability. In their offspring also, uterine growth and development, vaginal opening and hormonal secretion until puberty were not changed and there were no effects on estrous cyclicity and morphology of the reproductive organs after maturation, or on uterine carcinogenesis in animals initiated with ENNG[73].

BPA, a volume chemical used in the manufacture of polycarbonate plastics and found in canned foods, lacquered containers and composite dental sealant, is one of the most representative EDCs with weak estrogenic activity, and uterotropic potential has been demonstrated in the immature rat assay[74]. A study conducted by the National Toxicity Program (NTP) in the USA demonstrated that maternal exposure to high doses of BPA at 0.5 or 1.0% in the diet (approximately daily intakes of 875 and 1750 mg/kg/day) reduced the number of live pups per litter and litters per pair in first generation mice[75], although 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[76–78]. Recently, however, perinatal treatment with BPA at much lower doses has been described to influence male reproductive organ parameters such as weight of the testis, prostate, preputial gland and epididymis, and the efficiency of sperm production in rodents[79–81], and neonatal treatment advanced puberty in mice[82], although there are also some reports of no treatment-related effects at low dose levels when given to pregnant mice and rats[83–85], and to rats in a three-generation reproductive toxicity study[86]. To further assess the risk, we also investigated effects of maternal exposure to low-doses of BPA, including a human exposure-level, on growth and development of the female reproductive system, and also uterine carcinogenesis in Donryu rats. 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 (PND 21). The concentration of 0.006 mg/kg was selected as consistent with the 63 ppb defined as the average daily intake from canned food in human beings, and 6 mg/kg was selected as appropriate to simulate the maximum dose level

Table 5. Proliferative Uterine Endometrial Lesions in Rats Given Low-doses of BPA*

Dose	Incidence of lesions (%)			
	Hyperplasia			Adenocarcinoma**
	+	++	+++	
0 mg/kg/day	21	21	13	33
0.006 mg/kg/day	20	20	17	33
6 mg/kg/day	13	47	17	20

*: Yoshida *et al.*, J Reprod Dev 2004; 50: 349–360.
**: All adenocarcinomas in the three groups were well differentiated and limited to the uterus.

(80 ppm) detected in plastic plates[87]. The treatment did not exert any influences on the reproductive system of female offspring in either treated group, in terms of prepubertal uterine growth and gland-genesis, vaginal opening and gonadotropin secretion. After maturation also, no effects were evident with regard to estrous cyclicity, age-matched sequential changes of the reproductive organs, and uterine carcinogenesis until 15 months of age (Table 5). The results demonstrated that maternal exposure to BPA at human exposure-levels did not have any adverse effects on the female reproductive organs of offspring in rats[88].

For determination of effects of EDCs on offspring by maternal treatment, biotransfer of the chemicals from dam to offspring is crucial, because the impact is fundamentally related to the serum EDC level[40]. However, data for transfer of the test chemical via the placenta or milk to offspring, or toxicokinetics of low-dose EDCs are very limited[89]. In one of our studies, NP at 10 and 100 mg/kg doses was transferred from dams to their offspring via the milk, but the compound could not be detected in their serum or liver[73]. Furthermore, BPA levels in the milk of dams, and those in the serum and liver in offspring were comparable between control and treated groups, although the serum level of BPA in dams receiving 6 mg/kg was significantly elevated[88].

Quite recently, it was reported that cadmium has potent estrogen-like activity in vivo[90]. Thus, exposure to low-dose cadmium (a single ip injection at a dose of 5 μg/kg) increased uterine net weight accompanied by proliferation of the endometrium, promoted growth and development of the mammary glands, and induced hormone-regulated genes in ovariectomized rats. In utero exposure to the metal (i.p. injections of 0.5 or 5 μg/kg on days 12 and 17 gestation) mimicked also the effects of estrogens, and female offspring experienced an earlier onset of puberty and an increase in the epithelial area and the number of terminal end buds in the mammary gland. The amounts of cadmium used in the study were environmentally relevant, because the WHO-recommended Provisional Tolerable Weekly Intake Level is 7 μg/kg/week. Although the administration route was intraperitoneal, not oral, the ability of environmentally relevant amounts of cadmium to mimic the effects of estradiol is very important and the metal may represent a new class of EDC.

