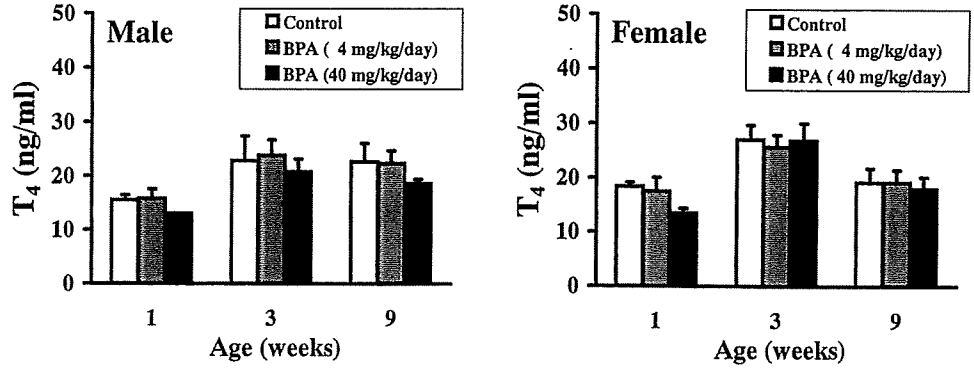

Fig. 1. Effects of maternal exposure to BPA on plasma T_4 levels in male and female offspring.
Each column and vertical bar represents the mean and SEM, respectively. There were no significant differences among groups.

weeks of age were injected with bovine TSH (bTSH; Sigma-Aldrich Corp., St. Louis, MO) intraperitoneally at 25.0 mIU/5 μl/g BW and intramuscularly at 12.5 mIU/5 μl/g BW. Blood samples were collected from the postcaval vein under euthanasia by ether inhalation at 6 h after bTSH administration and then stored at –20°C until the analysis. Plasma T_4 levels were measured as described above and are presented as the percentage of corresponding basal values.

Statistical analysis

For plasma T_4 determinations, the differences from the corresponding control group were statistically analyzed by analysis of variance followed by Dunnett's test (significance at $p<0.05$). In the TSH stimulation tests, the difference from the corresponding basal value was statistically analyzed using the Student's or Welch's *t*-test (significance at $p<0.05$).

Results

In male and female offspring, no statistically significant differences in plasma T_4 levels were observed between the control and BPA groups at 1, 3 and 9 weeks of age (Fig. 1). Plasma T_4 levels after TSH administration were significantly elevated compared with the corresponding basal values in the control and the BPA groups. No treatment-related differences were measured in the T_4 levels or in the relative T_4 increase in response to exogenous TSH in all groups of both sexes (Figs. 2, 3). In male offspring, plasma T_4 levels in the control, 4 and 40 mg/kg/day groups increased by 243%, 206% and 330%, respectively, compared with the corresponding basal values. Likewise, in female offspring, plasma T_4 levels increased by 285%, 311% and 275%, respectively (Fig. 3).

Discussion

Reproductive and developmental toxicity studies have been conducted using higher doses of BPA. Although maternal toxicity (reduction in maternal weight gain during gestation) was noted in rats exposed to BPA by gastric intubation at 160, 320 and 640 mg/kg/day from GD 6 through GD 15, there were no toxic effects on the development of their pups[17]. Kwon *et al.* showed that exposure at 320 mg/kg/day from GD 11 through PND 20 resulted in no apparent change in male and female pubertal development and reproductive function in SD rats[18]. We previously reported that exposure at 4 or 40 mg/kg/day from GD 6 through PND 20 did not cause changes in somatic growth or anogenital distance in rat offspring of both sexes[16], but these doses did affect testosterone homeostasis in the male testis[19]. Thus, much of the interest in BPA toxicity has focused on its putative effects on reproductive organs and genital glands. In the present study, we investigated whether *in utero* and lactational exposure to BPA affects endocrine status in the thyroid gland of rat offspring. Plasma T_4 levels were unaffected in the BPA groups (Fig. 1). A TSH stimulation test was then performed to examine thyroid function. Plasma T_4 levels were significantly elevated after exogenous TSH administration in the BPA-exposed groups, similar to the elevation seen in the control group (Fig. 2), suggesting that BPA exposure does not affect the synthesis and release of thyroid hormone in offspring *in vivo*. To our knowledge, this study represents the first attempt to better examine the effects of BPA on thyroid function *in vivo* in rat offspring exposed to relatively high levels of BPA.

