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Subchronic Inhalation Toxicity of Silver Nanoparticles

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The subchronic inhalation toxicity of silver nanoparticles was studied in Sprague-Dawley rats. Eight-week-old rats, weighing approximately 253.2 g (males) and 162.6 g (females), were divided into four groups (10 rats in each group): fresh-air control, low dose (0.6×10^6 particle/cm^3, 49 μg/m^3), middle dose (1.4×10^6 particle/cm^3, 133 μg/m^3), and high dose (3.0×10^6 particle/cm^3, 515 μg/m^3). The animals were exposed to silver nanoparticles (average diameter 18–19 nm) for 6 h/day, 5 days/week, for 13 weeks in a whole-body inhalation chamber. In addition to mortality and clinical observations, body weight, food consumption, and pulmonary function tests were recorded weekly. At the end of the study, the rats were subjected to a full necropsy, blood samples were collected for hematology and clinical chemistry tests, and the organ weights were measured. Bile-duct hyperplasia in the liver increased dose dependently in both the male and female rats. Histopathological examinations indicated dose-dependent increases in lesions related to silver nanoparticle exposure, including mixed inflammatory cell infiltrate, chronic alveolar inflammation, and small granulomatous lesions. Target organs for silver nanoparticles were considered to be the lungs and liver in the male and female rats. No observable adverse effect level of 100 μg/m^3 is suggested from the experiments.

Key Words: **lung; nanoparticles.**

The antimicrobial activity of silver nanoparticles has resulted in their widespread use in consumer products, such as disinfectants, deodorants, antimicrobial sprays and powders, bedding, washers, water purification, toothpaste, shampoo and rinse, nipples and nursing bottles, fabrics, deodorants, filters, kitchen utensils, toys, and humidifiers. Among 580 consumer nanotechnology-based products, the most common material mentioned in the product descriptions is silver-based nanoparticles (Woodrow Wilson International Center for Scholars, 2007). In a previously reported 28-day inhalation toxicity study of silver nanoparticles in Sprague-Dawley rats, we found no significant changes in the hematology and blood biochemical values for both male and female rats, and no distinct histopathology findings, indicating that exposure to silver nanoparticles at a concentration near to 1.32×10^6 particles/cm^3, 61 μg/m^3 for 28 days did not have any significant health effects (Ji *et al.*, 2007b). Based on this study, a subchronic 90-day inhalation toxicity study was designed to identify possible adverse effects not detected in the 28-day study and to identify a lowest observable adverse effect level (LOAEL).

The subchronic 90-day inhalation toxicity study of silver nanoparticles was conducted under test guideline 413 from the Organization for Economic Cooperation and Development (OECD) (1995) and Good Laboratory Practices. Test animals showed a decrease in the tidal volume and minute volume and other inflammatory responses after 90 days of exposure to silver nanoparticles. Thus, prolonged silver nanoparticle inhalation exposure would seem to induce lung function changes, along with inflammation, at much lower mass dose concentrations when compared with submicrometer particles (Sung *et al.*, 2008). This report presents a more extensive analysis of new and published data from our laboratory.

MATERIALS AND METHODS

Generation of silver nanoparticles. Silver nanoparticles were generated as described in previous reports (Ji *et al.*, 2007a,b; Jung *et al.*, 2006), and the rats exposed to the silver nanoparticles in a whole-body-type exposure chamber (1.3 m^3, Dusturbo, Seoul, South Korea). The generation consisted of a small ceramic heater connected to an alternating current power supply and housed within a quartz tube case. The heater dimensions were $50 \times 5 \times 1.5$ mm^3, and a surface temperature of about 1500°C within a local heating area of 5×10 mm^2 could be achieved within about 10 s (Jung *et al.*, 2006). For long-term testing, the source material (about 160 mg) was positioned at the highest temperature point. The quartz tube case was 70 mm in diameter and 140 mm long. Clean (dry and filtered) air was used as the carrier gas, and the gas flow maintained at 30.0 l/min (Re = 572, laminar flow regime) using a mass flow controller (MFC, AERA, FC-7810CD-4V, Japan; Ji *et al.*, 2007a,b). This generator has been shown to generate nanoparticles from 2 to 65 nm in diameter which do not agglomerate in air. X-ray diffraction analysis using an X-ray diffractometer utilizing CuK_2 radiation showed that particles generated are metallic silver, not silver oxides (Jung *et al.*, 2006).

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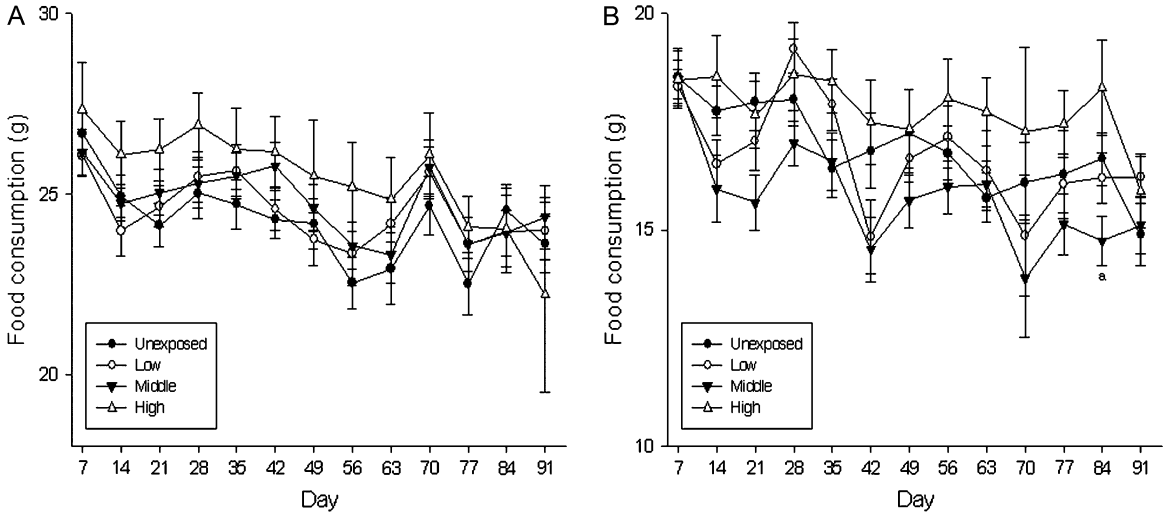

FIG. 1. Food consumption of rats exposed to silver nanoparticles. (A) Males, (B) females (a: $p < 0.05$, middle vs. high group).

