

Short Communication

Comparison of acute inhalation toxicity of sulfuric acid by the inhalation and intratracheal instillation methods

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Abstract: Recently, intratracheal instillation has been focused on as a simple, low-cost alternative to the inhalation method. In this study, intratracheal instillation of sulfuric acid, a typical acidic compound, was performed to compare the acute toxicity of acidic compounds that could cause damage to the respiratory system between intratracheal instillation and inhalation. Sulfuric acid was administered to male rats at doses of 0.7, 2, 7, 20, and 60 mg/kg by dividing the total dose into four doses. General condition and body weight were examined up to 14 days after administration, and macropathological and histopathological examinations were performed. The half-lethal dose was then estimated. All animals administered 20 and 60 mg/kg sulfuric acid and one animal administered 2 mg/kg sulfuric acid died within 4 h after administration. No abnormalities were observed in other animals. At 20 and 60 mg/kg, multiple red foci or diffuse red areas were macroscopically observed in the lungs. In these lesions, histopathologically, clefts between the mucosal epithelium and basement membrane and necrosis of the alveolar epithelium were observed. Deaths in these groups may have resulted from lung injury. No notable changes were observed in other animals. Therefore, the half-lethal dose of sulfuric acid by intratracheal instillation was estimated as 7–20 mg/kg. The acute toxicity by intratracheal instillation was evaluated with two-fold sensitivity since the exposure at the half-lethal sulfuric acid concentration in inhalation studies was calculated as 43.2 mg/kg. (DOI: 10.1293/tox.2020-0086; J Toxicol Pathol 2021; 34: 269–273)

Key words: acute inhalation toxicity, inhalation, intratracheal instillation, sulfuric acid

Inhalation is a major route of chemical exposure in industries. From the viewpoint of industrial hygiene, it is important to understand the acute inhalation toxicity properties of chemical substances and appropriately manage working environments to prevent health hazards to workers. Test methods for acute inhalation toxicity are defined in the OECD Guidelines for the Testing of Chemicals (OECD TG403, 436)^{1, 2}. In the typical acute inhalation toxicity study method, animals are exposed to the test substance in the form of gas, vapor, aerosol, or thier mixture using a specialized inhalation chamber connected to a test substance generator and using the whole-body or nose-only mode of exposure. However, it is not possible to assess the acute inhalation toxicity of all chemicals using the typical inhalation method because it can only be performed in a limited number of laboratories that have special techniques and equip-

ment and is quite costly.

In addition to the typical inhalation method described above, intratracheal instillation is another method used to evaluate toxicity by exposing the respiratory system to test substances³. In intratracheal instillation, an administration device is generally inserted into the trachea via the oral cavity of anesthetized animals, and the test substance is forcibly administered into the trachea. It is possible to uniformly expose the test substance to the trachea and lungs. This method can be performed with a simpler procedure and with a smaller amount of test substance than the inhalation method, and it requires no dedicated equipment or facility. Therefore, many studies using this method as an alternative to the inhalation method have been conducted to evaluate inhalation toxicity, especially in the field of nanomaterials^{3–5}.

The upper respiratory tract, trachea, bronchus, and lungs are naturally exposed to the test substance via continuous breathing over a certain time in the inhalation method, whereas only the bronchus and lungs are forcibly and rapidly exposed to the test substance in the intratracheal instillation method^{3, 6}. The risk of overlooking upper respiratory tract toxicity, such as nasal toxicity, is significant. Even if the total lung exposure is the same, intratracheal instillation causes a higher transient exposure than inhalation, and the distribution of test substances in the lungs differs between

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the two methods³. In addition, intratracheal instillation requires the test substance to be dissolved or suspended in an aqueous vehicle, and aggregation can be a problem with highly lipophilic and poorly soluble test substances³. Intratracheal instillation cannot completely replace inhalation due to these limitations, but given its advantages (such as simplicity of the procedure), it is a good screening method for inhalation toxicity. Therefore, to appropriately evaluate acute inhalation toxicity using the intratracheal instillation method as an alternative to the inhalation method, it is important to examine acute toxicity correlations between the two methods. A previous study has shown that intratracheal instillation demonstrated toxic effects at exposures several times lower than that in inhalation in studies of many chemical substances such as pharmaceuticals, pesticides, and hormones⁷.

