Intratracheal Instillation Methods and the Distribution of Administered Material in the Lung of the Rat

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Abstract: Intratracheal instillation is widely used for respiratory toxicity tests in experimental animals. However, there are wide variations in the techniques used for instillation, and it is thus difficult to compare the results obtained using different techniques. To examine the effect of instillation methods, we compared the distribution of a test substance in the lungs of rats after intratracheal instillations under various conditions. Rats received an intratracheal instillation of 0.3 mL of India ink suspension under different conditions as follows: 3 different angles of body restraint, 0° (supine horizontal), 45° (supine head up) and 90° (vertical head up); 2 instillation speeds, high (40 mL/min) and low (4 mL/min); and 2 different devices, a standard bulb-tipped gavage needle and an aerosolizing microsprayer designed for intratracheal instillation. One hour after treatment under these various conditions, rats were sacrificed, and the local distribution of the suspension in the lungs was observed. No animal restrained in the supine head-up or vertical head-up position died from the treatment; however, fatalities were observed when rats were restrained in the supine horizontal position except under high-speed dosing conditions with a microsprayer. Better distribution of the suspension in the lungs was observed in the rats restrained in the supine head-up position after instillation at high speed when compared with other conditions. These results indicated that high-speed instillation to the subject restrained in the supine head-up position is an appropriate condition for performing intratracheal instillation. (DOI: 10.1293/tox.2014–0022; J Toxicol Pathol 2014; 27: 197–204)

Key words: intratracheal instillation, lung, rat

Introduction

Inhalation of airborne materials induces various respiratory disorders, including rhinitis, asthma, pneumonia, emphysema, pneumoconiosis, and lung tumor. Inhalation exposure and intratracheal instillation have been applied to studies of respiratory toxicity in experimental animals. Inhalation exposure is a natural route, as is found in human exposure, but this technique cannot always be used because inhalation equipment and a large quantity of test substance are needed. As a result of these restrictions, intratracheal instillation has been widely used. A method for intratracheal instillation of carcinogens in rabbits and guinea pigs was described by Kimura in 1923\(^1\). Subsequently, Saffiotti \textit{et al.} reported a model of experimental bronchogenic carcinoma in hamsters using intratracheal instillation in 1968\(^2\). Since that time, many researchers have used these techniques in slightly modified ways\(^3–8\). Although intratracheal instillation is a more convenient method than inhalation exposure, there are variations in the techniques used for instillation\(^3–6, 9–11\), and it is thus difficult to compare the results obtained using different techniques. The important variables for intratracheal instillation are the volume of test substance\(^3, 4, 9\), instillation speed\(^9\), the method of instillation\(^10\), the specific vehicle containing the test material\(^10\), the angle of body restraint\(^9\) and the method of anesthesia\(^10\). However, these conditions have not been sufficiently investigated, and the most appropriate way to achieve the best distribution of test substances is unclear. Although Driscoll \textit{et al.} recommended several guidelines for appropriate use of intratracheal instillation\(^10\), the technique has not yet been standardized. To examine the effect of instillation methods and to determine appropriate conditions, we compared the distribution of test substances in the lungs after intratracheal instillation under various conditions of body restraint, instillation speed and devices used. This is our first trial to determine the most appropriate conditions.
Materials and Methods

Animals

Sixty-five 7-week-old male Crl:CD (SD) strain rats (200–230 g body weight) purchased from Charles River Laboratories Japan (Kanagawa, Japan) were used for the experiments after acclimation for 1 week. The rats were randomly allocated to 13 groups of 5 rats, housed in groups of 2 or 3 per cage under controlled conditions (22 ± 1°C, relative humidity 55 ± 5% and a 12:12-h light:dark cycle) and allowed free access to commercial food pellets (Rodent Diet CE-2, CLEA Japan) and water. Autoclaved bedding (Soft Chip, Japan SLC, Shizuoka, Japan) was provided for each cage and changed once a week.

Animal care and experimental procedures were approved by the Animal Research Committee of the National Institute of Occupational Safety and Health, Japan, and conducted according to the Regulations for Animal Experimentation at the National Institute of Occupational Safety and Health, Japan.

