Inflammogenic Effect of Polyacrylic Acid in Rat Lung Following Intratracheal Instillation

Chinatsu Nishida
University of Occupational and Environmental Health Japan: Sangyo Ika Daigaku
Taisuke Tomonaga
University of Occupational and Environmental Health Japan: Sangyo Ika Daigaku
Hiroto Izumi
University of Occupational and Environmental Health Japan: Sangyo Ika Daigaku
Ke-Yong Wang
University of Occupational and Environmental Health Japan: Sangyo Ika Daigaku
Toru Ishidao
University of Occupational and Environmental Health Japan: Sangyo Ika Daigaku
Jun-ichi Takeshita
National institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan
Ryohei Ono
The University of Kitakyushu - Hibikino Campus: Kitakyushu Shiritsu Daigaku - Hibikino Campus
Kazuki Sumiya
The University of Kitakyushu - Hibikino Campus: Kitakyushu Shiritsu Daigaku - Hibikino Campus
Shota Fujii
The University of Kitakyushu - Hibikino Campus: Kitakyushu Shiritsu Daigaku - Hibikino Campus
Shinichi Mochizuki
The University of Kitakyushu - Hibikino Campus: Kitakyushu Shiritsu Daigaku - Hibikino Campus
Kazuo Sakurai
The University of Kitakyushu - Hibikino Campus: Kitakyushu Shiritsu Daigaku - Hibikino Campus
Kei Yamasaki
University of Occupational and Environmental Health Japan: Sangyo Ika Daigaku
Kazuhiro Yatera
University of Occupational and Environmental Health Japan: Sangyo Ika Daigaku
Yasuo Morimoto (yasuom@med.uoeh-u.ac.jp)
University of Occupational and Environmental Health https://orcid.org/0000-0002-5339-6905

Research

Keywords: Cross-linked polyacrylic acid (CL-PAA), Organic chemicals, Pulmonary toxicity

Posted Date: January 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-147025/v1

Version of Record: A version of this preprint was published at Particle and Fibre Toxicology on January 21st, 2022. See the published version at https://doi.org/10.1186/s12989-022-00448-z.

Abstract

Background

Organic chemicals are known to cause allergic diseases such as bronchial asthma and hypersensitivity pneumonitis, and it has been considered that they do not cause irreversible pulmonary fibrosis. It has recently been reported, however, that cross-linked acrylic acid-based polymer, an organic chemical, might cause serious interstitial lung diseases, including pulmonary fibrosis. We investigated whether or not intratracheal instillation exposure to cross-linked polyacrylic acid (CL-PAA) can cause lung disorder in rats.

Methods

Male F344 rats were intratracheally instilled with dispersed CL-PAA at low (0.2 mg/rat) and high (1.0 mg/rat) doses, and were sacrificed at 3 days, 1 week, 1 month, 3 months and 6 months after exposure to examine inflammatory and fibrotic responses and related gene expressions in the lungs. Rat lungs exposed to crystalline silica, asbestos (chrysotile), and NiO and CeO_2 nanoparticles were used as comparators.

Results

Persistent increases in total cell count, neutrophil count and neutrophil percentage, and in the concentration of the cytokine-induced neutrophil chemoattractants (CINC)-1, CINC-2 and C-X-C motif chemokine 5 (CXCL5) (which had the highest expression of all chemokines by cDNA microarray using the lung tissue) were observed in bronchoalveolar lavage fluid (BALF) from 3 days until at least 1 month following CL-PAA intratracheal instillation. Persistent increases in heme oxygenase-1 (HO-1) in the lung tissue were also observed from 3 days to 6 months after exposure. Histopathological findings of the lungs demonstrated that extensive inflammation at 3 days was greater than that in exposure to silica, NiO nanoparticles and CeO_2 nanoparticles, and equal to or greater than that in asbestos (chrysotile) exposure, and the inflammation continued until 1 month. Fibrotic changes also progressed after 1 month postexposure.

Conclusion

Our results suggest that CL-PAA may potentially cause strong neutrophil inflammation in the rat and human lung.

Background

Inorganic chemicals such as asbestos and crystalline silica are known to cause irreversible interstitial pulmonary fibrotic lesions such as pneumoconiosis. Organic chemicals, conversely, cause allergic lung diseases such as bronchial asthma and hypersensitivity pneumonitis, but it is believed that they do not cause irreversible interstitial lesions like pulmonary fibrosis. Recent reports from South Korea, however,

have shown that the organic chemical polyhexamethyleneguanidine phosphate (PHMG-p), used as a humidifier disinfectant, caused lung disorders, including interstitial lung disease and acute respiratory distress syndrome (ARDS), in 5,955 people [1,2]. Occupational and environmental exposure to organic chemicals (e.g. exposure to wood dust, livestock and vegetable dust/animal dust) has also been identified as a potential risk factor for developing irreversible pulmonary fibrosis with very poor prognosis (5-year survival rate of 20 to 30%) [3]. It is fully conceivable, therefore, that exposure to other organic chemicals could also lead to irreversible pulmonary fibrosis. It has been reported that some workers at small and medium-sized enterprises in Japan that handle cross-liked acrylic acid-based polymers suffered from progressive lung disorder (Morimoto Y, et al. submitted), and the working group on occupational accident diseases of the Ministry of Health, Labour and Welfare compiled a report (Morimoto Y, et al. submitted). According to governmental reports (Morimoto Y, et al. submitted), in a workplace where acrylic acid-based polymers with cross-linked structures were handled, 6 out of tens of workers who had used a powder of the polymers developed pneumoconiosis, emphysema or pneumothorax. All of those workers were in their 20s-40s, and many of them developed lung disease in about two years after the beginning of exposure.

Cross-linked types of acrylic acid-based polymers have been used as intermediates in the manufacture of pharmaceuticals and cosmetics. Although pneumoconiosis caused by inorganic dusts has been known to progress slowly over 20 years or more after exposure to the causative substance [4–6], this organic chemical induced lung disorders in a surprisingly short period after exposure, and the disorders progressed significantly faster than pneumoconiosis due to inorganic chemicals such as asbestos and crystalline silica. Based on the above, the Japanese working group speculates that there is a causal relationship between exposure to cross-linked acrylic acid-based polymers and lung disorder, but so far there has not been sufficient evidence of lung disorder caused by cross-linked acrylic acid-based polymers.

The putative mechanism of lung disorder caused by inorganic chemicals is that the inhaled chemicals deposit in the lung and cause persistent inflammation, and eventually lead to the formation of chronic and irreversible lesions such as pulmonary fibrosis and tumors [7–11]. Asbestos and crystalline silica, which have high pulmonary toxicity, have been reported to cause persistent inflammation in the lungs, resulting in lung fibrosis and tumorigenesis [12,13]. Thus, in lung disorder caused by inhalable dust, persistent lung inflammation is considered to be an important process in the induction and progression of chronic and irreversible lesions in the lung [7,9–11]

In order to investigate lung disorder caused by cross-linked acrylic acid-based polymers, we performed intratracheal instillation of cross-linked polyacrylic acid (CL-PAA) in a rat model and analyzed the inflammatory and fibrotic responses in the lung. CL-PAA is a basic structure of cross-linked acrylic acid-based polymer, and polyacrylic acid (PAA) is an acrylic acid-based polymer obtained by homopolymerizing acrylic acid monomer. CL-PAA is widely used in various fields not only in Japan but also all over the world, and its production and quantity of import have been increasing over the years in Japan [14].

Results

Characterization of CL-PAA

The fundamental characteristics of CL-PAA are summarized in Table 1. The polymer used in our study had a weight average molecular weight (M_W) of 6.49 million by multiangle light scattering coupled with field flow fractionation (FFF-MALS) (Wyatt Technology Europe GmbH, Dernbach, Rheinland-Pfalz, Germany). The secondary diameter of the polymer in the testing suspension was 2.31 μm as measured by Mie scattering using xSight (Shoko Science Co., Ltd., Yokohama, Kanagawa, Japan). Figure 1 shows the scanning electron microscopy (SEM) by HITACHI S-4500 (Hitachi, Ltd., Tokyo, Japan) of the bulk polymer (A) and the dispersed polymer in the solution (B), respectively.

Body and lung weights

There were no significant changes in body weight in all of the groups, except for in the 1.0 mg-exposure group at 3 days after the instillation (Figure 2A). The relative lung weight (lung weight/body weight) increased in a dose dependent-manner during the observation period (Figure 2B). The lungs were edematous and mottled, especially in the 1.0 mg-exposure group, at 3days after the instillation (Figure 3).

Cell analysis and lactate dehydrogenase (LDH) activity in bronchoalveolar lavage fluid (BALF)

Figure 4 shows the results of inflammatory cell counts and LDH activity, an index of cell injury, in BALF. There was a statistically significant increase in the number of total cells in the 0.2 mg and 1.0 mg-exposure groups from 3 days to 1 month after exposure compared to the control group (Figure 4A). There were significant increases in the number of neutrophils (Figure 4B) and the percentage of neutrophils (Figure 4C) from 3 days to 3 months after exposure. Optical microscopic images of the BALF findings at 3 days after the instillation demonstrated that there were many neutrophils and many macrophages that had phagocytized CL-PAA in the exposure groups (Figure 5), indicating that CL-PAA induced persistent lung inflammation from an acute to sub-chronic phase after exposure. The results of released LDH activity in the 0.2 mg and 1.0 mg-exposure groups also showed statistically significant increases from 3 days to 1 month after exposure compared to the control group (Figure 4 D).

Concentration of cytokine-induced neutrophil chemoattractants (CINC) and C-X-C motif chemokine (CXCL5) in BALF and concentration of heme oxygenase (HO)-1 in lung tissue

Figure 6A-C shows the concentrations of CINC-1, CINC-2 and CXCL5 in BALF following the intratracheal instillation of CL-PAA. The concentrations of CINC-1, CINC-2 and CXCL5 increased persistently in a dose dependent-manner from 3 days until 1 month postexposure. The expression levels of these three chemokines in the exposed groups decreased with time, and no significant increase was observed after 3 months postexposure in general. Statistically significant persistent increases in the concentration of HO-1 in the lung tissues exposed to CL-PPA were observed during the observation time.

