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Review

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

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

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

Introduction

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

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

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

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

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

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

Main Spontaneous Uterine Tumors in Donryu and F344 Rats

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

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

Classification of EDCs

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

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

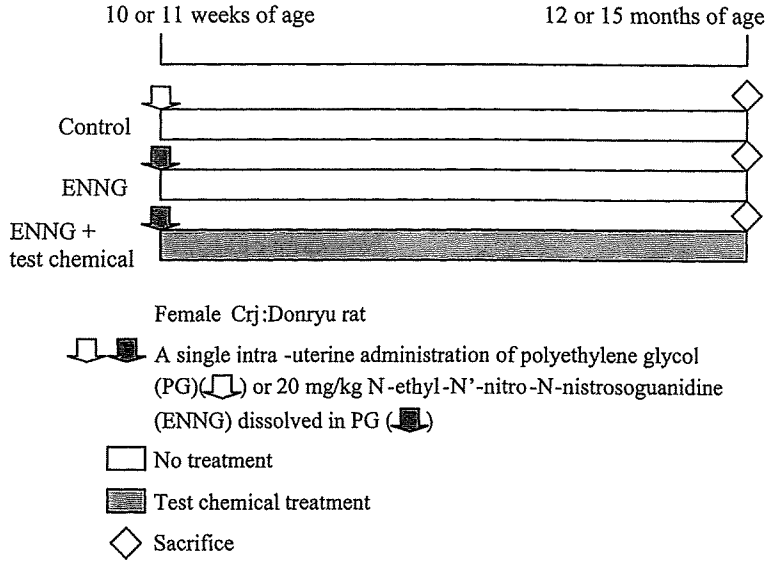

Fig. 1. Two-stage rat uterine carcinogenesis model

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

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

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

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

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

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

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

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

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

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,

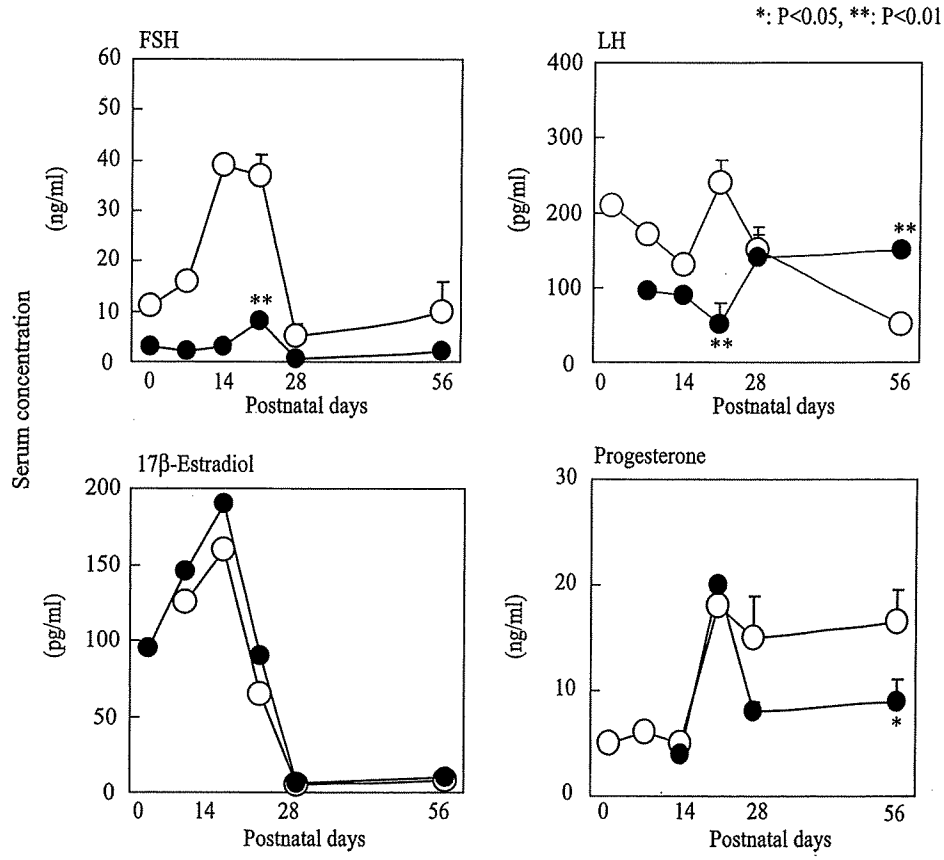

Fig. 2. Serem gonadotropins and sex steroid hormones in control and PNDs 1–15 OP-treated rats. Open circles (○), controls; black circles (●), PNDs 1–15 OP-treated. Katsuda *et al.*, Toxicol Appl Pharmacol 2000; 165: 217–226.

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 intra-uterine 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

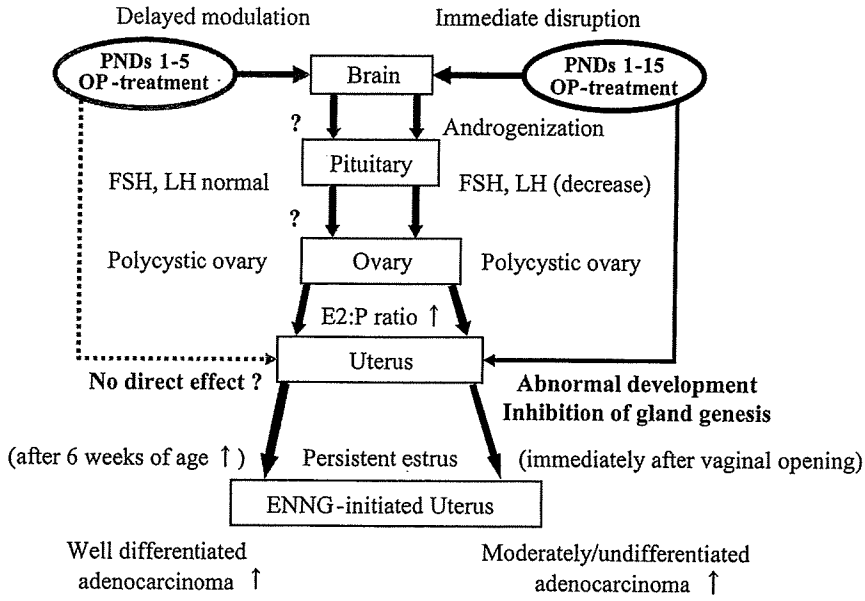

Fig. 3. Hypothesis of the scheme for "androgenization effects" or "delayed modulation effects" on the hypothalamo-pituitary-gonadal system in rats exposed neonatally to high-dose OP.

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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OECD validation of the Hershberger assay in Japan: Phase 3. Blind study using coded chemicals

