Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.2, No.3, 101-110, 2015

Letter

Positive and negative ions by air purifier have no effects on reproductive function or postnatal growth and development in rats

Dai Yamamoto, Yumi Wako, Shino Kumabe, Kiyoshi Wako, Yukari Sato, Mayumi Fujishiro, Yoshimasa Yasuda, Ikuo Matsuura and Yasuyuki Ohnishi

Nonclinical Research Center, Drug Development Service Segment, LSI Medience Corporation, 14-1 Sunayama, Kamisu-shi, Ibaraki 314-0255, Japan

(Received June 4, 2015; Accepted June 13, 2015)

ABSTRACT — Air purifiers, which release positive and negative ions generated by electric discharge, are widely used in a variety of places. In this study, male and female SD rats [Crl:CD(SD)] were exposed by whole-body inhalation (6 hr/day) to ionized air containing positive and negative ions for at least 10 weeks before mating and throughout the mating, gestation, and lactation periods over two generations, and the effects on the reproductive function of parental animals and development of offspring were assessed. The concentrations of the ionized air were set at 0 and 7,000,000 ions/cm^3 (280- to 1,000-fold higher than normally used in humans) and each group consisted of 24 F0 rats/sex/group and 20 to 23 F1 rats/sex/group. The ionized air had no general toxicological effects on parental animals in the observation for clinical signs, body weight and food consumption measurement, or pathological examination. As for the effects on the reproductive function, there were no exposure-related changes in mating ability, fertility, pregnancy, parturition, or nursing behavior, nor were there any changes in the estrous cycle or sperm parameters in either generation, nor in the ovarian follicle counts (only F1 females). Moreover, there were no effects on litter size, viability, growth, or development of F1 and F2 offspring, including sexual maturation. Therefore, it was suggested that the ionized air has no reproductive or developmental toxicity in rats.

Key words: Positive and negative ions, Whole-body inhalation, Reproductive toxicity, Two-generation

INTRODUCTION

Air purifiers, which release positive and negative ions generated by electric discharge, are used not only in common households, but also in offices and public facilities such as hospitals ever since the device was created (Nishikawa and Nojima, 2001; Nojima and Nishikawa, 2002). Air containing positive and negative ions have certain abilities such as purification of the atmosphere and deodorization, and inactivation of bacteria, mold, and other allergens (Nishikawa and Yagi, 2006; Kawamoto *et al.*, 2006). Furthermore, effectiveness of the ionized air includes: a decrease in allergenicities of atomized Japanese cedar pollen (Kawamoto *et al.*, 2006), a mitigation of the symptoms of atopic dermatitis in the model mice (Hiramoto *et al.*, 2011), and an improvement of skin moisture and comfort sensation (Nishitani *et al.*, 2010). In addition, the mechanism of the bacteria inactivation by ions was reported to be induced by fragmentation of the surface membrane proteins (Digel *et al.*, 2005; Nishikawa, 2013).

On the other hand, the ionized air has been reported to have no potential to induce DNA damage in lung cells of rats or mice (Takasawa *et al.*, 2011); however, there are not enough data concerning toxicity of ionized air. It is generally assumed that the results of toxicity studies using animals are relevant to human health. Associated with the expanded use of air purifiers in various locations, there is also an increase in the chance for young people giving birth to the next generation to be exposed to ions generated from these devices. In reproductive and developmental toxicity studies in animal models, only "no adverse effects on dams and embryo-fetal development (Yamamoto *et al.*, 2014)" have been reported. Therefore,

Correspondence: Dai Yamamoto (E-mail: Yamamoto.Dai@mb.medience.co.jp)

it is important to assess the reproductive and developmental toxicity of air containing positive and negative ions.

This study was conducted to assess the safety of air containing positive and negative ions on the integrity and performance of the reproductive systems of parental animals, and on the growth and development of the offspring when exposed by whole-body inhalation for two generations in rats.

MATERIALS AND METHODS

Test atmosphere

Air containing positive and negative ions generated by electric discharge devices for air purifiers (Nishikawa and Nojima, 2001; Nojima and Nishikawa, 2002) developed by Sharp Corporation (Osaka, Japan) is defined here as "ionized air (IONA)". The IONA is generated as follows. First, water and oxygen molecules are decomposed by applying positive and negative high voltages to each discharge electrode of the ion generation devices, and the devices generate positive hydrogen ions (H^+) and negative oxygen ions (O_2^-). Then, each ion is surrounded by water molecules like a bunch of grape to maintain stability. Accordingly, $[(H_2O)_mH]^+$ and $[(H_2O)_nO_2]^-$ as positive and negative ions, respectively, with "m" and "n" as natural numbers, are cluster-shaped and released into the air. The IONA are known as PlasmaCluster Ions® (Nojima and Nishikawa, 2002; Nishikawa and Yagi, 2006; Kawamoto *et al.*, 2006).

