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Changes over time in pulmonary inflammatory response in rat lungs after intratracheal instillation of nickel oxide nanoparticles

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Abstract

Objective: Nickel oxide nanoparticles (NiONPs) are representative metal oxide NPs and are categorized as an insoluble nickel compound. Our previous studies suggested that NiONPs have more pulmonary toxicity than micron-sized NiO because they may dissolve slowly and produce many more Ni ions. We confirmed the hypothesis that the slow dissolution of NiONPs induces a change in inflammatory response over time.

Method: We reanalyzed our previous data on intratracheally instilled NiONP to rats and focused on Ni retention in the lungs and the lung weight ratio for each rat to the mean of control rat lungs. We also measured the solubility of NiONPs and micron-sized NiO samples by means of an artificial lysosomal fluid (ALF, pH 4.5).

Results: The in vivo test of instilled NiONPs resulted in the biomarkers reaching their peak values at 1 week or 1 month, and not at 3 days, after instillation. We found that as the NiO mass in the lung increased, the lung weight ratios tended to increase. The relationships shifted to more toxic at 3 days to 1 month ($P < .01$). Compared to the dissolution of NiONPs in the ALF that took roughly 1 week, the dissolution of NiONPs in vivo was take about 1 month or more.

Conclusion: When intratracheally instilled NiONPs dissolve slowly in the phagolysosomes of alveolar macrophages (AM), the resulting Ni ions cause the AM to transform into foamy cells at 1 month, and the inflammatory response persists even at 3 months after instillation.

KEYWORDS
intratracheal instillation, nanoparticle, nickel oxide, rat, solubility

1 | INTRODUCTION

Along with advances in the field of nanotechnology, the toxic health effects of nanoparticles (NPs) have become a matter of concern in the last two decades. Many studies have reported that NPs are more toxic than larger-sized particles on an equal mass basis. Nickel oxide NPs (NiONPs) are a representative metal oxide NP and are manufactured for catalysts, batteries, additives, etc Nickel, which is widely used as a metal and in compounds, has been reported to have different toxicities and

health effects depending on its chemical form. In 2009, the Japan Society for Occupational Health revised its recommendation of the Occupational Exposure Limits (OEL) of nickel compounds and proposed an OEL of 0.01 mg Ni/m^3 (total dust) for water-soluble nickel compounds and 0.1 mg Ni/m^3 (total dust) for water-insoluble nickel compounds. In inhalation exposure to nickel sulfate or nickel chloride, the compounds are excreted in urine and have a biological half time (BHT) ranging from several hours to several days, but insoluble nickel compounds such as NiO are retained in the lungs after inhalation and have a BHT of several months or more.[1]

There have been many in vitro and in vivo studies of the biological effects of NiONPs. In vitro studies have suggested that NiONPs induce pulmonary inflammation and genotoxic effects in lung cells.[2-5] In our previous studies of intratracheal instillation of NiO particles administered to rats, samples of NiO particles with smaller diameters showed stronger inflammatory changes,[6,7] so we administered NiONPs to examine their inflammatory responses in rat lungs.[8,9] Inflammatory responses associated with NiONPs were stronger at three days after termination of a 4-week inhalation exposure than at three days after an intratracheal instillation.[10] The biological effects differed from those of typical nanomaterials such as TiO_2NPs nanoparticles, which elicit a strong inflammatory response following intratracheal instillation.[11-13] Horie et al[14] attempted to explain the reason behind this different biological effect between inhalation and intratracheal instillation based on the production of reactive oxygen species during the 4-week inhalation period. Nanoparticles have a much larger specific surface area; in the case of NiONPs, which dissolve slowly, this property conceivably leads to a greater production of Ni ions when the particle diameters are smaller, resulting in stronger inflammatory changes than that of micron-sized particles.[10]

We hypothesized that the inflammatory responses induced by NiONPs change over time due to their slow dissolution; thus in the present study we reexamined the relationship between the retention of intratracheally instilled NiONPs in rat lungs and biomarkers that we had examined in a previous study, this time with a focus on changes over time. The present study also examined the dissolution rate of different NiO samples in an artificial lysosomal fluid (ALF, pH 4.5).

2 | MATERIALS AND METHODS

In our previous intratracheal instillation test using samples of NiONPs, we examined total cell count, neutrophil count, and chemokines such as cytokine-induced neutrophil chemoattractant (CINC-1) in bronchoalveolar lavage fluid (BALF).[8] We also measured pulmonary retention of NiO, lung weight, phospholipids in BALF, and BALF surface tension (not yet published).