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Abstract

The Organization for Economic Co-operation and Development (OECD) has initiated the development of new guidelines for the screening and testing of potential endocrine disrupters. The Hershberger assay is one of the assays selected for validation based on the need for in vivo screening to detect androgen agonists or antagonists by measuring the response of five sex accessory organs and tissues of castrated juvenile male rats: the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis. The Phase 1 feasibility demonstration stage of the Hershberger validation program has been successfully completed with a single androgen agonist and a single antagonist as reference substances. The Phase 2 validation study was performed, employing a range of additional androgen agonists and antagonists. Recently, the Phase 3 validation study was conducted and performed in several International laboratories. Three Japanese laboratories have contributed to the blind study using coded materials of Phase 3 validation. Four coded test substances in the agonistic version and seven substances in the antagonistic version were orally administered by gavage for 10 consecutive days, respectively. In the antagonist version of the assay, 0.2 mg/kg/day of testosterone propionate (TP) was coadministered by subcutaneous injection. All five accessory sex reproductive organs and tissues consistently responded with statistically significant changes in weight within a narrow window in both versions. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

Keywords: Blind study; Endocrine; Hershberger assay; OECD validation

1. Introduction

Certain reproductive and developmental toxicants may have the potential to interfere with normal sexual differentiation and development in animals and humans by modulating or interfering with the endocrine system (McLachlan, 1993; McLachlan and Korach, 1995). The Organization for Economic Co-operation and Development (OECD) has initiated an activity to revise existing guidelines and develop new screening and testing guidelines to aid in the identification and assessment of such toxicants (OECD, 1998, 2000, 2002).