In the current study, the system produced different concentrations of nanoparticles (high, middle, and low) in three separate chambers. For the high-concentration chamber, the nanoparticle generator was operated at 47 l/min and mixed with 200 l/min of clean ambient air. A portion of the high nanoparticle concentration was then diverted to the middle-concentration chamber using the MFC for the first dilution (6.76 ± 0.16 liter per minute [lpm], mean ± SE), and a portion of the middle nanoparticle concentration then diverted to the low-concentration chamber using the second MFC (5.42 ± 0.18 lpm).

Monitoring of inhalation chamber and analysis of silver nanoparticles. In each chamber, the nanoparticle distribution with respect to size was measured directly in real-time using a differential mobility analyzing system (DMAS); combining a differential mobility analyzer (Short type-DMA, 4220, HCT Co., Ltd, Korea; range 5–150 nm) and condensation particle counter (CPC, 4312, HCT Co., Ltd, 0–10^8/cm^3 detection range). Nanoparticles were measured using sheath air at 5 l/min and polydispersed aerosol air at 1 l/min for DMA and CPC, respectively. The particle concentration in the fresh-air control chamber was measured using a particle sensor (4123, HCT Co., Ltd) that consisted of two channel; 300–1000 nm and over 1000 nm.

Transmission electron microscopy. The filters used to sample the fume particles were coated with carbon, mounted on an electron microscope grid (200 mesh, Veco, Eerbeek, the Netherlands), and visualized under a transmission electron microscope (TEM, Hitachi 7100, Japan). The diameters of 800 randomly selected particles were measured at a magnification of 100,000, and the silver particles analyzed using an energy-dispersive x-ray analyzer (EDX-200, Horiba, Japan) at an accelerating voltage of 75 kV.

Animals and conditions. Six-week-old male and female, specific-pathogen-free Sprague-Dawley rats (Slc:SD) (originally derived from the Charles River SD in 1968) were purchased from SLC (Tokyo, Japan) and acclimated for 2 weeks before starting the experiments. During the acclimation and experimental periods, the rats were housed in polycarbonate cages (five rats

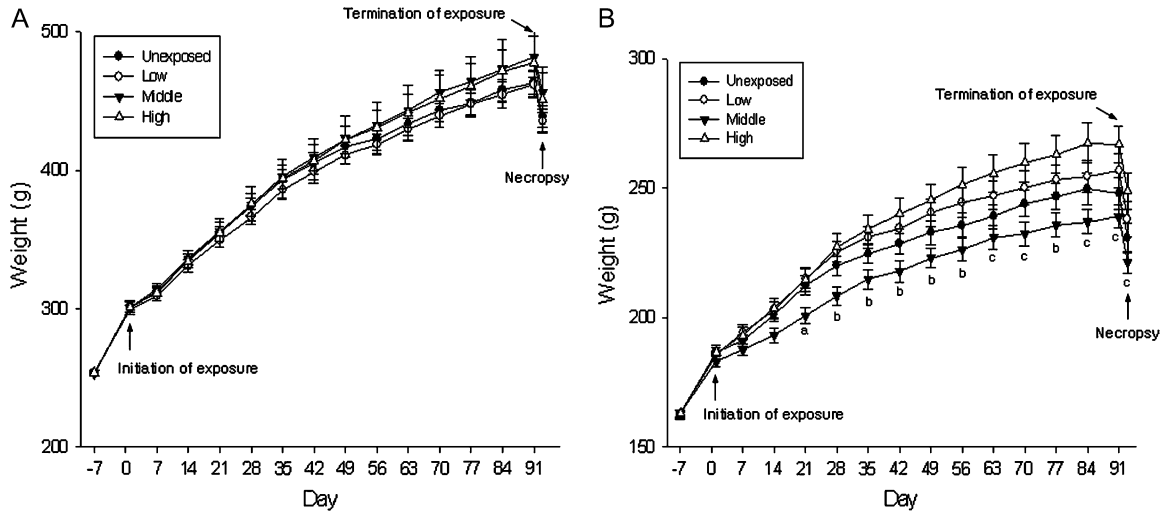

FIG. 2. Body weight changes for rats exposed to silver nanoparticles. (A) Males, (B) females (a: $p < 0.05$, middle vs. other groups, b: $p < 0.05$, middle vs. low and high groups, c: $p < 0.05$, middle vs. high group).

TABLE 1
Relative Organ Weights of Male Rats (Mean ± SE)

Group	Control, $N = 10$	Low, $N = 10$	Middle, $N = 10$	High, $N = 9$
Body weights[a]	437.05 ± 7.79	435.62 ± 8.86	456.22 ± 14.28	451.44 ± 23.55
Testis (left)[b]	0.40 ± 0.01	0.41 ± 0.02	0.39 ± 0.01	0.42 ± 0.02
Testis (right)[b]	0.41 ± 0.01	0.40 ± 0.01	0.39 ± 0.01	0.42 ± 0.02
Kidney (left)[b]	0.26 ± 0.00	0.26 ± 0.01	0.26 ± 0.01	0.26 ± 0.00
Kidney (right)[b]	0.27 ± 0.00	0.27 ± 0.01	0.26 ± 0.01	0.27 ± 0.01
Spleen[b]	0.17 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
Liver[b]	2.54 ± 0.04	2.51 ± 0.03	2.48 ± 0.03	2.50 ± 0.05
Adrenal gland (left)[b]	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Adrenal gland (right)[b]	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Heart[b]	0.27 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.27 ± 0.01
Thymus[b]	0.08 ± 0.01	0.09 ± 0.01	0.07 ± 0.00	0.09 ± 0.01
Lung (left)[b]	0.11 ± 0.00 (6)	0.11 ± 0.00 (6)	0.11 ± 0.00 (6)	0.11 ± 0.00 (5)
Lung (right)[b]	0.21 ± 0.01 (6)	0.22 ± 0.01 (6)	0.21 ± 0.00 (6)	0.22 ± 0.01 (5)
Brain[b]	0.48 ± 0.01	0.49 ± 0.01	0.46 ± 0.01	0.46 ± 0.02
Olfactory bulb[b]	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00

[a]Unit: g.
[b]Unit: % body weights. The lung weights of the four animals used for the bronchoalveolar lavage were not included. ( ): number of organs.

