Genes and Environment, Vol. 33, No. 3 pp. 96–102 (2011)

Regular article

Air Containing Ions Generated by Electric Discharge Has No Potential to Induce DNA Damage in Lung Cells in Rats and Mice

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(Received March 1, 2011; Revised May 18, 2011; Accepted June 6, 2011)

The comet assay is widely used as an *in vivo/in vitro* genotoxicity test to detect initial DNA damage in a single cell. Air containing ions generated by electric discharge was examined in lung comet assays in rats and mice. Using whole-body inhalation, the animals were continuously exposed to the ionized air (abbreviated as IONA) for 48 h at the concentrations range $100–700 \times 10^4$ ions/cm^3. Lung cell samples were prepared by mincing, and blood cells were also sampled and diluted with mincing buffer to confirm the results obtained with lung cells. Slide preparation and electrophoresis for the comet assay were conducted in accordance with the standard protocol (Version 14.2) provided by the Validation Management Team of the ''International Validation Study of *In Vivo* Alkaline Comet Assay'' controlled by the Japanese Center for the Validation of Alternative Methods (JaCVAM). Results of the lung comet assays in both rats and mice showed that the IONA exposure induced no significant differences in tail migration (tail length) or DNA-percentage in tail. In contrast, ethyl methanesulfonate used as the positive control gave clear positive responses in the assays. In the IONA-exposed groups, no abnormal changes were noted in clinical observations or body weight for rats or mice. Additionally, no IONA exposure-related abnormalities were detected in histopathological examinations in either species. Taken together, these results indicate that IONA has no potential to induce DNA damage in the lungs and has no effect on the general health conditions of animals, body weight or histopathology in the lungs under the conditions employed in this study.

Key words: *in vivo* comet assay, cluster ion, whole-body inhalation, electric discharge

Introduction

Air infused with positive and negative ions generated by electric discharge has been used in recent years for air cleaners (1,2) to inactivate bacteria, mold and other allergens (3,4), and toxicological assessment of this ionized air has become a point of interest among the general public. The comet assay is regarded as one of the most sensitive and effective methods for detecting initial DNA damage. The assay was first developed by Östling and Johanson (5) as a single cell gel electrophoresis technique for detecting DNA damage. Subsequently Singh *et al.* (6) introduced a single cell gel technique involving electrophoresis under alkaline (pH > 13) conditions, and the assay has been reviewed by a number of researchers (7–10). In the present study, slide preparation and electrophoresis conditions for the comet assays were in accordance with the methods prescribed in the standard protocol provided by the Validation Management Team (VMT) of the International Validation Study of *In Vivo* Alkaline Comet Assay (Version 14.2) (11), controlled by the Japanese Center for the Validation of Alternative Methods (JaCVAM), using single lung cells, the major tissue receiving the first contact on inhalation exposure to a chemical. The International Validation Study is expected to have an influence when issuing the comet assay guideline and in the regulatory sciences. Accordingly, the present investigation was intended to be conducted in accordance with the standard protocol (Version 14.2) (11) of the International Validation Study described above in as much as possible. However, the standard protocol describes only preparations for single cells of the liver and glandular stomach, as noted in previous reports related to the comet assay (12–15). Given that the comet assay can typically be conducted using any tissues from experimental animals

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provided a sufficiently high-quality single cell suspension can be prepared, we simply and slightly modified the liver suspension protocol (11) while making reference to the method of Sasaki *et al.* (16) to prepare the single lung cell samples. Here, lung comet assays were performed to evaluate the effects of ionized air in rats and mice exposed by whole-body inhalation. To our knowledge, this paper is the first report of no DNA damaging effects of cluster ions in experimental animals.