One proposed assay, referred to as the Hershberger assay, uses the androgen sensitivity of several accessory sex organs and tissues of the male reproductive tract. The assay was originally developed in the 1930s by Korenchevsky and coworkers, and a number of accessory sex organs and tissues were shown to be use-

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ful by these and other investigators including the ventral prostate (Deanesly and Parkes, 1936; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), the seminal vesicles and coagulating glands (Deanesly and Parkes, 1936; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), the preputial glands (Bülbring and Burn, 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), Cowper's glands (Wainman and Shipounoff, 1941), and the glans penis (Bülbring and Burn, 1935; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b). In the 1940s, it was discovered that the levator ani and bulbocavernosus muscles also responded to androgens, but in a differential way from the other tissues (Wainman and Shipounoff, 1941; Eisenberg et al., 1949; Eisenberg and Gordan, 1950). The basis for this differential sensitivity is the presence of 5α-reductase in most accessory tissues of the male reproductive tract, but its absence in the muscle complex (Di Salle et al., 1994). The capabilities of the assay were demonstrated in 1953 by Hershberger et al. when they analyzed the response of the ventral prostate, seminal vesicles and coagulating glands, and the levator ani without the bulbocavernosus muscle to a number of active chemicals, including estrogens and progesterones (Hershberger et al., 1953).

In the 1970s and 1980s, with the discovery of the androgen receptor and the first compounds such as cyprotone acetate that were antagonists of the receptor, the assay was modified to address antagonistic activity. Briefly, a set dose of a reference agonist was coadministered to several groups of animals to whom a set of doses of the purported antagonist was also administered. This modified system was successfully used by several investigators for assaying androgen antagonists (Peets et al., 1973; Raynaud et al., 1980, 1984; Wakeling et al., 1981).

Therefore, based upon the recommendation of scientific workshops, both the US Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) (USEPA, 1998) and the OECD Endocrine Disrupter Testing and Assessment Group (EDTA) of the OECD (OECD, 2000) have proposed this assay as a Tier-1 screen to identify possible reproductive and developmental toxicants acting through androgen agonist and antagonist mechanisms.

The OECD Phase 1 validation program for the Hershberger assay was completed in 2001. In this phase, a standardized protocol using the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis was successfully tested against a reference androgen compound, testosterone propionate (TP), and a reference antagonist, flutamide (OECD, 2002). The OECD proposed a Phase 2 validation program using additional androgen agonistic and antagonists as the next step to validate the assay, but the final results of Phase 2 studies were not opened by the OECD.

Recently, the OECD conducted a Phase 3 validation program as a final blind study using coded agonistic and antagonistic chemicals (OECD, 2003). In Phase 3, the coded test substances were to be used to investigate the reliability of the assay, including a demonstration of the protocol's transferability among laboratories and the reproducibility of the protocol's results. Three Japanese laboratories participated in the Phase 3 validation study using four coded agonistic test substances and seven antagonistic substances. The participation of the laboratories in the OECD Phase 3 validation study was performed as part of a national validation program in Japan.

2. Materials and methods

2.1. Laboratories

The three participating Japanese laboratories were: the Chemicals Evaluation and Research Institute (CERI); the Food Drug Safety Center; and the Japan Bioassay Research Center. Each laboratory performed the study in compliance with the principles of Good Laboratory Practice guidelines.

2.2. Test substance

All coded test substances except for TP were sent to each laboratory from a centralized chemical repository at TNO, Zeist, the Netherlands. TP and corn oil as vehicles were prepared in each laboratory. The coded substances A, B, L and E were used in the agonistic version, and F, G, I, C, K, D and H were used in the antagonistic version. We did not receive any information regarding the coded substances before all tests were started.

