Table 4
Effects of prior intrauterine position on estrous cycle in Sprague–Dawley rats

	Group		
	2F	1F	0F
No. of litters	17	27	25
No. of female offspring	23	36	26
Mean estrous cycle length (day)	4.16 ± 0.29[a]	4.08 ± 0.30	4.20 ± 0.42
No. of females showing Regular cycle (%)	18 (78.3)	28 (77.8)	21 (80.8)
No. of females showing Irregular cycle (%)	5 (21.7)	8 (22.2)	5 (19.2)

2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

3.1.4. Behavior and locomotor activity of offspring

Table 5 shows the results of an open field test at 4 weeks of age, and spontaneous activity within the wheel for 24 h at 7 weeks of age, for male and female offspring. There were no significant differences between groups 2M, 1M and 0M in latency, ambulation, rearing, grooming, defecation and urination, or number of revolutions for 24 h in a wheel cage. In the females, urination in group 0F was significantly increased as compared with that in group 2F, whereas other behavioral parameters, including the number of revolutions in a wheel cage were comparable between groups 2F, 1F and 0F.

3.1.5. Weights and histology of reproductive organs of offspring in adulthood

Table 6 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed in any of the reproductive organs, including the prostates, of the offspring.

3.2. Experiment II

3.2.1. AGD and body weights of fetuses at cesarean section

Table 7 shows the body weight, AGD, AGD/body weight, and AGD/$\sqrt[3]{\text{body weight}}$, of embryonic day 18 (PND 0) fetuses exposed to corn oil or 17β-estradiol. There were no significant differences in any of the parameters between the groups. No significant differences in viability of fetuses at cesarean section, or that from PND 0 to PND 21 were detected between the groups (the number of pups died from PND 0 to PND 21: see Tables 7 and 8).

3.2.2. Sexual maturation of offspring

Table 8 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups.

Table 5
Effects of prior intrauterine position on postnatal behavior in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Open field						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Latency (s)	20.4 ± 40.8[a]	17.9 ± 16.9	15.3 ± 16.2	12.0 ± 9.4	13.8 ± 12.3	16.9 ± 36.1
Ambulation (cm)	676.3 ± 411.3	627.1 ± 417.2	659.0 ± 501.9	940.6 ± 538.1	1039.8 ± 436.3	970.7 ± 449.8
No. of rearing	2.3 ± 3.1	3.0 ± 3.2	1.5 ± 1.4	3.5 ± 2.1	4.5 ± 3.4	3.8 ± 2.3
No. of grooming	0.6 ± 0.9	0.7 ± 0.7	1.1 ± 1.2	0.8 ± 0.9	0.4 ± 0.5	0.8 ± 0.8
No. of defecation	2.8 ± 1.9	2.1 ± 1.5	3.3 ± 2.2	1.9 ± 1.9	1.7 ± 1.8	1.8 ± 1.9
No. of urination	0.4 ± 0.6	0.4 ± 0.6	0.5 ± 0.5	0.2 ± 0.4	0.5 ± 0.5	0.7 ± 0.6**
Spontaneous activity						
Count/24 h	1547 ± 467	1789 ± 697	1559 ± 638	4107 ± 1140	4429 ± 1501	4746 ± 1831

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
**Significantly different from group 2F, $P < 0.01$ (by multiple comparison and Student t-test).
[a] Mean ± S.D.

Table 6
Effects of prior intrauterine position on reproductive organs after maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight at 10 weeks old						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Body weight (g)	417.2 ± 31.6[a]	416.1 ± 34.4	413.6 ± 36.9	270.0 ± 23.2	271.8 ± 28.9	273.6 ± 29.1
Testes (mg)[b]	3.00 ± 0.20	2.98 ± 0.15	3.00 ± 0.17			–
Testes[c]	0.72 ± 0.05	0.72 ± 0.06	0.73 ± 0.07			–
Epididymides (mg)[b]	0.77 ± 0.05	0.78 ± 0.07	0.76 ± 0.06			–
Epididymides[c]	0.19 ± 0.01	0.19 ± 0.02	0.18 ± 0.02			–
Ventral prostate (g)[b]	0.46 ± 0.08	0.44 ± 0.08	0.43 ± 0.10			–
Ventral prostate[c]	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.03			–
Dorsal prostate (g) + SV[b,d]	1.53 ± 0.28	1.56 ± 0.24	1.52 ± 0.27			–
Dorsal prostate + SV[c,d]	0.37 ± 0.07	0.38 ± 0.05	0.37 ± 0.07			–
Ovaries (mg)[b]				92.6 ± 13.3	91.8 ± 13.7	95.4 ± 16.9
Ovaries[c]				34.3 ± 3.6	33.8 ± 3.4	35.0 ± 5.8
Uterus (g)[b]				0.36 ± 0.06	0.38 ± 0.06	0.38 ± 0.05
Uterus[c]				0.13 ± 0.02	0.14 ± 0.03	0.14 ± 0.02

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (g or mg per 100 g body weight).
[d] Seminal vesicle.

Table 7
Effects of prior intrauterine position on anogenital distance in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	41	32	32	37	38	33	41	28
Body weight (g)	1.41 ± 0.08[a]	1.42 ± 0.05	1.32 ± 0.05	1.33 ± 0.04	1.42 ± 0.09	1.41 ± 0.10	1.32 ± 0.07	1.30 ± 0.11
AGD	1.92 ± 0.07	1.90 ± 0.06	0.95 ± 0.02	0.95 ± 0.03	1.92 ± 0.08	1.93 ± 0.06	0.93 ± 0.09	0.95 ± 0.05
AGD/body weight	1.36 ± 0.09	1.35 ± 0.10	0.75 ± 0.03	0.73 ± 0.05	1.40 ± 0.09	1.38 ± 0.10	0.75 ± 0.05	0.74 ± 0.07
AGD/$\sqrt[3]{\text{body weight}}$	1.71 ± 0.07	1.70 ± 0.10	0.88 ± 0.03	0.89 ± 0.05	1.70 ± 0.09	1.72 ± 0.11	0.89 ± 0.07	0.88 ± 0.08

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

Table 8
Effects of prior intrauterine position on sexual maturation in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	39	30	31	35	37	31	39	28
Day of preputial separation	27.2 ± 1.5[a]	27.3 ± 1.3			27.0 ± 1.8	26.9 ± 2.0		
Body weight (g)	30.3 ± 1.9	31.1 ± 1.5			30.0 ± 2.1	31.3 ± 1.8		
Day of vaginal opening			24.5 ± 1.6	25.1 ± 1.5			24.4 ± 1.7	24.9 ± 1.6
Body weight (g)			21.5 ± 0.9	21.6 ± 1.2			21.6 ± 1.1	22.0 ± 1.5

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

Table 9
Effects of prior intrauterine position on reproductive organs after maturation in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	34	25	26	30	32	26	34	23
Terminal body weight (g)	51.5 ± 4.2[a]	53.6 ± 4.4	40.3 ± 2.7	41.2 ± 3.4	55.1 ± 5.1	53.1 ± 6.2	41.8 ± 2.1	42.1 ± 3.1
Testes (g)	257.9 ± 28.4[b]	266.5 ± 23.5			259.3 ± 25.5	260.3 ± 19.9		
	486.3 ± 76.3[c]	484.2 ± 71.3			488.3 ± 62.3	479.1 ± 60.9		
Epididymides (mg)	89.5 ± 8.7	92.2 ± 9.3			94.6 ± 7.1	93.1 ± 7.1		
	175.2 ± 21.5	161.9 ± 16.9			161.2 ± 13.6	173.6 ± 4.9		
Seminal vesicle (mg)	413.9 ± 30.6	452.2 ± 13.9			431.3 ± 18.2	454.8 ± 21.0		
	812.8 ± 56.9	802.6 ± 44.2			811.5 ± 42.3	809.3 ± 33.5		
Ovary (mg)			15.3 ± 3.3	14.6 ± 4.2			15.5 ± 3.9	15.3 ± 4.5
			35.6 ± 7.5	33.2 ± 4.6			34.1 ± 6.9	33.6 ± 5.1

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
Five males in each group were processed to the transcardial perfusion. Male pups shown here were subjected to necropsy.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (mg per 100 g body weight).

3.2.3. *Weights and histology of reproductive organs of offspring in adulthood*

Table 9 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed.

4. Discussion

4.1. *Anogenital distance*

The AGD of newborn rats and mice is longer in males than in females, and it has been demonstrated that the AGD varies as a function of the intrauterine position of the animals [1–4]. The AGD is commonly regarded as a hormonally sensitive developmental measure in rodents [26], and it has been reported that a longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus [27,28]. Evidence supports the hypothesis that exposure to testosterone and estrogen in utero are critical components of the intrauterine position effect [29]. Female mouse fetuses located between two males have significantly higher serum testosterone levels and lower estradiol levels than their sisters that were located between two females. Male mice located between two females have significantly higher levels of estradiol and lower levels of testosterone than males located between two males [4,12]. The mechanism for these intrauterine position effects can be traced to amniotic fluid transport between adjacent fetuses in uterus [30,31]. However, our data were not consistent with previous reports showing a significant effect of intrauterine position on AGD in rats and mice [2,32–34].

A failure to replicate the effects of intrauterine position on AGD may have potentially arisen for a number of methodological reasons. A set of potential problems revolves around possible errors in the measurement of the AGD. One possibility was that our calipers were not accurate enough to detect small mean differences between females located in various positions in the uterus, found by other investigators [2,32–34]. However, as the calipers could be read to an accuracy of 0.01 mm, they were clearly accurate enough to detect differences of this magnitude. Another possibility is that of human error. Given the short distances being measured, it was absolutely essential that all fetuses or pups be oriented in exactly the same fashion, as even a slight arching of the animal's back could significantly distort the AGD measurements. Two attempts were made to minimize these sorts of errors: (i) efforts were made to orient all fetuses or pups in exactly the same fashion when measuring, and (ii) two independent measurements were taken for each fetus or pup and averaged to obtain the value used. In most cases, the different measurements were highly similar for the same animal.

Simon and Cologer-Clifford [35] reported an absence of an intrauterine position effect on AGD in CF-1 mice. Their finding is only the second study to examine AGD in CF-1 mice, and the original report was more than 10 years old [2]. Therefore, it is possible that either genetic drift, or differences in the source of the CF-1 breeding stock, may

underlie the discrepant findings. In this context, Jubilan and Nyby [6] also found no effect of intrauterine position on the AGD/body weight (AGDI) in CF-1 offspring, using stock from the same supplier employed by the Simon and Cologer-Clifford [35] report.

