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Original Article

Time-course comparison of pulmonary inflammation induced by intratracheal instillation of four different nickel oxide nanoparticles in male Fischer rats

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Abstract: Occupational exposure to nickel oxide (NiO) is an important cause of respiratory tract cancer. Toxicity is known to be associated with the dissociated component, i.e. nickel (II) ions. To address the relationship between physicochemical properties, including solubility in artificial lysosomal fluid, of NiO and time-course changes in the pulmonary response, we conducted an intratracheal instillation study in male Fischer rats using four different well-characterized NiO products, US3352 (NiO A), NovaWireNi01 (NiO B), I small particle (NiO C), and 637130 (NiO D). The NiOs were suspended in purified water and instilled once intratracheally into male F344 rats (12 weeks old) at 0 (vehicle control), 0.67, 2, and 6 mg/kg body weight. The animals were euthanized on days 3, 28, or 91 after instillation, and blood analysis, bronchoalveolar lavage fluid (BALF) testing, and histopathological examination were performed. The most soluble product, NiO B, caused the most severe systemic toxicity, leading to a high mortality rate, but the response was transient and surviving animals recovered. The second-most-soluble material, NiO D, and the third, NiO A, caused evident pulmonary inflammation, and the responses persisted for at least 91 days with collagen proliferation. In contrast, NiO C induced barely detectable inflammation in the BALF examination, and no marked changes were noted on histopathology. These results indicate that the early phase toxic potential of NiO products, but not the persistence of pulmonary inflammation, is associated with their solubility. (DOI: 10.1293/tox.2020-0066; J Toxicol Pathol 2021; 34: 43–55)

Key words: intratracheal instillation, nanomaterial, nickel oxide, pulmonary toxicity, rat

Introduction

Progress in technological development has led to the use of nanomaterials, which pose health hazards that are of serious concern. Generally, a nanomaterial is defined as a material that has at least one dimension in the range of 1 to 100 nm. The most likely route of exposure to nanomaterials is inhalation, and the Organisation for Economic Co-operation and Development (OECD) has revised its inhalation toxicity study guidelines to accommodate the evaluation of the health effects of nanomaterials[1, 2]. Another dosing method, intratracheal instillation, is considered acceptable for the purpose of hazard identification and comparative toxicity assessment of nanomaterials[3].

Intratracheal instillation is a useful delivery method that requires only a small amount of test material and simple experimental equipment; it is therefore cost effective[4–6]. We have investigated the effects of various study conditions[7] and established a standardized test procedure for obtaining reliable results[8, 9]. Accordingly, we have performed intratracheal instillation studies in rats to evaluate the comparative toxicities of several types of nano-sized titanium dioxide[10–12] and silicon dioxide materials (Kobayashi et al., in submission). In addition, we have reported on the *in vivo* kinetics of intratracheally instilled nickel oxide (NiO) nanomaterials[13]. Here, from the perspective of pulmonary inflammation, we provide further data obtained from the same instillation study of NiO.

NiO is an important cause of human health problems in occupational settings, including mining, refining, and manufacturing[14]. The main exposure route in occupational settings is inhalation, and inhaled nickel compounds can cause tumors in the lungs and nasal cavity of humans[14], and in the lungs and adrenals of laboratory rodents[15–17]. The toxic

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potential of nickel compounds is associated with their solubility; soluble nickel compounds are potentially more toxic than insoluble ones[18]. This is because nickel (II) ions (Ni^{2+}) predominantly account for the toxicity of nickel.

Despite these studies, no report has systematically compared the *in vivo* toxicities of several types of well-characterized NiO nanomaterials. In this study, we investigated the association between physicochemical properties and *in vivo* toxicity of four different NiO products. The NiOs were suspended in purified water and intratracheally instilled into male F344 rats. To assess time-course changes in toxic effects, the animals were euthanized on days 3, 28, or 91 after instillation, and examinations included blood evaluation, bronchoalveolar lavage fluid (BALF) analysis, and histopathological assessment.

Materials and Methods

Preparation and characterization of NiO dose suspensions

In the current study, we utilized four different NiO products, US3352 (NiO A; US Research Nanomaterials, Inc., Houston, TX, USA), NovaWireNi01 (NiO B; Novarials Corporation, Woburn, MA, USA), I small particles (NiO C; Kusaka Rare Metal Products, Tokyo, Japan), and 637130 (NiO D; Sigma-Aldrich, St. Louis, MO, USA). The procedure used to prepare the dose suspensions is described in our previous report[13]. Briefly, each raw NiO powder was suspended in purified water at a concentration of 1 g/mL, and the suspension was sonicated and centrifuged to remove sediments of large aggregated particles. The supernatant was then diluted to prepare dose suspensions at concentrations of 0.67, 2, and 6 mg/mL. Physicochemical properties were analyzed in detail and described in our previous report[13] (Supplementary Table 1 and 2). NiO A is a black powder with spherical particles in Scanning Electron Microscope (SEM) images. It has a primary particle size of 20 nm and a specific surface area (SSA) of 51 m^2/g. The secondary particle size, as determined by dynamic light scattering, was 49 nm. NiO B is a black powder on visual examination, but SEM images revealed the particles' fibrous shape (240 nm long and 29 nm in diameter), and the SSA was large (180 m^2/g). NiO C is a dark green powder. Its primary particles are an irregular cubic shape (SEM) 140 nm in size. The SSA was 6.6 m^2/g, and the secondary particle size was 1,600 nm. NiO D is a black powder appearing on SEM images as spherical particles. Notably, the primary particle size was obtained from the manufacturer's information because particle aggregation in the SEM specimen prevented us from determining the size. The SSA was 93 m^2/g and the secondary particle size was 39 nm.

In our previous study[13], we determined the solubility of these four materials in artificial lysosomal fluid (ALF: pH = 4.5) (Supplementary Table 2), artificial interstitium solution, hydrogen peroxide, saline, and pure water. Briefly, 2 mg/mL of NiO suspension and the solutions were combined at 1:3 (v/v) and mixed with a magnetic stirrer for up to 216 h at room temperature. The dissolved Ni^{2+} was then quantified by inductively coupled plasma – mass spectrometry. The solubility ranking of the tested NiOs was as follows: NiO B (100% soluble) >> NiO D (33%) > NiO A (11%) >> NiO C (0.7%). Compared with those in ALF, the solubilities of the tested NiO products in the other solutions were much lower (NiO A, 2.3% to 3.7%; NiO B, 3.5% to 6.5%; NiO C, 0.14% to 0.51%; and NiO D, 3.9% to 6.3%)[13].

Animals

Male F344/DuCrlCrlJ rats (specific pathogen-free) were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were housed in a barrier-system animal room, which was maintained at 21–25 °C, with a relative humidity of 40% to 70%, 10 to 15 air changes hourly, and a 12 h:12 h light:dark photoperiod. The study was conducted in accordance with the institutional animal welfare guidelines of the CERI Hita, which adhere to Japanese law, acts, and guidelines.

