Effects of administering different vehicles via single intratracheal instillation on responses in the lung and pleural cavity of Crl:CD(SD) rats

Takamasa Numano*, Mai Morioka, Hitomi Higuchi, Kazunari Uda, Taiki Sugiyama, Teruaki Hagiwara, Yuko Doi, Norio Imai, Mayumi Kawabe, Yukinori Mera and Seiko Tamano

DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya 491-0113, Japan

*Correspondence to: Takamasa Numano, DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya, Aichi 491-0113, Japan, E-mail: numano@dims.co.jp

Running head: Intratracheal instillation of different vehicles on rats
Abstract

Intratracheal instillation is the introduction of a substance directly into the trachea. Intratracheal instillation has been used to investigate the lung toxicity of several chemicals and requires the suspension or dissolution of test material in a vehicle for even dispersal throughout the lung. Importantly, the toxicities of vehicles used in intratracheal instillation studies are generally considered to be insignificant. Hence, evaluating the influence of different vehicles on the lung due to intratracheal instillation is crucial. We examined the toxic effects of pure water, saline, phosphate buffered saline (PBS), 0.5% Kolliphor® P188 (KP188), 0.1% Tween 20 in saline, and 1.0% BSA in PBS. These vehicles were administered to male Crl:CD(SD) rats by a single intratracheal instillation. On day 3, broncho-alveolar lavage fluid (BALF) from the right lung was collected and processed for cell counting and biochemical analysis, while the left lung was used for histopathological examination. Accumulation of alveolar macrophages was observed in all vehicle-treated groups but was minimal in the group administered saline, somewhat higher in the groups administered pure water, PBS, 0.1% Tween 20, and 1% BSA, and notably higher in the group administered 0.5% KP188. The results from BALF analysis indicated that intratracheal instillation of 0.5% KP188 also induced alveolar damage. Additionally, administering pure water did not appear to cause tissue damage. Eosinophil infiltration in the interstitial regions was
histopathologically observed. Altogether, the results of this study are helpful for the selection of appropriate vehicles for use in intratracheal instillation studies.

**Key words**

Intratracheal instillation, broncho-alveolar lavage fluid, lung toxicity
Introduction

Nanomaterials are materials smaller than 100 nanometers that have many unique properties, which contribute to their use as very promising materials for different applications. As a result of their remarkable potential in industrial and commercial applications, many nanomaterials have been produced. However, the production and use of nanomaterials, which continue to increase annually, have raised many concerns regarding their fate in biological systems. Therefore, there is a demand for a fast and reliable assessment of their health hazard potential. Inhalation toxicity studies involving whole body or nose-only provide standard routes to assess the toxicity of nanomaterials, thereby providing relevant and useful toxicological information. However, there are several drawbacks with using these inhalation methods as they may result in prolonged delay in the assessment of nanomaterial toxicity due to requirements for specialized facilities, equipment, techniques, and the quantity of test materials.

In addition to inhalation methods, another method of administering respirable materials to the lung for hazard identification is intratracheal instillation. Although intratracheal instillation disperses the test materials directly into the trachea and the lung, differences exist in the distribution, clearance, and retention of materials in the lung compared to inhalation. That is, intratracheal instillation does not require specialized facilities, equipment, or a large quantity of test
materials, thereby allowing the widespread use of this method. Additionally, intratracheal instillation is comparatively inexpensive, another factor enabling its widespread use. It is also important to note that particle deposition and dimensions are important factors in the toxicological potential of respirable materials. However, the nasal passages of rodents, which are obligate nasal breathers, are efficient particle filters, and restrict the amounts and the dimensions of the particles that penetrate into the lung during inhalation exposure. Consequently, there are limitations to the inhalation method. That is, the difference in particle deposition between rodents and humans can make it difficult to extrapolate the effects observed in rodent inhalation studies to those that may occur in humans\textsuperscript{2}. Furthermore, the short lifespan of rodents also makes it difficult to extrapolate the effects observed in rodent inhalation studies to those that may occur late in the lifespan of humans, e.g., development of mesothelioma\textsuperscript{2-4}. Therefore, while inhalation is a more natural route of exposure to respirable materials, studies in rodents using intratracheal instillation are a viable pulmonary hazard screening method\textsuperscript{5-7}.

Recent toxicity studies of a variety of engineered nanomaterials have been performed using intratracheal instillation as the administration method\textsuperscript{8-13}. The toxicity of nanomaterials depends on their size, shape, and surface properties\textsuperscript{14}. Toxicity is also influenced by the formation of agglomerates which can affect the physical properties of the material and lead to an erroneous
interpretation of the material's toxicity. Thus, using a vehicle where the test material can be well dispersed, could play a vital role in the results of toxicity studies using intratracheal instillation.

