

Original

Establishment of a Bioassay System for Detection of Lung Toxicity Due to Fine Particle Instillation: Sequential Histopathological Changes with Acute and Subacute Lung Damage Due to Intratracheal Instillation of Quartz in F344 Male Rats

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Abstract: In order to establish an appropriate bioassay for detection of lung damage after fine particle inhalation, sequential histopathological changes were here examined after intratracheal instillation of quartz (DQ-12, 4 mg/rat), as a typical lung toxic agent, in F344 male rats. A total of 50, 10-week-old animals, were separated into two groups. Twenty five were exposed to the material suspended in saline (0.2 ml) using a specially designed aerolizer and subgroups were sacrificed 1, 3, 7, 14, and 28 days thereafter. The remaining 25 rats were exposed by intratracheal instillation to saline (0.2 ml) as a control group and were sacrificed on the same days. Both groups received intraperitoneal injections of BrdU before sacrifice, and both groups underwent assessment of lung histopathology with immunohistochemical demonstrations of BrdU, iNOS and MMP-3 as end-point markers. The results suggest that Days 1 and 28 after intratracheal instillation of test fine particles are the most appropriate for detection of acute and subacute inflammatory changes, respectively. Furthermore, BrdU on Day 1 and iNOS on Day 28 proved to be suitable end-point markers for this purpose. Although instillation and inhalation models are different, the present instillation model can be used for detection of acute or subacute lung toxicity due to inhaled fine particles. (J Toxicol Pathol 2005; 18: 13–18)

Key words: instillation, quartz, lung, BrdU, iNOS, fine particles

Introduction

There are many toxicants in our environment, including air pollutants. Human investigations focusing on concentrated ambient particles have shown acute lung inflammation and changes in both blood indices and heart rate¹. It is thus an urgent priority to establish *in vivo* bioassays for the detection of hazards with fine particles, which can be inhaled into deep lung tissue by human beings.

In quartz dust exposed construction workers, obstructive and restrictive loss of lung function has been detected², as well as chronic obstructive pulmonary disease (COPD)^{3,4}. These are associated with an inflammatory cell

response characterized by alveolitis with recruitment of inflammatory cells, particularly neutrophils, and may result in pulmonary fibrosis and impaired lung function⁵. Intratracheal instillation of quartz into rats produces an inflammatory reaction followed by histological changes characteristic of lung fibrosis⁶, similar to the above noted human conditions.

In order to establish an appropriate bioassay for detection of lung damage after particle inhalation, sequential histopathological changes were here examined in rats using quartz as a typical lung toxic chemical. Additionally, for objective assessment, changes in 5-bromo-2'-deoxyuridine (BrdU) incorporation and expression of inducible NO synthase (iNOS) and matrix metalloproteinase 3 (MMP-3) were also immunohistochemically examined. These markers are generally associated with inflammation and are thought to increase at different times after particle inhalation.

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Materials and Methods

Chemical

Quartz dust (DQ-12) with a particle diameter of not more than 7 μm was obtained from Deutsche Montan Technologie (GmbH, Germany).

Animals

Male F344 rats (8 weeks of age), purchased from Japan Charles River (Atsugi, Japan), were maintained in the Kagawa Medical University Animal Facility according to the institutional animal care guidelines. All animals were housed in polycarbonate cages with white wood chips for bedding, and given free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity ($60 \pm 10\%$), lighting (12 h light/dark cycle) and temperature ($24 \pm 2^\circ\text{C}$).

After a 2-week acclimation period, a total of 50, 10-week-old rats, were randomly separated into two groups. Twenty-five rats were exposed by intratracheal instillation to quartz dust (4 mg/rat) suspended in 0.2 ml saline by a specially designed aerolizer (Penn Century, Philadelphia, USA), and subgroups of 5 rats were sacrificed 1, 3, 7, 14, and 28 days thereafter under ether anesthesia. The dose of quartz was determined from the study reported by Mercer *et al.*⁷ The remaining animals were exposed by intratracheal instillation to 0.2 ml saline as a control group and subgroups of 5 rats were similarly sacrificed on the same days (Fig. 1). All rats received an intraperitoneal injection of BrdU (100 mg/5 ml saline/kg rat) (Tokyo Kasei Kogyo Co., Ltd., Tokyo), one hour before sacrifice. At autopsy, their lungs and bronchi were rinsed with 10% neutral buffered formalin after excision, weighed and then infused with 10% neutral buffered formalin. Six hours later, the fixative solution was changed to 100% ether to preserve antigens. Other organs were immersed in 10% neutral buffered formalin for a week. Slices of lungs, liver and kidneys were routinely processed for embedding in paraffin for histopathological examination of hematoxylin and eosin staining and immunohistochemical studies.

