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Original Article

Analysis of Bronchoalveolar Lavage Fluid Adhering to Lung Surfactant

—Experiment on Intratracheal Instillation of Nickel Oxide with Different Diameters—

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Abstract: Nickel oxide with two different particle sizes, micron size (NiO) and submicron size (nNiOm), as well as crystalline silica as a positive control and titanium dioxide as a negative control, were intratracheally instilled in rats and the phospholipid concentration and the protein concentration and surface tension of bronchoalveolar lavage fluid (BALF), which are used in surfactant assessment, were measured to see if they could be effective biomarkers in toxicity assessment. The results showed that the NiO instilled group showed no significant difference compared to the control group throughout the observation period. In contrast, a significant difference was found in the nNiOm instilled group compared to the control group throughout the observation period. Moreover, a significant difference was found in the crystalline silica instilled group for each measurement compared to the control group while for the titanium dioxide group, almost no significant difference was found. These results indicate that submicronsized particles of nickel oxide with smaller median diameters potentially have a stronger biological effect than micron size particles. They also indicate that screening can be done by measuring the phospholipid concentration and the protein concentration and surface tension of BALF.

Key words: Nickel oxide, Lung surfactant, Instillation, Rat, BALF

Background and Objectives

The tens of thousands of new chemical substances have been produced each year. The most of these materials are introduced without assessment of the hazards they pose to living organisms. Moreover, with the rapid advancement of nanotechnology, the effects that can be caused by differences in particle size or crystal structure on living organisms is a cause of concern even in the case of known chemical substances. Particle size and surface area can play important roles in the response to inhaled particles, which is especially relevant for ultrafine or nano particles[1–3]. Our previous study using crystalline silica

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showed that even if the particles are of the same chemical composition, their biological effects vary depending on their particle size[4]. Hazard assessment of newly-generated respirable particles must therefore be conducted for such differences and various biomarker studies are currently being implemented to objectively and quantitatively assess the hazards of such substances[5–14].

Lung surfactant is a lipoprotein complex formed by type II alveolar cells and Clara cells and it plays an important role in the stability of the alveoli, within which gas exchange takes place. One function of lung surfactant is to control surface tension. Pulmonary disorders are believed to cause changes in the functioning of lung surfactant resulting in the collapse of the alveoli or peripheral respiratory tract and the development of inflammation and fibrosis. We reported the possibility that measuring phospholipid concentration can be a useful biomarker for assessing the toxicity of chemical substances in surfactant assessment using bronchoalveolar lavage fluid (BALF) in dust exposure animal models[15]. However, we could not find any reports that not only measured phospholipids, the primary component of surfactant, but also studied the surface tension of BALF in order to assess surfactant function. Our prvious study with different-sized nickel oxide showed that submicrometer nano-nickel oxide is associated with greater toxicity, as for crystalline silica, than micrometer-sized nickel oxide[16]. Using this model in this study, we explored whether or not it is possible to screen for toxicity/hazards by measuring the phospholipid concentration and the protein concentration and surface tension in BALF for surfactant assessment. Crystalline silica and titanium dioxide were also used as referential control.

Methods

Sample preparation

The details of materials used in this study were described elsewhere[16]. Briefly, thirty grams of crude nickel oxide (Vacuum Metallurgical Co., Ltd., Chiba, Japan) particles were gently suspended in 2,000 ml distilled water in a measuring cylinder and left to stand overnight. The upper 500 ml of suspension was collected and centrifuged. The supernatant was collected and dried to serve as the sample (nNiOm). This procedure was repeated until a sufficient amount for the sample was obtained. The particle size distribution was determined using a particle size analyzer (Microtrac FRA, Model FA500, Nikkiso Co. Tokyo, Japan). The median diameter of extracted fraction was 0.80 μm. Brunauer-Emmett-Teller (BET)-specific surface areas were determined from N_2 adsorption onto the powders using a Micromeritics FlowSorb 2300 (Micromeritics Co., Norcross, GA, USA) based on the flowing gas method. The average BET surface area of classified nNiOm particles was 32.6 m^2/g. The calculated primary particle size of nNiOm (density is 6.8 g/cm^3) was 27 nm in diameter.