Acidic compounds are widely used in industries and can cause damage to the respiratory system if inhaled. Thus, the assessment of acute inhalation toxicity of acidic compounds is critical. However, when the inhalation toxicity of acidic compounds is performed by the inhalation method, it may be necessary to modify the devices because it may corrode the device. In contrast, because intratracheal instillation requires no special devices, even acidic compounds can be evaluated. Intratracheal instillation may, thus, be more useful for evaluating acidic compounds. However, there are no reports comparing the intratracheal instillation and inhalation methods for the assessment of acute inhalation toxicity of acidic compounds. Therefore, in the present study, an intratracheal instillation study of sulfuric acid, a typical acidic compound, was conducted to compare acute inhalation toxicity between the two methods.

Sulfuric acid was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Male Sprague-Dawley rats (6 weeks old) were purchased from Charles River Japan (Yokohama, Japan). The animals were group-housed in plastic cages in an air-conditioned room (temperature, 22°C ± 3°C; humidity, 55% ± 15%; light cycle, 12 h/day). Feed (Oriental Yeast Co., Ltd., Japan) and water were available *ad libitum*. The animals were acclimatized for 1 week after receipt and used at 7 weeks of age. All studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Nissan Chemical Corporation (Approval No. A001794) and DIMS Institute of Medical Science, Inc. (Approval No. 17564).

Intratracheal instillation was performed according to a previously reported method^{8,9}. Briefly, rats that were anesthetized with isoflurane (Mylan Seiyaku Ltd., Tokyo, Japan; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were held on a board at an angle of approximately 50° using a rubber band, and the tip of the DIMS-type microsyringe aerosolizer (DIMS Institute of Medical Science, Inc., Aichi, Japan) connected to a disposable syringe was gently inserted into the trachea. Intratracheal instillation was performed by pressing the syringe plunger at a constant speed. Following intratracheal instillation, animals were retained in a similar position until they began to recover from

the intratracheal instillation procedure to prevent backflow and buildup of the administered solution in the bronchi and trachea. After administration, the animals were carefully monitored and immediately euthanized if any symptoms of moribundity or severe pain were observed.

Prior to intratracheal instillation of sulfuric acid, a preliminary study was conducted to investigate the administration frequency. For intratracheal instillation, a test substance is usually administered in a single dose, resulting in a transient higher exposure than that in inhalation. We tried dividing the total dose into several doses with repeated administration for 4 h, which is the guideline-recommended exposure time for acute inhalation studies, to reduce the bolus effect. Physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was administered intratracheally to two rats four times every hour or eight times every 30 minutes at a volume of 2 mL/kg each, and the effects on general condition and body weight were observed up to 14 days after administration. On the 14th day of administration, the animals were euthanized, and gross pathological examinations were performed. No abnormal findings were noted in the general condition or gross pathological examination in either group. In contrast, decreased body weight was observed only in the eight-times-dosing group 1 day after administration. Thereafter, weight gain was observed in both groups, and there was no difference in body weight between the two groups 14 days after administration. Therefore, the administration of physiological saline eight times in 4 h was deemed to have induced stress in the animals, and four times in 4 h was determined to be the appropriate frequency for administration.

The half-lethal concentration (LC₅₀) for 4 h sulfuric acid exposure in acute inhalation toxicity studies in the rat has been reported to be 0.375 mg/L¹⁰, and exposure at LC₅₀ was estimated to be 43.2 mg/kg with the following equation:

$$Exposure_{LC_{50}} = \frac{LC_{50} \times Q \times t \times a}{BW}$$

where Q, t, a, and BW represent rat respiratory volume, exposure time in an acute inhalation toxicity study, the absorption rate of sulfuric acid in inhalation, and rat body weight, respectively. Q, t, and BW were 7.2 L/h¹¹, 4 h, and 0.25 kg, respectively, while a was assumed to be 1 (maximum value). Given that sulfuric acid may result in higher toxicity with intratracheal instillation than with inhalation, the doses of sulfuric acid in our intratracheal instillation study were selected based on 20 mg/kg, which is approximately half of the exposure at LC₅₀ (Table 1). A vehicle control group was also established. In addition, sulfuric acid was administered by dividing the total dose into four doses in 4 h in the intratracheal instillation method based on the results of the preliminary study described above. General condition and body weight were examined for 14 days after administration, and the animals were euthanized under anesthesia with isoflurane on the 14th day. Gross pathological examinations of the major organs in the chest and abdominal cavities were performed in all animals. Subsequently, the lungs and