Thyroid hormones play important roles in normal growth, neuronal development and metabolism in animals. During

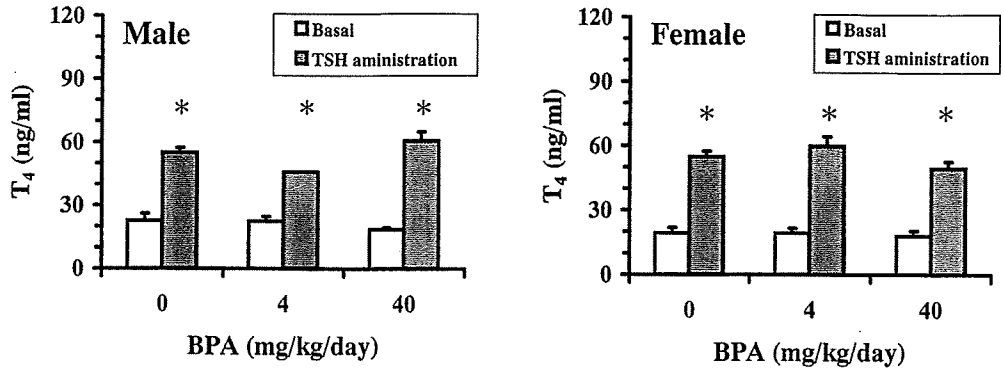

Fig. 2. Effects of maternal exposure to BPA on T_4 response to TSH in male and female offspring at 9 weeks of age.
Each column and vertical bar represents the mean and SEM, respectively. *Significantly different from the corresponding basal value ($p<0.05$).

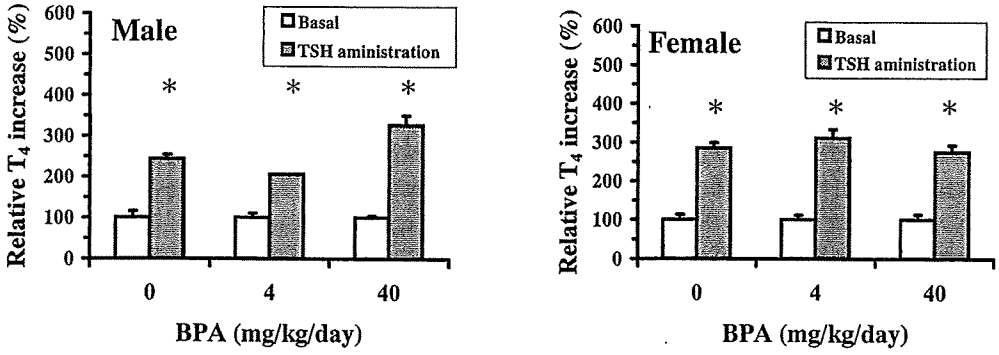

Fig. 3. Effects of maternal exposure to BPA on relative T_4 increase in response to TSH in male and female offspring at 9 weeks of age.
Each column and vertical bar represents the mean and SEM, respectively. *Significantly different from the corresponding basal value ($p<0.05$).

fetal and early neonatal periods, impaired thyroid hormone function may affect somatic growth[20]. BPA may act as an agonist or antagonist of the thyroid hormone receptor because of its structural similarity to thyroid hormone. Hence, given that thyroid hormone receptors are expressed ubiquitously and abundantly in various organs, BPA may perturb thyroid hormone action throughout the body tissue. Furthermore, BPA was observed to distribute rapidly in fetuses via placental transfer after a single BPA administration to pregnant female rats[21], mice and monkeys[22]; i.e., the placental barrier cannot block BPA transfer. Despite this high transplacental passage, there is sufficient glucuronyl transferase activity to metabolize BPA to BPA-monoglucuronide even in neonatal rats[23]. Although BPA may have the potential to disrupt thyroid hormone action through the thyroid hormone receptor, the results of this study confirmed that thyroid status was unaffected after *in utero* and lactational exposures of the offspring to BPA. The fact that normal somatic growth is observed in rat offspring following exposure of dams to BPA (even at high doses) [16] has led to the conclusion that the thyroid remains intact in the offspring.

In vitro studies have demonstrated the binding of BPA to the thyroid hormone receptor. Kitamura *et al.* reported that BPA does not inhibit the binding of T_3 to the thyroid hormone receptor and does not inhibit the hormonal activity of T_3 to induce growth and GH production of the rat pituitary cell line GH3[15]. On the other hand, Moriyama *et al.* used a competitive binding assay to confirm that BPA binds weakly to thyroid hormone receptors in rat liver nuclear extract[14]. Furthermore, BPA was shown to suppress T_3-stimulated transcriptional activity in transient expression assays[14]. The discrepancies between these *in vitro* studies indicate that further investigation is required to clarify the possible mechanism(s) of BPA action on the thyroid hormone receptor. Our results here are in accordance with an *in vitro* study reported by Kitamura *et al.* [15], however, we have no plausible

explanation for the differences between these *in vitro* studies and the *in vivo* outcome that we describe here.