2.1 | NiO samples

The NiONPs to install to rat lungs in this study was nano-sized NiO-n1. At the dissolution tests, nano-sized NiO-n2, submicron-sized NiO-s, and micron-sized NiO-m were used. NiO-n1 in suspension form, and the size of the particles was 26 nm, as measured by the dynamic light scattering method. The specific surface area was 172 m^2/g. The NiO-n2 had a nominal average particle size of 10−20 nm (Nanostructured & Amorphous Materials, Inc, Houston, TX, USA), and the specific surface area was 88 m^2/g. The NiO-n1 was obtained from the NiO-n2, from which the larger particles had been removed by centrifugal force and filtering. For classification, NiO-n2 was dispersed in purified water. The other NiO samples were NiO-s (Vacuum Metallurgical Co., Ltd., Chiba, Japan) and NiO-m (Nakalai Chemicals Ltd., Kyoto, Japan). The enough amount of NiO-n1 was not obtained in a powder form for the dissolution test. A detailed characterization of the NiO samples used here have been described elsewhere.[8,10]

2.2 | Animals

Male Wistar rats (9 weeks old) were purchased from Kyudo Co., Ltd. (Kumamoto, Japan). The rat body weights were 310 ± 15 g at the start of exposure (9 weeks old). All procedures involving the handling of animals followed 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.

2.3 | Administration and dissection

The NiO suspension (NiO-n1) was prepared with 0.1 mg (0.33 mg/kg) or 0.2 mg (0.66 mg/kg) of NiO in 0.4 mL distilled water, and then intratracheally instilled once to male Wistar rats. Control groups received distilled water in the same manner. After the instillation, all the animals were housed in cages at 20-25°C and 40-70% humidity, with free access to food and water, in the Laboratory Animal Research Center of University of Occupational and Environmental Health, Japan. Animals (10 in each group) were dissected at 3 days, 1 week, 1 month, 3 months, and 6 months after the instillation.[8]

2.4 | Tissue preparation for pathological observation

BALF collection and histopathological analysis were conducted using the removed lungs of five rats from each group. The left lung of each rat, that is, the clamped side at

BALF collection, was fixed with 10% buffered formalin at 25 cmH_2O overhead pressure. Paraffin sections of the left lung (5 μm thickness) were fixed on the slide glasses, rinsed by xylene to remove the paraffin and then observed by a scanning electron microscope (SEM, Hitachi GM3000, Tokyo).

2.5 | Pulmonary retention of Ni

The left lungs of five other rats were used to determine the mass of particles deposited in the lungs. As previously reported,[15] NiO particles in the lungs were digested with lung tissues into the element with H_2SO_4 and H_2O_2 by a microwave (Ethos 1600, Milestone, Italy). The amounts of Ni in 50 mL digested samples were determined by an inductively coupled plasma-atomic emission spectrometer (ICP-AES, SPS3500DD, SII NanoTechnology, Tokyo, Japan). The mass of NiO in each lung was calculated from the determined Ni amounts divided by the Ni content (78.6%) of the NiO and the weight ratio of left lung versus total lung of each sample. As the standard solution of 0.01 ppm Ni were detected to determine the calibration curve, we estimate that the detection limit of this method may be less than 0.5 μg Ni/lung sample, and Ni in control rat samples including livers was not detected by this method.

2.6 | Bronchoalveolar lavage fluid (BALF)

BALF was collected from five rats for each dose by inserting a cannula into the right lung via the respiratory tract, with the left main bronchus clamped, and pouring in a physiological saline (15 mL for each rat). After centrifuging the BALF (400 *g* 10 min), the supernatant was frozen and kept at −30° before being measured. The recovered cells in the BALF, such as alveolar macrophages (AM) and polymorphonuclear neutrophils (PMN), were also analyzed to determine cell numbers. Smears from the above cellular sample were prepared on glass slides by the Cytospin method and stained by a Diff-Quik kit (Sysmex Co., Kobe, Japan) to measure the ratio of PMN in total cell. The slides were observed by the same SEM (Hitachi GM3000, Tokyo) as above. Before SEM observation, we used lead-staining method for cells on the slide glasses to increase the contrast of SEM image.

2.7 | Measurement of pulmonary surfactants in BALF

We measured the concentrations of phospholipids, total protein, and BALF surface tension in order to evaluate any change in pulmonary surfactant in the rats exposed to the NiO-n1. The measurement methods for the analysis of the surfactant were basically the same as the method described in a previous paper.[16]

2.7.1 | Total protein concentration

The method for measuring total protein concentration was based on the same principle as the Bradford protein assay. A standard curve was made using Bovine Serum Albumin Standards (Thermo Scientific, USA) as the standard sample, and protein concentration was determined by measuring BALF absorption (at 595 nm) with a spectrum photometer, as above.

