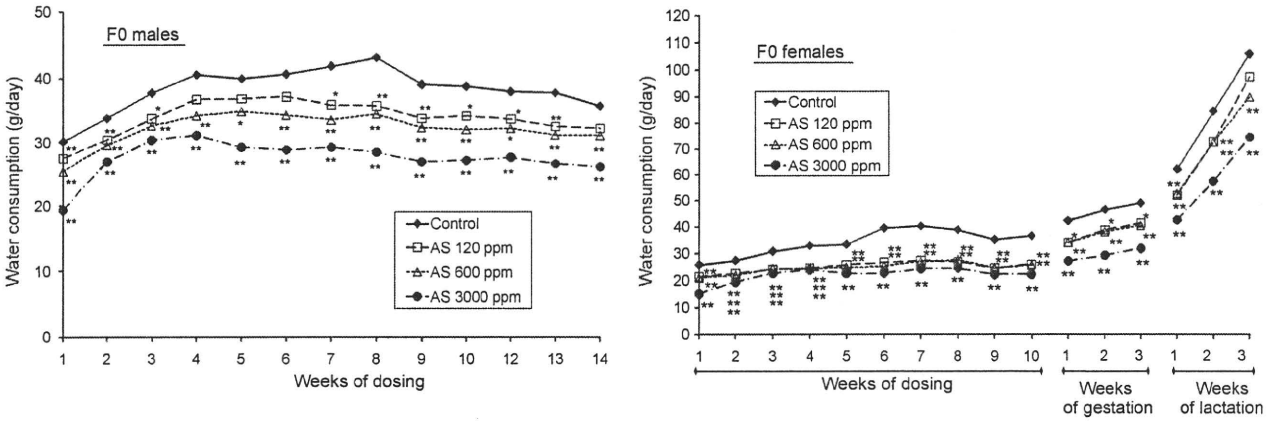

Fig. 1. Water consumption of F0 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

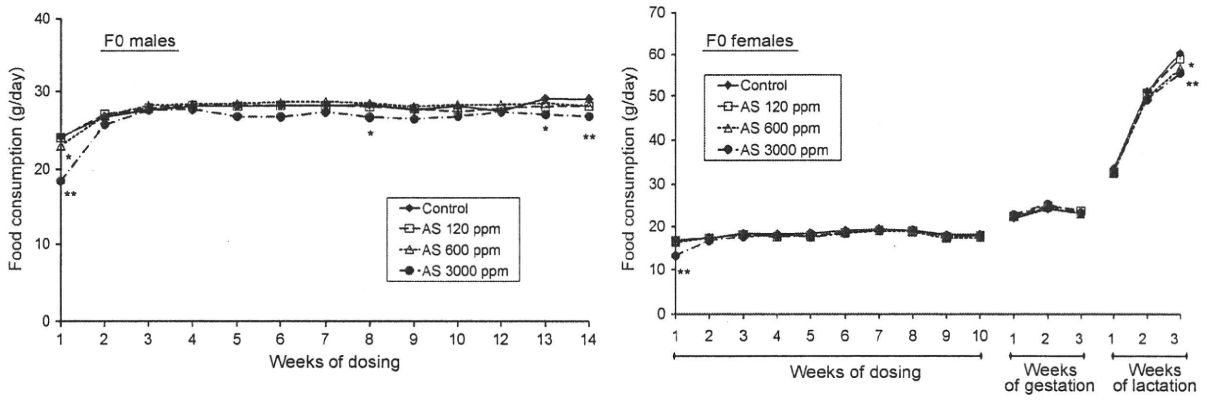

Fig. 2. Food consumption of F0 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

control and 600 ppm groups (100 ± 0.0% versus 98.4 ± 7.3%). The surface righting reflex on PND 5 and negative geotaxis reflex on PND 8 were achieved in all male and female F2 pups in all groups, and no significant changes were found in the response time (data not shown).

As for the sexual development of F1 male and female animals, vaginal opening was significantly delayed at 3000 ppm (31.4 ± 1.7, compared to 29.5 ± 2.1 in control). At this dose, body weight at the time of vaginal opening was slightly heavier than the control (119.0 ± 13.3 g versus 109.6 ± 11.6 g) although not statistically significant. No significant differences between control and AS-treated groups were noted in the age at preputial separation or body weight at the time of completion in males.

3.4. Behavioral effects (F1)

Spontaneous locomotor activity at 10-min intervals and for 60 min was not significantly different between control and AS-treated groups in male and female F1 rats. In the water-filled *T*-maze test, pre-test swimming trials in the straight channel

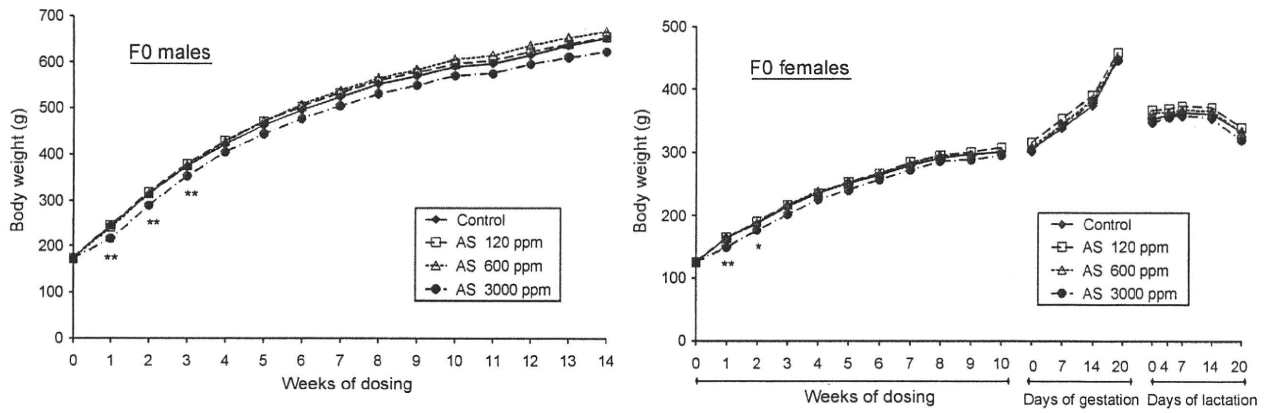

Fig. 3. Body weight of F0 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

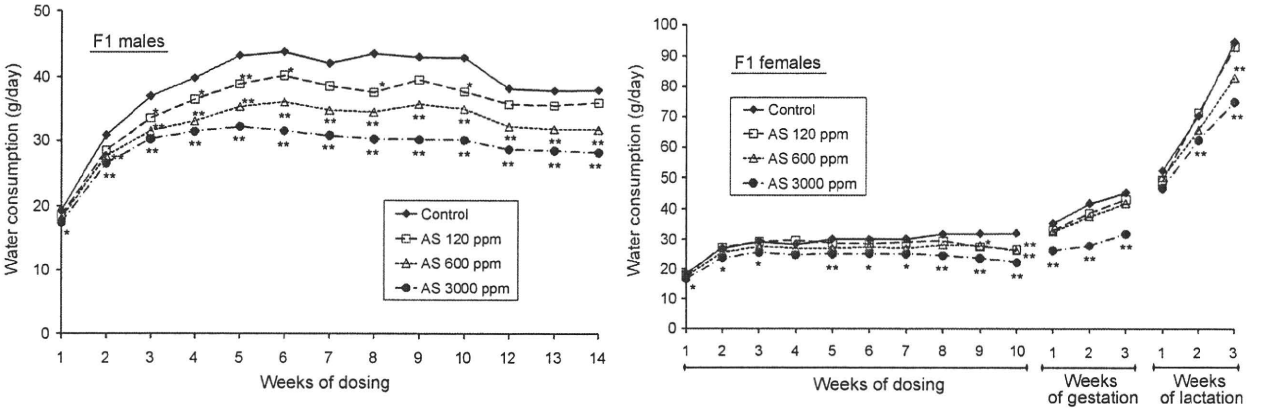

Fig. 4. Water consumption of F1 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

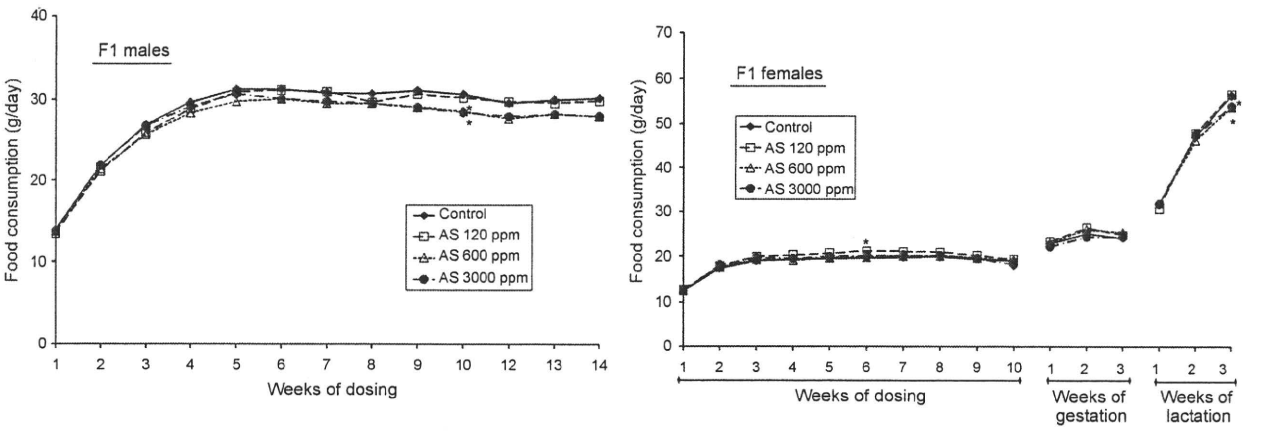

Fig. 5. Food consumption of F1 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

revealed that all male and female F1 rats in each group could swim satisfactorily, and no significant changes were observed in the elapsed time to traverse the straight channel. On days 2–4 of the *T*-maze test, no significant changes were observed in the elapsed time and number of errors in males. In females, the elapsed time and the number of errors on day 2 of the *T*-maze was significantly lowered at 600 ppm, but there were no significant differences in the elapsed time or number of errors on days 3 and 4 of the *T*-maze test between control and AS-treated groups (data not shown).

