Immature Rat Uterotrophic Assay of Bisphenol A

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We used the immature rat uterotrophic assay to determine the estrogenicity of bisphenol A (BPA). We administered BPA (in sesame oil) to rats subcutaneously (sc; 0, 8, 40, and 160 mg/kg/day) or orally (0, 40, 160, and 800 mg/kg/day) for 3 days beginning on postnatal day (PND) 18; rats were sacrificed 24 hr after the last administration. Uterine wet, blotted, and relative weights increased in all groups given BPA sc. After oral administration, uterine relative weight increased in 160 and 800 mg/kg BPA groups, and wet and blotted weights increased in the 800 mg/kg BPA group. Plasma concentrations of BPA at 1 hr after the last administration were detected in all groups given BPA sc and in groups given 160 and 800 mg/kg BPA orally, with a dose–response effect. The study was then reproduced under the same conditions. After sc injections, uterine wet and blotted weights increased in the 40 and 160 mg/kg BPA groups, and relative weight increased in all groups given BPA sc. By contrast, uterine wet, blotted, and relative weights increased only in the 160 and 800 mg/kg oral BPA groups. Also, to examine time-course changes in uterine weight, we administered BPA (in sesame oil) sc from PND 18 to PND 20 for 3 days at doses of 0, 8, 40, and 160 mg/kg/day; uterine weights were then measured at 6, 12, 18, and 24 hr after the last administration. Uterine wet, blotted, and relative weights increased in all BPA groups at 6 and 24 hr and in 40 and 160 mg/kg BPA groups at 12 hr. By contrast, at 18 hr, uterine wet, blotted, and relative blotted weights increased in all BPA groups and relative wet weight increased in 40 and 160 mg/kg BPA groups. The percentage increases in uterine wet and relative weights of 40 and 160 mg/kg BPA groups at 6 hr were higher than those at 24 hr relative to the controls, but the coefficient of variation in these weights in the group given 8 mg/kg BPA at 24 hr was smaller than that at 6 hr. These findings demonstrate BPA-induced uterotrophy in the immature uterotrophic assay in rats administered 8 mg/kg/day sc and in rats given 160 mg/kg/day orally, and suggest that the autopsy at 24 hr after the last administration is suitable.
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There is concern that certain chemicals may have the potential to disturb normal sexual differentiation and development in animals and humans (*1*,*2*). Recently, the Organisation for Economic Co-operation and Development (OECD) proposed the immature rat uterotrophic assay as one screening method for detecting the estrogenic properties of such chemicals, in which the chemical compounds are orally or subcutaneously administered for 3 days (*3*).

Bisphenol A (BPA) has been confirmed to have weak estrogenicity *in vitro* that is approximately 15,000 times less potent than 17 β-estradiol and interacts with both the α- and β-estrogen receptors (*4*–*7*). It is an important chemical used principally as a monomer in the manufacture of a multitude of chemical products including epoxy resins and polycarbonate, and its output has gradually increased from year to year. Uterotrophy measured by uterotrophic assay of BPA in the immature rat was previously detected after subcutaneous (sc) or oral administration of 400 mg/kg BPA (*8*), whereas no uterotrophy in this assay was observed in rats orally administered 5–150 mg/kg BPA (*9*). However, this assay has not yet been used to determine the lowest dose of BPA that can induce a uterotrophic response.

One purpose of this study was to determine the estrogenic effect of BPA at low dose levels using the immature rat uterotrophic assay. We also measured the plasma concentrations of BPA 1 hr after the last administration to examine the differences in plasma concentrations between sc and oral administration routes.

Because the reproducibility of the uterotrophic assay has been questioned (*10*), we performed a repeat study of BPA with the immature rat uterotrophic assay. Although time-course changes in uterine weight in the immature rat uterotrophic assay have been reported after the last administration of strong estrogenic compounds such as 17β-estradiol (*11*), there have been no reports of weak estrogenic compounds using this assay. By comparison, the OECD proposed autopsy in the immature rat uterotrophic assay at 24 hr after the last administration. Therefore, our other purpose in this study was to examine the time-course changes of uterine weight in the immature rat uterotrophic assay of BPA.