2.7.2 | Phospholipid concentration

Phosphatidylcholines and phosphatidylglycerols are major species of surfactant phospholipids. We used the enzymatic method to measure the total phospholipid concentration in the BALF. A standard curve was made using a standard sample of NESCAUTO PL Kit-K (Alfresa Pharma Corporation, Japan), and the concentration of phospholipids was determined by measuring BALF absorption (at 595 nm) with a spectrum photometer (Spectramax Plus 384; Molecular Devices, CA, USA).

2.7.3 | BALF surface tension

BALF surface tension directly indicates the functioning level of the pulmonary surfactant. We measured the surface tension by the du Nouy ring method (Taihei Rikakogyo, Tokyo, Japan) after diluting a 1.8 ml of BALF sample with 10 parts physiological saline. The BALF sample was poured into a disposable petri dish.

2.8 | Solubility of NiO

Microparticles deposited in the lungs are engulfed by alveolar macrophages, taken into macrophages as phagosomes, and fused with lysosomes to form phagolysosomes. The inside of a lysosome is considered to be acidic, with a pH of roughly 4.5. NiO is known to dissolve more readily under acidic conditions.[17] Assuming that NiO triggers inflammation due to slowly discharged Ni ions, the inflammatory response in the lungs can conceivably be associated not only with the dose of NiO administered but also with the amount of time elapsed.

We produced an ALF consisting primarily of citric acid, and conducted dissolution tests for 1 week using NiO samples with three different particle diameters: nanosized (NiO-n2), sub-micron-sized (NiO-s), and micron-sized (NiO-m).

The ALF (100 mL) was prepared by mixing NaCl (0.321 g), NaOH (0.60 g), and citric acid (2.08 g) with ultrapure water. Using a benchtop pH meter (Eutech pH700, ASONE, Osaka, Japan), we gradually added NaOH solution 1 mol/l solution to achieve a pH of 4.5. We then weighed 0.10 g of each of the three NiO samples and mixed them with 10 mL ALF. In a previous paper,[17] 0.1 g of NiO was mixed with 50 mL ALF, but in the present study we used a lower volume of fluid to achieve a higher sample to fluid ratio. Each mixture was preserved for 1 week at 37°C in a low temperature incubator (LTI 2000, EYELA, Tokyo, Japan) and stirred once daily. The samples were centrifuged at 12 300 *g* for 1 hour to separate particles ≥30 nm in diameter. We then collected the resulting supernatant and measured its pH. The dissolved nickel ions were similarly analytically quantified using the ICP-AES.

2.9 | Statistical analysis

Statistical analysis was carried out by the Mann–Whitney test. Analysis of variance (ANOVA) was conducted by IBM SPSS ver. 24 to determine the masses of NiO in the lungs and the responses to this exposure.

3 | RESULTS

Figure 1 shows the means and standard deviations for the biomarkers of inflammation (lung weight, total protein concentration in BALF, phospholipid concentration in BALF, and BALF surface tension) in the lungs of rats dissected at 3 days, 1 week, 1 month, 3 months, and 6 months after exposure to NiO by intratracheal instillation. In intratracheal instillation tests of particulate matter, changes in biomarkers are greatest immediately after instillation and gradually grow smaller over time in many cases. The NiO-n1 used in the present study resulted in biomarkers reaching their peak values at 1 week or 1 month after instillation, and not at 3 days.

In our previous study,[8] total cell number in BALF showed their peak at 3 months, and the numbers of PMN and the levels of the CINC-1 in the BALF reaching their peak at 1 week.

Figure 2 shows cells in BALF trapped on slides by the Cytospin method as observed with a SEM at 3 days, 1 week, 1 month and 3 months after administration of NiO-n1. Inset (a) shows an image of elemental mapping of Ni Kα of a macrophage (white square in the image of 3 days after) by an energy-dispersive X-ray spectrometry (QUANTAX 70; Bruker Co., Billerica, MA, USA). Inset (b) shows an imposed image of (a) and the original image at 3 days after. The reflection electronic image of the AM shows that some of the phagolysomes in the AMs engulfed particles with heavy Molecular Weight elements (white color images), and the image of inset (a) reveals that nickel was the main component of the particles. At 1 month, the AMs transformed into foamy cells, but the particles still retained. At 3 months, there still observed foamy cells but majority of AM showed smaller cytoplasm volume (white color area around the nuclei) than those observed at previous time points.

