Comparative Toxicity of Standard Nickel and Ultrafine Nickel in Lung after Intratracheal Instillation

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Abstract: Comparative Toxicity of Standard Nickel and Ultrafine Nickel in Lung after Intratracheal Instillation: Qunwei ZHANG, et al.; Department of Environmental Health, School of Medicine, Fukui Medical University—A comparison was made of the bronchoalveolar lavage fluid (BALF) response to ultrafine nickel (Uf-Ni) and standard-sized nickel (Std-Ni). Rats were intratracheally instilled with 0, 0.1, 0.5, 1 and 5 mg Uf-Ni and Std-Ni, respectively. At 3 d after instillation, the body weight and wet lung weight were determined. At the same time, BALF was analyzed for lactate dehydrogenase (LDH), total protein (TP), tumor necrosis factor-alpha (TNF-alpha), and total cell and differential cell counts. The results showed that indicators of lung injury and inflammation in BALF were markedly raised with increased Uf-Ni and Std-Ni for each from 0 to 1 mg, and there were no differences in the indices between instillation of Uf-Ni at 1 mg and 5 mg. The results also showed that the effects of Uf-Ni on the indices were significantly higher than those of Std-Ni. Additional groups of rats were intratracheally instilled with 1 mg of Uf-Ni or Std-Ni, and wet lung weight and BALF profiles were analyzed at 1, 3, 7, 15 and 30 d later. The effect of Uf-Ni and Std-Ni on indices that can be presumed to reflect epithelial injury and permeability (LDH or TP), and release of proinflammatory cytokine (TNF-alpha) were increased throughout the 30 d post-exposure and the effects of Uf-Ni on these indices were significantly higher than those of Std-Ni from 1 to 30 d after instillation. Moreover, the number of neutrophils and LDH activity in BALF of rats after exposure to Uf-Ni were significantly greater than those of Std-Ni-exposed rats up to 30 d after instillation. Our findings suggest that Uf-Ni has a much more toxic effect on the lung than Std-Ni, but the mechanism remains to be elucidated.

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Nickel is an important and sometimes essential commodity\textsuperscript{1}, but nickel and its compounds can cause skin sensitization and may cause lung reactions and inflammatory changes in the nasal mucosa\textsuperscript{2}. Various compounds of nickel, such as nickel subsulfide (Ni\textsubscript{3}S\textsubscript{2}), nickel sulfate (NiSO\textsubscript{4}), and nickel oxide have been shown to cause pulmonary inflammation, fibrosis, emphysema, alveolar proteinosis, and cancer\textsuperscript{3-6}. And nickel and its compounds are classified as human carcinogens by IARC\textsuperscript{7}.

Ultrafine metallic nickel particles (Uf-Ni) with a mean diameter of 20 nm is a new product made by the process of vacuum vapour deposition. Its characteristics include a high level of surface energy, high magnetism, low melting point, high surface area, and low burning point. Therefore, it is widely used in high magnetic tape, conducting paste, chemical catalysis, hot converter material in low temperature sintering promotion, microfilters, gas sensing equipment, combustion promotion, and light absorbance.

It is well known that the toxicity of particles to the lung in both occupational and environmental settings is not only related to exposure but also to the particle type. The present occupational exposure limit dose is based on mass and does not discriminate particle size and so takes no account of the likely enhanced toxicity of...
ultrafine particles in exposed workers. For instance, particulate titanium dioxide (TiO$_2$) is classified as a nuisance dust and so is considered to have few adverse effects on the lung except at high exposure concentration. Nevertheless, there are now reports showing that ultrafine titanium dioxide (Uf-TiO$_2$) with a diameter of 20 nm can induce a greater lung inflammatory reaction than 250 nm TiO$_2$ particles$^{13,14}$. Uf-TiO$_2$ has been shown to enter the pulmonary interstitium and this may be a feature of the pathogenicity of ultrafine particles$^{15}$. This may arise because of the much greater number of ultrafine particles deposited in the lung for a given mass than is seen with conventional fine particles$^{16}$. Additionally, several studies have suggested that active oxygen species associated with the surface of ultrafine particles play an important role in their toxicity$^{16,17}$. Our previous results have shown that the toxicity of three ultrafine metals could be ordered Uf-Ni>Uf-Co>Uf-TiO$_2$, and the difference in free-radical-generation activity could underlie the difference in toxicity of these three ultrafine metals$^{18}$. The aim of the present study is to compare the toxicity of standard nickel (Std-Ni) and ultrafine nickel (Uf-Ni) by using the technique of bronchoalveolar lavage (BAL) to study the inflammatory profile in the bronchoalveolar lavage fluid (BALF), a technique used extensively to study the effect of particles on lung$^{19,20}$. The data obtained from this study will be of benefit for predicting the acute and subacute effects of Uf-Ni on lungs.