4.2. Sexual maturation and estrous cycle

Since prenatal exposure of females to testosterone delays vaginal opening [36,37], it was predicted that females situated proximate to males in utero would display vaginal opening later than females not proximate to males during gestation. However, in the present study in rats and mice, there were no significant differences in days of vaginal opening or preputial separation between the groups (see Tables 3 and 8), suggesting that intrauterine position did not influence the sexual maturation in males and females. vom Saal [4] reported that 2F and 0F mice did not differ significantly in the age at vaginal opening, although 2F tended to exhibit vaginal opening at a slightly younger age than 0F (see categorization of the different intrauterine positions shown in Section 2).

Female mouse fetuses occupying an intrauterine position between male fetuses exhibit longer estrous cycles in adulthood than females formerly residing in utero next to other female fetuses [11,27]. Prior intrauterine position is therefore a source of individual variation in the production of, and sensitivity to, cues that modulate the timing of puberty and the length of subsequent estrous cycles in female mice, suggesting that prenatally androgenized females occupying an intrauterine position between male fetuses may have a reproductive advantage over other females at high population densities [4]. In the present study of rats, however, 0F and 2F did not differ significantly in the estrous cycle length, although the estrous cycle length of 2F (4.16 ± 0.29) tended to be shorter than that of 0F (4.20 ± 0.42). Prior studies have shown that, in the absence of males, vaginal estrus does not correlate with ovulation in peripubertal CF-1 female mice [38,39]. Further studies in which ovulation is confirmed by the presence of corpora lutea and tubal ova is thus required.

4.3. Behavior

Kinsley et al. [40] demonstrated that female mice located in utero between two female fetuses exhibited higher levels of regulatory running activity (locomotor activity) in adulthood than females located between two male fetuses. Male mice, which were less active than females, were also influenced by intrauterine contiguity, indicating that intrauterine position influences the behaviors involved in the maintenance of metabolic homeostasis. Previous work has shown that female rats and mice display higher levels of regulatory running activity than males, and that perinatal testosterone is responsible for this sex difference [41–43]. The present study also showed female rats displayed higher levels of running activity than males.

In the present study of rats, however, there were no significant differences in spontaneous activity in the wheel cage, or in ambulation in the circular area, as well as the frequency of rearing, grooming and defecation between the groups in both sexes, suggesting no intrauterine position effects on locomotor activity in rats. Interestingly, the frequency of urination for females that developed in utero between male fetuses was significantly increased, more than in females that developed in utero between female fetuses (see Table 5). Females that were located between female fetuses in utero were found to urine mark at higher rates than females that were located between male fetuses, in adulthood in CF-1 mice [2]. The frequency of urination in the circular area, observed in the present study, would relate to the emotionality of the animals when placed in a novel environment, and differ from urine marking. Female urine marking may play an important role in communication between female mice, as well as in inter-sexual communication. It has been suggested that in natural populations of mice, females urine mark to advertise their dominant breeding status to other females; urine-marking appears to be dependent on female social/reproductive status [44]. Taken together, these observations suggest that the intrauterine position did not affect behavior as evaluated by the open field test and the wheel cage.

4.4. Prostate development

Growth and differentiation of the prostate is primarily under the control of androgen. Expression of the androgen metabolizing enzyme, 5α-reductase, within prostatic mesenchyme cells is also necessary for normal development of the prostate [45]. The possibility that estrogen might be involved in modulating the effects of androgen on prostatic development during early life has been the subject of speculation for over 60 years [46–49]. Timms et al. [50] demonstrated that development of the urogenital system in male and female rat fetuses is influenced by their intrauterine proximity to fetuses of the same or opposite sex, and suggested that exposure to supplemental estradiol (due to being positioned between two female fetuses) induces prostatic bud development in females, and enhances the growth of prostatic buds in both males and females. An enlarged prostate in males located between two female fetuses was hypothesized to be mediated by an elevated level of serum estradiol, relative to males located between two males, due to the transport of estradiol from adjacent female fetuses [4,30]. This hypothesis was confirmed in a study in which estradiol was experimentally elevated by 50% in male mouse fetuses (via maternal administration), and the estrogen-treated males showed both a significant increase in prostatic glandular buds and significantly larger buds during fetal life, as well as enlarged prostates in adulthood [46].

In the present study, however, the weights of the prostates (with seminal vesicles) of the rats at PND 21, and the ventral and dorsal prostates (with seminal vesicles) of the rats at 10

weeks of age, were not significantly different between the groups. In addition, morphological observation of prostates in the rats and mice, in weanlings or adulthood, by light and electron microscope revealed no alteration in males located in any uterine position.

4.5. Developmental exposure to 17β-estradiol: interaction with endogenous estradiol during pregnancy in mice

In the present study we examined the effect of 17β-estradiol administration to pregnant mice on the early development of the prostate in male mouse fetuses, with attention being paid to the intrauterine position of the males. Timms et al. [17] reported that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) significantly reduced serum estradiol in males located between two females, but not males located between two males, and also significantly interfered with initial budding and subsequent growth of the prostate in males located between two females or two males. In sharp contrast, the seminal vesicles were larger in the control males located between two males than in control males located between two females, similar to prior findings in mice [29], and TCDD only decreased the size of the seminal vesicles in males located between two males. Taken together, the findings of Timms et al. [17] demonstrate that in utero exposure to TCDD disrupts the development of the prostate, but this disruption depends on an interaction with background levels of estradiol. Howdeshell and vom Saal [16] reported that fetal mouse exposure via the mother to an estrogen-mimicking chemical, bisphenol A, increased the rate of postnatal growth in males and females, and also advanced the timing of puberty in females. They also demonstrated that the greatest response to bisphenol A occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A treatment, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development.

In the present study, however, mouse fetal exposure via the mother to low-dose 17β-estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. Therefore, we concluded that exposure to low-dose estrogenic endocrine disrupting chemicals during fetal life does not contribute to the intrauterine position.

5. General discussion

We are at a loss to explain why we were unable to replicate the effects of intrauterine position on AGD, or to find intrauterine position effects upon sexual maturation, and the estrous cycle. However, we know the difficulty in demonstrating intrauterine position effects upon morphology and behavior [35]. In addition, in contrast to earlier work [11] which examined blood androgen titers in mouse fetuses, Baum et al. [51] reported that whole-body androgen levels in female rat fetuses did not vary as a function of intrauterine position, and suggested that intrauterine position effects upon rodent morphology and behavior may not have the robust generality that is generally assumed.

Howdeshell and vom Saal [16] demonstrated that one source of variability in the response of both male and female mouse fetuses to an estrogen-mimicking chemical, bisphenol A, is their background levels of endogenous sex hormones. They suggested that a very small increase in the level of endogenous estradiol may substantially increase the susceptibility of fetuses to endocrine disrupting chemicals consumed or absorbed through the skin or lungs by pregnant animals and humans.

Contiguous [1,52], caudal [53,54], and no effect [51,55–57], due to intrauterine position, have been reported. Hotchkiss et al. [55] in a study with Sprague–Dawley rats examined the effect of intrauterine position on concentrations of testosterone in several different tissues. No effect of either contiguous or caudal intrauterine position on testosterone concentration was detected in fetal carcasses, reproductive tracts, or amniotic fluid. Furthermore, no correlation was found between masculinization due to intrauterine position and increasing anogenital distance. It is unclear at this time why there is such a discrepancy between the previous findings and the present results in rats and mice. However, varied strains of rats and mice, multiple uncontrolled variables, and different criteria for defining the effects of intrauterine positioning, may all contribute to this uncertainty. In addition, the discrepancies in the data may be attributed to such factors as the dietary influences (such as background levels of phytoestrogens and caloric intake), caging (steel versus polycarbonate), bedding, housing (group versus individual), and seasonal variation, as well as differences among the studies in control body and prostate weights [58,59].

The results of the present study clearly showed that intrauterine position of embryos/fetuses did not influence postnatal development, including sexual maturation and behavior.

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References

[1] Clemens LG, Gladue BA, Coniglio LP. Prenatal endogenous influences on masculine sexual behavior and genital morphology in male and female rats. Horm Behav 1978;10:40–53.