Preparation of an India ink suspension

In this study, India ink (Kuretake, Nara, Japan) was used as a marker to determine the distribution of test substance in the lungs. An India ink suspension was prepared by dilution to 12.5% with sterilized saline (Otsuka Pharmaceutical Factory, Tokushima, Japan). This suspension was autoclaved at 121°C for 15 minutes, and cooled to room temperature.

Intratracheal instillation technique and conditions

To determine the distribution of India ink in stomachs, all animals were kept in a fasting condition beginning the evening before instillation. Before intratracheal instillation, the rats (240–275 g body weight) were anesthetized with 3.0% isoflurane (Forane, Abbott Japan, Tokyo, Japan) for 15 minutes using a face mask with an inhalation anesthetic system (Narcobit-E (type II), Natsume Seisakusho, Tokyo, Japan). As soon as anesthesia was accomplished, the animals were placed on an angled board (Hamri, Tokyo, Japan) by hanging the upper incisor teeth on an Incisor Loop (Solve, Kanagawa, Japan) in 3 different angles of body restraint, 0° (supine horizontal), 45° (supine head up) and 90° (vertical head up), respectively (Fig. 1). The device was inserted via the mouth to 2.5 cm from the epiglottis using a veterinary operating otoscope fitted with a speculum (Welch Allyn, Skaneateles, NY, USA), and the rats received intratracheal instillation of 0.3 mL of India ink suspension without air under different conditions as follows: 2 different devices, a standard bulb-tipped gavage needle (6202, Fuchigami, Kyoto, Japan) and an aerosolizing microsprayer designed for intratracheal instillation (MicroSprayer IA-1B, Penn-Century, Wyndmoor, PA, USA), and 2 instillation speeds, high (40 mL/min) and low (4 mL/min), set using a syringe driver (MCIP-III, Yuasa Bio Systems, Kanagawa, Japan) fitted with a 1 mL syringe connected to a 30 cm extension tube (Fig. 2). With this syringe driver, the instillation volume and speed of the test material were automatically controlled. After instillation, the animals were restrained to maintain the body position until recovery. The control group did not undergo anesthesia or intratracheal instillation. One hour after treatment under these various conditions, the rats received intravascular administration of India ink suspension without air under different conditions as follows: 2 different devices, a standard bulb-tipped gavage needle (6202, Fuchigami, Kyoto, Japan) and an aerosolizing microsprayer designed for intratracheal instillation (MicroSprayer IA-1B, Penn-Century, Wyndmoor, PA, USA), and 2 instillation speeds, high (40 mL/min) and low (4 mL/min), set using a syringe driver (MCIP-III, Yuasa Bio Systems, Kanagawa, Japan) fitted with a 1 mL syringe connected to a 30 cm extension tube (Fig. 2). With this syringe driver, the instillation volume and speed of the test material were automatically controlled. After instillation, the animals were restrained to maintain the body position until recovery. The control group did not undergo anesthesia or intratracheal instillation. One hour after treatment under these various conditions, the rats were euthanized and sacrificed by exsanguination from the abdominal aorta under isoflurane anesthesia, and their lungs and stomachs were removed to observe the local stereoscopic distribution of the India ink suspension.

Macroscopic analysis

The lungs were fixed in 10% neutral-buffered formalin for 24 hours without inflation, and surface images from the ventral aspect of the left lobe were obtained by scanning (Pictrostat Digital 400, Fujifilm, Tokyo, Japan). As the shape
of the right lobes shows a little more variation among animals when compared with the left lobe, the left side is generally used for histopathological observation when the other side is used for other types of testing. Hence, we selected the left side for the analysis. To determine differences in the distribution of India ink suspension in each group, scanned images from each lung were traced onto OHP sheets, and the OHP sheets of rats from the same group were superimposed and colored according to the degree of overlap: blue for 1 rat, purple for 2 rats, green for 3 rats, orange for 4 rats and red for 5 rats (Fig. 3).

**Histopathology**

The formalin-fixed left lobe of the lung was sliced in cross sections of approximately equal thickness (3–4 mm) and embedded in paraffin wax. The 4 μm paraffin sections were stained with hematoxylin and eosin (HE) and examined histopathologically.

**Results**

**Survival rate**

No animal retained in the supine head-up or vertical head-up position died from the treatment. However, fatalities were observed when rats were retained in the supine horizontal position, except when the dose was administered at high speed using the MicroSprayer (Table 1).