Materials and Methods

Study 1

Chemicals. We purchased BPA (lot 077H0666; > 98% pure) from Kanto Chemical Co., Tokyo, Japan, and sesame oil (lot 004RYY) from Fujimi Pharmaceutical, Co., Osaka, Japan.

Animals. Pregnant Crj:CD (SD) rats (at day 14 of gestation) were purchased from Charles River Japan, Inc., Shiga, Japan. At postnatal day (PND) 4, the litters were culled to eight per dam, and dams and pups were kept in polycarbonate pens until weaning. All rats were weaned at PND 17 and then housed individually in stainless steel wire-mesh cages during the study. The immature rats were weighed, weight-ranked, and assigned randomly to each of the treatment and control groups; each group consisted of 10 rats. Body weights and clinical signs were recorded on a daily basis throughout the study. Before weaning, rats were provided with tap water and a commercial diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) *ad libitum;* after weaning, rats received water automatically and a commercial diet (MF; Oriental Yeast Co., Tokyo, Japan) *ad libitum*. The animal room was maintained at a temperature of 23 ± 20°C and a relative humidity of 55 ± 5%, and was artificially illuminated with fluorescent light on a 12-hr light/dark cycle (0600–1800 hr).

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.

Study design. BPA (in sesame oil) was injected sc at 8, 40, and 160 mg/kg for 3 days beginning on PND 18, or administered orally via a stomach tube at 40, 160, and 800 mg/kg for 3 days beginning on PND 18. These doses were based on the results of preliminary studies using sc or oral doses of 40, 160, and 800 mg/kg/day. The concentration and stability of BPA were confirmed. Rats were administered 2 mL/kg body weight of sesame oil containing BPA sc or 5 mL/kg orally. A vehicle control group given only sesame oil was also established for each administration route. The animals were killed approximately 24 hr after the last administration by bleeding from the abdominal vein under deep ether anesthesia. After

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necropsy, the uteri were carefully dissected free of adhering fat and mesentery and then weighed. Plasma concentrations of BPA were measured in four female rats from each group 1 hr after the last administration. The time sampling point was determined by the results of preliminary study: mean plasma concentration of three rats given 1,000 mg/day BPA subcutaneously or orally for 3 days was higher 1 hr after the last administration than that at 0.5, 2, 4 and 6 hr after administration.

Plasma samples of each group were pretreated by solid extraction column and then measured using HPLC. Plasma samples (250 µL) were applied to the Sep-Pak C18 Cartridge (6 cc, 1 g, part WAT036905; Waters, Milford, MA, USA), washed twice with 1 mL distilled water and 1 mL 20% acetonitrile, and eluted with 500 µL 70% acetonitrile. A 20 µL aliquot of the effluent was analyzed using an HPLC system consisting of a pump (model L-7100; Hitachi, Tokyo, Japan), an auto sampler (model L-7200; Hitachi), a column oven (model L-7300; Hitachi), a detector (model L-7480 FL; Hitachi), and a D-7000 HPLC system manager (Hitachi). An L-column ODS (4.6 mm i.d. × 250mm; Chemicals Evaluation and Research Institute, Tokyo, Japan) was used for the analysis. For the mobile phase, we used 43% acetonitrile in water and a flow rate of 1 mL/min. Detection was performed at 224 nm (excitation wavelength) and 310 nm (emission wavelength).

Statistical analysis. Data of body weight and uterine weight were tested by Bartlett's test for homogeneity of variance. When the homogeneity of variance ($p < 0.05$) was evident from Bartlett's test, we performed analysis of variance (ANOVA). When significant ($p < 0.05$) treatment differences were indicated by ANOVA, we used the Student's *t*-test to compared each BPA group with the control.

Study 2

We performed a repeat test (study 2) approximately 1 month after study 1 using the same conditions as for study 1 except that plasma concentrations were not measured.

Study 3

For study 3, we used subcutaneous injection as the only administration route for this study, after the results of studies 1 and 2 revealed that sensitivity to sc-administered BPA was higher than with oral administration. We used the same conditions as for study 1, except the animals were sacrificed at 6, 12, 18, and 24 hr after the last administration by bleeding from the abdominal vein under deep ether anesthesia.