Materials and Methods

Laboratory animal

Specific pathogen-free Wistar male rats at the age of 7–8 weeks were supplied by Clear Japan, INC (JCL). The rats were housed in the animal center of Fukui Medical University, Japan, and fed conventional laboratory diet with free access to tap water.

Particles and characteristics of particles:

1. Nickel powder with an average diameter of 5 μm referred to as Std-Ni (Cat. No. 608D1307, Nickel powder Chemical Co., Japan);
2. Nickel powder particles with an average diameter of 20 nm; density, 0.19 g/cm$^3$ and 43.8 m$^2$/g of surface area, hereafter referred to as Uf-Ni (Ultra fine powder, Lot No.2237, Inabta and Co., Ltd., Vacuum Metallurgical Co., Ltd., Japan).

Size distribution Uf-Ni was determined with a transmission electron microscope (TEM) as previously described$^{19}$. The powders were suspended in physiological saline at concentration of 0, 0.1, 0.5, 1.0 and 5.0 mg/ml. The suspensions were ultrasonicated for about 30 min, then sterilized at 0.5 kgf/cm$^2$ and 120 °C for 15 min.

Treatment

Dose-response

All the rats were divided randomly into five groups of 5 to 7 rats. Each rat was injected intratracheally with 0.1, 0.5, 1.0 or 5 mg of Std-Ni or Uf-Ni in suspension in 1 ml of physiological saline under ether anesthesia. Hereafter, rats injected with physiological saline were called the control group. The rats were killed at 3 d after injection.

Time-response

All rats were divided randomly into five groups of 5 to 7 rats. Each rat was injected intratracheally with 1 mg of particles in suspension in 1 ml of physiological saline. Groups of 5–7 rats were killed at 1, 5, 7, 15 and 30 d after injection, respectively. Hereafter, rats injected with physiological saline alone are called the control group.

Bronchoalveolar lavage

Rats were killed by instillation of an overdose of phenobarbital solution (50 mg/ml) into the abdominal cavity, and the lungs and trachea were removed en bloc. The lung wet weight was measured after removing the heart, the mediastinal lymphoid and adipose tissue. Bronchoalveolar lavage fluid (BALF) was accomplished by 4 × 10 ml/ saline washes warmed at 37°C, with massage. The recovery of bronchoalveolar lavage fluid (BALF) for each rat was measured and calculated. The BALF was centrifuged (1,500 rpm, 10 min), and the first wash was used to measure the activity of the LDH and total concentration of protein (TP) and tumor necrosis factor (TNF-alpha). The total numbers of cells, macrophages, neutrophils, and lymphocytes were evaluated for the BALF pooled from each rat. The LDH activity was measured with an LDH C II-test kit (Wako Pure Chemical Industries Ltd.) by the lactate matrix method$^{21}$. The concentration of total protein in BALF was assessed by the Lowry$^{22}$ method. TNF-alpha was quantified with an enzyme-linked immunosorbent (ELISA) kit (Biosource International). The assay utilized a standard, 96 well quantitative immunometric “sandwich” enzyme technique according to the manufacturer’s recommended protocol. Cells from all BALF were counted, and cell differentiation was made by the Wright-Giemsa method.

Statistical analysis

Values were expressed as the means and standard errors. The difference among groups according to exposure to particles and doses were tested by one-way analysis of variance (ANOVA), followed by Bonferroni’s multiple comparison. If the P value was less than 0.05, the difference was considered to be significant.

Results

1) Dose-response of Uf-Ni and St-Ni

1. 1) The recovery of BALF (all >80%) did not differ significantly among the experimental groups and control (Data are not shown).

1. 2) Lung wet weight

Figure 1 shows that lung wet weights of rats instilled with 0.5, 1 and 5 mg Std-Ni or Uf-Ni were significantly
Fig. 1. Lung wet weight at 3 d after instillation with various doses of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Asterisks indicate significantly different from the control group, P<0.05. # indicates a significantly different from the Std-Ni groups, P<0.05.

Fig. 2. Number of total cells in BALF from rats at 3 d after instillation of various doses of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig.1.