Gene expression analysis

Table 2A shows the number of genes, among 20,174 genes examined by cDNA microarray, sorted by the fold change of mRNA expression levels in lung tissue at 1 month in the 1.0 mg CL-PAA-exposure group compared to the control group. More than 8-fold upregulated chemokine genes involved in “inflammatory response” are shown in Table 2B. Among them, the most upregulated gene was *CXCL5* (also known as *C-X-C motif chemokine* 6 (*CXCL6*) in rats), and there was a 69.02-fold amount in the 1.0 mg CL-PAA-exposure group compared with the control group. Supplemental Table 1 shows the upregulated genes related to “inflammatory response” (Table S1(A): 52 genes), “immune response” (Table S1(B): 47 genes) and “response to oxidative stress” (Table S1(C): 9 genes) with more than 2-fold upregulation compared to the control group.

Micro-CT imaging

Micro-CT revealed diffuse or centrilobular infiltration in both lungs at 3 days and 1 month after the exposure in a dose-dependent manner (Figure 7). An improvement of lung infiltrations was observed at 3 months as compared with those at 3 days and 1 month after the exposure.

Histopathological features in the lung

Representative histopathological findings in the lung at 3 days and 1month after the instillation of CL-PAA are shown in Figures 8 (A-C, H-J) and 9, respectively. Inflammatory cell infiltrations into the alveoli, mainly neutrophils, were remarkable in the lung in a dose-dependent manner at 3 days after exposure to CL-PAA (Figure 8A-C, H-J). The CL-PAA-induced inflammation in the lung at 3 days postexposure was greater than that in exposure to silica (Figure 8D, K), NiO nanoparticles (Figure 8F, M) and CeO_2 nanoparticles (Figure 8G, N), and equal to or greater than that in asbestos (chrysotile) exposure (Figure 8E, L). The pathological features of lung inflammation persisted even 1 month after exposure, but there were no granulomas or formation of giant cells. While inflammatory cell infiltration was persistent, alveolar fibrosis was observed from 1 month (Figure 9).

Lung immunohistochemistry at 1 month after exposure to 1.0 mg-CL-PAA demonstrated that the CXCL5-positive cells were macrophages around the neutrophil infiltration (Figure 10).

Discussion

The main findings obtained in the present study are as follows: (1) The CL-PAA caused severe lung inflammation and fibrosis. (2) The lung inflammation induced by CL-PAA occurred in a dose dependent-manner. (3) The HO-1 protein level in the lung tissue increased persistently during the observation period.

In the present study, there was marked neutrophil-based inflammatory cell infiltration in the alveoli in the lungs following intratracheal instillation of CL-PAA and it persisted until 1 month. We previously performed intratracheal instillations of various inorganic chemicals under the same experimental conditions of dose and observation period: crystalline silica, asbestos (chrysotile), and nanoparticles of nickel oxide (NiO) [15] and cerium oxide (CeO_2) [16], which has high pulmonary toxicity among

manufactured nanomaterials, and multi-walled carbon nanotube (MWCNT) [17] with lung tumorigenesis. Although all of these materials also induced persistent inflammation mainly due to neutrophil infiltration, the lung inflammation caused by CL-PAA was equal to or stronger than that of those materials. Actually, in the present study, we found more extensive distribution of inflammation in the lung than by the other substances with high pulmonary toxicity (Figure 8).

Regarding lung disorder caused by organic chemicals, there have been reports from South Korea of an animal model of exposure to PHMG-P, and severe lung inflammation which was mainly neutrophils was observed in all of the studies. Kim et al. performed an intratracheal instillation of PHMG-P (1.2 mg / kg BW, single exposure) in mice and observed them for 1 month after the exposure, and severe and persistent inflammation in the lung occurred until 1 month after the exposure [18]. Park S et al. conducted an inhalation exposure to PHMG-P in rats (1.6 mg/m^3, 6 hours/day, 5 days/week, for 4 weeks), and severe lung inflammation was observed [19]. The clinical characteristics of PHMG-P induced lung disorder in humans are a short duration (within one year) of its use before the onset of lung disorder, rapid development of fibrosis following severe pneumonia, and a high mortality rate [20,21]. In other reports, although the exposure doses were different from the present study, PHMG-p induced severe lung inflammation similarly to the present study, suggesting that CL-PAA has a high ability to cause lung inflammation.

The CINC-1 and CINC-2 concentrations in BALF increased persistently due to CL-PAA exposure. The CINC family are typical chemokines that induce and activate neutrophils and macrophages in rat lung. Intratracheal instillation of NiO and CeO_2 nanoparticles under the same exposure dose as in the present study also showed an increase in CINC-1 and CINC-2 concentrations in BALF [15,16,22], and the level of increase due to CL-PAA exposure was almost the same (Supplemental Figure 1A, B). On the other hand, the gene expression of *CXCL5* was significantly higher, approximately 70-fold, as compared with the control group. CXCL5 is a CXC chemokine with a glutamate-leucine-arginine (ELR) motif (ELR + chemokine) that has strong chemotaxis and activation functions for lung neutrophils [23]. In our previous study, which was performed by the instillation of 1.0 mg-NiO and 1.0 mg-CeO_2 nanoparticles, as in the present study, the gene expression of *CXCL5* in the lung tissue during the observation period of 3 days to 6 months was up to 20-fold higher than that in the control group [24]. The degree of neutrophil influx into the lungs by intratracheal instillation of CL-PAA was higher than that due to exposure to NiO and CeO_2 nanoparticles. These results suggest that CXCL5 has an enhancing effect of neutrophil influx in lung disorder caused by CL-PAA, in addition to CINC-1 and CINC-2 being involved in neutrophil influx into the lung.

Intratracheal instillation of CL-PAA caused lung fibrosis from 1 month after the exposure, and the extent was greater than that of crystalline silica and asbestos (chrysotile) in the lungs (Figure 11). This suggests that the CL-PAA used in the present study has a potent fibrotic ability. Similarly, as regards organic chemicals, exposure to PHMG-P induced more severe fibrosis than bleomycin at 1 month after the exposure by intratracheal instillation in a mice model [18]. The mechanism that caused the fibrosis due to exposure to CL-PAA is uncertain, but increases in the expression of some genes (e.g. CXCL5, C-C motif

chemokine 22 (CCL22), and C-C motif chemokine 17 (CCL17)) related to fibrosis were observed in the results of a comprehensive gene analysis (Table S1A, B). Since the expression of these genes have been reported to be involved in fibrosis in humans and animal exposure models [25–29], these may also have been related to the fibrosis in the present study.

As for the maximum dosage settings in this experiment, 1.0 mg/rat (equivalent to 4.0 mg/kg BW) was considered to be approximately the maximum dose that is necessary for evaluating the pulmonary toxicity of CL-PAA. The dose of 4.0 mg/kg BW is considered the maximum dose at which respirable chemical substances did not cause overload in intratracheal instillation studies. We previously injected doses in excess of 4.0 mg/kg BW, and they induced pulmonary surplus inflammation and the delay of the biological half time of the nanoparticles [30]. It was reported in toner studies by Morrow PE et al. and Bellmann B et al that a delayed clearance of alveolar macrophages occurred between 1.0 mg/rat (4 mg/kg BW in our study is equivalent to 1.0 mg/rat) and 3.0 mg/rat of lung deposition [31,32], indicating that the threshold of overload is between 1.0 and 3.0 mg/rat. We can conclude from these data that exposure to doses above 1.0 mg/rat might induce pulmonary toxicity by the excessive dose, in addition to the chemicals themselves. The dose of 4.0 mg/kg BW as the lung burden of respirable chemical substances after intratracheal instillation may correspond to a period of approximately 1.8 years of inhalation at a concentration of 3 mg/m^3 (the maximum concentration for humans of inhalable dust other than crystalline silica (working time 8 h/day, five days/week)), respectively, defined by the American Conference of Governmental Industrial Hygienists (ACGIH).

In the comprehensive gene analysis, the upregulation of HO-1 was higher among the group of “response to oxidative stress”, and 4.62 times compared to the control group. It was also found that there was a persistent increase in the concentration of HO-1 protein level in the lung tissue in the present study. In our previous study, we observed a persistent increase in the HO-1 protein level in lung tissue exposed to NiO nanoparticles [33], similar to a persistent increase in CINC-1 and CINC-2 in BALF. In the analysis of the HO-1 protein level in BALF, a persistent increase was shown in intratracheal instillation of the same NiO nanoparticles [15]. On the other hand, the HO-1 in BALF increased transiently in an intratracheal instillation of titanium dioxide (TiO_2) nanoparticles (Rutile) with low pulmonary toxicity [15]. The persistent increase in the HO-1 in the lung tissue in the present study is considered to reflect lung disorder. Furthermore, although we analyzed HO-1 in the lung tissue after the recovery of BALF, the level of HO-1 was higher than that in lung tissue exposed to NiO nanoparticles without recovery of BALF. CL-PAA exposure led to more extensive and severe lung inflammation and higher HO-1 levels in lung tissue than other particles with high pulmonary toxicity, suggesting that CL-PAA induced lung disorder through oxidative stress. It has been reported that oxidative stress made lung injury more progressive in knockout mice of class A scavenger receptors (SR-As) [34].

A limitation of this study is that, although intratracheal instillation studies can be useful for estimating the hazardous effects of inhalable chemicals, its exposure route is not physiological, in spite of the instillation of CL-PAA of a respirable size, unlike in inhalation studies. Therefore, inhalation studies are required to elucidate whether or not exposure to CL-PAA induces pulmonary inflammation and fibrosis.

Conclusions

In the present study, we performed intratracheal instillation of CL-PAA in rats and examined lung inflammation and fibrosis in an observation period of 3 days to 6 months. There was remarkable and persistent lung inflammation from just after the exposure, leading to fibrosis 1 month later. Chemokines such as CXCL5, CINC-1, and CINC-2, and oxidative stress were considered to be involved in the lung inflammation induced by CL-PAA. Taken together, these results suggest that CL-PAA has a high potential of induction of lung disorder.