Results

Study 1

Clinical signs, body weight, and gross findings. We detected no abnormal clinical signs, including immature vaginal opening or gross findings. We also found no significant differences in body weight between the controls and each BPA group (Table 1).

Organ weights. Uterine wet, blotted, and relative weights were increased in all groups given BPA sc. With oral administration, however, uterine wet and blotted weights were increased in the 800 mg/kg BPA group, whereas relative weight increased in the 160 and 800 mg/kg BPA groups (Table 2).

Plasma concentrations. Plasma concentrations of BPA were detected in all groups given BPA sc and in groups given 160 and 800 mg/kg BPA orally, with a dose–response effect (Table 3). Comparing plasma concentrations for the same dose between sc and oral routes, BPA values were much higher in the 160 mg/kg BPA sc group than in the group given the same dose of BPA orally.

Study 2

Clinical signs, body weight, and gross findings. No abnormal clinical signs were detected, and there were no significant differences in body weight between the controls and each BPA group (Table 1).

Uterine weight. With sc injection, uterine wet and blotted weights increased in 40 and 160 mg/kg BPA groups, whereas relative weights increased in all groups given BPA. With oral administration, uterine wet, blotted, and relative weights increased in groups given 160 and 800 mg/kg BPA orally (Table 2).

Table 1. Body weight of rats administered BPA for 3 days beginning on PND 18.

Route/study	Dose (mg/kg)	Start weight (g)	Final weight (g)
Subcutaneous			
Study 1	Vehicle control	36.8 ± 2.1	47.4 ± 2.5
	8	36.3 ± 2.2	46.1 ± 2.6
	40	36.7 ± 2.2	45.8 ± 2.3
	160	36.6 ± 2.0	45.4 ± 2.2
Study 2	Vehicle control	36.9 ± 1.2	47.5 ± 1.9
	8	37.4 ± 1.8	46.5 ± 1.8
	40	37.0 ± 1.0	46.8 ± 2.3
	160	37.1 ± 1.3	44.7 ± 3.3
Oral			
Study 1	Vehicle control	37.1 ± 2.6	46.5 ± 6.3
	40	36.5 ± 1.6	45.3 ± 2.2
	160	36.1 ± 2.2	45.4 ± 2.8
	800	37.1 ± 1.9	45.1 ± 4.5
Study 2	Vehicle control	36.9 ± 1.1	46.6 ± 2.3
	40	37.8 ± 1.1	47.2 ± 1.8
	160	36.8 ± 1.5	46.2 ± 3.7
	800	37.4 ± 1.5	44.5 ± 1.9

Values shown are mean ± SD.

Table 2. Uterine weight of rats administered BPA for 3 days beginning on PND 18.

Route/study	Dose (mg/kg/day)	Wet weight (mg)	Blotted weight (mg)	Relative wet weight (mg/100g)	Relative blotted weight (mg/100g)
Subcutaneous					
Study 1	Vehicle control	27.1 ± 3.6	26.5 ± 3.7	57.2 ± 6.4	55.8 ± 6.7
	8	31.1 ± 3.6*	30.3 ± 3.5*	67.8 ± 10.4*	66.1 ± 9.7*
	40	39.5 ± 4.6**	38.6 ± 4.4**	86.2 ± 8.7**	84.2 ± 8.4**
	160	55.7 ± 10.5**	54.7 ± 10.3**	122.1 ± 18.9**	119.8 ± 18.7**
Study 2	Vehicle control	27.6 ± 2.8	27.0 ± 2.5	58.4 ± 7.0	57.1 ± 6.4
	8	31.0 ± 4.5	30.4 ± 4.4	66.6 ± 8.3*	65.2 ± 8.2*
	40	40.7 ± 4.2**	39.7 ± 4.0**	87.1 ± 8.2**	84.8 ± 7.6**
	160	58.4 ± 8.5**	57.4 ± 8.0**	131.1 ± 21.1**	129.0 ± 20.0**
Oral					
Study 1	Vehicle control	27.7 ± 5.2	27.1 ± 5.1	59.5 ± 7.2	58.2 ± 7.3
	40	27.1 ± 2.5	26.4 ± 2.3	59.9 ± 6.0	58.4 ± 5.7
	160	30.5 ± 2.5	29.8 ± 2.6	67.3 ± 6.1*	65.8 ± 6.0*
	800	40.0 ± 6.6**	39.1 ± 6.7**	89.0 ± 14.6**	87.1 ± 14.4**
Study 2	Vehicle control	27.7 ± 3.0	27.1 ± 3.0	59.5 ± 6.0	58.2 ± 5.7
	40	29.8 ± 4.8	29.0 ± 4.8	63.1 ± 9.0	61.3 ± 9.2
	160	31.9 ± 5.1*	31.0 ± 5.1*	69.1 ± 10.3*	67.4 ± 10.2*
	800	41.0 ± 4.0**	39.9 ± 4.0**	92.2 ± 8.3**	89.5 ± 8.1**

