2M (the number of neonates in the 17β-estradiol exposed group and the control group: 38 and 41) and 0M (33 and 32), and 2F (41 and 32) and 0F (28 and 37). Fetuses adjacent to dead embryos, and fetuses that were closest to each ovary or the cervix, were discarded. In this experiment, fetuses of two intrauterine positions, 1M and 1F, were also discarded.

2.3.2. Observations of postnatal growth

2.3.2.1. Evaluation of sexual maturation. On PND 21, all male and female pups (2M, 0M, 2F, 0F) in each litter were weaned. For all male and female mice in each litter, as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 25), and preputial separation for males (beginning on PND 30), were assessed, and each pup was weighed when these criteria were achieved.

At 10 weeks of age, five males in each group were weighed and processed to the transcardial perfusion to observe the histological alteration of the prostate by electron microscope. The remaining males in each group were weighed and subjected to necropsy, and the testes, epididymides and seminal vesicles, were weighed. All females were weighed and subjected to necropsy. The ovaries were then weighed. These reproductive organs including prostates and uteri were fixed in 0.1 M phosphate-buffered 10% formalin solution and embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.4. Data analyses

Statistical analysis of the data for the offspring (AGD, body weight and organ weight, organ/body weight ratios, timing of vaginal opening and preputial separation) was performed using the litter as the unit [20,21]. The AGD, body weight and organ weight, organ/body weight ratios (relative organ weight), timing of vaginal opening and preputial separation, were analyzed using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect the significances among the groups. If a significant difference was detected among the groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous, or there was a group whose variance was zero, Kruskal–Wallis analysis of ranks was applied. If a significant effect was detected among the groups, Dunnett's test was applied for multiple comparisons. Comparisons between groups were made using $P \leq 0.05$ as the level of significance.

3. Results

3.1. Experiment I

3.1.1. AGD and body weights of fetuses at cesarean section and pups at PND 4

Table 1 shows the AGD, body weight, AGD/body weight (AGDI: anogenital distance index), and AGD/$\sqrt[3]{\text{body weight}}$ of fetuses at various intrauterine positions and pups at PND 4. It is reasonable to anticipate that the AGD might vary with body weight of fetus or pup. It has been proposed that the relationship between AGD and body weight should be more properly evaluated using the cube root of the body weight [22–25]. If it is desirable to normalize AGD to body weight, the AGD/$\sqrt[3]{\text{body weight}}$ seems to provide a more appropriate adjustment.

There were no statistically significant differences in any parameter evaluated at cesarean section (PND 0) or PND 4

Table 1
Effects of prior intrauterine position on anogenital distance in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
AGD of fetuses at cesarean section						
No. of litters	19	27	24	18	29	27
No. of pups	36	73	43	38	83	41
Body weight (g)	5.6 ± 0.4[a]	5.6 ± 0.3	5.7 ± 0.4	5.2 ± 0.3	5.4 ± 0.3	5.3 ± 0.4
AGD	2.43 ± 0.22	2.42 ± 0.22	2.42 ± 0.28	1.21 ± 0.20	1.23 ± 0.19	1.22 ± 0.24
AGD/body weight	0.43 ± 0.04	0.42 ± 0.04	0.42 ± 0.05	0.23 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
AGD/$\sqrt[3]{\text{body weight}}$	1.36 ± 0.12	1.36 ± 0.14	1.35 ± 0.18	0.69 ± 0.08	0.70 ± 0.07	0.70 ± 0.08
AGD of pups on PND 4						
No. of litters	19	27	24	18	29	27
No. of pups	34	69	41	37	79	38
Body weight (g)	10.9 ± 1.5	11.2 ± 1.5	10.8 ± 1.1	10.4 ± 1.4	10.3 ± 1.1	10.4 ± 1.3
AGD	4.57 ± 0.54	4.41 ± 0.48	4.43 ± 0.51	2.00 ± 0.22	1.99 ± 0.19	2.00 ± 0.21
AGD/body weight	0.42 ± 0.06	0.40 ± 0.05	0.41 ± 0.03	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.04
AGD/$\sqrt[3]{\text{body weight}}$	2.06 ± 0.22	1.99 ± 0.19	1.99 ± 0.19	0.92 ± 0.12	0.91 ± 0.11	0.92 ± 0.13

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.

Table 2
Effects of prior intrauterine position on reproductive organs before maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight on PND 21						
No. of litters	18	27	24	17	27	25
No. of offspring	13	37	11	14	43	12
Body weight (g)	40.9 ± 6.3[a]	40.5 ± 6.5	40.2 ± 9.6	38.8 ± 6.3	38.2 ± 7.0	40.7 ± 7.0
Testes (mg)[b]	169.3 ± 27.5	172.2 ± 22.2	164.9 ± 26.1			
Testes[c]	416.3 ± 48.4	429.2 ± 40.3	418.2 ± 46.7			
Epididymides (mg)[b]	23.3 ± 3.1	23.5 ± 4.8	21.9 ± 4.4			
Epididymides[c]	60.5 ± 10.8	58.2 ± 7.8	55.2 ± 7.0			
Prostate + SV (mg)[b,d]	47.2 ± 9.9	46.7 ± 10.3	45.9 ± 7.9			
Prostate + SV[c,d]	115.6 ± 18.0	115.7 ± 19.0	117.2 ± 19.5			
Ovaries (mg)[b]				24.3 ± 4.0	22.9 ± 3.9	24.8 ± 3.6
Ovaries[c]				63.5 ± 10.1	60.8 ± 9.5	61.5 ± 7.3
Uterus (mg)[b]				10.2 ± 2.0	11.2 ± 3.7	11.8 ± 2.9
Uterus[c]				26.4 ± 4.5	28.8 ± 6.8	29.1 ± 6.0

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.

between groups 2M, 1M and 0M in males, or groups 2F, 1F and 0F in females.