**Gross views of the lungs and stomachs**

In the supine head-up position, the suspension was detected in all lobes of the lungs under all conditions. Some of the animals in the vertical head-up position showed a poor distribution of the suspension when a standard gavage needle was used. In the supine horizontal position using a standard gavage needle at both speeds and using the MicroSprayer at low speed, there was insufficient distribution in the lungs. Complementarily to the distribution seen in the lungs, more suspension was distributed in the stomachs of rats held in the supine horizontal position when using a standard gavage needle than under other conditions, but various quantities of the suspension reached the mucosa of the stomach in all groups (Fig. 4 and Table 2).
Analysis of distribution patterns of india ink suspension in the left lobe

The overall lung distribution pattern was more basilar than apical. More even distribution of india ink suspension in the lungs was observed in rats retained in the supine head-up position after treatment with the MicroSprayer set at high speed when compared with other conditions. In the supine horizontal position and vertical head-up position, the suspension showed an insufficient or imbalanced distribution pattern in the lungs (Fig. 5).

Histopathology of the lungs

The histological appearance of the lung cross sections differed between the various instillation conditions and slice

Table 1. Survival

| Device          | Injection speed | Angle of body restraint | Survival |
|-----------------|-----------------|-------------------------|----------|
| Gavage needle   | High            | 0°                      | 4/5      |
|                 |                 | 45°                     | 5/5      |
|                 |                 | 90°                     | 5/5      |
|                 | Low             | 0°                      | 5/5      |
|                 |                 | 45°                     | 5/5      |
|                 |                 | 90°                     | 5/5      |
| Micro-Sprayer   | High            | 0°                      | 5/5      |
|                 |                 | 45°                     | 5/5      |
|                 |                 | 90°                     | 5/5      |
|                 | Low             | 0°                      | 5/5      |
|                 |                 | 45°                     | 5/5      |
|                 |                 | 90°                     | 5/5      |

Table 2. Distribution of the Suspension in Lungs and Stomachs (Number of Animals)

| Device          | Injection speed | Angle of body restraint | Distribution of the suspension in lungs | Distribution of the suspension in stomachs |
|-----------------|-----------------|-------------------------|----------------------------------------|------------------------------------------|
| Gavage needle   | High            | 0°                      | 2                                      | 2                                        |
|                 |                 | 45°                     | 2                                      | 2                                        |
|                 |                 | 90°                     | 2                                      | 2                                        |
|                 | Low             | 0°                      | 2                                      | 2                                        |
|                 |                 | 45°                     | 2                                      | 2                                        |
|                 |                 | 90°                     | 2                                      | 2                                        |
| Micro-Sprayer   | High            | 0°                      | 5                                      | 5                                        |
|                 |                 | 45°                     | 5                                      | 5                                        |
|                 |                 | 90°                     | 5                                      | 5                                        |
|                 | Low             | 0°                      | 5                                      | 5                                        |
|                 |                 | 45°                     | 5                                      | 5                                        |
|                 |                 | 90°                     | 4                                      | 5                                        |

Fig. 4. Macroscopic features of lungs and stomachs after using a standard gavage needle (A) and the MicroSprayer (B) under various body restraint and instillation speed conditions.
positions. In upper (proximal) slices, Indian ink suspension was observed on the surfaces of bronchi and peribronchial alveoli in rats from the supine head up and vertical head up groups, and collectively, these deposits extended only part way towards the peripheral margins of the lung. Macroscopically, the distribution of the suspension was poor in the supine horizontal position. By contrast, the instilled suspensions were more widely visible as heavy deposits in lower slices than in upper slices (Figs. 6, 7).

At high magnification, the deposits using both instillation devices were coarse and uneven, and within the region of deposition, alveolar exposure ranged between extremes of absence to complete filling. The patterns of the lung depositions using a standard gavage needle were focal, and the depositions using the MicroSprayer were dispersed (Fig. 8).

**Discussion**

The survival of animals and distribution of Indian ink suspension in lungs were significantly affected by the angles of body restraint and instillation speed. To our knowledge, there is little information regarding the angles of body restraint and their effects in the literature. On the other hand, intratracheal instillation at a fast speed has been recommended in some articles, however, this recommendation was only based on in-house experience, and the effects of speed on intratracheal instillation have hitherto remained unknown. From the results concerning the survival of animals and the distribution of the suspension in lungs, it is inferred that the suspension was retained in airways and that rats died as a result of airway obstruction by the suspension when they were restrained in the supine horizontal position and instillation was at a low speed.