Histopathological analysis

Each lung lobe was examined histopathologically for neutrophil infiltration in the walls and spaces of the alveoli, pulmonary edema, pulmonary fibrosis, alveolar macrophage infiltration and restructuring of the walls. The severity of each parameter was assessed as follows: 0, no change; 1, weak; 2, moderate; 3, severe.

Immunohistochemical analysis

Lungs were immunostained for BrdU, iNOS and MMP-3 by the avidin-biotin complex (ABC) method, with 3,3'-diaminobenzidine as the substrate, and counterstained with Gill's hematoxylin to facilitate orientation.

For exposure to anti-mouse BrdU monoclonal antibody, (code No. M0744 purchased from DAKO, Glostrup,

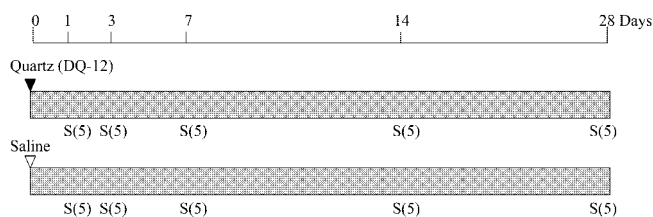


Fig. 1. Experimental design. ▼: intratracheal instillation of quartz (DQ-12), 4 mg/0.2 ml saline/rat. ▽: intratracheal instillation of saline, 0.2 ml/rat. S(5): sacrifice (5 rats).

Denmark), tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series, immersed in 0.5% hydrogen peroxide in absolute methanol for 30 min at room temperature to block endogenous peroxidase activity, and then in 37°C 4N HCl for 2 min for DNA degeneration and neutralized with borax boric acid solution. For enzymic activation, they were exposed at 37°C to 0.01% actinase for 5 min for antigen retrieval. Following incubation with normal horse serum at room temperature for 30 min, to block background staining, the sections were incubated with the antibody (diluted 1:100) for 12 h at 4°C .

For the anti-rabbit inducible nitric oxide synthase (iNOS) polyclonal antibody, NOS2 (N-20), (sc-650 purchased from SANTACRUZ, California, USA), tissue sections were deparaffinized, then antigen retrieval was performed by heating to 121°C for 5 min in an autoclave with citrate buffer solution. Blocking of endogenous peroxidase activity and background staining with goat serum were performed as described above and sections were incubated with the antibody (diluted 1:100) for 12 h at 4°C .

The same steps were performed for the anti mouse MMP-3 monoclonal antibody, (code No. 551117 from BD Biosciences, New Jersey, USA), except that horse serum was here applied and incubated, and the antibody was diluted 1:10.

Image analysis

Each immunohistochemically stained section was examined with the assistance of an image analyzer (IPAP; Image Processor for Analytical Pathology, Sumika Technoservice Co., Hyogo, Japan). More than 20×400 microscope images from lung lobes of the rats were assessed for numbers and areas of immunohistochemically positive cells as follows: %No; number of positive cells / total lung cells (all kinds of cells in the field) $\times 1000$, %Area; area of positive cells / total lung areas (except alveolar spaces) $\times 1000$. At least, 3500, 3700, 3000 cells were counted for labeling indices of BrdU, iNOS and MMP-3, respectively.

Statistics

Percentage data from immunohistochemistry were analysed by the post-hoc test (Tukey-Kramer) and by Spearman's rank correlation.

Results

The general condition of the rats in all groups demonstrated no remarkable change during the experimental period.

Macroscopic findings

The lungs of rats in the quartz treated group sacrificed on Day 1 showed discolored surfaces macroscopically. There were white small nodules in lungs of rats in the quartz treated group sacrificed on Day 28 (Fig. 2.). The bronchi, liver and kidneys demonstrated no remarkable change.