No significant differences in viability of fetuses at cesarean section (PND 0), or that from PND 0 to PND 4 (the number of pups died; 2M = 2, 1M = 4, 0M = 2, 2F = 1, 1F = 4, 0F = 3), were detected between the groups. In addition, there were no statistically significant differences in body weight at PND 0 and 4.

3.1.2. *Body weight and reproductive organ weight of offspring at PND 21*

The absolute and relative weights of testes, epididymides, and prostates with seminal vesicles in males, and ovaries and uteri in females, as well as body weight of offspring at PND 21 are shown in Table 2. Irrespective of the intrauterine position, no significant differences were detected between the groups in absolute or relative reproductive organ weights, or body weights of male and female weanlings, suggesting that the intrauterine position did not affect postnatal growth before weaning in rats.

3.1.3. *Sexual maturation and estrous cycle of offspring*

Table 3 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. The estrous cycle of female offspring from 6 to 10 weeks of age is shown in Table 4. No significant differences were detected between the groups in mean estrous cycle length, or the frequency of females showing each stage of estrous cycle.

Table 3
Effects of prior intrauterine position on sexual maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
No. of litters	18	27	24	17	27	25
No. of offspring	21	32	30	23	36	26
Day of preputial separation	43.3 ± 1.3[a]	43.4 ± 1.2	44.0 ± 1.8			
Body weight (g)[b]	211.8 ± 5.5	212.1 ± 4.3	212.9 ± 5.2			
Day of vaginal opening				33.8 ± 2.2	33.8 ± 1.8	34.1 ± 1.7
Body weight (g)[b]				125.6 ± 4.1	124.6 ± 4.4	126.1 ± 3.9

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] Body weight when the criterion was achieved.

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)[a]	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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Exacerbation of benzene pneumotoxicity in connexin 32 knockout mice: enhanced proliferation of CYP2E1-immunoreactive alveolar epithelial cells

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Abstract

The pulmonary pathogenesis triggered by benzene exposure was studied. Since the role of the connexin 32 (Cx32) gap junction protein in mouse pulmonary pathogenesis has been suggested, in the present study, we explored a possible role of Cx32 in benzene-induced pulmonary pathogenesis using the wild-type (WT) and Cx32 knockout (KO) mice. The mice were exposed to 300 ppm benzene by inhalation for 6 h per day, 5 days per week for a total of 26 weeks, and then sacrificed to evaluate the pneumotoxicity or allowed to live out their life span to evaluate the reversibility of the lesions and tumor incidence. Our results clearly revealed exacerbated pneumotoxicity in the benzene-exposed Cx32 KO mice, characterized by diffuse granulomatous interstitial pneumonia, markedly increased mucin secretion of bronchial/bronchiolar and alveolar epithelial cells, and hyperplastic alveolar epithelial cells positive for CYP2E1. But the results did not indicate any enhancement of pulmonary tumorigenesis in the Cx32 KO mice though the number of animals was small.

Keywords: Benzene; Connexin 32; Cx32 knockout mice; CYP2E1; Interstitial pneumonia; Pneumotoxicity

1. Introduction

Benzene has been reported to be a carcinogen capable of producing not only hemopoietic malignancies but also various solid tumors including lung cancers in mice, chronically exposed to it by ingestion or inhalation (Snyder et al., 1988; Huff et al., 1989; Farris et al., 1993).

Benzene toxicity and benzene-induced tumor development in the lung should be taken into consideration for the risk assessment in humans, since the lung is one of the benzene target organs and inhalation is the most common route through which humans are exposed to benzene. Furthermore, a strong relationship

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between benzene exposure and lung cancer development in humans has been assumed for the past decades (Aksoy, 1985, 1989). In addition, benzene metabolites such as benzene oxide, benzene dihydrodiol and diolepoxide have been shown to induce lung tumorigenesis in mouse neonates (Busby et al., 1990). However, little information is available on the pulmonary pathogenesis triggered by benzene exposure.

Intercellular communication through gap junction proteins (GJICs) plays an important role in cellular homeostasis by regulating cell growth, cell differentiation, and apoptosis (Yamasaki, 1996). Based on this concept, alteration in GJICs has been demonstrated to be closely associated with the pathogenesis and carcinogenesis induced by chemicals, particularly by nongenotoxic agents (Yamasaki et al., 1995; Kolaja et al., 2000). Furthermore, down modulation of GJICs is known to induce cytochrome P450s by other chemicals that may be involved in benzene metabolism (Neveu et al., 1994; Snyder and Hedli, 1996; Shoda et al., 2000). We, therefore, hypothesized that GJICs may contribute also to the processes of benzene-induced pneumotoxicity and lung carcinogenesis.

As the presence and the functional role of connexin 32 (C32) gap junction protein in the mouse lung tissue have been suggested in previous in vitro and in vivo studies (Albright et al., 1990; Lee et al., 1997; Ruch et al., 1998; Abraham et al., 1999, 2001), in the present study, we explored a possible role of Cx32 in the lung pathogenesis induced by chronic exposure to benzene, using Cx32 knockout (KO) mice. For this purpose, wild-type (WT) and Cx32 KO mice were exposed to 300 ppm benzene by inhalation for 6 h per day, 5 days per week for 26 weeks. Then the pathological changes were determined based on the results of histopathology, histochemistry for detecting mucin secretion, and immunohistochemistry for detecting CYP2E1 and proliferating cell nuclear antigen (PCNA). The tumor incidence in the pulmonary tissue was also compared between the benzene-exposed WT and Cx32 KO mice.