Animal experiments

Details of the procedure used for intratracheal instillation are given in our previous study[7]. Briefly, the rats (12 weeks old) were anesthetized with isoflurane, and intratracheal instillation was performed in a supine position. Doses were determined as 0 (vehicle control: purified water), 0.67, 2, and 6 mg/kg (body weight) according to the results of a preliminary study. We confirmed that 6 mg/kg of NiO A induced evident inflammation 3 days post-instillation, so the highest dose for all NiO samples was set at 6 mg/kg. For NiO A, NiO C, and NiO D, 30 animals per dose were used. For NiO B, an additional 50 rats were used (total 80 animals per dose). The additional animals were included to assess carcinogenicity at 52 (n=15) or 104 weeks (n=35) post-instillation (unpublished data), because its fibrous shape may raise serious health concerns similar to other fibrous nanomaterials[19, 20]. However, 20 out of 70 animals (not including the 10 animals in the day 3 group) that received 6 mg/kg of NiO B died from severe toxicity before the scheduled examinations at 28 or 91 days post-instillation. Therefore, 8 animals were assigned to the groups for days 28 and 91 post-instillation assessment of 6 mg/kg NiO B. After instillation, the clinical condition of each animal was examined daily. Body weights were measured on the day of instillation (Day 0), on days 3 and 7, and once weekly thereafter. On days 3, 28, and 91, 10 or 8 animals per dose were euthanized by being bled under anesthesia. Half of each group was used for BALF examination and kinetic analyses; the results have been reported previously[13]. Details of the procedure used for BALF collection are also described in a previous study[7]. Briefly, after euthanasia of the animal, the trachea was exposed and incised, and a gavage needle was inserted into the trachea and ligated. Then, 7 mL of physiological saline was instilled via the needle and the whole lungs were lavaged twice to obtain BALF. Total cell numbers in the BALF were measured using an ADVIA 120 hematology analyzer (Siemens Health Care Diagnostics, Tarrytown, NY, USA). The BALF was

then centrifuged (400× *g*, 10 min, 4°C) and the supernatant was removed. The cell pellet was resuspended in phosphate buffer solution, spread on a slide, and Giemsa stained for BALF cytological analysis. A differential leukocyte count was performed to identify the percentages of macrophages, neutrophils, lymphocytes, and eosinophils. The other half of the animals in each group were anesthetized with pentobarbital, and a blood sample was collected from the abdominal aorta for hematological and blood chemical analyses as follows: erythrocyte number, hemoglobin concentration (Hb), hematocrit value (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet number (PLT), reticulocyte percentage, leukocyte number and differential percentages (neutrophils, lymphocytes, eosinophils, basophils, monocytes, and others), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (GGT), total cholesterol (TC), triglycerides (TG), blood urea nitrogen (BUN), creatinine (Cre), total protein (TP), albumin (Alb), albumin to globulin ratio (A/G), glucose (Glu), total bilirubin (TB), inorganic phosphorus (IP), calcium (Ca), sodium (Na), potassium (K), and chlorine (Cl). After blood sampling, the animals were euthanized and the lungs, posterior mediastinal lymph nodes (PMLNs), liver, spleen, and kidneys were removed, fixed in 10% neutralized formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Additionally, the lung specimens were stained with Masson's trichrome stain. The histopathological findings and lung terminology underwent peer review externally by two expert pathologists.

Statistical analysis

For body weights, blood examinations, and BALF examinations, homogeneity of variance was analyzed using Bartlett's test. The differences between the mean values of treatment groups and the vehicle control were analyzed using Dunnett's test when variance was homogeneous ($P>0.01$ in the Bartlett test) or by using Steel's test when variance was heterogeneous ($P<0.01$ in the Bartlett test; two-tailed for body weights and blood examinations, and one-tailed for BALF parameters).

Results

Clinical signs

In animals treated with NiO A or NiO C, no abnormal changes were noted throughout the experimental period.

Animals receiving 6 mg/kg of NiO B showed serious clinical signs, such as anorexia, decreased spontaneous locomotion, no stool, and emaciation from days 4 to 23. In total, 20 out of 70 animals died between days 4 and 16. Surviving animals recovered after day 24. Animals receiving 2 or 0.67 mg/kg of NiO B showed abnormal signs, such as anorexia and decreased spontaneous locomotion from days 3 to 15, and one animal each receiving 2 mg/kg died on days 4 and 5. One animal receiving 0.67 mg/kg died on day 6. The dead animals were in the longer term carcinogenicity groups. Thereafter, surviving animals recovered, and no abnormal conditions were noted after day 16. The survival rates of NiO B-treated animals are summarized in Fig. 1.

Animals receiving 6 mg/kg of NiO D showed signs such as anorexia, decreased stool, and decreased spontaneous locomotion from days 3 to 20, and animals receiving 2 mg/kg showed anorexia and decreased stools from days 7 to 13. Thereafter, these animals recovered. No abnormalities were noted in animals treated with 0.67 mg/kg NiO D.

Body weights

Body weight changes are shown in Fig. 2. In NiO A-treated animals, the mean body weight in the 6 mg/kg group decreased from day 3 to 7, and was significantly lower than that in the vehicle controls. No significant changes were noted in the 2 or 0.67 mg/kg groups. In the NiO B-treated rats, mean body weight decreased from day 1 to 14 at 6 mg/kg and from day 1 to 7 at 2 mg/kg and 0.67 mg/kg. The values were significantly lower than those of the vehicle controls from days 3 to 35 and on day 49 at 6 mg/kg, from days 3 to 28 at 2 mg/kg, and from days 7 to 21 at 0.67 mg/kg. No significant changes were observed in the NiO C-treated groups. In the NiO D-treated animals, the mean body weight in the 6 mg/kg group decreased from day 1 to 7 and was significantly lower on days 3–56 and 84–91 than in the vehicle controls. The mean body weight in the 2 mg/kg group decreased from day 3 to 7 and was significantly lower than in the controls from day 7 to 21. No significant changes were noted in the 0.67 mg/kg group.

Hematological examination

The results of the hematological examinations on day 3 in the NiO B-treated groups are shown in Table 1; data for the other groups and examination times are provided in Supplementary Table 3. In NiO A-treated animals, a signifi-

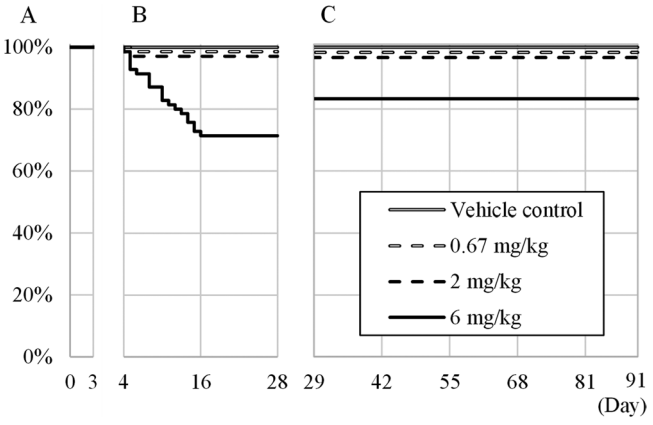

Fig. 1. Survival rate of NiO B-treated animals from day 0 to 91. The survival rate is calculated in total animal numbers, including carcinogenicity assessment groups. A: Day 0–3 (n=80), B: Day 4–28 (n=70), C: Day 29–91 (n=60). Note that the survival rates of C were raised due to the decrease of animal numbers.