In conclusion, the results of the present study suggest that *in utero* and lactational exposure to BPA does not have an effect on thyroid status in the F_1 generation of male and female rats under our experimental conditions.

Acknowledgments

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Original Article

Comparative Investigation of Several Sperm Analysis Methods for Evaluation of Spermatotoxicity of Industrial Chemical: 2-Bromopropane as an Example

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Abstract: Reproductive toxicity of 2-bromopropane (2BP), a substitute for ozone layer-depleting chloro-fluorocarbon, was found among the workers in an electronics factory in Korea in 1995. Furthermore the importance of testicular toxicity has been realized since the problem of endocrine disruptors arose all over the world, but manual methods must rely on subjective assessment. Recently, computer-assisted sperm analysis (CASA) was proposed but this system requires vast investment. We then investigated the applicability of the MTT method with a microplate and sperm quality analyzer (SQA) as simple, rapid, and economic instrumental methods for the examination of sperm quality in rats, comparing it with the manual microscopic method and CASA. Epididymal fluid derived from male F344/N Slc (Fischer) rats intraperitoneally injected with 2BP in the dose range of 125–1,000 mg/kg/d twice a week (total 8 times) were examined by these methods as a model experiment. Sperm count measured by the manual method and CASA in the epididymal fluid, absorbance by the MTT method and sperm motility index value by the SQA method were significantly lower in the 2BP 1,000 mg/kg administered group than in the control group. This result suggests that the MTT method can detect oligospermia. With the microplate and microplate reader, the efficiency of detection becomes much better. Sperm analyses by the MTT method with the microplate reader and the SQA method are available for reproductive toxicity study in rats.

Key words: 2-Bromopropane, Tetrazolium salt, MTT (3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl tetrazolium bromide), SQA (sperm quality analyzer), CASA (computer-assisted sperm analysis), Manual microscopic method, Reproductive toxicity, Rat

Introduction

Certain substances found in the environment can upset normal endocrine balance and become a health hazard. An example of growing concern is their effect on sperm[1]. Some workers in semiconductor factories in Korea were found to have affected in their reproductive functions after exposure to 2-bromopropane (2BP)[2,3]. Subsequently, the reproductive effects of 2BP were confirmed to the animal experimental studies[4–7]. These reports prompted close reappraisal of the efficacy and feasibility of mass screening for toxicity to male reproductive functions in industrial populations.

Surveying the method of investigating sperm activity, each method now in use has its own serious shortcomings. For instance, the conventional, manual method of sperm count and assessment of motility under the optical microscope is fraught with inevitable subjective variations which would make inter-institutional comparison of data practically

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impossible. The introduction of computer-assisted sperm analysis (CASA) eliminated variations due to subjective evaluation but this apparatus is expensive[8-13] and has not enjoyed wide acceptance. Meanwhile, the sperm quality analyzer (SQA), which measures sperm count and motility by the optical method, was introduced as a simple and inexpensive alternative[14, 15]. The third approach is the biochemical method (MTT method) which measures color changes in the tetrazolium reaction to mitochondrial reductase by absorption spectrometry reflecting the overall numerical and functional power of sperm activity[16, 17]. Although these methods of sperm testing have proliferated, their performance and efficacy have been evaluated individually and never collectively using the same test samples. In particular, comparative investigation of the MTT method with CASA has never been performed. Their performance in terms of mass handling of large numbers of samples has not been evaluated properly.

We attempted to develop a method for measuring toxicity to the sperm by combining an absorption spectrophotometer with a microplate reader so that a large number of specimen can be processed rapidly. The method is objective, simple, inexpensive and efficient and can be applied to mass screening of workers in suspicious environments. In the days when more and more clinical tests for male reproductive disability need to be performed on an everyday basis, the ability to processing a large number of samples will be an important prerequisite in the selection of test methods. Furthermore, the MTT method, with its speed and simplicity to deal with a large number of facilities, is a technique suitable for the animal studies of male reproductive disturbance induced by various chemicals. In this study, we induced reproductive toxicity with 2BP as a representative of bromopropanes, which are used widely in the industrial workplace.

We report the results of a study carried out on rats given repeated doses of 2BP using the MTT method with a microplate reader in comparison with other methods of sperm testing including CASA. The advantages and merits of various methods were compared and problems in performing the tests will be discussed.

Materials and Methods

Chemicals and supplements

2BP and MTT were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Dojin (Kumamoto, Japan), respectively. Olive oil, HCl and isopropyl alcohol were from Wako Pure Chemical (Osaka, Japan). Bovine serum albumin Fraction V (BSA), Medium 199 and phosphate buffered saline (PBS) were from Seikagaku Kogyo (Tokyo, Japan), GIBCO (Grand Island, USA) and Nissui Pharmaceutical (Tokyo, Japan), respectively.