Material And Methods

Sample polymer

CL-PAA (306223 Poly (acrylic acid)^{R}): average Mv~3,000,000) (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) was used. The polymer was mixed with distilled water, slowly stirred for 40 minutes (Mag-Mixer MF820 or MD300, Yamato Scientific co., Ltd., Tokyo, Japan) and then ultrasonically dispersed at 23 kHz for 10 minutes (ASU-10D, Taiyo Canpany Co., Ltd., Osaka, Japan).

Animals

Male Fischer 344 rats (8 weeks old) (Charles River Laboratories International, Inc., Kanagawa, Japan) were acclimated for 2 weeks in the Laboratory Animal Research Center of the University of Occupational and Environmental Health, Japan with free access to a commercial diet and water. All procedures and animal handling were done according to the guidelines described in the Japanese Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee, University of Occupational and Environmental Health, Japan (animal studies ethics clearance proposal number; AE17-009).

Intratracheal instillation

Doses of 0.2 mg (0.8 mg/kg BW) and 1.0 mg (4.0 mg/kg BW) of CL-PAA suspended in 0.4 ml distilled water were administered to rat lungs (12 weeks old) in single intratracheal instillations. The control group received distilled water.

Animals following intratracheal instillation

There were 5 rats in each exposure and control group at each time point. Animals were dissected at 3 days, 1 week, 1 month, 3 months and 6 months after intratracheal instillation under anesthesia with isoflurane (Pfizer Japan, Tokyo, Japan) inhalation. Body and lung weights were measured, then, at autopsy, blood was removed from the abdominal aorta and the lung was perfused with normal saline. The right lungs were repetitively inflated with normal saline under a pressure of 20 cm H_2O, following fluid recovery two times, while the left main bronchus was clamped. Between 7 and 14 mL of the recovered fluid (BALF) was collected in collection tubes by free fall, and then the right and left lungs were divided.

The homogenized third lobes of the right lungs after recovery of BALF were used for HO-1 and cDNA microarray. The left lungs were inflated and fixed by 10% formaldehyde under a pressure of 25 cm H2O for use in histopathological evaluation.

Cytospin analysis of inflammatory cells and measurement of LDH in BALF

BALF was centrifuged at 400 g at 4°C for 15 minutes, and the supernatant was transferred to a new tube for measurement of LDH and cytokines. The pellets were washed by suspension with polymorphonuclear leukocyte (PMN) Buffer (137.9 mM NaCl, 2.7 mM KCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 5.6 mM $C_6H_{12}O_6$) and centrifuged at 400 g at 4°C for 15 minutes. After removal of the supernatant, the pellets were resuspended with 1mL of PMN Buffer. The number of cells in BALF was counted by ADAM-MC (AR BROWN CO., LTD, Tokyo, Japan), and the cells were splashed on a slide glass using cytospin, fixed and stained with Diff-Quik (Sysmex CO., Kobe, Hyogo, Japan), then the number of neutrophils and alveolar macrophages were counted by microscopic observation. The released LDH activity in the BALF supernatant was measured by a Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Diagnostics GmbH, Mannheim, Nordrhein-Westfalen, Germany) according to the manufacturer's instructions. LDH activity was estimated using a standard curve obtained from known concentrations of recombinant LDH from rabbit muscle (Oriental Yeast Co., ltd., Tokyo, Japan).

Measurement of chemokines in BALF and HO-1 in lung tissue

Concentrations of CINC-1, CINC-2 and CXCL5 in BALF were measured by ELISA kits, #RCN100, #RCN200 (R&D Systems, Minneapolis, MN, USA), and LS-F23176 (LSBio, Seattle, WA, USA), respectively. All measurements were performed according to the manufacturer's instructions. The third lobes of the right lungs were homogenized with T-PER tissue protein extraction reagent (Thermo Scientific Inc., Rockford, IL, USA) including protein inhibitor cocktails (P8340, Sigma-Aldrich, St. Louis, MO, USA) and cOmplete Mini (Roche Diagnostics GmbH, Mannheim, Nordrhein-Westfalen, Germany), and then centrifuged (20,400 g at 4°C for 10 minutes). The protein concentration of the supernatant was measured by Pierce 660nm Protein Assay Reagent (Thermo Scientific Inc., Rockford, IL, USA), using bovine serum albumin as a standard. The total protein concentration was adjusted to a final concentration of 500 mg/mL for measuring the HO-1 by the ELISA kit, ADI-EKS-810A (Enzo Life Sciences, Farmingdale, NY, USA).

Total RNA extraction

Total RNA extraction was performed as described previously [24]. Briefly, the third lobes of the right lungs of the control group and 1.0 mg of the CL-PAA-exposure group at 1 month after the instillation were homogenized, and total RNA was extracted using a miRNAeasy Mini Kit (Qiagen, Hilden, Nordrhein-Westfalen, Germany). The RNA was quantified and quality checked for use in microarray analysis.

Microarray analysis

Microarray analysis was performed as described previously [24]. Briefly, A 3D-Gene Rat Oligo Chips 20K (version 1.1) (Toray Industries, Tokyo, Japan) containing 20,174 genes was used for a 3D-Gene array system (Toray Industries, Tokyo, Japan), according to the manufacturer's protocol. One μg of total RNA at 1 month was used to hybridize the Rat Oligo Chip 20K.The function of the enhanced expression genes was analyzed by Database for Annotation Visualization and Integrated Discovery 6.8 [35].

Micro-CT imaging

The X-ray micro-CT system (CosmoScan GX, Rigaku Co., Tokyo, Japan) was operated with the following parameters: a tube voltage of 90 kV, a tube current of 88 μA, chest CT, 6040 mm field of view (FOV) (the voxel matrix: μm, and the voxel size: μm). The lungs were scanned in the prone position under anesthetization with inhalation of mixed isoflurane (Pfizer Japan, Tokyo, Japan) and oxygen through a nose cone. The exposure time was 4.0 minutes, and images were retrospectively gated at the inspiration breathing phase with an average whole body exposure of 161.9 mGy/scan.

Histopathology and immunohistochemistry

Formaldehyde-fixed lung tissue was embedded in paraffin, sectioned at a thickness of 4μm, and then stained with hematoxylin and eosin (HE) and Masson trichrome (MT) staining. Immunostaining for CXCL5 was performed with rabbit anti-mouse CXCL5 polyclonal antibody (1:200 dilution, bs-2549R; Bioss Inc., Woburn, MA, USA), while using the lung tissue samples from the 1.0 mg CL-PAA-exposure group of one month after intratracheal instillation. The slides were assessed for histological changes by a board-certified pathologist.

Statistical analysis

Statistical analysis was carried out using JMP^{R} Pro software (JMP Version 14.2.0, SAS Institute Inc., Cary, NC, USA). *P* values <0.05 were considered statistically significant. Dunnett's tests were used appropriately to detect individual differences between those exposed to the cross-linked polyacrylate samples and the controls.

Abbreviations

PHMG-p: polyhexamethyleneguanidine phosphate; ARDS: Acute respiratory distress syndrome; CL-PAA: Cross-linked polyacrylic acid; PAA: Polyacrylic acid; Mw: Weight average molecular weight; FFF-MALS: Multiangle light scattering coupled with field flow fractionation; SEM: Scanning electron microscopy; LDH: Lactate dehydrogenase; BALF: Bronchoalveolar lavage fluid; CINC: Cytokine-induced neutrophil chemoattractant; CXCL5: C-X-C motif chemokine 5; Heme oxygenase-1: HO-1; CXCL6: C-X-C motif chemokine 6; NiO: Nickel oxide; CeO_2: Cerium dioxide; Multi-walled carbon nanotube: MWCNT; Glutamate-leucine-arginine: ELR; CCL22: C-C motif chemokine 22; CCL17: C-C motif chemokine 17; ACGIH: the American Conference of Governmental Industrial Hygienists; TiO2: Titanium dioxide; SR-As: Scavenger

receptors; PMN: Polymorphonuclear leukocyte; FOV: Field of view; HE: Hematoxylin and eosin; MT: Masson trichrome

Declarations

Ethics approval and consent to participate

All procedures and animal handling were done according to the guidelines described in the Japanese Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee, University of Occupational and Environmental Health, Japan (animal studies ethics clearance proposal number: AE17-009).

Consent for publication

Not required as no human data presented.

Availability of data and material

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was partially supported by JSPS KAKENHI Grant Number JP18K10060.

Authors' contributions

Authors CN, TT, HI, KY (Kazuhiro Yatera) and YM are responsible for the study design and writing of the manuscript. Authors CN, TT, HI, KW, TI, JT, KS (Kazuki Sumiya), SM, KS (Kazuo Sakurai), KY (Kei Yamasaki), KY (Kazuhiro Yatera) and YM are responsible for data and analysis. Authors CN, TT, HI, KW, KS (Kazuki Sumiya), KS (Kazuo Sakurai) and YM performed the experiments. All the authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank M. Shijo, R. Takai, M. Tashiro and T. Morimoto for technical support with the experiments.

