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Twenty-Eight-Day Repeated Inhalation Toxicity Study of Aluminum Oxide Nanoparticles in Male Sprague-Dawley Rats

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Abstract

Aluminum oxide nanoparticles (Al_2O_3 NPs) are among the most widely used nanomaterials; however, relatively little information about their risk identification and assessment is available. In the present study, we aimed to investigate the potential toxicity of Al_2O_3 NPs following repeated inhalation exposure in male Sprague-Dawley rats. Rats were exposed to Al_2O_3 NPs for 28 days (5 days/week) at doses of 0, 0.2, 1, and 5 mg/m^3 using a nose-only inhalation system. During the experimental period, we evaluated the clinical signs, body weight change, hematological and serum biochemical parameters, necropsy findings, organ weight, and histopathology findings. Additionally, we analyzed the bronchoalveolar lavage fluid (BALF), including differential leukocyte counts, and aluminum contents in the major organs and blood. Aluminum contents were the highest in lung tissues and showed a dose-dependent relationship in the exposure group. Histopathology showed alveolar macrophage accumulation in the lungs of rats in the 5 mg/m^3 group during exposure and recovery. These changes tended to increase at the end of the recovery period. In the BALF analysis, total cell and neutrophil counts and lactate dehydrogenase, tumor necrosis factor-α, and interleukin-6 levels significantly increased in the 1 and 5 mg/m^3 groups during exposure. Under the present experimental conditions, we suggested that the no-observed-adverse-effect level of Al_2O_3 NPs in male rats was 1 mg/m^3, and the target organ was the lung.

***Key words*:** Inhalation toxicity, Aluminum oxide, Target organ, No-observed-adverse-effect level

INTRODUCTION

Aluminum is a silver-white, soft, non-magnetic, ductile, and relatively abundant metal, accounting for approximately 8% of the earth's crust. This metal is used in aerospace, transportation, and building industries because of its low density and ability to resist corrosion (1). Aluminum has a relatively low toxicity and is not classified according to its carcinogenicity. Additionally, the time-weighted average (TWA) of aluminum is 5 mg/m^3 by inhalation. However, aluminum production has been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC) (2). The sources of human exposure to aluminum are very diverse, including food additives, pharmaceuticals, personal care products, paints, and fuel additives, amongst others (1). In particular, occupational exposure to aluminum occurs during the refining process of primary metals and in secondary industries utilizing aluminum products. Moreover, it is a common metal component in ultrafine airborne particles in the ambient environment, and is relatively stable in the form of aluminum oxide (Al_2O_3) (3). An example of occupational exposure to Al_2O_3 involves the use of sandpaper for polishing, where inhalable Al_2O_3 dust is generated when Al_2O_3 is used as an abrasive material on sandpaper.

Nanotechnology has advanced exponentially over the past decade, with nanoscale materials being exploited in several applications and disciplines. However, nano-sized materials exhibit different properties from those of conventional materials because the size and corresponding large specific surface area can influence the physiochemical properties (4).

Al_2O_3 nanoparticles (NPs) are among the most widely

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used nanomaterials owing to the advantages provided by their thermal, chemical, and physical properties (5,6). As such, Al_2O_3 NPs have been used for the modification of polymers, functionalization of textiles, heat transfer fluids, treatment of waste water, biosensors, biofiltration, drug delivery, and antigen delivery for immunization purposes.

Owing to the increasing use of Al_2O_3 NPs, concerns about their human health risks and associated need for safety research are increasing. Li *et al.* demonstrated that Al_2O_3 NPs induced mitochondrial-dependent apoptosis and oxidative stress *in vitro* and *in vivo*. Additionally, Kwon *et al.* evaluated the cytotoxicity of Al_2O_3 NPs in rat lung epithelial cells (3,7) and demonstrated that intratracheal instillation of Al_2O_3 NPs could trigger an inflammatory response in the lungs. Generally, NPs can be inhaled more deeply than large particles, leaving sediment on the surface of the trachea, bronchi, and alveoli. The lung is thus considered the primary target organ for inhaled NPs (3). Al_2O_3 NPs have previously been investigated; however, studies focused on inhalation exposure remain lacking. In a previous study, inhaled Al_2O_3 has been associated with pulmonary fibrosis (8). There is thus an urgent need for further occupational exposure toxicity data since workers can be directly exposed to Al_2O_3 NPs through inhalation. For this reason, we conducted a 28-day repeated inhalation toxicity study to assess the health and safety of Al_2O_3 NPs exposure, particularly as it relates to occupational exposure in the workplace. To achieve this, we examined the toxicity and determined the no-observed-adverse-effect level (NOAEL) after Al_2O_3 NPs inhalation in male Sprague-Dawley rats.

MATERIALS AND METHODS

Test materials. Al_2O_3 powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). Particle size was estimated by assuming the particles to have the same spherical shape and size, as per the formula used in a previous study (9). The Brunauer-Emmett-Teller (BET) method (Micromeritics ASAP 2420, Micromeritics Inc., Norcross, GA, USA) was used to determine the BET surface area under the following conditions: test materials were degassed for 4 hr at 300°C; adsorptive analysis used dinitrogen, analysis bath temperature was 77.300 K, and the equilibration interval was 10 s. The surface area of Al_2O_3 used in the present study was 127.25 m^2/g, and the particle size was 11.94 nm.

Test animals. Seventy specific pathogen-free (SPF) Sprague-Dawley (SD) male rats (6 weeks old) were purchased from Japan SLC Inc. (Tokyo, Japan), and acclimatized for two weeks before initial exposure, including restraining tube acclimatization. Rats were housed in a room maintained at 22 ± 3°C, with a relative humidity of 50 ± 20%, ventilation of 13-18 air changes/hr, and 12-hr light/12-hr dark cycle with 150~300 Lux. Four or less rats were housed in a solid bottom polysulfone cage (235 × 380 × 175 mm) containing sterilized bedding. Animals were provided with irradiation-sterilized pellet food (18% protein rodent diet 2918C, ENVIGO RMS Inc., IN, USA), and UV-sterilized and filtered water *ad libitum.* The study protocol was approved by the Institutional Animal Care and Use Committee of the Chemicals Toxicity Research Bureau (IACUC-1703).

Study design. After a 2 week acclimatization period, 64 healthy rats (8 weeks old) were used. Four groups of SD rats were exposed to Al_2O_3 NPs for 28 days (6 hr/day, 5 days/week) at doses of 0, 0.2, 1, and 5 mg/m^3. These doses were selected based on the results of a previous repeated inhalation toxicity study of metal NPs, including neodymium oxide, cerium oxide, and lanthanum oxide (10,11).

Each dose group consisted of 16 rats, with 8 rats/group sacrificed after the 28-day exposure period. The remaining rats were maintained as the recovery groups and sacrificed after a 28-day recovery period to identify reversibility, persistence, and delayed toxic effects.