2. Materials and methods

2.1. Animals

Cx32 KO mice, from the Institut für Genetik, Universität, Bonn, Germany (Moennikes et al., 2000), were maintained as heterozygous KO mice at the animal facility of National Institute of Health Sciences (NIHS), Japan. Because the *Cx32* gene is linked to the X-chromosome, we generated Cx32 WT ($Cx32^{+/Y}$) and KO male ($Cx32^{-/Y}$) mice for this study by cross breeding female $Cx32^{+/-}$ heterozygous mice and male C57BL/6 wild type mice. The Cx32 genotypes of the neonates were identified by the standard PCR assay (Moennikes et al., 2000). The WT and Cx32 KO mice aged 8–9 weeks were used in the study. During the study, the mice were housed within stainless-steel wire cages in inhalation chambers that were maintained on a 12-h light-dark cycle. The basal pellet diet (CRF-1; Funabashi Farm, Tokyo, Japan) was provided ad libitum, except during the 6-h daily inhalation of benzene when the food was withdrawn. Water was automatically supplied throughout the study.

2.2. Benzene exposure

Benzene was purchased from Wako Chemical Company (Osaka, Japan). The mice were randomized and exposed to benzene in 1.3 m^3 inhalation chambers, as described elsewhere (Yoon et al., 2001). Briefly, the benzene vapor was generated by heating liquid benzene to 16 °C and directed into the inhalation chambers (Sibata Scientific Technology Ltd., Tokyo, Japan) with a room temperature of 24 ± 1 °C. The flow rate of benzene was about 650 l/min, and the benzene concentration in the chambers was measured at 30-min intervals during the daily exposures using a gas chromatograph (Shimadzu Co., Kyoto, Japan). The temperature and humidity in the chambers were automatically controlled at 24 ± 1 °C and 55 ± 10%, respectively. As described in the previous Section 2.1, mice were supplied water ad libitum but withdrew the food pellets during the exposure.

The WT and Cx32 KO mice were, respectively, divided into the sham-exposed control group and the benzene-exposed groups; each group was composed of ten to twelve mice. The experimental group was exposed to 300 ppm benzene for 6 h per day, 5 days per week, for 26 weeks and the sham-exposed control group was maintained under the same conditions but without benzene inhalation. Five to six mice from each group were first sacrificed after the 26-week exposure to evaluate pneumotoxicity and the remaining five to seven mice from each group were allowed to

live out their lives to further evaluate their recovery from pulmonary lesions and the incidence of the pulmonary tumor.

2.3. Measurement of food consumption and body weight

Food consumption and body weight were measured every Friday throughout the 26-week benzene exposure.

2.4. Autopsy, organ weight measurement and histopathology

After the 26-week benzene exposure, five to six mice from each group were sacrificed under ethyl ether anesthesia for autopsy. Gross morphological examination of the mice was performed and the major visceral organs were weighed and analyzed. For the histopathological examination, tissues from both lungs were fixed in 10% neutral buffered formaldehyde for 24 h. Pulmonary tissues were sliced and immediately immersed in the fixative. After routine processing, the paraffin-embedded sections were stained with hematoxylin and eosin and then examined histopathologically under a light microscope.

2.5. Immunohistochemistry and histochemistry

The avidin–biotin–peroxidase complex (ABC) method was used for immunohistochemistry to detect the expression of the P450 CYP2E1 enzyme and PCNA. After the lung tissue sections mounted on poly-L-lysine-coated slides were deparaffinized and hydrated, endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for 15 min. The lung tissue sections in a Caplin jar containing 1 mM citric acid (pH 6.0) were microwaved for 10 min for retrieval of PCNA. After washing in phosphate-buffered saline (PBS, pH 7.4) for 15 min, the tissue sections were incubated with 10% normal serum at room temperature for 60 min to block nonspecific binding sites. The sections were then incubated with a mouse anti-PCNA monoclonal antibody (1:300, Sigma–Aldrich, Amherst, NY, USA) for 50 min at room temperature and a goat anti-rat CYP2E1 polyclonal antibody (1:1000, Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) overnight at 4 °C. The tissue sections were washed three times in PBS, incubated with the corresponding biotinylated secondary antibodies for 40 min at room temperature, and subsequently incubated with the ABC reagent for 30 min at room temperature. As a chromogen, 0.5% 3,3′-diaminobenzidine tetrahydrochloride was used, and the sections were counterstained with methylene blue. As a positive control for PCNA and CYP2E1, normal testis and kidney sections were used, respectively, and as a negative control, PBS instead of the primary antibodies was applied to the sections.

Periodic acid–Schiff (PAS) reaction was performed to detect mucus secretion. After deparaffinization, the tissue sections were immersed in 0.5% periodic acid solution. After washing with distilled water, the sections were incubated with the Schiff reagent for 15 min, washed with warm tap water for 10 min, and then counterstained with hematoxylin.

2.6. Statistical analysis

ANOVA was performed to evaluate the significant differences in food consumption and body weight between the nonexposed sham exposed control and benzene-exposed groups of WT and Cx32 KO mice as well as between WT and Cx32 KO mice of each group.