Another NiO particle (NiO: Nakalai Chemicals Ltd., Kyoto, Japan) with a median diameter of 4.8 μm was also used in this study. The average BET surface area of the NiO particles was 0.328 m^2/g. The calculated primary particle size of NiO (density is 6.8 g/cm^3) was 2,700 nm in diameter.

The following two substances were used to compare BALF phospholipid concentration and protein concentration and surface tension for surfactant assessment: crystalline silica (Min-U-5;U.S. Silica Co., Berkeley Springs, WV; geometric mean diameter 1.6 μm, geometric standard deviation [GSD] 2.0) and titanium dioxide (Wako Chemical Co., Osaka, Japan; geometric mean diameter 1.5 μm [GSD] 1.8). 2 mg of particles were suspended in each 0.4 ml of physiological saline aliquot, sonicated thoroughly, and intratracheally instilled into the animals (Wistar rats, males, 10 wk old, 25 animals in each group). Another 25 rats received physiological saline alone as the control. The dose was rather experimental, but determined through our previous results showing that nickel content in rat lungs was observed up to 2.6 mg after 12 months of exposure[17–19]. All rats had been fed a standard diet, and were maintained in the Animal Research Center of the University of Occupational and Environmental Health, in Japan. All of the experimental procedures were approved by the Experimentation Committee of the University of Occupational and Environmental Health, Japan.

Intratracheal instillation and BALF

Animals were sacrificed for dissection at the following time points from instillations: 3 d, 1wk, 1 month, 3 months, and 6 months. Five animals per group were examined at each time point. At autopsy, rats were anesthetized with intraperitoneal injection of ketamin (90 mg/kg) and xyladin (10 mg/kg). Fifteen milliliters of BALF was recovered by injection of saline into the right lung clumping the left main bronchus. After centrifuging the BALF (1,500 rpm; 10 min), the supernatant was frozen at minus 30ºC as a sample for measuring the phospholipid concentration and protein concentration and surface tension.

Measurement of the phospholipid concentration in BALF

We used the enzymatic method to measure the phospholipid concentration in BALF. A standard curve was made

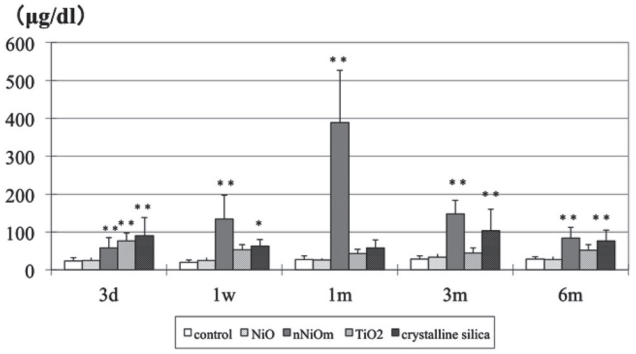

Fig. 1. Phospholipid concentration in BALF.
Date are mean ± SD. *p<0.05 **p<0.01 compared with control.

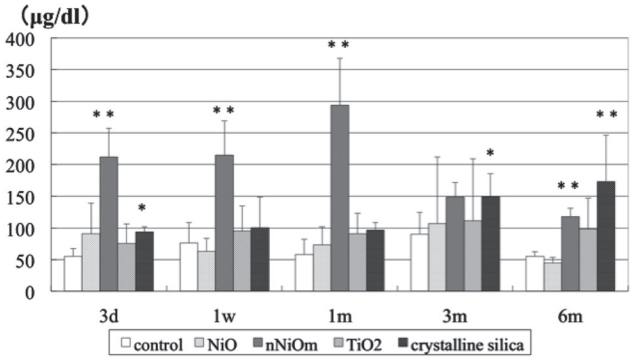

Fig. 2. Protein concentration in BALF.
Date are mean ± SD. *p<0.05 **p<0.01 compared with control.

using a NESCAUTO PL Kit-K (Alfresa Pharma Corporation, Japan) as the standard sample and phospholipid concentration was determined by measuring BALF absorption (absorption 595nm) with a spectrum photometer (Model 680, BIORAD).