Materials and Methods

Test compounds: Air containing positive and negative ions generated by electric discharge devices for air cleaners (1,2) developed by Sharp Corporation (Osaka, Japan) is defined here as ''ionized air (IONA)''. The IONA, which includes $[(H_2O)_mH]^+$ and $[(H_2O)_nO_2]^-$ as positive and negative ions, respectively, with ''m'' and ''n'' as natural numbers, are cluster-shaped and are known as ''PlasmaCluster Ions®'' (2–4). Animals were continuously exposed to the IONA by whole-body inhalation at concentrations of 0, 100×10^4, 500×10^4 and 700×10^4 ions/cm^3 for 48 h. The high exposure concentration of the IONA was set at a several hundred-fold higher than that normally used in human life ($0.5–2.0 \times 10^4$ ions/cm^3). Even the low concentration was 50- to 200-fold higher than that normally used. The exposure period was set for 48 h, corresponding to consecutive 2 days according to the method (12) referring to the standard protocol of the International Validation Study. IONA concentrations were measured using ion-counters placed at six points in the chamber housing animals during the exposure period. Ethyl methanesulfonate (EMS, CAS No.: 62–50–0) was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA) for use as a positive control in this investigation, given its clear positive response in a preliminary lung comet assay conducted. EMS was dissolved in physiological saline to prepare a 20-mg/mL solution, which was administered by oral gavage at 10 mL/kg (200 mg/kg) of animal body weight at 48, 24 and 3 h before sacrifice.

Animals: Male Crl:CD(SD) rats and male Crlj:CD-1(ICR) mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). After at least seven days of quarantine and acclimation, whole-body inhalation and EMS administration were started at the age of eight weeks. Throughout the experimental period, except during the whole-body inhalation period, each animal was housed individually in a polycarbonate cage with hard-wood bedding in an air-conditioned room (set at 22 ± 3°C and 55 ± 20% relative humidity) under a 12-h light-dark cycle and allowed free access to feed and drinking water. During the whole-body inhalation period, each animal was housed individually in a wire-netting cage in an inhalation chamber, and room temperature, relative humidity and oxygen concentration were monitored in the chamber. All animals were randomly assigned to treatment groups based on the body weight measured before the beginning of inhalation and administration. Animals were also weighed and checked for clinical signs just prior to sacrifice. In the EMS group, the animals were additionally weighed 24 h before sacrifice. All procedures involving live animals were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at the test facility and in accordance with the guidance of the animal experiments issued by the IACUC.

Experimental design and whole-body inhalation

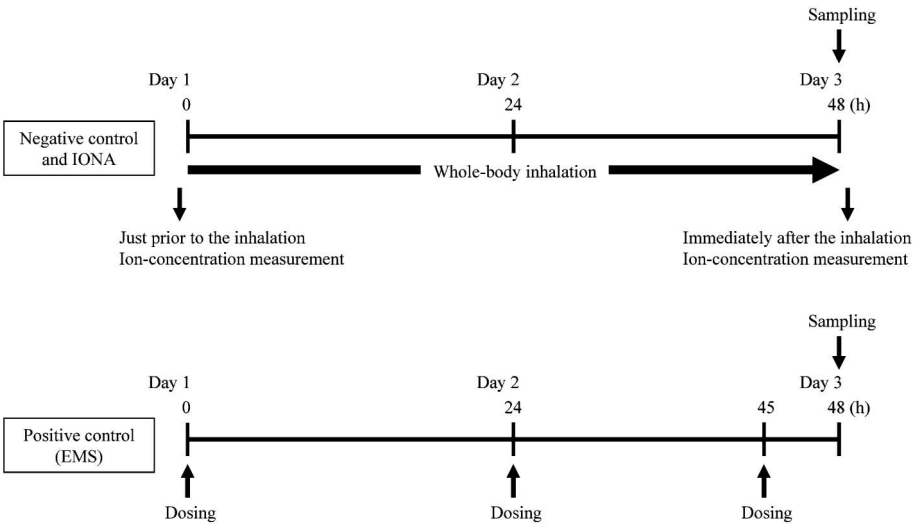

Fig. 1. Study schedule of the comet assay. The animals in the negative control and IONA groups were continuously exposed by whole-body inhalation for 48 h. In the positive control group, each animal received EMS by oral gavage at 3, 24 and 48 h before sampling. The lung cells and blood cells were collected from each animal 48 h after the beginning of the treatment.

exposure to IONA: The study schedule is shown in Fig. 1. Briefly, 5 animals per group were continuously exposed by whole-body inhalation for 48 h to either IONA or air not including ions, and 5 animals in the positive control group received EMS by oral gavage at 3, 24 and 48 h before sampling. Sampling was conducted 48 h after beginning of IONA exposure or EMS treatment. Ion concentrations of IONA were measured before and after the exposure.