Values shown are mean ± SD.
*Significantly different from corresponding vehicle control at $p < 0.05$. **Significantly different from corresponding vehicle control at $p < 0.01$.

Study 3

Clinical signs, body weight, and gross findings. In study 3, we observed no abnormalities in clinical signs, including immature vaginal opening and gross findings. In contrast, there were no significant differences in body weight between the control group and each BPA group.

Uterine weight. Uterine wet, blotted, and relative weights increased in all BPA groups at 6 or 24 hr after the last administration; these weights also increased in the 40 and 160 mg/kg BPA groups at 12 hr (Tables 4 and 5). At 18 hr, uterine wet, blotted, and relative blotted weights were increased in all BPA groups, whereas relative wet weight increased in 40 and 160 mg/kg BPA groups. Although the percentage increases in uterine wet and relative weights of the 40 and 160 mg/kg BPA groups were higher at 6 hr after the last administration than at 24 hr, the coefficient of variation in these weights in the 8 mg/kg BPA group was smaller at 24 hr than at 6 hr.

Discussion

In the immature rat uterotrophic assay, uterine weight has been used as a sensitive parameter for evaluating estrogenic activity (*12,13*). The OECD has proposed uterine weight change as an end point of estrogenic activity (*3*). Our results confirmed that uterine weight is a good marker for evaluating the estrogenic activity of BPA. We did not use parameters such as luminal cell height and cell proliferation because the usefulness of these parameters is unclear. Uterotrophy caused by 400 mg/kg BPA, either sc or oral administration, has been detected by the immature rat uterotrophic assay (*8*), but such a change has not yet been established for low doses. The present findings demonstrate that rats given a low dose of BPA, such as 8 mg/kg, show the endocrine-disrupting effect.

In studies 1 and 2, uterotrophy was estimated by both wet and relative weight changes, and reproducibility of the immature rat uterotrophic assay of BPA was confirmed. In study 2, uterine wet and blotted weights increased in groups administered 40 and 160 mg/kg BPA sc and in groups administered 160 and 800 mg/kg BPA orally, whereas in study 1 these weights increased in all sc BPA groups and in the group given 800 mg/kg BPA orally. Uterotrophy in the immature rat uterotrophic assay was usually determined by the change in wet weight because this weight was almost totally uninfluenced by the body weight changes (*14,15*). Because there were differences in uterine wet weights between studies 1 and 2, uterotrophy may be better estimated by changes in both wet weight and relative weight.

That uterotrophic activity was more sensitive to sc injection of BPA than oral administration reflects the differences in kinetics and bioavailability between these two routes. The mean plasma concentration of BPA in the sc 160 mg/kg BPA group was much higher than in the group given the same dose orally; this reflects the difference in extent of uterotrophy resulting from the two administration routes.

In this comparative study of the time-course changes in BPA-induced uterotrophy, the sensitivity to BPA as measured by both uterine wet weight and relative weight was higher at 6 or 24 hr after the last administration than at 12 and 18 hr. The percentage increases in wet weight and relative weight of 40 and 160 mg/kg BPA groups at 6 hr was higher than that at 24 hr relative to controls, but the coefficient of variation for both weights in the 8 mg/kg BPA group was lower at 24 hr than at 6 hr. We therefore suggest that autopsy at 24 hr after the last administration is more suitable, based on the coefficient of variation at low dose levels. Reel et al. (*11*) reported that uterine weights increased until 6 hr after 17β-estradiol administration and then decreased gradually; uterine weights also increased at 24 hr. The present results demonstrate that the OECD recommendation for autopsy at 24 hr after the last administration is reasonable for rat utertrophic assays.