Generation, analysis, and inhalation chamber monitoring. Al_2O_3 nanopowder was suspended in distilled water at concentrations of 0.08 to 0.49% w/v and sonicated for 30 min (5-s sonication/3-s rest cycle, 19 mm probe, 40% amplitude) using a probe-type ultrasonicator (VC750, Sonics & Materials Inc., Newtown, CT, USA) on ice to avoid heat generation. Hydrodynamic diameters were measured using dynamic light scattering (Zetasizer Nano ZS 90, Malvern, UK) to check particle size and dispersity. The resulting dispersion was aerosolized through a 0.6~0.8 mm diameter orifice at an airflow of 4~8 L/min in a nose-only inhalation chamber (NITC system, HCT Co., Icheon, Korea) under constant agitation. The total airflow for each chamber was set at 20 L/min to achieve a 1 L/min flow/rat. Chamber conditions (temperature, relative humidity, and oxygen concentration) were automatically measured.

The concentration of Al_2O_3 NPs were measured using a personal air sampling pump (Air Chek XR, SKC, Somerset, NJ, USA) and glass fiber filter (GF-C, HG00025C, HYUNDAI Micro, Korea). The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were measured using a cascade impactor (Model-135 Mini MOUDI™ Impactor, MSP Co., MN, USA). Samples were collected from the middle part of the port at a flow rate of 1 L/min. During the exposure period, the mass concentrations of the aerosols in the chamber were measured at least three times daily. Aerosol particles were also sampled for

transmission electron microscopy (TEM; H-7100FA, Hitachi, Tokyo, Japan). TEM was performed at a magnification of 10,000 ×, and the particles analyzed using an energy dispersive X-ray spectrometer (EDS, EX200, Horiba, Kyoto, Japan) at an accelerating voltage of 75 kV.

Clinical symptoms and body weight. All animals were observed daily for mortality and the development of clinical symptoms, including respiratory, dermal, behavioral, nasal, and genitourinary changes before and after exposure. The type, date of occurrence, and severity of these symptoms were individually recorded. Body weight was measured using an electronic balance (QUINTIX3102, Sartorius Co., Göttingen, Lower Saxony, Germany) once/week throughout the experimental period.

Hematology and serum biochemistry. Blood samples (approximately 2 mL each) were placed into a complete blood count (CBC) bottle (vacutainer 3 mL, BD, NJ, USA), containing dipotassium ethylenediaminetetraacetic acid (EDTA-2K) as an anticoagulant, and analyzed using an automatic hematology analyzer (ADVIA 2120i, Siemens Diagnostics, Tarrytown, NY, USA). Hematology analysis included the following parameters: red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, mean platelet volume, platelet count, white blood cell count, and white blood cell differential count (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). An additional 3 mL of blood was collected and placed into a 5-mL vacutainer tube (BD Pharmingen, San Diego, CA, USA), containing a clot activator. Blood samples were maintained at 20 ± 5°C for 15~20 min to allow coagulation, and then centrifuged at 3,000 rpm (LABMASTER ABC-CB200R, HANLAB, Cheongju, Korea) for 10 min. Using a serum biochemistry analyzer (TBA-120FR, Toshiba Co., Tokyo, Japan), the following parameters were measured: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatine phosphokinase, total bilirubin, glucose, total cholesterol, triglycerides, total protein, albumin, blood urea nitrogen, creatinine, inorganic phosphorus, lactate dehydrogenase (LDH), and calcium ions.

Necropsy and organ weight. Before the scheduled necropsy, all surviving rats were fasted overnight and euthanized using a small animal inhaler with isoflurane (Ilsung Pharm. Co., Seoul, Korea) on the day of necropsy. Blood was collected from the posterior *vena cava* for hematology and serum biochemistry analyses after confirming euthanasia. The abdominal aorta and posterior *vena cava* were cut for exsanguination. Gross examination of the body surface, subcutis, and all internal organs in the head, abdominal and thoracic cavities was performed. Thereafter the kidneys, spleen, lungs, brain, and liver were removed and weighed using an electronic balance (QUINTIX313, Sartorius Co.). The relative organ weight was determined based on the ratio of absolute organ weight to fasted body weight.

Histopathology. Microscopic examination of the weighed organs was performed. All gross lesions, as defined by the study pathologist, were included in the examination. Organs and tissues (the left lung was used for lung histopathology) were fixed in 10% neutral buffered formalin solution and stained with hematoxylin and eosin. The samples were examined under a light microscope at 100X or 200X magnification.

Bronchoalveolar lavage fluid (BALF) analysis. The right lung was lavaged five times with 3 mL cold sterile saline solution by tracheal cannulation with a PE-90 tube (Clay Adams, NJ, USA). Lavaged fluids were centrifuged at 1,500 rpm for 10 min (Micro 17R, Hanil Scientific, Gimpo, Korea). Supernatants from the first lavage fluid were stored at −80°C for subsequent albumin, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and LDH assays. Precipitated cells were counted using an automatic hematology analyzer (ADVIA 2120i, Siemens Diagnostics). The remaining pellet was used for differential leukocyte count determination after centrifugation at 1,500 rpm for 10 min (Hanil Cellspin, Incheon, Korea) and Diff-Quick staining (Sysmex, Kobe, Japan). Approximately 300 leukocytes (macrophages, neutrophils, lymphocytes, and eosinophils) were counted under a light microscope at 400X magnification. LDH and albumin concentrations in the lavage fluid were measured using a biochemistry analyzer (TBA-120FR, Toshiba Co.), while TNF-α and IL-6 concentrations were measured using commercial assay kits (RTA00 and R6000B, respectively, R&D systems, MN, USA).

Oxidative stress analysis. The right lung of animals was homogenized in five volumes of 50 mM phosphate buffer solution (pH 7.0), and the resulting supernatant was used for analysis after centrifugation (Micro 17R, Hanil Science). Catalase (Cayman catalase kit 707002, Cayman Chemical, Ann Arbor, MI, USA), reduced glutathione (GSH) (Cayman glutathione assay kit 703002, Cayman Chemical), and thiobarbituric acid reactive substance (TBARS) (Cayman TBARS assay kit 10009055, Cayman Chemical) were measured in the supernatant following adjustment to a final protein concentration of 2 mg/mL.

Aluminum content measurement. Aluminum content was measured using inductively coupled plasma mass spectrometry (ICP-MS; 7500CE, Agilent Technologies,

Table 1. Aluminum analysis condition of inductively coupled plasma mass spectrometry

Parameter	Unit	Value
RF power	W	1,600
Sampling depth	mm	8
Torch-H	mm	−0.4
Carrier gas	L/min	0.7
Makeup gas	L/min	0.5
S/C temp	°C	2
He gas	mL/min	5

RF, radio frequency; He, helium.

Table 2. Characterization of Al_2O_3 nanoparticles dispersion during the exposure period

Parameter	Dose (mg/m^3)		
	0.2	1	5
Concentration (%)	0.08 ± 0.02	0.26 ± 0.06	0.49 ± 0.04
Energy (Jule/mL)	279.95 ± 10.21	271.19 ± 9.27	269.47 ± 8.14
Size (nm)	186.90 ± 8.38	191.50 ± 9.95	193.69 ± 7.00
PDI	0.29 ± 0.03	0.33 ± 0.06	0.36 ± 0.06

PDI, polydispersity index.
Values are expressed as means ± SD (n = 20).