F0 animals were exposed to IONA by whole-body inhalation at 0 (control) and 7,000,000 ions/cm^3 for 6 hr a day from 6 weeks of age for 10 weeks before mating for males and females, throughout the mating period until the day before the necropsy in males, and throughout the mating, gestation, and lactation periods until the day before weaning of F1 offspring on postnatal day (PND) 21 in females. F1 animals were exposed from weaning for 10 to 12 weeks before mating for males and females, and thereafter, in the same way as F0 animals. However, maternal F0 or F1 females were not exposed from gestation day (GD) 21 to lactation day (LD) 4 to allow for parturition and to permit continuous maternal care to the early neonates. The day of copulation was designated as GD 0, and the day of parturition was LD 0 for the dams and PND 0 for the offspring. The high exposure concentration of IONA was set at the maximum feasible concentration, which is 280- to 1,000-fold higher than that usually applied to humans (7,000 to 25,000 ions/cm^3). The exposure period was set in accordance with the OECD Guidelines for the Testing of Chemicals (OECD TG 412, 2009; OECD TG 416, 2001). IONA concentrations were determined using an ion-counter (KEC-990, Kyoritsu Electronics Co., Ltd., Kawasaki, Japan, measuring range: 100 to 19,990,000 ions/cm^3) just before and after the exposure throughout the exposure period.

Animals and husbandry

F0 male and female Crl:CD(SD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) at 5 weeks of age. After quarantine and acclimation periods, 24 males and 24 females were assigned to the each group (control and IONA groups) by the stratified-by-weight randomization method on the basis of the body weights on the day before the start of exposure.

Animals were housed 2 animals of the same sex per cage, except for the mating, gestation, and lactation periods (1 male and 1 female, 1 dam, and 1 dam and her litter, respectively) in a polycarbonate cage with hard-wood chip bedding and an enrichment toy in an air-conditioned room (set at 22 ± 3°C and 55 ± 20% relative humidity) with artificial lighting from 7:00 to 19:00 and ventilation providing all fresh air. Food (CRF-1: Oriental Yeast Co., Ltd., Tokyo, Japan) and water were given *ad libitum*.

All procedures involving animals were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of the test facility and in accordance with the guidance of the animal experiments issued by IACUC. The facility has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Whole-body inhalation exposure

Animals were exposed to IONA in an approximately 1.6 m^3 stainless and glass whole-body inhalation chamber (Tokiwa Kagaku Kikai Co., Ltd., Tokyo, Japan). Each chamber was dedicated to one sex and one exposure group. The chambers were operated under a full ventilation system at an air flow of 15 air changes per hour (ventilation volume: 24 m^3 per hour). Environmental conditions within the chambers were monitored and maintained at temperature between 21.0°C and 26.3°C and relative humidity between 41% and 66%. The oxygen concentration was 20.7% to 21.3%.

The whole-body inhalation exposure was conducted on animals housed in a wire mesh cage, which was composed of 5 rooms (partitioned into 2 sub-rooms, 2 animals per room). The IONA generation device was constructed from 1 ion generator and 1 fan for 1 room, and each device was placed just above each room. The air speed of the fan was set approximately 1.5 m/sec (maximum air speed: approximately 2.3 m/sec) in the cage. In the chamber for the control group, the device without the ion gen-

erator was placed in the same manner.

Examination of parental animals (F0 and F1)

Clinical signs of all animals were observed twice a day (before and after exposure) from the first day of exposure to the day before necropsy and once a day during the other periods. Body weights were measured once a week for males and females, and on GD 0, 7, 14, and 20 and on LD 0, 4, 7, 14, and 21 for copulated females. Food consumption was measured until 10 weeks after the start of exposure, and during the gestation and lactation periods at the same intervals as the body weight.

The estrous cycle was examined by collecting vaginal smears every day in the morning for 2 weeks from 8 weeks after the start of exposure in the pre-mating period (F0 female: 14 weeks of age, F1 female: 11 weeks of age), and the mean estrous cycle length for each female and the incidence of females with irregular estrous cycles were calculated. Females that had an estrous cycle length other than 4 to 6 days were judged as having irregular estrous cycles. After the pre-mating period, males and females of the same group were cohabited day and night, except during the daily exposure period, on one-to-one basis avoiding siblings in F1 animals from 16:00 on the day of the start of mating for up to 14 days, and vaginal smears were collected every day in the morning. Copulation was confirmed by the presence of a vaginal plug or sperm in the vaginal smears, and the day on which the evidence of copulation was found was designated as GD 0. Pregnant females were allowed to delivery their pups and were observed twice a day (9:00 and 16:00) from GD 21 to 25. The females that delivered them completely by 16:00 were judged as dams giving birth on that day, which was designated as LD 0 for dams and PND 0 for pups. The dams were allowed to nurse their own pups until weaning on LD 21 and observed once a day for maternal behavior, including lactation, nest building, and cannibalism. At the necropsy on LD 21, the uterus was removed and examined for the number of implantations. The non-delivery animals were examined 26 days or later after copulation in the same way. The uterus without visible implantation sites was immersed in a 10 vol% ammonium sulfide solution to detect the presence of implantation sites (Salewski, 1964).

In males, the right testis and cauda epididymis were removed and weighed for sperm analysis at the necropsy. The sperm collected from the cauda epididymis was suspended in Medium 199 (Nissui pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1% BSA (bovine serum albumin, Sigma-Aldrich, MO, USA). The percentage of motile sperm (sperm motility) was determined with an automatic sperm analyzer (HTM-IVOS, Ver. 10.8, Hamilton-Thorne Research, Inc., MA, USA). The testis and cauda epididymis were homogenized in purified water and the number of homogenization-resistant testicular spermatids and cauda epididymal sperm were counted with the automatic sperm analyzer. The incidences of abnormal sperm (without hook, banana-like head, amorphous head, folded midpiece, and others) and tailless sperm were also determined by examining eosin Y-stained sperm smears prepared from the sperm suspension.