per cage) in a room with controlled temperature (23 ± 2?C) and humidity (55 ± 7%) with a 12-h light/dark cycle. The rats were fed a rodent diet (Harlan Teklab, Plaster International Co., Seoul, South Korea) and filtered water *ad libitum*. The 8-week-old rats, weighing approximately 253 g for the males and 162 g for the females, were then divided into four groups (10 rats in each group/sex): fresh-air control, low-dose group (target dose, 0.6×10^6 particles/cm^3, 1.0×10^9 nm^2/cm^3 surface area), middle-dose group (target dose, 1.4×10^6 particles/cm^3, 2.5×10^9 nm^2/cm^3 surface area), and high-dose group (target dose, 3.0×10^6 particles/cm^3, 5.0×10^9 nm^2/cm^3 surface area). The animals were exposed to silver nanoparticles for 6 h/day, 5 days/week, for 13 weeks. The animals were housed in individual wire cages during the exposure period, and examined daily on weekdays for any evidence of exposure-related effects, including respiratory, dermal, behavioral, nasal, or genitourinary changes suggestive of irritation. The animals were not provided food during the 6-h exposure period. The body weights were evaluated at the time of purchase, at the time of grouping, once a week during the inhalation exposure, and before necropsy. The experiment was approved by the KEMTI Institutional Animal Care and Use Committee.

Biochemistry and hematology. At the conclusion of the 13-week experiment, the rats were 21 weeks old. Before necropsy, food was withheld for 24 h and the rats anesthetized with pentobarbital. Blood was then drawn

TABLE 2
Relative Organ Weights of Female Rats (Mean ± SE)

Group	Control, $N = 10$	Low, $N = 10$	Middle, $N = 10$	High, $N = 9$
Body weights[a]	230.60 ± 5.75	237.95 ± 6.73	221.13 ± 4.32	248.70 ± 7.15
Ovary (left)[b]	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Ovary (right)[b]	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Kidney (left)[b]	0.30 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.29 ± 0.01
Kidney (right)[b]	0.31 ± 0.01	0.29 ± 0.01	0.31 ± 0.01	0.30 ± 0.01
Spleen[b]	0.22 ± 0.03	0.18 ± 0.01	0.20 ± 0.01	0.18 ± 0.01
Liver[b]	2.64 ± 0.10	2.43 ± 0.04	2.52 ± 0.03	2.59 ± 0.07
Adrenal gland (left)[b]	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Adrenal gland (right)[b]	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Heart[b]	0.34 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
Thymus[b]	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
Lung (left)[b]	0.16 ± 0.00 (6)	0.15 ± 0.01 (6)	0.16 ± 0.01 (6)	0.16 ± 0.01
Lung (right)[b]	0.29 ± 0.01 (6)	0.29 ± 0.01 (6)	0.30 ± 0.02 (6)	0.30 ± 0.01
Brain[b]	0.84 ± 0.03	0.82 ± 0.02	0.87 ± 0.02	0.79 ± 0.02
Olfactory bulb[b]	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.01 (10)	0.04 ± 0.00 (10)

[a]Unit: g.
[b]Unit: % body weights. The lung weights of the four animals used for the bronchoalveolar lavage were not included. ( ): number of organs.

TABLE 3
Hematological Values for Male Rats (Mean ± SE)

Parameter	Control, $N = 10$	Low, $N = 10$	Middle, $N = 10$	High, $N = 9$
WBC[1]	6.69 ± 0.66	6.52 ± 0.36	8.51 ± 2.65	6.31 ± 0.37
NE[2]	1.48 ± 0.42	1.15 ± 0.17	2.22 ± 1.16	1.23 ± 0.27
LY[3]	4.86 ± 0.29	5.02 ± 0.24	5.58 ± 1.09	4.78 ± 0.38
MO[4]	0.26 ± 0.05	0.23 ± 0.04	0.30 ± 0.09	0.23 ± 0.05
EO[5]	0.07 ± 0.03	0.09 ± 0.03	0.27 ± 0.22	0.05 ± 0.02
BA[6]	0.01 ± 0.01	0.03 ± 0.12	0.15 ± 0.14	0.01 ± 0.001
NE[7]	19.79 ± 3.69	17.16 ± 1.97	20.12 ± 4.24	19.33 ± 3.94
LY[8]	74.97 ± 3.36	77.52 ± 1.87	74.07 ± 4.45	75.83 ± 3.61
MO[9]	4.21 ± 0.92	3.68 ± 0.73	3.80 ± 0.78	3.89 ± 0.87
EO[10]	0.88 ± 0.27	1.20 ± 0.36	1.41 ± 0.67	0.81 ± 0.24
BA[11]	0.15 ± 0.06	0.44 ± 0.17	0.60 ± 0.42	0.15 ± 0.07
RBC[12]	8.87 ± 0.09	8.98 ± 0.20	8.95 ± 0.16	9.06 ± 0.09
Hb[13]	15.55 ± 0.24	15.39 ± 0.28	15.47 ± 0.23	15.48 ± 0.23
HCT[14]	36.72 ± 0.55	37.23 ± 0.70	37.33 ± 0.56	37.83 ± 0.59
MCV[15]	41.39 ± 0.46	41.54 ± 0.53	41.76 ± 0.60	41.78 ± 0.56
MCH[16]	17.54 ± 0.21	17.18 ± 0.18	17.32 ± 0.22	17.09 ± 0.25
MCHC[17]	42.38 ± 0.32	41.38 ± 0.54	41.44 ± 0.23	40.93 ± 0.43
RDW[18]	18.81 ± 0.25	18.84 ± 0.23	18.64 ± 0.34	18.50 ± 0.33
PLT[19]	780.70 ± 42.85	976.9 ± 72.45	801.40 ± 63.52	796.11 ± 60.65
MPV[20]	6.59 ± 0.22	6.75 ± 0.11	6.44 ± 0.11	6.41 ± 0.15

Note. Parameters: 1, white blood cells (k/μl); 2, neutrophils (k/μl); 3, lymphocytes (k/μl); 4, monocytes (k/μl); 5, eosinophils (k/μl); 6, basophils (k/μl); 7, percent of neutrophils (%); 8, percent of lymphocytes (%); 9, percent of monocytes (%); 10, percent of eosinophils (%); 11, percent of basophils (%); 12, red blood cells (M/μl); 13, hemoglobin (g/dl); 14, hematocrit (%); 15, mean corpuscular volume (fl); 16, mean corpuscular hemoglobin (pg); 17, mean corpuscular hemoglobin concentration (g/dl); 18, red-cell distribution width (%); 19, platelets (k/μl); 20, mean platelet volume (fl).