Table 1. Group Composition: Intratracheal Instillation Study of Sulfuric Acid in Rats

| Group | Dose per once (mg/kg) | Total dose ^{a)} (mg/kg) | pH of dosing solution | Number of animals |
|-----------|-----------------------|----------------------------------|-----------------------|-------------------|
| Control | 0 | 0 | - | 5 |
| 0.7 mg/kg | 0.175 | 0.7 | 2.8 | 5 |
| 2 mg/kg | 0.5 | 2 | 2.3 | 5 |
| 7 mg/kg | 1.75 | 7 | 1.9 | 5 |
| 20 mg/kg | 5 | 20 | 1.5 | 3 ^{b)} |
| 60 mg/kg | 15 | 60 | 1.0 | 3 ^{b)} |

a) Given that acute toxicity by intratracheal instillation may be more pronounced than that by inhalation, three lower doses and one higher dose were set with a common ratio of 3 from 20 mg/kg, which is approximately half of the exposure at LC₅₀ for sulfuric acid in the acute inhalation toxicity study. Moreover, 60 mg/kg was set as the maximum dose because 60 mg/kg is equal to or higher than the exposure at inhalation LC₅₀ for sulfuric acid, and it was assumed that administration at doses >60 mg/kg could cause severe distress to animals.

b) The doses of 20 and 60 mg/kg were determined to be above the LD₅₀ because three animals in these groups died within 4 h after administration. Further administration was discontinued considering animal welfare.

trachea were collected and fixed in 10% neutral-buffered formalin solution. For fixation, in surviving animals, the lungs were inflated and fixed by intrapulmonary formalin injection, whereas in dead animals, they were simply immersed in formalin solution. Hematoxylin and eosin-stained sections of the fixed tissues were prepared, and histopathological examinations were performed. Animals that demonstrated severe deterioration were euthanized and treated in the same manner as dead animals. The half-lethal dose (LD₅₀) for sulfuric acid in intratracheal instillation was estimated from the mortality in each group.

Mortality in each group is shown in Table 2. In the 60 mg/kg group, one animal was euthanized after the second administration due to severe dyspnea. Two other animals died after the third administration. In the 20 mg/kg group, three animals died after the first, third, and fourth administrations. These doses were determined to be above the LD₅₀, and their administration was discontinued in three animals. In addition, one animal in the 2 mg/kg group died after the first administration. No deaths were observed in the other groups.

General condition wise, decreased locomotor activity and delayed recovery from anesthesia were observed in the 20 and 60 mg/kg groups, and dyspnea and piloerection were observed in the 60 mg/kg group. No significant change in body weight was noted in surviving animals, and there was no difference in body weight gain over 14 days between the control group and the sulfuric acid groups. Macroscopically, multiple red foci or diffuse red areas in the lungs were scattered in dead or euthanized animals in the 20 and 60 mg/kg groups, respectively (Fig. 1). Further, no macroscopic abnormalities were observed in the dead animal in the 2 mg/kg group. No abnormalities related to the administration of the test substances were observed in surviving animals. Histopathologically, necrosis of the tracheal mucosa and cells desquamated into the bronchial lumen were identified in the 20 and 60 mg/kg groups (Fig. 2A). In the lungs, clefts between the mucosal epithelium and basement membrane in the bronchiole were observed (Fig. 2B), and this finding was deemed to be the initial image of desquamation of the

Table 2. Mortality: Intratracheal Instillation Study of Sulfuric Acid in Rats

| Group | Mortality ^{a)} |
|-----------|-------------------------|
| Control | 0% (0/5) |
| 0.7 mg/kg | 0% (0/5) |
| 2 mg/kg | 20% (1/5) |
| 7 mg/kg | 0% (0/5) |
| 20 mg/kg | 100% (3/3) |
| 60 mg/kg | 100% (3/3) |

a) The numbers in parentheses indicate the number of dead/treated animals.