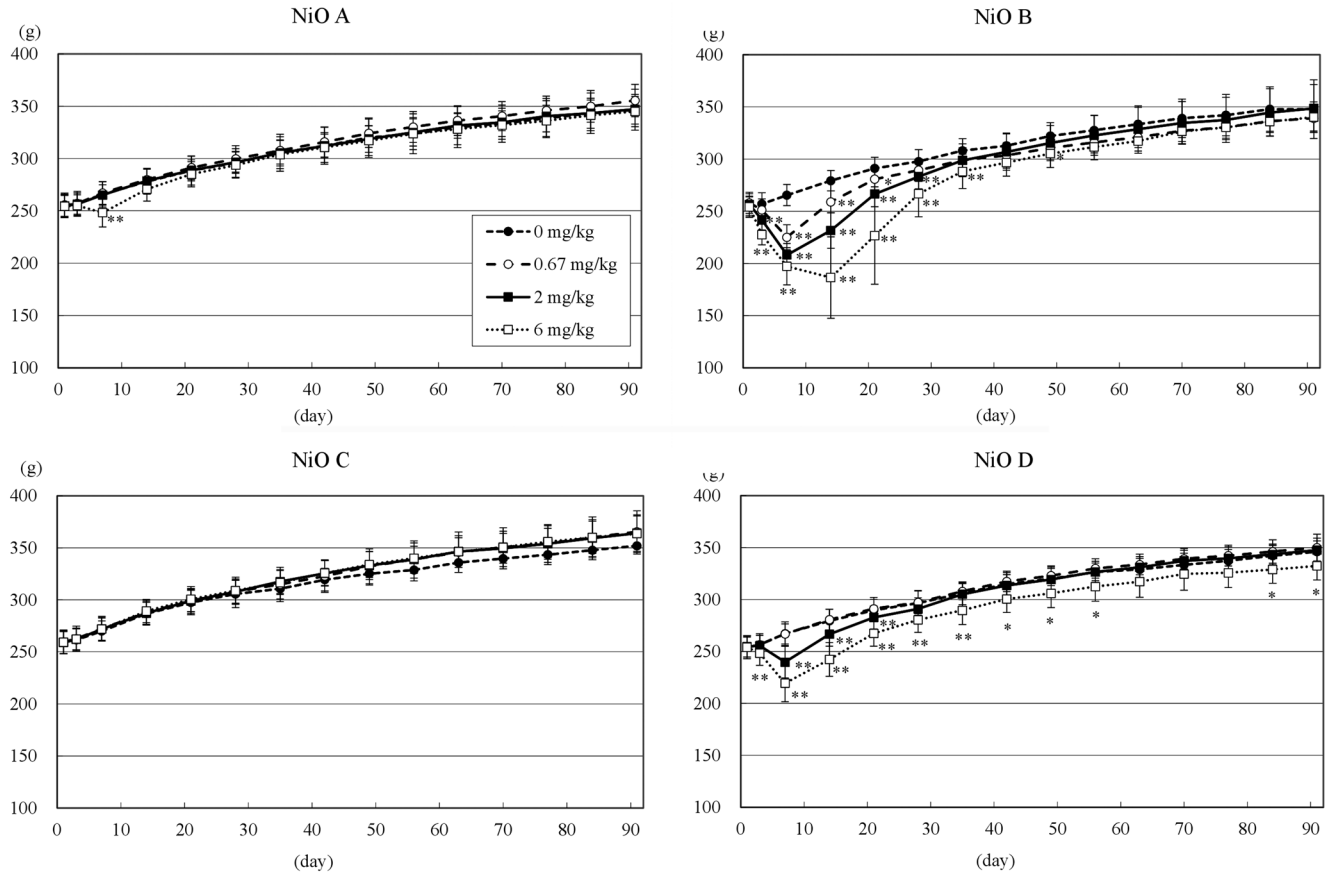

Fig. 2. Mean body weight changes in NiO-treated animals from instillation day (day 1) to terminal day (day 91). Values of all surviving animals at each time point are summarized. days 1 and 3: n=30, days 7 to 28: n=20, and days 35 to 91: n=10 in the NiO A, C, and D groups; and days 1 and 3: n=26, days 7 to 28: n=16, and days 35 to 91: n=8 in the NiO B group. Asterisks indicates significant differences compared with corresponding controls (*P<0.05; **P<0.01). Bars: standard deviation.

Table 1. Hematological Examination Results of NiO B-Treated Animals on Day 3

Items (unit)	Dose (mg/kg)	0	0.67	2	6
	Number of animals examined	5	5	5	5
Erythrocyte count (×10⁴/μL)		942.2 ± 11.4	997.2 ± 34.5*	1062.8 ± 24.3**	1110.2 ± 29.5**
Hb (g/dL)		15.76 ± 0.23	16.52 ± 0.57*	17.70 ± 0.38**	18.48 ± 0.52**
Ht (%)		46.58 ± 0.86	48.58 ± 1.52	51.58 ± 1.31**	53.46 ± 1.43**
MCV (fL)		49.44 ± 0.55	48.74 ± 0.48	48.52 ± 0.28*	48.18 ± 0.39**
MCH (pg)		16.74 ± 0.18	16.56 ± 0.05	16.66 ± 0.11	16.64 ± 0.09
MCHC (g/dL)		33.84 ± 0.21	33.98 ± 0.29	34.36 ± 0.21*	34.52 ± 0.29**
PLT (×10⁴/μL)		80.72 ± 3.40	89.86 ± 2.53*	96.14 ± 6.58**	98.32 ± 5.70**
Reticulocytes (%)		1.82 ± 0.19	2.08 ± 0.23	1.72 ± 0.16	1.36 ± 0.19**
Leukocyte number (×10²/μL)		55.00 ± 5.71	36.78 ± 7.07**	39.58 ± 0.90*	41.74 ± 3.69
Differentiation of leukocytes					
Neutrophil (%)		24.36 ± 1.65	36.74 ± 7.19*	50.48 ± 7.16**	65.94 ± 5.33**
Lymphocyte (%)		70.28 ± 1.50	57.82 ± 7.09**	43.38 ± 6.77**	28.14 ± 4.94**
Eosinophil (%)		1.44 ± 0.34	1.66 ± 0.85	1.18 ± 0.50	0.74 ± 0.09
Basophil (%)		0.76 ± 0.15	0.54 ± 0.11	0.74 ± 0.27	0.78 ± 0.22
Monocyte (%)		2.08 ± 0.13	2.16 ± 0.53	2.86 ± 0.57*	3.16 ± 0.44**
Others (%)		1.10 ± 0.21	1.06 ± 0.23	1.34 ± 0.38	1.22 ± 0.26

Values are shown as Mean ± S.D. Asterisk indicates significant difference from vehicle control (* P<0.05, ** P<0.01). Hb: hemoglobin concentration; Ht: hematocrit value; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet number.

cant decrease in the reticulocyte percentage in the 0.67 mg/kg group compared with the vehicle controls was observed on day 3. No significant changes were noted for any of the other items in this group or in the 6 or 2 mg/kg groups. On day 28, significant decreases in the eosinophil and monocyte percentages were noted in the 6 mg/kg group, and a significant percentage increase in neutrophils and decreases in lymphocytes and monocytes were noted in the 2 mg/kg group. No significant changes were noted in the 0.67 mg/kg group. On day 91, no significant changes were observed for any items in any dose group.

On day 3, the following significant changes were observed in NiO B-treated animals: increases in erythrocyte number, Hb, PLT, and neutrophil percentage, and a decrease in lymphocyte percentage in all dose groups; increases in Ht, MCHC, and monocyte percentage, and a decrease in MCV in the 6 and 2 mg/kg groups; a decrease in reticulocyte percentage in the 6 mg/kg group; and a decrease in leukocyte number in the 2 and 0.67 mg/kg groups. On day 28, a significant increase in neutrophil percentage and decrease in lymphocyte percentage was observed in the 6 mg/kg group, and increases in MCV and reticulocyte percentage were noted in the 2 mg/kg group. No significant changes were detected on day 91.

In the NiO C groups, significant changes were noted on day 3: decreases in monocyte percentage in all dose groups, decreases in the other lymphocyte percentages in the 6 and 0.67 mg/kg groups, and decreases in erythrocyte number and Hb in the 0.67 mg/kg group. On day 28, decreases in the monocyte percentage in all dose groups, a decrease in Hb in the 6 mg/kg group, and a decrease in MCH and increase in PLT in the 0.67 mg/kg group were observed. On day 91, an increase in PLT in the 6 mg/kg group and a decrease in monocyte percentage in the 2 and 0.67 mg/kg groups was detected.

In the NiO D groups, a decrease in the percentage of other lymphocytes in the 6 mg/kg group was observed on day 3. On day 28, a decrease in monocyte percentage in the 2 and 0.67 mg/kg groups, and decreases in PLT and other lymphocyte percentages in the 2 mg/kg group were noted. On day 91, leukocyte numbers decreased in all dose groups and decreased monocyte percentages in the 0.67 mg/kg group was observed.

Blood chemical examination

The results of the blood chemical examination on day 3 in the NiO B-treated groups are shown in Table 2; data for the other groups and examination times are provided in Supplementary Table 4. In the NiO A-treated animals, significant changes were detected on day 3: increases in TC, TG, BUN, IP, and Ca in the 6 mg/kg group and a decrease in Na in the 2 mg/kg group. On day 28, an increase in Cl in the 2 and 0.67 mg/kg groups, and a decrease in Glu in the 0.67 mg/kg group was noted. On day 91, an increase in GGT in the 6 and 2 mg/kg groups, a decrease in BUN in the 2 mg/kg group, and decreases in TG, Alb, and Na and increases in TB and K in the 0.67 mg/kg group were observed.

