

## PULMONARY EFFECTS OF SODIUM FLUORIDE AEROSOL ON RATS

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**SUMMARY:** Five-week-old male Fischer rats were exposed 3 hr per day for either 5 or 10 days to three concentrations of sodium fluoride aerosol (1.11, 3.33, and 10.3 mg F/m<sup>3</sup>). A significant increase in lung weight was observed at the highest concentration. The activity of lactate dehydrogenase (LDH) and total protein concentration in bronchoalveolar lavage (BAL) fluid were also significantly higher in rats exposed to 3.33 and 10.3 mg F/m<sup>3</sup>. Neutrophil phagocytic activity in the blood was stimulated by 10-day exposure, and a significant increase in bactericidal activity and superoxide production was observed by exposure to 3.33 mg F/m<sup>3</sup> but not to 10.3 mg F/m<sup>3</sup>.

Keywords: Aerosol fluoride, Airborne fluoride, Bronchoalveolar lavage, Lactate dehydrogenase, Neutrophil stimulation, Phagocytic activity, Pulmonary effects, Rat lungs.

### INTRODUCTION

Fluoride emissions are well known to occur as airborne pollutants during the production of aluminum, ceramics, glass, brick products, and phosphate fertilizers.<sup>1</sup> In one study fluoride concentrations in the plasma of workers in an aluminum plant increased significantly after exposure to 0.91 mg F/m<sup>3</sup> for 8 hr.<sup>2</sup> Another study reported a strong relation between inhaled HF and plasma fluoride concentrations in volunteers exposed to 0.2-5.2 mg HF/m<sup>3</sup> for only 1 hr.<sup>3</sup>

In China, coal with fluoride contents ranging from less than 100 ppm to several thousand ppm is a main source of fuel energy for both industrial and domestic purposes.<sup>4</sup> In some rural areas, coal containing high concentrations of fluoride is used for cooking, heating and crop drying without adequate ventilation. Fluoride from such coal burning is directly inhaled, and the airborne fluoride is easily absorbed in food drying and storage.<sup>4-8</sup> In these areas the incidence of respiratory symptoms (cough and chronic respiratory disease) is significantly higher than in non-polluted areas.<sup>9</sup> It has also been reported that the serum lactate dehydrogenase (LDH) activity in patients with skeletal fluorosis living in coal-burning areas is likewise higher than that in healthy adults.<sup>10</sup>

To obtain more adequate data for evaluating potential toxic effects of airborne fluoride in humans, the present study was designed as an extension of our earlier one with mice<sup>11</sup> to investigate biochemical changes in rats following exposure to various concentrations of NaF aerosols. Included are determinations of effects on certain organs, metabolic processes, neutrophil phagocytic activity, and superoxide production.

### MATERIALS AND METHODS

*Animals and exposure conditions:* Five-week-old SPF-grade male Fischer rats (Clea Japan Inc., Tokyo) were housed in stainless steel cages, 5 or 6 per cage,

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at  $22 \pm 1^\circ\text{C}$  and  $50 \pm 10\%$  relative humidity in a 12-hr light-dark cycle. The animals were given distilled water to drink and were fed AIN-93M purified diet (Oriental Yeast Co. Ltd., Tokyo) containing 1.0 mg F/kg diet. Water and food were given *ad libitum* before and after but not during exposure as previously reported for mice.<sup>11</sup>

All animals were 8 weeks old when exposure to airborne fluoride 3 hr/day for 5 or 10 days began. The concentrations of sodium fluoride (NaF) aerosol in a  $0.256\text{-m}^3$  inhalation chamber (Sibata Co., Tokyo) were 1.11, 3.33, and  $10.3\text{ mg F/m}^3$  prepared from 0.01, 0.03, and 0.1 M NaF as described below. Each of the four groups of 6 rats were sacrificed on the final day of exposure and used for bronchoalveolar lavage (BAL) and measurement of blood neutrophils, urinary excretion of fluoride, bone fluoride, and organ weights. Three groups of 5 rats each were separately exposed to  $10.3\text{ mg F/m}^3$  for 5 or 10 days to determine lung weight, urinary excretion of fluoride, and bone retention of fluoride. In this experiment, 5 rats were allowed to live 6 days after 10 days of exposure to determine further changes in lung weight and in urinary excretion and bone retention of fluoride. The three aerosol fluoride concentrations were generated by atomizing solutions of 0.01, 0.03 and 0.1 M NaF in distilled and deionized water and then dehydrating the aerosol to form submicron particles.<sup>11</sup>

*Collection of bronchoalveolar lavage fluid:* After 5 or 10 days exposure, the animals were weighed, anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and sacrificed by severing the abdominal aorta. The thorax was opened, and the lungs were lavaged three times with  $0.035\text{ mL/g}$  body weight of warm ( $37^\circ\text{C}$ )  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS (-) pH 7.3-7.65, Nissui Pharmaceutical Co. Ltd., Tokyo). The first lavage was kept separate, and the other lavage fluid were pooled for each rat. The BAL fluid was centrifuged at 400 g for 10 min at  $4^\circ\text{C}$ , and the first tube of supernatant was used to determine lactate dehydrogenase (LDH) activity and protein concentration.