3. Results

3.1. Changes in body weight during the 26-week benzene exposure

No significant difference was observed between WT and Cx32 KO mice of the nonexposed sham-control group throughout the study, even when the mean body weight of Cx32 KO mice was slightly less than that of WT mice at the late stage of this study (Fig. 1). Benzene exposure induced a significant decrease in the body weight of the benzene-exposed group of both WT and Cx32 KO mice compared with the nonexposed sham-control mice. The reduction was much more marked in Cx32 KO mice (Fig. 1), which was observed after seven weeks of exposure ($P < 0.05$). On the other hand, in WT mice, a significant difference in body weight was observed after the fourteenth week of exposure (Fig. 1). Furthermore, after the twelfth week

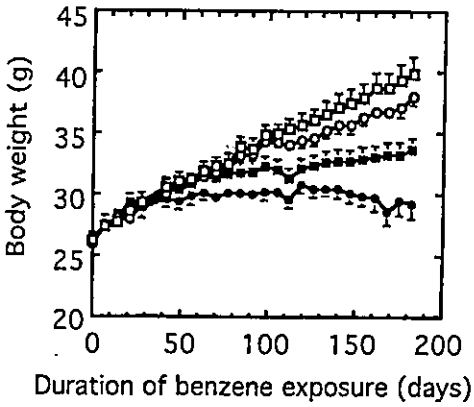

Fig. 1. Changes in body weights of WT and Cx32 KO mice during benzene exposure. Benzene (300 ppm) was inhaled for 6 h per day, 5 days per week for 26 weeks. Eleven to 12 mice per group were used. (□) WT-sham group; (○) Cx32 KO-sham group; (■) WT-benzene-exposed group; (●) Cx32KO-benzene-exposed group. There is significant difference between benzene-exposed group from the corresponding sham-control group after 10 weeks exposure for the Cx32 KO and 14 weeks exposure for WT. Vertical bars mean standard errors.

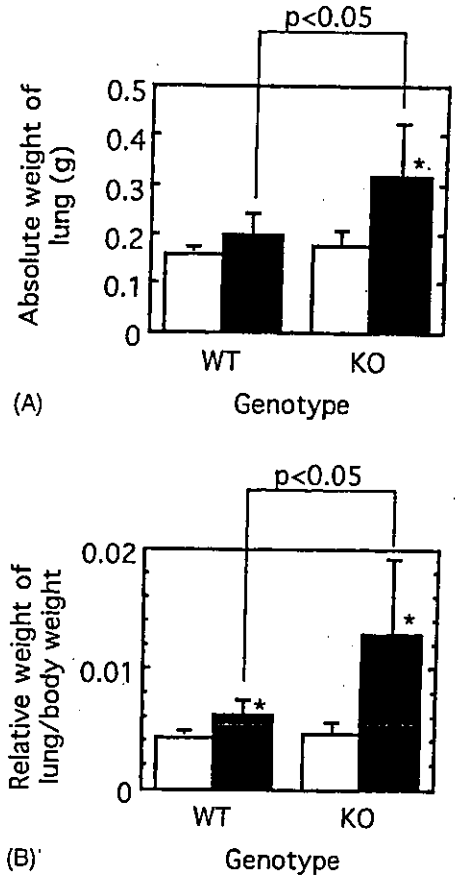

Fig. 2. Changes in weights of the lungs of mice exposed to 300 ppm benzene for 26 weeks. Open column; sham-control group, closed column; benzene-exposed group. Vertical bars mean standard deviations. Symbol (*) indicates significantly different from the corresponding sham-control group at $P < 0.05$.

of exposure, the mean body weight was significantly different between benzene-exposed WT and Cx32 KO mice ($P < 0.05$) (Fig. 1).

During the benzene exposure for 26 weeks, there had been no significant difference in food consumption between the nonexposed group and the benzene-exposed group of both WT and Cx32 KO mice and between WT and Cx32 KO mice of both groups (data not shown).

3.2. Weight of the lung

Significant increases were noted in the absolute lung weight of Cx32 KO mice and in the relative lung weights of both the WT ($P < 0.05$) and Cx32 KO mice ($P < 0.05$) after the twenty-sixth week of exposure to 300 ppm benzene (Fig. 2).

3.3. Histopathology and histochemistry

Severe diffuse interstitial pneumonia was observed in the lungs of the benzene-exposed Cx32 KO mice, which was comparable with that in the lungs of the WT mice showing much milder pulmonary lesions (Table 1, Fig. 3B and E). The alveolar walls were thickened by heavy infiltration of macrophages, the presence of a small number of lymphocytes and neutrophils, and a considerably increased number of type II alveolar epithelial cells (Fig. 3C). The proliferation of basophilic epithelial cells in the terminal bronchioles and alveolar ducts was frequently noted in the lungs of benzene-exposed Cx32 KO mice (Fig. 3F), while the lungs of benzene-exposed WT mice had mild and a few basophilic proliferating epithelial cell-proliferating foci. The numbers of mucus-secreting epithelial cells increased in the bronchi and bronchioli of both WT and Cx32 KO mice exposed to benzene for 26 weeks (Fig. 4C and D). In particular, in the benzene-exposed Cx32 KO mouse lungs, aggregates composed of mucin-secreting alveolar epithelial cells were occasionally detected (Fig. 4D).