References

1. Park DU, Ryu SH, Lim HK, Kim SK, Choi YY, Ahn JJ, et al. Types of household humidifier disinfectant and associated risk of lung injury (HDLI) in South Korea. Sci Total Environ. 2017; doi: 10.1016/j.scitotenv.2017.04.040.
2. Ryu S, Park D, Lee E, Park S, Lee S, Jung S, et al. Humidifier disinfectant and use characteristics associated with lung injury in Korea. Indoor Air. 2019; doi: 10.1111/ina.12585.
3. Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, et al. An Official ATS/ERS/JRS/ALAT Statement: Idiopathic pulmonary fibrosis: Evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med. 2011; doi: 10.1164/rccm.2009-040GL.
4. O'reilly KMA, Mclaughlin AM, Beckett WS, Sime PJ. Asbestos-Related Lung Disease. Am Fam Physician. 2007;75:683-8.
5. Roggli VL, Gibbs AR, Attanoos R, Churg A, Popper H, Cagle P, et al. Pathology of asbestosis - An update of the diagnostic criteria report of the asbestosis committee of the college of american pathologists and pulmonary pathology society. Arch Pathol Lab Med. 2010; doi: 10.1043/1543-2165-134.3.462.
6. Barnes H, Goh NSL, Leong TL, Hoy R. Silica-associated lung disease: An old-world exposure in modern industries. Respirology. 2019; doi: 10.1111/resp.13695.
7. Borm PJ, Driscoll K. Particles, inflammation and respiratory tract carcinogenesis. Toxicol Lett. 1996; doi: 10.1016/0378-4274(96)03725-3.
8. Shacter E, Weitzman SA. Chronic Inflammation and Cancer. Oncology (Williston Park). 2002; doi: 10.1201/b12696-15.
9. Bellmann B, Muhle H, Creutzenberg O, Ernst H, Müller M, Bernstein DM, et al. Calibration study on subchronic inhalation toxicity of man-made vitreous fibers in rats. Inhal Toxicol. 2003; doi: 10.1080/08958370390229843.
10. Kim H, Morimoto Y, Ogami A, Nagatomo H, Hirohashi M, Oyabu T, et al. Differential expression of EC-SOD, Mn-SOD and CuZn-SOD in rat lung exposed to crystalline silica. J Occup Health. 2007; doi: 10.1539/joh.49.242.
11. Nishi K, Morimoto Y, Ogami A, Murakami M, Myojo T, Oyabu T, et al. Expression of cytokine-induced neutrophil chemoattractant in rat lungs by intratracheal instillation of nickel oxide nanoparticles. Inhal Toxicol. 2009; doi: 10.1080/08958370802716722.
12. Pott F, Ziem U, Reiffer FJ, Huth F, Ernst H MU. Carcinogenicity studies on fibres, metal compounds, and some other dusts in rats. Exp Pathol. 1987; doi: 10.1016/s0232-1513(87)80044-0.
13. Muhle H, Bellmann B, Creutzenberg O, Dasenbrock C, Ernst H, Kilpper R, MacKenzie JC, Morrow P, Mohr U, Takenaka S et al. Pulmonary response to toner upon chronic inhalation exposure in rats. Fundam Appl Toxicol. 1991; doi: 10.1016/0272-0590(91)90219-t.
14. Japan CHEmicals Collabprative Knowledge database. Incorporated Administrative Agency National Institute of Technology and Evaluation. https://www.nite.go.jp/chem/jcheck/detail.action?request_locale=en&cno=9003-01-4&mno=6-0898 Accessed 10 Jan 2021.

15. Morimoto Y, Izumi H, Yoshiura Y, Tomonaga T, Lee BW, Okada T, et al. Comparison of pulmonary inflammatory responses following intratracheal instillation and inhalation of nanoparticles. Nanotoxicology. 2016; doi: 10.3109/17435390.2015.1104740.
16. Morimoto Y, Izumi H, Yoshiura Y, Tomonaga T, Oyabu T, Myojo T, et al. Pulmonary toxicity of well-dispersed cerium oxide nanoparticles following intratracheal instillation and inhalation. J Nanopart Res. 2015; doi: 10.1007/s11051-015-3249-1.
17. Morimoto Y, Hirohashi M, Ogami A, Oyabu T, Myojo T, Todoroki M, et al. Pulmonary toxicity of well-dispersed multi-wall carbon nanotubes following inhalation and intratracheal instillation. Nanotoxicology. 2012; doi: 10.3109/17435390.2011.594912.
18. Kim MS, Kim SH, Jeon D, Kim HY, Lee K. Changes in expression of cytokines in polyhexamethylene guanidine-induced lung fibrosis in mice: Comparison of bleomycin-induced lung fibrosis. Toxicology. 2018; doi: 10.1016/j.tox.2017.11.017.
19. Park S, Lee K, Lee EJ, Lee SY, In KH, Kim HK, et al. Humidifier disinfectant-associated interstitial lung disease in an animal model induced by polyhexamethylene guanidine aerosol. Am J Respir Crit Care Med. 2014; doi: 10.1164/rccm.201404-0710LE.
20. Hong SB, Kim HJ, Huh JW, Do KH, Jang SJ, Song JS, et al. A cluster of lung injury associated with home humidifier use: clinical, radiological and pathological description of a new syndrome. Thorax. 2014; doi: 10.1136/thoraxjnl-2013-204135.
21. Park D, Leem J, Lee K, Lim H, Choi Y, Ahn JJ, et al. Exposure characteristics of familial cases of lung injury associated with the use of humidifier disinfectants. Environ Health. 2014; doi: 10.1186/1476-069X-13-70.
22. Tomonaga T, Izumi H, Oyabu T, Lee BW, Kubo M, Shimada M, et al. Assessment of cytokine-induced neutrophil chemoattractants as biomarkers for prediction of pulmonary toxicity of nanomaterials. Nanomaterials (Basel). 2020; doi: 10.3390/nano10081563.
23. Jeyaseelan S, Manzer R, Young SK, Yamamoto M, Akira S, Mason RJ, et al. Induction of CXCL5 during inflammation in the rodent lung involves activation of alveolar epithelium. Am J Respir Cell Mol Biol. 2005; doi: 10.1165/rcmb.2005-0063OC.
24. Nishida C, Izumi H, Tomonaga T, Takeshita JI, Wang KY, Yamasaki K, et al. Predictive biomarkers for the ranking of pulmonary toxicity of nanomaterials. Nanomaterials (Basel). 2020; doi: 10.3390/nano10102032.
25. Keane MP, Belperio JA, Burdick MD, Lynch JP, Fishbein MC, Strieter RM. ENA-78 is an important angiogenic factor in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2001; doi: 10.1164/ajrccm.164.12.2104106.
26. Nakayama S, Mukae H, Ishii H, Kakugawa T, Sugiyama K, Sakamoto N, et al. Comparison of BALF concentrations of ENA-78 and IP10 in patients with idiopathic pulmonary fibrosis and nonspecific interstitial pneumonia. Respir Med. 2005; doi: 10.1016/j.rmed.2005.02.021.
27. Belperio JA, Dy M, Murray L, Burdick MD, Xue YY, Strieter RM, et al. The Role of the Th2 CC Chemokine Ligand CCL17 in Pulmonary Fibrosis. J Immunol. 2004; doi:

10.4049/jimmunol.173.7.4692.

28. Inoue T, Fujishima S, Ikeda E, Yoshie O, Tsukamoto N, Aiso S, et al. CCL22 and CCL17 in rat radiation pneumonitis and in human idiopathic pulmonary fibrosis. Eur Respir J. 2004; doi: 10.1183/09031936.04.00110203.
29. Yogo Y, Fujishima S, Inoue T, Saito F, Shiomi T, Yamaguchi K, et al. Macrophage derived chemokine (CCL22), thymus and activation-regulated chemokine (CCL17), and CCR4 in idiopathic pulmonary fibrosis. Respir Res. 2009; doi: 10.1186/1465-9921-10-80.
30. Oyabu T, Morimoto Y, Hirohashi M, Horie M, Kambara T, Lee BW, et al. Dose-dependent pulmonary response of well-dispersed titanium dioxide nanoparticles following intratracheal instillation. J Nanopart Res. 2013; doi: 10.1007/s11051-013-1600-y.
31. Morrow PE, Muhle H, Mermelstein R. Chronic Inhalation Study Findings as a Basis for Proposing a New Occupational Dust Exposure Limit. Int J Toxicol. 1991; doi: 10.3109/10915819109078637.
32. Bellmann B, Muhle H, Creutzenberg O, Mermelstein R. Irreversible pulmonary changes induced in rat lung by dust overload. Environ Health Perspect. 1992; doi: 10.1289/ehp.9297189.
33. Horie M, Yoshiura Y, Izumi H, Oyabu T, Tomonaga T, Okada T, et al. Comparison of the pulmonary oxidative stress caused by intratracheal instillation and inhalation of NiO nanoparticles when equivalent amounts of NiO are retained in the lung. Antioxidants (Basel). 2016; doi: 10.3390/antiox5010004.
34. Kobayashi H, Sakashita N, Okuma T, Terasaki Y, Tsujita K, Suzuki H, et al. Class A scavenger receptor (CD204) attenuates hyperoxia-induced lung injury by reducing oxidative stress. J Pathol. 2007; doi: 10.1002/path.2150.
35. 3D-Gene® [Toray DNA Chips] | TORAY - https://www.3d-gene.com/en/ Accessed 10 Jan 2021.

Tables

Table 1. Physiochemical characterization of the polymer used in the present study.

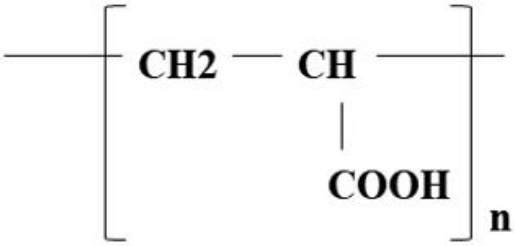
Name Polyacrylic acid	**Structural formula** 
CAS number	**9003-01-04**
Purity	**≦100 % (Benzene 0.5%)**
Molecular weight	
Weight average molecular weight (M_W)	**6,490,000**
Viscosity average molecular weight (Mv)	**3,000,000 (average)**
Cross-liking	**~ 0.1 %**
Appearance	**Solid, white powdered**
Odor	**None**
Size (Secondary particle diameter)	**2.31μm**

Table 1 shows the property of the polymer used in present study.

Secondary particle diameter: the particle diameter of agglomerate.

Table 2. (A) Number of genes by mRNA expression level in the polymer-high dose group at one month.

(B) Description of chemokine genes related to 'inflammatory response' among 58 genes upregulated ≧ 8-fold.

(A) mRNA level (fold change of control)		Number of genes
Up regulation	≧ 2-fold	788
	2~4-fold	620
	4~8-fold	110
	≧ 8-fold	58
Down regulation	≦-fold	668
	~-fold	648
	~-fold	17
	≦-fold	3

(B) Gene symbol	Gene description	Fold change
Inflammatory response		
GO: 0006954		
CXCL5	*Chemokine (C-X-C motif) ligand5*	69.02
CXCL11	*Chemokine (C-X-C motif) ligand11*	33.73
CCL7	*Chemokine (C-C motif) ligand7*	32.69
CCL2	*Chemokine (C-C motif) ligand2*	30.93
CXCL13	*Chemokine (C-X-C motif) ligand13*	29.57
CCL1	*Chemokine (C-C motif) ligand1*	20.32
CCL9	*Chemokine (C-C motif) ligand9*	16.24
CXCL10	*Chemokine (C-X-C motif) ligand10*	13.51
CCL12	*Chemokine (C-C motif) ligand12*	12.64

Table 2 (A) shows number of genes by mRNA expression level in the polymer-high dose group at one month after intratracheal instillation among 20,174 genes examined using cDNA microarray. (B) Upregulated chemokine genes among the genes involved in “inflammatory response” among 58 genes upregulated ≧ 8-fold.