[2] vom Saal FS, Bronson FH. In utero proximity to female mouse fetuses to males: effects on reproductive performance during later life. Biol Reprod 1978;19:843–53.
[3] Clark MM, Galef Jr BG. Effect of uterine position on rate of sexual development in female Mongolian gerbils. Physiol Behav 1988;42:15–8.
[4] vom Saal FS. Sexual differentiation in litter bearing mammals: influence of sex of adjacent fetuses in utero. J Anim Sci 1989;67: 1824–40.
[5] Clark MM, Galef Jr BG. Prenatal influences on reproductive life history strategies. Trends Ecol Evol 1995;10:151–3.
[6] Jubilan BM, Nyby JG. The intrauterine position phenomenon and precopulatory behaviors of house mice. Physiol Behav 1992;51:857–72.
[7] Hernandes-Tristan R, Arevalo C, Canals S. Effect of prenatal uterine position on male and female rats sexual behavior. Physiol Behav 1999;67:401–8.
[8] Sherry DF, Galef BG, Clark MM. Sex and intrauterine position influence the size of the gerbil hippocampus. Physiol Behav 1996;60: 1491–4.
[9] Tarraf CG, Knight JW. Effect of intrauterine position on conceptus development, placental and endometrial release of progesterone and estrone in vitro, and concentration of steroid hormones in fetal fluids throughout gestation in swine. Domest Anim Endocrinol 1995;12:179–87.
[10] vom Saal FS, Timms BG. The role of natural and manmade estrogens in prostate development. In: Naz RK, editor. Endocrine disruptors: effects on male and female reproductive systems. Boca Raton, FL: CRC Press; 1999. p. 307–27.
[11] vom Saal FS, Bronson FH. Variation in length of the estrous cycle in mice due to former intrauterine proximity to male fetuses. Biol Reprod 1980;22:777–80.
[12] vom Saal FS. Variation in infanticide and parental behavior in male mice due to prior intrauterine proximity to female fetuses. Elimination by prenatal stress. Physiol Behav 1983;30:675–81.
[13] Clark MM, Karpiuk P, Galef BG. Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. Nature (London) 1993;364:712.
[14] Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenberg JG, vom Saal FS. Exposure to bisphenol A advances puberty. Nature (London) 1999;401:763–4.
[15] Welshons WV, Nagel SC, Thayer KA, Judy BM, vom Saal FS. Low-dose bioactivity of xeno17β estradiols in animals: fetal exposure to low doses of methoxychlor and other xeno17β estradiols increases adult prostate size in mice. Toxicol Ind Health 1999;15:12–25.
[16] Howdeshell KL, vom Saal FS. Developmental exposure to bisphenol A: interaction with endogenous estradiol during pregnancy in mice. Am Zool 2000;40:429–37.
[17] Timms BG, Peterson RE, vom Saal FS. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin interacts with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses. Toxicol Sci 2002;67:264–74.
[18] Guide for the care and use of laboratory animals. NIH Publication 86–23, Bethesda, MD: National Institutes of Health; 1985.
[19] Everett JW. Neurobiology of reproduction in the female rat: a fifty-year perspective. New York: Springer-Verlag; 1989.
[20] Haseman JK, Hogan MD. Selection of the experimental unit in teratology studies. Teratology 1975;12:165–72.
[21] Holson RR, Pearce B. Principels and pitfalls in the analysis of prenatal treatment effects in multiparous species. Neurotoxicol Teratol 1992;14:221–8.
[22] Bliss CI. Statistics in biology, vol. I. New York: McGraw-Hill; 1967. p. 116–7.
[23] Wise LD, Vetter CM, Anderson CA, Antonello JM, Clark RL. Reversible effects of triamcinolone and lack of effects with aspirin or L-656224 on external genitalia of male Sprague–Dawley rats exposed in utero. Teratology 1991;44:507–20.
[24] Clark RL. Endpoints of reproductive system development. An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington, DC: ILSI Press; 1999. p. 10–27.
[25] Gallavan RH, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 1999;13:383–90.
[26] Heinrichs WL. Current laboratory approaches for assessing female reproductive toxicity. In: Dixon RL, editor. Reproductive toxicology. New York: Raven Press; 1985. p. 95–108.
[27] vom Saal FS. Variation in phenotype due to random intrauterine positioning of male and female fetuses in rodents. J Reprod Fertil 1981;62:633–50.
[28] Vandenbergh JG, Huggett CL. The anogenital distance index, a predictor of the intrauterine position effects on reproduction in female house mice. Lab Anim Sci 1995;45:567–73.
[29] Nonneman DJ, Ganjam VK, Welshons WV, vom Saal FS. Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. Biol Reprod 1992;47:723–9.
[30] Even MD, Dhar MG, vom Saal FS. Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. J Reprod Fertil 1992;96:709–16.
[31] vom Saal FS, Dhar MG. Blood flow in the uterine loop artery and loop vein is bidirectional in the mouse: implications for transport of steroids between fetuses. Physiol Behav 1992;52:163–71.
[32] Gandelman R, vom Saal FS, Reinisch JM. Contiguity to male fetuses affects morphology and behavior of female mice. Nature (London) 1977;266:722–4.
[33] McDermott NJ, Gandelman R, Reinisch JM. Contiguity to male fetuses influences ano-genital distance and time of vaginal opening in mice. Physiol Behav 1978;20:661–3.
[34] Zielinski WJ, Vandenbergh JG, Montano MM. Effects of social stress and intrauterine position on sexual phenotype in wild-type house mice (*Mus musculus*). Physiol Behav 1991;49:117–23.
[35] Simon NG, Cologer-Clifford A. In utero contiguity to males does not influence morphology, behavioral sensitivity to testosterone, or hypothalamic androgen binding in CF-1 female mice. Horm Behav 1991;25:518–30.
[36] Brown-Grant K, Sherwood MR. "The early androgen syndrome" in the guinea pig. J Endcrinol 1971;49:277–91.
[37] Goy RW. Experimental control of psychosexuality. Phil Trans R Soc London 1970;259:149–62.
[38] Stiff ME, Bronson FH, Stetson MH. Plasma gonadotropins in prenatal and prepubertal female mice: disorganization of pubertal cycles in the absence of a male. Endocrinology 1974;94:492–6.
[39] Perrigo GH, Bronson FH. Foraging effort, food intake, fat deposition and puberty in female mice. Biol Reprod 1992;29:455–63.
[40] Kinsley C, Miele J, Konen C, Ghiraldi L, Svare B. Intrauterine contiguity influences regulatory activity in adult female and male mice. Horm Behav 1986;20:7–12.
[41] Hitchcock FA. Studies in vigor: V. The comparative activity of male and female albino rats. Am J Physiol 1925;75:205–10.
[42] Gentry RT, Wade GN. Sex differences in sensitivity of food intake body weight and running wheel activity to ovarian steroids in rats. J Comp Physiol Psychol 1976;90:747–54.
[43] Broida J, Svare B. Sex differences in the activity of mice: modulation by postnatal gonadal hormones. Horm Behav 1984;18:65–78.
[44] Hurst JL. Urine marking in populations of wild house mice (*Mus domesticus* Rutty). II. Communications between females. Anim Behav 1990;40:223–32.
[45] Berman DM, Russell DW. Cell-type-specific expression of rat steroid 5α-reductase isozymes. Proc Natl Acad Sci USA 1993;90:9359–63.
[46] vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, et al. Prostate enlargement in mice due to fetal exposure

to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc Natl Acad Sci USA 1997;94:2056–61.
[47] Zuckerman S. The endocrine control of the prostate. Proc R Soc Med 1936;29:1557–68.
[48] Glenister TW. The development of the utricle and of the so-called 'middle' or 'median' lobe of the human prostate. J Anat 1962;96:443–55.
[49] Blacklock NJ. The development and morphology of the prostate. In: Ghanadian R, editor. The endocrinology of prostate tumours. Lancaster, England: MTP Press; 1983. p. 1–13.
[50] Timms BG, Petersen SL, vom Saal FS. Prostate gland growth during development is stimulated in both male and female rat fetuses by intrauterine proximity to female fetuses. J Urol 1999;161:1694–701.
[51] Baum MJ, Woutersen PJA, Slob K. Sex difference in whole-body androgen content in rats on fetal days 18 and 19 without evidence that androgen passes from males to females. Biol Reprod 1991;44:747–51.
[52] Tobet SA, Dunlap JL, Gerall AA. Influence of fetal position on neonatal androgen-induced sterility and sexual behavior in female rats. Horm Behav 1982;16:251–8.
[53] Houtsmuller EJ, de Jong FH, Rowland DL, Slob AK. Plasma testosterone in fetal rats and their mothers on day 19 of gestation. Physiol Behav 1995;57:495–9.
[54] Richmond G, Sachs BD. Further evidence for masculinization of female rats by males located caudally in utero. Horm Behav 1984;18:484–90.
[55] Hotchkiss A, Parks LG, Ostby J, Lambright C, Wolf C, Wilson VS. A quantitative determination of the environmental sources of variability in androgens of fetal Sprague–Dawley rats. Biol Reprod (Abstract) 2000;62:183.
[56] Meisel RL, Ward IL. Fetal female rats are masculinized by male littermates located caudally in the uterus. Science 1981;213:239–42.
[57] Slob AK, Van der Schoot P. Testosterone induced mounting behavior in adult female rats born in litters of different female to male ratios. Physiol Behav 1982;28:1007–10.
[58] NIEHS, National Toxicology Program (NTP). Endocrine disruptors low dose peer review report; 2001. p. 467.
[59] Witorsch RJ. Low-dose in utero effects of xenoestrogens in mice and their relevance to humans: an analytical review of the literature. Food Chem Toxicol 2002;40:905–12.

SHORT COMMUNICATION

An improved technique for repeated gavage administration to rat neonates

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ABSTRACT **The technique for gavage administration to rat nurslings was improved to allow determination of the direct effects of chemical substances in the nurslings. Rat neonates were treated with distilled water from postnatal day 1 through 20 using this technique. The viability of neonates during the administration period was comparable to that of untreated neonates. No adverse effects of this technique on the development of neonates were found, and no histological alterations of the esophagus or pharynx. Therefore, we conclude that use of our improved gavage administration method will contribute to ensuring successful neonatal development and thus allowing accurate assessment of the toxicological effects of test compounds on rat nurslings.**

Key words: gavage administration, rat neonates, histological changes, development

INTRODUCTION

In toxicological studies, a test substance is commonly administered to mature rats or mice to evaluate its adverse effects or to determine the no-observed adverse effect dose level (NOAEL). In particular, gavage administration is used in mature animals or immature animals after weaning for the above purpose. Hitherto, the toxicological effects of test substance on rat neonates have been evaluated via the milk of dams treated with the test substance during nursing. However, it is important to estimate the direct effects of test substances on nurslings in toxicological studies, using rodents in order to draw inferences about the effects on human infants. We reported the direct effects in male and female rats from neonatal exposure to environmental chemicals (Nagao *et al.*, 1999, 2000, 2001a; Kuwagata *et al.*, 2001). In addition, we demonstrated that direct neonatal exposure to phytoestrogen, genistein caused dysfunction of postpubertal reproductive performance as well as abnormal development of gonads in female but not in male rats (Nagao *et al.*, 2001b).

Problems encountered with daily gavage administration to neonatal rats include mortality due to cannibalism or neglect by the dam, resultant retardation of development, and injury of the esophagus or pharynx by the gastric tube. We aimed to develop a method of gavage administration which would enable us to test the effects of chemical compounds administered repeatedly to rat neonates. In this report, improved techniques for repeated gavage administration in rat neonates are described.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (Crj:CD, IGS) were purchased from Charles River, Atsugi, Japan, at 12 weeks of age. The animals were acclimated to the laboratory for 1 week prior to the start of the experiments. Animals were housed individually in metal cages in a room with controlled temperature (24 ± 1°C) and humidity (50 ± 5%), with lights on from 07:00 to 19:00 daily. Rats were given access to food (CE-2, Clea Japan) and tap water ad libitum.

Estrous female rats at 13 weeks of age were placed together overnight with a single male. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation. Once insemination was confirmed, the females were weighed. The dams were allowed to deliver naturally and nurse their pups until postnatal day (PND) 21 (PND 0: the day of birth). On PND 1, all pups were weighed and sexed and the litters were culled randomly to 12 (6 pups/sex/litter where possible). Litters of twelve pups or less were not reduced. The remaining pups were discarded and the nurslings were weighed daily.