Table 1
Pathological findings in the lungs of the wild-type (WT) and Cx32 knockout (KO) mice exposed to 300 ppm benzene for 26 weeks

Group (with or without benzene treatment)	Genotype			
	WT		Cx32 KO	
	Sham-exposed	300 ppm	Sham-exposed	300 ppm
Histopathology/no. of animals examined	6	5	5	5
Interstitial pneumonia granulomatous, diffuse	0 (0.0)	4 (80.0)	0 (0.0)	5 (100.0)
Moderate		4 (80.0)		1 (20.0)
Severe		0 (0.0)		4 (80.0)
Hyperplastic basophilic cell foci	0 (0.0)	1 (20.0)	0 (0.0)	4 (80.0)
Alveolar and bronchiolar epithelial cells		1 (20.0)		4 (80.0)
Mucin-secreting cells	0 (0.0)	5 (100.0)	1 (20.0)	5 (100.0)
Bronchial/bronchiolar epithelial cells		5 (100.0)	1 (20.0)	5 (100.0)
Alveolar epithelial cells		0 (0.0)	0 (0.0)	3 (60.0)

Number in parentheses represents the percentage (%) of the lesions.

3.4. Immunohistochemistry for PCNA and CYP2E1

The labeling indices for PCNA, compared with those of the corresponding control groups, significantly increased in both benzene-exposed WT and Cx32 KO mice; from 79.9 to 162.3‰ ($P < 0.005$) and 92.7 to 533.0‰ ($P < 0.002$), respectively (Fig. 5).

A few bronchial and bronchiolar epithelial cells of sham-control WT and Cx32 KO mice were positive for the CYP2E1 enzyme (Fig. 6A). The numbers of

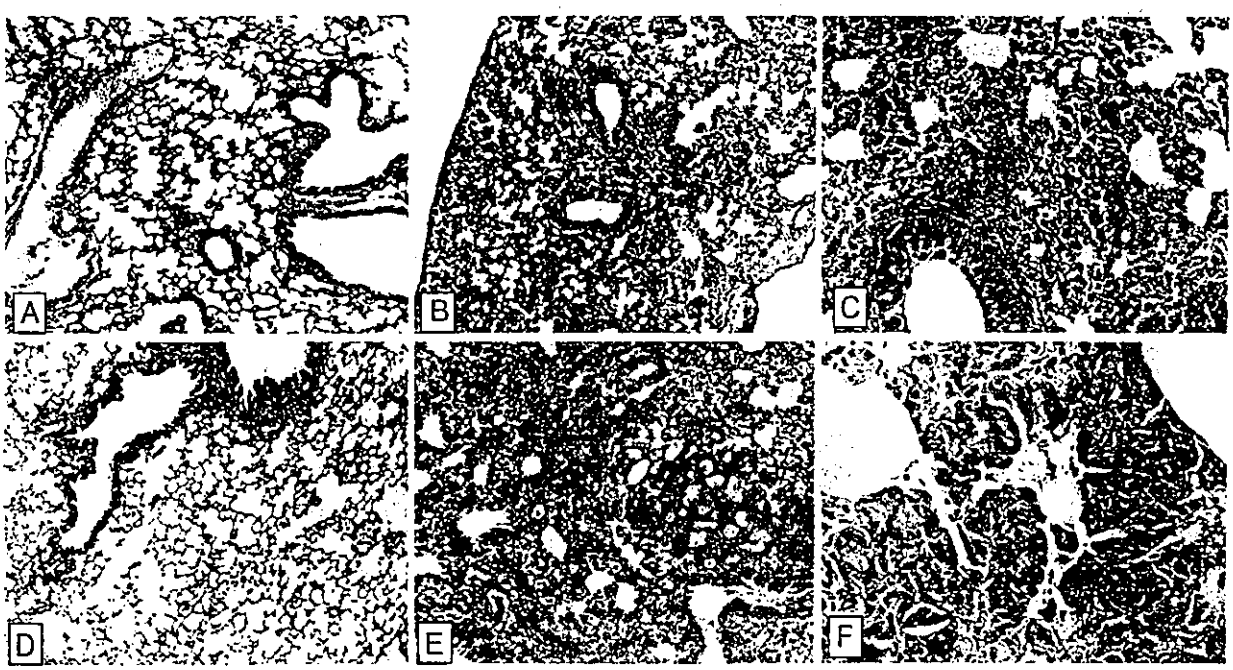

Fig. 3. Histopathological changes of the lungs of benzene-exposed WT and Cx32 KO mice exposed to 300 ppm benzene for 26 weeks. (A) sham-control WT mice, (B and C) benzene-exposed WT mice, (D) sham-control Cx32 KO mice, (E and F) benzene-exposed Cx32 KO mice. Note the granulomatous interstitial pneumonia in the lungs of benzene-exposed WT and Cx32 KO mice, and basophilic epithelial cell-proliferating foci frequently observed in the lungs of benzene-exposed Cx32 KO mice (F). Original magnification: (A) ×100; (B) ×100; (C) ×200; (D) ×100; (E) ×100; (F) ×400. Hematoxylin- and eosin-stained.