Pathological changes in the lungs as determined by H&E staining were shown in electron microscope images in a previous study.[8] Figure 3 shows lung tissue sections as observed using an SEM along with elemental mapping of Ni. Ni particles were present in the AM and interstitium at 1 week, but few were observed at 1 month, despite the large number of macrophages present then.

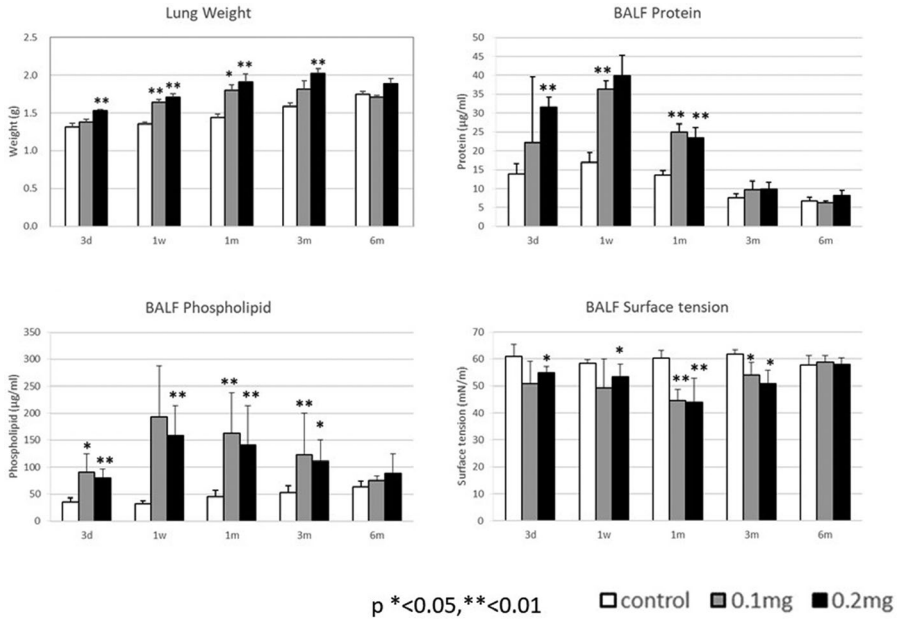

FIGURE 1 Lung weight, total protein concentration in BALF, phospholipid concentration in BALF, and BALF surface tension, in the lungs of rats at 3 days, 1 week, 1 month, 3 months, and 6 months after intratracheal instillation. Each column and bar represent mean + SE for five rats. Asterisk indicates significant difference at $P < .05$ versus each control group; double asterisk, at $P < .01$ versus each control group