After the mating period, males were euthanized under thiopental anesthesia, and abdominal organs/tissues were examined macroscopically. Females were necropsied in the same manner as males at the following time: on LD 21 for dams delivered, 26 days or later after copulation for non-delivery females, and 7 days after the mating period for non-copulated females. For dams sacrificed on LD 21, vaginal smears were collected in the morning on the day of the necropsy and examined for the estrous cycle stage. Dams that lost their entire litter were necropsied immediately upon discovery. The major organs/tissues including the lungs and the reproductive organs of males and females were weighed, stained with hematoxylin and eosin by standard methods, and examined histopathologically. The ovary of F1 females was cut at the maximum diameter and the paraffin-embedded sections from the maximum cut surface of the each ovary were subjected to immunohistochemical staining with anti-proliferating cell nuclear antigen. The ovarian follicles were classified (small, medium, and large) and counted based on their size (Pedersen and Peters, 1968; Bolon *et al.*, 1997; Bucci *et al.*, 1997).

Examination of offspring (F1 and F2)

The day of birth was designated as PND 0. On PND 0, the newborns were examined for the number of offspring (live or stillborn), sex, and presence of external anomalies. After that, the pups were observed daily for clinical signs and mortality until PND 21. On PND 4, litter size was randomly adjusted to 8 pups (equal sex ratio, in principle). Litter with less than 8 pups was maintained as is. The offspring were weighed by sex in each litter on PND 0 and 4 before culling and individually on PND 4, 7, 14, and 21 after culling. For F1 animals, 1 male and 1 female were randomly selected from each litter on PND 21 and were subjected to exposure as the animals for post-weaning examination (F1 parental animals).

The selected F1 offspring were examined for the day of vaginal opening from PND 27 for females and preputial separation from PND 35 for males as indices of sexual maturation with the body weight at the time. The oth-

er pups were necropsied on PND 21 in the same manner as parental animals. The selected organs/tissues including the brain, thymus, spleen, and uterus were weighed and the histopathological examination was carried out in 1 male and 1 female selected randomly from each litter. The pups that died, except for cannibalized ones, were examined for the presence of external anomalies and/or necropsied. The culled pups were euthanized in the same manner as parental animals.

Statistical analysis

The data of offspring obtained before weaning were analyzed on the basis of litter mean values except for the sex ratio. For the comparison of the metric data between the 0 and 7,000,000 ions/cm^3 groups, the homogeneity of variance was tested by the F test, and when the variance was homogeneous, Student's t test was performed for the statistical comparison; when heterogeneous, the Aspin-Welch t test was used. Non-parametric data such as gestation length, birth index, viability index, sexual maturation, and sperm motility were compared using Wilcoxon's rank sum test. The count data were analyzed by Fisher's exact probability test. The significance level was set at 5% for all statistical analyses (two tailed).

RESULTS AND DISCUSSION

Each concentration of positive and negative ions in the IONA group was 7,000,000 ions/cm^3 or more and was comparable between pre- and post-exposure. The coefficient of variation (C.V.) values of the daily ion concentrations were below 15% (Table 1). Accordingly, the animals were judged to have been properly exposed to the homogenous IONA throughout the exposure period.

As for the general toxicological effects on parental animals, no abnormalities in clinical signs were noted in any animals. The body weight, body weight gain, and food consumption in the IONA group were comparable to those in the control group (Table 2). There were no IONA-related changes in necropsy or histopathological examination (data not shown). In the organ weight measurement, statistically significant higher values were noted in absolute and relative uterine weights of F0 females (Table 3); however, the changes were considered to be estrous cycle-related and were not considered to be IONA-related because there were tendencies toward many females with diestrus in the control group and proestrus in the IONA group at the estrous stage at necropsy. The absolute and relative thyroids weights of F0 males showed statistically significant higher values in the IONA group; however, they were judged to be not IONA-related, because the changes were not seen in F1 parental males and no corresponding histopathological findings were observed in two generations. The other statistically significant changes noted in some organs were also judged to be not IONA-related, because they were found in absolute or relative weight only and there were no corresponding histopathological findings in any organs.

As for the effects on the reproductive function, there were no IONA-related changes in mating ability, fertility, pregnancy, gestation length, parturition, or nursing behavior, nor were there any changes in the estrous cycle, sperm parameters (sperm motility, spermatid counts, sperm counts, and incidences of morphological abnormal sperm and tailless sperm) in either generation (Tables 5 and 6). The ovarian follicle counts of F1 parental females in the IONA group were also comparable to that in the control group (Table 4).

As for the effects on offspring, there were no IONA-related changes in the viability index, number of offspring, external features, clinical signs, body weight, organ weights, necropsy, or histopathological findings. The body weight gain before culling showed a statistically significant higher value in F1 female offspring in the IONA group (Table 6). However, it was judged to be incidental, because the gain after culling was comparable to that in the control group and there were no significant differences in body weights at birth or throughout the pre-

Table 1. Actual concentration of cluster ions in the IONA group.