Administration

Three male and female pups per litter were given daily gavage administration of distilled water from PND 1 through 20, and the remaining littermates (3 males and 3 females) were not given any treatment. The number of males and females that were treated

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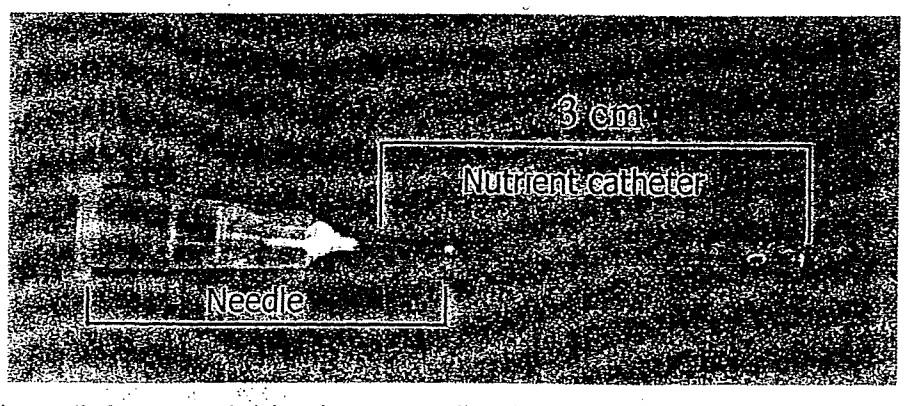

Fig. 1 Gastric tube with a needle for gavage administration to rat nurslings from PND 3 through 10.

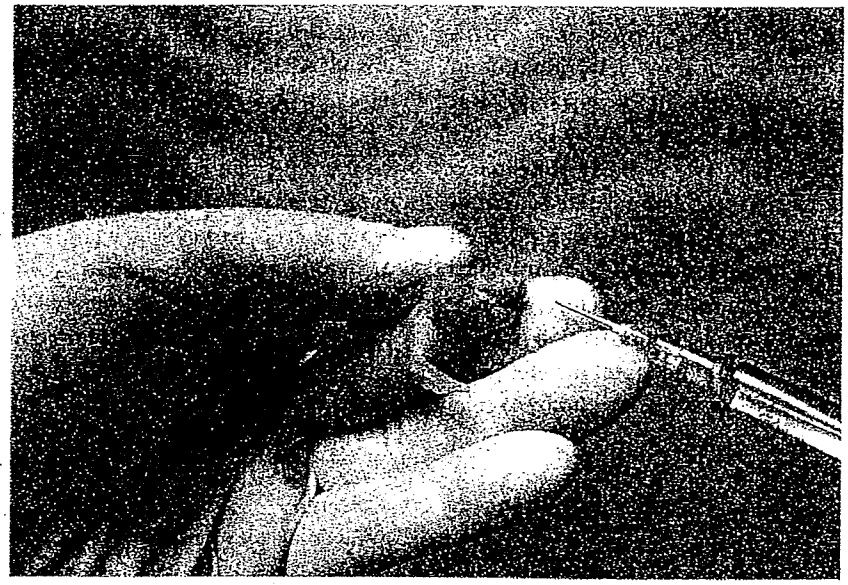

Fig. 2 Gavage administration to rat nursling on PND 1. The pup was held between the thumb and the 3rd finger.

with distilled water was 41 and 42, and the number of males and females that were not treated was 42 and 41, respectively (the total number of litters was 14).

We developed the following method for direct gavage administration to rat newborn pups on PND 1 through 20 in our laboratory. A needle (23G × 1", 0.65 × 25 mm, TERUMO Co., Tokyo) was cut off to a 10-mm length and the basal part of an indwelling feeding tube for infants (Nutrient catheter, Type 3Fr, Atom Medical Co., Tokyo) was attached to the needle (**Fig. 1**). This tube with the needle mounted to a microsyringe (Hamilton Gastight #1750, 500 μL, Hamilton Co., U.S.A.) or disposable syringe (1 mL, TERUMO Co., Tokyo) was used as a gastric tube. The length of the catheter attached to the needle was 2 cm for administration on PND 1 through 4, 3 cm for administration on PND 5 through 10, and 6 cm for administration on PND 11 and thereafter. The volume administered (10 μL/g body weight) was calculated based on the body weight measured daily. The rate of administration was approximately 10 μL/sec or less for administration on PND 1 through 7, 100 μL/sec or less for administration on PND 8 through 14, and 500 μL/sec or less for administration on PND 15 and thereafter. The pup was held between the thumb and the 3rd finger (**Fig. 2**) during the administration.

Observation

The numbers of live and dead pups were recorded for each litter on PND 1 through 21, and the viability from PND 1 to 21 was determined. Pups were observed daily for clinical signs and weighed daily. Developmental landmarks in the offspring were monitored on a daily basis for individual rats. All pups were examined for the development of neural reflexes (negative geotaxis and cliff-drop aversion) from PND 1 until the day of completion, for upper tooth eruption from PND 5 until the day of completion, and for eyelid opening from PND 10 until the day of completion as the endpoint of the physical milestones. Pups from each litter on PND 7 and 21 were weighed, anesthetized by ether and subjected to autopsy. The stomach was weighed. Subsequently,

the esophagus and pharynx were fixed in 0.1 M phosphate buffered 10% formalin solution and embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin (H&E), and examined histologically.

Animal care and use conformed to published guidelines (NIH, 1985).

RESULTS

Neonatal viability, body weight changes and developmental landmarks

No overt signs were apparent in any pups during the postnatal period including the administration period from PND 1 through 20. There were no significant differences in the number or viability of pups on PND 7 or 21 in the groups treated orally with distilled water as compared with those in the control (untreated) group. No significant differences were detected in the body weight of male pups between the treated and control groups throughout the study. In female pups, the body weights on PND 10, 11 and 14 in the treated group were significantly higher than those in the controls.

No significant differences in the PND on which the cliff-drop aversion response or righting reflex was completed were found between the treated and control groups in either sex. In addition, there were no significant differences in the PND on which teeth eruption, ear opening or eye opening was completed between the treated and control groups in either sex.

Organ weight and histological findings

There were no significant differences in body weight on PND 7 and 21 between the treated and control groups in either sex. In addition, no significant differences were detected in the absolute and relative weights of the stomachs of male and female pups treated with distilled water as compared with those of the controls.

There were no inflammatory changes such as hemorrhage and neutrophilia that were considered to be related to physical stimulation by the gavage administration in the esophagus and pharynx of neonates on PND 7 or 21.

DISCUSSION

In this report, an improved technique for repeated gavage administration from PND 1 in rat neonates was described. No adverse effects were observed on the growth or developmental landmarks of pups treated orally with distilled water. Significantly higher body weights of female pups were found in the treated group on PND 10, 11 and 14. However, this was a slight and transitory change, and not common to both sexes. In addition, the body weight gains from PND 1 through 21 were 44.6 g in male pups and 43.0 g in female pups of both groups. Thus, it is reasonable to consider that the higher body weights of female pups on PND 10, 11 and 14 were not related to the repeated gavage administration of distilled water. From these data, it would appear that our improved technique for repeated gavage administration does not affect the development of rat neonates, and will enable us to evaluate the toxicological effects of test substances administered to rat neonates.

REFERENCES

National Institute of Health (1985) *Guide for the Care and Use of Laboratory Animals*. NIH Publication 86-23, Bethesda.

Nagao T, Saito Y, Usumi K, Kuwagata M, Imai K (1999) Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. *Reprod Toxicol*, **13:** 303-311.

Nagao T, Saito Y, Usumi K, Nakagomi M, Yoshimura S, Ono H (2000) Disruption of the reproductive system and reproductive performance by administration of nonylphenol to newborn rats. *Human & Exp Toxicol*, **19:** 284-296.

Nagao T, Yoshimura S, Saito Y, Nakagomi M, Usumi K, Ono H (2001a) Reproductive effects in male and female rats of neonatal exposure to *p*-octylphenol. *Reprod Toxicol*, **15:** 683-692.

Nagao T, Yoshimura S, Saito Y, Nakagomi M, Usumi K, Ono H (2001b) Reproductive effects in male and female rats of neonatal exposure to genistein. *Reprod Toxicol*, **15:** 399-411.

Kuwagata M, Yoshimura S, Nagao T (2001) Reproductive effects of early neonatal exposure to genistein in Wistar rats. *Cong Anom*, **41:** 338-339.

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Low-dose bisphenol A does not affect reproductive organs in estrogen-sensitive C57BL/6N mice exposed at the sexually mature, juvenile, or embryonic stage

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Abstract

Bisphenol A (BPA) is used on a large scale in the manufacture of polycarbonate plastics. BPA has been shown to bind weakly to both estrogen receptor (ER) α and ER β. The objective of this study was to evaluate the effects of low-dose BPA on male sexual development after exposure at various stages of development. Mice of the estrogen-sensitive strain C57BL/6N were exposed to BPA orally at doses of 2, 20, or 200 μg/kg at various stages, i.e. adulthood, the immature stage just after weaning, or the embryonic/fetal stage, to evaluate the effects of low-dose BPA on male reproductive organs. Body weight changes, weights of reproductive organs (testes, epididymides, seminal vesicles), cauda epididymal sperm density, and histology of reproductive organs including the ventral prostate were not affected by exposure to BPA at any dose examined. The results of this study indicate that exposure of estrogen-sensitive C57BL/6N mice to low-dose BPA did not reduce sperm density or disrupt development of the male reproductive organs.

Keywords: Bisphenol A; Low-dose exposure; C57BL/6N mice; Reproduction; Testicular toxicity

1. Introduction

Bisphenol A (BPA) is an industrial compound that has generated a great deal of concern on the part of regulatory agencies and scientists due to its high level of production and widespread use. BPA is the monomer used in the manufacture of the resin used to line food and drink cans and from which polycarbonate plastic is made. BPA is also used to make dental sealants, which are often used to protect children's teeth [1]. BPA has been reported to be weakly estrogenic both in vitro and in vivo. Krishnan et al. [2] reported that BPA leached from polycarbonate flasks competed with [^{3}H]-estradiol for binding to estrogen receptors (ER) from rat uterus, induced progesterone receptor expression, and promoted cell proliferation in cultured human mammary cancer cells (MCF-7). BPA binds to both ER α and ER β with low affinity and transactivates reporter genes in vitro [3,4].

Recently, experiments by Nagel et al. [5] and vom Saal et al. [6] indicated that administration of low oral doses of BPA to pregnant mice on days 11 through 17 of gestation produced statistically significant increases in the weights of the prostate and preputial glands, a decrease in epididymis weight, and reduced efficiency of sperm production in male offspring. However, the low-dose effects of BPA have been controversial. Other researchers reported no treatment-related effects of BPA at the same and additional low-dose levels given at the same time of pregnancy to mice [7–12].