TABLE 4
Hematological Values for Female Rats (Mean ± SE)

Parameter	Control, $N = 10$	Low, $N = 10$	Middle, $N = 10$	High, $N = 9$
WBC[1]	4.22 ± 0.28	4.23 ± 0.36	3.74 ± 0.44	4.13 ± 0.47
NE[2]	0.92 ± 0.13	0.70 ± 0.11	0.58 ± 0.11	0.85 ± 0.14
LY[3]	3.10 ± 0.19	3.36 ± 0.26	3.04 ± 0.37	3.06 ± 0.41
MO[4]	0.19 ± 0.10	0.15 ± 0.03	0.11 ± 0.02	0.21 ± 0.06
EO[5]	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
BA[6]	0.00 ± 0.00	0.009 ± 0.006	0.00 ± 0.00	0.00 ± 0.000
NE[7]	21.43 ± 2.39	15.60 ± 1.51	15.53 ± 2.29	21.59 ± 3.22
LY[8]	74.05 ± 3.00	79.79 ± 0.97	81.09 ± 4.45	73.02 ± 2.80
MO[9]	4.05 ± 1.75	3.89 ± 0.82	3.03 ± 0.52	5.09 ± 1.37
EO[10]	0.88 ± 0.14	0.48 ± 0.22	0.32 ± 0.09	0.26 ± 0.10
BA[11]	0.10 ± 0.07	0.24 ± 0.16	0.050 ± 0.03	0.04 ± 0.02
RBC[12]	8.07 ± 0.159	8.22 ± 0.09	8.09 ± 0.12	8.07 ± 0.11
Hb[13]	14.56 ± 0.41	15.39 ± 0.16	15.22 ± 0.20	14.80 ± 0.22
HCT[14]	35.65 ± 1.00	37.07 ± 1.13	36.40 ± 1.12	35.14 ± 0.889
MCV[15]	44.23 ± 1.14	45.07 ± 1.12	45.03 ± 1.43	43.57 ± 1.22
MCH[16]	18.04 ± 0.34	18.73 ± 0.16	18.81 ± 0.17	18.35 ± 0.27
MCHC[17]	40.95 ± 1.14	41.77 ± 1.01	42.11 ± 1.18	42.31 ± 1.07
RDW[18]	16.85 ± 0.37	16.61 ± 0.16	16.56 ± 0.13	16.74 ± 0.12
PLT[19]	712.30 ± 74.13	683.90 ± 30.49	728.80 ± 17.61	768.70 ± 34.57
MPV[20]	6.28 ± 0.17	6.13 ± 0.12	6.07 ± 0.07	6.141 ± 0.13

Note. Parameters: 1, white blood cells (k/μl); 2, neutrophils (k/μl); 3, lymphocytes (k/μl); 4, monocytes (k/μl); 5, eosinophils (k/μl); 6, basophils (k/μl); 7, percent of neutrophils (%); 8, percent of lymphocytes (%); 9, percent of monocytes (%); 10, percent of eosinophils (%); 11, percent of basophils (%); 12, red blood cells (M/μl); 13, hemoglobin (g/dl); 14, hematocrit (%); 15, mean corpuscular volume (fl); 16, mean corpuscular hemoglobin (pg); 17, mean corpuscular hemoglobin concentration (g/dl); 18, red-cell distribution width (%); 19, platelets (k/μl); 20, mean platelet volume (fl).

from the abdominal aorta, collected in heparinized vacutainers, and analyzed for ALB (albumin), ALP (alkaline phosphatase), Ca (calcium), CHO (cholesterol), CRE (creatinine), gamma-GT (gamma-glutamyl transpeptidase), GLU (glucose), GOT (glutamic oxalacetic transaminase), GPT (glutamic pyruvic transaminase), IP (inorganic phosphorus), LDH (lactate dehydrogenase), Mg (magnesium), TP (total protein), UA (uric acid), BUN (blood urea nitrogen), TBIL (total bilirubin), CK (creatine phosphokinase), Na (sodium), K(potassium), Cl (chloride), TG (triglyceride), and A/G (ratio of albumin to globulin) using a biochemical blood analyzer (Hitachi 7180, Hitachi, Japan). The blood was also analyzed for the WBC (white blood cell count), RBC (red blood cell count), Hb (hemoglobin concentration), HTC (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RDW (red-cell distribution width), PLT (platelet count), MPV (mean platelet volume), NE# (number of neutrophils), NE% (percent of neutrophils), LY# (number of lymphocytes), LY% (percent of lymphocytes), MO# (number of monocytes), MO% (percent of monocytes), EO# (number of eosinophils), EO% (percent of eosinophils), BA# (number of basophils), and BA% (percent of basophils) using a blood cell counter (Hemavet 0950, CDC Technology, Irvine, CA).

Organ weights and histopathology. After collecting the blood, the rats were sacrificed by cervical dislocation, and the adrenal glands, bladder, testes, ovaries, uterus, epididymis, seminal vesicle, heart, thymus, thyroid gland, trachea, esophagus, tongue, prostate, lungs, nasal cavity, kidneys, spleen, liver, pancreas, and brain all removed carefully. These organs were then weighed and fixed in a 10% formalin solution containing neutral phosphate-buffered saline. The organs were embedded in paraffin, stained with hematoxylin and eosin. All organs from all animals were examined under light microscopy.

Determination of tissue silver. After wet digestion using a flameless method, the tissue concentrations of silver were analyzed using an atomic absorption spectrophotometer equipped with a Zeeman graphite furnace (Perkin Elmer 5100ZL, Zeeman Furnace Module, Waltham, MA) based on the NIOSH 7300 method (NIOSH, 1999).

Statistical analysis. All the results are expressed as the means ± SE. An ANOVA test and Duncan's multiple range tests were used to compare the body weights, bronchoalveolar lavage cell distributions, and parameters from the lung function test obtained for the three dose groups with those obtained for the control rats. Histopatholgical results were analyzed by Chi-squared analysis. Level of significance was set at $p < 0.05$ and $p < 0.01$.

