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Letter

Collection of background data for repeated dose toxicity studies by intratracheal instillation in rats

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ABSTRACT — Intratracheal instillation is a useful method for evaluating airway toxicity of various substances. However, there is limited information on this method in repeated dose toxicity studies. This study aimed to supplement existing background data by intratracheally administering distilled water for injection (DW) or phosphate-buffered saline (PBS) to four groups of six male SD rats each under anesthesia by inhalation of sevoflurane or isoflurane. Additionally, a non-intratracheal instillation group with inhalation anesthesia and a non-treatment control group with neither intratracheal instillation nor inhalation anesthesia were established. DW or PBS droplets were instilled via intratracheal intubation once a day, five days a week, for four weeks following inhalation anesthesia. The examination included hematology, blood chemistry, biochemical and cytological analysis of bronchoalveolar lavage fluid (BALF), organ weight measurement, gross necropsy, and histopathological examination of the lungs. No apparent abnormalities were observed in hematology, blood chemistry, or biochemical and cytological analysis of BALF. However, histopathological examination revealed perivascular/peribronchiolar eosinophil infiltration in the lungs induced by sevoflurane and isoflurane. The change was more pronounced with DW or PBS dosing, and was most severe in the DW groups, accompanied by focal inflammation. This study provides useful background data for conducting repeated dose toxicity studies via intratracheal instillation in rats.

Key words: Intratracheal instillation, Repeated dose toxicity study, Sevoflurane, Isoflurane

INTRODUCTION

Humans are exposed to various materials through the respiratory tract, including chemical products and agricultural chemicals, which have the potential to affect their health. Medical drugs are also frequently administered via the respiratory tract for therapeutic purposes or as one of the administration routes. Consequently, it has become necessary to conduct toxicity studies that include administration via the respiratory tract for these chemical compounds. There are two methods of administering substances via the respiratory tract: inhalation exposure, which occurs through the spontaneous respiration of animals, and intratracheal instillation, which involves the direct administration of the intended substance into the trachea. Inhalation exposure is a useful method that replicates human exposure routes, as chemical compounds are administered via the respiratory tract through spontaneous respiration. However, special equipment and facilities are required for inhalation exposure. In rodent studies, the inhalation exposure method presents challenges in precisely controlling the individual dose level due to variations in respiratory volume. In contrast, intratracheal instillation can be conducted using simpler equipment

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compared to the inhalation exposure method. Moreover, studies have reported that biological response is quantitatively similar between the intratracheal instillation and inhalation exposure methods (Baisch *et al.*, 2014; Yamano *et al.*, 2023). Therefore, intratracheal instillation is considered useful for screening studies involving administration to the respiratory tract (Driscoll *et al.*, 2000).

The intratracheal instillation method includes two non-invasive techniques: droplet instillation using an intratracheal cannula and spraying using a microsprayer. It has been reported that there are no differences in dosing substance dispersion or lung burden between the droplet instillation and microsprayer methods (Kobayashi *et al.*, 2016). The droplet instillation method is widely employed as it utilizes inexpensive and readily available equipment.

Repeated dose studies are required to clarify toxicological profiles of test compounds (i.e., OECD guideline No. 412). While there are several reports of repeated intratracheal instillation, these studies often employed administration intervals that differ from the frequency specified in the guidelines (Hojo *et al.*, 2023; Yamano *et al.*, 2023).

A light anesthesia is necessary to ensure reliable administration. Due to the importance of quickly restoring normal breathing after administration, short-acting anesthetics are preferred (Driscoll *et al.*, 2000). Isoflurane is one of the most used anesthetics in intratracheal instillation studies (Guan *et al*., 2022; Kobayashi *et al*., 2021; Numano *et al.*, 2020; Senoh *et al.*, 2020, etc.). Sevoflurane is also considered a valuable anesthetic for intratracheal instillation studies due to its brief duration of action and rapid wake-up time. However, the lung toxicity of these anesthetics in rodents is not well understood (Stevens *et al.*, 1975).

In this study, two types of vehicles were repeatedly instilled intratracheally into the lungs of rats over a four-week period under anesthesia induced by two types of inhalation anesthetics to obtain background data for repeated intratracheal instillation studies.