Though a relatively small amount of suspension (0.3 mL) was used in the present study, most of the rats showed depositions of the suspension in the lumen of their stomachs because the suspension flowed back into the stomach from the trachea. Such a backward flow of ink into the gastrointestinal tract as that observed in the present study was previously reported in a study performing intratracheal instillations in Syrian golden hamsters using a relatively large quantity of ink (1.5 mL), but it was not seen with smaller quantities of 0.2, 0.5 and 1.0 mL. This may be the result of differences in the intratracheal instillation technique and animal species, but it should be noted that the test substance can pass into the stomach when using intratracheal instillation.

The suspension instilled using a standard gavage needle showed a focal distribution in the peribronchial alveoli and bronchi, and this was similar to the distributions previously reported; the distribution was more dispersed in the lungs when using the MicroSprayer. These findings show that the physical state of the ejected suspension from each device is different. The distribution patterns of the suspension in the lungs suggested that the MicroSprayer was effective for achieving a more uniform distribution of the instilled material in the lung compared with a standard gavage needle. However, the MicroSprayer has a tendency to become clogged with test substances, and thus its use requires a little larger cost. Accordingly, the device for intratracheal instillation should be selected in accordance with the characteristics and required distribution of the test substance.

The difference in the suspension distributed in the lungs is consistent with a report by Brain et al. and supports the belief that gravity plays a major role in the distribution of instillate in the lungs, but this finding is contrary to that described in another report. Such results suggest that the distribution of test substance in the lungs may be...
Effects of Intratracheal Instillation Methods

affected by the method of intratracheal instillation and that when preparing a specimen from the lungs for histopathology, the direction of cutting and number of sections should be considered.

Even though intratracheal instillation is used as an alternative method for studying inhalation exposure in experimental animals, the localization of the test material in the lungs from inhalation and intratracheal instillation differs. Inhaled materials deposit thinly and uniformly throughout the entire lungs. By contrast, most of the test substance is unevenly distributed in the lungs when using intratracheal instillation. Moreover, Dorries et al. found that the number of lung macrophages containing test substances is greater when the test substances are inhaled than when the substances are instilled. This finding suggests that intratracheal instillation is not a satisfactory alternative to inhalation and may result in a markedly different distribution, transport and toxicity of test substances in the lungs.

In summary, we found that the angle of body restraint, the device used for instillation and the speed of instillation are very important variables when performing intratracheal instillation. The supine head-up position led to good survival of rats and distribution of the test substance suspension without being affected by the device used or speed of instillation. By contrast, restraining the rat horizontally in the supine position was inappropriate, because some rats died as a result of assumed accumulation of the suspension in the trachea. Moreover, in the vertical head-up position with a low instillation speed, the suspension was not sufficiently distributed in the lungs. A comparison of instillation at high and low instillation speeds showed that the high speed produced better survival and distribution of the suspension in lungs than the low speed. These results indicate that the supine head-up position and high-speed instillation are appropriate experimental conditions. Differences in the distribution of toxic substances may affect the pathogenesis and degree of lung lesions caused by the substances. Standardization of appropriate intratracheal instillation techniques is necessary to obtain more reliable test results.

Fig. 6. Representative microscopic views of the upper region of the left lobes instilled with India ink suspension at high speed using 2 devices, a standard gavage needle (A, C, E) and a MicroSprayer (B, D, F), and 3 different angles of body restraint, 0° (A, B), 45° (C, D) and 90° (E, F) (H-E staining, bar=200 μm).
Fig. 7. Representative microscopic views of the lower region of the left lobes instilled with India ink suspension at high speed using 2 devices, a standard gavage needle (A, C, E) and a MicroSprayer (B, D, F), and 3 different angles of body restraint, 0° (A, B), 45° (C, D) and 90° (E, F) (H-E staining, bar=200 μm).

Fig. 8. Intratracheal instillation in the lungs (1 hour after exposure). Suspension deposition along the alveolar duct is heavy and uneven (H-E staining, bar=200 μm). The deposition patterns obtained using a standard gavage needle (A) and the MicroSprayer (B) are different.
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