*Determination of LDH activity and protein concentration in the culture supernatant:* LDH activity in the supernatant was determined by kinetic analysis at  $35^\circ\text{C}$  (LDH-UV-Wako, Wako Pure Chemical Ltd., Osaka, Japan) using a computer-assisted spectrophotometer (UV-2200, Shimadzu Co. Kyoto, Japan), to measure the absorption decrease of the solution after 2 min at 340 nm. The activity of LDH was expressed as IU per liter of supernatant. The total protein concentration of each supernatant was determined at 562 nm using an assay kit (BCA Protein Assay Kit, Pierce Co., Rockford, IL, USA). Bovine serum albumin was used as the standard. The protein concentration was expressed as mg per liter of the culture supernatant.

*Measurement of phagocytic activity and superoxide productivity of neutrophils:* Phagocytic activity and superoxide productivity of neutrophils were measured by the nitroblue tetrazolium reduction test (NBT).<sup>12</sup> A  $25\text{-}\mu\text{L}$  sample of blood from tail veins and an equal volume of  $1\text{ }\mu\text{g/mL}$  NBT (Sigma Chem. Co., St.

Louis, USA) in 1/15 M phosphate-buffered saline (PBS, pH 7.2) with *Staphylococcus aureus* 209P ( $2 \times 10^9$  cfu[colony-forming units]/mL) were mixed in 75  $\mu$ L of heparinized capillary. The mixture was incubated at 37°C for 20 min. Then, smears were prepared, air-dried, and counterstained with Wright solution and Giemsa solution. The smears were observed under a microscope objectives of 1,000 magnifications (oil immersion). One hundred neutrophils of each smear were counted, and the neutrophil phagocytic activity was determined from the number of ingested particles per cell and the superoxide productivity from the NBT score as measured by the sum of the degree of formazan deposits.

*Determination of fluoride concentration:* Fluoride aerosol in the chamber was sampled by an AND sampler (Sibata Co., Tokyo). Fluoride concentrations in all samples were determined with a model 9609BN combination fluoride ion selective and a 720A pH/ISE meter (Orion Research, Boston, USA) as in our previous report.<sup>11</sup>

*Statistical Analysis:* All data was presented as means  $\pm$  SD, and analyzed using Stat-View software (Abacus Concepts, Inc. Berkeley, CA, USA). The statistical analyses were performed by Bonferroni/Dunn to compare mean values between control and F-exposed groups. Probability values less than 0.05 were accepted as indicative of a significant difference.

## RESULTS

The effects of exposure to sodium fluoride aerosol (1.11, 3.33 and 10.3 mg F/m<sup>3</sup>) on body, liver, kidney, and spleen weights were determined in rats following the treatment, but the effect of exposure to NaF on lung weight was determined only in rats exposed to 10.3 mg F/m<sup>3</sup> (Table 1). The relative lung weight increased significantly with exposure time, being 35.4% and 40.5% higher than that of controls following 5 and 10 days of exposure, respectively. After the exposure, the relative lung weight decreased during maintenance for 6 days in clean air and was only 7% higher than that of control rats, although the difference was statistically significant. The decrease in lung weight suggested that the animals recovered from the lung injury/edema. At the lower concentrations of 1.11 and 3.33 mg F/m<sup>3</sup>, no exposure-related changes in liver weights were found. With exposure to 10.3 mg F/m<sup>3</sup> for 10 days, however, significant decreases were observed in liver and kidney weights. Moreover, significant increases in both liver and kidney weights were noted in rats maintained 6 days in clean air after exposure. Spleen weights relative to body weights were not significantly affected in any group of F-exposed rats.

Exposure of animals also had an effect on urinary fluoride excretion. As seen in Figure 1, among the animals exposed to 3.33 and 10.3 mg F/m<sup>3</sup> there was a significant increase in urinary fluoride excretion by the first day after exposure. The urinary fluoride excretion was  $33.3 \pm 5.8$   $\mu$ g F/day in rats following 10 days of exposure to 10.3 mg F/m<sup>3</sup>. In clean air, it was reduced by 58% 4 days after the exposure ended ( $13.9 \pm 3.2$   $\mu$ g F/day). On the sixth day after exposure ended, it was  $11.5 \pm 1.4$   $\mu$ g F/day, the same as the mean value ( $11.5 \pm 1.9$   $\mu$ g

F/day) of rats following one day exposure to the lowest concentration of NaF (1.11 mg F/m<sup>3</sup>). Similar results were found in bone fluoride retention (Table 2). Bone fluoride retention significantly increased in rats exposed to 3.33 and 10.3 mg F/m<sup>3</sup>. It was 544 ± 16 mg/kg in rats exposed to 10.3 mg F/m<sup>3</sup> for 10 days, and six days after exposure ended it decreased to 468 ± 48 mg/kg (group E).