Measurement of protein concentration in BALF

The method for measuring protein concentration was based on the same principle as the Bradford protein assay. A standard curve was made using bovine serum albumin (nacalai tesque) as the standard sample and protein concentration was determined by measuring BALF absorption (absorption 595 nm) with the same spectrum photometer as above.

Measurement of BALF surface tension

After diluting the 1.8 ml sample with ten parts physiological saline, BALF surface tension was measured with a du Nouy surfactometer (Taihei Rikakogyo) using the du Nouy ring method. The correction factor was obtained from the water temperature and surface tension of the distilled water on the day the experiment was conducted and actual surface tension was calculated by adding the correction factor to the value obtained for each sample.

Histopathological examination

After BALF was extracted, the left lung was fixed with 10% buffered formalin at 25 cm H2O pressure overnight, and was used for histopathological examination. The paraffin sections of the left lung (3 μm thickness) were stained with hematoxylin and eosin (HE).

Statistical analysis

SPSS was used as the statistical analysis software. When examining the mean value of the two groups, the significant difference was judged to be a p value of less than 0.05 using the Mann-Whitney U-test.

Results

BALF Phospholipid concentration

Figure 1 shows the results for phospholipid concentration. Values for the control group fluctuated between 20–60 μg/ml. No significant difference was noted in the NiO group compared to the control group during the observation period. In the nNiOm group, phospholipid concentration rose and peaked at the 1-month mark and was observed to be significantly high compared to the control group. It was also significantly higher than the group instilled with crystalline silica as a positive control except at the 3-d point. In the group instilled with titanium dioxide, a significantly high concentration compared to the control group was only observed at the 3-d point and no significant difference was observed for any other period.

BALF protein concentration

Figure 2 shows the results for protein concentration. Values for the control group fluctuated between 50–90 μg/ml. No significant difference was found in the NiO group during the observation period when compared to the control group. In the nNiOm group, on the other hand, protein concentration rose and peaked at the 1-month mark and was observed to be significantly high compared to the control group except at the 3-month point. In the crystalline silica group, protein concentration was significantly high at the 3-d, 3-month and 6-month points compared to the control group and it showed a tendency to increase with the passage of time. In the case of the titanium dioxide group, no significant difference was observed compared to the control group throughout the observation period.

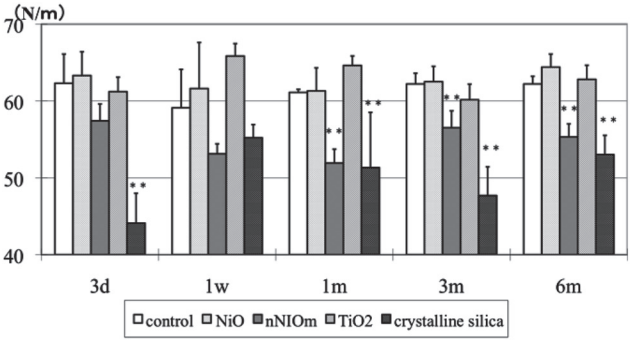

Fig. 3. BALF surface tension.
Date are mean ± SD. *p<0.05 **p<0.01 compared with control.