Species Differences in Toxicologic/carcinogenic Effects of EDCs, Effects of EDCs based on the Molecular Biology and Extrapolation of the Effects to Humans

It is well known that the toxicokinetics of chemicals in animals are influenced by many factors, including species, strain, sex, age, dosage and/or administration method, as mentioned above. In particular, species differences are very important for the risk assessment in humans. Species differences in occurrence of toxicologic/carcinogenic effects of EDCs may be an indication of variation in endogenous hormonal factors, in addition to susceptibility to exogenous agents. Mice are generally more sensitive to estrogens than rats, and uterine adenocarcinomas can be induced in mice by estrogen alone, but not in rats. In mice, perinatal exposure to estrogens was found to induce ovary-independent proliferation of the vaginal epithelium, which could not be abolished by ovariectomy[91]. On the other hand, the vagina in rats neonatally exposed to high-dose t-OP became atrophic immediately after ovariectomy[34]. Adenocarcinoma development in the ENNG-initiated endometrium of Donryu rats exposed to high-dose t-OP was also ovary-dependent[64]. In mice, however, E2 promoted uterine adenocarcinoma development ovary-independently[56]. Differences in the reaction to estrogens or EDCs with estrogenic activity may help to explain species differences in toxicity/carcinogenicity, though further studies on this point, focusing on metabolism of estrogens or EDCs and localization of ER expression, are needed.

The prenatal and/or neonatal periods are more sensitive to estrogens, and also EDCs, than the adult period. As mentioned above, it has been pointed out that tamoxifen increases the risk of endometrial cancer in women. In rodents, TAM can induce uterine carcinomas when given to newborn animals, but not adults. Similarly, 2-OHE2 has weak estrogenic, but not carcinogenic effects, although 4-OHE2 is a potent estrogenic catechol causing uterine tumors in adult mice. However, both catechols induced tumors when given on days 1–5 of neonatal life, although carcinogenic activity of 4-OHE2 is stronger than that of 2-OHE2[92]. Further studies on age-dependent differences in the mechanism of EDCs' effects on the female genital organs are also needed.

Various methods such as DNA micro-array techniques based on gene expression levels have recently been used for the evaluation of hazardous effects of various chemicals, because they should reveal very early changes. Up till now, however, there have only been a few reports concerning EDCs. The fact that changes in ontogenic expression of ER alpha and not of ER beta occur in the fetal female rat reproductive tract, provides fundamental information critical for clarifying species-specific physiological roles of ER subtypes during fetal development and for investigating the tissue-specific mechanisms underlying prenatal responses to estrogen and E2 agonists[93]. Genome-wide analysis of early gene expression has furthermore suggested a basis for the

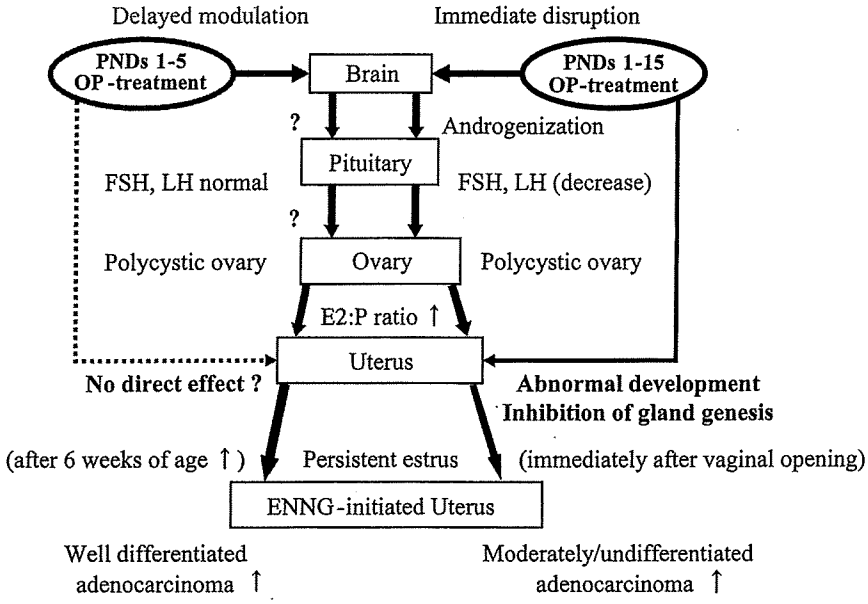

Fig. 3. Hypothesis of the scheme for "androgenization effects" or "delayed modulation effects" on the hypothalamo-pituitary-gonadal system in rats exposed neonatally to high-dose OP.