Instruments

The semen analyzer (HTM-IVOS Ver. 10.9i) was from Hamilton Thorne Research (Beverly, MA, USA). SQA was the product of Medical Electronic Systems (Migdal Haemek, Israel). The microplate reader (Immunoreader NJ2000) was purchased from Nalge Nunc (Tokyo, Japan). The optical microscope (Eclipse E600) was from Nikon (Tokyo, Japan).

Experimental protocol

F344/N Slc (Fischer) male rats (11 wk of age) from Japan SLC (Shizuoka, Japan) were kept in cages under standard conditions and received pellets (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. The body weight was monitored just before each administration and sacrifice. Each of 4–5 rats (12 wk of age)/group received intraperitoneal instillations of 2BP dissolved in olive oil twice a week for 24 d in doses of 125, 250, 500 and 1,000 mg/kg. Control rats received an equal volume of olive oil. So each rat received a total of 8 injections. After a one week rest period following the last dose, the animals were sacrificed under ether anesthesia and the testes, epididymis and epididymal cauda were separated and weighed immediately. And then relative organ weights were calculated.

Preparation of epididymal cauda sample

Epididymal cauda was minced with scissors to release sperm in 2 ml of Medium 199 containing 0.5% BSA at 37°C. This sperm suspension sample served for the MTT and SQA methods. The aliquot of this sample was stored at −80°C. Before sperm count analysis, this aliquot was diluted 1:4 with PBS, further homogenized at room temperature, and served as a sample for the manual method and CASA.

Manual method

After staining with trypan blue the specimen was spread on a hemocytometer and the sperm heads were counted manually under the optical microscope. The data were expressed as the total number of sperm per one cauda epididymal tissue.

CASA

Each sample was stained with the attached staining kit (Supra Vital IDENT Stain Kit, Hamilton-Thorne Research, Bevely, MA, USA), dropped into a disposable counting chamber CELL-VU (Millennium Sciences Corp. NY, USA) and

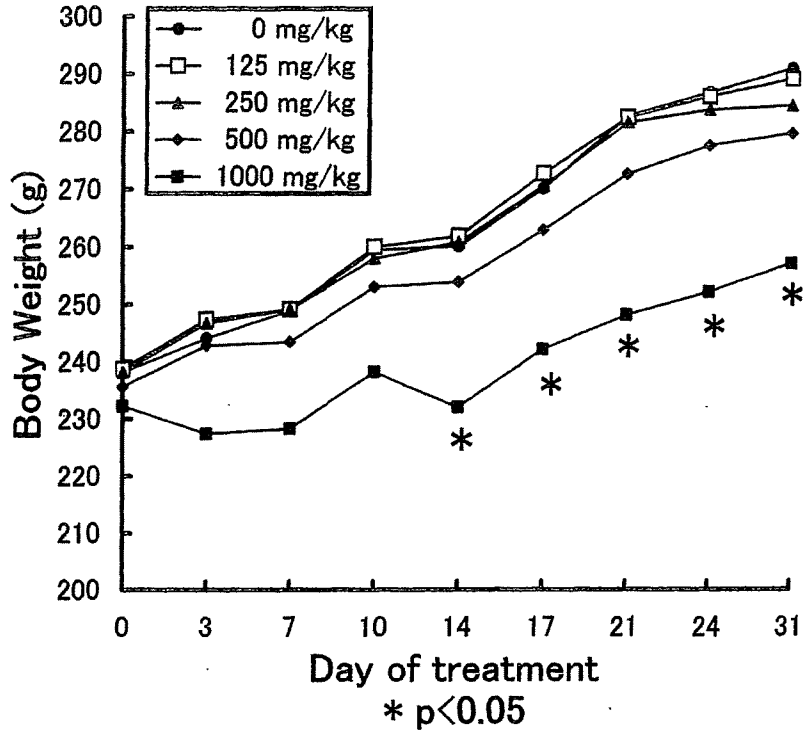

Fig. 1. Mean body weight of F344 rats exposed to 2BP ip.

mounted on the Semen Analyzer. Sperm heads fluorescence under an ultra-violet beam were counted in RAT-IDENT mode automatically. The data were expressed as the total number of sperm per cauda epididymal tissue sample.

SQA method

The disposable SQA capillary (Medical Electronic Systems, Migdal Haemek, Israel) containing the specimen was inserted into the slot in the SQA and the sperm motility index (SMI) was determined.

MTT method

Fifty microliters of the sperm suspension sample in the sterile 96-microplate well was incubated with MTT reagent (5 mg/ml in PBS, 25 micro liter) at 37°C for two hours. Then the reaction was stopped by rapid cooling. After the addition of 0.04 M HCl-isopropylalcohol and pipeting exhaustively to dissolve the formazan thus formed in the process, the absorbance in each well was estimated at 574 nm by the microplate reader.