Histopathological observation

The sequential histopathological changes in lungs of rats treated with quartz are illustrated in Fig. 3. The main findings were neutrophil infiltration in the walls and spaces of the alveoli, pulmonary edema, pulmonary fibrosis, alveolar macrophage infiltration and restructuring of the alveolar walls. In addition, lungs of rats in the quartz treated group sacrificed on Day 28 demonstrated a granulation-like change with giant cells and macrophages in the alveoli. Scoring indices for each lesion are summarized in Table 1. Lungs of rats in the quartz treated group sacrificed on Day 1 already exhibited neutrophil infiltration, and lungs of rats in the quartz treated group sacrificed on Day 28 scored highly for all of the findings.

The bronchi, liver and kidneys did not demonstrate significant alterations, histopathologically.

Immunohistochemical analysis

Numbers and areas of BrdU positive cells were the highest in lungs of rats in the quartz treated group sacrificed on Day 1 and then decreased with time (Table 2). Data for iNOS immunohistochemistry are shown in Table 3. In the

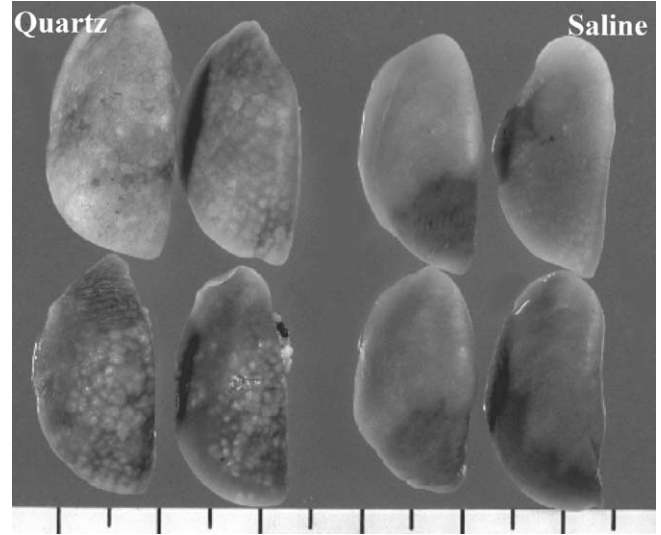


Fig. 2. Lungs (Day 28) after instillation of quartz and saline. Only left lobes of 4 rats in each group are shown. With instillation of quartz multiple small nodules are evident.