Type of ion		Actual conc. (ions/cm^3) [a]	
		Mean (Range)	C.V. (%) [b]
Pre-exposure	Positive ion	7,880,000 (7,020,000 - 9,230,000)	0.0 - 10.4
	Negative ion	7,900,000 (7,440,000 - 9,430,000)	0.0 - 9.3
Post-exposure	Positive ion	7,960,000 (7,020,000 - 9,360,000)	0.0 - 7.1
	Negative ion	7,910,000 (7,440,000 - 9,430,000)	0.0 - 12.2

[a] The actual concentrations were calculated from daily mean concentrations of 24 devices at a maximum.
[b] Coefficient of variation for daily ion concentration.

Table 2. Body weighs and food consumption of F0/F1 parental animals.

Generation:	F0		F1	
Group (concentration, ions/cm³):	Control (0)	IONA (7,000,000)	Control (0)	IONA (7,000,000)
Male				
Number of animals examined	24	24	21	23
Body weight (g)				
Pre-mating period				
Day 0/21 [b]	189.53 ± 6.76 [a]	189.73 ± 5.20	56.38 ± 7.63	58.71 ± 5.64
Day 70/91	497.18 ± 40.10	509.48 ± 43.27	519.02 ± 38.03	519.16 ± 38.59
Body weight gain (g)				
Pre-mating period				
Day 0 - 7/21 - 28 [b]	57.26 ± 5.50	55.93 ± 8.79	42.32 ± 5.28	44.19 ± 4.12
Day 0 - 70/21 - 91	307.65 ± 38.59	319.75 ± 39.99	462.64 ± 37.13	460.44 ± 37.34
Food consumption (g/day)				
Pre-mating period				
Day 7/28 [b]	22.26 ± 0.99	22.21 ± 1.10	10.26 ± 0.69	10.58 ± 0.43
Day 70/91	24.24 ± 1.47	24.36 ± 1.45	28.15 ± 1.17	27.73 ± 1.57
Female				
Number of animals examined	24	24	20	23
Body weight (g)				
Pre-mating period				
Day 0/21 [b]	151.95 ± 6.40	151.21 ± 7.11	53.91 ± 6.47	55.27 ± 6.20
Day 70/91	301.22 ± 28.28	295.47 ± 27.18	304.05 ± 24.86	290.76 ± 35.10
Gestation period				
Day 0	305.18 ± 29.31	300.77 ± 29.32	318.53 ± 29.69	303.79 ± 37.77
Day 20	442.05 ± 45.71	437.30 ± 32.71	460.64 ± 38.94	432.31 ± 50.26
Lactation period				
Day 0	346.64 ± 40.84	330.85 ± 30.09	362.84 ± 38.71	349.84 ± 42.45
Day 14	372.66 ± 32.20	367.42 ± 24.53	381.72 ± 29.91	363.52 ± 29.67
Day 21	351.87 ± 29.21	342.79 ± 24.24	365.05 ± 28.29	352.45 ± 29.07
Body weight gain (g)				
Pre-mating period				
Day 0 - 7/21 - 28 [b]	28.46 ± 6.44	26.84 ± 7.35	34.72 ± 4.15	36.03 ± 4.18
Day 0 - 70/21 - 91	149.27 ± 24.02	144.26 ± 23.70	250.15 ± 24.89	235.49 ± 32.47
Gestation period				
Day 0 - 20	136.87 ± 27.00	136.53 ± 19.13	142.11 ± 16.93	128.52 ± 25.33
Lactation period				
Day 0 - 14	26.02 ± 20.38	36.57 ± 20.27	18.88 ± 17.56	12.78 ± 23.77
Day 0 - 21	5.22 ± 24.74	11.94 ± 18.55	2.21 ± 19.01	1.72 ± 25.04
Food consumption (g/day)				
Pre-mating period				
Day 7/28 [b]	16.35 ± 0.93	16.24 ± 1.19	9.33 ± 0.57	9.54 ± 0.58
Day 70/91	17.76 ± 1.00	17.86 ± 0.80	19.33 ± 1.93	18.08 ± 1.13
Gestation period				
Day 20	24.67 ± 2.40	24.79 ± 2.25	25.15 ± 2.22	24.16 ± 2.71
Lactation period				
Day 14	49.22 ± 4.85	51.40 ± 4.45	48.36 ± 5.55	47.95 ± 3.51
Day 21	61.03 ± 5.90	63.61 ± 5.61	60.60 ± 7.82	61.31 ± 4.09

[a] Mean ± S.D.

[b] Day of the pre-mating period, F0 after the start of exposure/F1 postnatal day (exposed from PND 21).

Table 3. Organ weights of F0/F1 parental animals.