Comet assay: Reagents and single cells were prepared in accordance with the standard protocol of the International Validation described above. Animals were euthanized after 48-h whole-body inhalation or at 3 h after the third administration of EMS as follows. Blood was first collected from the vena cava under anesthesia using a syringe rinsed with sodium heparin, and then the animals were subjected to euthanasia. The lungs were then removed, and the left lung was excised for the comet assay while the right lung was fixed with 10% phosphate buffered formalin for histopathological examination. The removed left lung was minced in ice-cold mincing buffer (20 mM EDTA-Na_2 and 10% DMSO in Hank's balanced salt solution, pH 7.5) using scissors to release the lung cells. The cell suspension was strained through a BD Falcon™ cell strainer (pore size: 40 μm, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the number of cells in the suspension was provided to approximately 2.0×10^5 cells/mL (lung sample). Collected blood was diluted 10-fold with ice-cold mincing buffer (blood sample). Single cell preparation was conducted within 1 h after euthanasia. The lung and blood samples were coded to avoid revealing the group or concentration to the microscopic observer. A 40-μL aliquot of each sample for each tissue type was mixed with 360 μL of 0.5% (w/v) low-melting agarose gel (Lonza, Basel, Switzerland), and 40 μL of the resulting mixture was then mounted onto wells of 20-well slides (CometSlide HT, 20-well slide; Trevigen Inc., Gaithersburg, MD, USA). The prepared slides were immersed in a chilled lysing solution (100 mM EDTA-Na_2, 2.5 M NaCl, 10 mM Tris hydroxymethyl aminomethane, 1% Triton X-100, 10% DMSO, pH 10.0) overnight ($\geq$14 h) shielded from light in a refrigerator. After the lysing treatment, the slides were immersed in an alkaline solution (300 mM NaOH, 1 mM EDTA-Na_2, pH $>$ 13) for 20 min to unwind double strand DNA, and then electrophoresed with a constant voltage at 0.7 V/cm for 20 min at approximately 0.3 A. The temperature of the alkaline solution was maintained at $\leq$10°C during the unwinding and electrophoresis processes. At the comet analysis, the slides were stained with SYBR Gold (Invitrogen-Molecular probes™; Life Technologies Corporation, Carlsbad, CA, USA), and the cells (comets) were measured using a charge-coupled device digital camera linked to an image analyzer system (Comet Assay IV™; Perceptive Instruments Ltd., Suffolk, UK) and a fluorescence microscope (×200). Comet parameters were measured with regard to tail length and the DNA-percentage (% DNA) in the tail in 100 comets for each sample (tissue type/animal). ''Hedgehogs,'' defined as heavily damaged cells whose microscopic image consists of small or non-existent heads with large and diffuse tails were excluded from the data collection; however, the frequency of ''hedgehogs'' was still determined separately in scoring 100 cells per sample.

Necropsy and histopathology: The thoracic-abdominal cavities of the treated animals were examined at necropsy. The right lung was fixed with 10% neutral phosphate buffered formalin and processed by the standard method for paraffin embedding and hematoxylin and eosin staining. Histopathological examination was conducted on the right lung under a light microscope.

Statistical analysis: Statistical analyses were conducted for comet assay parameters and body weight. The negative control group and IONA groups were compared using the multiple comparison test. With regard to variation, homo- or heterogeneity was initially determined using Bartlett test. One-way analysis was then applied in cases of homogeneous variance and the Kruskal-Wallis test for heterogeneous variance. If statistical significance was detected, Dunnett's multiple comparison test was used. Negative and positive control groups were compared by first examining the between-group variance using the F test. Student's *t*-test was then applied in cases of homogeneous variance and Aspin-Welch's *t*-test for heterogeneous variance.

Results

Concentration of exposed ions: Measured ions actually exposed to animals are shown in Table 1. Numbers of the counted ions in the IONA groups were actually higher than the nominal concentrations, and the actual concentrations were comparable between pre- and post-exposure in all IONA groups. Additionally, the coefficient variance (C.V.) values were $<$20% in the low-concentration group and $<$10% in the middle- and high-concentration groups. In contrast, no ions were detected in the negative control groups for rats or mice.

Environmental housing conditions during whole-body inhalation: In the rat and mouse experiments during whole-body inhalation, actual room temperatures were approximately 24°C and relative humidity ranged from 40% to 50%. Measured oxygen concentrations were around 21%.