Surfactants are often added to the vehicle to promote dispersion. However, high concentrations of surfactants can cause cytolysis or organic changes. As it is imperative to use a vehicle that does not interfere with the outcome of the study, it is essential to know the in vivo toxic effects of the vehicle itself, and to avoid using unsuitable vehicles regardless of the dispersibility of the test material in the vehicle. In the present study, we examined the difference in acute responses in the lung and pleura due to intratracheal instillation of six commonly used vehicles: pure water, saline, phosphate buffered saline (PBS), Kolliphor®P188 (KP188) diluted in saline to 0.5%, Tween 20 diluted in saline to 0.1%, and BSA diluted in PBS to 1.0%.

Materials and Methods

Chemicals and preparation of test vehicles

Physiological saline (saline) was supplied by Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Pure water was prepared using Elix®UV3 (Sigma-Aldrich Japan Ltd., Tokyo, Japan). Phosphate buffered saline (PBS) was purchased from Life Technologies Japan (Tokyo, Japan). Kolliphor®P188 (KP188) and Polysorbate 20 (Tween 20) were purchased from
Sigma-Aldrich Japan Ltd. (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Carbochem Co., Ltd. (Jiangsu, China). Solutions of 0.5% KP188 and 0.1% Tween 20 in physiological saline (154 mM NaCl in H$_2$O) and 0.5% BSA in PBS were prepared and frozen until use.

**Animals and Husbandry**

Six-week-old pathogen-free male Crl:CD(SD) rats were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The animals were housed in an animal room maintained under standard conditions (temperature 22±3 °C, humidity 55±15%, 12-h light-dark cycle) and received oriental CRF-1 pellet diet sterilized by 30 kGy $\gamma$ rays irradiation (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. After a two-week quarantine and acclimation period, the 8 week-old rats were randomized by body weight and assigned to one of seven groups (6 rats/group). There were no significant differences in the body weights between groups, Bartlett test ($p < 0.05$) and Tukey test ($p < 0.05$, two-sided). There were no abnormalities in the general condition of the animals.

The study was approved by the animal experimental committee at the DIMS Institute of Medical Science, Inc. and conducted in accordance with the “Law for the Humane Treatment and Management of Animals” (Law No. 46, May 2014), “Standards Relating to the Care and
Management of Laboratory Animals and Relief of Pain” (Notice No. 84 of the Ministry of the Environment, September 2013), “Basic policies for the conduct of animal experiment in academic research institutions under the jurisdiction of the Ministry of Health, Labour and Welfare” (Notice No. 0220-1 of the Ministry of Health, Labour and Welfare, February 2015), “Guidelines for Proper Conduct of Animal Experiments” (Science Council of Japan, June 2006), and “Standards for Care and Use of Laboratory Animals of DIMS Institute of Medical Science, Inc.” (June 1, 2016).

*Intratracheal instillation and the collection of Bronchoalveolar lavage fluid and Pleural cavity lavage fluid*

Animals were placed under 3% isoflurane anesthesia using the NARCOBIT-E inhalation anesthesia system for small laboratory animals (Natsume Seisakusho Co., Ltd., Tokyo, Japan). A single intratracheal instillation was performed using a volume of 1 ml/kg with a DIMS-type micro-sprayer aerosolizer (for rats) connected to a 1 mL disposable syringe (OSAKA CHEMICAL Co., Ltd., Osaka, Japan). The dosing volume was calculated for each individual rat based on their body weight on the day of administration. Briefly, the rats placed under 3% isoflurane anesthesia were held on a board at an angle of approximately 50 degrees using a rubber band. The tip of the micro-sprayer aerosolizer was then gently inserted into the trachea of rats via palpation of tracheal
cartilages, at about 6 cm from the angulus oris. Intratracheal instillation was performed by pressing the syringe plunger at a constant speed during inhalation. Thereafter, about 5-15 s, the animal was retained in a similar position until it began to recover from the intratracheal instillation procedure to prevent backflow and buildup of the administered solution in the bronchi and trachea. The animals in the untreated control group did not undergo either isoflurane anesthesia or intubation with the micro-sprayer aerosolizer on the day of administration. The condition of rats was checked twice on the day of intratracheal instillation, once immediately post-intratracheal instillation and once in the afternoon, as well as twice on the following day, once in the morning and again in the afternoon.

Three days after the intratracheal instillation procedure, all animals were placed under deep anesthesia and exsanguinated by exsanguination from the abdominal aorta. After blood collection, the pleural cavity lavage fluid (PLF) was collected. Briefly, 8 mL of room temperature sterilized physiological saline was injected into the right pleural cavity through the diaphragm using a 10 mL syringe connected to a 21G-needle. The rat was then gently rolled from side to side and the PLF collected using the same 10 mL syringe. Bronchoalveolar lavage fluid (BALF) was subsequently collected from the right lung. Thereafter, the sternum was removed, the left bronchus was ligated just below the bifurcation, and the right bronchus was cannulated through the trachea. Four mL of room temperature sterilized physiological saline was then infused into the right lungs through a
3-way stopcock by gravity feed from a height of about 30 cm. When influx stopped, the inlet cock of the 3-way stopcock was closed and the vent cock was opened to collect discharge from the right lungs by gravity feed. This operation was repeated twice. Finally, the left lung was removed and weighed, then used for histopathological examination. BALF and PLF were used in the assessment of lung and pleural cavity toxicity\textsuperscript{16}.