In the NiO B-treated animals, significant changes were noted as follows on day 3: an increase in TC in all dose groups, an increase in ALT and decreases in A/G ratio, IP, and Na in the 6 and 2 mg/kg groups, increases in AST, BUN, Cre, and TP, and decreases in K and Cl in the 6 mg/kg group. On day 28, a decrease in Glu and increase in IP in all dose groups, decreases in TP and Alb and an increase in Cl in the 6 and 2 mg/kg groups, an increase in ALP and decrease in Cre in the 6 mg/kg group, and an increase in BUN in the 0.67 mg/kg group were observed. On day 91, a decrease in Na and an increase in K in the 0.67 mg/kg group was noted.

In the NiO C-treated animals, significant changes were noted as follows on day 3: decreases in ALT, Cre, and Glu in the 6 mg/kg group, an increase in Cl in the 2 and 0.67 mg/kg groups, and an increase in GGT in the 0.67 mg/kg group. On day 28: a decrease in TP in the 6 mg/kg group was noted, and on day 91, an increase in Ca in the 6 mg/kg group, decreases in ALP and Alb in the 2 mg/kg group, and a decrease in Glu and increase in K in the 0.67 mg/kg group were observed.

In the NiO D-treated animals, significant changes were noted as follows on day 3: increases in TC and Ca in the 6 and 2 mg/kg groups, increases in GGT, Na, and K in the 6 mg/kg group, and decreases in the A/G ratio and Cl in the 2 mg/kg group. On day 28, decreases in TP and Alb and an increase in IP in the 6 mg/kg group, and a decrease in TC in the 0.67 mg/kg group were detected. On Day 91, a decrease in TC in the 6 and 2 mg/kg groups, a decrease in TP in the 6 mg/kg group, and an increase in IP in the 2 and 0.67 mg/kg groups were noted.

BALF examination

Results of the BALF examination are shown in Fig. 3. In NiO A-treated animals, significant increases compared with those in the vehicle controls were detected on day 3, including neutrophil numbers in all dose groups, lymphocyte numbers in the 6 and 2 mg/kg groups, and macrophage number in the 6 mg/kg group. On day 28, macrophage, neutrophil, and lymphocyte numbers in all dose groups increased, and on day 91, macrophage and lymphocyte numbers in all dose groups and neutrophil numbers in the 6 and 2 mg/kg groups increased.

In NiO B-treated animals, significant increases in macrophage, neutrophil, and lymphocyte numbers in all dose groups and eosinophil numbers in the 6 and 2 mg/kg groups were detected on day 3. On day 28, significant increases in macrophage, neutrophil, and lymphocyte numbers in all dose groups were observed, and on day 91, macrophage numbers in the 6 and 2 mg/kg groups were significantly increased.

In NiO C-treated animals, significant increases in macrophage, neutrophil, and lymphocyte numbers in the 6 mg/kg group were noted on days 3 and 28. No significant changes were noted on day 91.

In NiO D-treated animals, significant increases were detected on day 3: macrophage and neutrophil numbers in

Table 2. Blood Chemical Examination Results of NiO B-Treated Animals on Day 3

Items (unit)	Dose (mg/kg)	0	0.67	2	6
	Number of animals examined	5	5	5	5
AST (IU/L)		63.4 ± 3.4	61.4 ± 2.9	74.6 ± 11.8	76.6 ± 5.0*
ALT (IU/L)		27.2 ± 2.0	35.2 ± 7.8	41.0 ± 5.1**	40.6 ± 3.1**
ALP (IU/L)		415.6 ± 20.7	418.2 ± 27.6	391.2 ± 16.2	429.4 ± 31.0
GGT (IU/L)		0.48 ± 0.20	0.48 ± 0.16	0.84 ± 0.39	0.78 ± 0.37
TC (mg/dL)		45.8 ± 1.5	63.4 ± 5.3*	81.6 ± 3.0*	96.4 ± 13.8*
TG (mg/dL)		32.6 ± 4.8	39.2 ± 6.8	39.2 ± 4.8	40.0 ± 10.8
BUN (mg/dL)		18.04 ± 0.93	18.68 ± 1.28	20.24 ± 1.28	33.78 ± 10.42**
Cre (mg/dL)		0.212 ± 0.008	0.218 ± 0.016	0.218 ± 0.011	0.264 ± 0.055*
TP (g/dL)		5.54 ± 0.11	5.54 ± 0.17	5.54 ± 0.09	6.06 ± 0.24**
Alb (g/dL)		2.70 ± 0.00	2.64 ± 0.05	2.60 ± 0.00	2.84 ± 0.11
A/G ratio (-)		0.950 ± 0.037	0.912 ± 0.040	0.888 ± 0.027*	0.882 ± 0.011**
Glu (mg/dL)		131.2 ± 6.6	136.2 ± 14.3	135.2 ± 18.1	154.0 ± 22.9
TB (mg/dL)		0.048 ± 0.004	0.044 ± 0.005	0.044 ± 0.009	0.050 ± 0.000
IP (mg/dL)		8.06 ± 0.53	7.56 ± 0.68	7.02 ± 0.47*	6.78 ± 0.23**
Ca (mg/dL)		9.58 ± 0.15	9.60 ± 0.27	9.50 ± 0.16	9.58 ± 0.22
Na (mEq/L)		142.6 ± 0.5	140.8 ± 1.5	139.6 ± 1.8*	137.4 ± 1.7**
K (mEq/L)		3.96 ± 0.18	4.14 ± 0.15	4.30 ± 0.41	3.46 ± 0.27*
Cl (mEq/L)		109.20 ± 0.70	104.66 ± 3.40	102.68 ± 2.00	94.70 ± 4.52**

Values are shown as Mean ± S.D. Asterisk indicates significant difference from vehicle control (* $P<0.05$, ** $P<0.01$). AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: γ-glutamyltranspeptidase; TC: total cholesterol; TG: triglycerides; BUN: blood urea nitrogen; Cre: creatinine; TP: total protein; Alb: albumin; A/G: albumin to globulin ratio; Glu: glucose; TB: total bilirubin; IP: inorganic phosphorus; Ca: calcium; Na: sodium; K: potassium; CL: chlorine.

all dose groups and lymphocyte numbers in the 6 and 2 mg/kg groups; and on days 28 and 91, macrophage, neutrophil, and lymphocyte numbers in all dose groups.

In addition, considering the solubility of NiO B, NiO D, NiO A, and NiO C (i.e., 100%, 33%, 11%, and 0.7%), the three dose levels for each NiO can be transformed to proportions of dissolved NiO. Fig. 4 shows the dissolved NiO dose–response curve of the BALF total cell numbers. On day 3, regardless of the type of NiO, similar amounts of dissolved NiO induced comparable magnitudes of pulmonary inflammation, except for the insoluble NiO C. The pulmonary response of NiO B-treated animals tended to subside beginning on day 28, whereas NiO A- and NiO D-treated animals maintained increased BALF total cell numbers until day 91.

Histopathological examination

Histopathological findings in the lung are summarized in Table 3, and representative images of the lung in the 6 mg/kg groups are shown in Fig. 5. The findings in PMLNs are summarized in Table 4, and representative PMLN images from the 6 mg/kg groups on day 91 are shown in Fig. 6. Additionally, representative images of the kidney in 6 mg/kg NiO B-treated animals on day 3 are shown in Fig. 7.

In the lungs of the NiO A-treated groups, acute inflammatory responses, such as inflammatory cell infiltration of the alveolar space and the peribronchiolar and perivascular areas were noted in the 6 and 2 mg/kg groups on day 3, with particle deposition in the alveolar macrophages and alveolar space (Fig. 5A-1 black arrowhead), and type II cell hyperplasia around the area. The inflammation was still present on day 28, and was accompanied by macrophage degeneration and/or necrosis (Fig. 5A-2 arrow). On day 91, the inflammatory response had turned granulomatous and proliferation of collagen was detected by Masson's trichrome staining (Fig. 5A-3). Note that the change was not expressed as fibrosis, but as granulomatous inflammation, based on H&E stained section morphology.