Clinical observations and body weights: Clinical signs in all animals were checked on Day 3 before sacrifice, and no abnormal clinical signs were observed in rats and mice. The initial mean body weights in the examination groups ranged from 327–336 g and 35.9–36.7 g in rats and mice, respectively. On the day of

Table 1. Concentration of cluster ions exposed by whole-body inhalation to rats and mice

Group	Nominal conc. ($\times 10^4$ ions/cm^3)	Type of ion	Rats				Mice			
			Pre-exposure		Post-exposure		Pre-exposure		Post-exposure	
			Actual conc.* (Mean ± S.D.)	C.V. (%)	Actual conc.* (Mean ± S.D.)	C.V. (%)	Actual conc.* (Mean ± S.D.)	C.V. (%)	Actual conc.* (Mean ± S.D.)	C.V. (%)
Negative control	0	Positive	0	—	0	—	0	—	0	—
		Negative	0	—	0	—	0	—	0	—
Low conc.	100	Positive	137 ± 21	15	144 ± 22	15	137 ± 21	15	171 ± 17	10
		Negative	147 ± 0	0	172 ± 27	16	163 ± 25	15	196 ± 31	16
Middle conc.	500	Positive	554 ± 22	4	547 ± 21	4	581 ± 31	5	547 ± 21	4
		Negative	572 ± 40	7	580 ± 48	8	572 ± 51	9	580 ± 37	6
High conc.	700	Positive	745 ± 17	2	738 ± 52	7	745 ± 17	2	745 ± 17	2
		Negative	760 ± 27	4	751 ± 40	5	743 ± 20	3	743 ± 20	3

*Mean concentration calculated from 6 measurement-points (Unit of the actual concentration is "$\times 10^4$ ions/cm^3"). C.V.: Coefficient variation.

Table 2. Results of comet assay in rats; lung and blood

Group	Nominal conc. ($\times 10^4$ ions/cm^3)	Lung			Blood		
		Tail length (μm)	% DNA in tail	Frequency of hedgehog (%)	Tail length (μm)	% DNA in tail	Frequency of hedgehog (%)
Negative control	0	19.63 ± 1.95	4.71 ± 0.46	0.0–0.0	5.79 ± 1.50	0.77 ± 0.16	0.0–0.0
Low conc.	100	16.77 ± 1.03	4.12 ± 0.42	0.0–1.0	6.24 ± 1.77	0.79 ± 0.20	0.0–1.0
Middle conc.	500	17.75 ± 0.25	4.57 ± 0.80	0.0–2.0	5.75 ± 0.70	0.82 ± 0.15	0.0–0.0
High conc.	700	17.90 ± 1.17	4.23 ± 1.07	0.0–1.0	5.60 ± 1.47	0.93 ± 0.38	0.0–1.0
Positive control	EMS (200 mg/kg)	47.37 ± 2.83	25.12 ± 2.24	0.0–1.0	42.42 ± 2.71	19.34 ± 0.97	5.0–10.0

Table 3. Results of comet assay in mice; lung and blood

Group	Nominal conc. ($\times 10^4$ ions/cm^3)	Lung			Blood		
		Tail length (μm)	% DNA in tail	Frequency of hedgehog (%)	Tail length (μm)	% DNA in tail	Frequency of hedgehog (%)
Negative control	0	14.45 ± 2.16	3.46 ± 0.51	0.0–1.0	10.01 ± 1.50	0.69 ± 0.23	0.0–0.0
Low conc.	100	13.29 ± 2.26	2.51 ± 0.81	0.0–0.0	10.02 ± 1.61	0.74 ± 0.11	0.0–0.0
Middle conc.	500	14.02 ± 2.36	2.63 ± 0.52	0.0–0.0	11.09 ± 1.82	0.69 ± 0.21	0.0–0.0
High conc.	700	13.67 ± 3.09	3.30 ± 0.99	0.0–0.0	11.24 ± 1.68	0.89 ± 0.17	0.0–1.0
Positive control	EMS (200 mg/kg)	36.27 ± 5.45	12.81 ± 1.29	0.0–3.0	34.02 ± 1.66	14.74 ± 3.06	0.0–3.0

sacrifice, the mean body weights in the negative control and the IONA exposure groups were in the ranges of 338–343 g and 35.6–37.0 g, respectively. No exposure-related changes were observed in the mean body weights of any IONA groups of rats and mice. However, in the positive control group of rats, the mean body weight decreased during the experimental period and was significantly lower than in the negative control group before sacrifice (the mean body weight: 287 g), whereas the change was not observed in the positive control group of mice (the mean body weight: 36.6 g).