2.3. Animals

Laboratory details regarding rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage are summarized in Table 1. Two laboratories used Crj:CD (SD) rats castrated at 6-weeks old, and the test substances were administered 1 week after castration. One laboratory used Brl Han: WIST Jcl (GALAS) rats castrated at 6-weeks old, and the test substances were administered 2 weeks after castration. In all the laboratories, the rats were weighed, weight-ranked, and assigned randomly to each of the experimental and control groups after they had recovered from their operation. Body weight and clinical signs were recorded daily throughout the study. Rats were provided with water and

Table 1
Laboratory detail for rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage

Lab	Rat strain	Age of castration	Age at start of dosing	Day of autopsy	Diet	Number of rats per cage
1	Brl Han: WIST Jcl (GALAS)[a]	6-weeks old	8-weeks old	10-weeks old	MF[b]	3
2	Crj:CD (SD)[c]	6-weeks old	7-weeks old	9-weeks old	CE-2[d]	1
3	Crj:CD (SD)[c]	6-weeks old	7-weeks old	9-weeks old	CRF-1[d]	1

[a] Clear Japan Inc., Tokyo, Japan.
[b] Oriental Yeast Co., Ltd., Tokyo, Japan.
[c] Charles River Japan, Kanagawa, Japan.
[d] Clear Japan Inc.

a commercial diet ad libitum. The animals were kept under SPF conditions. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by The Japanese Association for Laboratory Animal Science.

2.4. Administration

We performed each test according to the protocol proposed by the OECD (OECD, 2000, 2002, 2003). Each test substance was orally administered via a stomach tube for 10 consecutive days at approximately the same time each day. A vehicle control group receiving only corn oil was used in both versions. For the antagonistic version, 0.2 mg/kg/day of TP was coadministered each day by subcutaneous injection in the dorsal region after the oral administration of each chemical. The volume of the corn oil solution containing the TP was 0.5 ml/kg. In the agonistic version, a positive control group of animals received TP injections alone. The group size in all cases was six rats. The volume of the corn oil solutions containing each of the test chemicals was 5 ml/kg. The animals were killed by bleeding from the abdominal vein under deep ether anesthesia approximately 24 h after receiving their final dosage. The five mandatory tissues, the ventral prostate and fluid, seminal vesicle and fluid, LABC, glans penis, and Cowper's gland, were carefully dissected free of adhering fat and weighed to the nearest 0.1 mg. We also weighed the liver in three laboratories, and paired kidney and adrenal weights were measured in one laboratory.

2.5. Statistical analysis

We received the information from the coordinator of this Phase 3 validation after all tests were finished that the participating laboratories received pairs of the test chemicals (i.e. L and E, F and G, I and C, or K and D), so we analyzed the data using the following analytical methods between the vehicle control group and the same chemical groups in the agonistic version, and the TP group and the same chemical groups in the antagonistic version. In addition, coded A and F were nonylphenol, B and G were dinitrophenol, E and L were trenbolone, C and I were *p,p'*-DDE, and D and K were linurone. Body weight and organ weight data were analyzed by Bartlett's test for homogeneity of variance. When the variance was homogeneous at a significance level of 5%, one-way analysis of variance was performed. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed with Dunnett's test. If the variance was not homogeneous, the Kruskal–Wallis test was used. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed by the non-parametric Dunnett's test. On the other hand, differences in body weight and organ weight between the control group and the TP group, coded A or B in the agonistic version and between TP group and the group using coded H, G or F in the antagonistic version were assessed for statistical significance by the two-tailed Student's *t*-test. For graphical presentation, the sex accessory organ data were normalized to visually compare the shapes of the responses produced by each laboratory. For this normalization, the control value was set to 100% in the agonistic study, and 100% in the TP without coded compound in the antagonistic study. Analyses of variance were performed on the data from each laboratory and for the pooled laboratory data; these normalized values were not analyzed statistically.