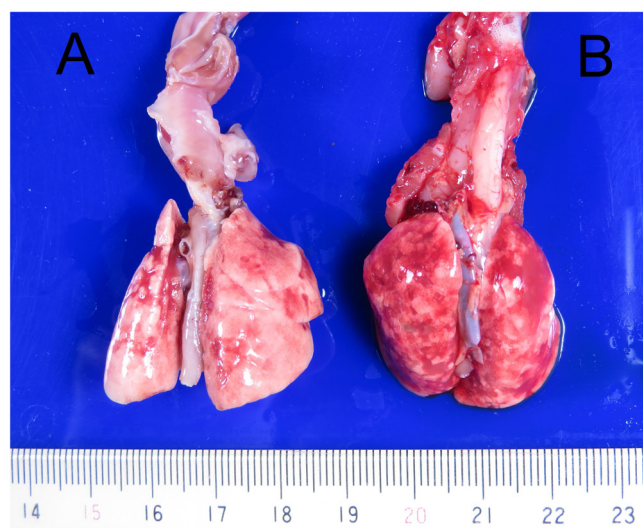


Fig. 1. Macroscopic findings in the lungs. (A) The 20 mg/kg group, multiple red foci in the lung; (B) the 60 mg/kg group, diffuse red areas in the lung.

mucosal epithelium. In the 60 mg/kg group, necrosis of the alveolar epithelium in the alveolar ducts was observed in addition to the findings in the bronchiole (Fig. 2C). Other findings such as alveolar hemorrhage and congestive edema