Figures

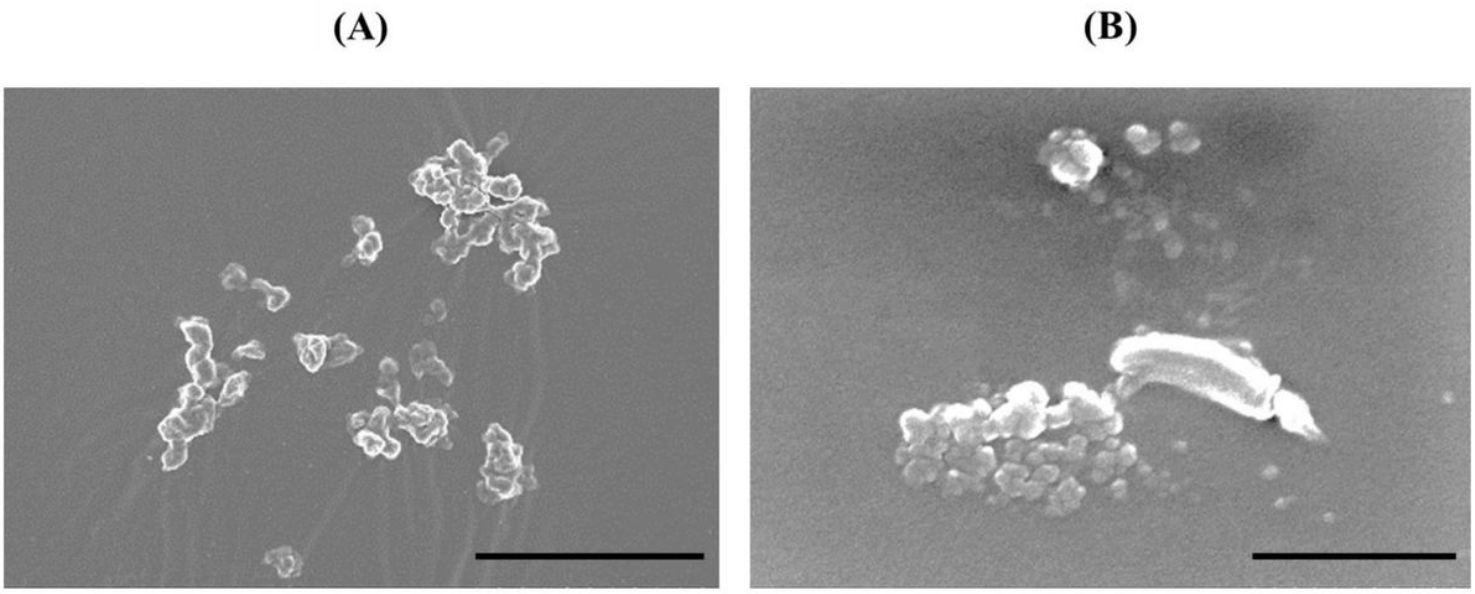


Figure 1

SEM images of the CL-PAA. Both the bulk polymer powder (A) and the suspended polymer in distilled water (B) made up agglomerates. (internal scale bar = 20 μm for (A) and 0.5 μm for (B))

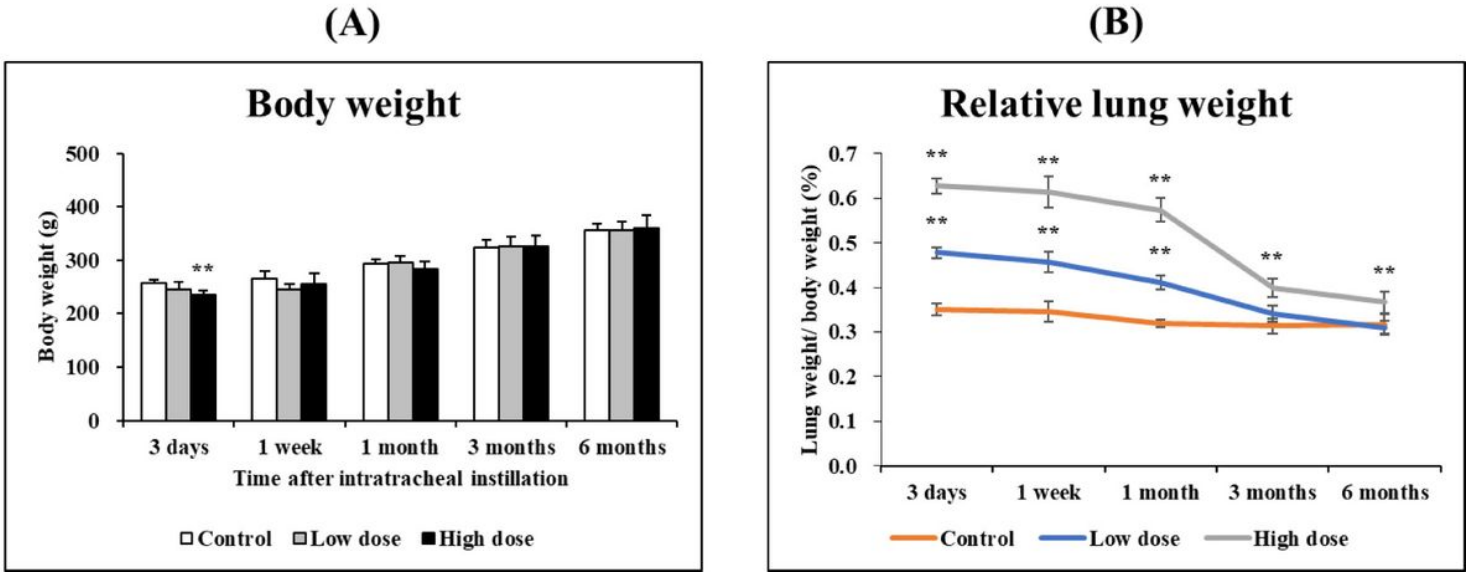


Figure 2

Body weight and relative lung after the instillation. (A) Time course of changes in the body weights of rats in each group. (B) Relative weight of the whole lung was calculated as a ratio of whole lung weight (g) to body weight (g) for each rat. Data are presented as mean ± SE. (* $p < 0.05$, ** $p < 0.01$)

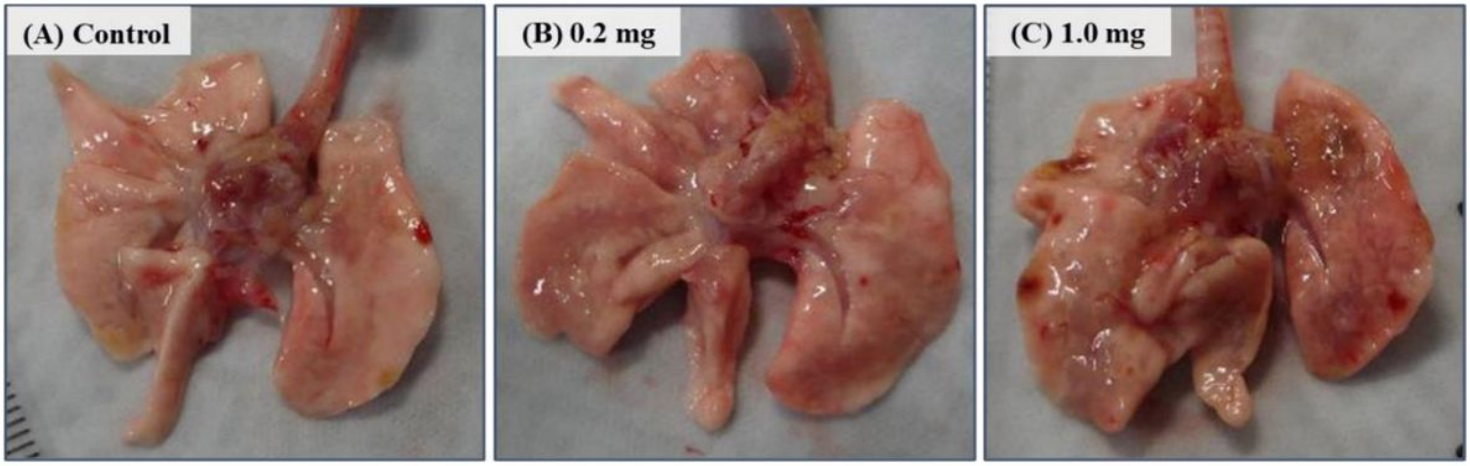


Figure 3

Gross findings at 3 days after the instillation. The lungs in the exposed groups showed ballooning at 3 days following intratracheal instillation. (A) control group. (B) 0.2 mg-exposure group. (C) 1.0 mg-exposure group.