3.5. Necropsy, organ weight and histopathology of adults (F0 and F1)

In F0 males, absolute and relative liver weights were significantly decreased at 3000 ppm. Absolute spleen weight was also

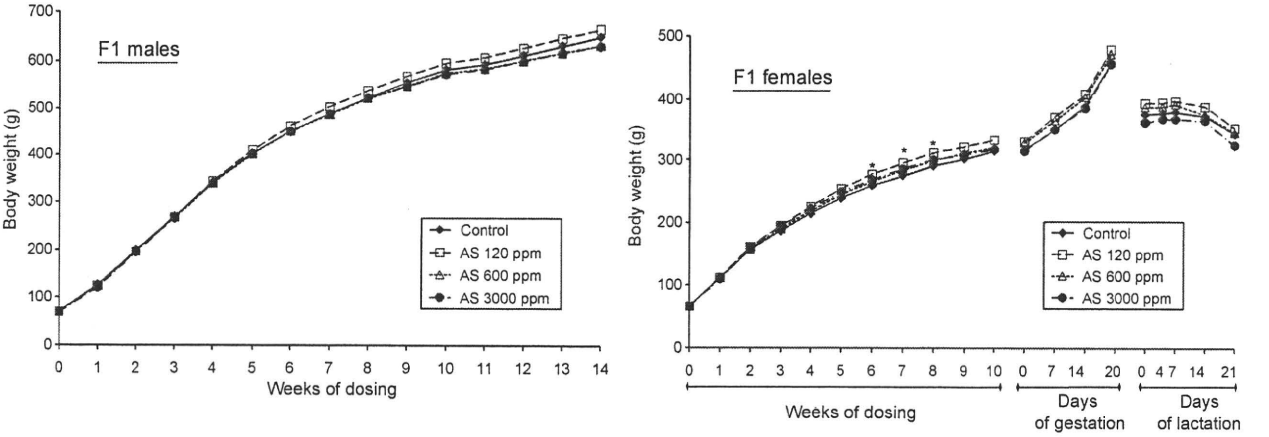

Fig. 6. Body weight of F1 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

Table 1
Reproductive performance of F0 and F1 parental animals.

AS (ppm)		0 (control)	120	600	3000
F0 generation					
No. of rats (male/female)		24/24	24/24	24/24	24/24
Copulation index (%)[a]	Males	91.7	91.7	100	91.7
	Females	95.8	100	100	100
Precoital interval (days)[b]		3.2 ± 1.1	3.2 ± 1.8	2.9 ± 1.3	2.8 ± 1.6
Fertility index (%)[c]	Males	95.5	90.9	100	95.5
	Females	95.7	91.7	100	95.8
Gestation index (%)[d]		100	95.5	95.7	95.7
Gestation length (days)[b]		22.4 ± 0.5	22.5 ± 0.6	22.1 ± 0.4	22.3 ± 0.5
Delivery index (%)[b,e]		94.3 ± 5.6	88.6 ± 21.0	90.7 ± 20.8	92.0 ± 20.5
F1 generation					
No. of rats (male/female)		24/24	23/24	24/24	24/24
Copulation index (%)[a]	Males	95.8	91.3	95.8	87.5
	Females	100	95.8	100	95.8
Precoital interval (days)[b]		3.3 ± 3.2	3.0 ± 2.0	2.7 ± 1.5	2.3 ± 1.1
Fertility index (%)[c]	Males	91.3	81.0	91.3	95.2
	Females	91.7	82.6	91.7	91.3
Gestation index (%)[d]		100	94.7	100	100
Gestation length (days)[b]		22.4 ± 0.5	22.3 ± 0.5	22.2 ± 0.4	22.2 ± 0.4
Delivery index (%)[b,e]		94.0 ± 9.9	87.5 ± 22.6	91.4 ± 10.7	94.6 ± 6.8

[a] Copulation index (%) = (no. of animals with successful copulation/no. of animals paired) × 100.
[b] Values are given as the mean ± S.D.
[c] Fertility index (%) = (no. of animals that impregnated a female or were pregnant/no. of animals with successful copulation) × 100.
[d] Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e] Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.

decreased significantly in this group, but no significant change was found in the relative weight. In F1 males, the absolute weights of the adrenals at 3000 ppm and the testes at 600 ppm were significantly decreased without significant changes in the relative weight. There were no significant changes in the absolute and relative weights of any organ in F0 and F1 female adults (data not shown).

No dose-related gross lesions were found in F0 or F1 adults. Histopathological examination of the reproductive organs revealed no compound-related alterations. There was no significant difference in the number of primordial follicles in the ovary of F1 females between control and 3000 ppm groups (data not shown).

3.6. Necropsy, organ weight and histopathology of weanlings (F1 and F2)

Absolute and relative organ weights of male and female F1 weanlings are shown in Table 3. The 3000 ppm treated males and females had a significantly lower body weight at scheduled sacrifice than the controls. In this group, absolute and relative liver weights were significantly lower than the controls. Absolute spleen weight was also decreased significantly in both sexes of the 3000 ppm group, accompanied by a significant decrease in the relative weight in males. In addition, significant decreases in the absolute weight were found for the thymus in both sexes and for the kidneys, testes and epididymides in males at 3000 ppm, and for the uterus in females at 600 and 3000 ppm. Relative brain weight was significantly increased in both sexes of the 3000 ppm group.

Table 4 presents absolute and relative organ weights of male and female F2 weanlings. The mean body weight at scheduled sacrifice was significantly lowered in both sexes of the 3000 ppm group. In males, the absolute and relative weights of the thymus and spleen were significantly decreased in the 3000 ppm group. Significant decreases were also found in the absolute weight of the liver and epididymides at 3000 ppm. The relative brain weight was significantly increased at this dose. At 120 ppm, the only significant change was a non-dose-related decrease in the relative thymus weight. In F2 females, there were significant decreases in the absolute and relative weights of the liver, and the absolute weight of the spleen, ovary and uterus, and a significant increase in the relative brain weight at 3000 ppm. In addition, a significant decrease in the absolute brain weight was observed only in the 600 ppm group.

External and internal gross observations revealed no compound-related alterations either in F1 and F2 weanlings or in pups found dead during the preweaning period. There were no dose-related histopathological changes in the liver and spleen of male and female F1 and F2 weanlings.

4. Discussion

AS administered via the drinking water to male and female rats resulted in decreased water consumption for both sexes in all treatment groups. Since the dosing solution containing AS was pH 3.57–4.20, the acidity would decrease the palatability of drinking water in AS-treated groups. Decreased water consumption was associated with decreased food consumption by F0 and F1 males and females in the 600 and 3000 ppm groups and decreased body weight in F0 male and females in the 3000 ppm group. Since water-deprived animals typically reduce their levels of feed consumption and consequently lower their body weight [46], decreased food consumption and body weight observed in the present study could be considered secondary to the decreased water consumption. In the present study, food consumption and body weight fell notably during the early dosing period in F0 males and females. Food consumption also decreased in F0 and F1 females at the end of the lactation period, when F1 or F2 pups would commence eating and drinking for themselves [37]. Campbell et al. [46] reported that animals have a certain amount of "buffering" capacity in the form of physiological mechanisms acting to reduce fluid loss. This might explain notable changes around the time when rats start drinking AS-containing water.

Continuous drinking of AS-contained water for two generations did not result in changes in copulation, fertility or gestation indices, pre-coital or gestation length, the number of implantations or pups delivered, or the incidence of pups with malformations or variations. In addition, adverse effects were not found in estrous cyclicity or sperm parameters, and the histopathology of reproductive tissues in male and female parental animals. Previous studies have demonstrated that parenterally administered aluminium affected

Table 2
Sex ratio, viability and body weight for F1 and F2 pups.