MATERIALS AND METHODS

Regents

Sevoflurane (Sevo., Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) and isoflurane (Iso., Mylan Pharmaceutical Co., Ltd., Tokyo, Japan) were used as anesthetics. Water for injection (DW, Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) and phosphate-buffered saline (PBS, Takara Bio Co., Ltd., Shiga, Japan) were used as vehicles.

Animal

Crl:CD(SD) male rats were purchased at seven weeks of age from Jackson Laboratory Japan, Inc. (Kanagawa, Japan). After a 7-day quarantine and acclimatization period, they were assigned to study groups by stratified weight randomization. Food (CR-LPF: Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided *ad libitum*. The rats were maintained in the animal room under the following conditions: temperature of 22 ± 3°C, relative humidity of 55 ± 20%, 12-hr light cycle, and air ventilation of ten to fifteen times per hour. The rats were housed in groups of two per cage. This study was reviewed and approved by the Animal Care and Use Committee (ACUC) and the Institutional Official in accordance with the "Institutional Guidelines for Animal Study".

Animal experiment

The study groups consisted of seven groups: a non-treatment group (control), two anesthesia groups (Sevo or Iso), and four groups that received intratracheal instillation of DW or PBS after being anesthetized with either sevoflurane or isoflurane (Sevo + DW, Sevo + PBS, Iso + DW, and Iso + PBS). Each group consisted of six males. The study design is shown in Table 1.

The anesthetic concentrations were set based on widely recommended guideline (MOE, 2013); sevoflurane was administered at 5% for both induction and maintenance, while isoflurane was administered at 5% for induction and 2.5% for maintenance. Intratracheal instillation was performed using an outer tube of an 18-gauge catheter (indwelling needle, Terumo Corp., Tokyo, Japan) inserted into the trachea after anesthesia. The vehicle was administered in drops at a volume of 1 mL/kg. The repeated dosing was conducted once daily, five days per week for four weeks.

Observation and examinations

Clinical signs were observed twice daily on the day

Table 1. Study Group Design.

Anesthetic	Dosing solution	Group abbreviation	Number of rats
None	None	Non-treatment	6
Sevoflurane	None	Sevo	6
	DW	Sevo + DW	6
	PBS	Sevo + PBS	6
Isoflurane	None	Iso	6
	DW	Iso + DW	6
	PBS	Iso + PBS	6

Table 2. Biochemical and Cytological Analysis of Bronchoalveolar Lavage Fluid.

Group	n	LDH (U/L)	Total protein (μg/mL)	Total cell (10^3 cells)	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)	Macrophage (%)
Non-treatment	6	8.5 ± 9.7	0.0 ± 0.0	1373.8 ± 872.0	3.38 ± 1.6	9.45 ± 5.5	2.55 ± 1.4	84.62 ± 8.2
Sevo	6	4.7 ± 7.5	0.0 ± 0.0	1802.7 ± 330.8	2.20 ± 0.3	8.03 ± 1.3	1.70 ± 1.0	88.07 ± 2.2
Sevo + DW	5	4.3 ± 8.5	0.0 ± 0.0	3097.3 ± 1213.1	3.35 ± 1.9	5.00 ± 0.6	4.78 ± 2.5	86.88 ± 2.8
Sevo + PBS	6	7.0 ± 11.0	0.0 ± 0.0	3512.7 ± 1967.3	2.13 ± 1.2	8.23 ± 6.2	2.68 ± 2.6	86.95 ± 9.5
Iso	6	14.6 ± 15.5	0.0 ± 0.0	1887.6 ± 668.1	2.24 ± 0.8	9.68 ± 3.0	2.82 ± 2.6	85.26 ± 5.8
Iso + DW	5	21.5 ± 13.1	43.8 ± 87.5	2138.0 ± 1319.8	2.35 ± 0.7	8.10 ± 2.7	3.43 ± 1.3	86.13 ± 3.8
Iso + PBS	6	16.4 ± 6.9	0.0 ± 0.0	1828.6 ± 918.7	3.16 ± 2.9	8.50 ± 6.1	1.04 ± 0.4	87.30 ± 9.2