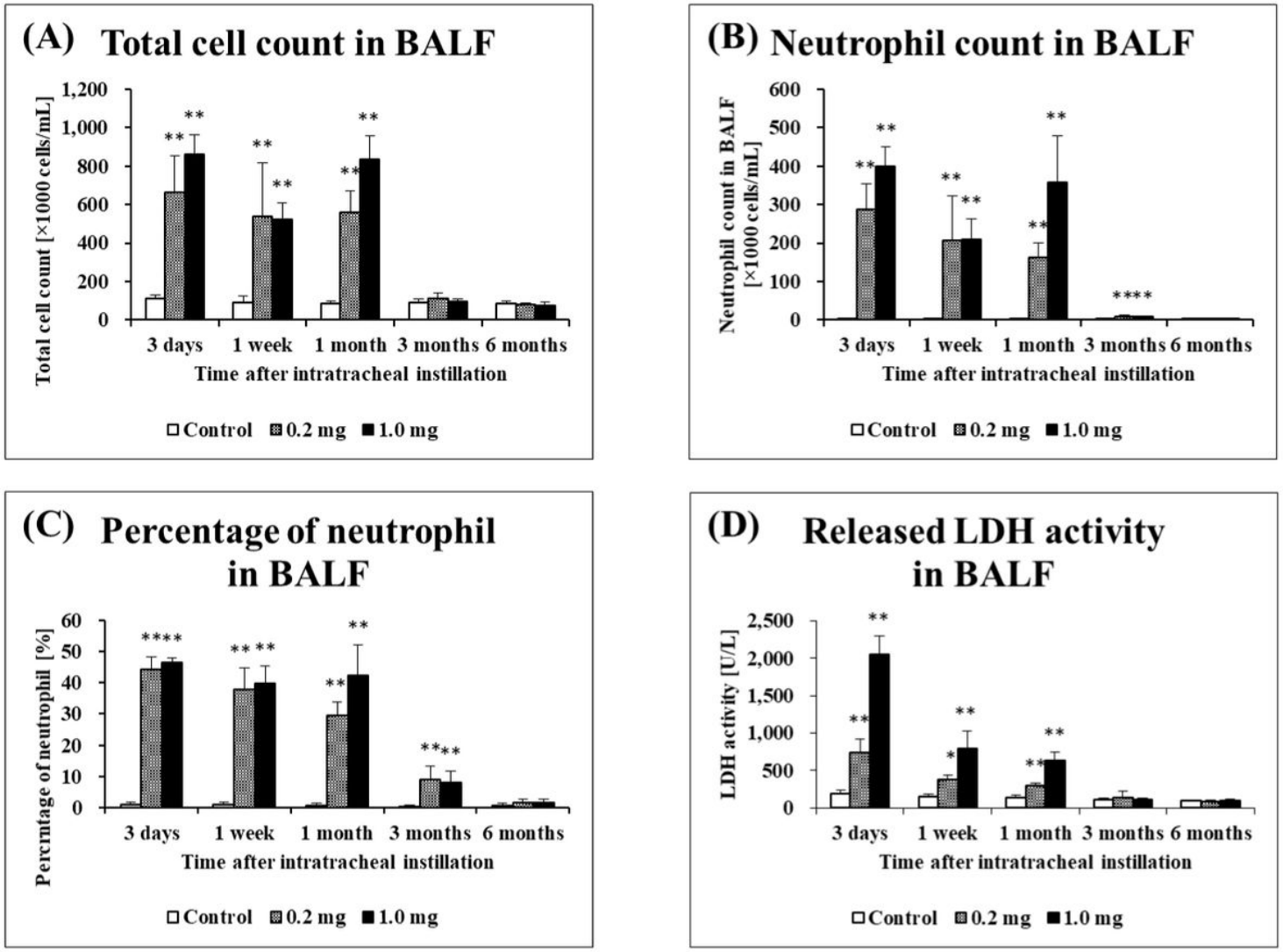


Figure 4

Analysis of cell number and released LDH activity in BALF following intratracheal instillation. (A) total cell number in BALF. (B) neutrophil count in BALF. (C) percentage of neutrophil in BALF. (D) released LDH activity in BALF. Data are presented as mean ± SE. (* $p < 0.05$, ** $p < 0.01$)

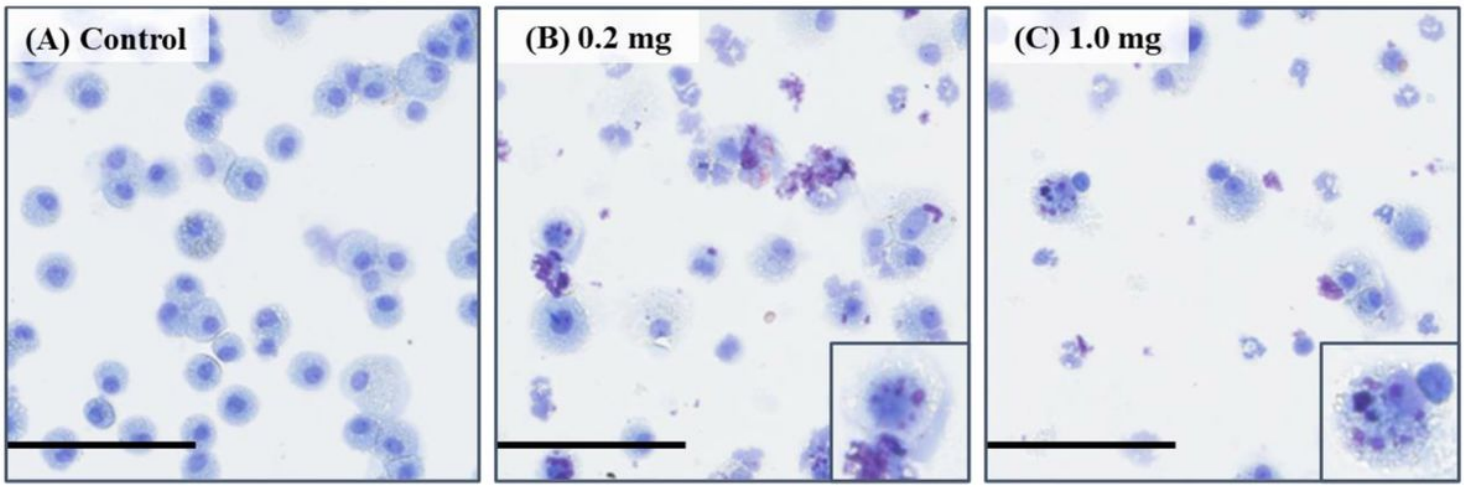


Figure 5

Cells in BALF at 3 days after the instillation. The images of polymer phagocytosis by macrophages are shown in the insets. (A) control group. (B) 0.2 mg-exposure group. (C) 1.0 mg-exposure group. (internal scale bar = 100 μm for all)

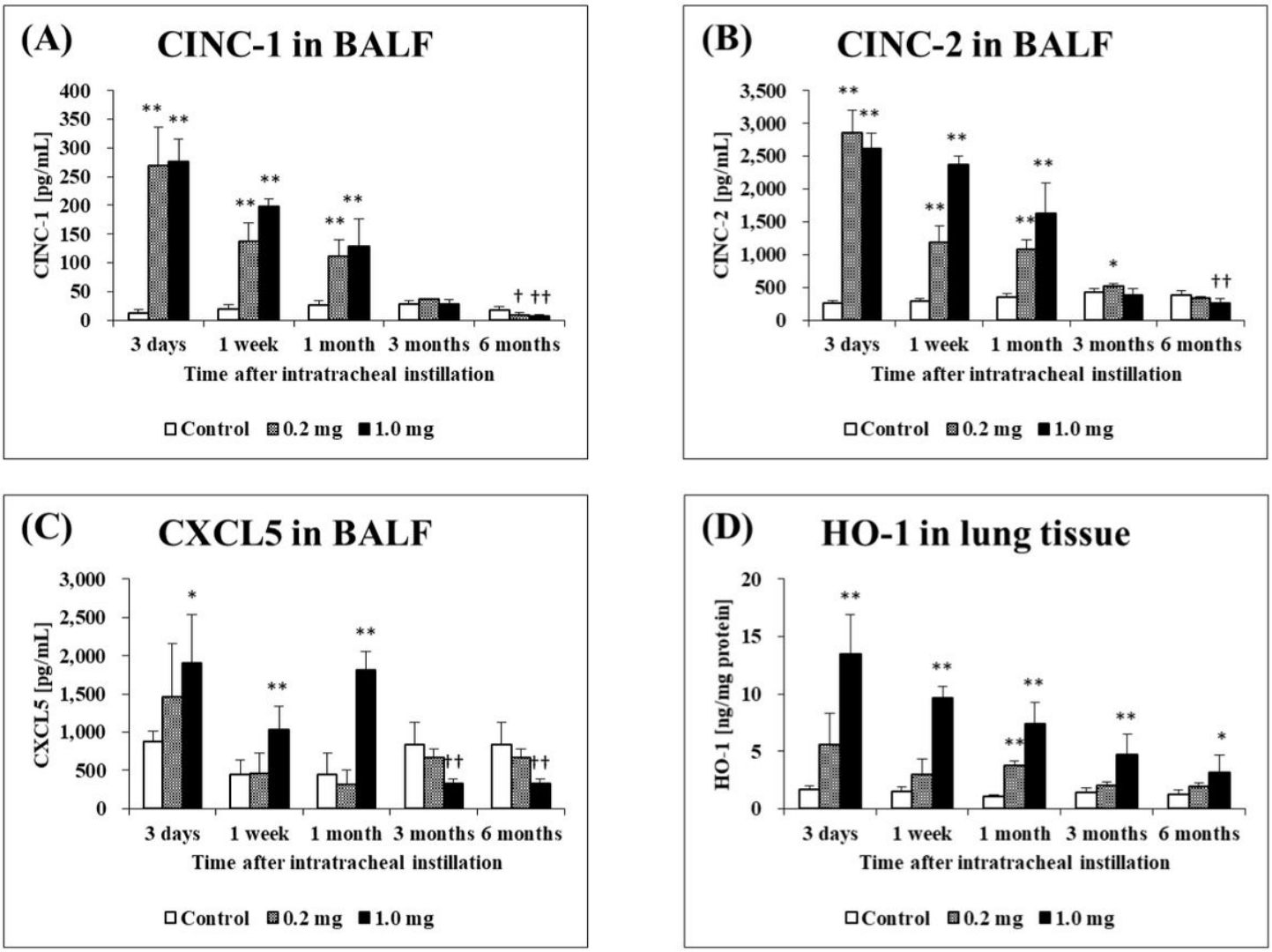


Figure 6

Analysis of cytokines in BALF and HO-1 in lung tissue following intratracheal instillation. (A) CINC-1/CXCL1 concentration in BALF. (B) CINC-2/CXCL3 concentration in BALF. (C) CXCL5 concentration in BALF. (D) HO-1 concentration in lung tissue. Data are presented as mean ± SE. (* $p < 0.05$ and ** $p < 0.01$ indicate that the values are significantly higher than control group. † $p < 0.05$ and †† $p < 0.01$ indicate that the values are significantly lower than control group.)