AS (ppm)	0 (control)	120	600	3000
F1 offspring				
No. of litters	22	21	22	22
No. of pups delivered[a]	13.9 ± 1.7	12.4 ± 4.7	13.1 ± 4.1	13.1 ± 3.4
Sex ratio of pups[b]	0.503	0.462	0.513	0.536
Viability index of pups (%)[a]				
On PND 0[c]	100.0 ± 0.0	99.3 ± 2.3	99.7 ± 1.6	99.5 ± 2.4
On PND 4[d]	98.7 ± 2.9	95.2 ± 21.8	98.8 ± 2.6	98.0 ± 5.4
On PND 21[e]	99.4 ± 2.7	100.0 ± 0.0	100.0 ± 0.0	99.4 ± 2.7
Male pup weight during lactation (g)[a]				
On PND 0	7.05 ± 0.61	7.25 ± 0.99	6.74 ± 0.69	6.96 ± 0.76
On PND 4	11.04 ± 0.85	11.41 ± 1.99	10.86 ± 1.37	11.00 ± 1.06
On PND 7	18.91 ± 1.29	19.36 ± 2.77	18.59 ± 1.71	18.47 ± 1.35
On PND 14	37.70 ± 2.63	37.97 ± 3.08	37.39 ± 2.59	36.34 ± 2.41
On PND 21	62.48 ± 4.50	62.63 ± 6.14	60.77 ± 4.01	57.34 ± 4.86**
Female pup weight during lactation (g)[a]				
On PND 0	6.61 ± 0.55	6.89 ± 0.83	6.35 ± 0.57	6.60 ± 0.64
On PND 4	10.46 ± 0.89	11.06 ± 1.71	10.27 ± 1.33	10.43 ± 0.83
On PND 7	18.03 ± 1.27	18.56 ± 2.31	17.69 ± 1.61	17.61 ± 1.21
On PND 14	36.29 ± 2.71	36.94 ± 3.03	35.67 ± 2.60	35.31 ± 2.24
On PND 21	60.17 ± 4.16	60.87 ± 5.68	57.68 ± 4.33	55.60 ± 4.34**
F2 offspring				
No. of litters	22	18	22	21
No. of pups delivered[a]	13.1 ± 3.6	13.2 ± 3.8	12.6 ± 3.9	14.0 ± 1.9
Sex ratio of pups[b]	0.528	0.502	0.536	0.457
Viability index of pups (%)[a]				
On PND 0[c]	99.68 ± 1.51	99.49 ± 2.14	98.42 ± 3.57	98.69 ± 3.60
On PND 4[d]	94.72 ± 14.54	98.07 ± 5.45	99.07 ± 3.15	99.01 ± 2.49
On PND 21[e]	100.00 ± 0.00	98.61 ± 4.04	100.00 ± 0.00	100.00 ± 0.00
Male pup weight during lactation (g)[a]				
On PND 0	6.97 ± 0.68	6.92 ± 0.81	6.87 ± 0.74	6.89 ± 0.60
On PND 4	10.73 ± 1.62	10.53 ± 1.27	11.27 ± 1.81	10.52 ± 1.15
On PND 7	17.96 ± 2.05	17.51 ± 2.12	18.83 ± 2.39	17.72 ± 1.60
On PND 14	35.79 ± 3.52	36.18 ± 3.63	37.32 ± 4.15	35.44 ± 2.73
On PND 21	59.61 ± 5.45	59.44 ± 5.67	60.12 ± 7.12	56.36 ± 4.47
Female pup weight during lactation (g)[a]				
On PND 0	6.66 ± 0.69	6.38 ± 0.78	6.41 ± 0.65	6.50 ± 0.49
On PND 4	10.22 ± 1.63	9.70 ± 1.23	10.36 ± 1.54	9.98 ± 0.91
On PND 7	17.03 ± 1.99	16.36 ± 2.35	17.40 ± 2.18	16.89 ± 1.23
On PND 14	34.82 ± 3.52	34.17 ± 3.58	34.96 ± 4.24	34.01 ± 2.09
On PND 21	57.33 ± 4.90	56.11 ± 5.54	56.41 ± 6.04	54.16 ± 2.82*

[a] Values are given as the mean ± S.D.
[b] Sex ratio = total no. of male pups/total no. of pups.
[c] Viability index on PND 0 (%) = (no. of live pups on PND 0/no. of pups delivered) × 100.
[d] Viability index on PND 4 (%) = (no. of live pups on PND 4/no. of live pups on PND 0) × 100.
[e] Viability index on PND 21 (%) = (no. of live pups on PND 21/no. of live pups on PND 4 after cull) × 100.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

male reproductive systems, causing decreases in testicular and epididymal sperm counts, necrosis of spermatocytes/spermatids in the testes, and reduction of fertility, etc., in rats and mice [19,20,47]. Although aluminium has extremely low oral bioavailability (less than 1%) [48,49], male reproductive toxicity was also observed in oral gavage studies [22,23,50]. In the 6-month oral gavage study in rats, changes in the number of spermatozoa and motility, and the substantial proliferation of interstitial cells in the testes were observed at doses as low as 2.5 mg Al/kg/day (as aluminium chloride) [50]. The primary reason why such effects were not detected in the present study might be a difference in the administration method because the toxicokinetic behavior of chemicals given as a bolus dose by gavage must differ significantly from those after continuous administration via drinking water. Another possible factor is aluminium content in the laboratory animal feed, for which substantial brand-to-brand and lot-to-lot variations, ranging from 60 to 8300 ppm, have been reported [51]. Since the dietary intake of aluminium was not considered in these oral gavage studies, toxic effects of aluminium could be overestimated. As for continuous exposure studies, taking into account the aluminium content in the basal diet, Hicks et al. [52] demonstrated that 28-day continuous dietary exposure to basic sodium aluminium phosphate or aluminium hydroxide did not affect the testicular histopathology up to 302 mg Al/kg/day in Sprague–Dawley rats. In the 26-week feeding study of basic sodium aluminium phosphate in beagle dogs, germinal epithelial cell degeneration and atrophy in the seminiferous tubules were observed at 75 mg Al/kg bw/day [21], but such effects on male reproductive organs were not detected up to 88 mg Al/kg/day in the similar subchronic dietary study for acidic sodium aluminium phosphate in beagle dogs [53]. These dietary studies used water-insoluble or sparingly soluble forms of aluminium [8,51]. Since it is widely assumed that insoluble aluminium compounds are less bioavailable than soluble compounds, such as AS, aluminium chloride and aluminium lactate [8], there is a possibility that the male reproductive toxicity of aluminium was underestimated in these previous dietary exposure studies. The present two-generation study provided useful information that the male reproductive system is not affected even after continuous exposure to a water-soluble aluminium compound, at least up to around 50 mg Al/kg bw/day.

In the present study, some developmental effects were observed. Male and female F1 pups and female F2 pups in the

Table 3
Absolute and relative organ weight of F1 male and female weanlings.

AS (ppm)		0 (control)	120	600	3000
Males					
No. of animals		22	20	22	22
Body weight	(g)	90.8 ± 6.9	93.4 ± 10.5	89.7 ± 6.1	79.4 ± 7.5**
Brain	(g)	1.69 ± 0.06	1.73 ± 0.08	1.72 ± 0.07	1.68 ± 0.05
	(g/100 g bw)	1.88 ± 0.13	1.87 ± 0.19	1.92 ± 0.09	2.14 ± 0.17**
Thymus	(mg)	375 ± 55	384 ± 86	357 ± 58	305 ± 51**
	(mg/100 g bw)	414 ± 56	409 ± 64	398 ± 59	383 ± 36
Liver	(g)	4.33 ± 0.43	4.40 ± 0.60	4.22 ± 0.45	3.49 ± 0.53**
	(g/100 g bw)	4.77 ± 0.30	4.71 ± 0.33	4.70 ± 0.27	4.37 ± 0.30**
Kidney[a]	(g)	1.06 ± 0.09	1.09 ± 0.14	1.03 ± 0.11	0.95 ± 0.13**
	(g/100 g bw)	1.17 ± 0.06	1.16 ± 0.07	1.15 ± 0.08	1.20 ± 0.07
Spleen	(mg)	394 ± 49	410 ± 68	388 ± 74	301 ± 43**
	(mg/100 g bw)	436 ± 63	437 ± 40	432 ± 73	379 ± 37**
Testis[a]	(mg)	596 ± 65	583 ± 67	569 ± 65	539 ± 51*
	(mg/100 g bw)	657 ± 64	626 ± 49	635 ± 64	682 ± 58
Epididymis[a]	(mg)	81.8 ± 8.6	76.8 ± 10.9	76.5 ± 8.4	72.0 ± 9.9**
	(mg/100 g bw)	90.4 ± 10.3	82.0 ± 6.1	85.4 ± 8.4	91.5 ± 14.6
Females					
No. of animals		22	20	22	21
Body weight	(g)	84.3 ± 6.3	85.9 ± 9.2	80.5 ± 7.0	75.8 ± 6.4**
Brain	(g)	1.64 ± 0.06	1.66 ± 0.06	1.63 ± 0.05	1.63 ± 0.07
	(g/100 g bw)	1.96 ± 0.12	1.95 ± 0.18	2.04 ± 0.17	2.16 ± 0.14**
Thymus	(mg)	383 ± 66	373 ± 74	345 ± 46	313 ± 33**
	(mg/100 g bw)	453 ± 63	433 ± 64	429 ± 57	415 ± 41
Liver	(g)	3.83 ± 0.47	3.92 ± 0.48	3.61 ± 0.35	3.24 ± 0.34**
	(g/100 g bw)	4.53 ± 0.30	4.57 ± 0.31	4.48 ± 0.30	4.27 ± 0.25*
Kidney[a]	(g)	0.99 ± 0.11	0.99 ± 0.09	0.93 ± 0.10	0.93 ± 0.10
	(g/100 g bw)	1.17 ± 0.08	1.15 ± 0.07	1.15 ± 0.09	1.23 ± 0.09
Spleen	(mg)	337 ± 62	356 ± 55	341 ± 64	292 ± 43*
	(mg/100 g bw)	400 ± 67	415 ± 44	422 ± 53	386 ± 47
Ovary[a]	(mg)	25.3 ± 4.8	25.3 ± 3.8	22.5 ± 4.6	24.7 ± 3.2
	(mg/100 g bw)	30.1 ± 5.1	29.7 ± 5.0	27.9 ± 5.0	32.5 ± 4.2
Uterus	(mg)	70.6 ± 16.6	74.2 ± 32.0	59.2 ± 11.9*	55.4 ± 13.4**
	(mg/100 g bw)	83.8 ± 19.2	85.5 ± 32.4	73.3 ± 11.9	73.3 ± 18.0