\textit{Analysis of inflammatory cells and clinical chemistry in BALF}

BALF was centrifuged at 1500 rpm for 10 min at 2 °C, and the supernatant removed and stored at -80 °C until use. Cell pellets were re-suspended in 1 mL of sterilized physiological saline and processed for differential leucocyte count using an automatic multi-item blood cell analyzer (XT-2000i, Sysmex Corporation, Hyogo, Japan). The supernatants were used for analysis of alkaline phosphate (ALP), lactate dehydrogenase (LDH), protein concentration (total protein), and albumin using an automatic analyzer (Hitachi 7070, Hitachi, Ltd., Tokyo, Japan). LDH and ALP were used as indicators of general cytotoxicity and alveolar type II epithelial cell toxicity, respectively. Additionally, albumin and total protein were used as indicators of alveolar capillary permeability.

\textit{Total cell counts and clinical chemistry in PLF}
The PLF was centrifuged at 1500 rpm for 10 min at 2 °C and the supernatant was removed and frozen at -80 °C until use. Cell pellets were then re-suspended in 1 mL of sterilized physiological saline and processed for total cell counts using an automatic multi-item blood cell analyzer (XT-2000i, Sysmex Corporation, Hyogo, Japan). The supernatants were then used for analysis of total protein using an automatic analyzer (Hitachi 7070, Hitachi, Ltd., Tokyo, Japan). Total protein was used as an indicator of capillary permeability.

**Histopathological examination**

The left lung, heart, diaphragm, and thoracic wall were fixed in 10% neutral buffered formalin solution. The fixed tissues were cut 5-μm thick, embedded in paraffin, and then processed for hematoxylin and eosin (H&E) staining and histopathological analysis. However, the right lung following BALF collection was not used for histopathological examination. The terminology used in this report conforms with the INHAND Project.

**Statistical analysis**

Homogeneity of variance of the non-treated group and each vehicle-treated group was analyzed using the F-test, whereas the statistical significance of the difference between the non-treated group and the vehicle administered group when the variance was homogeneous, was derived using the student's t-test (one-sided). The Welch’s t-test (one-sided) was used when the
variance was heterogeneous. The frequency of the findings from the histopathological examinations was evaluated using Fisher’s test (1-tailed) and the significance of the grade of the lesions was evaluated using Wilcoxon’s rank-sum test (2-tailed). P values less than 0.05 were considered significant.

Results

General condition

In the present study, no deaths were observed in the untreated and vehicle-administered groups. However, on the day of administration, moist rale was observed in all vehicle-administered animals, but not in the untreated rats. Additionally, the day after intratracheal instillation, moist rale was no longer present in any of the rats. As such, moist rale was concluded to be caused by intratracheal instillation, however, it was considered to have a low toxicological significance. Besides moist rale, there were no abnormalities in the general condition of any of the untreated or treated rats.

Macroscopic pathological examination, body weight and organ weight

Macroscopically, no abnormalities were observed in any of the organs or tissues in rats from the different groups. Body weights on days 0 and 3 along with the absolute and relative lung
weights at autopsy are shown in Table 1. No differences in body weights were found between any of the vehicle-administered groups and the untreated group. However, there was a significant increase in absolute and relative lung weights in the 0.5% KP188-administered group compared to the untreated group. There was no significant difference in absolute or relative lung weights between the other vehicle-administered groups and the untreated group.

**Comparison of differential leucocyte counts in BALF**

Fig. 1 shows the counts of total cells (A), lymphocytes (B), neutrophils (C), macrophages (D), and eosinophils (E) in BALF collected from the right lung. The administration of 0.5% KP188 induced a significant increase in neutrophil count compared to the untreated group. In addition, a significant decrease in eosinophils was observed in the saline and PBS-treated groups; however, a decrease in eosinophil count in the BALF had no known clinical relevance and was thus concluded to be an incidental effect rather than a toxic effect. No other differences were observed between the vehicle-administered groups and the untreated group.

**Clinical chemistry in the BALF**

The levels of ALP, LDH, total protein, and albumin in BALF are presented in Table 2. Results in this table revealed significantly higher levels of ALP, total protein, and albumin in the 0.5% KP188-treated group compared to the untreated group. LDH levels were also elevated in the 0.5%
KP188-treated group compared to the untreated group but this increase was not statistically significant. No significant differences in these BALF parameters were observed between the other vehicle-treated groups and the untreated group.