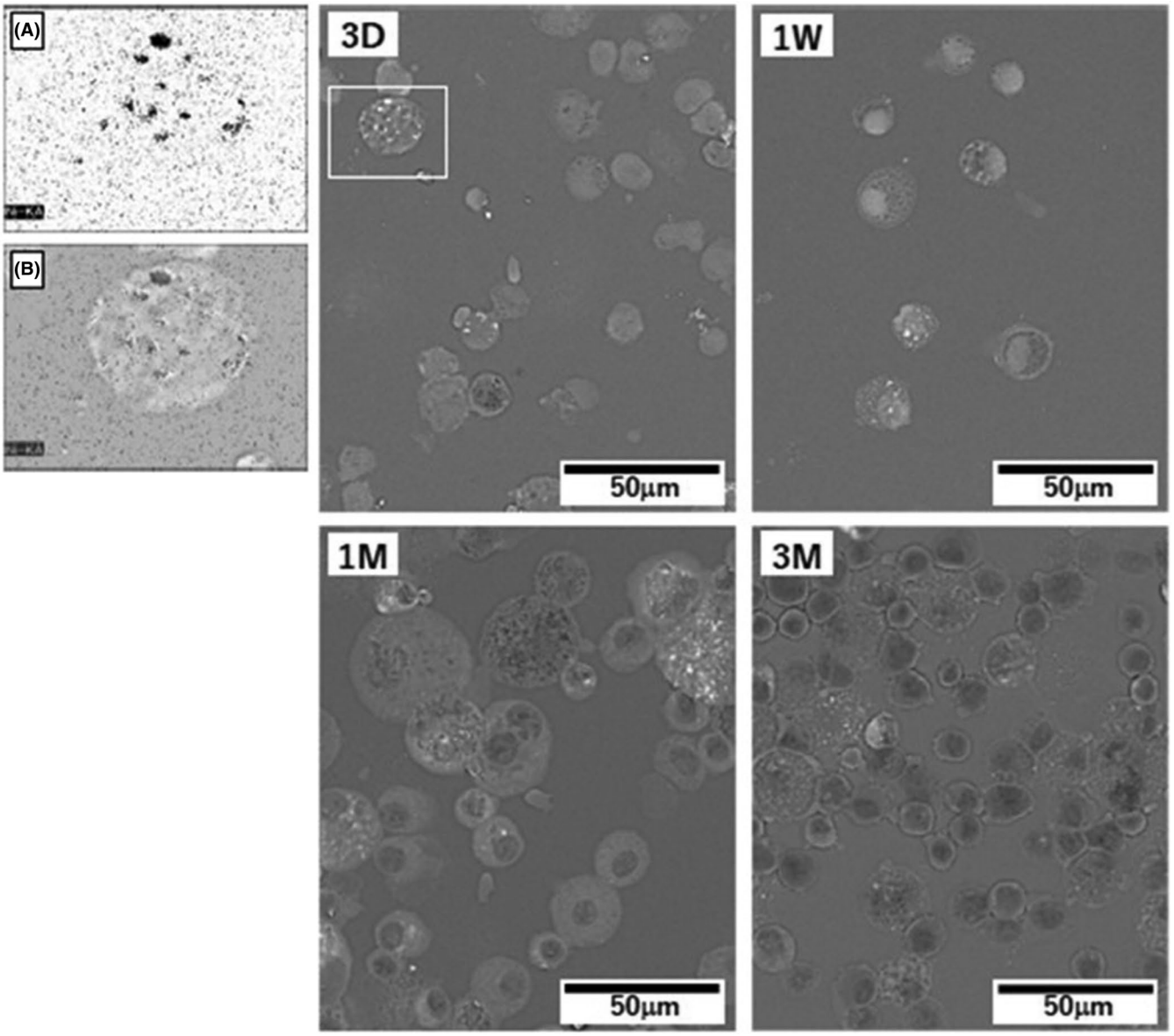

FIGURE 2 Cell images in BALF by the Cytospin method as observed with a SEM at 3 days, 1 week, 1 month, and 3 months after instillation of NiONPs (0.2 mg). Inset (A) shows an image of elemental mapping of Ni Kα of a macrophage. Inset (B) shows an imposed image of (A) and the original image at 3 days after (white square in the image of 3 days after)

Figure 4 shows SEM images of particles remaining in the samples harvested prior to dissolution and at 1 week, as well as the pH of the solution samples and masses of NiO. In the ALF, 61.4% of nanosized NiO particles (NiO-n2) had dissolved, and the pH of the ALF had changed to approximately 7.0. In contrast, only 10% of the sub-micron-sized particle sample (NiO-s) and almost none of the micron-sized particle sample (NiO-m) had dissolved, and the pH of the ALFs had not changed. The SEM images showed that the nanosized particles of NiO-n2 disappeared after 1 week.

We measured the pulmonary retention of NiO nanoparticles in rats exposed to NiO. During this measurement process, we also determined the lung weights in all the groups of rats exposed to NiO and in the control group. Figure 5A shows the ratios of lung weight for rats of the exposure groups to the mean lung weight of the control group rats, and the relationships of these ratios to the pulmonary NiO mass. Masses of NiO in the lungs are shown as logs. The key shows the mean and variance in the 0.1 mg group and the 0.2 mg group at all points in time. As shown in Figure 1, pulmonary inflammation increases lung weight. The change in lung weight compared with control lung weight is widely used as a biomarker of pulmonary inflammation. A kind of dose-response relationship is one in which lung weight increases in proportion to the logarithm of NiO mass in the lung. The dose-response relationships shifted to the left in the figure until 1 month, but then showed a decrease over the prolonged period from 1 month to 3 months (see arrows in Figure 5A).