Fig. 3. Percentage of neutrophils in BALF from rats at 3 d after instillation of various doses of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig. 1.

Fig. 4. LDH activity in BALF from rats at 3 d after instillation of various doses of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig. 1.
Fig. 5. Total protein in BALF from rats at 3 d after instillation of various doses of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig. 1.

Fig. 6. Lung wet weight of rats up to 30 d after instillation of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Asterisks indicate significantly different from the control group, P<0.05. # indicate significantly different from the Std-Ni groups, P<0.05. Comparisons by ANOVA with Bonferroni’s comparison test.

Fig. 7. Total numbers of cells in BALF of rats up to 30 d after instillation of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig. 1.

Fig. 8. Percentage of neutrophils in BALF of rats up to 30 d after instillation of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig. 1.
Fig. 9. Percentage of macrophages in BALF of rats up to 30 d after instillation of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig. 1.

Fig. 10. LDH activity in BALF of rats up to 30 d after instillation of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig. 1.

Fig. 11. Total protein in BALF of rats up to 30 d after instillation of Std-Ni and Uf-Ni. Values are the mean ± SE for five to seven rats. Significant differences as in Fig. 1.

Fig. 12. Concentration of TNF-alpha in BALF of rats up to 30 d after instillation of Std-Ni and Uf-Ni. Values are mean ± SE of five to seven rats. Significant differences as in Fig. 1.
greater than those of controls at 3 d after injection. There was a dose-related increase in the lung wet weight after instillation of various doses of Std-Ni or Uf-Ni. The results also showed that the lung wet weights of rats instilled with 1 mg Uf-Ni were significantly greater than those instilled with the same dose of Std-Ni.

1.3) Cellular constituents in BALF

Instillation of only 0.1 mg of Uf-Ni caused a detectable inflammatory response, as measured by the increase in the total numbers of cells and neutrophils in BALF (Fig. 2 and Fig. 3). There was a dose-related increase in total cells and neutrophils after instillation of increasing doses of Std-Ni or Uf-Ni, but the increase in neutrophils was about 3 to 7 times greater with Uf-Ni than with St-Ni at the same mass.

1.4) Biochemical markers in lavage

Std-Ni had a very modest effect on LDH activity in the lavage but Uf-Ni caused a slight increase at 0.5 mg and a very dramatic increase at 1.0 mg that was not greatly increased by increasing the dose to 5 mg (Fig. 4). Total protein showed the same pattern as the LDH (Fig. 5).

2) Time-effect

2.1) Recovery of BALF

The recovery of BALF (all >80%) did not differ significantly among the experimental and control groups (data not shown).

2.2) Lung wet weight

The lung wet weights of rats in the Std-Ni and Uf-Ni groups were significantly greater than those of the controls at 1, 3, 7, 15 and 30 d after instillation; the Std-Ni group had heavier lungs than the control except at 30 d after instillation. The wet lung weights of rats in the Uf-Ni group were significantly greater than those in the Std-Ni group at 1 and 3 d (Fig. 6).

2.3) Cellular and biochemical constituents in BALF

Std-Ni and Uf-Ni instillation initially caused a marked increase in the total numbers of cells and neutrophils and macrophages in BALF. Std-Ni and Uf-Ni induced an increase in total numbers of cells and neutrophils that peaked at day 1 and declined over the succeeding 30 d, but at 30 d after instillation, the induced increases were still significant (Fig. 7, Fig. 8). Nevertheless, Std-Ni and Uf-Ni induced an increase in the percentage of macrophages in BALF from day 1 to day 30 (Fig. 9). The effect caused by Uf-Ni was marked and greater than that of Std-Ni at all time points. The results also showed that LDH activity of the Uf-Ni exposed rats was much greater than that of Std-Ni; it was more than 10 times the amount of LDH seen in the BAL of Std-Ni-exposed rats at most time points (Fig. 10). The protein showed a similar pattern as LDH; Uf-Ni also caused a remarkable and sustained increase in the level of protein in BALF, at more than 7 to 12 times the levels seen in the Std-Ni-exposed rats (Fig. 11). The TNF levels in the BALF (Fig. 12) show a remarkable agreement with the extent of inflammation caused by the Uf-Ni and Std-Ni particles.

Discussion

Nickel and its compounds are known to cause lung inflammation, fibrosis, emphysema, alveolar proteinosis and tumors. The aim of this study is to compare the toxicity of Uf-Ni, one of the new categories of ultrafine particles, with that of Std-Ni on the lungs in terms of dose and time response.