Santa Clara, CA, USA). The right lung, liver, spleen, kidneys, whole blood, and brain tissues were digested in 5 or 10 volumes of 69% nitric acid (Merck, Whitehouse Station, NJ, USA) before analysis. ICP-MS analytical conditions are shown in Table 1.

Statistical analysis. Data are presented as the means ± standard deviation (SD). Body weight, hematology, serum biochemistry, organ weights, BALF analysis, and aluminum content were assumed to be normally distributed and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's T3 *post hoc* test. SPSS 22.0K software was used for all statistical analyses (IBM SPSS Statistics, Armonk, NY, USA). A p-value < 0.05 indicated statistical significance.

RESULTS

Monitoring of inhalation chamber and analysis of Al_2O_3. The concentration of Al_2O_3 was maintained as constant by daily adjustment of dispersion concentration and aeration flow in the inhalation exposure system throughout the exposure period. Al_2O_3 dispersion was prepared daily during the exposure period, with its associated properties shown in Table 2. The concentration of Al_2O_3 disper-

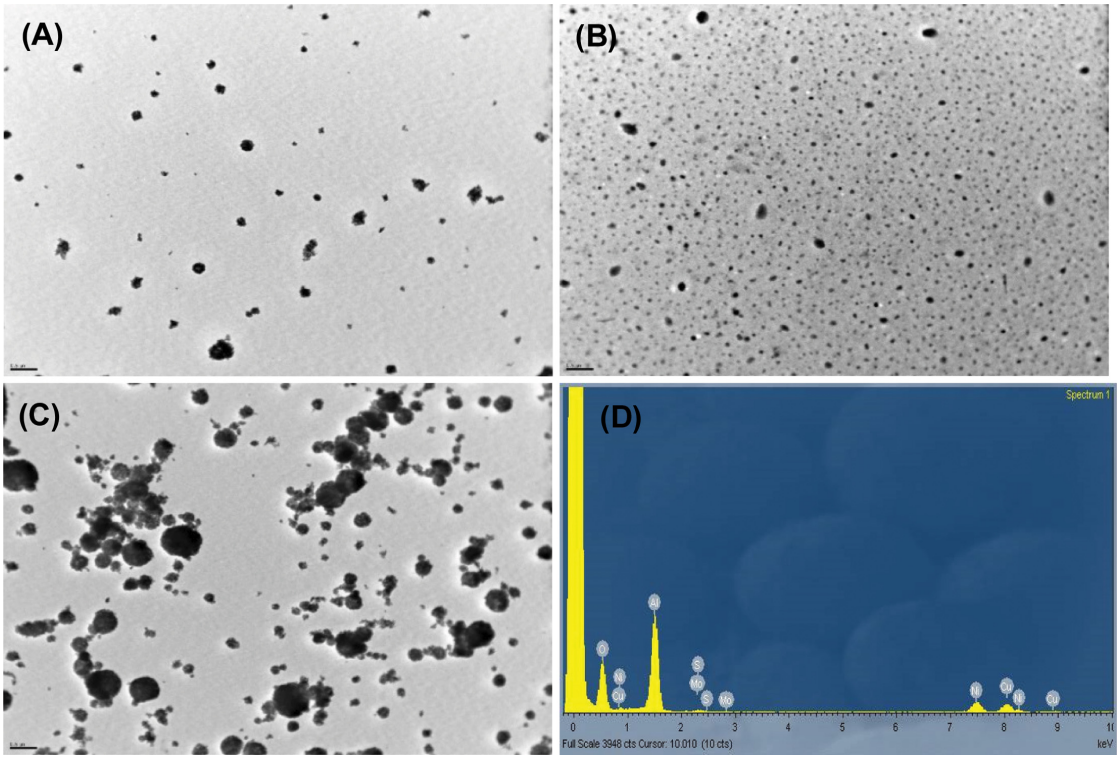

Fig. 1. Transmission electron microscopy (TEM) images (A-C, 10,000×) and energy dispersive spectroscopy (EDS) analysis (D) of Al_2O_3 nanoparticles collected from the exposure chambers. TEM images were taken from the 0.2 mg/m^3 (A), 1 mg/m^3 (B) and 5 mg/m^3 (C) exposure group. Bar = 0.5 μm.

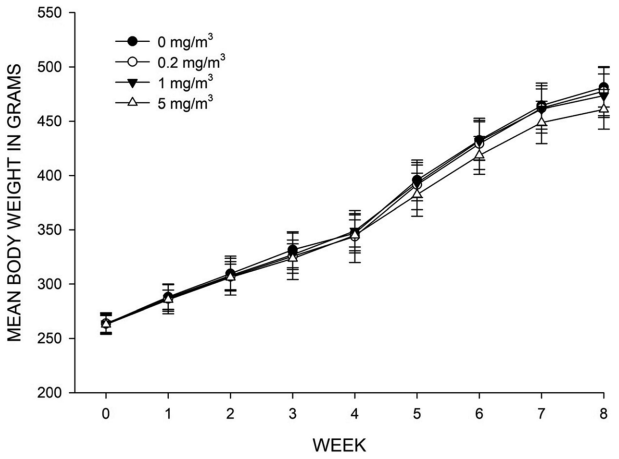

Fig. 2. Mean body weight of rats exposed to 0 mg/m^3, 0.2 mg/m^3, 1 mg/m^3 and 5 mg/m^3 Al_2O_3 nanoparticles during the experimental period. Values are expressed as means ± SD (n = 16, 0~4 week; n = 8, 5~8 week; n = 8).

sion was 0.08 ± 0.02, 0.26 ± 0.06, and 0.49 ± 0.04% for the 0.2, 1, and 5 mg/m^3 groups, respectively. After sonication, the dispersion particle sizes were 206 ± 31, 267 ± 14.8, and 273 ± 13.2 nm, while the polydispersity indices were 0.29 ± 0.03, 0.33 ± 0.06, and 0.36 ± 0.06 for the 0.2, 1, and 5 mg/m^3 groups, respectively. The applied energy to dispersions was 279.95 ± 10.21, 271.19 ± 9.27, and 269.47 ± 8.14 J/mL for the 0.2, 1, and 5 mg/m^3 groups, respectively. The actual concentrations for the 0.2, 1, and 5 mg/m^3 groups were 0.24 ± 0.13, 1.27 ± 0.43, and 4.99 ± 1.2 mg/m^3, respectively. The MMAD was 0.378, 0.48, and 0.515 μm, and the GSD 1.7, 4.61, and 3.28 for the 0.2, 1, and 5 mg/m^3 groups, respectively. TEM of particles collected from the inhalation chamber showed aciniform aggregates and agglomerates (Fig. 1).

Clinical symptoms and body weight. No treatment-related clinical symptoms were observed during the exposure and recovery periods (data not shown). Similarly, no significant differences were observed in body weight among groups (Fig. 2).