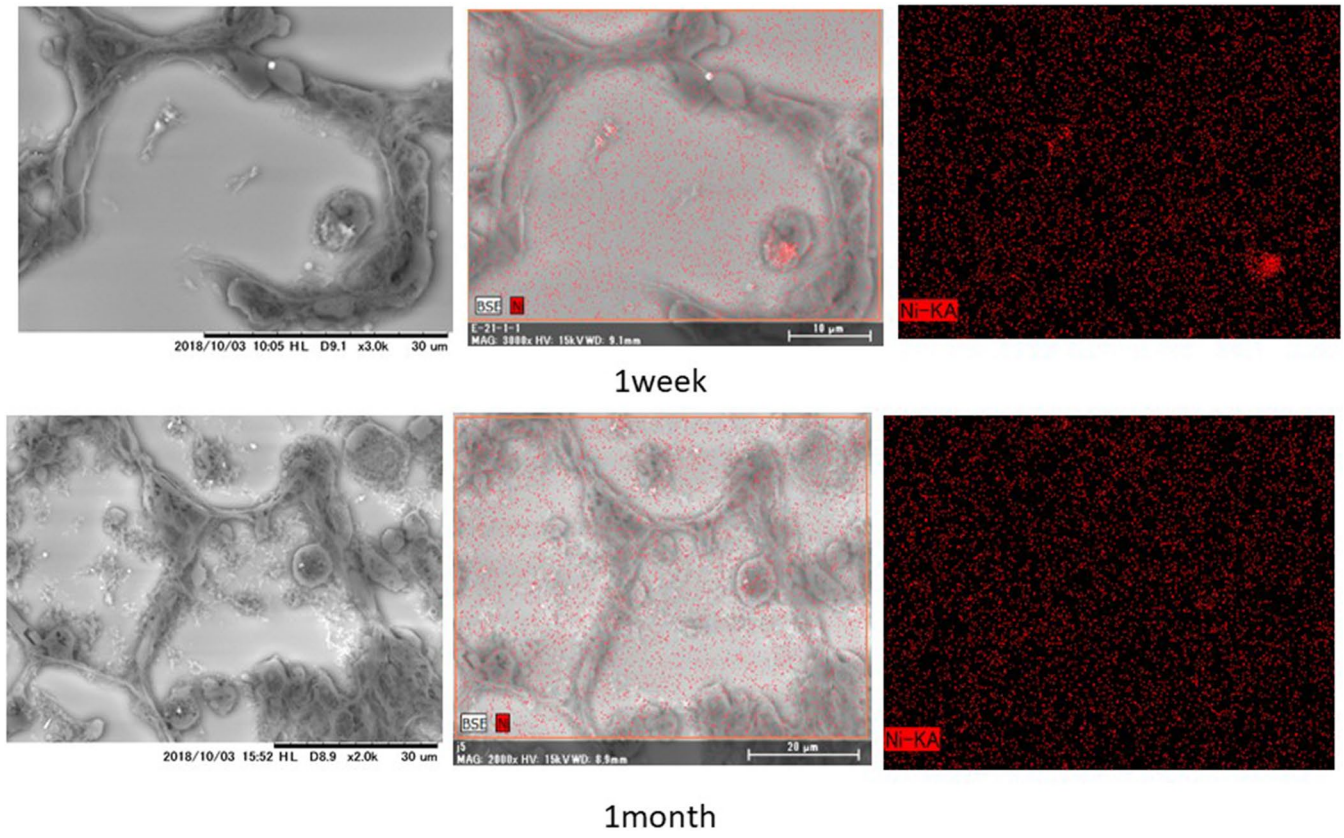

FIGURE 3 Lung tissue sections at 1 week and 1 month after instillation (0.2 mg) as observed using an SEM along with elemental mapping of Ni. Left images show original SEM images, middle images show imposed images of left images and right images, and right images show images of elemental mapping

4 | DISCUSSION

In intratracheal instillation tests of TiO_2NPs,[13-15] most biomarker responses peak at 3 days after instillation and gradually decrease over time. In contrast, with crystalline silica dust, biomarker responses gradually worsen over time.[18] In our study, as shown in Figure 1, the biomarkers peaked at 1 week or 1 month following intratracheal instillation of NiO-n1 and then gradually decreased, unlike with other types of nanoparticles. Cao et al [2] also evaluated inflammatory responses of SD rats at 3-28 days after intratracheal instillation of NiONPs and observed sustained pulmonary inflammation, as in our study.

Based on the SEM images in Figure 2 of macrophages and other cells in the BALF, we observed that NiO-n1 remained in cells 3 months after instillation. Similarly, NiO-n1 was observed in the SEM images of lung sections shown in Figure 3. However, as shown in Figure 4, nanosized portion of NiO-n2 had dissolved and the pH of the solution had reached approximately 7.0 at 1 week in the dissolution tests using ALF. The NiO-n1 sample may have been equivalent to the dissolved part of NiO-n2. Thus, there was a difference in the dissolution tests in lysis in the phagolysosomes in the AM in the rat lungs.