RESULTS

Silver Nanoparticle Distribution

Silver nanoparticle distribution measurements were as previously reported by Sung *et al*. (2008). For the high-concentration chamber, the geometric mean diameter, total number concentration, and surface area of silver nanoparticles measured by the DMAS were 19 (geometric standard deviation [GSD], 2) nm, 2.85×10^6 particles/cm^3, and 6.61×10^9 nm^2/

TABLE 5
Serum Biochemical Values for Male Rats (Mean ± SE)

Parameter	Control, $N = 10$	Low, $N = 10$	Middle, $N = 10$	High, $N = 9$
ALB[1]	2.54 ± 0.04	2.64 ± 0.04	2.51 ± 0.04	2.54 ± 0.05
ALP[2]	230.50 ± 18.2	205.30 ± 10.98	248.60 ± 10.43	242.67 ± 16.10
CA[3]	10.52 ± 0.77	10.94 ± 0.72	11.08 ± 0.78	11.17 ± 0.82
CHO[4]	54.70 ± 6.64	53.50 ± 3.43	56.40 ± 8.08	50.11 ± 5.15
CRE[5]	0.84 ± 0.04	1.01 ± 0.05	0.98 ± 0.05	0.86 ± 0.05
γ-GT[6]	1.60 ± 0.31	2.00 ± 0.42	2.20 ± 0.33	2.22 ± 0.43
GLU[7]	138.40 ± 9.08	123.40 ± 7.40	142.60 ± 10.93	141.67 ± 7.81
GOT[8]	156.00 ± 16.74	160.00 ± 11.87	148.90 ± 13.25	134.67 ± 10.70
GPT[9]	50.60 ± 4.72	45.20 ± 1.69	51.40 ± 2.98	49.33 ± 3.00
IP[10]	6.97 ± 0.28	7.39 ± 0.36	7.18 ± 0.28	6.72 ± 0.19
LDH[11]	2746.40 ± 278.36	3120.20 ± 315.45	2224.60 ± 371.64	2010.11 ± 288.96
MCONTROL[2]	2.43 ± 0.07	2.59 ± 0.11	2.47 ± 0.08	2.28 ± 0.05
TP[13]	6.37 ± 0.04	6.52 ± 0.08	6.27 ± 0.09	6.21 ± 0.07
UA[14]	1.40 ± 0.11	1.65 ± 0.12	1.50 ± 0.13	1.31 ± 0.09
BUN[15]	18.87 ± 0.68	18.35 ± 0.56	18.53 ± 0.46	16.77 ± 0.43
TBIL[16]	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Na[17]	139.00 ± 0.63	138.40 ± 0.64	138.90 ± 0.67	138.00 ± 0.37
K[18]	4.37 ± 0.11	4.31 ± 0.07	4.43 ± 0.14	4.33 ± 0.14
Cl[19]	95.80 ± 0.73	94.10 ± 0.59	95.30 ± 0.37	96.33 ± 0.47
A/G[20]	0.66 ± 0.01	0.68 ± 0.02	0.67 ± 0.01	0.70 ± 0.02

Note. Parameters: 1, albumin (g/dl); 2, alkaline phosphatase (IU/l); 3, calcium (mg/dl); 4, cholesterol (mg/dl); 5, creatinine (mg/dl); 6, gamma-glutamyl transpeptidase (IU/l); 7, glucose (mg/dl); 8, glutamic oxaloacetic transaminase (IU/l); 9, glutamic pyruvic transaminase (IU/l); 10, inorganic phosphorus (mg/dl); 11, lactate dehydrogenase (IU/l); 12, magnesium (mg/dl); 13, total protein (g/dl); 14, uric acid (mg/dl); 15, blood urea nitrogen (mg/dl); 16, total bilirubin (mg/dl); 17, creatine phosphokinase (U/l); 18, sodium (mmol/L); 19, potassium (mmol/l); 20, chloride (mmol/l); 21, ratio of albumin and globulin.

TABLE 6
Serum Biochemical Values for Female Rats (Mean ± SE)

Parameter	Control, $N = 10$	Low, $N = 10$	Middle, $N = 10$	High, $N = 9$
ALB[1]	2.73 ± 0.11	2.80 ± 0.05	2.67 ± 0.05	2.80 ± 0.07
ALP[2]	138.70 ± 15.82	116.70 ± 11.19	142.30 ± 11.81	126.70 ± 19.26
CA[3]	9.75 ± 0.09	9.70 ± 0.06	9.56 ± 0.11	9.74 ± 0.10
CHO[4]	97.30 ± 7.59	88.30 ± 4.24	87.10 ± 3.46	95.20 ± 4.98
CRE[5]	0.90 ± 0.03	0.97 ± 0.04	0.93 ± 0.03	0.86 ± 0.05
γ-GT[6]	1.10 ± 0.23	0.60 ± 0.16	1.00 ± 0.00	3.50 ± 2.08
GLU[7]	110.40 ± 7.63	115.90 ± 5.86	108.00 ± 5.44	110.10 ± 6.04
GOT[8]	170.10 ± 3.019	121.70 ± 13.61	110.90 ± 4.85	299.70 ± 87.82
GPT[9]	61.60 ± 12.40	38.90 ± 2.38	38.30 ± 1.21	116.30 ± 40.14
IP[10]	6.48 ± 0.27	6.88 ± 0.21	6.54 ± 0.36	6.03 ± 0.26
LDH[11]	2139.10 ± 398.60	1924.40 ± 367.27	1760.30 ± 191.56	2120.64 ± 294.64
MG[12]	2.48 ± 0.09	2.41 ± 0.08	2.47 ± 0.06	2.44 ± 0.08
TP[13]	6.32 ± 0.13	6.31 ± 0.10	6.30 ± 0.08	6.51 ± 0.10
UA[14]	1.44 ± 0.14	1.30 ± 0.12	1.39 ± 0.06	1.33 ± 0.06
BUN[15]	19.53 ± 1.03	19.60 ± 0.73	19.41 ± 1.18	18.93 ± 1.22
TBIL[16]	0.03 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.01
Na[17]	140.20 ± 0.49	140.70 ± 0.56	141.00 ± 0.54	139.80 ± 0.25
K[18]	4.29 ± 0.11	4.30 ± 0.05	4.26 ± 0.10	4.35 ± 0.07
Cl[19]	97.10 ± 0.67	97.40 ± 0.48	98.10 ± 0.43	97.70 ± 0.50
A/G[20]	0.76 ± 0.03	0.80 ± 0.02	0.74 ± 0.03	0.76 ± 0.02

Note. Parameters: 1, albumin (g/dl); 2, alkaline phosphatase (IU/l); 3, calcium (mg/dl); 4, cholesterol (mg/dl); 5, creatinine (mg/dl); 6, gamma-glutamyl transpeptidase (IU/l); 7, glucose (mg/dl); 8, glutamic oxaloacetic transaminase (IU/l); 9, glutamic pyruvic transaminase (IU/l); 10, inorganic phosphorus (mg/dl); 11, lactate dehydrogenase (IU/l); 12, magnesium (mg/dl); 13, total protein (g/dl); 14, uric acid (mg/dl); 15, blood urea nitrogen (mg/dl); 16, total bilirubin (mg/dl); 17, creatine phosphokinase (U/l); 18, sodium (mmol/l); 19, potassium (mmol/l); 20, chloride (mmol/l); 21, ratio of albumin and globulin.