**Total cell counts and total protein in the PLF**

Total cell counts in the PLF are presented in Fig. 2, which showed no significant differences between the vehicle-treated groups and the untreated group. Total protein levels are presented in Table 3, which showed a significantly lower level of total protein in the saline-treated group than the untreated group. As total protein in the BALF was not affected by intratracheal instillation of saline (see Table 2) and although blood chemistry was not examined in the present study, this lower protein level was considered to be an incidental effect rather than a toxic effect.

**Histopathological examination**

The results of histopathological examination for the left lungs are summarized in Table 4. The lungs of the untreated group showed normal histology (Fig. 3A and 3B). However, alveolar macrophage accumulation was significantly increased in all vehicle-treated groups compared to the untreated group. The degree of alveolar macrophage accumulation was minimal in the group administered saline; somewhat higher in the groups administered pure water, PBS, 0.1% Tween 20, and 1% BSA; and notably higher in the group administered 0.5% KP188 (Fig. 3C). There was no
degeneration or necrosis of macrophages in any of the vehicle-treated groups.

Inflammatory cell infiltration, such as infiltration of neutrophils and macrophages expressed as “Infiltration, mixed” in Table 4 was observed in the alveolar area and in the alveolar/peribronchial area in one of the rats administered pure water as well as in one of the rats administered 1% BSA. In addition, eosinophilic infiltration in the peribronchial/perivascular area was observed in one or more rats in all vehicle-treated groups except for the PBS-treated group. Eosinophilic infiltration was statistically significant in the pure water-treated group.

Proliferation of alveolar type II epithelial cells was seen in two of the rats administered pure water (Fig. 3E), one of the rats administered 0.5% KP188, and two of the rats administered 1% BSA.

Discussion

As aforementioned, exposure to respirable materials by inhalation requires specialized equipment for aerosolization of the test material and whole body inhalation requires specialized facilities. However, nose-only inhalation is stressful to the animal and prolonged exposures are difficult. The availability of newly developed materials may be insufficient in quantity for an inhalation exposure study. These drawbacks can result in a prolonged delay in assessing the toxicity
of respirable materials. Thus, as new materials are being developed, particularly respirable nanomaterials, procedures that can expedite risk assessment of newly developed materials, are becoming ever more crucial. Risk assessment has four basic steps; hazard identification, hazard characterization, exposure assessment, and risk characterization. Based on this point of view, intratracheal instillation is useful for hazard identification and hazard ranking\(^5\)-\(^7\). Importantly, intratracheal instillation does not require specialized facilities or equipment, enabling its utility by many research groups. Intratracheal instillation studies therefore make a valuable contribution to the process of risk assessment.

In the study, six commonly-used vehicles in intratracheal instillation studies were administered to rats: pure water, physiological saline, PBS, 0.5% KP188 in saline, 0.1% Tween 20 in saline, and 1% BSA in PBS. Based on the findings, lung weight was significantly higher in rats administered 0.5% KP188. Macrophage accumulation in the alveoli was seen in all treated groups: accumulation was slight in the rats administered saline; somewhat high in the groups administered pure water, PBS, Tween 20, and BSA; and notably higher in the rats administered 0.5% KP188. Eosinophilic infiltration in the interstitial region around the blood vessels and the bronchi was histopathologically observed in rats administered pure water. In BALF, elevated numbers of neutrophils and elevated levels of ALP, total protein, and albumin were observed in rats
administered 0.5% KP188. LDH levels in the BALF were also increased in these rats, but this was not statistically significant compared to the untreated group. In PLF, there was no change in total cell count or total protein levels in the groups administered pure water, PBS, 0.5% KP188, 0.1% Tween 20, or 1% BSA. Additionally, in the saline-treated rats, there was a decrease in total protein in the PLF. Overall, our results suggest that 0.5% KP188 had higher acute lung toxicity than the other vehicles and pure water induced different histopathological change compared to the other vehicles.