In the lungs of the NiO B groups, inflammatory responses were induced, with macrophage degeneration and/or necrosis (Fig. 5B-1) and hemorrhage on day 3. Particle deposition was not evident. On day 28, degenerated and/or necrotic macrophages remained, although the inflammation had subsided (Fig. 5B-2), and no marked changes were noted on day 91 (Fig. 5B-3).

In the lungs of NiO C-treated groups, no inflammatory changes were observed, although particle deposition was detected in the alveolar macrophages and/or alveolar space at all time points (Fig. 5C-1–3).

In the lungs of NiO D groups, acute inflammation was induced on day 3, with particle deposition and type II cell hyperplasia (Fig. 5D-1 white arrowhead). The inflammatory response persisted on days 28 and 91, and fibrosis occurred as early as day 28 (Fig. 5D-2). The intensity and incidence of fibrosis increased by day 91 (Fig. 5D-3).

In PMLNs, paracortical hypertrophy was observed in the 6 and 2 mg/kg NiO A-treated groups and in all NiO D dose groups from day 3 onward, in all NiO B dose groups on day 3, and in the 6 mg/kg NiO B group on day 28 (Table 4). Particle deposition was observed in the 6 and 2 mg/kg NiO A groups and the 6 mg/kg NiO C and D groups on day 28, as well as in the NiO A and NiO D dose groups and the 6 mg/

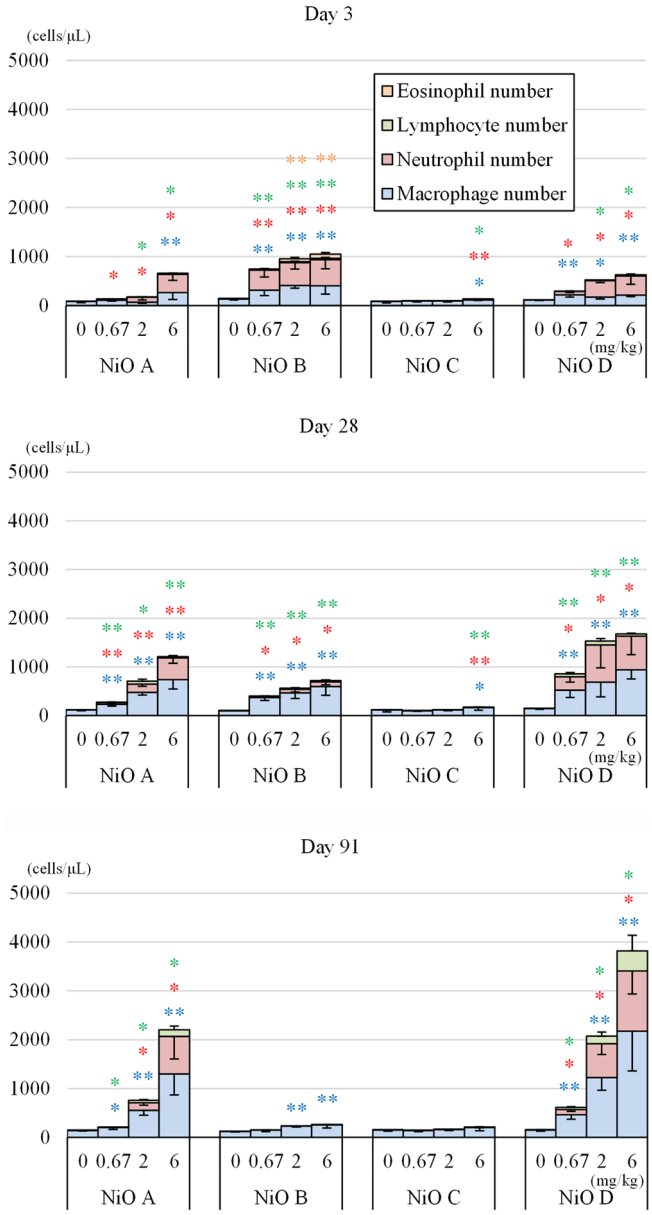

Fig. 3. Results of bronchoalveolar lavage fluid cytological examinations on days 3, 28, and 91 (n=5, except for days 28 and 91 in the NiO B group, where n=4). Colored asterisks indicate significant differences in corresponding parameters compared with each control value (*P<0.05; **P<0.01). Bars: standard deviation.

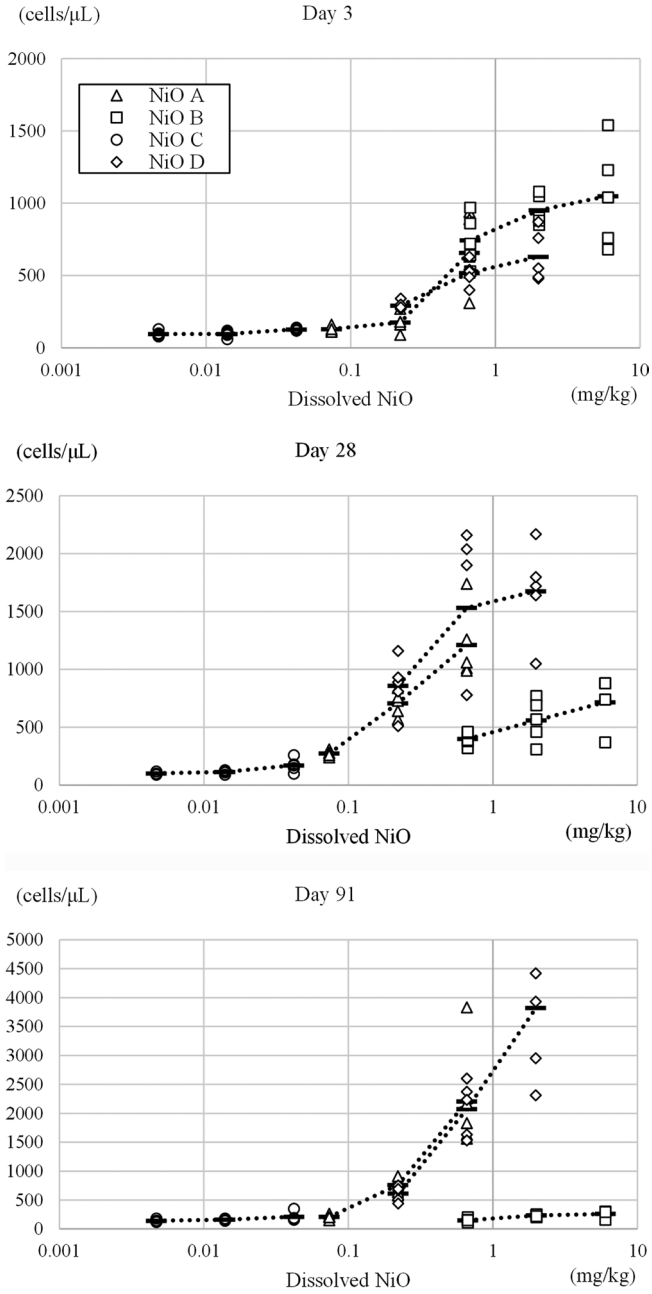

Fig. 4. Dose-response analysis of BALF total cell numbers relative to proportion of dissolved NiO. Individual values are plotted. Bars represents the average for each group and are connected by a dashed line for each NiO.

kg NiO C group on day 91 (Fig. 6A, C, and D). No inflammatory or degenerative changes were noted. In the NiO B groups, particle deposition was not observed (Fig. 6B).

Additionally, in the kidneys of 6 mg/kg NiO B-treated animals, necrosis of the proximal tubules (moderate: 2/5 animals; slight: 3/5) was observed on day 3 (Fig. 7). No abnormal changes were noted in the 2 and 0.67 mg/kg groups on day 3, or in any of the groups on days 28 and 91. No abnormal changes were observed in the NiO A, NiO C, or NiO D groups.

In the spleen of 6 mg/kg NiO B-treated animals, atrophy (slight: 5/5 animals) was observed on day 3, which is considered to be a stress-related change. No abnormal changes were detected in the 2 and 0.67 mg/kg groups on day 3, or in any of the groups on days 28 and 91. No abnormal changes were noted in the NiO A, NiO C, or NiO D groups. No treatment-related changes were noted in the liver (data not shown).