**Table 1.** Effect of inhalation exposure to NaF on organ weights in rats

Group	Exposure mg F/m <sup>3</sup> days		Relative organ weights (mg/g, body weight)			
			Lung	Liver	Kidney	Spleen
<i>Control-1</i>	0		ND	36.9 ± 2.27	6.33 ± 0.28	2.34 ± 0.10
Exp-A	1.11	10	ND	40.5 ± 2.48	6.51 ± 0.21	2.22 ± 0.14
Exp-B	3.33	10	ND	36.1 ± 3.42	6.30 ± 0.21	2.22 ± 0.14
<i>Control-2</i>	0		3.41 ± 0.07	36.2 ± 1.63	6.46 ± 0.17	2.38 ± 0.25
Exp-C	10.3	5	4.62 ± 0.11 <sup>†</sup>	35.3 ± 1.58	6.36 ± 0.16	2.48 ± 0.67
Exp-D	10.3	10	4.79 ± 0.28 <sup>†</sup>	32.8 ± 1.22 <sup>*</sup>	6.21 ± 0.11 <sup>*</sup>	2.32 ± 0.19
Exp-E <sup>a</sup>	10.3	10	3.65 ± 0.07 <sup>†</sup>	38.8 ± 1.66 <sup>*</sup>	6.70 ± 0.09 <sup>*</sup>	2.56 ± 0.27

Values are means ± SD of 5 to 6 rats of each group. <sup>a</sup>The animals of group E were maintained 6 days in clean air after 10 days of exposure. ND: not determined.  
<sup>\*</sup>p<0.01 and <sup>†</sup>p<0.001 compared to control group.

**Figure 1.** Effects of fluoride aerosol inhalation on urinary fluoride excretion

Animals were exposed to 1.11 (filled circles), 3.33 (triangles) or 10.3 mg F/m<sup>3</sup> (squares) for 10 days, or non-exposure (open circles), respectively.

**Table 2.** Effect of inhalation exposure to NaF on bone fluoride retention

Group	Exposure (mg F/m <sup>3</sup> )	No. of days	Bone F (mg/kg)
Control-1	0		373 ± 47
Exp-A	1.11	10	389 ± 19
Exp-B	3.33	10	426 ± 20*
Control-2	0		365 ± 35
Exp-C	10.3	5	459 ± 18 <sup>†</sup>
Exp-D	10.3	10	544 ± 16 <sup>†</sup>
Exp-E <sup>a</sup>	10.3	10	468 ± 48 <sup>†</sup>

Values are means ± SD of 5 to 6 rats. <sup>a</sup>The animals of group E were maintained 6 days in clean air following 10 days of exposure. \*p<0.05, <sup>†</sup>p<0.01 and <sup>‡</sup>p<0.001 compared to control group.

As shown in Table 3, the LDH activity in the supernatant of BAL fluid was significantly increased in F-exposed animals except the rats exposed to the lowest concentration of NaF. The LDH activity was 48% higher than that of controls in rats exposed to 3.33 mg F/m<sup>3</sup>, and 4.2 to 5.2 times higher than that of controls in rats exposed to the highest concentration of NaF for 5 or 10 days, respectively. A linear correlation was observed between exposure and the LDH activity in BAL fluid (Figure 2). A significant increase of total protein concentration was also found in the higher F-exposed animals but not in the rats exposed to the lowest NaF aerosol concentration. It was 1.4 times higher than that of controls in rats exposed to 3.33 mg F/m<sup>3</sup>. Following 5 and 10 days of exposure at 10.3 mg F/m<sup>3</sup>, the total protein concentration was 2.2 and 3.2 times higher than that of control rats, respectively. A linear positive correlation was found between exposure and total protein concentration in BAL fluid (Figure 3).

**Table 3.** Effect of inhalation exposure to NaF on LDH activity and total protein concentration (TP) in BAL fluid of rats

Exposure (mg F/m <sup>3</sup> )	No of days	LDH (IU/L)	TP (mg/L)
0		60 ± 12	177 ± 22
1.11	10	68 ± 14	179 ± 24
3.33	10	89 ± 16*	419 ± 38 <sup>†</sup>
10.3	5	314 ± 48 <sup>†</sup>	559 ± 66 <sup>†</sup>
10.3	10	372 ± 40 <sup>†</sup>	742 ± 79 <sup>†</sup>

Values are means ± SD of 5 to 6 rats. \*p<0.01 and <sup>†</sup>p<0.001 compared to control group.