Values are given as the mean ± S.D.
[a] Values represent the total weights of the organs on both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

3000 ppm group had a lower body weight on PND 21 while no difference was found in the birth weight. Such inhibition of the preweaning body weight gain may be simply attributable to the decreased palatability of the drinking water, which would decrease the water intake of pups themselves or might decrease the amount of maternal breast milk; however, similar developmental effects of aluminium were reported in the previous three dietary exposure studies, in which aluminium lactate was mixed in the diet and fed to mice from day 0 of gestation throughout the lactation period [28,30,32], and the lowest effect level was 500 ppm, which was estimated to be equal to 94–273 mg Al/kg bw/day based on the body weight and food consumption during the lactation period [28]. In these dietary exposure studies, food consumption was decreased, but Golub et al. [28] indicated that the mean body weight of pups on PNDs 15 and 20 in the 1000 ppm group was lower than that of the pair-fed control. In contrast, the effects on body weight of preweaning mice were not found in the other two dietary exposure studies [29,31], which were conducted using a similar study design by the same research group. Donald et al. [29] discussed differences in the constituents of the diet as a possible cause of the inconsistent results, but this speculation appears to be contradicted by a subsequent study [30]. Although it is still unclear why different results were obtained in these dietary exposure studies, these results suggest that the fall in body weight around weaning in the present study might not be explained only by decreased water intake. Aluminium ingested by pups themselves and/or taken via maternal milk may affect preweaning growth, or impairment of maternal nursing behavior or the lactation status could be considered possible factors.

In F1 and F2 weanlings, various organ weight changes were found in the 3000 ppm group. Among them, an increase in the relative brain weight is considered to be a secondary change that occurs with the fall in body weight because the absolute weight did not change. Similarly, decreased absolute weights of the kidneys, testes, epididymides, uterus, etc., without changes in the relative weight, were thought to be associated with decreased body weight. On the other hand, the effects on the liver and spleen, the absolute and relative weights of which were decreased in both generations, could not be explained only by the fall of body weight. Since similar effects were observed in the above-mentioned dietary exposure study of aluminium lactate [28], the effects on the liver and spleen weight might be a direct effect of aluminium ingested by pups themselves and/or taken via maternal milk; however, in the present study, no histopathological changes were detected in the liver and spleen. Furthermore, the changes in the liver or spleen weight were not detected in adults, except for F0 males in the 3000 ppm group; therefore, organ weight changes observed in F1 and F2 weanlings in the 3000 ppm group were not deemed to be adverse effects. Organ weight changes in the 120 and 600 ppm groups were not considered to have toxicological significance because these changes were not dose-dependent or were inconsistent across generations.

As for effects on the developmental landmarks, vaginal opening was slightly delayed in F1 females in the 3000 ppm group while no compound-related changes were found in the other developmental landmarks, including male preputial separation. Vaginal opening generally occurs around the time of first ovulation in response to an increase in serum estradiol levels as females enter puberty [54], and therefore, it is widely used as a marker of female puberty.

Table 4
Absolute and relative organ weight of F2 male and female weanlings.

AS (ppm)		0 (control)	120	600	3000
Males					
No. of animals		21	18	22	21
Body weight	(g)	87.7 ± 5.8	89.0 ± 8.7	87.0 ± 9.6	79.2 ± 6.8**
Brain	(g)	1.66 ± 0.05	1.69 ± 0.06	1.70 ± 0.06	1.67 ± 0.06
	(g/100 g bw)	1.90 ± 0.13	1.91 ± 0.17	1.97 ± 0.16	2.13 ± 0.17**
Thymus	(mg)	382 ± 50	348 ± 49	357 ± 66	305 ± 36**
	(mg/100 g bw)	439 ± 70	392 ± 52*	411 ± 57	386 ± 40**
Liver	(g)	3.93 ± 0.37	4.04 ± 0.64	3.91 ± 0.39	3.45 ± 0.41**
	(g/100 g bw)	4.49 ± 0.34	4.52 ± 0.44	4.50 ± 0.24	4.36 ± 0.23
Kidney[a]	(g)	1.02 ± 0.09	1.01 ± 0.13	0.99 ± 0.13	0.94 ± 0.10
	(g/100 g bw)	1.16 ± 0.08	1.14 ± 0.06	1.14 ± 0.07	1.19 ± 0.06
Spleen	(mg)	368 ± 54	381 ± 62	361 ± 49	296 ± 48**
	(mg/100 g bw)	421 ± 64	427 ± 50	416 ± 48	372 ± 42**
Testis[a]	(mg)	559 ± 67	549 ± 98	543 ± 77	534 ± 54
	(mg/100 g bw)	637 ± 60	615 ± 81	624 ± 47	680 ± 92
Epididymis[a]	(mg)	75.3 ± 6.9	78.3 ± 8.8	75.1 ± 10.7	70.5 ± 5.7*
	(mg/100 g bw)	86.1 ± 8.3	88.4 ± 9.0	86.5 ± 9.0	89.4 ± 8.2
Females					
No. of animals		22	18	21	21
Body weight	(g)	80.8 ± 6.0	80.0 ± 7.2	80.8 ± 9.1	73.8 ± 4.4**
Brain	(g)	1.60 ± 0.06	1.61 ± 0.05	1.64 ± 0.05*	1.61 ± 0.04
	(g/100 g bw)	1.99 ± 0.14	2.03 ± 0.16	2.05 ± 0.20	2.19 ± 0.15**
Thymus	(mg)	337 ± 45	364 ± 36	347 ± 49	312 ± 37
	(mg/100 g bw)	419 ± 61	457 ± 50	431 ± 47	424 ± 54
Liver	(g)	3.56 ± 0.35	3.61 ± 0.39	3.61 ± 0.48	3.07 ± 0.26**
	(g/100 g bw)	4.41 ± 0.21	4.51 ± 0.26	4.47 ± 0.26	4.17 ± 0.29**
Kidney[a]	(g)	0.95 ± 0.07	0.93 ± 0.10	0.92 ± 0.10	0.88 ± 0.08
	(g/100 g bw)	1.18 ± 0.08	1.16 ± 0.09	1.14 ± 0.06	1.20 ± 0.07
Spleen	(mg)	320.9 ± 46.7	331.8 ± 59.3	331.3 ± 57.1	269.9 ± 55.2**
	(mg/100 g bw)	398.4 ± 59.0	414.8 ± 64.3	409.0 ± 42.2	365.0 ± 67.4
Ovary[a]	(mg)	23.9 ± 3.7	22.8 ± 3.6	23.2 ± 3.5	20.2 ± 2.3**
	(mg/100 g bw)	29.7 ± 4.9	28.8 ± 5.6	29.0 ± 4.7	27.5 ± 3.5
Uterus	(mg)	60.5 ± 17.0	63.8 ± 18.4	65.0 ± 41.7	49.3 ± 11.6*
	(mg/100 g bw)	74.6 ± 19.2	79.3 ± 19.3	78.7 ± 40.4	67.0 ± 16.2

Values are given as the mean ± S.D.
[a] Values represent the total weights of the organs on both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

On the other hand, vaginal opening is closely related to body weight, and growth retardation can delay the day of acquisition [55]. In the present study, body weight at the time of vaginal opening was slightly heavier in F1 females of the highest dose group, compared with the control. However, other hormone-dependent events, including estrous cyclicity and AGD, were not changed in AS-treated groups. It seems unlikely that aluminium have a clear impact on the hormonal event. Further studies are required to draw a definitive conclusion on the effects of aluminium on the sexual maturation.

Aluminium has been reported to affect the developing nervous system [56,57]. In continuous exposure studies using rodents, the neurobehavioral effects were reported as follows: dietary exposure of pregnant rats to aluminium chloride or lactate at more than 96 mg Al/kg bw/day impaired the righting reflex, gasping reflex, negative geotaxis and/or locomotor coordination of preweaning pups [25,26]. Impaired performance of operant conditioning tasks, accompanied by a decrease in locomotor activity, was observed on PND 65 in the offspring of rats receiving a diet containing aluminium lactate at 400 mg Al/kg bw/day during gestation [58], suggesting that the effects continue after maturation. Unfortunately, aluminium concentration in the basal diet was not reported in these feeding studies using rats. Similar neurobehavioral effects were found in the preweaning, juvenile and adult periods of the offspring after dietary exposure of mice to aluminium lactate throughout the gestation and lactation periods [28–32]. These mouse studies took the basal diet content of the aluminium diet into consideration, and the lowest observed effect level was 500 ppm, corresponding to 100 mg Al/kg bw/day at the beginning of pregnancy and 210 mg Al/kg/day near the end of lactation [29]. In the present study, no changes were found in the reflex ontogeny of F1 and F2 pups and in spontaneous locomotor activity tested at 4 weeks of age for F1 animals. As for the performance in a water-filled multiple *T*-maze, a transient decrease in the elapsed time and the number of errors were found in F1 females in the 600 ppm group, but this change was not considered to be treatment-related because of the lack of dose-dependency. These findings indicate that previous findings of developmental neurotoxic effects were possibly related to the toxic effects of aluminium given at higher doses than those given in this study.