cm^3, respectively, whereas the measurements for the middle-concentration chamber were 18 nm, 1.43×10^6 particles/cm^3, and 2.37 x 10^9 nm^2/cm^3, respectively, and those for the low-concentration chamber were 18 nm, 6.64×10^5 particles/cm^3, and 1.08×10^9 nm^2/cm^3, respectively. The silver nanoparticles observed by TEM were spherical in shape and nonaggregated/agglomerated in form, with diameters under 55 nm. The diameters were log normally distributed between 6 and 55 nm, and the count median diameter and GSD were 18 nm and 1.5, respectively (Sung *et al*., 2008).

Animal Observation, Food Consumption, and Effect on Body and Organ Weights

No gross effects were observed during the 90-day exposure period. One animal from the high-dose group died during the ophthalmological examination. No significant differences were observed in food consumption between the exposed rats and the control group (Figs. 1A and 1B). There were no significant changes in body weights of male rats (Fig. 2A). Although female rats showed a significant body weight difference between the high and middle dose groups, there were no significant dose-related changes (Fig. 2B). No significant organ weight changes were observed in either the male or female rats after the 90 days of silver nanoparticle exposure (Tables 1 and 2).

Effects on Hematology and Blood Biochemistry

There were no significant dose-related differences in the hematology values among the groups (Tables 3 and 4), and no significant dose-related differences in the blood biochemical parameters (Tables 5 and 6).

Silver Distribution in Tissue

Silver concentration in lung tissue from groups exposed to silver nanoparticles for 90 days were a statistically significant ($p < 0.01$) and increased with dose (Tables 7 and 8). There was also a clear dose-dependent increase in the silver concentration in the blood, and dose-dependent increase in the liver silver concentration for both genders. Silver concentration in the olfactory bulb was higher than in brain, and increased in a dose dependent manner in both the male and female rats ($p < 0.01$). Interestingly, silver concentrations in the kidneys showed a gender difference

TABLE 7
Tissue Content of Silver in Male Rats (Mean ± SE) (Unit: ng/g tissue wet weight)

	Control	Low	Middle	High
Liver	0.70 ± 0.20 (3)	3.52 ± 0.98 (5)	13.75 ± 2.28 (5)	132.97 ± 22.87* (4)
Kidneys	0.85 ± 0.20 (5)	1.63 ± 0.33 (5)	3.58 ± 0.41** (5)	9.49 ± 0.86* (4)
Olfactory bulb	0.51 ± 0.38 (3)	6.44 ± 0.77 (5)	17.10 ± 1.61 (5)	30.48 ± 2.15*** (4)
Brain	1.12 ± 0.34 (2)	3.45 ± 0.73 (4)	7.89 ± 0.95** (5)	18.63 ± 1.24* (4)
Lungs	0.77 ± 0.25 (5)	613.57 ± 66.03† (5)	5450.29 ± 904.17** (5)	14645.42 ± 2630.24* (4)
Whole blood	0.09 ± 0.02 (7)	0.68 ± 0.08 (10)	1.82 ± 0.20** (10)	4.31 ± 0.37* (9)

Note. (): number of samples.
*$p < 0.01$, high-dose versus other groups.
**$p < 0.01$, middle-dose versus unexposed and low-dose groups.
***$p < 0.01$, high-dose versus other groups (dose dependent).
†$p < 0.05$, male versus female in low-dose group.

(Tables 7 and 8), with the female kidneys containing two to three times more silver accumulation than in male kidneys.

Histopathological Examination

In liver, minimal bile-duct hyperplasia was identified in 0/10, 0/10, 1/10, and 4/9 of the control, low, middle, and high-dose males, respectively (Table 9, Figs. 3A and 3C). One high-dose male had minimal bile-duct hyperplasia with minimal portal mineralization. The higher incidence of bile-duct hyperplasia in the high-dose males, with or without mineralization, suggested a minimal test article–related effect at the high dose. Minimal bile-duct hyperplasia was also present in 3/10, 2/10, 4/10, and 8/10 of the control, low, middle, and high-dose females, respectively (Table 10, Figs. 3B and 3D). Single-cell hepatocellular necrosis, characterized by increased cellular eosinophilia and shrunken condensed nuclei, was noted in 3/10 of the high-dose females. One high-dose female exhibited moderate bile-duct hyperplasia with concurrent moderate centrilobular fibrosis, minimal single-cell hepatocyte necrosis, mild pigment accumulation, and moderate multifocal necrosis. The higher incidence of bile-duct hyperplasia, with or without necrosis, fibrosis, and/or pigmentation, in the high-dose females suggested a test article–related effect at the high dose, which was slightly more obvious than in the males.

Histopathological examination of lung samples revealed a high incidence of minimal alterations, including some chronic alveolar inflammation, a mixed cell perivascular infiltrate, and alveolar macrophage accumulation in the high-dose male and female animals when compared with the controls (Tables 9 and 10). No histopathologic findings in the nasal pathways were considered test article related.

In kidneys, a similar incidence of minimal tubular basophilia was noted in all the groups, including the controls, and thus not considered to be test article exposure related. However, the tubular basophilia was more prevalent in males compared with the females. Although tubular dilatation, cast formation, mineralization, and inflammation were noted occasionally in the control and/or treated animals, they were considered to be

TABLE 8
Tissue Content of Silver in Female Rats (Mean ± SE) (Unit: ng/g tissue wet weight)

	Control	Low	Middle	High
Liver	0.90 ± 0.31 (5)	4.55 ± 1.40 (5)	12.07 ± 2.50 (5)	71.08 ± 24.50* (5)
Kidneys	0.94 ± 0.18 (4)	2.61 ± 0.57 (5)	11.81 ± 4.27 (4)	37.66 ± 7.04*† (5)
Olfactory bulb	2.26 ± 0.74 (4)	7.43 ± 0.75 (5)	13.75 ± 1.32 (5)	32.84 ± 2.74*** (5)
Brain	0.66 ± 0.26 (4)	4.09 ± 0.46 (5)	10.22 ± 1.19** (5)	19.97 ± 2.41* (5)
Lungs	1.01 ± 0.10 (3)	295.92 ± 78.50 (5)	4241.17 ± 641.10** (5)	20585.63 ± 1880.31* (5)
Whole blood	0.05 ± 0.01 (5)	0.85 ± 0.14 (10)	2.10 ± 0.22** (10)	6.86 ± 0.60*‡ (10)

Note. ( ): number of samples.
*$p < 0.01$, high-dose versus other groups.
**$p < 0.01$, middle-dose versus unexposed and low-dose groups.
***$p < 0.01$, high-dose versus other groups (dose dependent).
†$p < 0.05$, female versus male in low-dose group.
‡$p < 0.01$, female versus male in high-dose group.