Data are presented as mean ± SD.
LDH: lactose dehydrogenase

of dosing and once daily on the other days. Body weight was measured twice weekly and food consumption was measured once weekly. Blood samples were obtained from the posterior vena cava under anesthesia after four weeks of administration. Hematology and blood chemistry items were evaluated according to OECD test guideline (No. 412, 2018). The rats were euthanized by exsanguination from the abdominal aorta. The lung weight was measured using an electronic balance after gross necropsy. Bronchoalveolar lavage was performed on the right lung using PBS at approximately 37°C. The lavage was carried out by moving the catheter up and down, positioning it 15 cm above and 8 cm below the rat to obtain constant pressure using gravity. The obtained bronchoalveolar lavage fluid (BALF) was subjected to biochemical analysis (total protein: pyrogallol red method, LDH: UV-rate method) using an auto analyzer (TBA-2000FR: CANON Medical Systems, Corp., Tochigi, Japan) and cytological analysis using a flow cytometric hematology system (XT-2000iv: Sysmex, Corp., Hyogo, Japan). Histopathological examination was conducted on a hematoxylin and eosin-stained specimen of the left lung.

Statistical analysis

Multiple comparisons were conducted between the non-treatment control group and each treatment group for each anesthetic separately. Initially, Bartlett's test was used to assess the homogeneity of variance. If the variance was homogeneous, Tukey's multiple comparison test was conducted. If the variance was heterogeneous, Steel-Dwass multiple comparison test was used. For the same treatment groups of sevoflurane and isoflurane, an F-test was conducted, followed by a t-test for equal variances if the variances were homogeneous, and an Aspin-Welch's test for unequal variances if the variances were heterogeneous.

Table 3. Lung Weight.

Group	n	Absolute weight (g)		Body Weight ratio (%)	
Non-treatment	6	1.761 ± 0.077		0.386 ± 0.027	
Sevo	6	1.570 ± 0.061		0.355 ± 0.014	
Sevo + DW	5	1.940 ± 0.084	b	0.452 ± 0.024	a,b
Sevo + PBS	6	1.781 ± 0.159	b	0.398 ± 0.032	c
Iso	6	1.625 ± 0.135		0.370 ± 0.019	d
Iso + DW	5	1.861 ± 0.102	b	0.451 ± 0.021	a,b
Iso + PBS	6	1.732 ± 0.117		0.397 ± 0.023	c

Data are presented as mean ± SD.
BW ratio: body weight-relative ratio
a: Significantly different from the non-treatment group ($p<0.05$, Tukey/Steel-Dwass test)
b: Significantly different from the Sevo/Iso group ($p<0.05$, Tukey/Steel-Dwass test)
c: Significantly different from the Sevo/Iso + DW group ($p<0.05$, Tukey/Steel-Dwass test)
d: Significantly different from the Sevo group ($p < 0.05$, Student/Aspin-Welch t-test)

RESULTS

Clinical signs, body weight, and food consumption

In the Svo + DW and Iso + DW groups, one rat each died. Rale and irregular respiration related to intratracheal instillation were observed. In the Sevo + DW and Iso + DW groups, respiration stopped after instillation several times, requiring artificial respiration for recovery; however, no instances of respiration cessation occurred in the groups administered PBS. No noteworthy changes were detected in body weight or food consumption.

Hematology and blood chemistry

No noteworthy changes were observed between the groups, and the data were within the background data

range of the test facility.

Biochemical and cytological analysis of BALF

Table 2 summarizes the results of biochemical and cytological analysis of BALF. The total cell counts in the Sevo + DW and Sevo + PBS groups tended to be higher; however, no statistically significant difference was observed compared to the non-treatment control group. The LDH level in the Iso + DW and Iso + PBS groups tended to be higher; however, no statistically significant difference was observed compared to the non-treatment group.

Lung weight and gross necropsy

Table 3 summarizes the lung weight. The absolute lung weight was statistically significantly higher than the non-treatment control group in the Sevo + DW and Sevo + PBS groups. The body weight-relative lung weight was statistically significantly higher than the Sevo group in the Sevo + DW group. No abnormal gross findings were observed.

Histopathology of lungs

Table 4 and Fig. 1 indicate the lung histopathology. Eosinophil infiltration in the perivascular/peribronchiolar areas was observed in all groups except for the non-treatment control group. The incidence of this finding was increased in the groups instilled with DW and PBS, and the severity of this finding increased in the animals instilled with DW. In addition, focal inflammatory cell infiltration was observed in the Sevo, Sevo + DW, and Sevo + PB groups, and Iso + DW and Iso + PBS groups, and its severity increased in the animals instilled with DW. Neutrophil/macrophage infiltration in the alveolar was observed in the inflammatory focus, a few animals had enlargement of the alveolar epithelium.