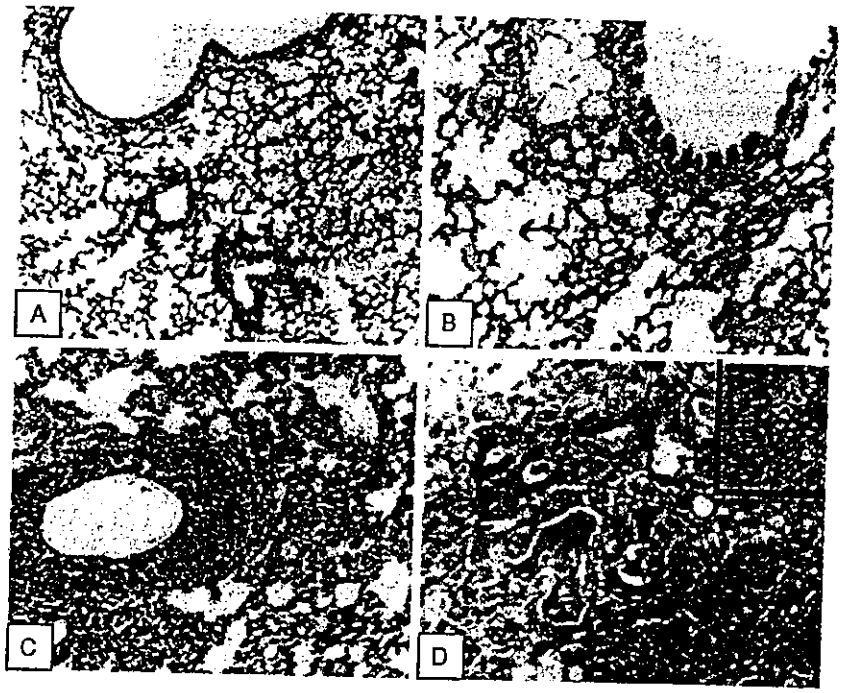

Fig. 4. Histochemistry for detection mucin secretion. (A) Sham-control WT mice, (B) sham-control Cx32 KO mice, (C) benzene-exposed WT mice, (D) benzene-exposed Cx32 KO mice. Note the enhanced mucin secretion from bronchiolar epithelial cells of WT mice (C) and Cx32 KO mice (Inset in D), and the aggregated cells releasing mucin occasionally observed in the benzene-exposed Cx32 KO mice (D). Original magnification: (A) ×200; (B) ×200; (C) ×200; (D) ×400.

CYP2E1-positive bronchial and bronchiolar epithelial cells considerably increased following long-term benzene exposure in both WT and Cx32 KO mice (Fig. 6B). The proliferating basophilic alveolar epithelial cells frequently observed in the benzene-exposed Cx32 KO mice were strongly positive for CYP2E1 (Fig. 6D), which was significantly comparable with the WT mice in which these findings were rarely observed.

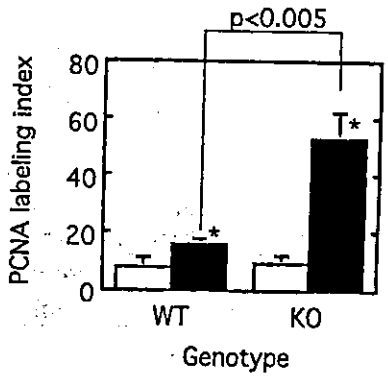

Fig. 5. PCNA labeling indices in the mouse lung tissues exposed to 300 ppm benzene for 26 weeks. Values represent the number of PCNA-positive cell per 1000 cells. More than 3,000 alveolar epithelial cells were counted under a light microscope at a high magnification (×400).

3.5. Survival curves for life time observation

Five to seven mice were randomly selected and allowed to live their life span to evaluate their recovery from pulmonary lesions and the incidence of pulmonary tumor. Survival curves for each group are shown in Fig. 7. In each group the number of mice were limited to about five to seven mice per group. There was no intermittent death during the exposure time up to 182 days (26 weeks). The sham-exposed control group indicated by open symbols, circles for WT mice and squares for Cx32 KO mice, show a longer life span than the benzene-exposed group indicated by closed symbols, circles for WT mice and squares for Cx32 KO mice. Interestingly, in the exposed group, Cx32 KO mice showed a longer life span than the wild-type mice, although the sham-exposed group does not show much difference between wild-type mice and Cx32 KO mice. During the observation period, all the mice that

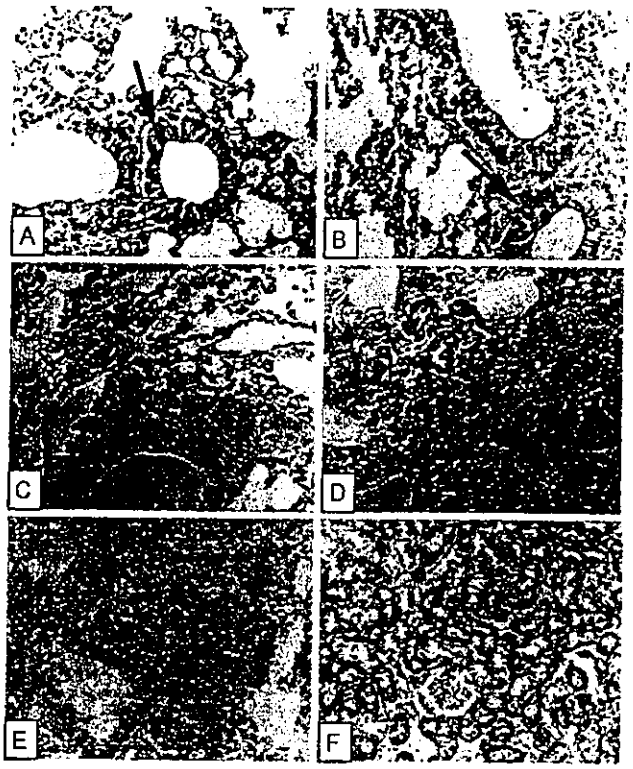

Fig. 6. Immunohistochemistry for P450 CYP2E1 in the lungs of WT and Cx32 KO mice exposed to 300 ppm benzene for 26 weeks. (A) Sham-control WT mice, (B) benzene-exposed WT mice, (C) sham-control Cx32 KO mice, (D) benzene-exposed Cx32 KO mice, and (E and F) negative and positive control, respectively. A few bronchiolar epithelial cells of sham-control WT and Cx32 KO mice were positive for CYP2E1 (arrows). Benzene exposure induced increases in the numbers of the CYP2E1-producing bronchial/bronchiolar and alveolar epithelial cells in WT and Cx32 KO mice. Note the proliferating basophilic alveolar epithelial cells positive for CYP2E1 in the benzene-exposed Cx32 KO mice (D). Original magnification: (A) ×200; (B) ×200; (C) ×200; (D) ×400; (E) ×200; (F) ×200.

died were immediately autopsied, whenever possible, and histopathological examinations were performed.