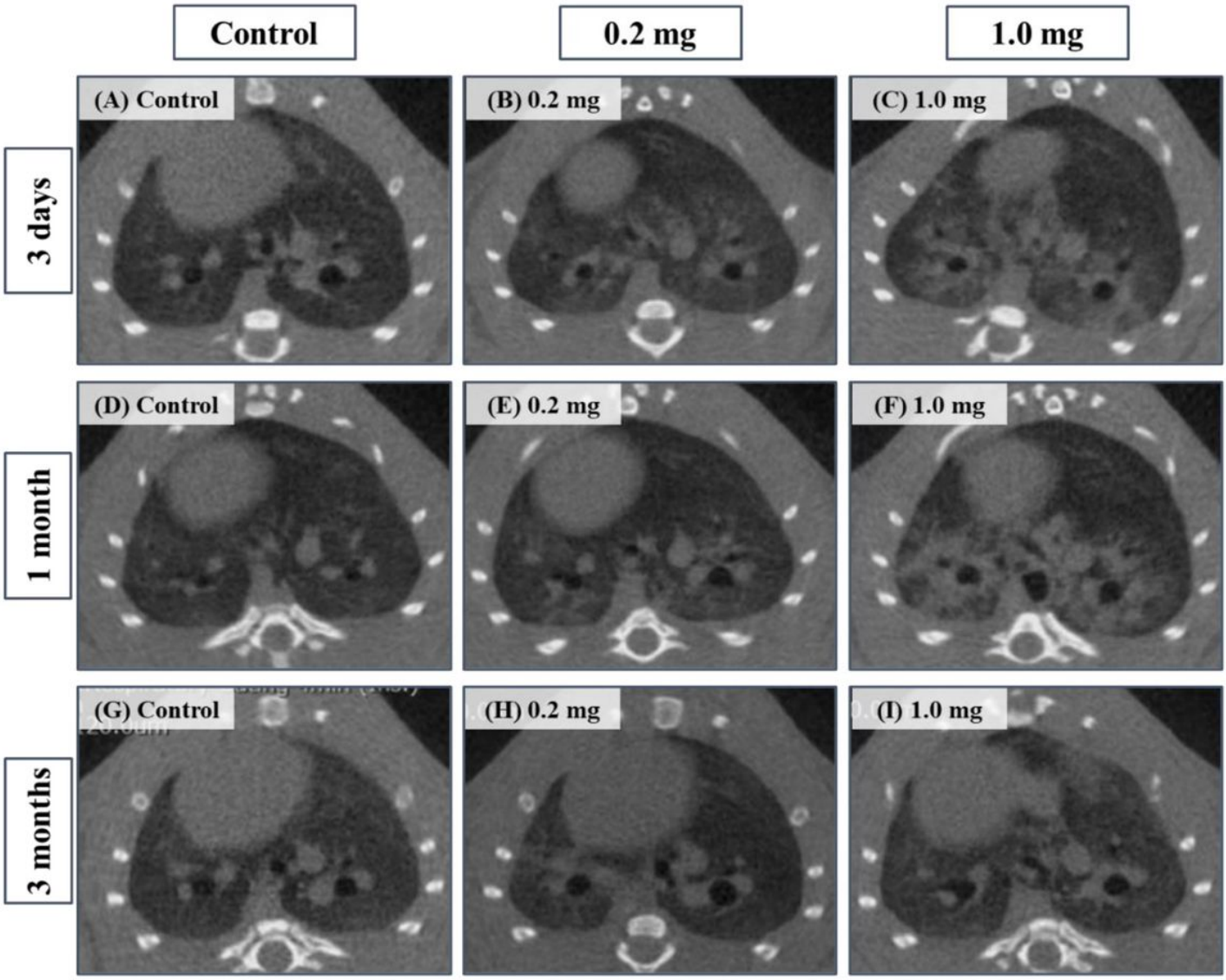


Figure 7

3D micro-CT imaging following intratracheal instillation. At 3 days: (A); control group. (B) 0.2 mg-exposure group. (C) 1.0 mg-exposure group. At 1 month: (D); control group. (E) 0.2 mg-exposure group. (F) 1.0 mg-exposure group. At 3 monthss: (G); control group. (H) 0.2 mg-exposure group. (I) 1.0 mg-exposure group.

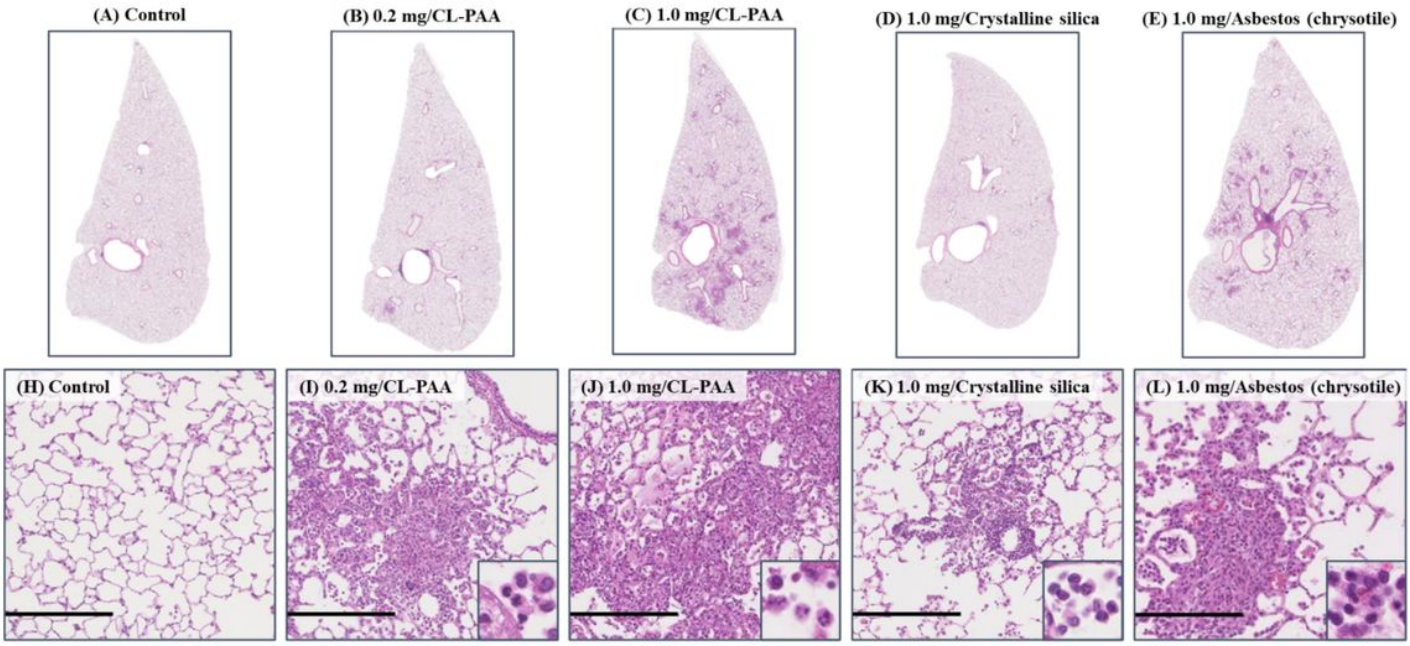


Figure 8

Histological findings at 3 days following the instillation (HE staining). (A)(H) distilled water as a negative control, (B)(I) 0.2 mg CL-PAA-exposed lung, (C)(J) 1.0 mg CL-PAA-exposed lung, (D)(K) 1.0 mg crystalline silica-exposed lung, (E)(L) 1.0 mg asbestos (chrysotile)-exposed lung, (F)(M) 1.0 mg NiO nanoparticle-exposed lung, (G)(N) 1.0 mg CeO2 nanoparticle-exposed lung. (A)-(G) are low magnification images of (H)-(N), respectively. (internal scale bar = 250 μm for all)