drastic uterotropic effects following estrogen administration[94]. Although DNA micro-array techniques presently demonstrate problems with reliability and reproducibility, future precise analysis should facilitate understanding of the mechanisms underlying effects of estrogenic EDCs[95].

Almost all EDCs exist at only very low concentrations in the environment, but humans may be exposed for long periods. In many animal studies, various toxicologic effects of EDCs on the female genital organs were demonstrated when very high doses were given, but no obvious effects were detected with low-doses. In humans also, low doses may show no adverse effects, because of homeostasis, although there are notable exceptions in animal and human studies. In fish, the ova-testis is known to be a good indicator of estrogenic effects of EDCs in females, but in female rodents, we are still lacking a consensus regarding equivalent reliable endpoint markers. Thus, more comprehensive studies of the endocrinological, morphological and also biomolecular aspects are necessary in animal studies using rodents for extrapolation of EDCs' effects to humans.

Conclusion

It is well known that the prenatal and/or neonatal period is particularly sensitive to various chemicals, including EDCs, in humans and rodents. Inappropriate exposure may exert irreversible influence, resulting in androgenization of the female genital system. In addition, it has also been reported that a delayed influence may be exerted. Neonatal exposure to a high dose of t-OP (100 mg/kg s.c. injection every other day from 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 shown by vaginal cytology and polycystic ovaries. Neonatal treatment of high-dose EDCs having estrogenic activity can thus affect gonadotropin secretion during the developmental period of sexual maturation with direct masculinization of the hypothalamic function. Abnormal differentiation in the developing rat uteri may be induced via abnormal ER expression and subsequent alteration of cell proliferating activity. However, exposure limited to the first 5 days after birth to 100 mg/kg t-OP caused "delayed" influence which was characterized by accerelated appearance of atrophic ovary, manifested by an early-occurring and long-term continuing persistent estrus status after puberty, whereas no abnormalities could be found with regard to growth and development of the reproductive organs and the hypothalamo-pituitary-gonadal control system up to maturation. The hypothetic scheme for "androgenization effects" or "delayed modulation effects" on the hypothalamo-pituitary-gonadal system in rats exposed neonatally to high-dose OP is shown in Fig. 3.

On the other hand, the most notable effect on the female reproductive system when normal cycling rats were exposed to a high-dose of t-OP for a short time (28 days), was disappearance of the estrous cycle, and no clear changes were detected in other parameters such as uterine weight and morphology. These results indicate that the vaginal smear is the most sensitive parameter for the detection of effects of EDCs in normal cycling rats.

Well or moderately differentiated adenocarcinomas

were increased in Donryu rats initiated by ENNG, when high dose t-OP was given subcutaneously during adulthood. Neonatal exposure to a high dose of t-OP also showed promoting effects on uterine adenocarcinoma development in a two-stage rat uterine carcinogenesis model using Donryu rats, with slight to higher malignancy with more prolonged treatment.

For the risk assessment of EDCs to human health, it is very important to investigate the effects of low doses at actual human exposure levels, because the concentrations of agents, including alkylphenols and BPA, in the environment are very low. In addition, the main exposure route to EDCs is oral, not subcutaneous, in humans. Thus, we have focused on effects of maternal exposure to low doses of EDCs, such as NP and BPA, by the oral route, which have shown no effects on growth and development of the female reproductive system or uterine carcinogenesis. Transfer of low doses of BPA from dams to offspring via the placenta and/or milk was not unequivocal, although NP was transferred when relatively high doses were given.

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, because of the effects of homeostasis and clearance from the blood stream on first passage through the liver.

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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.
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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].