LDH and ALP were used as indicators of general cytotoxicity and alveolar type II epithelial cell toxicity, respectively, whereas albumin and total protein were used as indicators of alveolo-capillary permeability. The increase in ALP in the 0.5% KP188 group suggests that KP188 resulted in damage to alveolar type II epithelial cells. This postulation is supported by the increase in LDH, which was not statistically significant. It also suggests that this damage was not extensive. The increase in total protein and albumin in the BALF suggests increased capillary permeability; a typical inflammatory response to tissue damage. The increase in macrophages in the alveoli and the increased number of neutrophils in the BALF from the 0.5% KP188-treated rat also showed a very early stage of inflammatory response in the lung. That is, the increase in lung weight supports the notion that intratracheal instillation of KP188 induced an inflammatory response in the lung.
consequently causing tissue damage. Importantly, no degeneration or necrosis was observed in the macrophages that accumulated in the lungs of the KP188-treated rats 3 days after intratracheal instillation. This suggests that these macrophages were not actively producing cytotoxic mediators such as reactive oxygen and nitrogen species, but may have been active in the resolution of inflammation and tissue repair. This was evident in the accumulation of macrophages and possibly neutrophils with immune suppressive activity seen during the resolution of inflammation and tissue repair\textsuperscript{18-20}. The lack of an inflammatory cell infiltrate in these rats and the relative lack of an increase in alveolar type II epithelial cell proliferation compared to the other vehicle-administered groups support the idea that the tissue damage induced by KP188 was relatively mild and readily repaired. Overall, the result obtained from the 0.5% KP188-treated group indicates that intratracheal instillation of this vehicle caused tissue damage and subsequent inflammation, but that this effect may be transitory. This conclusion is supported by another study where KP188 (also known as Poloxamer 188 which is used as an alternative to Pluronic F-68: MDL number MFCD00082049) was used as the vehicle, and 4 weeks after the final intratracheal instillation of 0.5 ml of 0.5% KP188 in saline (a total of 8 intratracheal instillation procedures), the vehicle administered group did not differ from the untreated group in any of the parameters examined\textsuperscript{21}. Importantly, neither LDH, ALP, albumin, nor total protein was elevated in the BALF of pure water,
saline; PBS, Tween 20 or BSA-treated groups, thereby suggesting that intratracheal instillation of
these vehicles caused little tissue damage in the lung. An observable accumulation of macrophages
was however seen in the lung alveoli in rats in these groups, though macrophage accumulation was
not as marked as that in the KP188-treated rats (see Table 4). Furthermore, since macrophages are a
primary defense against foreign materials that reach the alveoli, it is reasonable that intratracheal
instillation would induce at least a mild macrophage response regardless of the material that is
being introduced into the lung. The only other significant response was eosinophilic infiltration in
the peribronchial/perivascular area in the pure water-treated group. All other responses were
sporadic where inflammatory cell infiltration was observed in the alveolar area and in the
alveolar/peribronchial area in one of the rats in the pure water and the BSA groups as mentioned
above. An eosinophilic infiltration in the peribronchial/perivascular area was observed in three rats
in the saline group, one rat in the Tween 20 group, and two rats in the BSA group whereas
proliferation of alveolar type II epithelial cells was observed in two rats in the pure water group and
two rats in the BSA group. There was a significant decrease in eosinophils in the BALF of the
saline- and PBS-treated rats. However, a low BALF eosinophil count has no known clinical
relevance and because three rats in the saline group and two rats in the BSA group had an
eosinophilic infiltration in the peribronchial/perivascular area, this decrease was concluded to be
incidental rather than toxic. Finally, there was a statistically significant decrease in total protein in PLF in the saline-treated group. This decrease was also concluded to be incidental as protein levels in the BALF were not affected by intratracheal instillation of saline. Overall, these results suggest that pure water elicited a slightly stronger response in the lung than the four saline based vehicles and simple physiological saline elicited the mildest response.

The six vehicles tested in this study evoked varying responses in the lung, with 0.5% KP188 in saline being the most toxic of the vehicles and pure water having a slightly more adverse effect than the saline-based vehicles which included physiological saline, PBS, 0.1% Tween 20 in saline, and 1% BSA in PBS. The choice of vehicle depends on both the toxicity of the vehicle and the solubility/dispersibility of the test agent in the vehicle. For example, polyoxyethylene-polyoxypropylene copolymers such as Pluronic F-68 (discontinued) and KP188 have been used for dispersal of hydrophobic carbon nanotubes and potassium octatitanate fibers11. KP188 is used as the vehicle; however, it is water-soluble and not bio-persistent in the lung. As mentioned before, the present study showed that intratracheal instillation of KP188 induced acute toxic changes in the lung. A further study is therefore required to elucidate the recovery process of the damage. However, it has been reported that the toxic effects of KP188 were transient21.
Pure water has been used in previous intratracheal instillation studies\textsuperscript{10, 16, 24-27}. In the present study, while there were no notable increases in inflammatory cells or clinical markers in the BALF of rats administered pure water, intratracheal instillation of pure water induced eosinophilic infiltration in the interstitial area. Therefore, if possible, physiological saline-based vehicles as opposed to pure water should be used for intratracheal instillation studies.

Importantly, none of the six vehicles tested in the present study affected the pleural parameters that were assessed. Therefore, neither the intratracheal instillation procedure itself nor the formulation of the vehicles tested caused a tissue reaction in the pleura. Vehicles that cause a reaction in the pleura or a lasting response in the lung should not be used regardless of the dispersibility of the test material in the vehicle.

In summary, physiological saline evoked the mildest response when instilled into the lung; this was followed by the saline-based vehicles PBS, 0.1% Tween 20 in saline, and 1% BSA in PBS. On the other hand, pure water evoked a stronger response than the saline-based vehicles whilst 0.5% KP188 in saline evoked the strongest response. None of the vehicles evoked a response in the pleura or a long-term response in the lung. These findings indicate that pure water and 0.5% KP188 in saline are appropriate for use as vehicles but should be used with caution and only when the dispersibility of the test material or the activity of the test material in the vehicle requires their use.
Acknowledgments

The authors thank Dr. David B. Alexander from Nagoya City University for reading our manuscript.