Large (more than 16-fold) differences in sensitivity to disruption of juvenile male reproductive development by 17β-estradiol (E_2) were found between strains of mice. Spermatid maturation was eliminated by low doses of E_2 in strains such as C57BL/6J and C17/Jls. In contrast, mice of the widely used CD-1 line showed little or no inhibition of spermatid maturation even in response to 16-fold higher doses of E_2 [13].

In the present study, we examined the effects on development of male reproductive organs in C57BL/6N mice, which were confirmed to be sensitive to estrogens similarly to C57BL/6J, as a result of embryonic or fetal exposure to environmentally relevant doses of BPA. In addition, the effects on reproductive organs in males exposed to low

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doses of BPA at the sexually mature or immature stages after weaning were also investigated in this strain.

2. Materials and methods

2.1. Animals and treatment

C57BL/6N and ICR mice were purchased from Charles River, Atsugi, Japan, at 3 or 8 weeks of age. The animals were acclimated to the laboratory for 3 days to 2 weeks prior to the start of the experiments. Animals were housed individually in polycarbonate cages in a room with controlled temperature (24 ± 1°C) and humidity (50 ± 5%), with lights on from 07:00 to 19:00 daily. Mice were given access to food (PLD, phytoestrogen-low diet, Oriental Japan) and tap water (distilled water) ad libitum. The contents of phytoestrogens in the diet, tap water, and wood bedding were determined, and genistein and daidzein levels were below 0.5 mg/100 g.

2.2. Experiment I (exposure of C57BL/6N and ICR juvenile male mice to E_2)

To compare the susceptibility to 17β-estradiol (E_2, Sigma Chemical Co., St. Louis, MO) between C57BL/6N and ICR males, 6 to 8 males from each strain were treated subcutaneously (s.c.) with E_2 at 10 μg/kg from postnatal day 27 to 48. The administration period was determined according to the protocol of the study by Spearow et al. [13]. Administration was performed at a defined time (12:00). Ten males of each strain were given corn oil (2 ml/kg) as controls. On postnatal day 43, male mice were weighed and subjected to necropsy. Subsequently, the testes, epididymides and seminal vesicles with coagulating glands were weighed. These reproductive organs were fixed in Bouin's solution for histologic observation.

2.3. Experiment II (exposure of C57BL/6N adult males to BPA)

Groups of twenty C57BL/6N male mice at 10 weeks of age were exposed to bisphenol A (BPA, Tokyo Kasei, purity, GC min. 99.0%) at 2, 20, or 200 μg/kg by oral gavage for 6 consecutive days. The dosages were determined on the basis of body weight on the day of the treatment. Administration was performed at a defined time (12:00). Twenty males were given 0.5% carboxymethyl cellulose (5 ml/kg) as controls. Six weeks after the final administration, male mice were weighed and 15 were subjected to necropsy. The administration period and the day of necropsy after the last administration were determined based on the results of the previous study by Ohsako et al. [14]. Subsequently, the testes, epididymides, and seminal vesicles with coagulating glands were weighed. The ventral prostate was not weighed in the present study since it was difficult to sample only the prostate in mice, and to determine the precise weight of this organ. The left cauda epididymis was homogenized in 1 ml distilled water. The homogenates were stained with an IDENT staining kit (Hamilton Thorne prepackaged DNA-specific dye based on Hoechst 33342). The stained samples were placed onto Cell-Vu slides (Fertility Technologie, MA, USA), and the numbers of sperm were counted using an HTM-IVOS analyzer (Hamilton Thorne Research, MA, USA) and the IDENT software supplied with the HTM-IVOS. Other reproductive organs were fixed in Bouin's solution for histologic evaluation. The remaining mice (5 mice per group) were anesthetized. Transcardiac perfusion was carried out with a mixture of 0.1 M phosphate-buffered 1.25% glutaraldehyde and 2% paraformaldehyde. Following fixation, the testes, epididymides, seminal vesicles, and prostates of these mice were rinsed three times in phosphate buffer, postfixed for 2 h at 4°C in 2% osmium tetroxide, and dehydrated in alcohol; these organs were embedded in epoxy resin. Tissue sections (1 μm thick) were stained with toluidine blue for light microscopy. Ultrathin sections stained with uranyl acetate and lead citrate were observed with an electron microscope (H-7100, Hitachi, Japan).

2.4. Experiment III (exposure of C57BL/6N juvenile males to BPA)

To obtain pregnant animals, 10-week-old virgin C57BL/6N females were cohabited overnight on a 1:1 basis with males of the same strain at 11 weeks of age or older. The next morning, females with vaginal plugs were regarded as pregnant, and the day of gestation was designated as day 0. All pregnant mice were allowed to give birth. On postnatal day 0, all female pups were discarded, and the number of males per litter was adjusted to 3. Male pups (30 males from 10 litters/group) were weaned on postnatal day 21, and exposed to BPA at 2, 20, or 200 μg/kg by oral gavage from postnatal day 21 to 43. The dosages were determined on the basis of body weight on the day of treatment. Administration was performed at a defined time (12:00). Thirty males from 10 litters were given 0.5% carboxymethyl cellulose (5 ml/kg) as controls. At 6 weeks old, all males were weighed. Five mice per group were anesthetized and transcardiac perfusion was carried out. The remaining males were subjected to necropsy. Subsequently, the testes, epididymides, and seminal vesicles with coagulating glands were weighed, and the left cauda epididymis of each male was homogenized to determine the sperm density. Finally, weighed organs were fixed in Bouin's solution for histologic evaluation.

2.5. Experiment IV (exposure of C57BL/6N embryos/fetuses to BPA)

Pregnant mice were obtained as described in Experiment III. Groups of 10 C57BL/6N mice were exposed to BPA at

Table 1
Reproductive organ weights in male ICR and C57BL/6N mice exposed to E_2

Dose (μg/kg/day)	No. males treated	Start of treatment (Administration period)	Age at termination	Final body weight (g)	Testes (g)	Epididymides (g)	Seminal vesicles (g)
ICR							
0	10	27 days old (21 days)	7 weeks old	38.52 ± 0.77[a]	0.221 ± 0.011[b] 0.577 ± 0.037[c]	0.074 ± 0.002 0.192 ± 0.007	0.288 ± 0.023 0.743 ± 0.051
10	10	27 days old (21 days)	7 weeks old	35.69 ± 0.78	0.203 ± 0.012 (92%)[d] 0.563 ± 0.034 (98%)	0.069 ± 0.003 (93%) 0.194 ± 0.007 (101%)	0.263 ± 0.015 (91%) 0.739 ± 0.042 (99%)
C57BL/6N							
0	10	27 days old (21 days)	7 weeks old	19.15 ± 0.45	0.130 ± 0.005 0.673 ± 0.024	0.042 ± 0.002 0.216 ± 0.008	0.100 ± 0.008 0.520 ± 0.030
10	10	27 days old (21 days)	7 weeks old	19.61 ± 0.34	0.108 ± 0.017* (83%) 0.548 ± 0.083* (81%)	0.028 ± 0.004** (67%) 0.141 ± 0.018** (65%)	0.057 ± 0.011** (57%) 0.284 ± 0.050** (55%)

[a]mean ± S.E.
[b]absolute weight.
[c]relative weight (organ weight/terminal body weight) × 100.
[d]percentage of control.
*Significantly different from the control, $P < 0.05$.
**Significantly different from the control, $P < 0.01$.

2, 20, or 200 μg/kg by oral gavage from gestational day 11 through 17. The administration period was determined according to the protocol of the study by Cagen et al. [8]. The dosages were determined on the basis of body weight on the day of treatment. Administration was performed at a defined time (12:00). Ten pregnant mice were given 0.5% carboxymethyl cellulose (5 ml/kg) as controls. Forty untreated female mice were allowed to give birth. All the BPA-treated and control dams were subjected to cesarean section on day 18 of gestation. The day of cesarean section was considered as postnatal day 0. The neonates obtained by cesarean section were fostered to untreated C57BL/6N females, one litter per female. On postnatal day 4, all female pups were discarded, and the number of males per litter was adjusted to 3 for each foster female. The males were weaned on postnatal day 21 and then individually housed in polycarbonate cages, where they were allowed to grow and sexually mature. At 12 weeks of age, all males were weighed. Five males per group were anesthetized and transcardiac perfusion was carried out. The remaining males (25 males per group) were subjected to necropsy. Subsequently, the testes, epididymides, and seminal vesicles with coagulating glands were weighed, and the left cauda epididymis of each male was homogenized to determine the sperm density. Finally, weighed organs were fixed in Bouin's solution for histologic evaluation.

Animal care and use conformed to published guidelines [15].

2.6. Analysis of data

Data were analyzed, where appropriate, to determine the statistical significance of differences between the control and E_2- or BPA-treated groups; $P < 0.05$ and $P < 0.01$ were taken to indicate statistical significance. Individual data or mean values of each litter were treated as single samples, and homogeneity of variance of these samples among groups was analyzed initially using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect significance of differences among groups. If a significant difference was detected among groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous or there was any group in which the variance was zero, Kruskal-Wallis analysis of ranks was applied. If significance was detected among groups, Dunnett's test was applied for multiple comparisons.

3. Results

3.1. Susceptibility to E_2 between C57BL/6N and ICR mice

Table 1 shows the reproductive organ weights of C57BL/6N and ICR males treated s.c. with E_2 from postnatal days 27 to 48. There were no significantly different in the absolute or relative weights (testes, epididymides, and seminal vesicles) between the E_2-treated males and the controls in ICR strain. In marked contrast, significant decreases in the absolute and relative weights of reproductive organs were detected in the E_2-treated males of the C57BL/6N strain as compared with those of the controls. In particular, relative weight of seminal vesicles in the E_2-treated males of this strain was 55% that of the controls. At necropsy, three and five C57BL/6N males treated with E_2 showed marked atrophy of the testes and seminal vesicles, respectively, while no macroscopic changes of the reproductive organs were observed in the ICR males treated with

Table 2
Histopathologic findings in ICR and C57BL/6N male mice exposed to E_2

Strain	ICR																C57BL/6N													
Dose (μg/kg/day)	0							10									0							10						
Animal No.	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	1	2	3	4	5		6
Testis																														
Multinucleated giant cell, unilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	±	–	–	–	–		–
Decrease in elongate spermatid, unilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	±	–	–	–	+		–
Decrease in elongate spermatid, bilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–		++
Atrophy of Leydig cell, bilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	±	–	–	±	+		++
Epididymis																														
Cell debris in lumen, unilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		+	–	–	–	–	–	+	–	–	–	–	±		–
Cell debris in lumen, bilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	+	+	–	–	–	–	–	–	–		+
Decrease in sperm, unilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		+	–	–	–	–	–	+	–	–	–	–	–		–
Decrease in sperm, bilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	+	+	–	–	–	–	–	–	+		+++
Seminal vesicle																														
Atrophy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	+	–	–	–	++		+++

–, Negative; ±, Very slight; +, Slight; ++, Moderate; +++, Severe.