Correlation among data from various methods

In an attempt at evaluation of various methods, we carried out the following experiments. Epididymis cauda obtained from an untreated rat (17 wk of age) was dissected with a pair of scissors in 2 ml of medium to suspend sperm. The sample consisted of this undiluted suspension and its dilutions with Medium 199 containing 0.5% BSA to 4 strength. Data on sperm count, SMI and absorbance obtained by pair of investigating methods were compared and correlation was sought.

Statistical analysis

The data were analyzed by one-way ANOVA. The statistical significance of difference between the control and 2BP-treated groups was determined with Fisher's PLSD test. In all cases, $P < 0.05$ was considered statistically significant.

Results

Body and organ weights

Body weight decreased in the groups with a dose of 1,000 mg/kg (Fig. 1) on and after day 14 as compared to the control group. The relative weights of testis (TE, right (R) and left (L)), epididymis (EP, right (R) and left (L)), and epididymis cauda (EPC, right (R) and left (L)) are shown in Table 1. The weight of both the right and left testis decreased in the 500 and 1,000 mg/kg dose groups. In the 250 mg/kg dose group, only the left testis weight decreased significantly, but no significant difference was found in the right testis.

Table 1. Relative weight (%) of reproductive organ in 2BP-treated (mg/kg) rats

2BP (mg/kg)	TER	TEL	EPR	EPL	EPCR	EPCL
0	0.503 ± 0.010	0.521 ± 0.018	0.172 ± 0.007	0.176 ± 0.011	0.080 ± 0.004	0.076 ± 0.005
125	0.505 ± 0.020	0.512 ± 0.020	0.186 ± 0.012	0.176 ± 0.017	0.089 ± 0.003*	0.082 ± 0.006
250	0.493 ± 0.023	0.480 ± 0.025*	0.183 ± 0.011	0.184 ± 0.014	0.087 ± 0.006	0.089 ± 0.011*
500	0.407 ± 0.021*	0.409 ± 0.038*	0.168 ± 0.012	0.179 ± 0.008	0.086 ± 0.006	0.076 ± 0.003
1000	0.193 ± 0.010*	0.199 ± 0.019*	0.131 ± 0.009*	0.130 ± 0.007*	0.056 ± 0.006*	0.055 ± 0.004*

TER: Right Testis, TEL: Left Testis, EPR: Right Epidydymis, EPL: Left Epididymis. EPCR: Right Epididymal Cauda, EPCL: Left Epididymal Cauda. Each value represents the mean ± SD. *: Significantly different at $p<0.05$.