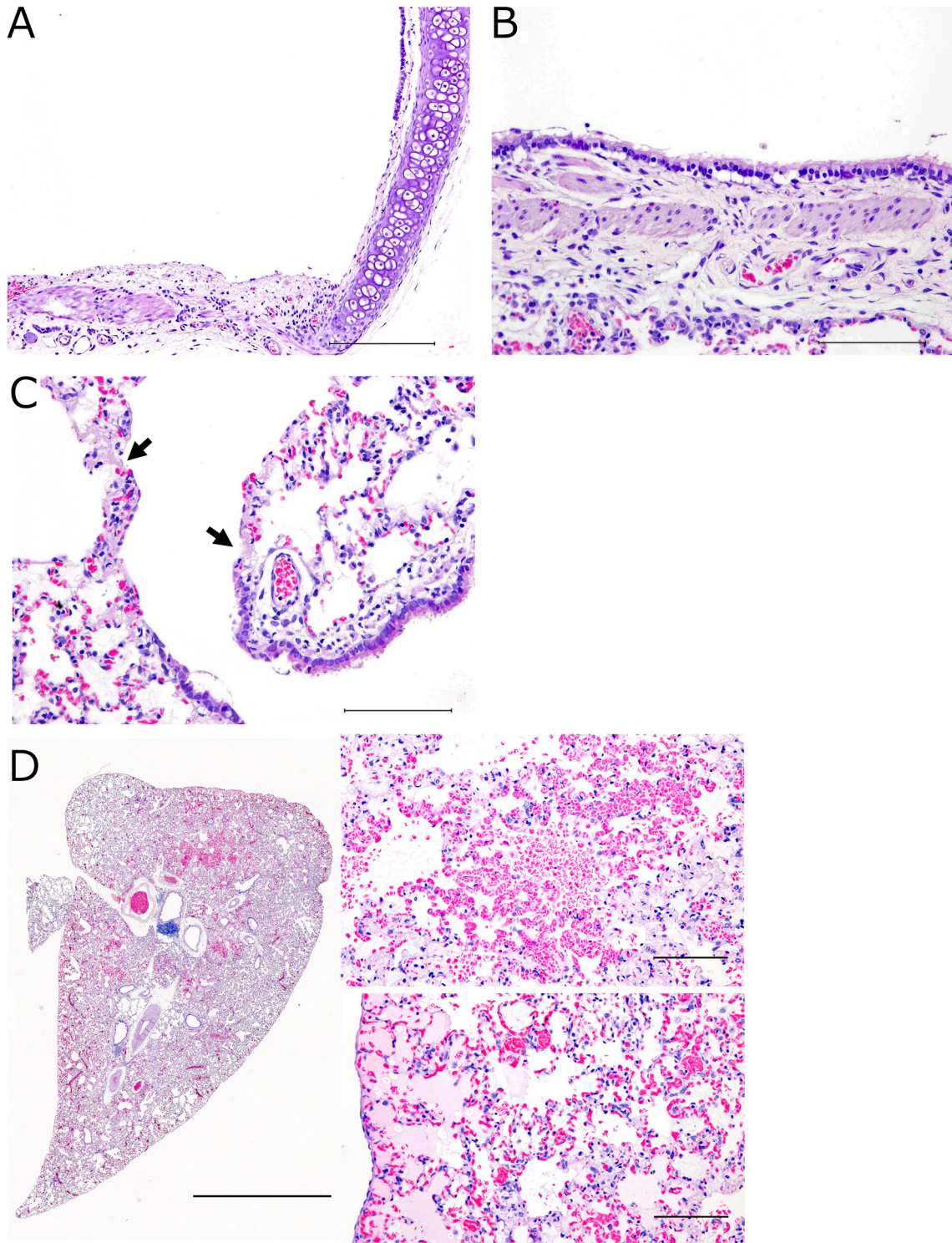


Fig. 2. Histopathological findings in the bronchi and lungs. (A) Necrosis and desquamation of tracheal mucosa in the trachea in the 20 and 60 mg/kg groups (Bar=150 μ m). (B) The cleft between mucosal epithelium and basement membrane in bronchioles of the 60 mg/kg group (Bar=200 μ m). (C) Necrosis of the alveolar epithelium (arrow) in alveolar ducts in the 60 mg/kg group (Bar=200 μ m). (D) Left; Loupe image of the lung in the 60 mg/kg group (Bar=3,000 μ m). Upper right; Congestion and alveolar hemorrhage (Bar=100 μ m). Lower right; Congestive edema (Bar=100 μ m). Hematoxylin and eosin stain.

were also noted (Fig. 2D). No abnormalities were observed in the lungs and trachea in the 0.7, 2, and 7 mg/kg groups.

Sulfuric acid was administered intratracheally to rats

at doses of 0.7, 2, 7, 20, and 60 mg/kg by dividing the total dose into four doses to evaluate the acute inhalation toxicity by intratracheal instillation. One animal died in the 2

mg/kg group (Table 2). However, the death was considered accidental because there were no histopathological lung changes in this animal or in the other animals in the 2 mg/kg group, and no dose-mortality relationship was observed. Death was observed in all animals in the 20 and 60 mg/kg groups, whereas no death was observed in the <7 mg/kg groups, except for one animal in the 2 mg/kg group (Table 2). Therefore, the LD₅₀ for sulfuric acid with intratracheal instillation was estimated to be 7–20 mg/kg.

Multiple red foci or diffuse red areas in the lungs were observed by macropathological examination in animals that died in the 20 and 60 mg/kg groups (Fig. 1). In addition, impaired changes in the bronchi and lungs were observed by histopathological examination (Fig. 2). In contrast, no abnormalities were observed at doses <7 mg/kg. Since there was a correlation between the deaths and pathological findings, we believe that the deaths were caused by lung injury due to the administration of sulfuric acid.

The LC₅₀ for sulfuric acid in inhalation toxicity studies has been reported to be 0.375 mg/L¹⁰, and exposure at the LC₅₀ was calculated to be 43.2 mg/kg. In the current study, the LD₅₀ was estimated to be 7–20 mg/kg. Given the higher exposure at the LC₅₀ by inhalation than at the LD₅₀ by intratracheal instillation, acute sulfuric acid toxicity by the latter method is evaluable at more than two-fold sensitivity. In addition, sulfuric acid was administered by dividing the total dose into four doses in intratracheal instillation to avoid the bolus effects of transient high exposure. Even so, acute toxicity by intratracheal instillation was still evaluated with more than twice the sensitivity. These results were consistent with those of a previous study, which demonstrated that intratracheal instillation showed more toxic effects at lower exposures than inhalation in studies of many chemical substances⁷.

Previous reports have shown that inhalation of sulfuric acid causes pathological changes in the lungs, such as hemorrhage and edema¹², which were similar to the lung findings observed by intratracheal instillation in this study. However, an inhalation study of sulfuric acid also showed ulceration of the turbinate, trachea, and larynx¹², although this toxicity could not be evaluated by intratracheal instillation. When using intratracheal instillation as an alternative to inhalation, the risk of overlooking upper respiratory tract toxicity (such as nasal toxicity) should be fully understood.

Although intratracheal instillation has some limitations and the dose should be chosen carefully from the viewpoint of animal welfare, intratracheal instillation has the advantages of being a simple method that is suitable for test materials that are difficult to assess by inhalation, such as acidic compounds. Therefore, by further examining the correlations between these two methods, we posit that intratracheal instillation would be a good alternative to inhalation.

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