DISCUSSION

Droplet intratracheal instillation is a well-known useful method for the safety assessment of several substances that are comonly inhaled by humans. Therefore, it has become necessary that toxicological profiles of various chemicals, chemical products, agricultural chemicals, or medicines be assessed by conducting repeated dose toxicity studies. This study was conducted to obtain background data for a repeated dose intratracheal instillation study. Basic vehicles such as DW and PBS were intratracheally instilled once a day, five days a week, for consecutive four weeks under standard inhalation anesthetics such as sevoflurane and isoflurane.

Instillation of DW resulted in animal mortality, irrespective of the type of anesthetics used. Moreover, DW resulted in respiratory arrest after instillation, making artificial respiration necessary for recovery. These respiratory changes were not observed after PBS instillation. It was suggested that these adverse effects resulted from airway obstruction by DW instillation. Viscosity of DW and PBS at 25°C was approximately 1.0 mPa·s and 1.1 mPa·s, respectively. Since there was no significant difference in viscosity, it was estimated that the difference in airway obstruction between DW and PBS was not caused by their viscosity. Regarding the surface tension of DW and PBS, PBS is slightly lower than DW. Therefore, it was considered that differences in absorbency and wettability due to surface tension in the respiratory mucosa, a biological membrane, might be related to airway obstruction.

In the histopathological examination of the lungs, eosinophil infiltration in the perivascular/peribronchiolar areas was observed in all groups excluding the non-treatment group. Its incidence increased in the DW and PBS groups, and its severity increased in the DW group. No histopathological changes in the lungs were reported in a former study (Fukushima *et al.*, 1987), where iso-

Table 4. Histopathological Findings of Lungs - Incidence and Severity.

Finding		Non-treatment	Sevo	Sevo + DW	Sevo + PBS	Iso	Iso + DW	Iso + PBS
Number of animals examined		6	6	5	6	6	5	6
	Grade							
Eosinophil infiltration, Perivascular/peribronchiolar	minimal	3		4	4		6	
	mild		3	2		4		
	moderate		1					6
Infiltrate, inflammatory cell, Focal	minimal	1	1	1		3	1	
	mild		1					1