In summary, AS administered via drinking water at 120, 600 or 3000 ppm resulted in decreased water consumption. This change was associated with decreased food consumption in the 600 and 3000 ppm groups and decreased body weight in the 3000 ppm group. In the 3000 ppm group, male and female pups had a lower body weight on PND 21. At this dose, vaginal opening was slightly delayed. No definitive effects were found in the other reproductive/developmental parameters, including developmental neurobehavioral toxicity. Although there is a possibility that observed developmental effects are results of decreased water consumption, more definitive conclusions could not be reached since paired-comparison data are not available to assess the effects of decreased water intake in the absence of AS exposure. Based on these considerations, a conservative evaluation of the data led to the conclusion that the no observed adverse effect level of AS in this two-generation study is 600 ppm (41.0 mg/kg bw/day) for parental systemic toxicity and reproductive/developmental toxicity. The total ingested dose of aluminium from food and drinking

water combined in this 600 ppm group was calculated to be 8.06 mg Al/kg bw/day.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Original Article

Effects of perfluorooctanoic acid (PFOA) exposure to pregnant mice on reproduction

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ABSTRACT — Perfluorooctanoic acid (PFOA) has similar characteristics to perfluorooctane sulfonate (PFOS) in reproduction toxicity featured by neonatal death. We found that PFOS exposure to mice during pregnancy led to intracranial blood vessel dilatation of fetuses accompanied by severe lung collapse which caused neonatal mortality. Thus, we adopted the corresponding experimental design to PFOS in order to characterize the neonatal death by PFOA. Pregnant ICR mice were given 1, 5 and 10 mg/kg PFOA daily by gavage from gestational day (GD) 0 to 17 and 18 for prenatal and postnatal evaluations, respectively. Five to nine dams per group were sacrificed on GD 18 for prenatal evaluation; other 10 dams were left to give birth. No maternal death was observed. The liver weight increased dose-dependently, with hepatocellular hypertrophy, necrosis, increased mitosis and mild calcification at 10 mg/kg. PFOA at 10 mg/kg increased serum enzyme activities (GGT, ALT, AST and ALP) with hypoproteinemia and hypolipidemia. PFOA treatment reduced the fetal body weight at 5 and 10 mg/kg. Teratological evaluation showed delayed ossification of the sternum and phalanges and delayed eruption of incisors at 10 mg/kg, but did not show intracranial blood vessel dilatation. Postnatal evaluation revealed that PFOA reduced the neonatal survival rate at 5 and 10 mg/kg. At 5 mg/kg pups were born alive and active and 16% died within 4 days observation, while all died within 6 hr after birth at 10 mg/kg without showing intracranial blood vessel dilatation. The cause of neonatal death by PFOA may be different from PFOS.

Key words: PFOA, Mice, Neonatal death, Biochemical

INTRODUCTION

Perfluorooctanoic acid (PFOA), a synthetic perfluorinated eight-carbon organic chemical has broad industrial applications. As a result of the prevalence and persistence of PFOA in organisms, the potential adverse effects of this compound on diverse species, especially mammals, have been extensively examined. PFOA is well absorbed following oral exposure. Once absorbed in the body, it distributes predominantly to the liver and plasma and to a lesser extent to the kidney and lungs.

Numerous repeated-dose studies have been conducted with a variety of species (mice, rats, and monkeys), and these have recently been reviewed (Kennedy *et al.*, 2004; Lau *et al.*, 2007). They demonstrate that liver is the primary target organ for PFOA toxicity. The hepatotoxicity manifests as increased liver weights, hepatocellular hypertrophy, liver degeneration and necrosis, increases in plasma transaminases, and proliferation of smooth endoplasmic reticulum and peroxisomes in rodents. Hypolipidemia has also been reported in some rodent studies. The relation between maternal toxicity and specific developmental toxicity was found to be species-specific. The developmental effects were agent-specific, ranging from complete resorption to lack of effect (Rogers and Kavlock, 2008). It is well known that significant changes are observed in biochemical parameters in experimental animals during pregnancy (Wells *et al.*, 1999; Honda *et*

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al., 2008; Mizoguchi *et al.*, 2010; Urasako *et al.*, 2009). However, the biochemical parameters of liver injury caused by PFOA during pregnancy have not been reported yet.

The developmental toxicity induced by exposure to PFOA during gestation includes dose-related full litter resorptions, reduced fetal weight, reduced postnatal survival, delays in postnatal growth and development in offspring (Butenhoff *et al.*, 2004; Lau *et al.*, 2004, 2006). A cross-foster study indicates that the postnatal effects on survival, eye opening and weight gain are a consequence of gestational exposure and that exposure via lactation is not a major factor (Wolf *et al.*, 2007). However, the exact cause of neonatal death has not been explained yet.

Perfuorooctane sulfonate (PFOS), a similar compound to PFOA, also induces developmental and reproductive effects, such as reduced birth weight, decreased gestational length, structural defects, developmental delays, and increased neonatal mortality (Grasty *et al.*, 2003; Lau *et al.*, 2003; Luebker *et al.*, 2005a, 2005b; Thibodeaux *et al.*, 2003). In our previous study on neonatal death in mice exposed to PFOS, we found that PFOS exposure during pregnancy lead to intracranial blood vessel dilatation accompanied by severe lung collapse which caused neonatal mortality (Yahia *et al.*, 2008). However, the neonatal death caused by PFOA has not been examined in relation to the intracranial blood vessel dilatation.

The aim of this study is to examine the biochemical, hepatic and developmental effects of PFOA in mice exposed during pregnancy and the cause of neonatal death.

MATERIALS AND METHODS

Animals

Adult ICR male and female mice were purchased from CLEA Japan, Inc., Tokyo, Japan at seven weeks of age and used for the experiment after one week of acclimatization. Animals were housed in aluminum cages with cedar shavings for bedding and provided pellet chow (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water *ad libitum*. Animal facilities were controlled for temperature (20-24°C) and operated under a 12 hr light-dark cycle. Animal experiments were conducted according to the Guidelines for Animal Experiments in Iwate University, Morioka, Japan.

Mating

Female mice were checked for estrous cycle stage and each proestrus female was placed with an individually housed male overnight, and those animals with spermatozoa in a vaginal smear and/or with a copulatory plug were considered to be at gestational day (GD) 0. Pregnant dams were housed individually.

Animal treatment and sampling

PFOA (90% pure) was purchased from Fluka Chemie GmbH. PFOA solutions were prepared at 0.1, 0.5 and 1 mg/ml by deionized water and administered to the pregnant mice at 1, 5 and 10 mg/kg by gavage at a volume of 10 ml/kg once daily from GD 0 to the end of the study (GD 17 for prenatal study, GD 18 for postnatal study). Controls received an equivalent volume of water.

Maternal body weights as well as food and water consumption were monitored daily throughout gestation. Five to nine dams per group were euthanized on GD 18 (24 hr after the last treatment) under diethyl ether anesthesia for serum biochemical analysis, histopathological examination of maternal liver, kidneys, lungs and brain, and teratological evaluation. The other 10 dams per group were left to give birth.

Serum analysis

Serum samples were used for determination of blood serum levels of lactate dehydrogenase (LDH), gamma glutamyl transfers (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, blood urea nitrogen (BUN), total bilirubin, total protein, albumin, globulins, calcium, inorganic phosphorus, glucose, triglycerides, phospholipids, total cholesterol, free fatty acids and hydroxy butyric acid by using an auto analyzer (Accute TBA-40FR, Toshiba Medical Systems Corporation, Tokyo, Japan).

Histopathological examination of maternal organs

Maternal liver, kidney, lung and brain were kept in 10% buffered formalin at room temperature, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin.

Prenatal evaluation

The gravid uterus was removed and examined, and the numbers of the live or dead fetuses as well as resorptions were recorded. Live fetuses were weighed individually and examined for external abnormalities. All fetuses removed from the deeply anesthetized dam were immediately euthanized by 70% ethanol, eviscerated, fixed in 95% ethanol and subsequently stained with alizarin red and alcian blue to visualize bone and cartilage, respectively. Skeletal morphology was evaluated as described by Narotsky and Rogers (2000).

Postnatal evaluation

We examined neonatal lungs, because Grasty *et al.* (2003) suggested that the cause of neonatal death in rats exposed to PFOS was lung immaturity. Neonatal head was examined because we found intracranial blood vessel dilatation in mice exposed to PFOS in our previous study related to neonatal death in mice exposed to PFOS (Yahia *et al.*, 2008). Five dams (out of 10) per group were treated from GD 0 to GD 18 and left to give birth, after delivery the neonates were observed during day time for 4 days to determine the neonatal mortality. The other 5 dams per group were treated from GD 0 to GD 18 and left to give birth, all neonates were collected and kept in Bouin's solution and examined pathologically. Two samples per litter were randomly selected and were routinely embedded in paraffin wax, sectioned at 4 μm in thickness and stained with hematoxylin and eosin.

Statistical analysis

The differences in any parameter of the fetuses, pups or dams between the average of treated dams and the concurrent control dams were compared with Dunnett test after one way ANOVA. A p-value less than 0.05 was considered statistically significant.

RESULTS

Maternal effects

Maternal exposure to PFOA during pregnancy did not cause any apparent toxic signs or mortalities in all the exposed groups. A significant reduction in weight gain was observed at 10 mg/kg group from GD 12 till the end of gestation. There was significant decrease in food intake in that group in early stage of gestation but not in late of gestation. At 5 mg/kg there was a significant increase in feed consumption and water intake late in gestation (data not shown).