Conflict of interests

The authors have no conflict of interest.
References

1. T Kasai, K Gotoh, T Nishizawa, T Sasaki, T Katagiri, Y Umeda, T Toya and S Fukushima. Development of a new multi-walled carbon nanotube (MWCNT) aerosol generation and exposure system and confirmation of suitability for conducting a single-exposure inhalation study of MWCNT in rats. Nanotoxicology. 8: 169-178. 2014.

2. LC Chen and M Lippmann. Inhalation toxicology methods: the generation and characterization of exposure atmospheres and inhalational exposures. Curr Protoc Toxicol. 63: 24 21-23. 2015.

3. OECD. GUIDANCE DOCUMENT ON INHALATION TOXICITY STUDIES Series on Testing and Assessment No. 39 (Second Edition) 2018.

4. S Fukushima, T Kasai, Y Umeda, M Ohnishi, T Sasaki and M Matsumoto. Carcinogenicity of multi-walled carbon nanotubes: challenging issue on hazard assessment. J Occup Health. 60: 10-30. 2018.

5. Y Morimoto, H Izumi, Y Yoshiura, Y Fujisawa and K Fujita. Significance of Intratracheal Instillation Tests for the Screening of Pulmonary Toxicity of Nanomaterials. J UOEH. 39: 123-132. 2017.

6. GaK Oberdörster, T.A. In vivo effects: methodologies and biokinetics of inhaled nanomaterials. NanoImpact. 10: 38-60. 2018.

7. OECD. DRAFT GUIDANCE DOCUMENT ON INHALATION TOXICITY TESTING #39 2nd WNT Commenting Round. 2017.

8. T Kobayashi, Y Oshima, Y Tsubokura, N Hashizume, S Ajimi, T Kayashima, M Nakai, T Sasaki, K Kawaguchi and N Imatanaka. Effects of dose volume and delivery device on bronchoalveolar lavage parameters of intratracheally administered nano-sized TiO2 in rats. Regul Toxicol Pharmacol. 81: 233-241. 2016.

9. T Numano, J Xu, M Futakuchi, K Fukamachi, DB Alexander, F Furukawa, J Kanno, A Hirose, H Tsuda and M Suzui. Comparative study of toxic effects of anatase and rutile type nanosized titanium dioxide particles in vivo and in vitro. Asian Pac J Cancer Prev. 15: 929-935. 2014.

10. H Senoh, H Kano, M Suzuki, M Ohnishi, H Kondo, K Takanobu, Y Umeda, S Aiso and S Fukushima. Comparison of single or multiple intratracheal administration for pulmonary toxic responses of nickel oxide nanoparticles in rats. J Occup Health. 59: 112-121. 2017.

11. M Suzui, M Futakuchi, K Fukamachi, T Numano, M Abdelgied, S Takahashi, M Ohnishi, T Omori, S Tsuruoka, A Hirose, J Kanno, Y Sakamoto, DB Alexander, WT Alexander, X Jiegou and H Tsuda. Multiwalled carbon nanotubes intratracheally instilled into the rat lung induce
development of pleural malignant mesothelioma and lung tumors. Cancer Sci. 107: 924-935. 2016.
12. J Xu, M Futakuchi, M Iigo, K Fukamachi, DB Alexander, H Shimizu, Y Sakai, S Tamano, F Furukawa, T Uchino, H Tokunaga, T Nishimura, A Hirose, J Kanno and H Tsuda. Involvement of macrophage inflammatory protein 1alpha (MIP1alpha) in promotion of rat lung and mammary carcinogenic activity of nanoscale titanium dioxide particles administered by intra-pulmonary spraying. Carcinogenesis. 31: 927-935. 2010.
13. J Xu, M Futakuchi, H Shimizu, DB Alexander, K Yanagihara, K Fukamachi, M Suzuki, J Kanno, A Hirose, A Ogata, Y Sakamoto, D Nakae, T Omori and H Tsuda. Multi-walled carbon nanotubes translocate into the pleural cavity and induce visceral mesothelial proliferation in rats. Cancer Sci. 103: 2045-2050. 2012.
14. A Albanese, PS Tang and WC Chan. The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annu Rev Biomed Eng. 14: 1-16. 2012.
15. M Roursgaard, KA Jensen, SS Poulsen, NE Jensen, LK Poulsen, M Hammer, GD Nielsen and ST Larsen. Acute and subchronic airway inflammation after intratracheal instillation of quartz and titanium dioxide agglomerates in mice. ScientificWorldJournal. 11: 801-825. 2011.
16. H Takehara, M Makita, R Tanaka, M Tsuchiya, M Naya and M Hayashi. Lung toxicity assessment using bronchoalveolar lavage fluid and pleural lavage fluid cytology by intratracheal treatment in rats. J Toxicol Sci. 39: 141-145. 2014.
17. R Renne, A Brix, J Harkema, R Herbert, B Kittel, D Lewis, T March, K Nagano, M Pino, S Rittinghausen, M Rosenbruch, P Tellier and T Wohrmann. Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. Toxicol Pathol. 37: 5S-73S. 2009.
18. CEM Aarts and TW Kuijpers. Neutrophils as myeloid-derived suppressor cells. Eur J Clin Invest. 48 Suppl 2: e12989. 2018.
19. S Sendo, J Saegusa and A Morinobu. Myeloid-derived suppressor cells in non-neoplastic inflamed organs. Inflamm Regen. 38: 19. 2018.
20. TA Wynn and KM Vannella. Macrophages in Tissue Repair, Regeneration, and Fibrosis. Immunity. 44: 450-462. 2016.
21. M Abdelgied, AM El-Gazzar, DB Alexander, WT Alexander, T Numano, M Iigou, A Naiki-Ito, H Takase, KA Abdou, A Hirose, Y Taquahashi, J Kanno, H Tsuda and S Takahashi. Potassium octatitanate fibers induce persistent lung and pleural injury and are possibly carcinogenic in male Fischer 344 rats. Cancer Sci. 109: 2164-2177. 2018.
22. T Ohba, J Xu, DB Alexander, A Yamada, J Kanno, A Hirose, H Tsuda and Y Imaizumi. MWCNT causes extensive damage to the ciliated epithelium of the trachea of rodents. J Toxicol Sci. 39: 499-505. 2014.
23. JP Ryman-Rasmussen, MF Cesta, AR Brody, JK Shipley-Phillips, JI Everitt, EW
Tewksbury, OR Moss, BA Wong, DE Dodd, ME Andersen and JC Bonner. Inhaled carbon nanotubes reach the subpleural tissue in mice. Nat Nanotechnol. 4: 747-751. 2009.