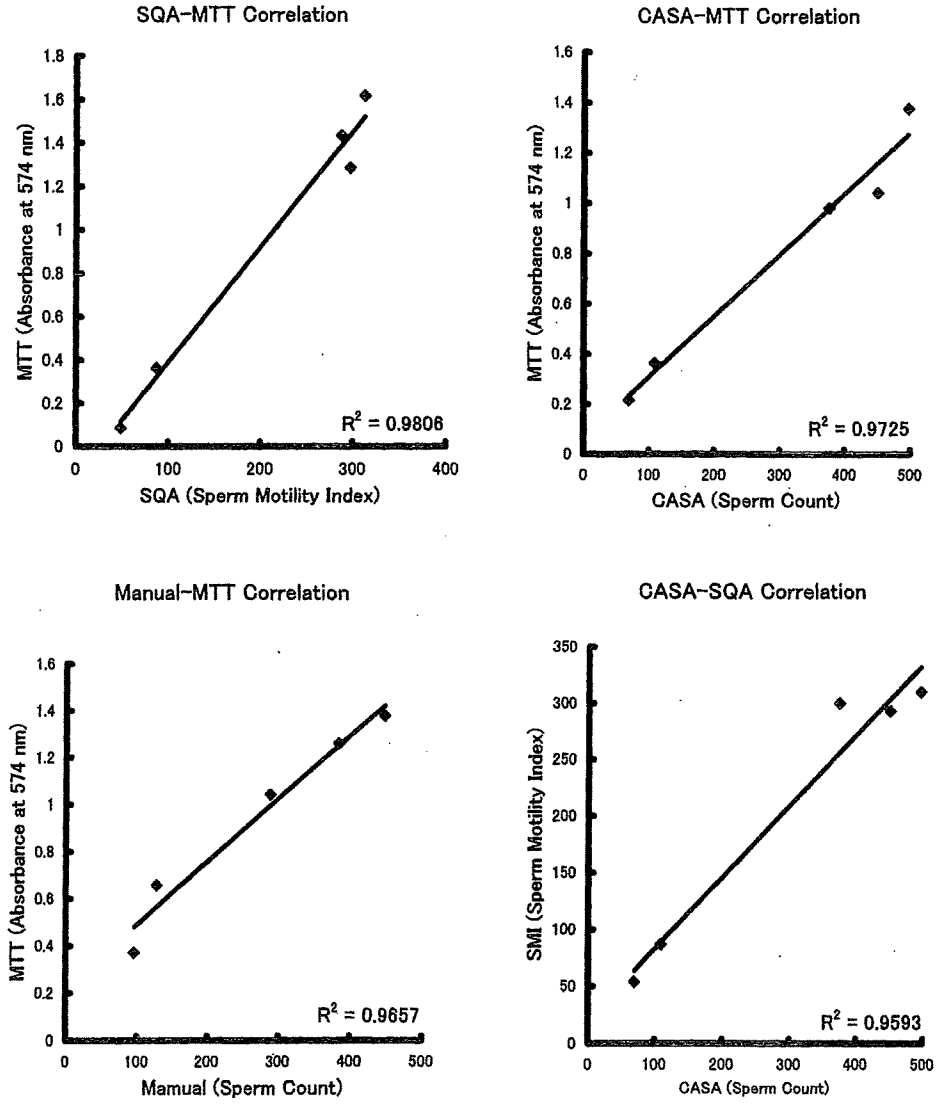

Fig. 2. Relationship between parameters of several methods.

And the weights of the epididymis and epididymal cauda decreased in the 1,000 mg/kg group, but some reverse results were found in lower dose groups (EPCR: 125 mg/kg, EPCL: 250 mg/kg), but they were not in both sides of the tissues.

Correlation between the sperm analysis methods

Figure 2 shows the correlation between the two methods. A high correlation was found between the SMI value found by the SQA method and absorption by the MTT method

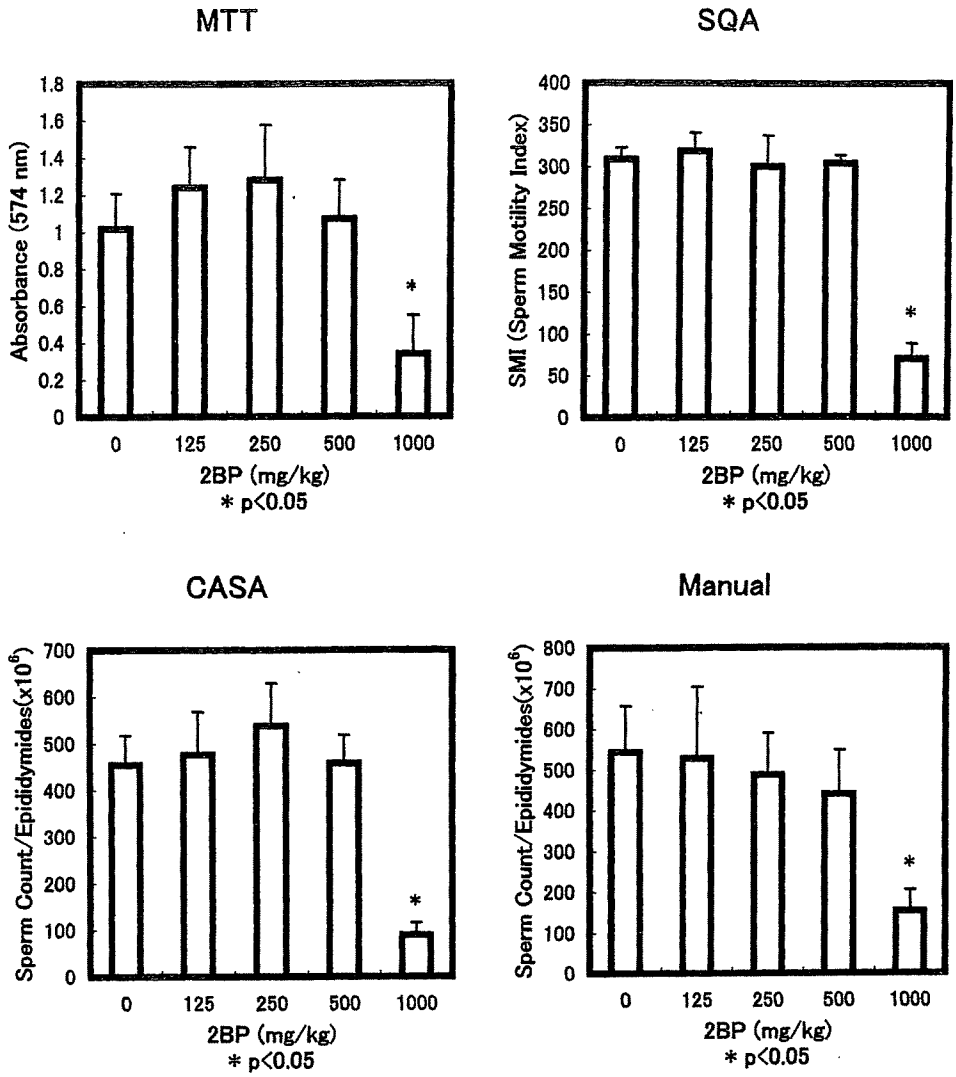

Fig. 3. Effect on rat sperm of 2BP (Comparison of results obtained by the MTT, SQA, CASA and Manual methods).