3. Results

3.1. Agonistic version

3.1.1. Body weights, clinical observations, and optional organ weights

The body weights and the optional organ weight changes are shown in Table 2. Terminal body weights in rats given L were significantly lower than in rats given vehicle alone in Labs 2 and 3, and tendency towards lowering of the terminal body weights was observed in Lab 1. No abnormal clinical signs were observed in any of the rats that were treated with each substance. The paired kidney weights in rats given substance A and TP were significantly higher than in rats given only the vehicle in Lab 3, and the liver weights in rats given A and TP were also higher than in rats given the vehicle only in Lab 2.

Table 2
Optional organ weights including the liver, adrenal, and kidney in agonistic version

Lab	Body weights/organ weights	Substances					
		V.C.	A	B	L	E	TP
1	Starting body wt. (g)	214.8 ± 10.6	214.4 ± 11.0	213.1 ± 10.1	219.1 ± 10.5	215.0 ± 8.0	214.9 ± 9.3
	Terminal body wt. (g)	262.9 ± 17.0	250.6 ± 12.0	252.4 ± 12.8	243.7 ± 9.2	249.4 ± 12.9	266.0 ± 12.7
	Liver (g)	10.0 ± 1.1	10.2 ± 1.3	9.6 ± 0.5	11.1 ± 0.7	9.7 ± 0.7	10.1 ± 0.8
2	Starting body wt. (g)	231.0 ± 5.3	227.5 ± 5.0	229.6 ± 6.9	229.1 ± 3.9	226.8 ± 7.9	230.9 ± 6.9
	Terminal body wt. (g)	280.8 ± 7.9	275.9 ± 6.3	285.2 ± 12.4	261.5 ± 7.1*	281.8 ± 15.0	305.6 ± 15.0*
	Liver (g)	11.1 ± 0.6	12.1 ± 0.8*	10.7 ± 1.1	11.5 ± 0.6	11.5 ± 0.9	12.8 ± 1.2*
3	Starting body wt. (g)	257.1 ± 8.9	256.7 ± 8.9	256.0 ± 8.3	257.4 ± 7.9	256.8 ± 10.1	255.0 ± 12.2
	Terminal body wt. (g)	303.2 ± 15.5	297.3 ± 17.6	308.3 ± 14.9	264.6 ± 26.4*	300.8 ± 14.9	320.0 ± 22.7
	Liver (g)	12.9 ± 0.9	14.0 ± 1.9	13.0 ± 1.3	12.2 ± 1.6	13.0 ± 1.3	13.6 ± 2.0
	Adrenals (mg)	58.8 ± 9.3	57.0 ± 9.2	61.5 ± 10.6	49.9 ± 7.8	50.2 ± 5.1	51.0 ± 11.7
	Kidneys (mg)	2110 ± 72	2344 ± 138*	2290 ± 162	2229 ± 226	2189 ± 192	2435 ± 244*

V.C., vehicle control; TP, testosterone propionate. $n = 6$ rats/group/Lab.
* Significantly different from control group at $P < 0.05$.

3.1.2. Accessory sex organ weights

Five accessory sex organ and total five organ weight changes are shown in Table 3, and normalized organ weight changes are shown in Fig. 1. The accessory sex organ weights of rats given TP only in all laboratories were higher than these of rats given the vehicle alone, confirming the reliability of this study. Almost all accessory sex organ weights and total five organs in rats given L were higher than in rats given the vehicle in all laboratories. The LABC weights in rats given E was significantly higher than in rats given the vehicle in Lab 2, but the normalized change in this organ was