TABLE 9
Histopathological Findings for Male Rats

Group				Control		Low		Middle		High	
Number of animals				10		10		10		9	
				N	%	*N*	%	*N*	%	*N*	%
Liver	No microscopic findings			10/10	100	10/10	100	9/10	90	5/9	55.6
	Abnormality*			0/10	0	0/10	0	1/10	10	4/9	44.4
	Necrosis	Multifocal	Minimum	0/10	0	0/10	0	0/10	0	1/9	11.1
	Hyperplasia*	Bile duct	Minimum	0/10	0	0/10	0	1/10	10	4/9	44.4
	Vacuolation	Hepatocellular	Minimum	0/10	0	0/10	0	0/10	0	1/9	11.1
	Mineralization	Portal	Minimum	0/10	0	0/10	0	0/10	0	1/9	11.1
Lungs	No microscopic findings			5/10	50	3/10	30	3/10	30	0/9	0
	Abnormality			5/10	50	7/10	70	7/10	70	9/9	100
	Accumulation	Macrophage, alveolar	Minimum	3/10	30	5/10	50	5/10	50	8/9	88.9
	Inflammation**	Chronic, alveolar	Minimum	2/10	20	3/10	30	2/10	20	8/9	88.9
	Infiltrate	Mixed cell perivascular	Minimum	3/10	30	4/10	40	6/10	60	7/9	77.8
	Hemorrhage	Alveolar	Minimum	1/10	10	0/10	0	0/10	0	0/9	0
	Osseous foreign body			0/10	0	0/10	0	0/10	0	1/9	11.1
	Hyperplasia	Respiratory epithelium level I		0/10	0	0/10	0	0/10	0	1/9	11.1

*$p < 0.05$, compared with control, **$p < 0.01$, compared with control.

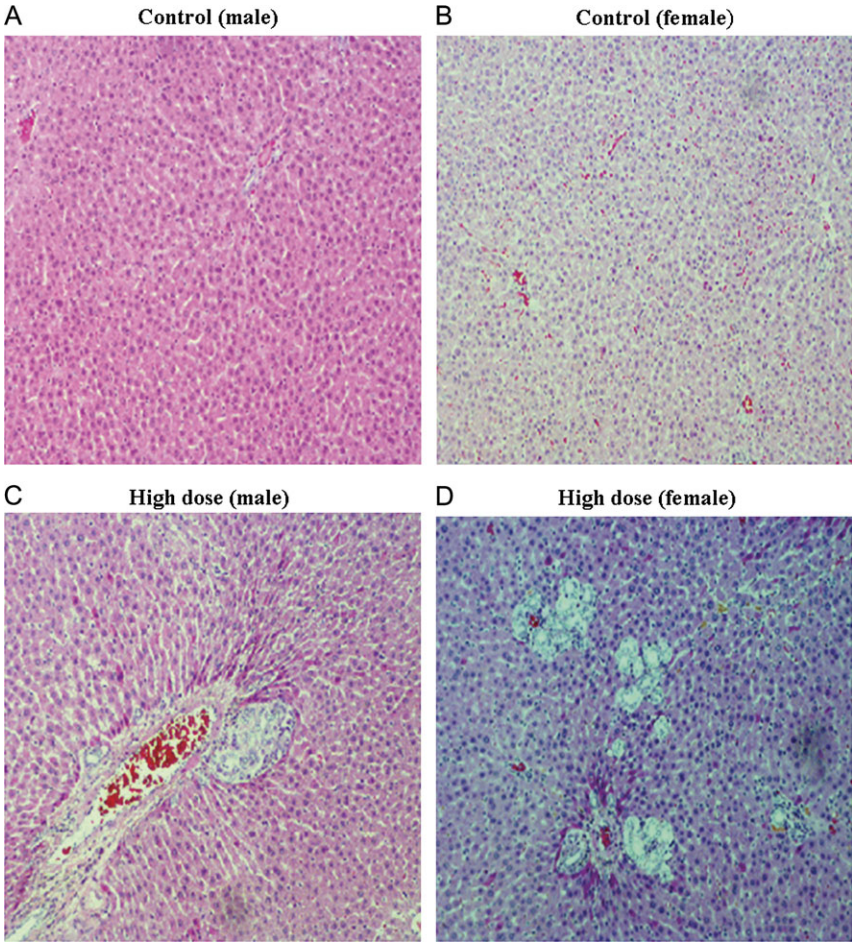

FIG. 3. Bile-duct hyperplasia in liver. (A) Control (male), (B) Control (female), (C) High dose (male), (D) High dose (female).

TABLE 10
Histopathological Findings for Female Rats

Group				Control		Low		Middle		High	
Number of animals				10		10		10		10	
				N	%	*N*	%	*N*	%	*N*	%
Liver	No microscopic findings			7/10	70	5/10	50	5/10	50	1/10	10
	Abnormality*			3/10	30	5/10	50	5/10	50	9/10	90
	Necrosis	Multifocal	Minimum	2/10	20	0/10	0	0/10	0	0/10	0
			Moderate	0/10	0	0/10	0	0/10	0	1/10	10
		Focal	Minimum	0/10	0	0/10	0	1/10	10	0/10	0
		Single-cell* hepatocellular	Minimum	0/10	0	0/10	0	0/10	0	3/10	30
	Hyperplasia*	Bile duct	Minimum	3/10	30	2/10	20	4/10	40	8/10	80
			Moderate	0/10	0	0/10	0	0/10	0	1/10	10
	Granuloma	Multifocal	Minimum	0/10	0	2/10	20	0/10	0	0/10	0
	Vacuolation	Hepatocellular	Minimum	0/10	0	1/10	10	0/10	0	0/10	0
	Fibrosis	Centrilobular	Mild	0/10	0	0/10	0	0/10	0	1/10	10
	Pigment	Centrilobular	Mild	0/10	0	0/10	0	0/10	0	1/10	10
Lungs	No microscopic findings			3/10	30	5/10	50	6/10	60	2/10	20
	Abnormality			7/10	70	5/10	50	4/10	40	8/10	80
	Accumulation	Macrophage, alveolar	Minimum	7/10	70	4/10	40	4/10	40	6/10	60
	Inflammation**	Chronic, alveolar	Minimum	3/10	30	2/10	20	0/10	0	8/10	80
	Infiltrate**	Mixed cell perivascular	Minimum	0/10	0	0/10	0	1/10	10	7/10	70

*$p < 0.05$, compared with control, **$p < 0.01$, compared with control.

within the range of expected background spontaneous change (data not shown).