(R^2=0.9806). Similarly, MTT absorbance correlates well with the Sperm count by CASA (R^2=0.9725) and the manual method (R^2=0.9657). All the methods had common linearity mutually.

Detection of sperm toxicity of 2BP by several methods

As shown in Fig. 3, sperm count by the manual method, sperm count by CASA, SMI by the SQA method and absorbance by the MTT method were similarly low only in the 1,000 mg/kg group as compared with the control group. There was no significant reduction in the parameters in the groups given 2BP at the dose of less than 500 mg/kg.

Discussion

Only the testes weight could indicate sperm toxicity in the 500 mg/kg dose group, whereas while other methods failed to detect the change (Table 1 and Fig. 3). Although the weight of testes was the most sensitive parameter indicating the sperm toxicity, this requires autopsy and therefore has no clinical usefulness. A non-invasive method such as sperm count under an optical microscope is more practical and is widely accepted, but inherent shortcomings of this method are obvious: counts are susceptible to subjective variations and their inter-institutional or inter-observer comparisons and analyses are unreliable if ever possible, and it is not suitable for processing a large number of specimens in mass screening.

SQA is a simple and practical method, but it is no match in efficiency for the MTT method. In comparison, the MTT method excels SQA on account of rapidity and simplicity and therefore its ability to handle a large number of samples simultaneously. CASA gives not only the sperm count, but other information such as the motility rate and even morphological indexes[8–13]. Unfortunately this requires rather expensive equipment and has not achieved wide acceptance. MTT, one of the tetrazolium salts, is known to form formazan and is turned blue in the somatic cells by mitochondrial reductase[16]. The same reaction is observed in a suspension of sperm and the extent of coloring reflects the number and viability of the sperm[17]. The MTT method is utilized in many toxicity studies on somatic cells, but its use in sperm cell studies has been reported in only one paper. We introduced the use of the microplate and established a distinctly more efficient measurement system. If only qualitative analysis is required, direct observation may suffice, dispensing with absorption spectrometry. Possibly other tetrazolium salts may be found equally or more useful as the substrate and may replace MTT. We proposed the use of the MTT method for sperm analysis and established the protocol with microplates to facilitate processing a large number of samples rapidly as is required in mass screening. In the sperm count we obtained high correlation between the results by the MTT method and those by SQA, CASA, and the manual method as shown in Fig 2. We believe that the MTT method can replace these other methods where only the sperm count is required, but the MTT reaction is dependent on the activity of mitochondrial reductase in the sperm. This method cannot be expected to distinguish those sperm with abnormal morphology or diminished motility from normal, healthy sperm, as long as they have metabolic activity. On the other hand, the SMI value which is obtained by SQA has a positive correlation with both sperm count and sperm motility and is recognized as a strong predictor of fertility of the semen. The manual method can distinguish sperm deformity as well as give the sperm count. The advantage of the MTT method, on the other hand, is the efficiency in processing a large number of specimens and therefore may be a powerful tool for preliminary screening. The results of our animal experiments also established that the MTT method could detect sperm toxicity caused by introduced chemical agents to an extent comparable to other methods such as the manual method or CASA.

In conclusion we assert that the MTT method using the microplate reader provide a new tool in detecting sperm toxicity with sensitivity comparable to conventional or more expensive methods and is especially suitable for workplace mass screening. But manual dissolution of formazan is an extra step required in the MTT method. It is not readily amenable to automation. And this is a cause of errors in measurement. Recently tetrazolium salts which produce water-soluble formazan have been developed and their usefulness in toxicity tests on somatic cells has been reported. When these newer salts are used the process of dissolution of formazan is not necessary. Enhanced simplicity and improved accuracy of the method are expected. We plan to continue further studies on sperm toxicity using various tetrazolium salts to replace MTT.

In our experiments, distinct sperm toxicity was observed only in the group of rats given a large dose (1,000 mg/kg) of 2BP whereas a significant reduction in the weight of testes had already been found in the 500 mg/kg group (Table 1). This suggests that 2BP exerts its effect in the spermatogenesis stage. And reverse effects were observed in several cases in organ weight (EPCR: 125 mg/kg, EPCL: 250 mg/kg). This phenomenon is thought to result in a transitory effect.