Table 3. Summary of Histopathological Examination of the Lung

Findings	Dose	NiO A			NiO B			NiO C			NiO D		
	[mg/kg]	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91
Number of animals examined	0.67	5	5	5	5	5	5	5	5	5	5	5	5
	2	5	5	5	5	5	5	5	5	5	5	5	5
	6	5	5	5	5	4	4	5	5	5	5	5	5
Particle deposition/ alveolar macrophage	0.67	−	−	−	−	−	−	±: 1	−	−	−	−	−
	2	±: 5[a]	±: 5	±: 5	−	−	−	±: 5	±: 4	±: 3	±: 3	±: 1	±: 1
	6	±: 4 +: 1	±: 4 +: 1	±: 5	−	−	−	+: 5	±: 4 +: 1	+: 5	+: 5	+: 5	+: 5
Particle deposition/ alveolar space	0.67	−	−	−	−	−	−	−	−	−	−	−	−
	2	±: 5	−	−	−	−	−	−	−	−	−	−	±: 1
	6	±: 4 +: 1	±: 4 +: 1	−	−	−	−	±: 4	−	±: 1	−	−	±: 1 +: 3
Inflammatory cell infiltration/ alveolar space	0.67	−	±: 1	−	±: 2 +: 3	−	−	−	−	−	±: 3 +: 2	±: 2	±: 1 +: 3
	2	±: 3 +: 2	±: 4 +: 1	±: 2 +: 3	+: 4 ++: 1	−	−	−	−	−	+: 5	±: 3 +: 2	+: 4 ++: 1
	6	+: 5	+: 4 ++: 1	±: 1 +: 4	++: 5	±: 1	−	−	−	−	+: 4 ++: 1	++: 5	+: 2 ++: 3
Inflammatory cell infiltration/ peribronchiolar	0.67	−	−	−	±: 3 +: 2	−	−	−	−	−	±: 2	±: 2	+: 2
	2	±: 1 +: 1	−	−	±: 1 +: 3 ++: 1	−	−	−	−	−	±: 3 +: 2	±: 3 +: 2	+: 1
	6	+: 3	−	−	+: 2 ++: 2	−	−	−	−	−	++: 5	++: 5	−
Inflammatory cell infiltration/ perivascular	0.67	−	−	−	±: 3 +: 1	−	−	−	−	−	−	−	−
	2	±: 2	±: 2	+: 3	±: 1 +: 2 ++: 1	−	−	−	−	−	±: 3 +: 1	±: 3 +: 1	−
	6	±: 2 +: 1	±: 1 +: 1	+: 3	±: 1 +: 2 ++: 1	±: 1	−	−	−	−	+: 2	++: 2	−
Degeneration and necrosis/ alveolar macrophages	0.67	−	+: 3	±: 3	−	−	−	−	−	−	−	±: 1 +: 2	±: 5
	2	−	+: 4 ++: 1	+: 5	±: 1 +: 4	±: 3	−	−	−	−	−	+: 5	+: 3 ++: 2
	6	−	+: 2 ++: 3	+: 5	+: 4	+: 4	−	−	−	−	−	+: 3 ++: 2	++: 5
Increase/ foamy macrophages	0.67	−	−	±: 2	−	±: 3	−	−	−	−	−	−	±: 1 +: 4
	2	−	−	+: 3 ++: 2	−	±: 5	−	−	−	−	−	−	+: 5
	6	−	−	++: 5	−	±: 1 +: 2	±: 2	−	−	−	−	−	+: 5
Hyperplasia/ type II cells	0.67	−	−	±: 1	−	−	−	−	−	−	±: 1 +: 1	±: 2	−
	2	−	±: 2	±: 1 +: 3	−	−	−	−	−	−	+: 2	−	1 ++: 2
	6	±: 2	±: 1	+: 4	−	−	−	−	−	−	++: 5	−	−
Inflammation/ granulomatous	0.67	−	−	−	−	−	−	−	−	−	−	−	+: 1
	2	−	±: 1	±: 1	−	−	−	−	−	−	−	−	+: 1
	6	−	−	±: 1 +: 2	+: 1	−	−	−	−	−	−	−	+: 1
Fibrosis	0.67	−	−	−	−	−	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	−	−	−	−	−	−	−	−	+: 1 ++: 2	+: 3 ++: 2

Table 3. Continued

Findings	Dose	NiO A			NiO B			NiO C			NiO D		
	[mg/kg]	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91
Hemorrhage	0.67	–	–	–	±: 1 +: 1	–	–	–	–	–	–	–	–
	2	–	–	–	+: 1	–	–	–	–	–	–	–	–
	6	–	–	–	+: 2	–	–	–	–	–	+: 1	–	–

a: Number of animals with lesions. −: normal, ±: very slight, +: slight, ++: moderate, +++: severe.

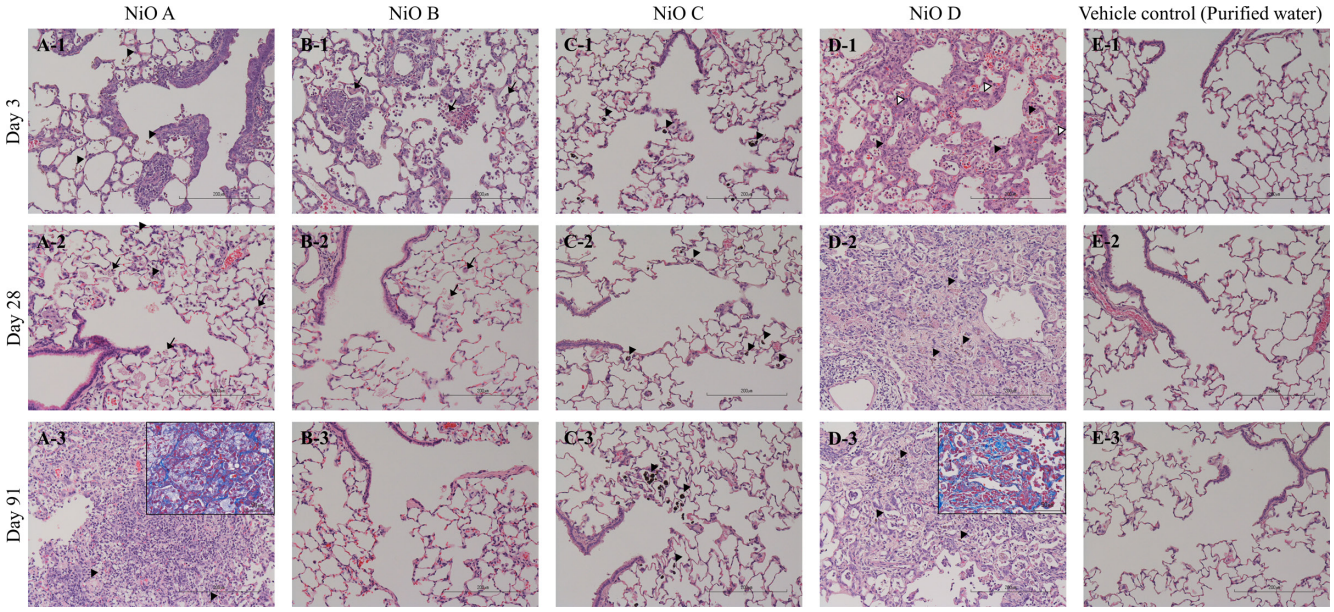

Fig. 5. Representative histopathological images of lungs from rats treated with 6 mg/kg of NiO or vehicle (purified water). NiO A induced acute inflammation on day 3 (A-1), and inflammation persisted on day 28 with degeneration/necrosis of macrophages (A-2), and on day 91 with granulomatous inflammation (A-3). NiO B induced acute inflammation with degeneration/necrosis of alveolar macrophages on day 3 (B-1); this was followed by gradual recovery by days 28 (B-2) and 91 (B-3). NiO C resulted only in particle deposition in the macrophages or alveolar space with no inflammatory response (C-1–3). NiO D induced acute inflammation on day 3 (D-1); this persisted on days 28 (D-2) and 91 (D-3), with fibrosis. Hematoxylin and eosin staining. Bars: 200 µm. Insets in A-3 and D-3: Masson's trichrome staining. Arrows: degeneration/necrosis of macrophages. Black arrowheads: particle deposition. White arrowheads: type II cell hyperplasia.