Generation:	F0		F1	
Group (concentration, ions/cm^3):	Control (0)	IONA (7,000,000)	Control (0)	IONA (7,000,000)
Male				
Number of animals examined	24	24	21	23
Final body weight (g)	536.01 ± 40.96 [a]	542.60 ± 46.02	593.27 ± 46.31	586.43 ± 48.54
Brain (g)	2.177 ± 0.074 [b]	2.204 ± 0.073	2.191 ± 0.087	2.211 ± 0.081
	0.408 ± 0.026 [c]	0.409 ± 0.032	0.371 ± 0.030	0.380 ± 0.036
Pituitary (mg)	16.90 ± 2.07	16.34 ± 1.91	17.10 ± 1.92	16.64 ± 1.94
	3.16 ± 0.32	3.02 ± 0.32	2.88 ± 0.26	2.84 ± 0.28
Thyroids (mg)	27.76 ± 4.49	32.92 ± 4.55 **	29.44 ± 5.27	32.24 ± 4.63
	5.20 ± 0.93	6.07 ± 0.70 **	4.98 ± 0.89	5.51 ± 0.73 *
Thymus (mg)	305.8 ± 86.7	308.3 ± 78.2	407.7 ± 120.7	364.9 ± 67.4
	57.32 ± 16.46	57.34 ± 15.58	68.82 ± 19.78	62.59 ± 12.59
Lungs (g)	1.667 ± 0.130	1.673 ± 0.174	1.700 ± 0.095	1.679 ± 0.114
	0.312 ± 0.021	0.309 ± 0.028	0.287 ± 0.016	0.287 ± 0.017
Heart (g)	1.550 ± 0.149	1.558 ± 0.133	1.630 ± 0.137	1.620 ± 0.141
	0.289 ± 0.022	0.288 ± 0.019	0.275 ± 0.019	0.277 ± 0.019
Liver (g)	17.304 ± 1.973	16.928 ± 2.006	20.523 ± 2.885	20.126 ± 2.523
	3.224 ± 0.219	3.115 ± 0.183	3.448 ± 0.287	3.426 ± 0.235
Spleen (g)	0.845 ± 0.125	0.858 ± 0.141	0.955 ± 0.175	0.905 ± 0.104
	0.158 ± 0.019	0.159 ± 0.027	0.161 ± 0.023	0.155 ± 0.016
Kidneys (g)	3.647 ± 0.367	3.748 ± 0.534	3.876 ± 0.348	3.845 ± 0.431
	0.680 ± 0.043	0.690 ± 0.073	0.654 ± 0.038	0.656 ± 0.052
Adrenals (mg)	56.47 ± 7.50	59.63 ± 9.64	62.03 ± 9.08	61.48 ± 8.55
	10.58 ± 1.51	11.02 ± 1.72	10.48 ± 1.44	10.54 ± 1.67
Testes (g)	3.528 ± 0.303	3.466 ± 0.216	3.639 ± 0.298	3.670 ± 0.310
	0.660 ± 0.059	0.642 ± 0.054	0.617 ± 0.068	0.630 ± 0.077
Prostate (g)	0.823 ± 0.188	0.796 ± 0.169	0.766 ± 0.163	0.732 ± 0.145
	0.154 ± 0.034	0.147 ± 0.030	0.130 ± 0.032	0.125 ± 0.024
Seminal vesicle (g)	3.152 ± 0.445	3.099 ± 0.326	2.877 ± 0.377	2.901 ± 0.272
	0.590 ± 0.082	0.573 ± 0.064	0.487 ± 0.068	0.498 ± 0.064
Epididymides (g)	1.363 ± 0.097	1.360 ± 0.109	1.344 ± 0.103	1.361 ± 0.072
	0.255 ± 0.020	0.252 ± 0.025	0.228 ± 0.023	0.234 ± 0.023
Female				
Number of animals examined	21	23	17	19
Final body weight (g)	351.87 ± 29.21 [a]	342.79 ± 24.24	365.05 ± 28.29	352.45 ± 29.07
Brain (g)	1.992 ± 0.066 [b]	1.993 ± 0.102	1.989 ± 0.099	2.027 ± 0.088
	0.569 ± 0.046 [c]	0.584 ± 0.047	0.547 ± 0.044	0.578 ± 0.046 *
Pituitary (mg)	21.73 ± 2.16	21.09 ± 2.29	21.98 ± 2.79	20.06 ± 2.60 *
	6.23 ± 0.88	6.17 ± 0.69	6.04 ± 0.78	5.70 ± 0.69
Thyroids (mg)	22.93 ± 3.79	22.07 ± 2.92	23.74 ± 4.42	25.76 ± 3.82
	6.58 ± 1.38	6.47 ± 0.97	6.51 ± 1.16	7.31 ± 0.92 *
Thymus (mg)	190.2 ± 74.4	177.0 ± 47.9	214.5 ± 65.4	197.4 ± 67.3
	53.90 ± 19.06	51.54 ± 13.13	58.66 ± 16.80	56.45 ± 20.02
Lungs (g)	1.361 ± 0.100	1.334 ± 0.130	1.364 ± 0.117	1.327 ± 0.117
	0.387 ± 0.018	0.389 ± 0.027	0.375 ± 0.033	0.377 ± 0.022
Heart (g)	1.149 ± 0.087	1.188 ± 0.089	1.245 ± 0.078	1.224 ± 0.105
	0.328 ± 0.026	0.347 ± 0.024 *	0.342 ± 0.021	0.348 ± 0.026
Liver (g)	15.645 ± 1.440	15.663 ± 1.406	17.229 ± 1.269	16.980 ± 1.644
	4.457 ± 0.366	4.572 ± 0.311	4.736 ± 0.400	4.823 ± 0.328
Spleen (g)	0.742 ± 0.177	0.670 ± 0.071	0.710 ± 0.114	0.642 ± 0.119
	0.211 ± 0.047	0.196 ± 0.018	0.195 ± 0.032	0.181 ± 0.024

Table 3. (Continued.)