Hematology and serum biochemistry. Hematology analysis showed no significant differences among the test and control groups during exposure (Table 3). By contrast, the mean corpuscular hemoglobin concentration significantly decreased in the 1 mg/m^3 recovery group compared with the control group ($p < 0.01$). Additionally, neutrophil count significantly increased in the 1 mg/m^3 recovery group, compared with the control group ($p < 0.05$) (Table 4). Serum biochemistry analysis indicated no significant differences among the test and control groups during both exposure and recovery (Table 5, 6).

Necropsy and organ weight. There were no treatment-related gross findings in all sacrificed animals. Compared with the control group, the absolute and relative lung weights showed a significant increase by 11.5 and 10.6% respectively in the 5 mg/m^3 group during exposure ($p < 0.01$) (Table 7). However, compared with the control group, only the absolute kidney weight in the 1 mg/m^3 group was significantly increased during recov-

Table 3. Hematological values of rats after 28-day exposure of Al_2O_3 nanoparticles

Parameter	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals	8	8	8	8
RBC (10^6/μL)	8.10 ± 0.29	8.49 ± 0.49	8.42 ± 0.31	8.28 ± 0.23
Hemoglobin (g/dL)	14.49 ± 0.38	14.96 ± 0.77	14.94 ± 0.44	14.73 ± 0.32
Hematocrit (%)	41.54 ± 1.06	43.35 ± 2.21	43.05 ± 1.39	42.76 ± 1.12
MCV (fL)	51.29 ± 1.28	51.10 ± 1.11	51.18 ± 1.00	51.65 ± 0.63
MCH (pg)	17.89 ± 0.61	17.64 ± 0.54	17.75 ± 0.40	17.79 ± 0.30
MCHC (g/dL)	34.86 ± 0.31	34.50 ± 0.37	34.69 ± 0.16	34.43 ± 0.36
RDW (%)	13.21 ± 0.78	12.85 ± 0.92	13.20 ± 1.00	12.78 ± 0.77
Platelet (10^3/μL)	1032.63 ± 115.25	1045.38 ± 106.94	1035.88 ± 62.60	980.38 ± 59.89
MPV (fL)	7.36 ± 0.44	7.35 ± 0.40	7.44 ± 0.37	7.64 ± 0.29
WBC (10^3/μL)	5.08 ± 1.21	4.78 ± 0.78	5.41 ± 1.11	5.52 ± 0.64
Neutrophils (10^3/μL)	1.17 ± 0.29	1.19 ± 0.32	1.31 ± 0.20	1.51 ± 0.33
Lymphocytes (10^3/μL)	3.73 ± 1.01	3.42 ± 0.97	3.89 ± 0.91	3.81 ± 0.53
Monocytes (10^3/μL)	0.08 ± 0.04	0.07 ± 0.02	0.09 ± 0.03	0.09 ± 0.02
Eosinophils (10^3/μL)	0.06 ± 0.02	0.06 ± 0.02	0.07 ± 0.01	0.06 ± 0.01
Basophils (10^3/μL)	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00

RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; WBC, white blood cell count.
Values are expressed as means ± SD.

Table 4. Hematological values of rats after 28-day recovery of Al_2O_3 nanoparticles

Parameter	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals	8	8	7	8
RBC ($10^6/\mu L$)	8.47 ± 0.28	8.49 ± 0.44	8.58 ± 0.20	8.49 ± 0.28
Hemoglobin (g/dL)	14.61 ± 0.31	14.79 ± 0.50	14.80 ± 0.26	14.71 ± 0.52
Hematocrit (%)	43.01 ± 0.92	42.78 ± 1.48	43.09 ± 0.65	42.83 ± 1.06
MCV (fL)	50.83 ± 1.43	50.45 ± 0.96	50.25 ± 1.30	50.46 ± 1.16
MCH (pg)	17.28 ± 0.53	17.44 ± 0.39	17.26 ± 0.55	17.33 ± 0.43
MCHC (g/dL)	33.98 ± 0.23	34.55 ± 0.23**	34.34 ± 0.41	34.36 ± 0.63
RDW (%)	15.68 ± 1.28	15.69 ± 0.88	15.79 ± 1.30	15.68 ± 1.58
Platelet ($10^3/\mu L$)	1018.75 ± 67.21	1051.75 ± 127.74	994.75 ± 90.67	1004.25 ± 81.44
MPV (fL)	8.56 ± 0.57	8.15 ± 0.68	8.70 ± 0.44	8.28 ± 0.41
WBC ($10^3/\mu L$)	6.28 ± 0.96	5.22 ± 0.89	6.11 ± 0.69	6.26 ± 0.81
Neutrophils ($10^3/\mu L$)	1.62 ± 0.54	1.12 ± 0.20*	1.43 ± 0.32	1.63 ± 0.16
Lymphocytes ($10^3/\mu L$)	4.32 ± 0.62	3.86 ± 0.87	4.39 ± 0.57	4.32 ± 0.78
Monocytes ($10^3/\mu L$)	0.16 ± 0.03	0.11 ± 0.04	0.13 ± 0.04	0.16 ± 0.06
Eosinophils ($10^3/\mu L$)	0.11 ± 0.03	0.08 ± 0.02	0.09 ± 0.03	0.08 ± 0.04
Basophils ($10^3/\mu L$)	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00

RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; WBC, white blood cell count.
Values are expressed as means ± SD.
*,** Significantly different from vehicle control at $p < 0.05$ and $p < 0.01$.

Table 5. Serum biochemical values of rats after 28-day exposure of Al_2O_3 nanoparticles

Parameter	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals	8	8	8	8
AST (IU/L)	105.06 ± 23.01	110.49 ± 43.87	100.30 ± 21.83	113.68 ± 23.94
ALT (IU/L)	51.41 ± 9.90	51.24 ± 9.05	46.46 ± 7.17	51.68 ± 6.35
ALP (IU/L)	971.38 ± 223.04	1110.54 ± 26.71	979.93 ± 87.44	1010.43 ± 20.96
CPK (IU/L)	533.18 ± 271.21	631.91 ± 220.95	782.96 ± 323.25	682.56 ± 266.52
LDH (IU/L)	1789.49 ± 942.80	2088.69 ± 838.93	2317.25 ± 957.19	2385.49 ± 1021.01
TBil (mg/dL)	0.17 ± 0.04	0.17 ± 0.02	0.16 ± 0.04	0.17 ± 0.03
Glucose (mg/dL)	142.20 ± 47.45	128.56 ± 12.83	130.38 ± 16.83	129.09 ± 18.48
TCho (mg/dL)	80.19 ± 40.16	68.93 ± 8.48	74.34 ± 10.43	68.41 ± 6.04
Triglyceride (mg/dL)	70.91 ± 42.38	48.49 ± 9.25	49.80 ± 14.73	46.36 ± 15.96
TP (g/dL)	5.89 ± 0.29	5.94 ± 0.13	5.93 ± 0.26	5.89 ± 0.15
Albumin (g/dL)	4.04 ± 0.27	4.11 ± 0.10	4.11 ± 0.08	4.19 ± 0.10
BUN (mg/dL)	15.46 ± 3.20	15.83 ± 1.49	14.99 ± 2.92	15.65 ± 2.16
Creatinine (mg/dL)	0.41 ± 0.04	0.40 ± 0.03	0.40 ± 0.02	0.41 ± 0.02
IP (mg/dL)	7.84 ± 0.55	7.98 ± 0.74	7.84 ± 0.94	8.01 ± 0.42
Ca (mg/dL)	9.19 ± 0.25	9.31 ± 0.24	9.35 ± 0.28	9.38 ± 0.28

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; LDH, lactate dehydrogenase; TBil, total bilirubin; TCho, total cholesterol; TP, total protein; BUN, blood urea nitrogen; IP, inorganic phosphorus; Ca, calcium.
Values are expressed as means ± SD.

ery ($p < 0.01$) (Table 8).