Table 3
Mean body weights and mean organ weights in agonistic version

Lab	Body weights/organ weights	Substances					
		V.C.	A	B	L	E	TP
1	Terminal body wt. (g)	262.9 ± 17.0	250.6 ± 12.0	252.4 ± 12.8	243.7 ± 9.2	249.4 ± 12.9	266.0 ± 12.7
	Ventral prostate (mg)	16.8 ± 1.0	17.0 ± 3.2	15.6 ± 3.4	34.1 ± 8.0	14.9 ± 1.2	93.6 ± 11.0*
	Seminal vesicles (mg)	27.9 ± 5.6	26.5 ± 1.9	25.7 ± 4.9	61.2 ± 9.6*	29.0 ± 3.5	190.4 ± 19.1*
	LABC (mg)	136.8 ± 22.2	128.2 ± 19.2	128.3 ± 11.0	298.5 ± 28.1*	141.5 ± 15.2	312.3 ± 26.9*
	Glans penis (mg)	29.5 ± 5.6	29.9 ± 2.8	28.3 ± 7.2	49.8 ± 6.5*	32.6 ± 5.1	64.4 ± 6.0*
	Cowper's glands (mg)	4.1 ± 1.3	4.4 ± 1.4	3.9 ± 1.5	10.3 ± 2.5*	4.5 ± 1.1	20.4 ± 3.5*
	Total of five organs (mg)	215.0 ± 21.3	206.0 ± 23.5	201.9 ± 21.3	453.8 ± 48.3*	222.4 ± 19.0	681.0 ± 42.3*
2	Terminal body wt. (g)	280.8 ± 7.9	275.9 ± 6.3	285.2 ± 12.4	261.5 ± 7.1*	281.8 ± 15.0	305.6 ± 15.0*
	Ventral prostate (mg)	16.0 ± 5.2	19.8 ± 5.1	15.8 ± 6.5	33.4 ± 6.3*	18.4 ± 3.2	121.8 ± 25.6*
	Seminal vesicles (mg)	42.0 ± 14.5	40.9 ± 11.2	38.8 ± 12.2	178.7 ± 60.4*	41.6 ± 11.5	420.4 ± 32.1*
	LABC (mg)	163.6 ± 38.3	178.6 ± 23.5	189.9 ± 30.4	426.8 ± 46.2*	216.3 ± 17.3*	527.5 ± 23.5*
	Glans penis (mg)	44.1 ± 4.3	42.9 ± 2.2	41.8 ± 2.3	58.5 ± 3.7*	45.5 ± 2.5	73.9 ± 3.9*
	Cowper's glands (mg)	5.8 ± 1.3	5.6 ± 1.1	4.4 ± 1.7	10.1 ± 2.6*	5.8 ± 1.8	34.4 ± 8.1*
	Total of five organs (mg)	271.6 ± 51.3	287.8 ± 29.7	290.7 ± 35.3	707.6 ± 104.7*	327.7 ± 14.6	1177.9 ± 35.1*
3	Terminal body wt. (g)	303.2 ± 15.5	297.3 ± 17.6	308.3 ± 14.9	264.6 ± 26.4*	300.8 ± 14.9	320.0 ± 22.7
	Ventral prostate (mg)	22.0 ± 3.1	20.6 ± 1.4	24.0 ± 1.7	43.7 ± 11.5*	26.2 ± 3.8	186.5 ± 48.4*
	Seminal vesicles (mg)	61.2 ± 5.9	58.1 ± 7.0	58.4 ± 8.2	165.5 ± 37.1*	61.2 ± 10.9	431.3 ± 55.1*
	LABC (mg)	191.3 ± 16.0	178.6 ± 25.2	190.7 ± 6.6	452.3 ± 34.5*	221.1 ± 35.8	543.5 ± 83.5*
	Glans penis (mg)	53.0 ± 8.0	54.6 ± 5.4	54.8 ± 5.6	72.9 ± 3.2*	52.0 ± 2.6	95.1 ± 8.0*
	Cowper's glands (mg)	8.5 ± 2.2	7.4 ± 1.8	8.0 ± 1.3	18.2 ± 5.2*	8.8 ± 2.4	37.1 ± 6.6*
	Total of five organs (mg)	336.0 ± 19.9	319.3 ± 29.6	336.0 ± 13.7	752.5 ± 66.4*	369.4 ± 45.8	1293.6 ± 112.7*

V.C., vehicle control; TP, testosterone propionate. $n = 6$ rats/group/Lab.
* Significantly different from control group at $P < 0.05$.