Generation:	F0		F1	
Group (concentration, ions/cm³):	Control (0)	IONA (7,000,000)	Control (0)	IONA (7,000,000)
Kidneys (g)	2.503 ± 0.125	2.521 ± 0.207	2.653 ± 0.216	2.557 ± 0.242
	0.714 ± 0.051	0.737 ± 0.058	0.729 ± 0.061	0.727 ± 0.062
Adrenals (mg)	74.65 ± 8.26	78.76 ± 10.34	77.80 ± 7.75	74.81 ± 10.38
	21.39 ± 3.20	22.99 ± 2.59	21.46 ± 2.88	21.25 ± 2.51
Ovaries (mg)	104.36 ± 15.22	103.93 ± 15.49	105.33 ± 15.47	100.51 ± 16.09
	29.76 ± 4.47	30.28 ± 3.79	28.97 ± 4.35	28.64 ± 4.85
Uterus (g)	0.485 ± 0.114	0.601 ± 0.132 **	0.521 ± 0.118	0.512 ± 0.129
	0.138 ± 0.034	0.177 ± 0.044 **	0.143 ± 0.034	0.144 ± 0.030
Estrous stage at necropsy				
Proestrous	5	12	5	7
Estrous	1	1	2	1
Metestrous	2	3	1	1
Diestrous	13	7	9	10

[a] Mean ± S.D.
[b] Absolute organ weight (upper row)
[c] Relative organ weight (lower row, g% or mg%)
Significantly different from control: *, $P < 0.05$; **, $P < 0.01$.

Table 4. Ovarian follicle count of F1 parental females.

Generation:	F1	
Group (concentration, ions/cm³):	Control (0)	IONA (7,000,000)
Number of animals examined	20	23
Number of follicles [a]		
Small follicles	17.2 ± 8.2 [b]	19.1 ± 11.0
Medium follicles	3.8 ± 2.6	3.3 ± 2.0
Large follicles	11.9 ± 6.3	12.9 ± 3.6
Total	32.9 ± 14.0	35.3 ± 12.4

[a] One section in maximum diameter was examined for each side of the ovary.
[b] Mean ± S.D.

weaning period. Both age and body weight at preputial separation in males and vaginal opening in females of F1 offspring were comparable between the control and IONA groups (Table 7); therefore, there were no IONA-related changes in sexual maturation of either male and female offspring. In the organ weight measurement at weaning, a statistically significant higher value was noted in absolute uterine weight in females of F1 offspring (Table 8); however, no change was found in relative weight and there were no corresponding histopathological findings, nor abnormal changes in reproductive function such as early puberty, irregular estrous cycles, or decreased fertility. Therefore, it was judged to be not IONA-related. Consequently, it was considered that IONA had no effects on the development of offspring.

As mentioned above, IONA had no general toxicological parameters on parental animals. There were no effects on reproductive function of parental animals or development of offspring even at the concentrations of 280- to 1,000-fold higher than that normally used in humans. Therefore, it was suggested that the ionized air containing positive and negative ions has no reproductive or neonatal toxicity in rats.

Conflict of interest---- The authors declare that there is no conflict of interest.

Table 5. Estrous cycle and sperm analysis of F0/F1 parental animals.

Generation:	F0		F1	
Group (concentration, ions/cm^3):	Control (0)	IONA (7,000,000)	Control (0)	IONA (7,000,000)
Estrous cycle				
Number of females examined	24	24	20	23
Mean estrous cycle length (days)	4.21 ± 0.41 [a]	4.09 ± 0.23	4.19 ± 0.39	4.26 ± 0.42
Number of females with abnormal estrous cycles	3	0	2	0
Sperm analysis				
Number of males examined	24	24	21	23
Sperm motility (%)	92.5 ± 5.2	91.0 ± 6.7	93.4 ± 6.8	93.4 ± 4.5
Homogenization-resistant spermatids (10^6/g testis)	79.86 ± 7.96	78.23 ± 9.82	66.54 ± 12.24	61.03 ± 8.17
Sperm count (10^6/g cauda epididymis)	469.50 ± 91.82	447.39 ± 81.99	412.27 ± 100.09	474.97 ± 109.10
Abnormal sperm (%)	0.25 ± 0.33	0.35 ± 0.58	0.21 ± 0.41	0.28 ± 0.47
Tailless sperm (%)	2.83 ± 2.46	2.46 ± 2.26	2.33 ± 4.09	1.43 ± 1.20

[a] Mean ± S.D.

Table 6. Reproductive data of F0/F1 parental and F1/F2 offspring.