Histopathology. Results of the histopathological examination are presented in Table 9 and Fig. 3. Alveolar macrophage accumulation in the lungs was observed in 4 and 8 cases of the 5 mg/m^3 group, during exposure and recovery respectively. Lesion severity increased during recovery, compared to that observed during exposure. No treatment-related lesions were observed in other organs.

Analysis of BALF. Results of BALF cellular analysis are presented in Table 10, Fig. 4 and 5. Compared with the control group, the total number of cells were significantly increased in the 1 and 5 mg/m^3 groups during exposure,

Table 6. Serum biochemical values of rats after 28-day recovery Al_2O_3 nanoparticles

Parameter	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals	8	8	7	8
AST (IU/L)	100.78 ± 43.70	91.93 ± 9.36	95.56 ± 22.58	89.59 ± 21.93
ALT (IU/L)	60.80 ± 22.10	53.38 ± 7.35	52.61 ± 10.29	50.68 ± 6.70
ALP (IU/L)	647.04 ± 111.41	642.69 ± 92.87	624.91 ± 189.49	628.45 ± 116.61
CPK (IU/L)	560.05 ± 190.30	598.21 ± 219.25	546.59 ± 206.11	539.86 ± 257.62
LDH (IU/L)	1520.29 ± 615.41	1647.25 ± 486.74	1699.54 ± 528.25	1567.85 ± 738.63
TBil (mg/dL)	0.18 ± 0.02	0.18 ± 0.02	0.19 ± 0.02	0.17 ± 0.02
Glucose (mg/dL)	178.89 ± 30.84	184.49 ± 26.45	177.95 ± 24.11	183.06 ± 22.98
TCho (mg/dL)	80.98 ± 12.20	96.05 ± 6.32	78.71 ± 9.74	79.35 ± 9.37
Triglyceride (mg/dL)	103.83 ± 24.68	107.68 ± 32.74	97.73 ± 31.74	92.55 ± 24.60
TP (g/dL)	6.64 ± 0.20	6.51 ± 0.12	6.64 ± 0.22	6.65 ± 0.24
Albumin (g/dL)	4.23 ± 0.12	4.21 ± 0.11	4.27 ± 0.13	4.27 ± 0.17
BUN (mg/dL)	16.81 ± 2.60	18.95 ± 2.79	19.10 ± 2.43	18.03 ± 2.76
Creatinine (mg/dL)	0.48 ± 0.04	0.49 ± 0.03	0.49 ± 0.04	0.48 ± 0.03
IP (mg/dL)	7.39 ± 0.66	7.26 ± 0.48	7.08 ± 0.43	7.25 ± 0.48
Ca (mg/dL)	10.51 ± 0.30	10.49 ± 0.18	10.43 ± 0.18	10.44 ± 0.25

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; LDH, lactate dehydrogenase; TBil, total bilirubin; TCho, total cholesterol; TP, total protein; BUN, blood urea nitrogen; IP, inorganic phosphorus; Ca, calcium.
Values are expressed as means ± SD.

Table 7. Absolute and relative organ weights of rats after 28-day exposure of Al_2O_3 nanoparticles

Parameter	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals	8	8	8	8
Body weight (g)	331.07 ± 19.77	326.94 ± 24.48	333.43 ± 12.98	335.11 ± 12.24
Lung	1.829 ± 0.142	1.761 ± 0.119	1.839 ± 0.093	2.040 ± 0.073**
per body weight (%)	0.615 ± 0.048	0.592 ± 0.040	0.619 ± 0.031	0.686 ± 0.024**
Liver (g)	10.381 ± 1.034	9.783 ± 0.788	10.031 ± 0.899	9.822 ± 0.705
per body weight (%)	3.492 ± 0.348	3.291 ± 0.265	3.375 ± 0.302	3.304 ± 0.237
Kidney	2.172 ± 0.267	1.997 ± 0.182	2.022 ± 0.121	2.028 ± 0.137
per body weight (%)	0.731 ± 0.090	0.672 ± 0.061	0.680 ± 0.041	0.682 ± 0.046
Spleen (g)	0.602 ± 0.034	0.579 ± 0.044	0.623 ± 0.111	0.651 ± 0.046
per body weight (%)	0.202 ± 0.011	0.195 ± 0.015	0.210 ± 0.037	0.219 ± 0.016
Brain (g)	1.976 ± 0.074	2.012 ± 0.086	1.941 ± 0.067	2.053 ± 0.170
per body weight (%)	0.665 ± 0.025	0.677 ± 0.029	0.653 ± 0.023	0.691 ± 0.057

Values are expressed as means ± SD.
**Significantly different from vehicle control at $p < 0.01$.

while polymorphonuclear leukocytes (PMNs) increased significantly in the 0.2, 1, and 5 mg/m^3 groups. In addition, lymphocytes increased significantly in the 5 mg/m^3 group during exposure. However, compared with the control group, only PMNs increased significantly in the 5 mg/m^3 group during recovery.

Results of the LDH, albumin, IL-6, and TNF-α levels in BALF supernatant are also presented in Table 10. Compared to the control group, LDH, IL-6, and TNF-α levels increased significantly in the 5 mg/m^3 group during exposure. Similarly, LDH and IL-6 levels were significantly increased in the 5 mg/m^3 group during recovery. Albumin was not detected in all groups during both exposure and recovery.

Oxidative stress analysis. There was no dose-dependent nature and/or statistical significance in catalase, GSH and TBARS detection (data not shown).