Treatment of pregnant mice with PFOA increased the maternal absolute and relative liver weight in a dose dependent manner and reduced the absolute brain weight at the 10 mg/kg group. PFOA increased the relative kidney weight in all treated groups (Table 1).

Pathological examination of maternal organs showed hepatocellular hypertrophy and increased mitosis in all the treated groups and single cell necrosis and mild calcification at 10 mg/kg group (Fig.1). Hypertrophied hepatocytes were observed in central lobular area at low and middle doses, and diffusely spread throughout lobules at high dose. The kidney showed slight hypertrophy in the outer medulla and proximal tubular cells in all the treated mice. There were no changes in maternal lung and brain in all the exposed mice.

At 10 mg/kg group, PFOA caused changes in serum biochemical parameters such as increased GGT, AST, ALP and albumin globulin (A/G) ratio, but reduced the total protein, albumin, globulins, triglycerides, phospholipids, total cholesterol and free fatty acids (Table 2).

Prenatal study

PFOA treatment reduced the fetal body weight at 5 and 10 mg/kg groups. There were no significant effects on the prenatal survival, in addition to absence of gross abnor-

Table 1. Whole body weight and organ weight of dams on GD18

		Control (n = 7)	1 mg/kg (n = 9)	5 mg/kg (n = 5)	10 mg/kg (n = 8)
Whole body	g	68.3 ± 1.7	63.7 ± 1.3	60.2 ± 1.7*	56.7 ± 2.7**
Liver	g	2.77 ± 0.05	3.58 ± 0.28	5.17 ± 0.23**	6.39 ± 0.24**
	(%)	(4.0 ± 0.1)	(5.4 ± 0.3)*	(8.6 ± 0.3)*	(11.4 ± 0.6)**
Kidney	g	0.43 ± 0.05	0.47 ± 0.02	0.43 ± 0.02	0.45 ± 0.17
	(%)	(0.62 ± 0.01)	(0.72 ± 0.02)**	(0.71 ± 0.01)*	(0.79 ± 0.01)**
Lung	g	0.21 ± 0.01	0.21 ± 0.01	0.19 ± 0.01	0.19 ± 0.01
	(%)	(0.30 ± 0.01)	(0.31 ± 0.01)	(0.31 ± 0.01)	(0.34 ± 0.01)
Brain	g	0.48 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.45 ± 0.01**
	(%)	(0.69 ± 0.02)	(0.73 ± 0.02)	(0.78 ± 0.02)	(0.80 ± 0.04)

Data represent the mean ± S.E. of examined dams. *: $p < 0.05$. **: $p < 0.01$.
%: relative organ weight to the body weight.

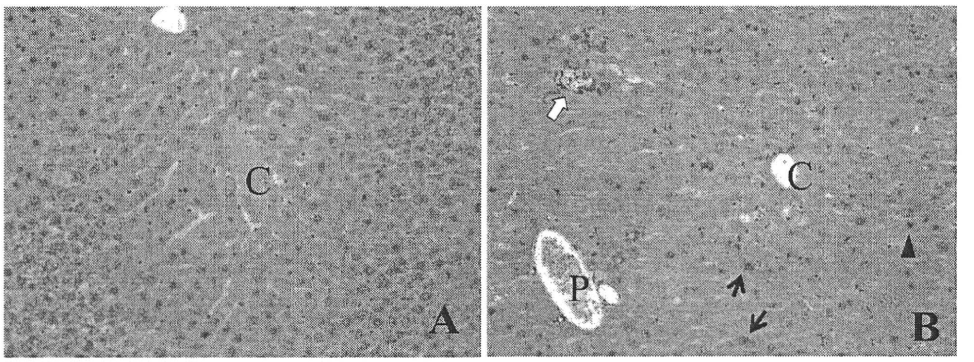

Fig. 1. A: Liver at 1 mg/kg group showing hypertrophied hepatocytes in centrilobular area. B: Liver at 10 mg/kg group showing diffuse hepatocellualr hypertrophy with single cell necrosis (black arrows) or mitosis of hepatocytes (arrowhead) or calification (white arrow). C: central vein. P: portal vein.

Table 2. Effects of PFOA on serum biochemical parameters in dams on GD18

	Control (n = 7)	1 mg/kg (n = 5)	5 mg/kg (n = 5)	10 mg/kg (n = 8)
AST (U/l)	104 ± 10	105 ± 10	428 ± 80	950 ± 190**
ALT (U/l)	24.6 ± 8.4	15.8 ± 6.2	61.6 ± 13.8	116.2 ± 31.9**
GGT (U/l)	0	0	0	2.5 ± 1.1*
LDH (U/l)	1802 ± 263	1476 ± 276	1308 ± 205	2811 ± 618
ALP (U/l)	195 ± 24	217 ± 20	257 ± 32	773 ± 158**
Creatinine (mg/dl)	0.15 ± 0.01	0.21 ± 0.04	0.14 ± 0.05	0.15 ± 0.02
BUN (mg/dl)	22.6 ± 1.4	27.8 ± 1.3*	25.4 ± 1.7	20.5 ± 1.0
Total bilirubin (mg/dl)	0	0	0	0.08 ± 0.05
Total protein (mg/dl)	3.95 ± 0.15	3.94 ± 0.17	3.35 ± 0.16*	3.09 ± 0.09**
Albumin (g/dl)	2.36 ± 0.18	2.45 ± 0.13	2.11 ± 0.09	2.00 ± 0.07*
Globulin (g/dl)	1.58 ± 0.02	1.49 ± 0.04	1.24 ± 0.06**	1.08 ± 0.04**
A/G ratio	1.49 ± 0.05	1.60 ± 0.05	1.70 ± 0.04	1.86 ± 0.07**
Phosphorus (mg/dl)	6.05 ± 0.91	9.26 ± 0.62*	8.80 ± 0.86*	7.96 ± 0.51
Calcium (mg/dl)	8.96 ± 0.67	9.34 ± 0.51	9.26 ± 0.44	8.05 ± 0.40
Glucose (mg/dl)	112 ± 19	130 ± 8	105 ± 9	70 ± 10
Triglyceride (mg/dl)	175 ± 29	132 ± 31	92 ± 21	32 ± 8**
Phospholipid (mg/dl)	76.1 ± 4.6	84.5 ± 10.5	68.2 ± 7.6	50.8 ± 4.7*
Total cholesterol (mg/dl)	50.0 ± 2.6	54.4 ± 7.8	45.8 ± 5.6	34.2 ± 3.7*
Free fatty acid (mmol/l)	1266 ± 143	1237 ± 238	832 ± 156	704 ± 99*
Hydroxybutyric acid (μmol/l)	191 ± 27	416 ± 155	287 ± 64	178 ± 26

Data represent the mean ± S.E. of examined dams. * $p < 0.05$. **: $p < 0.01$.

Table 3. Effects of PFOA on fetuses on GD 18

	Control	1 mg/kg	5 mg/kg	10 mg/kg
Number of fetuses/dams	81/7	88/9	68/5	70/8
Live fetuses (%)	99.0 ± 1.0	99.3 ± 0.7	97.1 ± 1.8	94.8 ± 2.7
Live fetus weight (g)	1.47 ± 0.01	1.53 ± 0.01**	1.33 ± 0.01**	1.06 ± 0.01**
Cleft palate (%)	0	0	0	5.0 ± 3.3
Cleft sternum (%)	1.3 ± 1.3	0	8.7 ± 6.9	55.2 ± 18.2*
Delayed ossification of phalanges (%)	2.5 ± 2.5	1.1 ± 1.1	16.0 ± 11.2	47.7 ± 17.2*
Delayed eruption of incisors (%)	2.5 ± 2.5	0	15.9 ± 12.4	44.8 ± 19.9*
Extra ribs (%)	6.8 ± 3.7	1.4 ± 1.4	21.0 ± 6.7	15.7 ± 7.7
Abnormal tail (%)	11.3 ± 7.0	2.3 ± 1.5	4.4 ± 1.8	9.6 ± 8.1

Data represent the mean ± S.E. of examined dams. *: $p < 0.05$. **: $p < 0.01$.

Table 4. Survival rate and body weight of neonates

	Control	1 mg/kg	5 mg/kg	10 mg/kg
Number of pups/dams	76/5	70/5	53/5	51/5
Body weight (g)	1.63 ± 0.01	1.66 ± 0.01	1.50 ± 0.02**	1.16 ± 0.02**
Survival rate on PND4 (%)	98.0 ± 2.0	100	84.4 ± 5.5**	0**

Data represent the mean ± S.E. of examined dams. *: $p < 0.05$. **: $p < 0.01$.

malities in all the examined fetuses, while there were few skeletal anomalies as delayed ossification of the sternum and phalanges accompanied by delayed eruption of incisors at 10 mg/kg group. The results of the prenatal study were summarized in Table 3.

Postnatal study

PFOA at 10 mg/kg caused delayed delivery as reported elsewhere (Lau *et al.*, 2006), all the observed 5 dams delivered in early morning or during the day time, which made us possible to observe the pup's behavior. Surprisingly, about 58% of pups were still born, though about 95% of fetuses had been alive when examined on GD 18 at 10 mg/kg group. The remaining live pups at 10 mg/kg group were active at parturition but all died within 6 hr after birth. At 5 mg/kg group 16% of neonates died during the 4 days observation. Significant reductions in neonatal body weight were detectable in the 5 and 10 mg/kg groups (Table 4). After delivery, no abnormal nursing behavior was observed in the dams.