Comet assay: Analytical results of the lung and blood comet assays in rats are shown in Table 2, while results in mice are shown in Table 3. Additionally, the results for the lung assays in rats and mice are also shown in Fig. 2. Of the comet, tail length and % DNA in tail in the lung cells of negative control group of rats were 19.63 ± 1.95 μm and 4.71 ± 0.46, respectively, and those of mice were 14.45 ± 2.16 μm and 3.46 ± 0.51. No significant differences in both of the comet parameters were observed between the IONA groups and the negative control group in the lung cells of rats and mice. Negative results were also obtained in the blood cells of rats and mice. Frequencies of hedgehogs scored in rats and mice are shown in Tables 2 and 3, respectively, ranging from 0% to 2% in all of the samples including lung cell and blood cells of the IONA exposed and negative control groups of both species. This finding there-

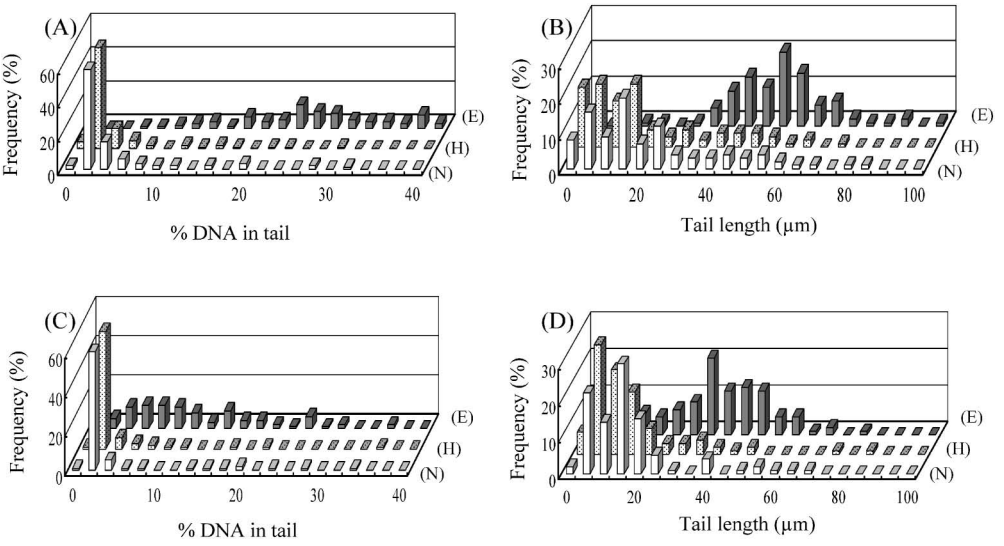

Fig. 2. Histogram of the lung comet assay on Plasma Cluster Ions. The lung cells were collected from rats (A and B) and mice (C and D). The distributions of the figures are shown using the data obtained from 500 cells/group (100 cells/animal × 5 animals). (N): Negative control group at 0 ions/cm^3; (H): High concentration group at 700×10^4 ions/cm^3; (E): EMS-treated group at 200 mg/kg/day.