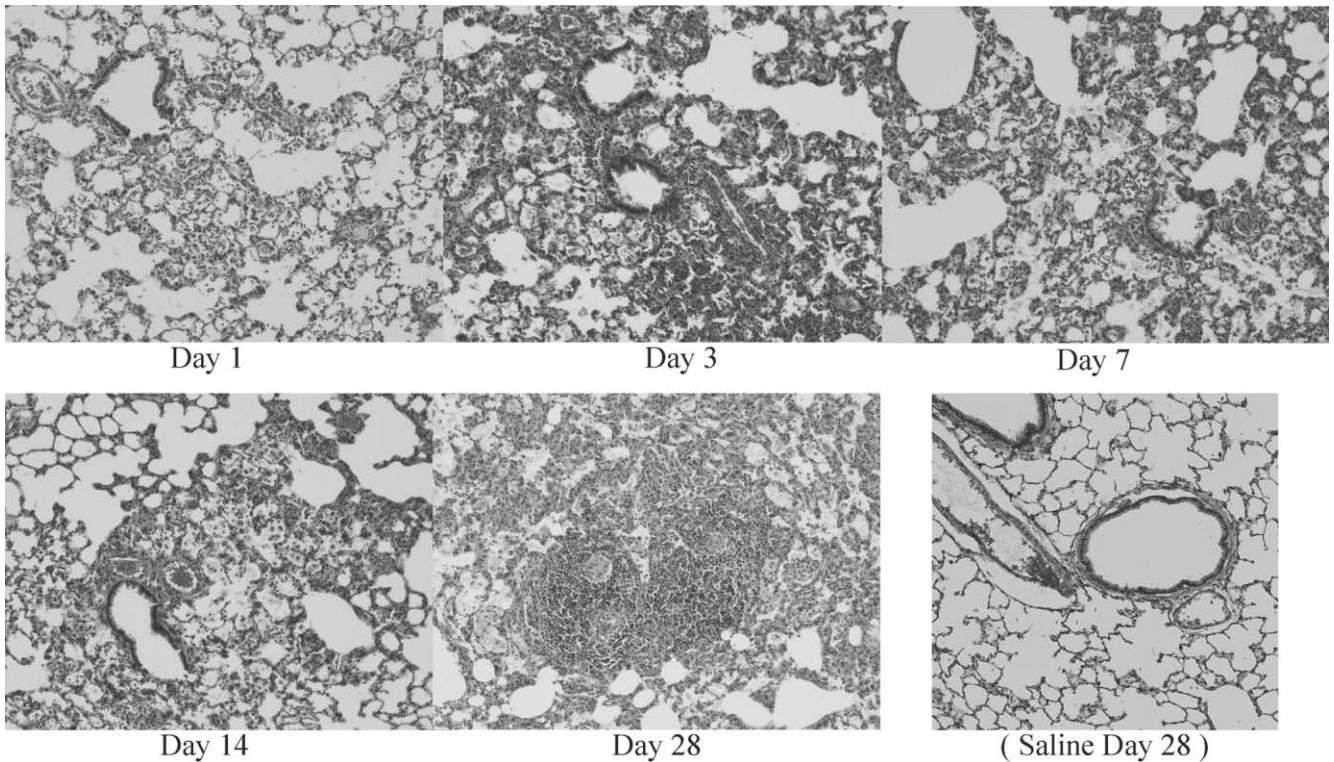


Fig. 3. Sequential histopathological changes in lungs from quartz treated rats ($\times 100$).

Table 1. Scoring Indices for Histopathological Parameters of Inflammatory Change

| Chemical | Days after treatment | No. of rats | Scoring Indices of Histopathological Changes | | | |
|----------|----------------------|-------------|--|--|--------------------------|----------------------------|
| | | | Neutrophil infiltration in the walls | Neutrophil infiltration in the spaces of alveoli | Pulmonary edema | Pulmonary fibrosis |
| Quartz | 1 | 5 | 2.6 ± 0.9 ^{a,b} | 1.4 ± 0.6 ^b | 1.0 ± 0.7 | 0.6 ± 0.6 |
| Saline | 1 | 5 | 0.6 ± 0.6 | 0.0 ± 0.0 | 0.4 ± 0.6 | 0.2 ± 0.5 |
| Quartz | 3 | 5 | 2.4 ± 0.9 ^b | 2.4 ± 0.9 ^b | 1.8 ± 0.5 ^b | 2.2 ± 0.8 ^{b,c} |
| Saline | 3 | 5 | 0.8 ± 0.8 | 0.0 ± 0.0 | 0.6 ± 0.6 | 0.2 ± 0.5 |
| Quartz | 7 | 5 | 1.6 ± 0.6 | 1.4 ± 0.6 ^b | 2.2 ± 0.5 ^{b,c} | 1.6 ± 0.6 ^b |
| Saline | 7 | 5 | 0.6 ± 0.6 | 0.0 ± 0.0 | 0.4 ± 0.6 | 0.0 ± 0.0 |
| Quartz | 14 | 5 | 2.2 ± 0.5 ^b | 1.8 ± 0.8 ^b | 2.4 ± 0.6 ^{b,c} | 2.6 ± 0.6 ^{b,c} |
| Saline | 14 | 5 | 0.8 ± 0.5 | 0.2 ± 0.5 | 0.8 ± 0.5 | 0.0 ± 0.0 |
| Quartz | 28 | 5 | 2.8 ± 0.5 ^b | 2.8 ± 0.5 ^{b,c,e} | 2.6 ± 0.6 ^{b,c} | 2.8 ± 0.6 ^{b,c,e} |
| Saline | 28 | 5 | 0.6 ± 0.6 | 0.6 ± 0.6 | 1.0 ± 0.0 | 0.6 ± 0.6 |