Pathological examination revealed that there were no changes between the control and exposed mice either in the lung or brain except that some neonates at 10 mg/kg group showed whole body edema.

DISCUSSION

Results of the current study for maternal effects such as reduced weight gain and feed intake, and remarkable increase in liver weight are similar to but stronger than those of previous studies in mice (Lau *et al.*, 2006; Wolf *et al.*, 2007). Since PFOA is an agonist of peroxisome proliferator-activated receptor (PPAR)-alpha, the PFOA-induced liver enlargement is attributed to the effect on PPAR-alpha (Chevalier *et al.*, 2000; Wolf *et al.*, 2008).

The significant increases in serum AST, GGT and ALP, which reflect mainly the status of the liver (Bogin *et al.*, 1986), confirmed the gross and histopathological findings recorded in the present study. The increase in hepatic enzymes was consistent with those observed in male mice (Son *et al.*, 2008). The hypolipidemia that was observed in this study (reduction in serum levels of triglycerides,

cholesterol, phospholipids and free fatty acids) is similar to the previous studies, where feeding a diet containing 0.02% PFOA for 7 days to male mice caused severe adipose tissue atrophy and hypolipidemia (Xie *et al.*, 2003). Thus, the pregnant state did not remarkably affect the effect of PFOA on the biochemical parameters. The effects of PPAR-alpha agonist include increased beta-oxidation of fatty acids, increase in several cytochrome P450-mediated reactions, and inhibition of the secretion of very low density lipoproteins and cholesterol from the liver (Kennedy *et al.*, 2004; Peraza *et al.*, 2006). Thus, in addition to liver hypertrophy, the changes in biochemical parameters are also attributed to the effect of PFOA on PPAR-alpha. Hypoproteinemia observed at 10 mg/kg might reflect the maternal body weight loss.

Maternal exposure to PFOA reduced the neonatal body weight and survival rate at 5 and 10 mg/kg groups. At 10 mg/kg PFOA group, about 95% of fetuses had been alive when examined on GD 18, but about 58% of neonates were still born, and the others died within 6 hr after birth. These results are reminiscent of the findings reported by Wolf *et al.* (2007), where exposure to a high dose of PFOA (20 mg/kg) from GD 15 to 17 was sufficient to produce neonatal mortality and birth weight reduction.

Few skeletal anomalies as delayed ossification of the sternum and phalanges accompanied by delayed eruption of incisors were observed in fetuses exposed to PFOA at 10 mg/kg. These changes are similar to those reported by Lau *et al.* (2006). However, pathological examination of neonatal lungs and brain revealed that PFOA did not cause any changes from the control, and we couldn't detect the cause of neonatal death.

Although the mechanism is not clear, Abbott *et al.* (2007) showed PFOA induced developmental toxicity was dependent on expression of PPAR-alpha using PPAR-alpha knockout mice. Because PFOA crosses the placenta (Apelberg *et al.*, 2007), effects on the fetal PPAR-alpha might be involved in the fetal or neonatal death of mice.

We (Yahia *et al.*, 2008) found that PFOS exposure to mice during pregnancy led to intracranial blood vessel dilatation of fetuses accompanied by severe lung collapse, which caused sudden neonatal mortality without causing still born. In spite of the fact that PFOS and PFOA are similar in causing neonatal death, the mechanism of the neonatal death may be different.

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Letter

Air purifiers that diffuse reactive oxygen species potentially cause DNA damage in the lung

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ABSTRACT — Several appliance manufacturers have recently released new type air purifiers that can disinfect bacteria, fungi and viruses by diffusing reactive oxygen species (ROS) into the air. In this study, mice were exposed to the outlet air from each of 3 air purifiers from different manufacturers (A, B, C), and the lung was examined for DNA damage, lipid peroxidation and histopathology to confirm the safety of these air purifiers. Neither abnormal behavior during exposure nor gross abnormality at necropsy was observed. No histopathological changes were also observed in the lung. However, significant increase of DNA damage was detected by the comet assay in the lung immediately after the direct exposure for 48 hr to models A and B, and for 16 hr to model B. As for model B, DNA migration was also increased by 2 hr exposure in a 1 m^3 plastic chamber but not by 48 hr exposure in a room (12.6 m^3). Model C did not cause DNA damage. Lipid peroxidation and 8-hydroxy deoxyguanosine (8-OH-dG) was not increased under the conditions DNA damage was detected by the comet assay. The present results revealed that some models of air purifiers that diffuse ROS potentially cause DNA damage in the lung although the mechanism was left unsolved.

Key words: DNA damage, Air purifier, Reactive oxygen species, Air ion, Lung

INTRODUCTION

Air purifiers are commonly used in a house, office, hospital, and so on. Conventional air purifiers only remove particulates such as house dust, pollens, and cigarette smoke by a filter, whereas new type ones have a function to disinfect bacteria, fungi and viruses. For these purposes, some models are equipped with an antibacterial filter or photocatalytic device, and some models diffuse air-ions into the room.

There are several studies on the bactericidal or virucidal effects of air-ions (Mitchell and King, 1994; Fan *et al.*, 2002; Tyagi *et al.*, 2008), and the underlying mechanism is suggested to be degeneration of surface proteins of organisms (Digel *et al.*, 2005). According to the manufacturer's information, active substance of the air purifiers is superoxide or hydroxyl radical, which is a member of reactive oxygen species (ROS). ROS are potentially toxic to living matters. There are few published studies on the safety of these air purifiers although several manufacturers have officially announced on the website that various toxicity tests including genotoxicity test have been conducted by contract research organizations.

In this study, therefore, mice were exposed to the outlet air from these air purifiers and the lung was examined for DNA damage, lipid peroxidation and histopathology to confirm their safety.

MATERIALS AND METHODS

Animals and apparatus

Seven weeks old ICR male mice were purchased from CLEA Japan (Tokyo, Japan), and randomly divided into groups of 5 mice each. They were fed commercial feed (MF, Oriental Yeast, Tokyo, Japan) and tap water throughout the acclimation (1 week) and experimental periods freely. The animal room was kept at 22-24°C with 12 hr light/dark cycle. Animal experiments were conducted according to the guidelines for animal experiments of Iwate University.

Three models of household air purifiers from different manufacturers, A, B and C were tested. According to the manufacturer's information, model A diffuses negative (O_2^-) and positive (H^+) cluster ions, model B diffuses nanoparticles of water including hydroxyl radical, and model C diffuses electrolytic water mist that includes hydroxyl rad-

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ical and hypochlorous acid. Chemicals used without special mention were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Exposure and sampling

For models A and B, exposure was conducted in an air duct connected to their outlet (Fig. 1). Air flow was 0.8 m^3/min for A and 1.0 m^3/min for B at low mode (wind velocity < 0.5 m/sec). For model C, exposure was conducted in an incubator (45 × 46 × 46 cm), because it was not equipped with a fan; and fresh air was introduced into the incubator (0.03 m^3/min) by an air pump during exposure. All of the air purifiers were set to the low mode. The exposures were conducted for 16 hr or 48 hr in respective rooms.

Model B was also tested under two other exposure conditions. (I) The air purifier was put on a side of a small room (12.6 m^3, 2.1 × 2.5 × 2.4 m), and mice were exposed at the opposite side of the room for 48 hr. (II) The air purifier and animal cage were put in an air-tight plastic chamber (1.0 m^3, 0.9 × 1.2 × 0.9 m) to conduct the exposure for 2 hr. Oxygen concentration in the plastic chamber remained practically the same (20.9-20.7%) during exposure.

Immediately after exposure, mice were sacrificed by cervical dislocation to collect the lung for the assessment of its damage.

Assessment of lung damage

DNA damage was assessed by the *in vivo* comet assay and 8-hydroxy deoxyguanosine (8-OH-dG). The comet assay was conducted according to Tsuda *et al.* (2000) and Hashimoto *et al.* (2007), where 50 nuclei/tissue were measured for DNA migration and the mean migration was regarded as the individual level of DNA damage. 8-OH-dG was determined by HPLC equipped with an electrochemical detector. Detailed procedures were described in the next section. Lipid peroxidation was estimated by thiobarbituric acid reactive substances (TBARS) and 8-isoprostane. TBARS were determined according to the method of Kikugawa *et al.* (1992) with a little modification. We omitted the solvent extraction with butanol-pyridine, because transparent samples could be obtained by addition of propanol followed by centrifugation at 3,000 rpm. 8-Isoprostane was determined by an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Left lobes of the lung were fixed in 10% neutral buffered formalin, routinely processed, and stained with hematoxylin eosin for histopathological examination. Dunnett's test or Student's t test was employed for the statistical analysis, and P value less than 0.05 was considered statistically significant.

8-OH-dG assay

Tissue sample (100-200 mg) was gently homogenized at 0°C with 2 ml of lysing solution (1% Triton X-100, 320 mM saccharose, 5 mM $MgCl_2$, 0.005% BHT, 10 mM Tris, pH 7.5) using a Potter homogenizer. A portion of 1 ml was centrifuged at 600 g for 10 min at 4°C, and the supernatant was carefully discarded. The precipitate was resuspended in 1 ml of the lysing solution and centrifuged under the same conditions. This step was repeated once more. The precipitate was suspended in 0.3 ml of reaction solution (1% SDS, 5 mM Na_2EDTA, 0.005% BHT, 10 mM Tris, pH 8.0) and incubated with 10 µl of proteinase K (17 mg/ml) at 37°C for 60 min. During the incubation, the sample was shaken vigorously every 10 min to facilitate the enzyme reaction.