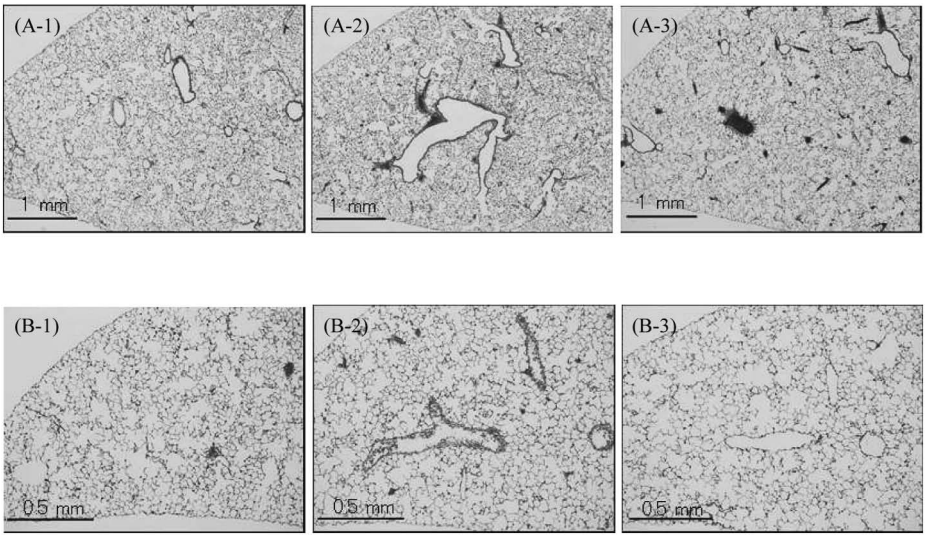

Fig. 3. Histopathological photographs of lung/bronchus in rats (A-1 to A-3) and mice (B-1 to B-3). (A-1) and (B-1): Negative control group at 0 ions/cm^3; (A-2) and (B-2): High concentration at 700×10^4 ions/cm^3; (A-3) and (B-3): EMS-treated group at 200 mg/kg/day. No abnormal findings were detected in any groups.

fore indicates that IONA had no cytotoxic effects on the lungs or blood.

In contrast, EMS induced clear positive results in both of the comet parameters, tail length and % DNA in tail, of lung cells and blood cells of rats and mice. The frequency of hedgehogs was increased in the blood cells of rats, but not in the lungs of rats and mice or blood of mice, indicating that EMS was slightly cytotoxic to the blood cells of rats.

Histopathology: No abnormalities were observed in the histopathological examination of the lung of rats or mice in any of control or experimental groups (Fig. 3).

Discussion

In this investigation, rats and mice were exposed to air containing ions generated by electric discharge devices (1,2) at the high concentration atmosphere by whole-body inhalation. The concentrations of IONA were measured with ion-counters commonly used for the environmental assessment, and it was confirmed that the positive- and negative-ion concentrations were ap-

proximately $100–700 \times 10^4$ ions/cm^3. It was reported that no other ions than intended were generated from the same electric discharge devices for the air cleaners (2). When the devices are working in operation, the ion-counter shows a high ion-count, but when the devices are not, the ion-counter indicates zero. Thus, the air exposed to the animals in this investigation was defined as ''ionized air (IONA)''. The results of ion count indicated that the animals in the present examination were successfully and stably exposed to uniform ions via the whole-body inhalation (Table 1). The housing conditions during the whole-body inhalation exposure were considered appropriate for the animals, and the environmental housing conditions during the exposure were considered not to have had any adverse effects on the experimental results.

Blood comet assays are nearly completely devoid of non-specific factors or artifacts in tissue sampling or single cell preparation procedures, because blood is usually collected directly from the vein and simply diluted with mincing buffer to prepare single cells. Therefore, we performed comet assay in the lung where cells directly first contact to the IONA and blood cells where intervening of the non-specific factor can be excluded. We detected no DNA damaging activity of IONA in the lung cells of rats and mice and also in blood cells of rats and mice. Frequencies of hedgehogs in the negative control group for the lung comet assays in rats and mice were comparable to those in the blood comet assays, and they did not increase by exposure to IONA, indicating that IONA did not show cytotoxic effects in the lung cells and blood cells of rats and mice (Tables 2 and 3). Further, no inhalation-related abnormalities were detected by the histopathological examination of the lung tissues of rats and mice (Fig. 3).

As for comet parameters in the lung cells and blood cells of negative controls, the values obtained were different between these tissues; tail length were 19.63 ± 1.95 μm and 5.79 ± 1.50 μm, and % DNA in tail were 4.71 ± 0.46 and 0.77 ± 0.16, in lung cells and blood cells, respectively. Although the values obtained in these two tissues were relatively different, variation coefficients of the parameters in the lung cells were less than 10% in rats. Similar results were obtained in mice, and the variation coefficients of the comet parameters were less than 15% in the lung cells. On a separate note, it was admitted that some blood may have contaminated the lung samples; however, based on the low variation coefficient, it was ascertained that this possibility does not merit extensive consideration.