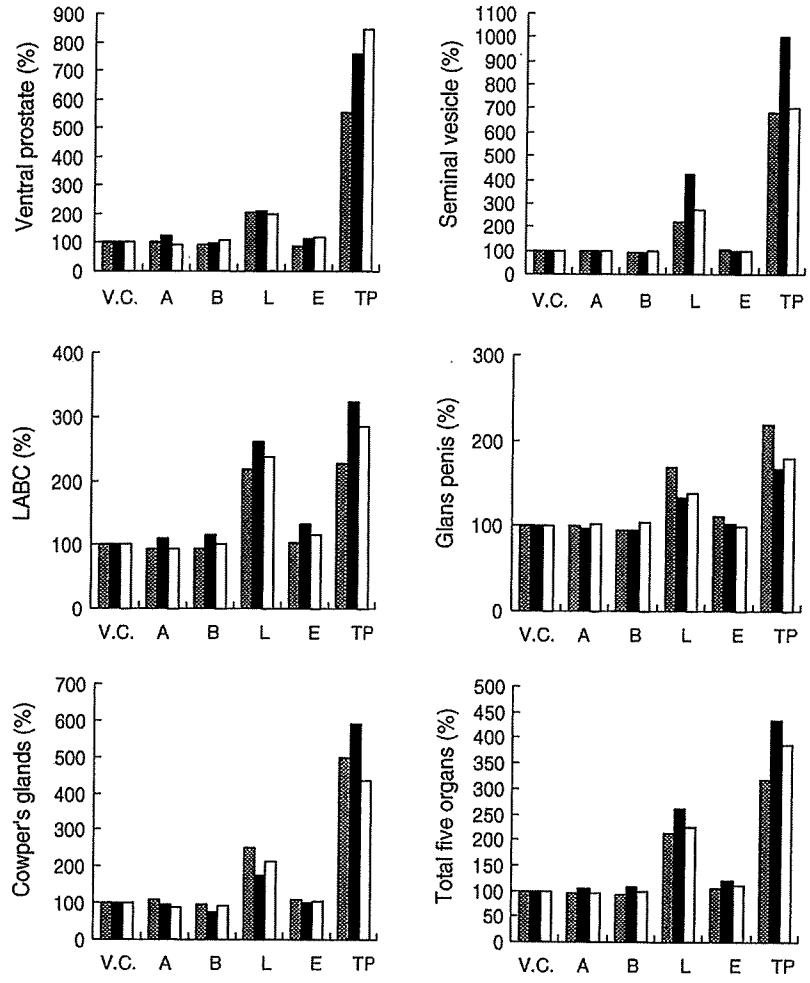

Fig. 1. Organ weights of accessory sex organs in agonistic version. Values from each laboratory were normalized to the control value set equal to 100%. LABC: levator ani and bulbocavernosus muscle; V.C.: vehicle control; A, B, L, and E: coded chemicals; TP: testosterone propionate. $n = 6$ rats/group/Lab (▩, Lab 1; ■, Lab 2; □, Lab 3).

not apparent. Normalized weight changes of the glans penis in rats given coded L showed the weakest response among five organs (Fig. 1).

3.2. Antagonistic version

3.2.1. Body weights, clinical general observations, and optional organ weights

The body weight changes and the optional organ weight changes are shown in Table 4. Two rats given I plus TP died with toxic signs such as decreasing body weight, soft feces, reddish urine, and weakness at 7–10 days after the administration in Labs 2 and 3, respectively. The terminal body weights in rats given I plus TP or K plus TP were significantly lower than in rats given TP only in two laboratories. The paired adrenals in rats given K plus TP were significantly higher than in rats given TP in Lab 3. The liver weights in rats given I plus TP were higher than in rats given TP in all laboratories, and increased liver weights were also observed in rats given C in Lab 1.

3.2.2. Accessory sex organ weights

Five accessory sex organ and total organ weight changes are shown in Table 5, and normalized organ weight changes are shown in Fig. 2. All accessory sex organ weights of rats given H, which is a positive compound, flutamide, plus TP were lower than those of rats given TP, confirming the reliability of this version. Almost all the accessory sex organ weights in rats given I plus TP and K plus TP were significantly lower than in rats given TP in all laboratories. Some accessory sex organ weights in rats given C plus TP and D plus TP were also lower than in the rats given TP. Although the