In the first part of this report, we presented the results of a dose-response study. These results showed that Uf-Ni induced more severe pulmonary inflammation than Std-Ni, on a mass basis. Analysis of the cellular and biochemical profile of the BALF after exposure to pulmonary toxins is a useful method for characterizing the inflammatory response of the lung\textsuperscript{19–20}; it can be used to assess lung injury induced by mineral dusts or metallic compounds\textsuperscript{19–20, 23–24}. All of the indices of lung inflammation, lung weight, total cells, PMN, LDH and protein were all increased and remained increased in a mass dose-related manner that was significantly greater in magnitude for Uf-Ni than for Std-Ni. Instillation of 0.1 mg Uf-Ni, a low dose, produced massive edema, as shown by increased absolute and relative lung wet weight, together with an increase in leukocyte influx. This was accompanied by cell damage, as measured by LDH activity and protein in BALF. These effects of Uf-Ni were dose-related and were most severe at doses of 1 and 5 mg of Uf-Ni. Furthermore, these effects of Uf-Ni greatly exceeded those elicited by Std-Ni.

In the second part of this paper, we presented results of the time course of pulmonary inflammation with Uf-Ni and Std-Ni. Instillation of 1 mg Uf-Ni induced pulmonary inflammation, as shown by increased lung weight, sustained macrophage and neutrophil influxes, and severe injury as measured by LDH and protein in BALF. These effects of Uf-Ni greatly exceeded those elicited by Std-Ni. The BALF indicators suggested that this Uf-Ni instillation may cause a sequence of events commencing with acute damage to the epithelium and resulting in LDH released. Epithelial injury or stimulation is likely to be an important component of the whole process after instillation, because of prolonged interaction between the ultrafine particles and the epithelium resulting in failure of the macrophages to phagocytose the very large number of deposited particles\textsuperscript{19}. Interstitialization of particles is also a very striking feature of the ultrafine particle-exposed lung\textsuperscript{20} which may arise from the prolonged interaction between particles and epithelial cells. The data here are in general support of this contention, since epithelial injury would be anticipated to produce LDH accumulation and leakage of protein into the airspaces.

It is generally accepted that macrophages play a key role in starting the cascade of adverse reaction to toxic
particles. Neutrophils normally perform an important defensive role against microbial infection, but when macrophages increase in number and become activated in response to particle deposition, they can release chemotactic factors that attract polymorphonuclear leukocytes and monocytes. These activated macrophages and neutrophils can release a variety of cytokines, toxic oxygen metabolites and proteinase that can damage the lung parenchyma and also stimulate fibroblast proliferation.

TNF-alpha is a proinflammatory cytokine produced by macrophages in response to various stimuli. Neutrophils are activated by TNF-alpha to a state where they are more likely to promote inflammation and cause injury to epithelial cells. Our results showed that one striking feature of the Uf-Ni inflammation is the high levels of TNF, more than twice the levels found with Std-Ni at most time points. Interestingly, the increase in TNF-alpha in BALF was accompanied by an increase in the total numbers of cells and neutrophils in BALF. TNF could contribute to the uncoupling of some aspects of the inflammatory response by causing increased activation of neutrophils in the Uf-Ni exposed lungs so that they become more adherent to the epithelium, increasing their ability to cause epithelial injury. In addition, there is evidence showing that TNF causes direct increases in epithelial permeability and this also could be an important factor in the increased protein leak seen in Uf-Ni-exposed lungs.

This study did not elucidate the mechanism whereby Uf-Ni is able to cause higher pulmonary toxicity than Std-Ni, but the difference between Uf-Ni and Std-Ni in particle diameter is one of the most striking characteristics, and Uf-Ni has a much greater surface area than Std-Ni. Particle size appears to be very important in terms of inducing lung disease after deposition in the lung. Furthermore, our previous studies also found that some ultrafine particles have the potential to generate free radical activity, and exposure to Uf-Co and Uf-Ni can stimulate pulmonary leukocytes to release TNF-alpha and NO, which may underlie the effects reported here.

In conclusion, our study confirmed that Uf-Ni is much more toxic to the lungs than Std-Ni. The data obtained from this study demonstrate the special toxicity of Uf-Ni. Since the present occupational exposure limit for metal is based on mass only and does not take ultrafine particle size into account, these findings have important implications for hygiene regulation.

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