Aluminum content measurement. Aluminum content in the major organs and blood are presented in Table 11. Aluminum content in the lungs significantly increased in all treated groups during both exposure and recovery. However, it decreased during recovery by approximately

Table 8. Absolute and relative organ weights of rats after 28-day recovery Al_2O_3 nanoparticles

Parameter	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals	8	8	7	8
Body weight (g)	475.87 ± 19.51	471.83 ± 21.29	466.94 ± 20.11	455.20 ± 17.99
Lung	2.236 ± 0.134	2.256 ± 0.212	2.159 ± 0.108	2.204 ± 0.118
per body weight (%)	0.470 ± 0.019	0.478 ± 0.042	0.463 ± 0.015	0.484 ± 0.023
Liver (g)	14.759 ± 1.476	14.457 ± 0.910	13.914 ± 1.384	13.338 ± 0.916
per body weight (%)	3.098 ± 0.240	3.062 ± 0.086	2.975 ± 0.188	2.932 ± 0.196
Kidney	2.591 ± 0.125	2.529 ± 0.148	2.750 ± 0.276**	2.498 ± 0.154
per body weight (%)	0.544 ± 0.015	0.537 ± 0.042	0.589 ± 0.058	0.549 ± 0.025
Spleen (g)	0.819 ± 0.114	0.797 ± 0.099	0.770 ± 0.077	0.803 ± 0.103
per body weight (%)	0.172 ± 0.022	0.169 ± 0.018	0.165 ± 0.013	0.176 ± 0.022
Brain (g)	2.085 ± 0.069	2.079 ± 0.089	2.083 ± 0.071	2.072 ± 0.069
per body weight (%)	0.438 ± 0.017	0.441 ± 0.011	0.447 ± 0.022	0.456 ± 0.023

Values are expressed as means ± SD.
**Significantly different from vehicle control at $p < 0.01$.

Table 9. Histopathologic findings of rat lungs treated with Al_2O_3 nanoparticles

Finding (after 28-day exposure)	Grade	Dose (mg/m^3)			
		0	0.2	1	5
No. of animals		8	8	8	8
Normal appearance		8	5	8	3
Alveolar macrophage accumulation	±	0	1	0	4
Inflammatory cells infiltration	±	0	1	0	1
Granulomatous inflammation	±	0	1	0	0
Finding (after 28-day recovery)	Grade	Dose (mg/m^3)			
		0	0.2	1	5
No. of animals		8	8	8	8
Normal appearance		8	6	6	0
Alveolar macrophage accumulation	±	0	1	0	0
	+	0	0	0	8
Inflammatory cells infiltration	±	0	1	1	0
Granulomatous inflammation	±	0	0	1	0

Grade: ±, minimal; +, slight.

20, 14, and 19% in the 0.2, 1, and 5 mg/m^3 groups, respectively. Aluminum content only increased significantly in the kidneys of the 0.2 mg/m^3 group during exposure. No statistically significant changes were observed in the other groups.

DISCUSSION

As a result of their specific physiochemical properties, nanomaterial applications are increasing. Concerns regarding the health and environmental effects of these nanomaterials are consequently increasing, particularly for workers handling nanomaterials. Al_2O_3 NPs are among the most widely used nanomaterials (5); however, limited information is available regarding their risk identification and assessment, including inhalation toxicology data. In this study, we evaluated the inhalation toxicity of Al_2O_3 NPs using a nose-only inhalation system in SD rats. Exposure conditions to Al_2O_3 NPs were in accordance with the OECD test guidelines for 28-day inhalation toxicity studies (12), with exposure concentrations selected based on the results of a previous inhalation study of metal nanomaterials that included rare earth metals and Al_2O_3 (10,11). Male rats were preferentially used since men primarily work in environments prone to nanomaterial exposure.

There were no clinical symptoms or abnormal body weight changes related Al_2O_3 NP exposure during the experimental period. In addition, hematology and serum biochemistry results showed that there were no systemic effects related to the exposure to Al_2O_3 NPs. Kwon *et al.* showed similar results in SD rats, although the exposure to Al_2O_3 NPs was through intratracheal instillation (7). Although no systemic effects were observed in the present study after Al_2O_3 NP exposure, including body weight loss and clinical symptoms, the toxicological effects related to Al_2O_3 NPs exposure were mainly observed in the lungs. More specifically, this was reflected by an increase in the absolute and relative lung weights in the 5 mg/m^3 group during exposure. This was in contrast to a previous study where intratracheal instillation of Al_2O_3 NPs induced no changes in organ weight (7).

In the BALF analysis, we observed a significant increase in total cell counts, PMNs, and lymphocytes during exposure, whereas only PMNs increased during recovery. Kwon *et al.* similarly reported an increase in the counts of total cells, PMNs, and lymphocytes after intratracheal instillation of Al_2O_3 NPs at 1, 20, and 40 mg/kg (7). The levels of LDH, IL-6, and TNF-α increased significantly in the 5 mg/m^3 group during exposure; additionally, LDH and IL-6