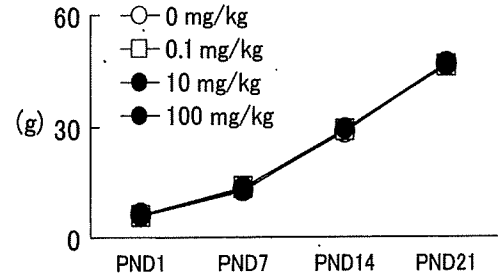

Fig. 1. Growth curve of female offspring up to the weaning. The body weights are comparable among the groups.

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

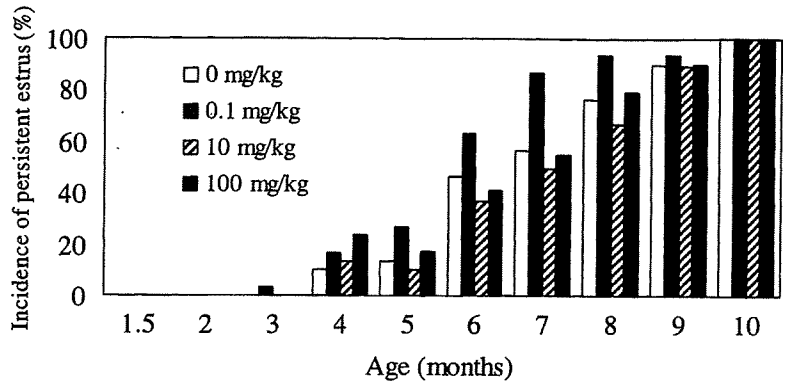

Fig. 2. The incidence (%) of animals showed persistent estrus (PE) at their vaginal cytology. The number of the animals with PE is increased with advanced age and their incidence is not significantly different among the groups.

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

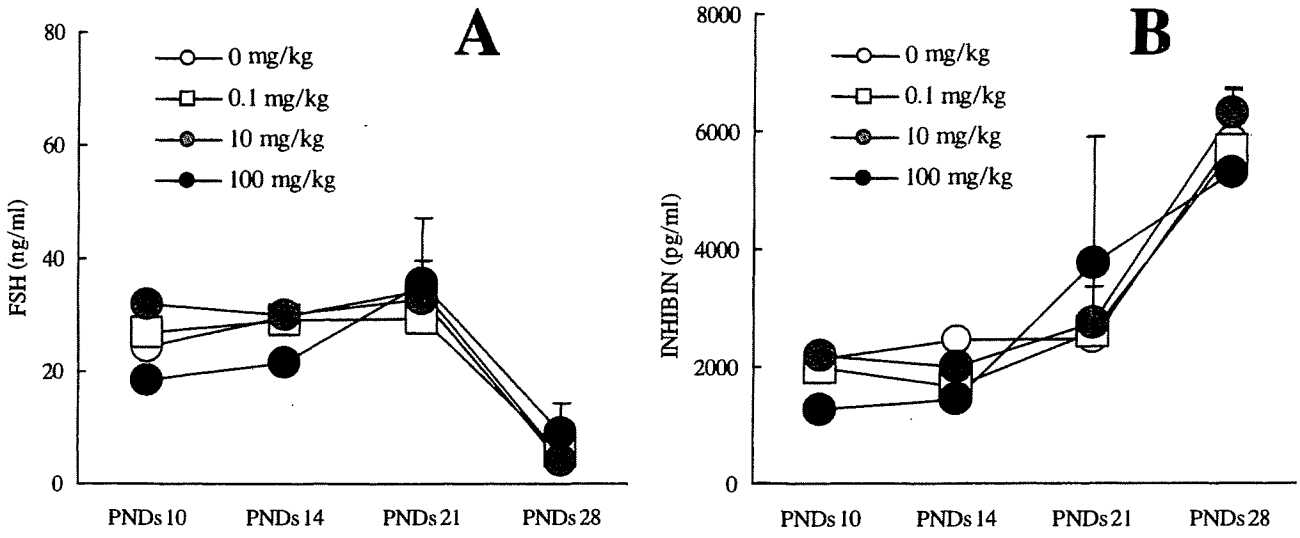

Fig. 3. Serum hormone profiles of follicle stimulating hormone, gonadotropin (A), and inhibin, a hormone secreted in the ovary (B) up to puberty. In all treated groups, FSH levels are comparable to that in controls. Inhibin levels were anti-related with levels of FSH.

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.

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