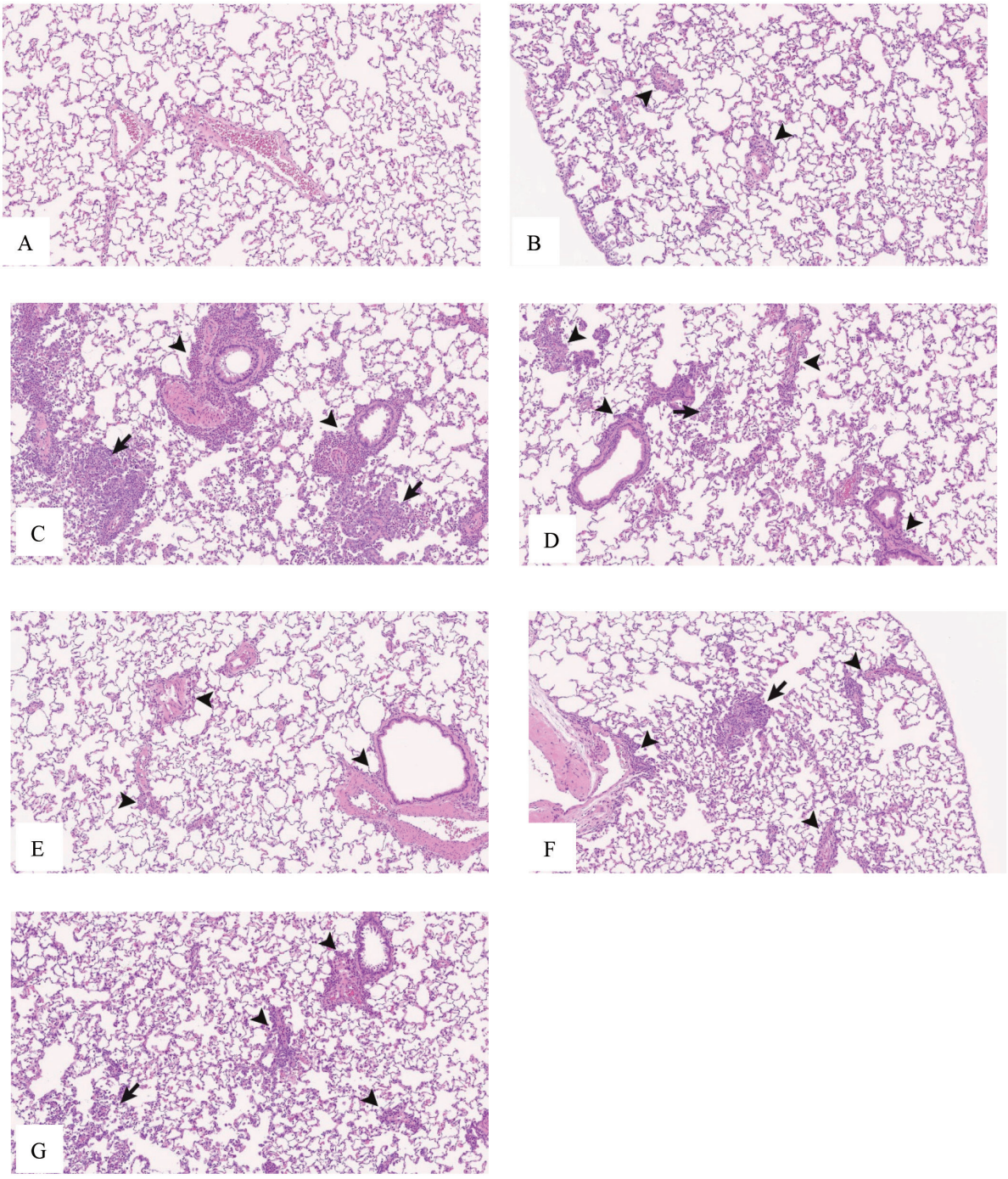

Fig. 1. Histopathological Findings of Lungs. No remarkable change in the non-treatment group (A). Perivascular/peribronchiolar eosinophil infiltration (arrow head) and/or inflammatory cell infiltration (arrow) in the Sevo group (B), in the Sevo + DW group (C), in the Sevo + PBS group (D), in the Iso group (E), in the Iso + DW group (F), and in the Iso + PBS group (G).

flurane exposure was at maximum of 1.2%, three hours a day, three days a week for ten weeks. As to sevoflurane, no changes in the lungs were reported when it was exposed at maximum of 2.2%, three hours a day, one day interval for eight weeks (Tamada *et al.*, 1986). The anesthesia concentrations used for intratracheal instillation are reported as 3% to 4.5% for isoflurane (Baisch *et al.*, 2014; Hasegawa-Baba *et al.*, 2014; Hojo *et al.*, 2023; Numano *et al.*, 2020) and 3.5% for induction, and 2.6% for maintenance for sevoflurane (Fortis *et al.*, 2012). In this study, based on widely recommended concentrations (MOE, 2013), the anesthesia concentration used was 5% for induction and 2.5% for maintenance with isoflurane, and 5% for both induction and maintenance with sevoflurane. The eosinophil infiltration of the lungs was considered to be the results of repeated exposure to high concentrations and frequencies of anesthesia, compared with those in the toxicology reports. Similar eosinophil infiltration was reported in a single intratracheal instillation of DW (Numano *et al.*, 2020); therefore, it is suggested that the intratracheal instillation of DW has the potential to induce damage in the lungs.

While histopathological alterations were observed in the lungs, no statistically significant differences were noted in the BALF chemistry or cytology compared with the non-treatment control group. LDH is a common indicator of cellular toxicity and alveolar epithelial cell injury, and total protein is a common indicator of membrane permeability. It was considered that the alterations in the whole lung were slight, since there were no changes suggesting any toxicity in these parameters.

This study has shown that it is possible to conduct repeated intratracheal droplet instillation under inhalation anesthesia in accordance with the OECD guideline (i.e. No. 412).

The results of this study suggest that repeated intratracheal instillation induces eosinophil infiltration of the lung that possibly originated from the inhalation of anesthesia This histological alteration was increased by DW instillation. If DW is chosen as the vehicle for an intratracheal instillation study, caution should be taken, as DW has resulted in mortalities due to airway obstruction and/or respiratory arrest. In conclusion, the findings of this study provide valuable background information for repeated intratracheal instillation toxicity studies involving various chemical substances.

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

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