3.6. Tumor incidence and recovery of pulmonary lesions

Results of histopathological observation are shown in Table 2. No pulmonary tumors were observed in WT and Cx32 KO C57BL/6 mice sacrificed after the 26th week of exposure to 300 ppm benzene. Pulmonary adenoma developed in one Cx32 sham-control mouse. Pulmonary adenoma and adenocarcinoma developed only in two out of the seven benzene-exposed WT

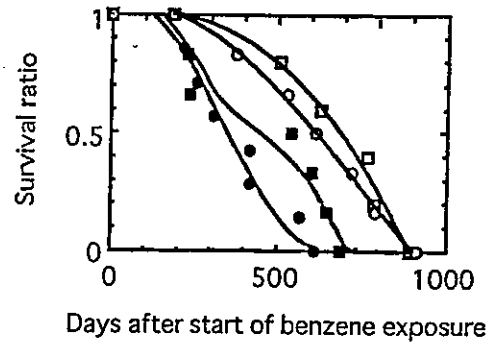

Fig. 7. Survival curves of groups for lifetime observation. The number of mice was limited about five to seven mice per group. There was no intermittent death during the exposure time up to 182 days (26 weeks). The sham-exposed group, indicated by open symbols, circles for wild-type mice and squares for Cx32 KO mice, show a longer life span than the 300-ppm benzene-exposed group indicated by closed symbols, circles for wild-type mice and squares for Cx32 KO mice.

mice, at 59.7 weeks and 87.3 weeks of the study, respectively (Table 2). The pulmonary lesions observed in the WT and Cx32 KO mice exposed to benzene for 26 weeks were considerably attenuated and regressed with time after cessation of the exposure (data not shown).

Most of the benzene-exposed WT and Cx32 KO mice, which were allowed to live out their lives after termination of benzene exposure, died far earlier than the sham-control mice of each genotype due to malignant lymphomas, squamous cell carcinomas, spindle cell sarcoma and hepatomas or a combination of these tumors (Table 2).

The incidence of hemopoietic neoplasia in C57BL/6 mice was enhanced by 300 ppm benzene exposure as previously reported elsewhere (Snyder et al., 1980; Cronkite et al., 1985; Kawasaki et al., unpublished observation). In the Cx32 KO group, incidences of hemopoietic neoplasia were identical for both the benzene-exposed and sham-exposed control mice, although peak incidences were earlier in the benzene-exposed mice than in the sham-exposed control mice. (see Fig. 7).

4. Discussion

Benzene has been suspected for years as an agent that induces human pulmonary cancer (Aksoy, 1989)

Table 2
Tumor development in the wild-type (WT) and Cx32 knockout (KO) mice that were allowed to live out their life span after termination of 26-week exposure to 300 ppm benzene

Tumor/group (with or without benzene treatment)	Genotype			
	WT		Cx32 KO	
	Sham-exposed	300 ppm	Sham-exposed	300 ppm
No. of animals examined	6	7	5	6
Animals bearing tumor(s)	3 (50.0)	6 (85.7)	4 (80.0)	6 (100.0)
Pulmonary adenoma/adenocarcinoma	0 (0.00)	2 (28.6)	0 (0.0)	0 (0.0)
Hemopoietic neoplasia	2 (33.3)	5 (71.4)	4 (80.0)	5 (83.3)
Hepatoma	2 (33.3)[a]	0 (0.0)	1 (20.0)[a]	1 (16.7)
Squamous cell carcinoma	0 (0.0)	2 (28.6)[b]	0 (0.0)	4 (66.7)[c]
Spindle cell sarcoma	0 (0.0)	1 (14.3)	0 (0.0)	1 (16.7)
Animals without tumor(s)[d]	3 (50.0)	1 (14.3)	1 (20.0)	0 (0.0)

Number in parentheses represents the percentage (%) of the lesions.
[a] Concomitant with malignant lymphomas.
[b] Concomitant with pulmonary adenocarcinoma and spindle cell sarcoma.
[c] Concomitant with spindle cell sarcoma for one mouse, granulocytic leukemia for two mice and hepatoma for one mouse.
[d] Mice without tumor in the WT sham-control and the 300-ppm benzene-exposed groups had auricular thrombosis and one mouse without tumor in the Cx32 KO-control group died of ascending nephritis and renal infarction.

and the long-term exposure of mice to benzene had been shown to notably increase the incidence of pulmonary adenoma and adenocarcinoma (Huff et al., 1989; Maltoni et al., 1989; Farris et al., 1993). However, little information is available on the mechanism by which benzene exerts its pneumotoxicity and induces lung cancer.

Experimental conditions of benzene exposure and the incidence of hemopoietic neoplasia occurring in groups for lifetime observation were identical to those previously reported by Snyder et al. (1980, Fig. 4, p. 326 in their article) and also to our separate large-scale study (Kawasaki et al., unpublished observation). In the present study, we specifically focused on a possible role of Cx32 in benzene-induced pneumotoxicity and the pathogenesis of pulmonary tumor using bioengineered Cx32 KO and the WT mice.