Table 3. Plasma concentration of BPA 1 hr after last administration.

Route	Dose (mg/kg)	Plasma concentration (ng/mL)
Subcutaneous	Vehicle control	ND
	8	94.6 ± 58.0
	40	886.3 ± 56.4
	160	2948.8 ± 768.8
Oral	Vehicle control	ND
	40	ND
	160	198.8 ± 88.2
	800	2879.0 ± 2328.3

ND, not detected. Values shown are mean ± SD.

Table 4. Absolute uterine weights of rats 6, 12, 18, and 24 hr after last sc administration of BPA.

Hour	Dose (mg/kg/day)	Wet weight (mg)[a]	Blotted weight (mg)[a]	Wet weight (% of control)	CV (wet weight)
6	Vehicle control	27.8 ± 5.2	27.2 ± 5.0	100	18.7
	8	36.5 ± 8.5*	34.9 ± 7.4*	131	23.2
	40	55.6 ± 12.9**	53.4 ± 12.3**	200	23.2
	160	75.9 ± 11.1**	72.5 ±10.8**	273	14.6
12	Vehicle control	28.9 ± 6.2	28.0 ± 6.1	100	21.4
	8	32.2 ± 1.8	31.1 ± 1.7	111	5.5
	40	50.1 ± 5.8**	48.6 ± 5.3**	173	11.6
	160	63.4 ± 9.8**	62.0 ± 9.5**	219	15.4
18	Vehicle control	28.3 ± 4.7	27.3 ± 4.5	100	16.6
	8	33.1 ± 5.3*	32.4 ± 5.4*	117	16.1
	40	41.1 ± 5.6**	39.9 ± 5.4**	145	13.7
	160	52.8 ± 7.4**	51.6 ± 7.0**	186	14.1
24	Vehicle control	27.6 ± 4.2	27.0 ± 4.1	100	15.2
	8	36.6 ± 5.1**	35.7 ± 5.2**	133	14.1
	40	40.1 ± 5.5**	39.4 ± 5.1**	146	13.7
	160	53.5 ± 9.4**	52.3 ± 9.5**	194	17.7

CV, coefficient of variation.
[a]Values shown are mean ± SD. *Significantly different from corresponding vehicle control at $p < 0.05$. **Significantly different from corresponding vehicle control at $p < 0.01$.

Table 5. Relative uterine weights of rats 6, 12, 18 and 24 hr after last sc administration of BPA.

Hour	Dose (mg/kg/day)	Wet weight (mg/100g)[a]	Blotted weight (mg/100g)[a]	Wet weight (% of control)	CV (wet weight)
6	Vehicle control	66.4 ± 8.1	65.0 ± 8.0	100	12.1
	8	87.5 ± 18.2**	82.8 ± 15.8**	132	20.8
	40	132.4 ± 23.3**	127.2 ± 22.0**	200	17.6
	160	189.8 ± 25.8**	181.2 ± 25.8**	286	13.6
12	Vehicle control	66.4 ± 12.1	67.3 ± 11.4	100	18.2
	8	78.0 ± 7.4	75.4 ± 6.7	112	9.4
	40	122.2 ± 17.6**	118.5 ± 16.1**	176	14.4
	160	152.2 ± 20.2**	148.8 ± 19.8**	219	13.3
18	Vehicle control	62.2 ± 10.9	59.8 ± 10.5	100	17.5
	8	70.7 ± 8.8	69.2 ± 9.0*	114	12.5
	40	87.3 ± 10.8**	84.8 ± 10.4**	140	12.4
	160	115.7 ± 15.8**	113.0 ± 14.7**	186	13.7
24	Vehicle control	62.0 ± 7.1	60.7 ± 7.2	100	11.5
	8	76.3 ± 12.2**	74.3 ± 12.4**	123	16.0
	40	88.9 ± 10.5**	87.3 ± 9.8**	143	11.8
	160	118.0 ± 19.4**	115.2 ± 19.5**	191	16.4

CV, coefficient of variation.
[a]Values shown are mean ± SD. *Significantly different from corresponding vehicle control at $p < 0.05$. **Significantly different from corresponding vehicle control at $p < 0.01$.

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