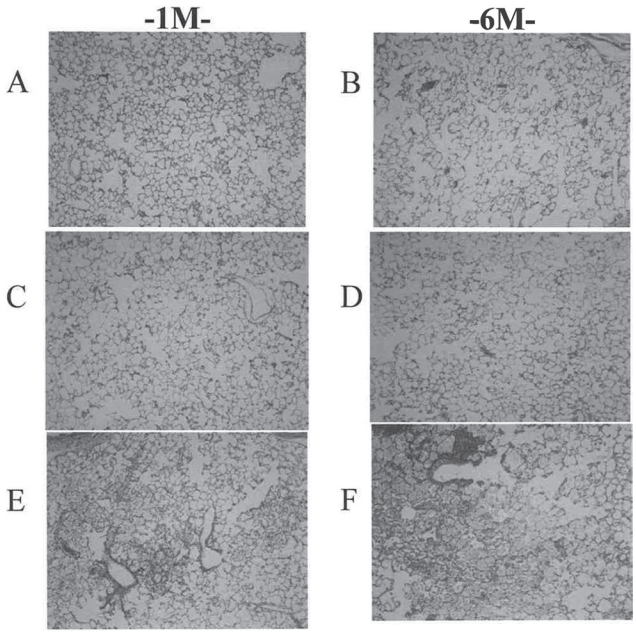

Fig. 4. Representative image of rat lung sections with HE stain at 1 month (A, C, E) and 6 months (B, D, F) after intratracheal instillation (×100).
A, B: control, C, D: NiO, E, F: nNiOm.

BALF surface tension

Figure 3 shows the results for surface tension. Values for the control group fluctuated between 59–64 mN/m. As for both phospholipid and protein concentrations, no significant difference was found in the NiO group during the observation period when compared to the control group. In contrast, surface tension in the nNiOm group was lowest at the 1-month point and significantly lower compared to the control group at the 1-month, 3-month and 6-month points. In the crystalline silica group, on the other hand, the lowest surface tension occurred at the 3-d point and surface tension was significantly lower than for the control group at every period except the 1-wk point. No significant difference was observed for the titanium dioxide group compared to the control group throughout the observation period.

Histology

Representative features at 1 month and 6 months after instillation are shown in Fig. 4A–4F. Histopathological findings in nNiOm groups showed features similar as follows: the infiltration of polymorphonuclear cells (PMN), foamy macrophages in the alveolar space, and debris of cells or alveolar lipoproteinosis were remarkable from 6 months after instillation. In addition, multifocal to multifocal moderate alveolitis with associated moderate epithelial cell alveolar hypertrophy and hyperplasia also occurred. In contrast to the effects observed with nNiOm, inflammatory changes were not as obvious at all postexposure points in the NiO group.

Discussion

In this experiment, nickel oxide with two different particle sizes, NiO (micron size) and nNiOm (submicron size), as well as crystalline silica as a positive control and titanium dioxide as a negative control, were intratracheally instilled in rats and the phospholipid concentration and the protein concentration and surface tension, which are used in surfactant assessment, were measured to see if they could be effective biomarkers in toxicity assessment. The results showed that the NiO instilled group showed no significant difference compared to the control group throughout the observation period. In contrast, a significant difference was found in the nNiOm instilled group compared to the control group throughout the observation period. Moreover, a significant difference was found in the crystalline silica instilled group for each measurement compared to the control group while for the titanium dioxide group, almost no significant difference was found. In addition, the results of simultaneously conducted total cell counts and neutrophil counts in BALF longitudinally/over time showed the same results. Furthermore, with regards to the inflammation ratio obtained by the point counting method that quantitatively assesses inflammation as a pathologic feature/findings, the NiO group showed almost no difference compared to the control group whereas the nNiOm group exhibited a significant difference throughout the observation period compared to the control group, although peak inflammation was slightly delayed[16]. These results indicate that

nanosized particles of nickel oxide with smaller median diameters potentially have a stronger biological effect than microsize particles. They also indicate that screening can be done by measuring the phospholipid concentration and the protein concentration and surface tension of BALF.