Table 4. Summary of Histopathological Examination of the Posterior Mediastinal Lymph Node

Findings	Dose	NiO A			NiO B			NiO C			NiO D		
	[mg/kg]	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91	Day 3	Day 28	Day 91
Number of animals examined	0.67	5	5	5	5	5	5	5	5	5	5	5	5
	2	5	5	5	5	5	5	5	5	5	5	5	5
	6	5	5	5	5	4	4	5	5	5	5	5	5
Particle deposition	0.67	–	–	±: 3[a]	–	–	–	–	–	–	–	–	±: 2
	2	–	±: 1 +: 1	±: 1 +: 4	–	–	–	–	–	–	–	–	±: 1 +: 4
	6	–	±: 3 +: 2	±: 2 +: 3	–	–	–	–	±: 4	+: 5	–	+: 5	+: 3 ++: 2
Paracortical hypertrophy	0.67	–	–	–	+: 5	–	–	–	–	–	±: 3	+: 1	+: 1
	2	–	±: 1 +: 2 ++: 1	±: 1	+: 5	–	–	–	–	–	±: 1 +: 4	±: 1 +: 3	+: 4
	6	±: 1 +: 1	±: 1 +: 2 ++: 1	+: 5	±: 2 +: 1 ++: 1	+: 1	–	–	–	–	+: 5	±: 2 +: 2	+: 4

a: Number of animals with lesions. −: normal, ±: very slight, +: slight, ++: moderate, +++: severe.

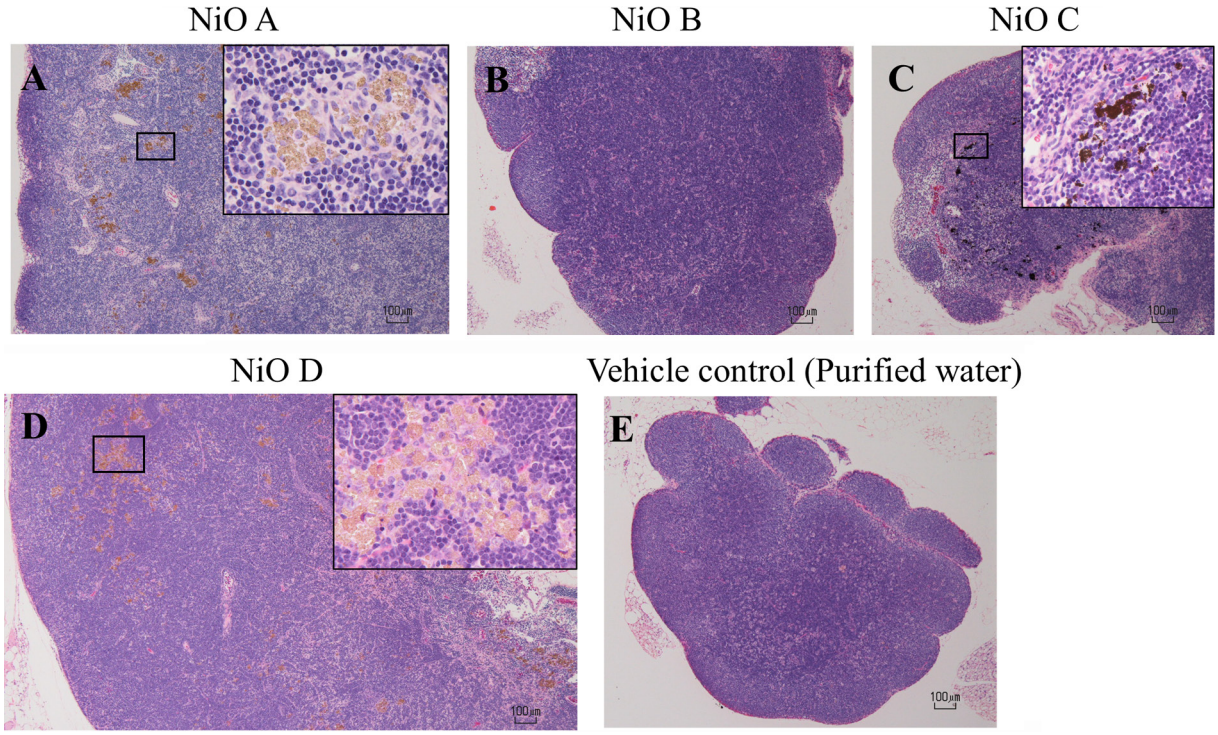

Fig. 6. Representative histopathological images on day 91 of posterior mediastinal lymph nodes from rats treated with NiO or vehicle (purified water). Particle deposition and paracortical hypertrophy were observed in the NiO A- and NiO D-treated groups. No marked changes were noted in the NiO B group (B). Particle deposition was observed in the NiO C group (C). Inset: higher magnification. Hematoxylin and eosin staining. Bars: 100 μm.

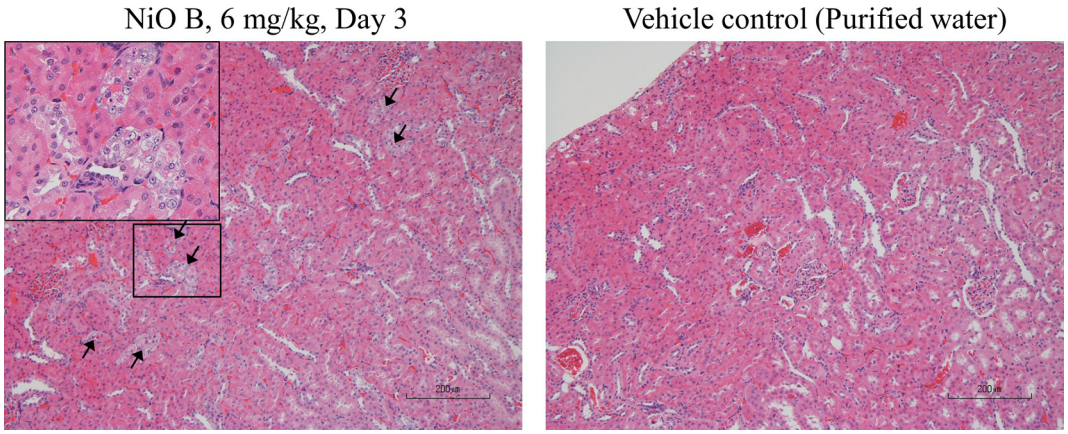

Fig. 7. Representative histopathological images of the kidney in the 6 mg/kg NiO B-treated group on day 3. Left: necrosis of proximal tubules (arrow). Inset: higher magnification. Right: vehicle control (purified water) group. Hematoxylin and eosin staining. Bars: 200 μm.

Discussion

Because nickel compounds, including NiO, are important causes of occupational disease, numerous studies have been conducted to explore the mechanisms of their toxicity. The toxicity is dictated by Ni^{2+} and, accordingly, solubility is a critical toxicity factor. The solubility of NiO is associated with particle size (specific surface area) and stoichiometry[18]. Smaller particles are much more soluble than larger ones. This is explained on the basis of surface area — larger surface area particles show high solubility, as is the case for NiO B (Supplementary Table 1 and 2). The stoichiometry of NiO reflects an unstable ratio of nickel to oxygen. NiO with a low nickel-to-oxygen ratio is black and is more soluble than NiO with a high ratio, which tends to be green[18]. Although we did not analyze the stoichiometry of our test

samples, based on its appearance, NiO C, unlike the others, was dark green and likely has a high nickel-to-oxygen ratio. Accordingly, NiO C was the most insoluble of the four materials in lysosomal fluid. Green nickel oxide has a rock-salt stoichiometric structure and is poorly able to release Ni^{2+}; it thus has low toxicity[18]. Therefore, the relatively safe profile of NiO C is attributable to its insolubility.