E_2. Histopathologic findings of the reproductive organs in ICR and C57BL/6N mice treated with E_2 are shown in Table 2, and the representative changes observed in C57BL/6N mice are shown in Fig. 1. Only a few elongate spermatids were observed in seminiferous tubules of C57BL/6N mice treated with E_2. The number of size of Leydig cells of C57BL/6N mice treated with E_2 was decreased as compared with the controls. In addition, no sperm were observed in the lumen of the epididymal ducts, and the lumen of the seminal vesicles contained no secretions. Three other C57BL/6N males treated with E_2 showed similar but slight changes in the testis, epididymis, and seminal vesicles. C57BL/6N males in the control group showed no abnormalities except for cell debris and a slight decrease in sperm in the epididymal duct. In ICR males treated with E_2, no histopathologic changes in the reproductive organs were observed.

3.2. Low-dose effects of BPA in C57BL/6N male mice

No adult or immature C57BL/6N male mice exposed to BPA for 6 days or 3 weeks, respectively, died during the study period. In addition, there were no significant differences in body weight gain from the day of commencement of administration to the day of necropsy between the BPA-treated groups and the controls. No significant differences were found in embryo mortality or viability after birth between the BPA-treated groups and the control, nor in the body weight gain until necropsy (data not shown).

Table 3 shows the percentages of control values for the reproductive organ weights in C57BL/6N males exposed to BPA at various stages. In males exposed to BPA at the mature or immature stage after weaning there were no significant differences between the BPA-treated groups and the controls in terminal body weight or reproductive organ weights (testes, epididymides, seminal vesicles), or their relative weights.

In C57BL/6N males exposed to BPA as embryos at the organogenic stage, a significant decrease in the absolute weight of seminal vesicles in the 2 μg/kg group was found as compared with the controls, but the effect was not dose-dependent, suggesting that the decrease in the weight of the seminal vesicles was not related to BPA administration.

Fig. 2 shows the numbers of sperm per gram cauda epididymis of C57BL/6N males exposed to BPA at various stages. There were no significant differences in the density of sperm between the BPA-treated groups and the controls.

With regard to the histopathologic observations of the testes, epididymides, seminal vesicles, and prostate by light and electron microscopy, slight atrophy of the seminiferous tubules and multinucleated giant cells in immature seminiferous tubules were observed in males exposed to BPA after weaning for 3 weeks in all of the BPA-treated groups and the controls. In one of 30 males exposed to BPA at 2 μg/kg after weaning for 3 weeks, diffuse atrophy of the seminiferous tubules was found. In C57BL/6N males exposed to BPA at the mature stage or embryonic stage, no histopathologic changes that may have resulted in a decreased number of germ cells including mature sperm were observed in any parts of the reproductive tract.

4. Discussion

Significant decreases in weights of reproductive organs were detected in C57BL/6N male mice treated with E_2 from postnatal day 27 to 48 as compared with those of the controls. In particular, relative weight of seminal vesicles in the E_2-treated males of the C57BL/6N was 55% of that of the controls. Male C57BL/6N mice treated with E_2 showed

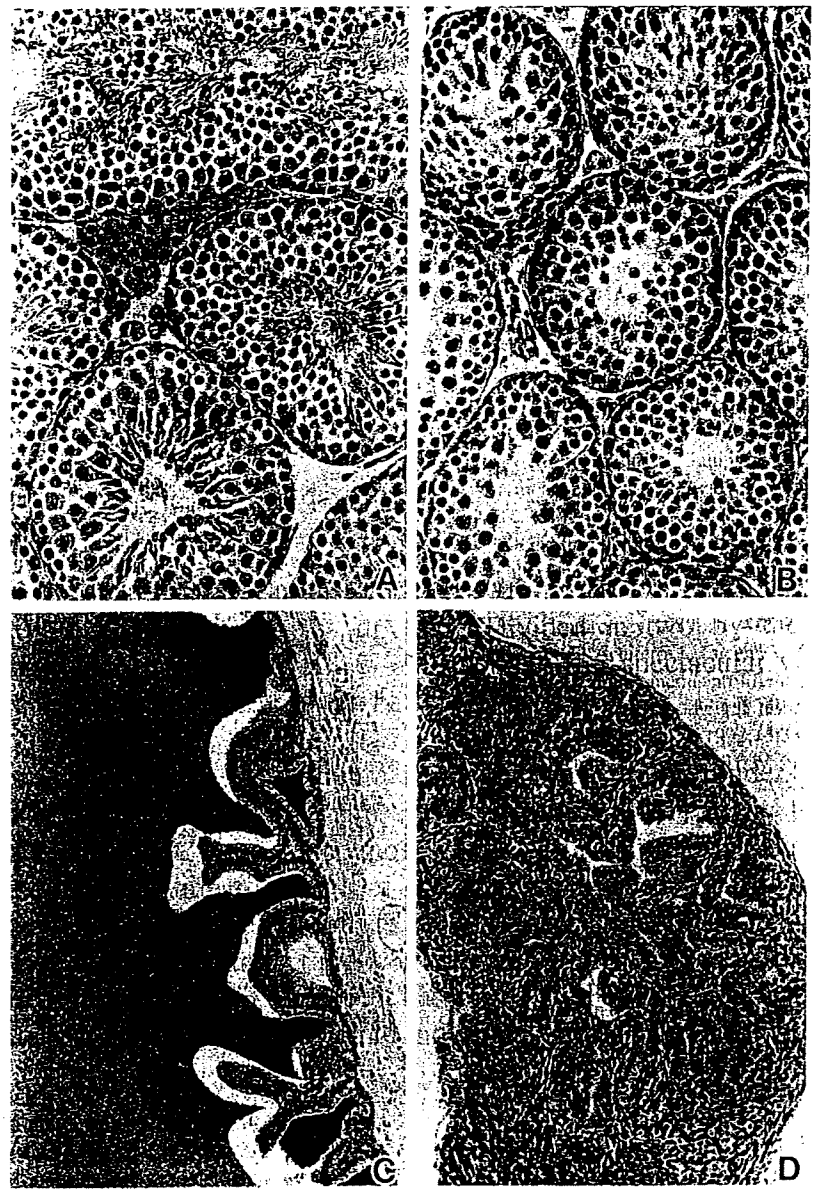

Fig. 1. Histopathologic changes in C57BL/6N male mice exposed s.c. to E_2 from postnatal day 27 to 48. (A) Control male testis. No abnormalities were observed. H&E, ×350. (B) Testis from BPA-treated male. Only a few elongate spermatids were observed in the seminiferous tubules.The number and size of Leydig cells was decreased. H&E, ×350. (C) Control seminal vesicle. No abnormalities were observed. H&E, ×175. (D) Seminal vesicle from BPA-treated male. The lumen contained no secretions. H&E, ×175.

marked atrophy of the testes and seminal vesicle as well as histopathologic changes of the reproductive organs (see Table 2). In contrast, no macroscopic or microscopic changes of the reproductive organs were detected in males of ICR mice treated with E_2. Taken together, these observations suggested that male C57BL/6N mice are extremely sensitive to the effects of E_2 similar to the male C57BL/6J mice reported previously [13], while male ICR mice are extremely resistant to E_2.

In the present study, reproductive organ weights (testes, epididymides, seminal vesicles) in C57BL/6N males exposed to BPA at the mature or immature stage did not differ from those of controls. Ohsako et al. [14] reported that testes weight and daily sperm production of Sprague-Dawley rats 36 days after the first treatment with BPA at 20 μg/kg/day for 6 days were significantly decreased, and concluded that even at very low doses BPA affected spermatogenesis of mature rats. These results were incompatible with those of the present study. There were no significant differences in the density of sperm between the groups exposed to BPA at various stages and the controls. In addition, the numbers of sperm in the BPA-treated and control groups were within the range of the other control data based on information gathered in reproductive and developmental toxicity studies using C57BL/6N mice in our laboratory over a period of 3 years, suggesting that exposure of mice to low-dose BPA at various stages did not affect sperm production.

Table 3
Reproductive organ weights in male C57BL/6N mice exposed to BPA at various stages

Dose (μg/kg/day)	No. mice treated	Start of treatment (Administration period)	Age at termination	No. males/ No. litters	Final body weight (g)	Testes (g)	Epididymides (g)	Seminal vesicles (g)
0	20 males	12 weeks old (6 days)	17 weeks old	15	29.77 ± 0.59[a]	0.150 ± 0.008[b] 0.502 ± 0.042[c]	0.071 ± 0.003 0.237 ± 0.015	0.419 ± 0.016 1.395 ± 0.033
2	20 males	12 weeks old (6 days)	17 weeks old	15	29.00 ± 0.47	0.157 ± 0.001 0.536 ± 0.013	0.072 ± 0.001 0.246 ± 0.006	0.417 ± 0.030 1.431 ± 0.128
20	20 males	12 weeks old (6 days)	17 weeks old	15	29.81 ± 0.77	0.159 ± 0.005 0.539 ± 0.026	0.078 ± 0.003 0.262 ± 0.013	0.407 ± 0.008 1.366 ± 0.050
200	20 males	12 weeks old (6 days)	17 weeks old	15	29.75 ± 0.75	0.159 ± 0.004 0.545 ± 0.017	0.072 ± 0.001 0.246 ± 0.011	0.411 ± 0.020 1.382 ± 0.083
0	30 males	21 days old (21 days)	6 weeks old	25/10	17.15 ± 0.29	0.119 ± 0.002 0.713 ± 0.014	0.031 ± 0.001 0.181 ± 0.006	0.045 ± 0.003 0.259 ± 0.015
2	30 males	21 days old (21 days)	6 weeks old	25/10	18.22 ± 0.67	0.117 ± 0.017 0.663 ± 0.084	0.033 ± 0.003 0.187 ± 0.013	0.044 ± 0.006 0.247 ± 0.028
20	30 males	21 days old (21 days)	6 weeks old	25/10	16.08 ± 0.83	0.116 ± 0.012 0.701 ± 0.056	0.030 ± 0.003 0.184 ± 0.011	0.043 ± 0.008 0.253 ± 0.041
200	30 males	21 days old (21 days)	6 weeks old	25/10	16.83 ± 0.34	0.115 ± 0.006 0.677 ± 0.038	0.030 ± 0.001 0.173 ± 0.007	0.045 ± 0.003 0.263 ± 0.014
0	10 dams	Gestational day 11 (7 days)	12 weeks old	25/10	24.70 ± 0.28	0.149 ± 0.003 0.617 ± 0.014	0.060 ± 0.001 0.249 ± 0.004	0.248 ± 0.006 1.028 ± 0.027
2	10 dams	Gestational day 11 (7 days)	12 weeks old	25/10	23.99 ± 0.50	0.146 ± 0.003 0.612 ± 0.012	0.058 ± 0.002 0.242 ± 0.006	0.234 ± 0.003* 0.968 ± 0.038
20	10 dams	Gestational day 11 (7 days)	12 weeks old	25/10	23.85 ± 0.25	0.142 ± 0.002 0.594 ± 0.009	0.057 ± 0.002 0.239 ± 0.007	0.243 ± 0.011 1.009 ± 0.047
200	10 dams	Gestational day 11 (7 days)	12 weeks old	25/10	24.75 ± 0.52	0.149 ± 0.003 0.608 ± 0.012	0.060 ± 0.001 0.240 ± 0.005	0.250 ± 0.008 1.008 ± 0.022