24. NR Jacobsen, T Stoeger, S van den Brule, AT Saber, A Beyerle, G Vietti, A Mortensen, J Szarek, HC Budtz, A Kermanizadeh, A Banerjee, N Erkal, U Vogel, H Wallin and P Moller. Acute and subacute pulmonary toxicity and mortality in mice after intratracheal instillation of ZnO nanoparticles in three laboratories. Food Chem Toxicol. 85: 84-95. 2015.

25. Y Morimoto, M Hirohashi, A Ogami, T Oyabu, T Myojo, K Nishi, C Kadoya, M Todoroki, M Yamamoto, M Murakami, M Shimada, WN Wang, K Yamamoto, K Fujita, S Endoh, K Uchida, N Shinohara, J Nakanishi and I Tanaka. Inflammogenic effect of well-characterized fullerenes in inhalation and intratracheal instillation studies. Part Fibre Toxicol. 7: 4. 2010.

26. Y Morimoto, H Izumi, Y Yoshiura, T Tomonaga, T Oyabu, T Myojo, K Kawai, K Yatera, M Shimada, M Kubo, K Yamamoto, S Kitajima, E Kuroda, K Kawaguchi and T Sasaki. Evaluation of Pulmonary Toxicity of Zinc Oxide Nanoparticles Following Inhalation and Intratracheal Instillation. Int J Mol Sci. 17. 2016.

27. T Okada, A Ogami, BW Lee, C Kadoya, T Oyabu and T Myojo. Pulmonary responses in rat lungs after intratracheal instillation of 4 crystal forms of titanium dioxide nanoparticles. J Occup Health. 58: 602-611. 2016.
Figure legends

**Fig. 1** Total and differential leucocyte counts in BALF.

Total numbers of leucocytes (A), Lymphocytes (B), neutrophils (C), Macrophages (D), and Eosinophils (E) in BALF. Values are presented as mean ± S.D.

**Fig. 2** Total leucocyte counts in PLF

Total numbers of leucocytes (A) in PLF. Values are presented as mean ± S.D.

**Fig. 3** Histopathological observations

Normal histology in the alveolar area of the lung in the untreated group (A) and normal histology in the peribronchial and perivascular area in the untreated group (B). Accumulation of alveolar macrophages in the 0.5% KP188 group (C). Eosinophilic infiltration in the peribronchial and perivascular area in the pure water group (D). The proliferation of alveolar type II cell (Red arrows) in the pure water group (E).
Table 1. Body weights, organ weights, and organ to body weight ratios of rats.