It was also considered that the procedures for tissue sampling and single cell preparation had no adverse effects on the results of the lung comet assay. The lung comet assays in rats and mice in the negative control groups showed approximately 4–5% DNA in the tail (Tables 2 and 3). According to the standard protocol (Version 14.2), the negative control values for average % DNA in tails should be 1–8% and 1–20% in the liver and glandular stomach, respectively. Values in the liver and stomach are typically around 1–2% and 10% at the test facility, respectively. However, we maintain that the values of 3–5% obtained from the negative control groups in the present study were reasonable given the tissue characteristics of the lung compared to the liver; substantial organ and glandular stomach using mucous membrane.

These results from the negative and positive control groups underscored that the validity of this examination.

Airborne particles such as molds and bacteria are assumed to be inactivated by radicals generated on combining positive and negative cluster ions at the surface of the particles (3). The concentrations of ions usually used in air cleaners range $0.5–2.0 \times 10^4$ ions/cm^3. The radicals become extinct at the moment these radicals collide with something such as an airborne particle possibly preventing them from entering the animal body, including humans. The negative results obtained in the present study were therefore expected.

A potential limitation of the present study was the fact that the positive control was administered by oral gavage and not inhalation exposure. Achieving exposure by a route similar to that of the test compound, inhalation exposure or intratracheal route is an issue that must be addressed in future studies.

Given the results of this lung comet assay in rats and mice exposed by whole-body inhalation, it was determined that IONA had no effects on clinical signs, body weight or histopathological examination results under the conditions employed in the present study; using the concentrations 350- to 1400-fold higher than concentrations usually applied to humans (700×10^4 ions/cm^3 vs. $0.5–2.0 \times 10^4$ ions/cm^3). It was reported that the life span of the generated ions is approximately 5 sec (2); however it is unknown whether the cluster ions directly reach the lung of exposed animals.

It was concluded that under the conditions employed, IONA showed no DNA damaging potential in the lungs of rats and mice.

References

1 Nishikawa K, Nojima H. Air purification effect of positive and negative charged ions generated by discharge plasma at atmospheric pressure. Jpn J Appl Phys. 2001; 40: 835–7.
2 Nojima H, Nishikawa K. Air purification technology using ions generated by discharge plasma at atmospheric pressure (in Japanese). J Inst Electrostatics Jpn. 2002; 26: 153–7.
3 Nishikawa K, Yagi H. Bactericidal effects of positively

and negatively charged cluster ions generated by plasma discharge at atmospheric pressure. Proc UT Sympo NanoBio Integration (NANOBIO-TOKYO). 2006; 337–40.
4 Kawamoto S, Oshita M, Fukuoka N, Shigeta S, Aki T, Hayashi T, Nishikawa K, Ono K. Decrease in the allergenicity of Japanese cedar pollen allergen by treatment with positive and negative cluster ions. Int Arch Allergy Immunol. 2006; 141: 313–21.
5 Östling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun. 1984; 123: 291–8.
6 Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988; 175: 184–91.
7 McKelvey-Martin VJ, Green MHL, Schmezer P, Pool-Zobel BL, De Méo MP, Collins A. The single cell gel electrophoresis assay (comet assay): a European review. Mutat Res. 1993; 288: 47–63.
8 Fairbairn DW, Olive PL, O'Neill KL. The comet assay: a comprehensive review. Mutat Res. 1995; 339: 37–59.
9 Tice RR. The single cell gel/comet assay: A microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In: Phillips DH, Venitt S, editors. Environ mutagen. Oxford: BIOS Scientific Publishers; 1995. p. 315–39.
10 Anderson D, Yu T-W, McGregor DB. Comet assay responses as indicators of carcinogen exposure. Mutagenesis. 1998; 13: 539–55.
11 The Standard Protocol of the International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Version 14.2) issued by the Validation Management Team (VMT), November 30, 2009.
12 Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. Recommendations for conducting the *in vivo* alkaline comet assay. Mutagenesis. 2003; 18: 45–51.
13 Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A. Fourth International Workgroup on Genotoxicity Testing: Results of the *in vivo* comet assay workgroup. Mutat Res. 2007; 627: 31–5.
14 Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen. 2000; 35: 206–21.
15 Wiklund SJ, Agurell E. Aspects of design and statistical analysis in the comet assay. Mutagenesis. 2003; 18: 167–75.
16 Sasaki FY, Tsuda S, Izumiyama F, Nishidate E. Detection of chemically induced DNA lesions in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow) using the alkaline single cell gel electrophoresis (Comet) assay. Mutat Res. 1997; 388: 33–44.