[a]mean ± S.E.
[b]absolute weight.
[c]relative weight: (organ weight/terminal body weight) × 100.
*Significantly different from the control, $P < 0.05$.
Five males in each group were anesthetized and transcardiac perfusion was performed for histologic observance testes, epididymides, seminal vesicles, and prostates with an electron microscope.

Developmental and reproductive toxicity of high doses of BPA have been demonstrated in rats and mice. Persistent estrus was observed in ovariectomized rats injected twice/day for 3 consecutive days with 100 mg BPA [16]. Intraperitoneal (i.p.) injection of BPA at 125 mg/kg on gestational day 1 through 15 also interfered with the maintenance of pregnancy and reduced the number of live fetuses per litter in Sprague-Dawley rats [17]. However, no significant developmental toxicity of BPA was observed in CD rats or CD-1 mice exposed to BPA (rats, 640 mg/kg; mice 1000 mg/kg) by gavage from gestational day 6 through 15 [18]. In addition, neonatal exposure to BPA at 300 mg/kg did not produce detectable effects on the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA), nor in development of male reproductive organs or reproductive function after puberty in Sprague-Dawley rats [19]. Oral administration of BPA at much lower doses has also been reported to affect male reproductive organ parameters such as the prostate gland (increase in fresh tissue weight at 2 or 20 μg/kg/day), preputial glands (increase in tissue weight at 2 μg/kg/day), and epididymis (decrease in tissue weight at 2 μg/kg/day), and the efficiency of sperm production (decrease in daily sperm production per g testis at 20 μg/kg/day) in CF-1 mice exposed to BPA during prenatal development from GD 11 to GD 17 [5,6]. However, the low-dose effects of BPA have been controversial. Other groups reported no treatment-related effects of BPA at the same and additional low-dose levels given at the same time during pregnancy to CF-1 mice [7,8]. In the experiments in Sprague-Dawley rats, Welsch et al. [12] demonstrated the lack of effects of perinatal exposure to low doses of BPA on ventral prostate weight of male offspring. Elswick et al. [9] also reported that rats exposed to low doses of BPA during the perinatal period did not display significant differences in hormone levels, sperm counts, or immunohistochemical ventral prostate androgen receptor (AR) levels. Recently, two- or three-generation reproductive toxicity studies of BPA administered by gastric intubation or in the diet were performed. The results of these multigeneration studies indicated that low doses of BPA between 0.2 and 200 μg/kg or between 0.015 and 75 ppm over 2 or 3 generations did not cause significant compound-related changes in reproductive or developmental parameters in rats [10,20]. At present, we cannot explain the differences between the results of the present study and those of vom Saal [6] and others [5] and the rat study of Ohsako et al. [14]. These

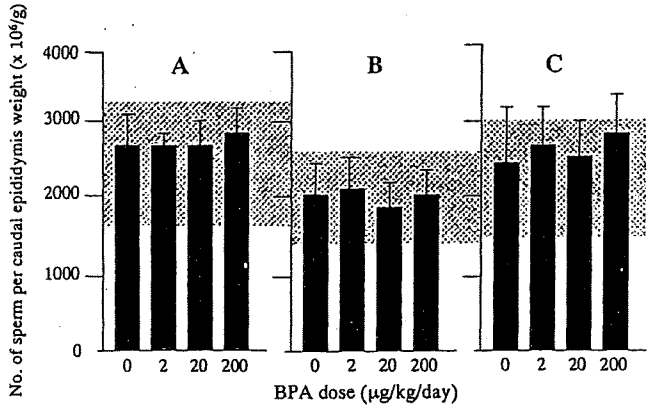

Fig. 2. Numbers of sperm per gram cauda epididymis of male C57BL/6N mice exposed to low-dose BPA at various developmental stages; (A) sexually mature, (B) juvenile, or (C) embryonic/fetal stage. The shadowed regions represent the range of sperm number per gram cauda epididymis in the historical controls in C57BL/6N male mice (left, 17–18 weeks old; middle; 6 weeks old; right, 12–13 weeks old).

discrepancies make the assessment of xenoestrogenic compounds extremely controversial, while concomitantly highlighting the need to resolve these potential important public health concerns [21]. Ashby suggested that some of the failures to repeat observations are probably associated with the many subtle differences that exist between formally identical studies conducted in different laboratories (radio playing quietly in the animal room, soy content of the animal diet, average body weight of the control CF-1 mice, genetic drift of CF-1 mice) [7]. Some chemicals produce small changes in sex-related biologic endpoints that may be difficult to reproduce among laboratories.

Diffuse atrophy of the seminiferous tubules was observed in one C57BL/6N male mouse exposed to BPA at 2 μg/kg after weaning for 3 weeks in the present study. As this was the only case in which an abnormality was found in the testes from males exposed to BPA at various stages, it was not clear whether this change had arisen spontaneously or as a consequence of BPA exposure. In C57BL/6N males exposed to BPA at the mature stage or embryonic stage, no microscopic changes that may have resulted in the decreased number of germ cells including mature sperm were observed in any parts of the reproductive tract. These histopathologic findings were consistent with the lack of effects on sperm density following BPA administration.

When assessing the biologic activity of putative estrogenic compounds in animal model systems, it is important to consider species and strain differences. Strain differences in response to estrogenic stimuli have been demonstrated previously. Gorski and coworkers [22–24] found that the potent estrogens diethylstilbestrol and E_2 induce an overgrowth of lactotropes in the pituitary glands of F344 rats but not in those of outbred strains of rats. Spearow et al. [13] found marked strain-related differences in the susceptibility of mice to estradiol-induced disruption of testicular development. Estradiol treatment during juvenile development resulted in the suppression of testis weight in mouse strains CD-1, C57BL/6J, C17/Jls, S15/Jls, E/Jls and CN-/Jls [13]. Among these, C57BL/6J mice were extremely sensitive with the lowest E_2 dose used (2.5 μg) producing 60% suppression of testis weight in this strain. Unlike C57BL/6J mice in which low to moderate doses of E_2 completely blocked spermatogenesis, CD-1 mice showed very little inhibition of spermatogenesis in response to increasing doses of E_2. Other studies have shown differences in the efficacy of E_2 in stimulation of uterine DNA synthesis between strains of mice [25,26]. In our preliminary study, female ICR and C57BL/6N mice were exposed to various estrogens and endocrine disrupting chemicals during pregnancy, and C57BL/6N embryos were more sensitive (10- to 100-fold) to the lethal effects and disruptive effects of all of these chemicals on development of the reproductive organs than ICR embryos (Nagao, unpublished data).

5. Concluding remarks

In the present study, C57BL/6N mice that are highly susceptible to endocrine disruption by estrogen were used to evaluate the effects of low-dose BPA following exposure at various stages. Based on the results of the present study and the considerable body of literature on the effects of BPA at similar and much higher doses, low-dose BPA should not be considered as a testicular toxicant.

References

[1] Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM, Sonnenschein C. Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect 1996;104:298–305.

[2] Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 1993;132:2279–86.

[3] Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ, McDonnell DP. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol Appl Pharmacol 1997;143:205–12.

[4] Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139:4252–63.

[5] Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ Health Perspect 1997; 105:70–6.

[6] vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Ind Health 1998;14:239–60.

[7] Ashby J, Tinwell H, Haseman J. Lack of effects for low dose levels of bisphenol A and diethylstilbestrol on the prostate gland of CF1 mice exposed in utero. Regul Toxicol Pharmacol 1999;30:156–66.

[8] Cagen SZ, Waechter JM, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, Harris LR. Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicol Sci 1999;50:36–44.

[9] Elswick BA, Janszen DB, Gould JC, Stedman DB, Welsch F. Effects of perinatal exposure to low doses of bisphenol A in male offspring of Sprague-Dawley rats. Toxicol Sci 2000;54(Suppl.):256A.

[10] Ema M, Fujii S, Furukawa M, Kiguchi M, Ikka T, Harazono A. Rat two-generation reproductive toxicity study of bisphenol A. Reprod Toxicol 2001;15:505–23.

[11] Tyl RW, Myers CB, Marr MC, Chang TY, Seely JC, Brine DR, Veselica MM, Fail PA, Joiner RL, Butala JH, Dimond SS, Shiotsuka RN, Stropp G, Veenstra GE, Waechter JM, Harris LR. Three-generation reproductive toxicity study of bisphenol A (BPA) administered in the diet to CD® (Sprague-Dawley) rats. Reprod Toxicol 2001;15:596.

[12] Welsch F, Elswick BA, Janszen DB, Robinette CL. Lack of effects of perinatal exposure to low doses of bisphenol A on male rat offspring ventral prostate glands. Reprod Toxicol 2001;15:597.

[13] Spearow JL, Doemeny P, Sera R, Leffler R, Barkley M. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science 1999;285:1259–61.