| Chemical | Days after treatment | No. of rats | Scoring Indices of Histopathological Changes | |
|----------|----------------------|-------------|--|---|
| | | | Histiocyte infiltration in the alveoli | Restructuring of the walls of the alveoli |
| Quartz | 1 | 5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Saline | 1 | 5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Quartz | 3 | 5 | 0.8 ± 0.5 ^c | 0.0 ± 0.0 |
| Saline | 3 | 5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Quartz | 7 | 5 | 1.4 ± 0.6 ^{b,c,d} | 0.0 ± 0.0 |
| Saline | 7 | 5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Quartz | 14 | 5 | 3.0 ± 0.0 ^{b,c,d,e} | 2.0 ± 0.7 ^b |
| Saline | 14 | 5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Quartz | 28 | 5 | 3.0 ± 0.0 ^{b,c,d,e} | 3.0 ± 0.0 ^{b,c,f} |
| Saline | 28 | 5 | 0.0 ± 0.0 | 0.0 ± 0.0 |

a: Mean ± SD. Severity of each parameter assessed as follows: 0, no change; 1, weak; 2, moderate; 3, severe.

b: P<0.05 vs. the respective saline control.

c: P<0.05 vs. Quartz Day 1.

d: P<0.05 vs. Quartz Day 3.

e: P<0.05 vs. Quartz Day 7.

f: P<0.05 vs. Quartz Day 14.

quartz treated group, numbers and areas of iNOS positive cells were the lowest in lungs of rats sacrificed on Day 1 and significantly increased with time. No significant variation in immunohistochemical staining for MMP-3 was evident between the quartz treated and control groups at any time (Table 4).

Discussion

In the present experiment, lungs of rats in the quartz treated group sacrificed on Day 1 demonstrated acute inflammatory changes with neutrophil infiltration, while granulation-like change with giant cells and macrophages in the alveoli were evident on Day 28. The results are generally in line with the pronounced accumulation of neutrophils in the alveoli⁸. Furthermore, numbers of BrdU positive cells were found to gradually decrease after the initial peak on Day 1. Monoclonal antibodies specific for BrdU provide a sensitive method for detecting DNA replication for DNA repair and cell proliferation *in situ*⁹.

Numbers and areas of iNOS positive cells, in contrast, increased with time, throughout the experimental period.

Under normal physiological conditions, endogenous NO is produced by the constitutive NOS isoforms, eNOS (endothelial NOS) and nNOS (neuronal NOS). The inducible form is expressed by many cells within the lung parenchyma after exposure to various inflammatory stimuli^{10,11}, and is also associated with neovascularization and proliferation¹². Furthermore, iNOS activity in primary tumor tissues in fresh human gynecological and breast cancers correlates positively with the tumor grade^{13,14}. The generation of oxidants and nitric oxide, in particular, is temporally and anatomically associated with the development of lung damage, inflammation, granulomas and fibrosis induced by inhalation of silica¹⁵.

MMP-3 cleaves proteoglycans, collagens (type II, IX, XI), gelatin, laminin, and fibronectin^{16,17} and may degrade cell matrix materials in areas with active inflammation¹⁸. After quartz instillation, however, there was no association with any of the subchronic changes, such as granulation, collagenization or fibrosis.

Based on the results of this study, we propose a bioassay method to detect toxic effects of inhaled fine particles as follows. Test particles are suspended in

Table 2. Labelling Indices from BrdU Immunohistochemistry of the Pulmonary Epithelium after Exposure to Quartz by Intratracheal Instillation

| Days after treatment | Quartz | | | Saline | | |
|----------------------|-------------|--------------------------|--------------------------|-------------|-----------|-----------|
| | No. of rats | % No. | % Area | No. of rats | % No. | % Area |
| 1 | 5 | 42.1 ± 32.7 ^a | 14.8 ± 11.0 ^a | 5 | 0.4 ± 0.5 | 0.2 ± 0.3 |
| 3 | 5 | 14.5 ± 7.1 ^a | 12.8 ± 5.7 ^a | 5 | 0.3 ± 0.5 | 0.3 ± 0.5 |
| 7 | 5 | 1.6 ± 2.6 ^{b,c} | 1.0 ± 1.5 ^{b,c} | 5 | 0.1 ± 0.3 | 0.2 ± 0.4 |
| 14 | 5 | 3.8 ± 3.1 | 3.8 ± 4.3 | 5 | 0.1 ± 0.1 | 0.0 ± 0.1 |
| 28 | 5 | 1.8 ± 1.5 | 2.9 ± 2.7 | 5 | 1.2 ± 2.6 | 0.1 ± 0.2 |

At least 3500 cells (total areas of nuclei: 0.102 mm²) were counted for each sample.

a: P<0.05 vs. the respective saline control.

b: P<0.05 vs. Quartz Day 1.

c: P<0.05 vs. Quartz Day 3.