Furthermore, we have started the study of reproductive toxicity induced by bromopropanes other than 2BP and have found that the MTT method is equally applicable to in these studies. We plan to present additional data on 2BP and other chemicals and further discussion on the mechanism of reproductive toxicity in our ensuing reports.

Acknowledgement

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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.

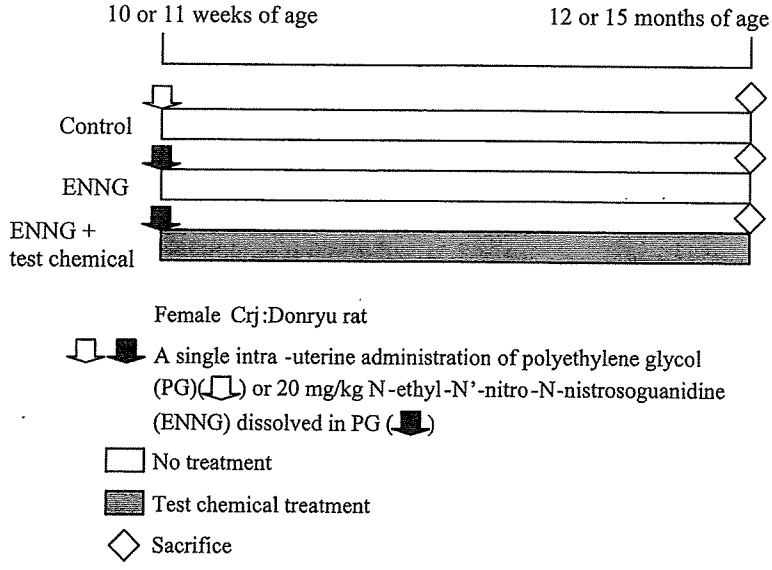

Fig. 1. Two-stage rat uterine carcinogenesis model

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,

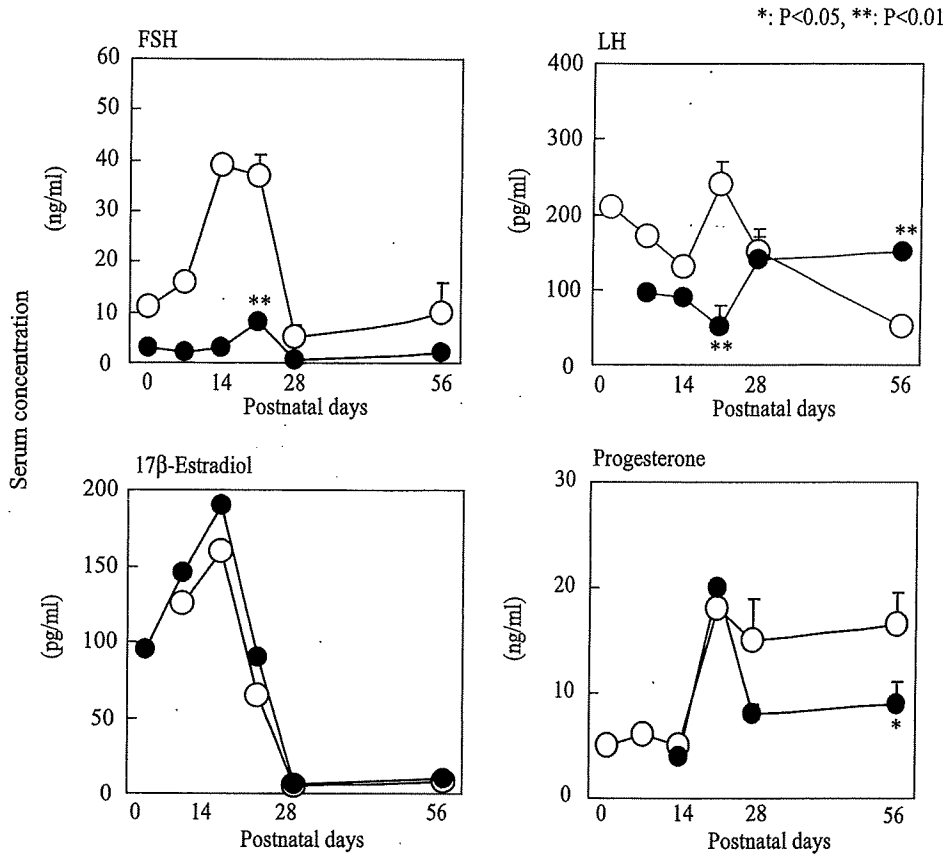

Fig. 2. Serem gonadotropins and sex steroid hormones in control and PNDs 1–15 OP-treated rats. Open circles (○), controls; black circles (●), PNDs 1–15 OP-treated. Katsuda *et al.*, Toxicol Appl Pharmacol 2000; 165: 217–226.

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