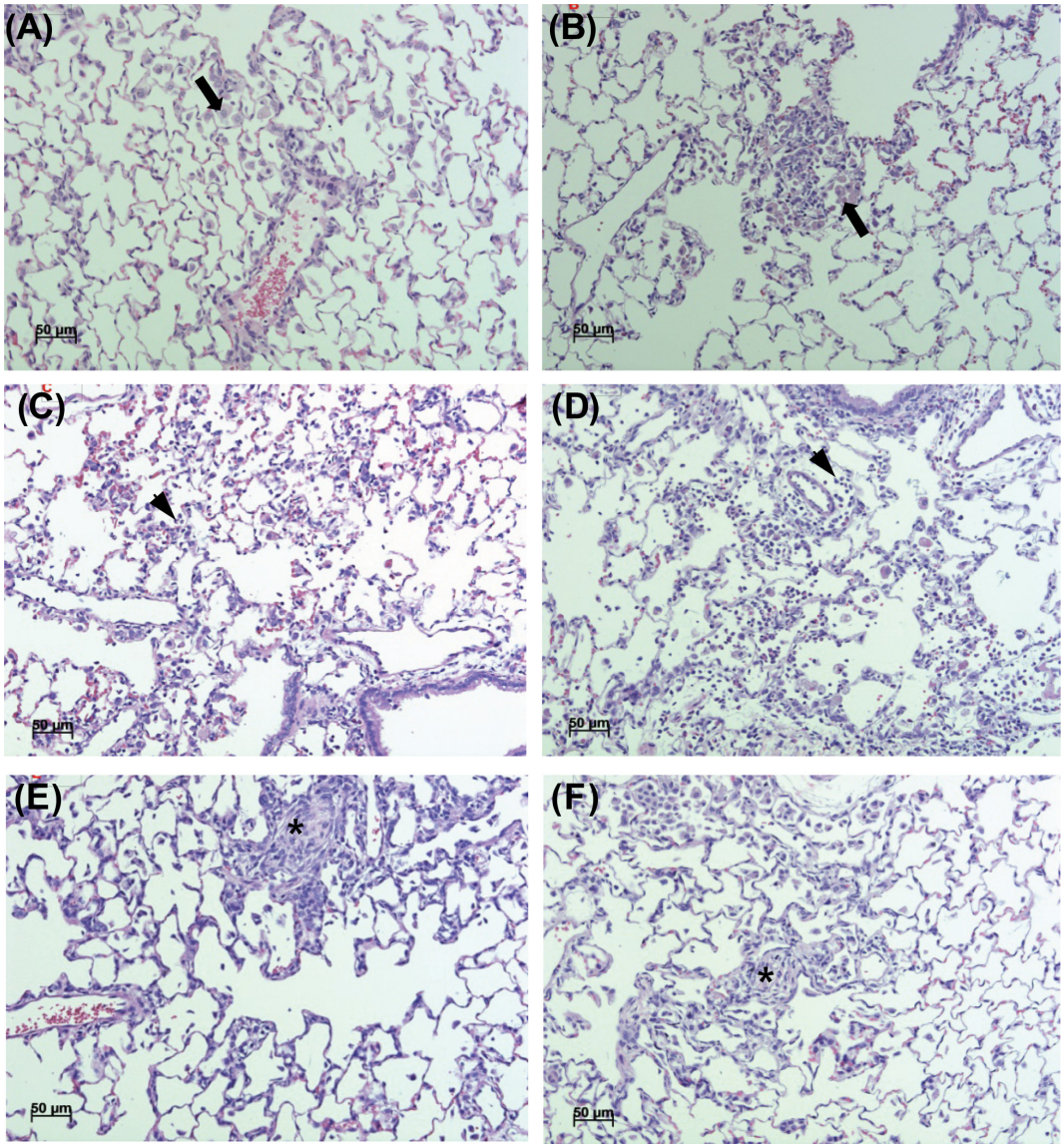

Fig. 3. Representative photographs of lung sections from the 5 mg/m^3 in exposure group (A), 5 mg/m^3 in recovery group (B), 1 mg/m^3 in exposure group (C), 1 mg/m^3 in recovery group (D), 0.2 mg/m^3 in exposure group (E) and 0.2 mg/m^3 in recovery group (F) stained with hematoxylin and eosin (200 ×). Alveolar macrophage accumulation (A, B; arrow), Inflammatory cells infiltration (C, D; arrowhead) and Granulomatous infiltration (E, F; asterisk) were shown.

concentrations increased significantly in the 5 mg/m^3 group during recovery. By contrast, Kwon *et al.* (7) showed there were no significant changes in LDH, IL-6, and TNF-α levels in BALF, although they tended to increase in the middle and/or high dose-exposed groups. These opposing results might be attributed to the difference in the amount and/or particle size of Al_2O_3 NPs that reach deep into the lungs *via* inhalation compared to tracheal instillation.

Aluminum content in the major organs were determined after the 28-day exposure and subsequent 28-day recovery periods. Most of the inhaled aluminum was deposited in the lungs after the 28-day exposure, with a significant increase in the levels that showed a dose-dependent manner. However, aluminum was not significantly deposited in other organs, except for the kidneys in the 0.2 mg/m^3 exposure group.

Zhang *et al.* (13) measured aluminum contents in the brain (cortex and midbrain) and lungs of mice following exposure to Al_2O_3 NPs for 28 consecutive days at a concentration of 0.5 mg/m^3. Aluminum levels in the cortex and lungs were significantly increased; however, levels in the midbrain did not change. Direct comparison with aluminum levels measured in our study was not possible owing to the limited results of the previous study and dif-

Table 10. Analysis of bronchoalveolar lavage fluid from rats treated with Al_2O_3 nanoparticles

Parameter (after 28-day exposure)	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals	8	8	8	8
Total cells ($10^3/\mu L$)	0.81 ± 0.26	0.91 ± 0.27	1.21 ± 0.28**	2.53 ± 0.80**
Macrophages ($10^3/\mu L$)	0.76 ± 0.24	0.82 ± 0.23	0.92 ± 0.32	0.70 ± 0.22
Neutrophils ($10^3/\mu L$)	0.02 ± 0.02	0.06 ± 0.04**	0.25 ± 0.10**	1.78 ± 0.65**
LDH (IU/L)	22.03 ± 5.41	21.94 ± 5.47	34.96 ± 22.12	81.88 ± 37.02**
Albumin (mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
IL-6 (pg/mL)	126.4 ± 11.5	121.4 ± 10.2	128.9 ± 6.87	138.9 ± 6.0*
TNF-α (pg/mL)	3.87 ± 2.71	2.92 ± 2.04	3.63 ± 1.70	9.26 ± 3.68**
Parameter (after 28-day recovery)	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals	8	8	8	8
Total cells ($10^3/\mu L$)	1.28 ± 0.40	1.19 ± 0.32	1.29 ± 0.39	1.61 ± 0.27
Macrophages ($10^3/\mu L$)	1.19 ± 0.40	1.11 ± 0.32	1.15 ± 0.38	1.06 ± 0.25
Neutrophils ($10^3/\mu L$)	0.05 ± 0.04	0.05 ± 0.04	0.11 ± 0.11	0.51 ± 0.15**
LDH (IU/L)	18.56 ± 8.19	19.94 ± 7.85	18.38 ± 9.00	38.28 ± 12.98**
Albumin (mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
IL-6 (pg/mL)	114.2 ± 10.0	110.9 ± 6.26	113.0 ± 5.94	121.4 ± 5.69*
TNF-α (pg/mL)	3.02 ± 1.66	3.38 ± 1.22	2.42 ± 1.69	4.68 ± 1.76

LDH, lactate dehydrogenase; IL-6, interleukin 6; TNF-α, tumor necrosis factor-alpha.
Values are expressed as means ± SD.
*,**Significantly different from vehicle control at $p < 0.05$ and $p < 0.01$.

Table 11. Tissue content of aluminum from rats treated with Al_2O_3 nanoparticles

Organ (after 28-day exposure)	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals examined	8	8	8	8
Lung (μg/g)	0.01 ± 0.00	0.10 ± 0.02**	0.59 ± 0.08**	2.29 ± 0.24**
Brain (ng/g)	6.63 ± 0.61	7.52 ± 1.89	7.02 ± 2.99	7.06 ± 0.94
Liver (ng/g)	4.67 ± 1.79	4.74 ± 0.92	5.09 ± 1.56	4.62 ± 1.05
Spleen (ng/g)	38.9 ± 16.4	36.0 ± 22.7	35.1 ± 4.12	39.0 ± 32.7
Kidney (ng/g)	4.22 ± 0.71	5.77 ± 0.74**	5.11 ± 1.44	3.62 ± 0.38
Whole blood (ng/g)	4.13 ± 1.64	3.31 ± 4.02	3.80 ± 3.14	2.35 ± 0.49
Organ (after 28-day recovery)	Dose (mg/m^3)			
	0	0.2	1	5
No. of animals examined	8	8	8	8
Lung (μg/g)	0.01 ± 0.00	0.08 ± 0.02**	0.51 ± 0.11**	1.85 ± 0.27**
Brain (ng/g)	7.30 ± 2.12	8.62 ± 1.43	7.26 ± 2.28	5.92 ± 0.78
Liver (ng/g)	3.93 ± 0.69	4.51 ± 0.59	3.20 ± 1.13	5.70 ± 1.38
Spleen (ng/g)	35.9 ± 17.2	46.9 ± 6.72	28.8 ± 9.19	22.3 ± 3.13
Kidney (ng/g)	6.70 ± 1.53	9.40 ± 4.94	5.11 ± 2.75	5.24 ± 2.18
Whole blood (ng/g)	3.13 ± 2.44	2.41 ± 1.35	4.50 ± 4.22	3.08 ± 1.22