Table 3. Labelling Indices from iNOS Immunohistochemistry of the Pulmonary Epithelium after Exposure to Quartz by Intratracheal Instillation

| Days after treatment | Quartz | | | Saline | | |
|----------------------|-------------|----------------------------|----------------------------|-------------|-----------|-----------|
| | No. of rats | % No. | % Area | No. of rats | % No. | % Area |
| 1 | 5 | 4.6 ± 2.9 ^a | 15.2 ± 17.6 ^a | 5 | 1.4 ± 1.2 | 2.6 ± 1.4 |
| 3 | 5 | 7.7 ± 0.0 ^a | 12.5 ± 6.4 | 5 | 0.3 ± 0.2 | 0.8 ± 0.7 |
| 7 | 5 | 23.1 ± 11.0 ^a | 28.7 ± 13.2 ^{a,b} | 5 | 1.2 ± 1.4 | 1.0 ± 0.9 |
| 14 | 5 | 18.1 ± 8.6 ^a | 36.1 ± 16.1 ^{a,b} | 5 | 1.6 ± 1.0 | 2.1 ± 0.5 |
| 28 | 5 | 43.0 ± 19.0 ^{a-d} | 77.6 ± 41.2 ^{a,b} | 5 | 1.2 ± 0.6 | 3.0 ± 2.1 |

At least 3700 cells (total areas of nuclei: 0.072 mm²) were counted for each sample.

a: P<0.05 vs. the respective saline control.

b: P<0.05 vs. Quartz Day 1.

c: P<0.05 vs. Quartz Day 3.

d: P<0.05 vs. Quartz Day 14.

Table 4. Labelling indices from MMP-3 Immunohistochemistry of the Pulmonary Epithelium after Exposure to Quartz by Intratracheal Instillation

| Days after treatment | Quartz | | | Saline | | |
|----------------------|-------------|----------------------------|----------------------------|-------------|--------------|-------------|
| | No. of rats | % No. | % Area | No. of rats | % No. | % Area |
| 1 | 5 | 164.5 ± 52.9 | 27.6 ± 11.8 | 5 | 127.8 ± 54.4 | 43.3 ± 28.0 |
| 3 | 5 | 128.6 ± 61.2 ^a | 31.5 ± 20.6 | 5 | 70.1 ± 15.9 | 14.9 ± 7.7 |
| 7 | 5 | 164.8 ± 79.0 | 44.0 ± 27.3 | 5 | 177.3 ± 48.4 | 44.7 ± 13.6 |
| 14 | 5 | 156.2 ± 49.8 ^a | 23.6 ± 12.4 | 5 | 103.5 ± 33.3 | 15.2 ± 4.4 |
| 28 | 5 | 89.9 ± 82.2 ^{a-c} | 14.2 ± 12.0 ^{b-c} | 5 | 58.0 ± 31.3 | 8.3 ± 5.3 |

At least 3000 cells (total areas of nuclei: 0.080 mm²) were counted for each sample.

a: P<0.05 vs. the respective saline control.

b: P<0.05 vs. Quartz Day 1.

c: P<0.05 vs. Quartz Day 3.

d: P<0.05 vs. Quartz Day 7.

physiological saline, when possible, and applied to 10-week-old male F344 rats by intratracheal instillation using our specially designed aerolizer. Lung histopathology, as well as immunohistochemical analyses using BrdU and iNOS, can then be employed to assess lung injury on Day 1 and Day 28 after treatment. Although instillation and inhalation models are different, the present instillation model can be

used for detection of acute or subacute lung toxicity caused by inhaled various fine particles.

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