The inside of a lysosome is acidic, and NiO is presumed to dissolve more readily under acidic conditions. However, in an in vitro experiment, the uptake of basic matter (silver particles) triggered dysfunction in phagolysosomes, which were thus neutralized and did not become sufficiently acidic.[19] NiO-n1 trigger a similar phenomenon in the lungs. Compared to the dissolution of NiO-n2 in an ALF, which takes roughly 1 week, the dissolution of NiO-n1 in vivo is considered to take about 1 month or more. The masses of NiO-n1 in the phagolysosomes shown in Figure 2 are roughly the same size as the phagolysosomes themselves. The ratio of particulate mass to solution volume was presumed to be larger than that in the dissolution test in the present study, and the dissolution rate was considered to decrease.

Figure 5B shows the ratios of lung weight for each rat of the exposure groups to the mean lung weight of the control group rats to the NiO mass in lung as Figure 5A. The keys in the figure show the values for all 10 rats in both the 0.1 and 0.2 mg administration groups at 3 days, 1 week, and 3 months, and 9 rats at 1 month. In Figure 5B, one rat at 1 month and one rat at 3 months in 0.1 mg administration group showed relatively lower pulmonary NiO mass than other rats. One possibility is incomplete administration at intratracheal instillation but we combined these data for the statistical analysis as lower NiO mass and lower response.

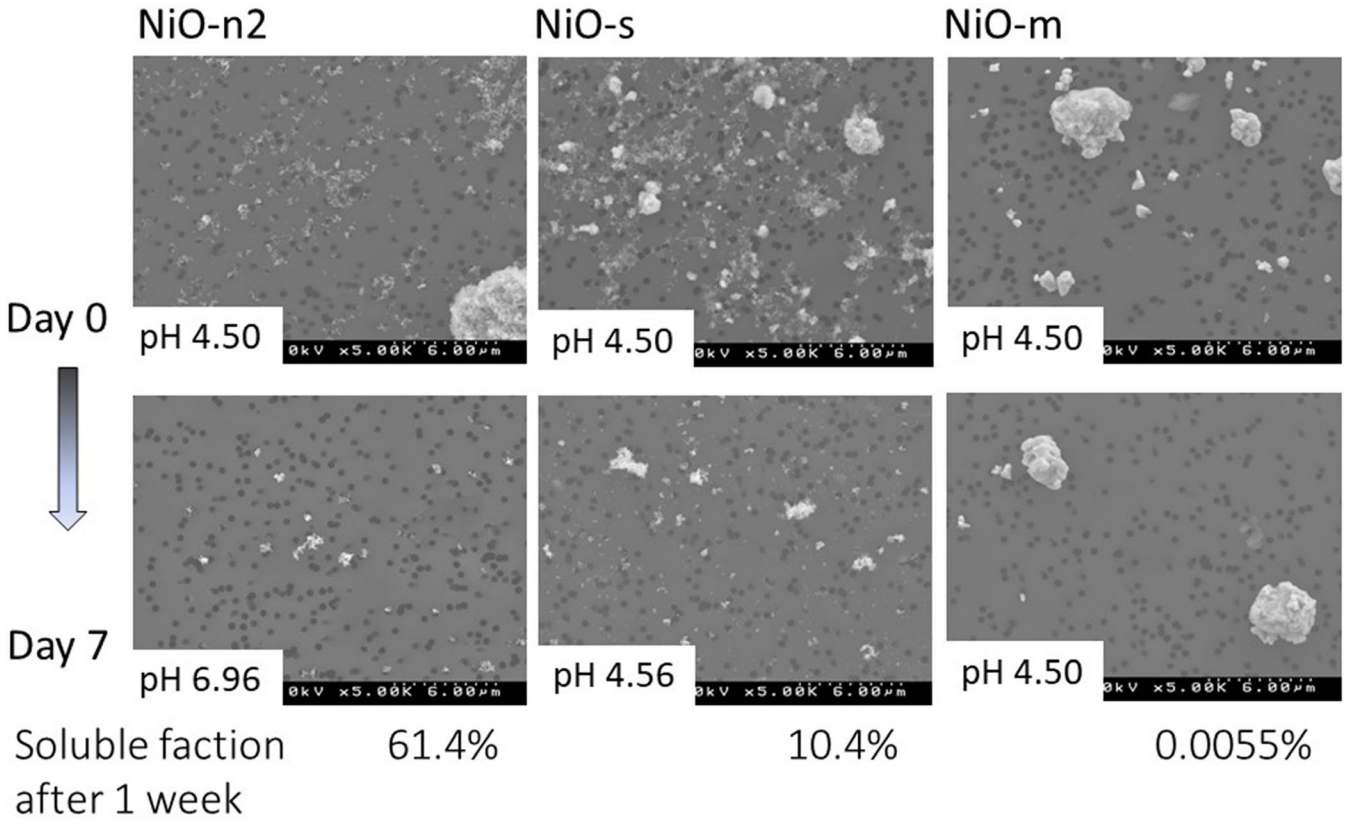

FIGURE 4 SEM images of particles remaining in the samples harvested prior to dissolution and at 1 week after, as well as the pH of the solution samples and soluble fraction of each NiO sample