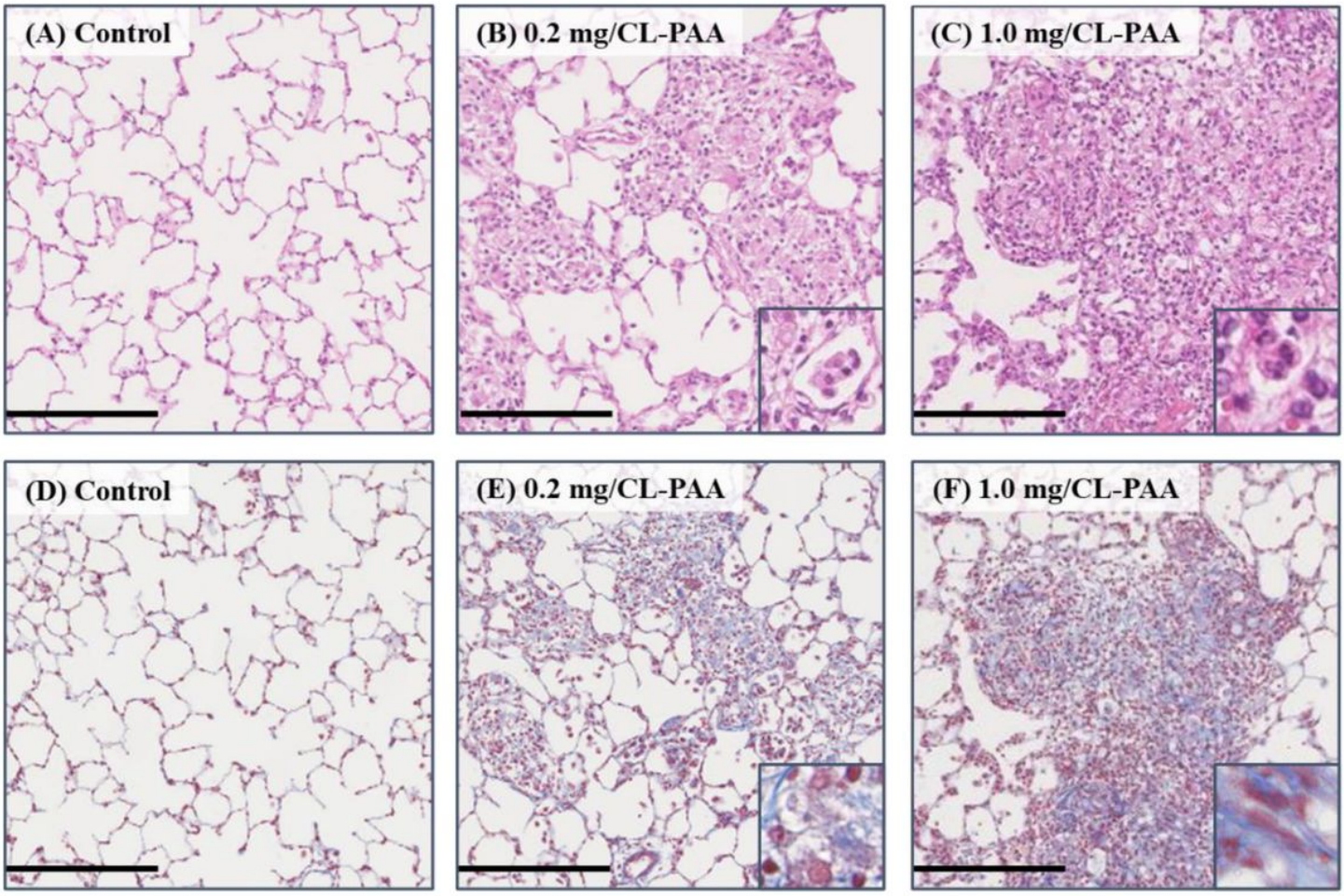


Figure 9

Histological findings at 1 month following the instillation (HE and MT staining). (A)-(C) and (D)-(F) show HE staining and MT staining images, respectively, at 1 month after the instillation of 1.0 mg-CL-PAA.

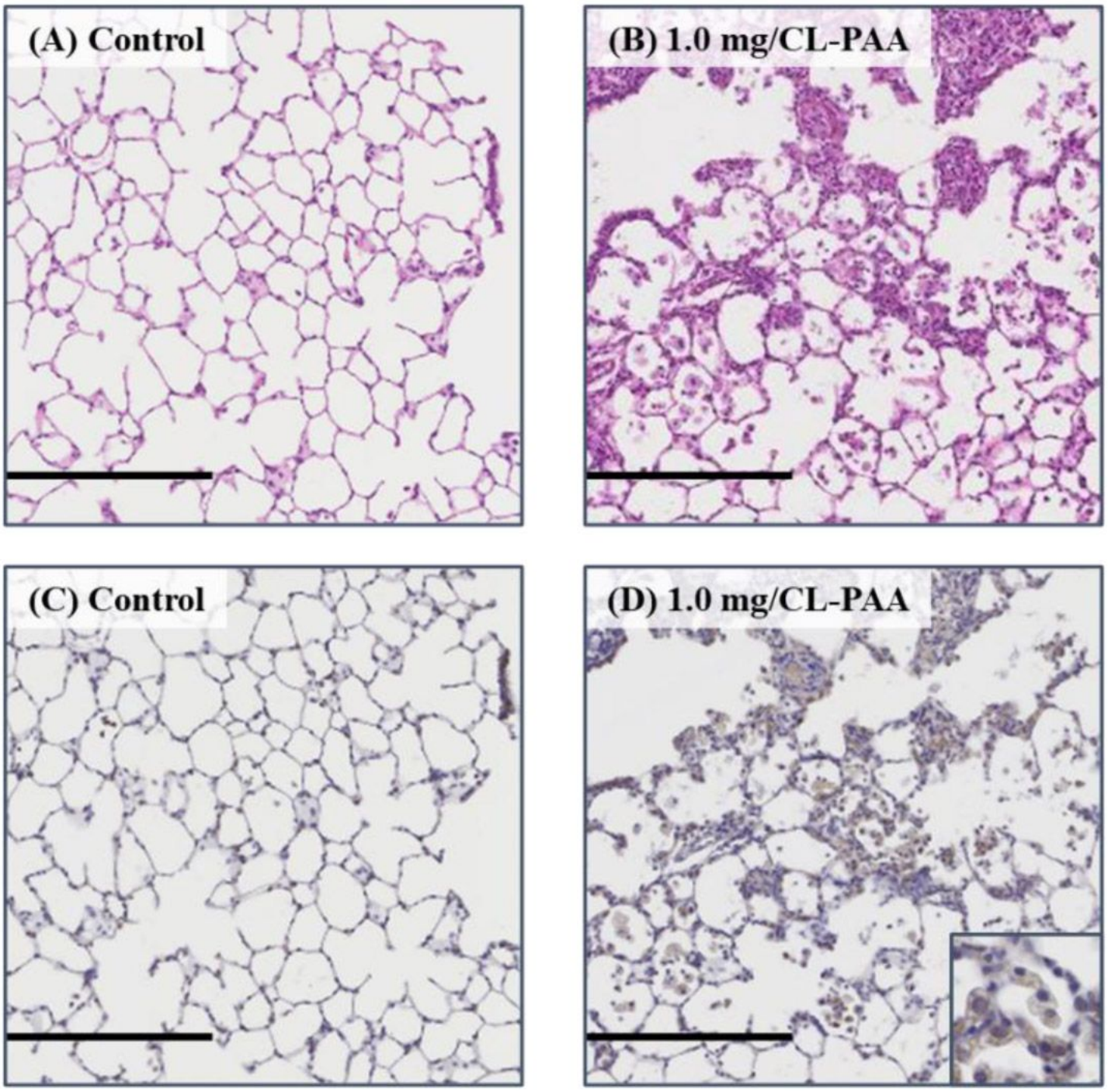


Figure 10

Representative images of CXCL5 immunostaining in lung tissue at 1 month after exposure to CL-PAA. (A) control lung (HE staining), (B) 1.0 mg CL-PAA-exposed lung (HE staining), (C) control lung (CXCL5 immunostaining), (D) 1.0 mg CL-PAA-exposed lung (CXCL5 immunostaining). Positive cells of CXCL5 immunostaining on 1.0 mg CL-PAA-exposed lung were mainly macrophages. (internal scale bar = 250 μm for all)

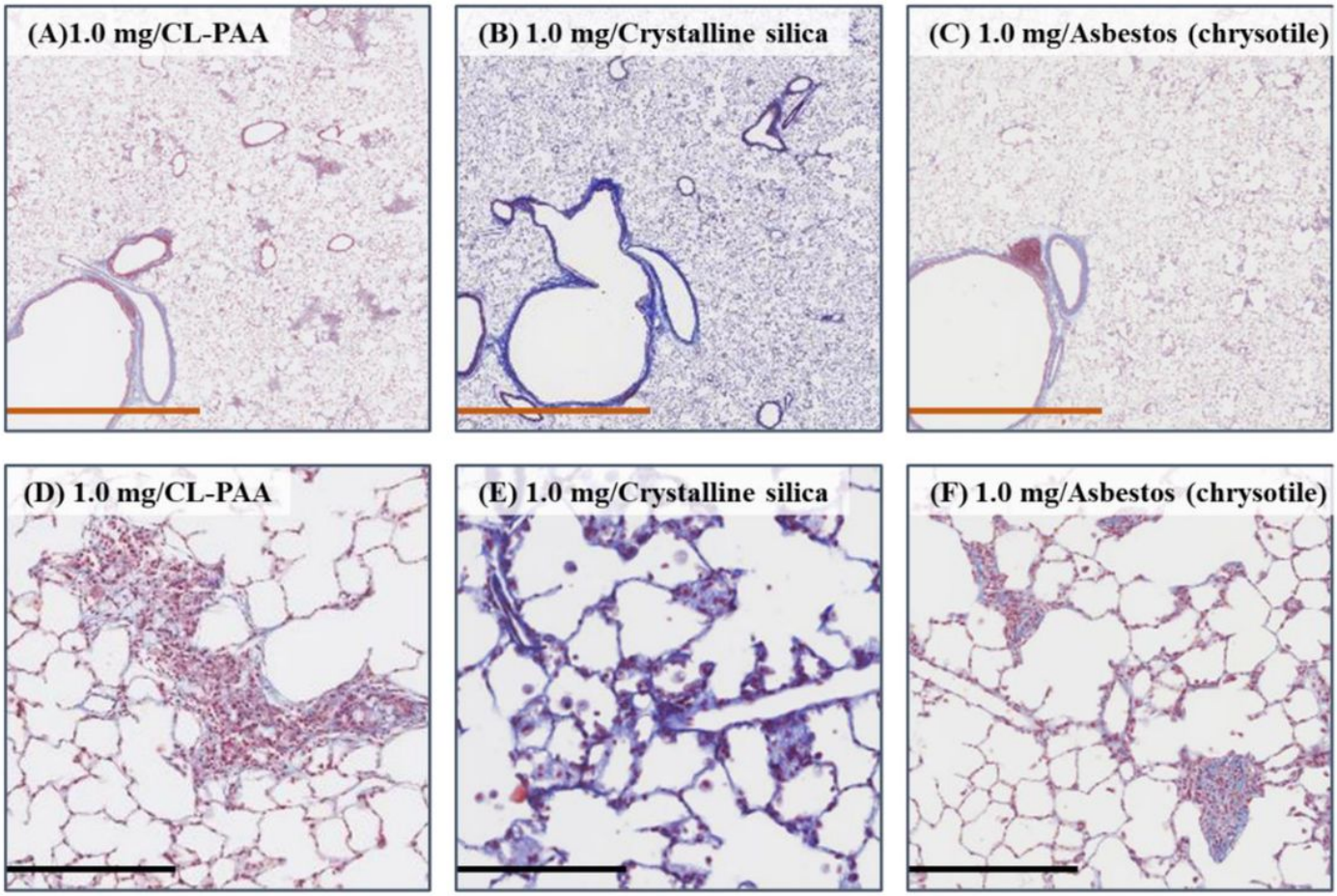


Figure 11

Histological findings at 3 months after the instillation (MT staining). (A)(D) 1.0 mg CL-PAA-exposed lung, (B)(E) 1.0 mg crystalline silica-exposed lung, (C)(F) 1.0 mg asbestos (chrysotile)-exposed lung. (A)-(C) are low magnification images of (D)-(F), respectively. (internal scale bar = 2.5 mm for brown, 250 μm for black)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementtable1.docx
- 42.SupplementalFigure1.pdf