Although Cx32-deficient mice have a late-onset peripheral neuropathy, a condition with features similar to those of Charcot–Marie–Tooth disease in humans, their gross morphology had been reported to be normal independent of age (7–28 weeks) and gender, except for a slightly lower body weight than the wild-type mice of the same genetic background (Nelles et al., 1996; Anzini et al., 1997). In agreement with a previous report, the body weight of Cx32 KO mice was lower than that of WT mice at the late stage of this study, although this difference was not significantly different (Fig. 1). The organ weight and histological findings consistently indicated that the decrease in body weight observed during long-term exposure to benzene closely correlated with the development of pulmonary lesions, characterized by diffuse granulomatous interstitial pneumonia, regenerating alveolar epithelial cell proliferation, and increased mucus secretion (Table 1, Figs. 2–5). The pulmonary lesions were far severer in Cx32 KO mice than in WT mice, strongly suggesting that Cx32 prevents the benzene-induced lung pathogenesis.

It has generally been accepted that the metabolism of benzene by the CYP2E1 enzyme to phenolic metabolites is a critical event in its toxic and carcinogenic mechanisms. A noteworthy finding of our study was the active proliferation of bronchiolar–alveolar epithelial cells expressing the CYP2E1 enzyme in the lungs of benzene-exposed Cx32 KO mice (Figs. 4D and 6F). This suggests that benzene exposure stimulates CYP2E1-producing epithelial cells in the lungs through a pathway that is regulated by the Cx32 gap junction protein. The activation of CYP2E1-producing epithelial cells may enhance the metabolism of benzene to metabolites that are potentially pneumotoxic such as benzene oxide, phenol and hydroquinone, resulting in exacerbation of

benzene-induced pneumotoxicity. CYP2E1 has been detected in the lungs of humans and rats (Tindberg and Ingelman-Sundberg, 1989; Carlson and Day, 1992; Wheeler et al., 1992). Moreover, recent studies have shown the important role of the enzyme in benzene metabolism resulting in its pneumotoxicity (Powley and Carlson, 2000, 2001), which is also supported by the finding of benzene metabolism inhibition by a CYP2E1 inhibitor, diethyldithiocarbamate (Chaney and Carlson, 1995). The metabolic level of benzene in the pulmonary tissue has not been evaluated yet. However, a previous study showed that pulmonary microsomes can metabolize benzene at similar rates to those of hepatic microsomes, and that they are likely more efficient in generating hydroquinone (Chaney and Carlson, 1995). Recently, with regards to CYP2E1-mediated 1,1-dichlroethylene-induced lung toxicity, Forkert et al. (2001) reported good correlations among the amount of the enzyme, metabolism of 1,1-dichlroethylene to a toxic metabolite, and lung cytotoxicity.

Activation of alveolar pneumocytes by benzene was considered to be another possible important event for the pneumotoxicity of benzene observed in the present study, as shown in the lungs of benzene-exposed groups of WT and Cx32 KO mice (Fig. 3). Alveolar epithelial cells, containing a large amount of peroxidase, are capable of metabolizing phenolic compounds to genotoxic reactive species that can induce DNA adducts and generate oxygen-free radicals (Brieland et al., 1987; Schlosser et al., 1989; Smith et al., 1989) and of producing nitric oxide by themselves (Laskin et al., 1995). The production of reactive oxygen intermediates has been implicated in cytotoxicity and carcinogenesis, by inhibiting GJICs as well as causing DNA damage (Kuo et al., 1998; Upham et al., 1997, 1998). Several investigators have shown that oxygen radicals from benzene-activated alveolar epithelial cells play an important role in the genotoxic and nongenotoxic mechanisms of benzene-target organs (Subrahmanyam et al., 1991; Kolachana et al., 1993; Laskin et al., 1995). In the lung, Suleiman (1987) showed that benzene induces lipid peroxidation and increases the amount of the lysosomal enzyme released by activating alveolar epithelial cells, contributing to the pathological changes. The formation of oxygen radicals and related reactive oxygen species is highly controlled in a biological system by physiological antioxidant defense mechanisms. In a study by Kojima et al. (1996), a potential role of Cx32 in the regulation of oxygen radical production in cultured hepatocytes has been suggested based on the correlation found between the expression of Cx32 and the effect of oxygen radical scavengers. Therefore, it can also be hypothesized in our present study that the disregulation of reactive oxygen species production by benzene in lung tissues due to a dysfunction of GJICs caused by Cx32 might contribute to the exacerbation of pulmonary lesions in Cx32 KO mice. Further studies will be required to prove this hypothesis.

Despite the finding that the Cx32-mediated disruption of GJICs enhanced the pneumotoxicity of benzene, our present study, though with a small number of animals did not indicate any enhancement of the development of pulmonary tumor in the Cx32 KO mice (Table 2).

Therefore, the pathological lesions exacerbated in Cx32 KO mice may not seem to be critical changes for pulmonary carcinogenesis of benzene. This was supported by their recovery from the pulmonary lesions after removal of benzene and the absence of tumor incidence in benzene-exposed Cx32 KO mice.

In conclusion, our present study indicates that Cx32 attenuates the pneumotoxicity of benzene, particularly in the case of chronic exposure in vivo, most likely by regulating proliferation of CYP2E1-producing lung cells population. However, the role of Cx32 in benzene-induced pulmonary tumorigenesis was not clarified in the present study.

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