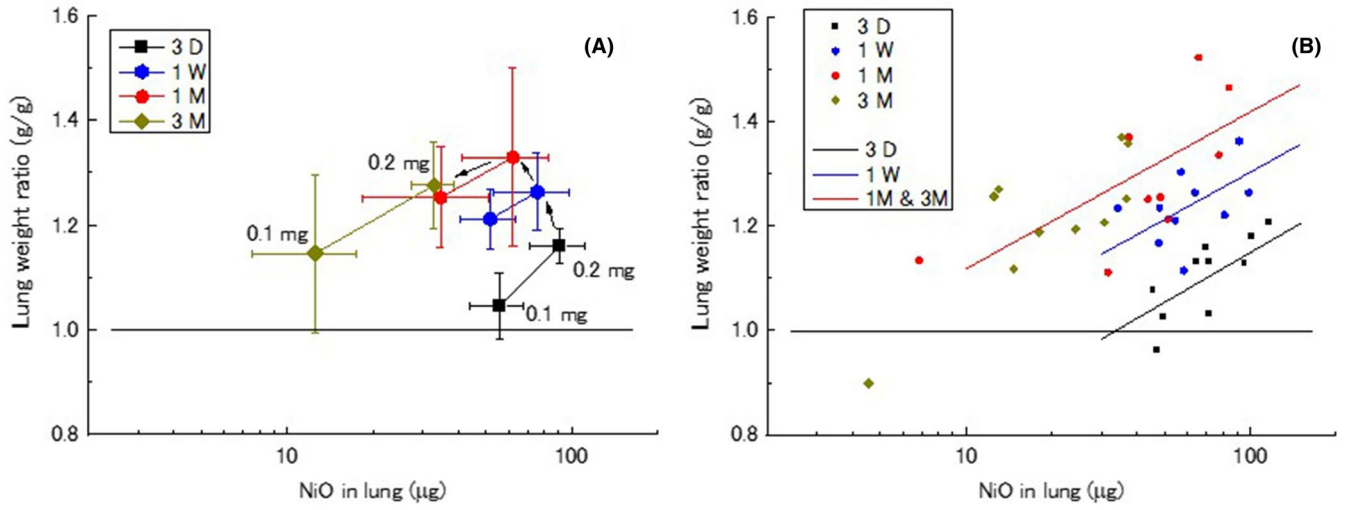

FIGURE 5 Relationship between the logarithm of NiO mass in the lung and lung weight ratios. A, shows means and variances of each group at 3 days to 3 months after 0.1 mg and 0.2 mg administered to each rat. B, shows individual rat data at 3 days to 3 months after both of 0.1 mg and 0.2 mg administered

Using IBM SPSS ver. 24, we conducted one-way ANOVA for rats in the 3 days, 1week, 1 month, and 3 months groups. Significant differences were observed among rats dissected at 3 days, 1 week, and 1 month ($P < .01$), whereas the difference between the 1 month and 3 months groups was not significant ($P = .63$). Figure 5B shows the lines yielded by ANOVA for the 3 days, 1 week, 1 month, and 3 months groups. Each line shows the dose-response relationship for NiO-n1, and thus demonstrates that the biological effects of NiO-n1 become more pronounced with not only increased mass in the lungs but also with the passage of time. The lines obtained from ANOVA show the same

trend as the lines connecting the 0.1 mg group and 0.2 mg group at each point in Figure 5A.

In summary, the above findings suggest that NiO, which is typically poorly soluble, dissolves under acidic conditions when it is nanosized. Conceivably, when intratracheally instilled NiO-n1 dissolve, the resulting Ni ions cause AM to transform into foamy cells at 1 month, and the inflammatory response persists even at 3 months after instillation. The long BHT of NiO-n1 in the lungs compared with that of TiO_2NPs, which elicit a relatively weak inflammatory response, may be due to impaired removal of NiO-n1 associated with damage to AM, as mentioned above.[20] NiO exerts greater biological effects the smaller its particles are. Furthermore, these effects appear to persist for long periods of time after exposure. The chronic lung inflammation may be considered a surrogate for tumor-enhancing effects. Stricter measures for occupational hygiene management are needed for nanoparticles of not only nickel but also other metal compounds.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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