Generation:		F0 parental/F1 offspring		F1 parental/F2 offspring	
Group (concentration, ions/cm^3):		Control (0)	IONA (7,000,000)	Control (0)	IONA (7,000,000)
Number of pairs		24	24	20	23
Days until copulation [b]		2.79 ± 2.13 [a]	2.83 ± 1.20	2.25 ± 1.07	2.64 ± 1.09
Number of estrus stages without copulation		0.04 ± 0.20	0.00 ± 0.00	0.05 ± 0.22	0.17 ± 0.65
Copulation index (%) [c]		100.0 (24/24)	100.0 (24/24)	100.0 (20/20)	95.7 (22/23)
Fertility index (%) [d]		91.7 (22/24)	100.0 (24/24)	85.0 (17/20)	95.5 (21/22)
Gestation length (days)		21.8 ± 0.5	22.1 ± 0.7	22.1 ± 0.2	22.0 ± 0.4
Gestation index (%) [e]		95.5 (21/22)	95.8 (23/24)	100.0 (17/17)	95.2 (20/21)
Birth index (%) [f]		89.74 ± 21.17	85.58 ± 22.03	91.38 ± 4.64	83.67 ± 24.51
Number of implantation sites		14.0 ± 4.0	15.1 ± 3.1	15.4 ± 1.8	14.1 ± 3.7
Number of offspring at birth		13.8 ± 3.4	14.2 ± 2.3	14.2 ± 1.8	13.2 ± 3.4
Number of live offspring at birth		13.8 ± 3.4	14.0 ± 2.2	14.0 ± 1.5	13.0 ± 3.5
Sex ratio (males/females)		1.16 (156/134)	1.29 (184/143)	1.02 (122/120)	0.80 (117/146)
Viability index (%)					
Day 0 [Live birth index] [g]		99.66 ± 1.55	98.61 ± 4.10	98.55 ± 2.70	97.93 ± 4.99
Day 4 [Viability index on Day 4] [h]		98.07 ± 4.24	98.86 ± 3.17	94.32 ± 16.69	92.53 ± 22.38
Day 21 [Weaning index] [i]		99.40 ± 2.73	100.00 ± 0.00	97.35 ± 7.52	98.68 ± 5.74
Body weight of offspring (g)					
Male	Day 0	6.74 ± 0.64	6.66 ± 0.51	6.60 ± 0.46	6.69 ± 0.60
	Day 4	10.53 ± 1.95	10.89 ± 1.00	10.09 ± 1.32	10.35 ± 1.43
	Day 21	56.18 ± 6.86	57.61 ± 5.07	54.26 ± 5.70	53.24 ± 4.61
Female	Day 0	6.24 ± 0.60	6.27 ± 0.53	6.35 ± 0.44	6.34 ± 0.59
	Day 4	9.64 ± 1.68	10.36 ± 1.02	9.74 ± 1.38	9.92 ± 1.31
	Day 21	53.24 ± 6.47	55.17 ± 4.83	52.71 ± 5.14	52.29 ± 4.45
Body weight gain of offspring (g)					
Male	Day 0 - 4	3.79 ± 1.39	4.23 ± 0.64	3.49 ± 1.08	3.66 ± 0.97
	Day 4 - 21	45.37 ± 5.38	46.54 ± 4.69	43.97 ± 4.60	42.70 ± 3.86
Female	Day 0 - 4	3.40 ± 1.31	4.09 ± 0.59 *	3.39 ± 1.09	3.54 ± 0.90
	Day 4 - 21	43.40 ± 5.22	44.60 ± 4.42	42.80 ± 4.28	42.03 ± 4.02

[a] Mean ± S.D.

[b] Number of days from the start of mating to detection of copulation

[c] Copulation index (%) = Number of animals with successful copulation/number of animals paired × 100

[d] Fertility index (%) = Number of pregnant animals/number of animals with successful copulation × 100

[e] Gestation index (%) = Number of females with live offspring/number of pregnant females × 100

[f] Birth index (%) = Number of offspring born alive/number of implantations × 100

[g] Live birth index (%): Number of live offspring at birth/number of offspring at birth × 100

[h] Viability index on Day 4 (%): Number of live offspring on Day 4/number of live offspring at birth × 100

[i] Weaning index (%) = Number of live weanlings/number of live offspring after culling × 100

Significantly different from control: *, $P < 0.05$

Table 7. Sexual maturation of F1 offspring.

Generation:	F1	
Group (concentration, ions/cm³):	Control (0)	IONA (7,000,000)
Male		
Preputial separation		
Number of animals examined	21	23
Age (day) [b]	41.1 ± 1.7 [a]	40.8 ± 1.3
Body weight (g) [c]	214.19 ± 13.34	215.06 ± 14.35
Female		
Vaginal opening		
Number of animals examined	20	23
Age (day)	34.0 ± 2.5	33.8 ± 2.0
Body weight (g)	127.47 ± 16.26	127.20 ± 17.46

[a] Mean ± S.D.
[b] Day of completion
[c] Body weight at the completion

Table 8. Organ weights of F1/F2 offspring at weaning.