Among the moderately soluble NiOs, NiO D induced a greater inflammatory response and systemic toxicity than NiO A. This is consistent with the solubility rank mentioned above. Thus, the predominant toxic component likely released is Ni^{2+}. The respiratory toxicity of soluble nickel compounds has been well studied. For example, rapidly soluble nickel compounds reach the alveolar region, where they immediately dissociate into Ni^{2+}, which is rapidly excreted in the urine[21]. In contrast, moderately soluble compounds, such as NiO, are engulfed by alveolar macrophages or taken into pulmonary epithelial cells; they then dissociate into Ni^{2+} in acidic environments such as lysosomal fluid[22]. Accordingly, intracellular Ni^{2+} induces cytotoxicity and nuclear damage and consequently has carcinogenic potential[22]. As previously mentioned, NiO A, NiO B, and NiO D were highly to moderately soluble in ALF, but less soluble in the other solutions. This implies that the NiOs did not dissolve immediately at the instilled site; instead, they dissolved after they were phagocytosed by alveolar macrophages. Alveolar macrophage degeneration and/or necrosis detected in the NiO A, NiO B, and NiO D groups might have been a consequence of these intracellular toxic mechanisms.

In the present study, comprehensive data on toxicological parameters, including body weight changes, BALF examinations, and histology, indicated that the most soluble material, NiO B, induced the most severe toxicity during the early phase of the experimental period. However, the surviving animals clearly recovered. This pattern can be explained by the release of Ni^{2+} and its rapid excretion. Highly soluble nickel compounds can cause severe toxicity with high mortality rates, and it is therefore difficult to assess their tumorigenic effects[23]. An unintendedly similar situation occurred here; however, we were able to confirm that at least 23 and 13 animals in the 6 mg/kg group were still alive at more than 52 and 104 weeks after instillation, respectively, and the lower dose groups did not show carcinogenic effects in the lung (unpublished data). Although the number of animals examined was quite low, we considered this finding to be reliable, because our previous lung burden analysis indicated that little NiO B remained in the lungs, even on day 91[13]. In addition, NiO B is fibrous, but no shape-related effects, such as pleural cell alteration[20], were noted. This is because NiO B shows high solubility, which is associated with a large surface area. Regarding the other NiOs, physicochemical parameters, such as particle diameter and surface area, were also associated with solubility, so the contributions of particle size or shape were unclear.

Eosinophil infiltration of the BALF occurred only in the NiO B-treated groups and only on day 3. Pulmonary eosinophilia is induced by the instillation of soluble zinc oxide and copper oxide[24], as well as by nickel compounds, including $NiCl_2$ and NiO[25]. Eosinophilia is reportedly triggered by the release of eotaxin from ruptured cells[25], although its toxicological significance remains unclear. Other noticeable findings on day 3 in the NiO B groups were the degeneration and/or necrosis of alveolar macrophages and hemorrhage. These findings may further elucidate the pulmonary eosinophilia caused by NiO nanomaterials. In contrast, the BALF parameters of NiO A and NiO D included dose-related increases in macrophages, neutrophils, and/or lymphocytes. The BALF values reflected the intensity of pulmonary inflammation, given that BALF was collected through whole-lung lavage. When comparing the results between materials on day 3, the mean total cell number of 6 mg/kg NiO B-treated animals was the highest, and 2 and 0.67 mg/kg were the second and third, respectively (Fig. 4). These results demonstrate that in our experimental model, solubility in ALF plays a critical role in NiO-induced pulmonary inflammation. However, the factor responsible for the persistence of the pulmonary inflammation observed in NiO A- and NiO D-treated animals remains unclear. Our results reveal that this persistence cannot be explained solely by solubility.

Paracortical hypertrophy in PMLNs occurred in animals that showed pulmonary inflammation; that is, in the NiO A, NiO B, and NiO D groups. This is likely a change related to the reaction to inflammation, because with the subsidence or cessation of pulmonary inflammation in the NiO B groups, paracortical hypertrophy virtually disappeared. Particle deposition in lymph nodes, including the PMLNs, has been reported in several instillation studies[26, 27]; although, like in our study, these studies detected no degenerative changes in the lymph nodes. Additionally, NiO B, the most soluble material, was not deposited in the PMLNs, so the histological changes of PMLNs reflect excretion of the instilled materials via the lymphatic system.

In addition to the pulmonary toxicity of NiO, several secondary effects were detected in terms of a variety of toxicological parameters. In the most soluble (NiO B) groups, serious pulmonary inflammation was induced at 6 mg/kg in the early phase of the experimental period. Consequently, 20 out of 70 animals died between days 4 and 16; the dose was apparently excessive. Accordingly, blood examinations of 6 mg/kg NiO B animals on day 3 revealed hemoconcentration, high erythrocyte numbers and Hb and Ht values, as well as high BUN, TP, and Glu (although not significantly so), and electrolyte imbalance. Hemoconcentration has been well studied in food restriction models in rats[28–30]. For example, Moriyama et al. examined the effects of 2 weeks of food restriction on various toxicological parameters in Sprague-Dawley rats[30]; the erythroid parameters were similar to those in our NiO B-treated animals. These food restriction models caused moderate depression of hematopoiesis in the bone marrow, and this in turn affected related parameters, such as the number of leukocytes and reticulocytes. Leukocyte numbers were not affected in our 6 mg/kg NiO B rats, but the neutrophil percentage was increased on days 3 and 28 in this group, possibly due to pulmonary

inflammation. Additionally, in the food restriction model, low values of Glu and TP resulting from poor nutrition were reported[30]. Our animals were not fasted before blood sampling, so the (non-significant) high Glu values on day 3 in the NiO B-treated animals may have been associated with food consumption. However, taking into account the signs of anorexia in the 6 mg/kg NiO B group, these rats might have been dehydrated, as food intake reduction can also cause a reduction in water intake, leading to hemoconcentration[29, 30]. In such a situation, glucocorticoid release is stimulated by the reduced body fluid volume[31], so the Glu results may have been a secondary effect of glucocorticoid release in response to stress.

The animals treated with 6 mg/kg NiO B showed slight to moderate proximal tubular necrosis of the kidney on day 3. Similar kidney lesions have been reported in a food restriction model[30]; however, the changes were accompanied by hyaline droplets that resembled hemoglobin. The authors found a reddish hemoglobinuria and concluded that renal toxicity was induced by a hemolytic effect. In contrast, hyaline droplets were not detected in our rats on histopathology, and no abnormal changes suggestive of hemolysis were detected in the blood of NiO B-treated animals. Additionally, although the urinary system is a major route of excretion of Ni^{2+}, this excretion is reportedly not accompanied by histopathological changes[15–17, 32]. Obone et al. examined the 13-week oral toxicity of soluble nickel sulfate in male SD rats via drinking water[32]. They found Ni retention in the kidneys and an increase in relative kidney weights and high BUN values, but no histopathological changes were detected. Moreover, no histological changes were reported in the kidneys of F344 rats in studies of NiO inhalation for up to two years[16]. In the case of human death through accidental high-concentration exposure to nickel-coated respirable particles in metal arc processing, acute tubular necrosis was reported[33]. However, considering the aforementioned toxicity profiles, this seemed to have been caused by the severe systemic state of toxicity that was induced, rather than by the renal toxicity of nickel. These findings together suggest that the proximal tubular necrosis observed in the NiO B-treated animals was a secondary effect caused by the serious clinical condition of the animals, including hypovolemic shock. This may also have contributed to the high BUN and Cre values observed.

Here we showed that various NiO materials induced pulmonary toxicity upon intratracheal instillation in male F344 rats, and revealed that the intensity of inflammation was associated predominantly with the solubility of the material. NiO B, the most soluble material, induced severe inflammation but was rapidly excreted, after which inflammation subsided. NiO C, the least soluble material, had the safest profile. The moderately soluble NiO A and NiO D both induced persistent and progressive pulmonary inflammation, leading to collagen proliferation in the lungs. Further experiments are needed to clarify how the solubility of NiO products and other factors are associated with the persistence of inflammation and potentially with carcinogenic effects.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no competing interests.

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