| Treatment         | No. of rats | Day 0 | Day 3 | Absolute (g) | Relative (%) |
|-------------------|-------------|-------|-------|--------------|--------------|
|                   | examined    | body weight (g) |          |              |              |
| Untreated         | 6           | 312.8 ± 13.9    | 339.7 ± 14.5 | 0.437 ± 0.025 | 0.129 ± 0.009 |
| Saline            | 6           | 310.5 ± 13.8    | 334.5 ± 15.3 | 0.454 ± 0.035 | 0.136 ± 0.007 |
| Pure water        | 6           | 311.2 ± 12.2    | 333.7 ± 15.9 | 0.465 ± 0.036 | 0.139 ± 0.007 |
| PBS               | 6           | 310.7 ± 15.3    | 335.3 ± 15.9 | 0.43 ± 0.012  | 0.128 ± 0.006 |
| 0.5% KP188        | 6           | 310.5 ± 10.2    | 337.2 ± 13.6 | 0.48 ± 0.032  | * 0.142 ± 0.009 * |
| 0.1% Tween 20     | 6           | 310.8 ± 13.1    | 334.7 ± 13.9 | 0.451 ± 0.026 | 0.134 ± 0.009 |
| 1% BSA            | 6           | 313.5 ± 14.1    | 338.7 ± 15.4 | 0.438 ± 0.026 | 0.142 ± 0.009 |

*: Significantly different from the untreated group at p<0.05.

a: The day of administration

b: The day of autopsy
Table 2. Alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total protein, and albumin in BALF.

| Treatment   | No. of rats examined | ALP (U/L) ± | LDH (U/L) ± | Total protein (mg/dL) ± | Albumin (g/mL) ± |
|-------------|----------------------|-------------|-------------|--------------------------|------------------|
| Untreated   | 6                    | 185.3 ± 59.8| 11.3 ± 2.4  | 7.87 ± 1.93              | 11.47 ± 2.94     |
| Saline      | 6                    | 204.2 ± 43.1| 11.8 ± 4.8  | 7.17 ± 1.72              | 12.5 ± 2.11      |
| Pure water  | 6                    | 225.7 ± 18.7| 11.5 ± 7.9  | 8.97 ± 1.89              | 12.93 ± 1.69     |
| PBS         | 6                    | 207.5 ± 50.5| 11 ± 4.9    | 7.17 ± 1.12              | 11.62 ± 2.83     |
| 0.5% KP188  | 6                    | 351.8 ± 66.8**| 22.2 ± 11.1| 13.05 ± 2.48**          | 20.53 ± 2.86**   |
| 0.1% Tween 20 | 6                | 195.8 ± 38.8| 16 ± 6      | 6.74 ± 1.86              | 12.24 ± 3.39     |
| 1% BSA      | 6                    | 189.7 ± 27.6| 17.2 ± 8.5  | 9.05 ± 1.88              | 14.77 ± 3.55     |

**: Significantly different from the untreated group at p<0.01.
Table 3. Total protein in the PLF

| Treatment      | No. of rats examined | Total protein (mg/dL) |
|----------------|----------------------|-----------------------|
| Untreated      | 6                    | 48.47 ± 5.78          |
| Saline         | 6                    | 39.7 ± 6.72 *         |
| Pure water     | 6                    | 45.27 ± 8.7           |
| PBS            | 6                    | 40.47 ± 7.97          |
| 0.5% KP188     | 6                    | 41.75 ± 7.59          |
| 0.1% Tween 20  | 6                    | 40.82 ± 6.99          |
| 1% BSA         | 6                    | 44.1 ± 6.65           |

*: Significantly different from the untreated group at p<0.05.
Table 4. Histopathological findings in the lung on day 3 after instillation

| Findings                                      | Untreated | Saline | Pure water | PBS | 0.5% | 0.1% | 1% |
|-----------------------------------------------|-----------|--------|------------|-----|------|------|----|
| Treatment                                     |           |        |            |     |      |      |    |
| Lung/bronchial                                |           |        |            |     |      |      |    |
| Alveolar macrophage aggregation/(1)\(^{a}\)   | 0         | 5      | 2          | 3   | 0    | 2    | 2  |
| /(2)                                          | 0         | 1      | **4        | **3 | **0  | **4  | **3 |
| /(3)                                          | 0         | 0      | 0          | 0   | 6    | 0    | 1  |
| Infiltration, mixed: alveolar/(1)              | 0         | 0      | 0          | 0   | 0    | 0    | 1  |
| /(2)                                          | 0         | 0      | 1          | 0   | 0    | 0    | 0  |
| Infiltration, mixed: alveolar/peribronchial/(1)| 0         | 0      | 0          | 0   | 0    | 0    | 1  |
| /(2)                                          | 0         | 0      | 1          | 0   | 0    | 0    | 0  |
| Infiltration, eosinophilic: peribronchial/perivascular/(1)| 0         | 3      | 3          | 0   | 1    | 0    | 2  |
| /(2)                                          | 0         | 0      | 2          | **0 | **1  | **0  | **0 |
| /(3)                                          | 0         | 0      | 1          | 0   | 0    | 0    | 0  |
| Proliferation, alveolar type II cell/(1)       | 0         | 0      | 2          | 0   | 1    | 0    | 2  |

\(^{a}\): Numbers in parentheses indicate the grade of the lesion: (1) Minimal, (2) Slight, (3) Moderate

\(^{**}\): Significantly different from untreated group at p<0.01.
Fig. 1
Fig. 2