The International Agency for Research on Cancer (IARC) categorizes nickel compounds as Group 1 (carcinogenic) substances and groups them together with nickel oxide, nickel sulfide and nickel carbonyl. Of these, nickel oxide is extremely insoluble and is thought to remain longer in the lungs. The carcinogenic potential of nickel oxide is estimated to be only one-tenth as nickel subsulfide. However, the results presented in existing reports on toxicity in the lung for nickel oxide of the same composition but with smaller particles correspond to the results of our study.

In an experiment by Toya *et al*. in which rats were intratracheally instilled with nickel oxide particles (median diameter 1.7 μm) and nickel fumes (containing 97% nickel oxide with a cohesive median diameter of 0.6 μm), results showed stronger toxicity in the lungs for the group instilled with nickel fumes than for the group instilled with larger particle nickel oxide[20].

In our experiment, the BALF phospholipid and protein concentrations rose and surface tension dropped in the group instilled with nNiOm, which had a higher ratio of inflammation, compared to the control group and the NiO group. We can surmise that this is caused by accelerated production and secretion of lung surfactant to prevent lung collapse due to inflammation. These mechanisms for maintaining homeostasis in the lung are the physiological and biochemical basis for the premise that the measurement of BALF phospholipid concentration and protein concentration and surface tension used in this experiment are useful in bioeffects screening. Steinberg *et al.* showed the relationship between the spread of inflamed cells and the amount of phospholipid in BALF in rats with fibrosis caused by exposure to radiation[21]. These experiments also support using lung surfactant assessment for hazard assessment of chemical substances.

In addition, we assessed the surface tension control effect of surfactant, in other words, surfactant function, using BALF surface tension measurements. In our experiment, the surface tension of BALF did not decrease even though the BALF had a high concentration of phospholipids, the main component of surfactant. Moreover, despite the fact that phospholipid concentration in the crystalline silica group was not very high, the surface tension decreased. These results indicate that when assessing surfactant *in vivo* it is necessary to evaluate production and function separately.

The composition of fatty acid, which comprises phospholipids, is known to affect the function of surfactant surface tension. Schmidt *et al.* pointed out changes in the ratio of saturated fatty acid and unsaturated fatty acid in the BALF of 39 patients with acute respiratory distress syndrome (ARDS) and reported a decrease in the patients' lung surface tension with administration of phosphatidylcholine, the main component of saturated fatty acid[22]. In addition, Davidson *et al.* pointed out reduced lung compliance in rats with acute lung obstructions and reported a difference in unsaturated fatty acid concentration[23]. We could not find any experiments like ours that assessed surfactant function by BALF surface tension.

It is also noteworthy that in this experiment phospholipid concentration, protein concentration, protein surface tension, total cell counts and neutrophil counts, which were the screening indicators used in this experiment, all changed in similar ways at the same points in time[16]. This indicates that this is a highly reliable screening method. Moreover, while inflammation occurred slightly later compared to pathological features, they followed the same trend, demonstrating that these screenings are extremely useful and supporting their reliability. Recently, Schleh *et al.* investigated the effect of titanium dioxide (TiO_2) nanosized particles (NSP) and microsized particles (MSP) on biophysical surfactant function after inhalation to rats. Their data did not suggest that inhalation of TiO_2 nanoparticles cause a significant disturbance of the pumonary surfactant system[24].

In conclusion, we intratracheally instilled nickel oxide with different particle diameters in rats, measured the phospholipid concentration and the protein concentration and surface tension of BALF and compared these to total cell counts and neutrophil counts in BALF and to pathological features. We showed that submicronsized particles of nickel oxide with smaller median diameters potentially have a stronger biological effect than microsize particles. The results indicate that these measurements can act as biomarkers in animal models of dust exposure. Further research is necessary to determine whether BALF analysis of surfactant adhering to the lungs can serve as a biomarker in hazard assessment of chemicals.

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