**Figure 2.** Relationship between fluoride exposure and LDH activity in BAL fluid  
Values are mean  $\pm$  SDs of 6 rats.

**Figure 3.** Relationship between fluoride exposure  
and protein concentration in BAL fluid  
Values are mean  $\pm$  SDs of 6 rats.

The neutrophil phagocytic activity and superoxide productivity in blood are shown in Table 4. Phagocytic activity increased with NaF exposure concentration, and a significant increase was found in rats exposed to 3.33 mg F/m<sup>3</sup>. However, a decreasing trend was observed at the highest concentration of NaF, and the phagocytic activity in rats following 10 days of exposure was lower than that of controls. Superoxide productivity also showed the same tendency as the phagocytic activity.

**Table 4.** Effect of inhalation exposure to NaF on neutrophil phagocytic activity and superoxide productivity in blood of rats.

Exposure (mg F/m <sup>3</sup> )	No of days	Phagocytic activity (particles/cell)	Superoxide production (score/cell)
0		2.83 ± 1.30	0.11 ± 0.06
1.11	10	3.60 ± 0.58	0.10 ± 0.04
3.33	10	4.91 ± 0.95 <sup>†</sup>	0.18 ± 0.07*
10.3	5	3.91 ± 1.76	0.23 ± 0.13*
10.3	10	2.43 ± 1.48	0.16 ± 0.08

Values are means ± SD of 6 rats. \*p<0.05 and <sup>†</sup>p<0.01 compare to control group.

## DISCUSSION

Inhalation of toxic substances in the workplace can result in a variety of respiratory disorders. One relatively rare sequel of the inhalation of toxic fumes is bronchiolitis obliterans, a condition characterized by fibrosis and narrowing of the small airways. Several substances have been reported to cause bronchiolitis obliterans, including ammonia, chlorine, hydrogen fluoride, etc.<sup>13</sup> Risk of occupational exposure to hydrogen fluoride by inhalation has been studied in humans and animals. HF has been shown to cause respiratory symptoms including lung inflammation.<sup>3,13,14</sup> However, reports on inhalation exposure of animals to low concentrations of fluoride aerosol are limited.<sup>11</sup>

The present results show that NaF aerosol increased wet lung weight, in agreement with previous published data from our and other laboratory studies.<sup>11,15,16</sup> Stavert *et al* reported a significant increase of lung wet weight in mouth breathing (MB) rats exposed to 1300 ppm HF (~823 mg/m<sup>3</sup>) for 30 min.<sup>15</sup> Similar results were also found in a study of short-term exposures to airborne HF. The lung weight significantly increased in MB rats exposed to 4887 or 8621 ppm HF for 2 min, and a significant increase was found in spleen weight in MB rats exposed to 8621 ppm HF for 2 min.<sup>16</sup> Furthermore, the decrease in relative lung weight in post-exposure animals in this study suggested that the lung edema might gradually disappear with lung function recovery from damage by inhalation exposure.

With fluoride exposure there should be an increase in the concentration of protein in lavage fluid and in the various mediators of inflammation released by neutrophils and activated macrophages. If cell lysis occurs, or if cell membranes are damaged, leakage of cytoplasmic enzymes such as LDH should occur.<sup>17</sup> Hence analysis of the cellular and biochemical profile in the BAL fluid after exposure is a useful method to characterize the inflammatory response of the lung. This technique has been used to assess induction of lung injury by mineral dusts and metallic compounds.<sup>18,19</sup> LDH is a cytoplasmic enzyme that is released extracellularly by damaged cells, and therefore increased LDH activity in BAL fluid reflects a degree of damage of lung cells and tissue.<sup>20,21</sup> The present results showed that biochemical parameter markedly increases in BAL fluid, which confirms similar findings in studies of rats. Dalbey *et al*<sup>22</sup> exposed MB rats to different concentrations of HF and found significant increases of protein and LDH in BAL fluid in rats exposed to 1764 ppm HF for 10 min, and 1589, 4887 and 8621 ppm HF for 2 min.

Moreover, our study showed that the capacity of neutrophils to ingest increased significantly along with bactericidal activity in rats exposed to 3.33 mg F/m<sup>3</sup>, which might be explained in part by any stimulation. At the highest fluoride concentration, the exposure also induced phagocytic activity, but its decrease was probably due to an impaired capacity of neutrophils to ingest and control bactericidal activity.

In conclusion, the increased LDH activity in BAL fluid reflected the fact that the lung cells and tissue were damaged by exposure to airborne NaF and that the neutrophil phagocytic activity in blood increased following exposure to the fluoride aerosol.

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