[14] Ohsako S, Sakaue M, Ishimura R, Kurosawa S, Yonemoto J, Tohyama C. Effects of low-dose bisphenol-A on rat spermatogenesis. International Symposium on Environmental Endocrine Disruptor's 99. Abstract: 107 (Japanese).

[15] Guide for the Care and Use of Laboratory Animals. NIH Publication 86–23. Bethesda, MD: National Institute of Health, 1985.

[16] Dodds EC, Lawson W. Synthetic oestrogenic agents without the phenanthrene nucleus. Nature 1936;137:996.

[17] Hardin BD, Bond GP, Sikov MR, Andrew FD, Beliles RP, Niemeier RW. Testing of selected workplace chemicals for teratogenic potential. Scand J Work Environ Health 1981;7:66–75.

[18] Morrissey RE, George JD, Price CJ, Tyl RW, Marr MC, Kimmel CA. The developmental toxicity of bisphenol A in rats and mice. Fundam Appl Toxicol 1987;8:571–82.

[19] Nagao T, Saito Y, Usumi K, Kuwagata M, Imai K. Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. Reprod Toxicol 1999;13:303–11.

[20] Kwon S, Stedman DB, Elswick BA, Cattley RC, Welsch F. Pubertal development and reproductive functions of Crl:CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. Toxicol Sci 2000;55:399–406.

[21] Ashby J, Elliott BM. Editorial. Reproducibility of endocrine disruption data. Regul Toxicol Pharmacol 1997;26:94–5.

[22] Wiklund J, Wertz N, Gorski J. A comparison of estrogen effects on uterine and pituitary growth and prolactin synthesis in F344 and Holtzman rats. Endocrinology 1981;109:1700–7.

[23] Wiklund J, Gorski J. Genetic differences in estrogen-induced deoxyribonucleic acid synthesis in the rat pituitary: correlations with pituitary tumor susceptibility. Endocrinology 1982;111:1140–9.

[24] Wendell DL, Gorski J. Quantitative trait loci for estrogen-dependent pituitary tumor growth in the rat. Mamm Genome 1997;8:823–9.

[25] Lee AE. Cell division and DNA synthesis in the mouse uterus during continuous oestrogen treatment. J Endocrinol 1972;55:507–13.

[26] Martin L. Estrogens, anti-estrogens and the regulation of cell proliferation in the female reproductive tract in vivo. In: McLachlan JA, editor. Estrogens in the environment. New York: Elsevier, 1980. p. 103–29.

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Maternal and developmental toxicity in mice by aminophenylnorharman, formed from norharman and aniline

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9-(4′-Aminophenyl)-9*H*-pyrido [3,4-*b*] indole (aminophenylnorharman, APNH) is a novel mutagenic heterocyclic amine, produced by the reaction of norharman with aniline in the presence of S9 mix. In the present study, the maternal and developmental toxicity of APNH were investigated in ICR mice administered oral doses of 0, 0.625, 1.25, 2.5 or 5 mg/kg/day on gestational days (GD) 6 through 15 or 0, 5, 10, or 20 mg/kg on GD 12. Maternal and foetal parameters were evaluated on day 18 of gestation. Foetuses of dams treated on GD 6–15 were examined for external and skeletal malformations and variations, and foetuses of dams treated on GD 12 were inspected for cleft palate. Maternal death occurred when APNH was administered at 5 mg/kg/day on GD 6–15. No significant decrease in body weight gain during the administration period was observed at doses of 2.5 mg/kg/day or less when applied on GD 6–15. Adverse changes in general condition of dams were observed in the groups treated at doses of 2.5 mg/kg/day and above on GD 6–15, whereas no adverse effects on dams were noted even when APNH was applied at a fairly high dose on GD 12. Intracytoplasmic vacuolation in hepatocytes, necrosis of proximal tubular epithelial cells and desquamation of necrotic epithelial cells in the tubular lumen were observed in dams treated with APNH at 2.5 or 5 mg/kg/day on GD 6–15. Increased preimplantation loss was observed at 5 mg/kg/day and post-implantation loss was observed at 2.5 mg/kg/day and above when applied on GD 6–15, or at 20 mg/kg when applied on GD 12. Foetal body weight was decreased by APNH in a dose-dependent manner. The frequency of external malformations (cleft palate) was significantly increased in the group treated with APNH at 2.5 mg/kg/day on GD 6–15 compared to the controls. However, there were no foetuses with cleft palate even when APNH was given at 20 mg/kg on GD 12. No significant increases in skeletally malformed foetuses were found in any APNH-treated group. The frequency of lumbar ribs was increased dose dependently. This study demonstrated the developmental toxicity of a mutagenic compound, APNH, in mice at maternally toxic doses, and that cleft palate observed in term foetuses resulted from the adverse effect of APNH on the maternal environment during organogenesis. More detailed studies are warranted to assess the possible risks to pregnant women from exposure to APNH. *Human & Experimental Toxicology* (2002) **21**, 147–151.

Key words: aminophenylnorharman; developmental toxicity; human exposure; maternal toxicity; mice; teratogenicity

Introduction

Norharman (9*H*-pyridol[3,4-*b*]indole) is widely distributed in our environment, including cigarette smoke and cooked foodstuffs.[1–3] Norharman alone was reported to have non-mutagenic activity in *Salmonella* strains, but it enhanced the mutagenicities of Trp-P-1 and Trp-P-2, and of several typical mutacarcinogens such as benzo[*a*]pyrene, dimethylamino-azobenzene[4] and 2-acetyl-aminofluorene derivatives.[5]

Recently, norharman was shown to become mutagenic to *Salmonella typhimurium* TA98 and YG1024 with S9 mix in the presence of non-mutagenic aromatic amines such as aniline and *o*-toluidine. Totsuka *et al.* isolated the compound produced by a reaction between norharman and aniline with S9 mix.[6] The structure of this compound was determined to be a coupled compound of norharman and aniline, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman, APNH). APNH is converted to the hydroxyamino derivative and forms DNA adducts to induce mutations in TA98 and YG1024. In addition to its mutagenic property, APNH showed marked testicular toxicity when orally administered to F344 rats at 90 mg/kg.[7]

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Since humans including pregnant women are simultaneously exposed to norharman and aniline, possible maternal and developmental toxicity of APNH *in vivo* should be elucidated. Therefore, we examined the developmental toxicity of APNH including teratogenicity when given orally to pregnant ICR mice.

Materials and methods

Animals

The studies were performed on random-bred ICR mice (Charles River, Atsugi, Japan). The animals were acclimated to the laboratory for at least 2 weeks prior to initiation of the experiment. Animals were housed in polycarbonate cages with hardwood chip bedding in a room in which the temperature (24±1°C) and relative humidity (50±5%) were controlled. Lights were on in the room housing the mice from 07:00 to 19:00 hours daily. Mice were allowed food (CE-2, CLEA, Yokohama, Japan) and water *ad libitum*. Female mice weighing 29–35 g were caged with males overnight and examined for a vaginal plug the next morning. The day on which a plug was found was termed day 0 of gestation (GD 0). Animal care and use conformed to published guidelines.[8]

Chemical

9-(4′-Aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman [APNH], $C_{17}H_{13}N_3$) as shown in Figure 1, is synthesized according to the method of Totsuka *et al.*[6,7] In the present study, APNH·HCl with purity more than 99% by HPLC analysis was obtained from the Nard Institute, Osaka, Japan.

Teratology

APNH·HCl was dissolved in distilled water, and mice were orally administered an aqueous solution of APNH (10 mL/kg) on GD 6–15. Animals administered distilled water were set up as controls. To determine appropriate doses for further study, groups of five pregnant mice were administered APNH orally at doses of 5, 10, 20 and 40 mg/kg/day on GD 6–15. All animals given APNH at 20 and 40 mg/kg/day, and four of five animals given APNH at 10 mg/kg/day died during the early administration period (GD 8–10). Based on these results, doses used in the present study were 0, 0.625, 1.25, 2.5 and 5 mg/kg/day. All females were weighed on each day of treatment (GD 6–15), GD 16 (the day after the last treatment) and 18 (the day of cesarean section). Dosage levels for each female were individually adjusted by weight on each day of dosing. In addition, pregnant mice were treated with APNH at doses of 0, 5, 10, and 20 mg/kg on GD 12 to evaluate the induction of cleft palate by this compound. On GD 18, all female mice were killed by cervical dislocation. The uterus including its contents were obtained, and implantation sites, early and late resorptions, and live foetuses were counted. Live foetuses were individually weighed, sexed and inspected for external malformations including abnormalities of the oral cavity under a dissecting microscope. Subsequently, viable foetuses in each litter in the groups treated on GD 6–15 were fixed in 95% ethanol and skeletal malformations were examined after staining with alizarin red S according to the modified Dawson's technique.[9] Abnormal organs observed at cesarean section of dams were saved in 10% neutral-buffered formalin. Tissues were further processed to slides and examined microscopically.

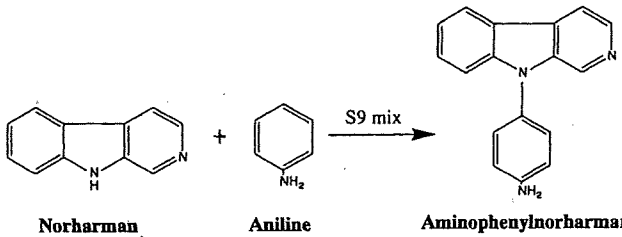

Figure 1 Formation of mutagenic aminophenylnorharman by a reaction of norharman with aniline in the presence of S9 mix

Statistical analysis

Comparisons between the APNH-treated and control groups were made using $p \leq 0.05$ and $p \leq 0.01$ as levels of significance. Maternal body weight gain, mean number of implants, live foetuses and mean foetal body weights were compared by analysis of variance (one way), Bartlett's test for homogeneity of variance, and the appropriate *t* test (for equal or unequal variance) as described by Steel and Torrie[10] using Dunnett's[11] multiple comparison tables or heterogeneous Student's *t* test with Bonferroni correction to determine the significance of differences.

The proportions of litters with malformations and developmental variations were compared using the chi-square test with Yates' correction for 2×2 contingency tables and/or Fisher's exact probability test as described by Siegel[12] to determine the significance of differences. The proportions of early and late embryonic death were compared by the Mann–Whitney *U* test as described by Siegel[12] to determine the significance of differences.

Results and discussion

The numbers of pregnant animals in the control, 0.625, 1.25, 2.5 and 5 mg/kg/day groups treated on GD 6–15 were 10, 9, 9, 11 and 16, respectively. The numbers in the control, 5, 10 and 20 mg/kg groups