Values are expressed as means ± SD.
**Significantly different from vehicle control at $p < 0.01$.

ferent animal species being used. It is postulated that NPs may enter the bloodstream *via* the lungs, and aluminum might directly enter the brain from the nasal cavity, bypassing the systemic circulation; however, convincing evidence is lacking (1,14,15). Aluminum is poorly absorbed following inhalation, and approximately 1.5~2% of inhaled aluminum is absorbed; however, absorption efficiency is dependent on the chemical form and particle size. Aluminum binds to various ligands in the blood and thereby distributes to every organ, with the highest con-

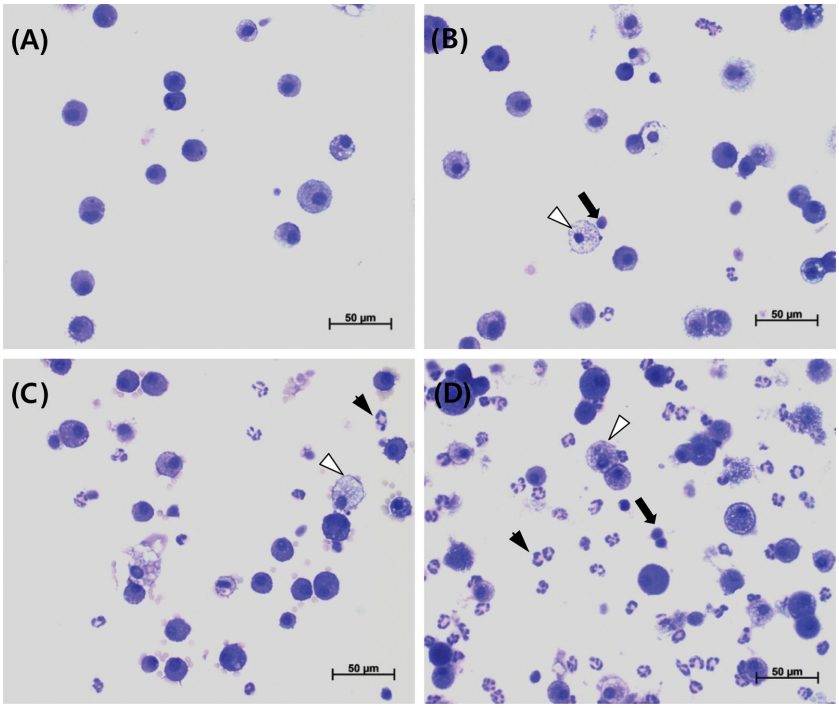

Fig. 4. Representative photographs of bronchoalveolar lavage fluids (BALF) from the control (A), 0.2 mg/m^3 (B), 1 mg/m^3 (C), and 5 mg/m^3 (D) in exposure groups stained with a Diff-Quick staining solution (400 ×). Lymphocyte infiltration (arrow), neutrophil infiltration (arrowhead), and morphological changes of alveolar macrophages (foamy; white arrowhead) were shown.

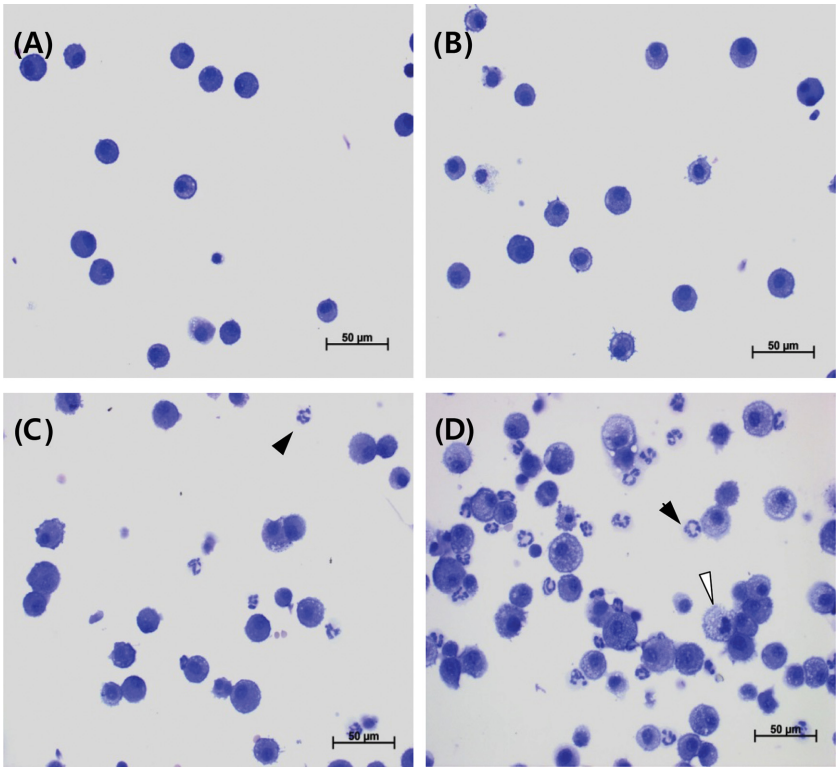

Fig. 5. Representative photographs of bronchoalveolar lavage fluids (BALF) from the control (A), 0.2 mg/m^3 (B), 1 mg/m^3 (C), and 5 mg/m^3 (D) in recovery groups stained with a Diff-Quick staining solution (400 ×). Neutrophil infiltration (arrowhead), and morphological changes of alveolar macrophages (foamy; white arrowhead) were shown.

centrations ultimately found in the bones and lungs. Absorbed aluminum is principally excreted in the urine and, to a lesser extent, in the bile (16).

Pathological findings showed marked alveolar macrophage accumulation in the lungs in the 5 mg/m^3 exposure group. Importantly, the severity of these lesions was found to increase during recovery. This is in line with the results of our previous study, using nanosized metals, including lanthanum oxide and neodymium oxide (10,11). It is believed that this change is caused by a constantly stimulated immune response to foreign metals as a result of slow test material elimination. Nanomaterials could thereby damage the immune system and cause pathological changes (17).

NP toxicity may be induced by reactive oxygen species (ROS) generation, oxidative stress, DNA damage, inflammation, and cell death (3,18). In this study, we measured GSH, catalase, and TBARS in lung tissues; however, no Al_2O_3 NP-related changes were observed.

In this study, 28-day repeated inhalation exposure to 5 mg/m^3 Al_2O_3 NPs in male rats increased lung weight and resulted in histopathological changes, including alveolar macrophage accumulation, increased total cell counts and neutrophils, as well as increased levels of IL-6, TNF-α, and LDH in BALF. After a 28-day recovery period, these changes generally recovered, except for the pathological changes. Under the present experimental conditions, the NOAEL of Al_2O_3 NPs in male rats was 1 mg/m^3. Additionally, the target organ was the lung; however, further toxicity and kinetic studies are required for a precise assessment of the health risks.

CONFLICT OF INTEREST

Declaration of interests.

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