Generation:	F1		F2	
Group (concentration, ions/cm³):	Control (0)	IONA (7,000,000)	Control (0)	IONA (7,000,000)
Male				
Number of animals examined	21	23	17	19
Final body weight (g)	56.43 ± 7.54 [a]	57.72 ± 4.74	55.85 ± 5.38	54.34 ± 5.16
Brain (g)	1.510 ± 0.088 [b]	1.517 ± 0.050	1.506 ± 0.056	1.509 ± 0.085
	2.710 ± 0.279 [c]	2.644 ± 0.217	2.718 ± 0.240	2.791 ± 0.166
Thymus (mg)	249.1 ± 40.7	258.0 ± 35.6	246.1 ± 36.9	258.3 ± 83.2
	441.62 ± 44.97	447.30 ± 53.25	440.94 ± 51.35	473.11 ± 133.79
Spleen (g)	0.286 ± 0.074	0.287 ± 0.060	0.281 ± 0.061	0.272 ± 0.056
	0.501 ± 0.093	0.494 ± 0.077	0.498 ± 0.078	0.498 ± 0.080
Female				
Number of animals examined	20	23	17	19
Final body weight (g)	53.66 ± 6.32	55.95 ± 4.66	53.96 ± 6.11	52.77 ± 5.60
Brain (g)	1.447 ± 0.073	1.478 ± 0.047	1.452 ± 0.066	1.471 ± 0.052
	2.724 ± 0.261	2.658 ± 0.228	2.720 ± 0.283	2.813 ± 0.273
Thymus (mg)	262.1 ± 53.7	264.4 ± 41.3	250.8 ± 33.0	250.2 ± 34.6
	485.71 ± 70.65	472.66 ± 61.75	466.37 ± 49.14	475.49 ± 53.72
Spleen (g)	0.275 ± 0.063	0.295 ± 0.061	0.285 ± 0.063	0.275 ± 0.061
	0.507 ± 0.074	0.524 ± 0.085	0.523 ± 0.071	0.517 ± 0.084
Uterus (mg)	40.40 ± 6.70	47.10 ± 9.96 *	42.40 ± 11.45	43.53 ± 10.31
	75.92 ± 12.91	84.65 ± 19.12	78.66 ± 18.80	83.33 ± 22.17

[a] Mean ± S.D.
[b] Absolute organ weight (upper row)
[c] Relative organ weight (lower row, g% or mg%)
Significantly different from control: *, $P < 0.05$

REFERENCES

Bolon, B., Bucci, T.J., Warbritton, A.R., Chen, J.J., Mattison, D.R. and Heindel, J.J. (1997): Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: Results from continuous breeding bioassays. Fundam. Appl. Toxicol., **39**, 1-10.

Bucci, T.J., Bolon, B., Warbritton, A.R., Chen, J.J. and Heindel, J.J. (1997): Influence of sampling on the reproducibility of ovarian follicle counts in the mouse toxicity studies. Reprod. Toxicol., **11**, 689-996.

Digel, I., Temiz Artmann, A., Nishikawa, K., Cook, M., Kurulgan, E. and Artmann, G.M. (2005): Bactericidal effects of plasma-generated cluster ions. Med. Biol. Eng. Comput., **43**, 800-807.

Hiramoto, K., Orita, K., Yamate, Y., Sato, E.F., Okano, H., Nishikawa, K. and Inoue, M. (2011): Plasma cluster ions decrease the antigenicity of mite allergens and suppress atopic dermatitis in NC/Nga mice. Arch. Dermatol. Res., **303**, 367-370.

Kawamoto, S., Oshita, M., Fukuoka, N., Shigeta, S., Aki, T., Hayashi, T., Nishikawa, K. and Ono, K. (2006): Decrease in the allergenicity of Japanese cedar pollen allergen by treatment with positive and negative clusters ions. Int. Arch. Allegy Immunol., **141**, 313-321.

Nishikawa, K. and Nojima, H. (2001): Air purification effect of positively and negatively charged ions generated by discharge plasma at atmospheric pressure. Jpn. J. Appl. Phys., **40**, L835-L837.

Nishikawa, K. and Yagi, H. (2006): Bactericidal effects of positively and negatively charged cluster ions generated by plasma discharge at atmospheric pressure. Proc. UT Sympo. NanoBio. Integration (NANOBIO-TOKYO), 337-340.

Nishikawa, K. (2013): Air purification technology of positively and negatively charged cluster ions by plasma discharge at atmospheric pressure. J. Plasma Fusion Res., **89**, 164-168.

Nishitani, M., Sugino, T., Kadowaki, T., Sakikawa, N., Nishikawa, K. and Kajimoto, O. (2010): Effects of plasma cluster ions on moisturizing skin and increasing comfort sensation (in Japanese). Jpn. J. Compl. Alternative Med., **7**, 113-116.

Nojima, H. and Nishikawa, K. (2002): Air purification technology using ions generated by discharge plasma at atmospheric pressure (in Japanese). J. Inst. Electrostat. Jpn., **26**, 153-157.

Pedersen, T. and Peters, H. (1968): Proposal for a classification of oocytes and follicles in the mouse ovary. Reprod. Fertil., **17**, 555-557.

Salewski, E. (1964): Färbemethode zum makroskopischen Nachweis von Implantations-stellen am Uterus der Ratte. Arch. Exp. Pathol. Pharmakol., **247**, 367.

Takasawa, H., Wako, K., Sato, Y., Takashima, R., Hattori, A., Kawabata, M., Tsuchitani, M., Wako, Y., Yamada, N. and Hamada, S. (2011): Air containing ions generated by electric discharge has no potential to induce DNA damage in lung cells in rats and mice. Genes and Environment., **33**, 96-102.

Yamamoto, D., Wako, K., Sato, Y., Fujishiro, M., Matsuura, I. and Ohnishi, Y. (2014): Positive and negative ions by air purifier have no effects on embryo-fetal development in rats. J. Toxicol. Sci., **39**, 447-452.