After the incubation, the sample was centrifuged at 10,000 g for 5 min at 4°C. The supernatant (0.15 ml) was mixed with 0.3 ml of NaI (7.6 M NaI, 20 mM Na_2EDTA, 40 mM Tris, pH 8.0) and 0.6 ml of isopropanol, stirred until filamentous DNA was deposited, and centrifuged at 10,000 g for 5 min at 4°C. The precipitate was resuspended in 1 ml of 70% ethanol and centrifuged under the same

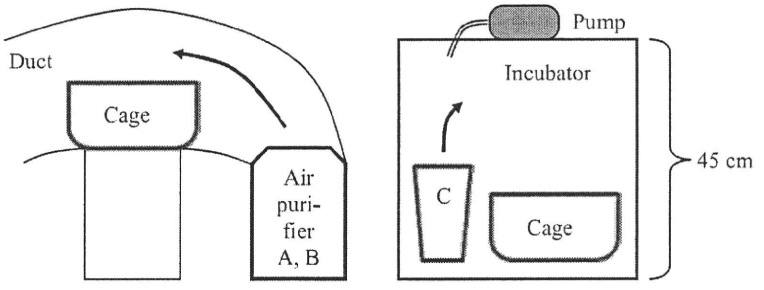

Fig. 1. Schematic diagram of the direct exposure.

conditions. This step was repeated once more. The supernatant was completely discarded to remove ethanol.

The precipitated DNA was dissolved in 0.1 ml of pure water, denatured in boiling water for 3 min, then cooled in ice water. Ten microliters of 200 mM acetic acid buffer (pH 5.3, 1 mM $ZnCl_2$), nuclease P1 (100 U/ml, Yamasa Corporation, Chiba, Japan) and acid phosphatase (100 U/ml) were added to the DNA solution, incubated at 37°C for 30 min, then filtered with a centrifugal filter device (Ultrafree-MC, 0.45 um, Millipore, Bedford, MA, USA) to obtain analytical sample for HPLC. Conditions of HPLC were as follows.

Apparatus : Shiseido Nanospace (Shiseido, Tokyo, Japan)
Column : Inertsil ODS, 3 μm, 3.0 × 50 mm (GL Science, Tokyo, Japan), 35°C
Eluent : 50 mM acetic acid buffer, pH 5.3, 5% methanol, 0.4 ml/min
Detector : UV (275 nm), ECD (Ox 0.6 V)

Ozone measurement

Ozone concentration in the outlet air (model A and B) or in the incubator (model C) was measured by detection tubes (Komyo Rikagaku Kogyo, Kanagawa, Japan).

RESULTS

Neither abnormal behavior during exposure nor gross abnormality at necropsy was observed. No changes were also detected by histopathological examination of the lung directly exposed to model A, B or C for 48 hr (pictures were omitted). The other exposure conditions were not allocated for histopathology.

DNA migration of the lung was significantly increased by 48 hr direct exposure to model A and B, and by 16 hr exposure to model B (Fig. 2). Sixteen hours exposure to model C was not examined, because this model did not increase DNA migration even after 48 hr exposure. As for model B, DNA migration was also increased by 2 hr exposure in a 1 m^3 plastic chamber but not by 48 hr expo-

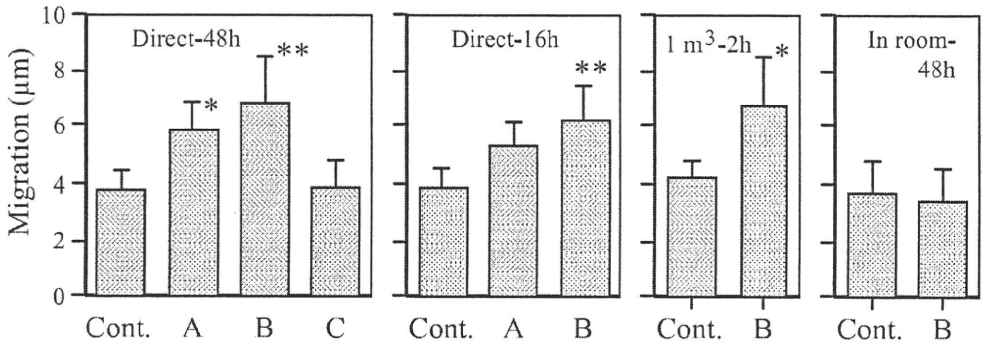

Fig. 2. DNA damage in the lung measured by the comet assay. Mean ± S.D. for 5 mice. *: $p < 0.05$, **: $p < 0.01$.

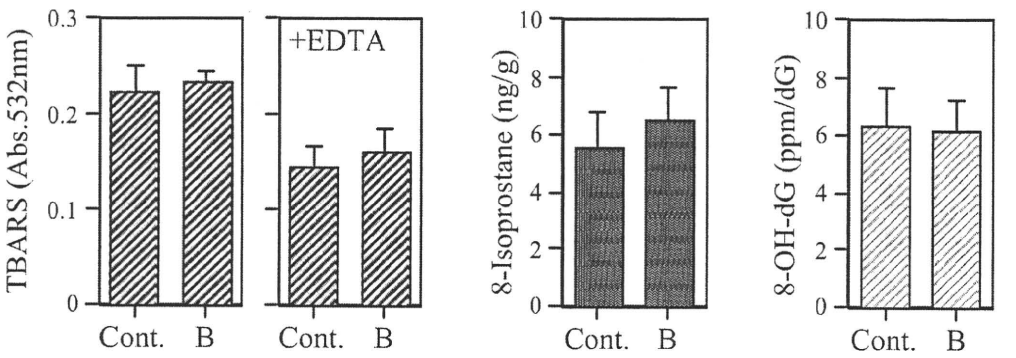

Fig. 3. Lipid peroxidation and 8-OH-dG in the lung of mice exposed directly to model B for 48 hr. Mean ± S.D. for 5 mice.

sure in a small room (Fig. 2).

TBARS, 8-isoprostane and 8-OH-dG were not affected by 48 hr direct exposure to model B (Fig. 3). The other exposure conditions were not examined for these endpoints because above condition caused most severe DNA damage.

Ozone concentration in the exposing air was lower than the detection limit (25 ppb) for all air purifiers tested.

DISCUSSION

According to the instruction manuals, models A and B collaterally generate small amount of ozone. Ozone is a powerful oxidant that causes DNA damage as well as inflammatory reaction in the lung or pulmonary cultured cells (Victorin, 1992; Bornholdt *et al.*, 2002). An *in vivo* study using guinea pig has demonstrated that 72 hr exposure to 1 ppm ozone caused DNA single strand break (SSB) in tracheobronchial epithelial cells, but the same duration at 0.45 ppm did not cause SSB (Ferng, 2002). Occupational exposure limit for ozone recommended by the Japan Society for Occupational Health is 0.1 ppm (JSOH, 2009). In this study, ozone levels in the exposing air were less than 0.025 ppm. Thus, ozone is not involved in the DNA damage caused by air purifiers. It was also demonstrated by histopathological examination that the DNA damage was not attributable to the cellular degeneration or necrosis.

Models A and B diffuse superoxide and hydroxyl radical, respectively, either of which can cause oxidative DNA damage such as modified base and strand break if it reaches the target site. However, they may not react directly with nuclear DNA of pulmonary cells because of their low membrane permeability and/or high reactivity. Thus, inhaled superoxide and hydroxyl radical might have primarily reacted with the epithelial cell lining fluid or cell membrane to cause lipid peroxidation, and indirectly attacked DNA through genotoxic products such as alkylperoxyl radicals, alkoxyl radicals and reactive carbonyls (Burcham, 1998; Blair, 2008). Similar mechanism has been suggested for the development of ozone toxicity (Mehlman and Borek, 1987; Pryor and Church, 1991). However, there was no increase in the levels of TBARS and 8-isoprostane when mice were directly exposed to model B for 48 hr, while the DNA damage was observed. TBA method is a widely used conventional method to evaluate lipid peroxidation in living tissues and foods. 8-Isoprostane is a degradation product of arachidonic acid, and is reported to increase in high-oxygen environment (Vacchiano and Tempel, 1994). The negative results of both TBARS and 8-isoprostane suggest that lipid peroxidation may not be the main cause of the DNA damage. Another possibility may be hydrogen peroxide, a stable and penetrable ROS, which may have been generated before or after inhalation causing oxidative DNA damage of the lung. However, there was no change in the levels of 8-OH-dG, a marker of oxidative DNA damage, under the same conditions as mentioned above.

Model C, which diffuses electrolytic water mist by ultrasonic nebulizer, did not cause DNA damage. However, it is unclear whether the negative result was due to the unique mechanism of this model, because exposure conditions including ROS density were not equalized in this study.

Digel *et al.* (2005) examined bactericidal effects of plasma-generated air ions on several gram-positive strains. They found that the air ions did not damage bacterial DNA but denatured surface proteins. Bacterial cells are enveloped by thick cell wall which consists chiefly of peptidoglycan and teichoic acid. Therefore, the present results do not necessarily contradict their findings and revealed that some models of air purifiers that diffuse ROS potentially cause DNA damage in the lung under strong exposure conditions. However, the mechanism of DNA damage of the lung caused the air purifiers remains to be solved.

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