In heart, minimal degeneration/necrosis was observed in all the groups, including the controls, indicating that this alteration was not test article related. The change was more obvious in the males. This finding is a common spontaneous background change, usually considered to be stress-related, secondary to the handling of animals during inhalation studies. Although inflammation was also noted occasionally in the Harderian gland and prostate, it is also a common background spontaneous finding and thus not considered to be test article related (data not shown).

Erythrocyte Aggregation and Kidney Function Test

To evaluate aggregation of red blood cells or blood coagulation attributable to silver nanoparticles, erythrocyte aggregation, activated partial thromboplastin time, and prothrombin time were tested. Only the percent of aggregation in the high-dose females

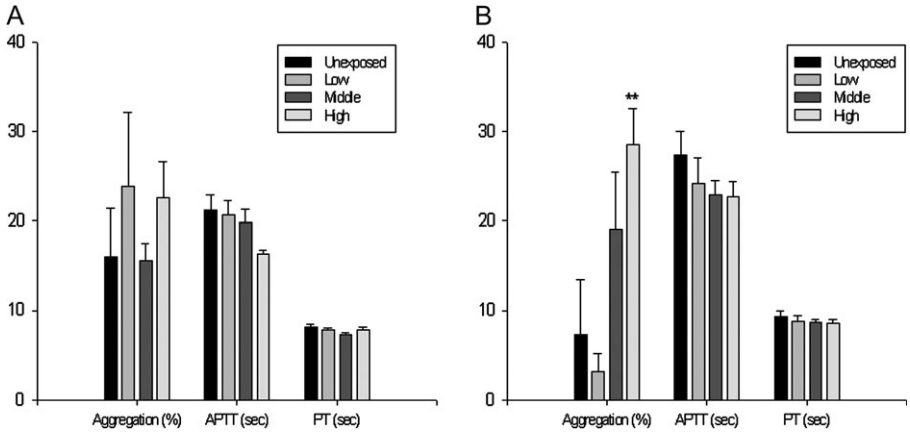

FIG. 4. Erythrocyte aggregation, APTT, and PT for rats exposed to silver nanoparticles, (A) males, (B) females. (**$p < 0.01$, high vs. low group).

TABLE 11
Kidney Function Test for Male Rats Exposed to Silver Nanoparticles (Mean ± SE)

Group	Control	Low	Middle	High
NAG[a]	10.10 ± 1.44 (5)	13.94 ± 2.70 (5)	33.06 ± 22.52 (5)	12.88 ± 2.25 (5)
Protein[b]	1.89 ± 0.11 (5)	1.58 ± 0.32 (5)	2.39 ± 0.23 (5)	2.57 ± 0.13* (5)

Note. ( ): number of samples. *$p < 0.05$, high group versus low group.
[a]N-acetyl-β-D-glucosaminidase (unit/g creatinine).
[b]g/g creatinine.

TABLE 12
Kidney Function Test for Female Rats Exposed to Silver Nanoparticles (Mean ± SE)

Group	Control	Low	Middle	High
NAG[a]	12.25 ± 1.60 (5)	13.81 ± 0.48 (5)	12.28 ± 2.83 (5)	15.70 ± 0.17 (5)
Protein[b]	3.88 ± 2.48 (5)	0.90 ± 0.13 (5)	2.93 ± 1.11 (5)	2.26 ± 0.90 (5)

Note. ( ): number of samples.
[a]N-acetyl-β-D-glucosaminidase (unit/g creatinine).
[b]g/g creatinine.

showed a statistically significant difference compared with the controls (Fig. 4). Because a gender difference in silver accumulation was noted in kidneys, the kidney function was measured based on the N-acetylglutamate and protein in urine. Surprisingly, no significant difference was noted among the dose groups and between genders (Tables 11 and 12), except for an increase of protein in the urine from the high-dose male rats.

DISCUSSION

We are unaware of any previous *in vivo* studies of inhaled silver nanoparticles with which to compare our data. The results of this subchronic 90-day silver nanoparticle inhalation toxicity indicated that lungs and liver were the major target tissues for prolonged silver nanoparticle accumulation. Effects on the lungs have been previously reported as a decrease in the tidal volume and minute volume and other inflammatory responses, such as a mixed inflammatory cell infiltrate and chronic alveolar inflammation (Sung *et al*., 2008). Furthermore, although on a minimal level, silver nanoparticle exposure-related bile-duct hyperplasia was noted in both the male and female animals. Histopatholgical observations in a different 28-day oral-dose study also showed a similar dose-dependent increased incidence of bile-duct hyperplasia around the central vein to the hepatic lobule with the infiltration of inflammatory cells, including eosinophils, in the hepatic lobule and portal tract (Kim *et al*., 2008). No bile-duct hyperplasia was noted in the previous 28-day inhalation toxicity study by the present authors, indicating that a higher dose with prolonged exposure is needed to induce these responses.

The higher accumulation of silver nanoparticles in the female kidneys reported in the present study was also previously observed in the oral-dose study by Kim *et al*. (2008) indicating that the gender-different accumulation was not apparently dependent on route of administration. Most of the silver nanoparticles in the kidneys were located in the basement membranes of the medulla and cortex of the kidneys (unpublished data), yet the exact functional meaning of the gender difference in silver nanoparticle accumulation is unclear, because no significant kidney function or histopathologic changes in the female kidneys were found in the present inhalation study.

As previously observed in the 28-day inhalation and 28-day oral-dose studies, silver nanoparticles were distributed in all the tissues examined in the present study. However, when compared with the results of the oral-dose study, the present study found that the olfactory bulb accumulated more silver than brain. In contrast to the 28-day inhalation study, the present study found a clear dose-dependent increase in the blood silver nanoparticle concentration, indicating a systemic distribution of silver nanoparticles by the circulating blood.

The different sizes of nanoparticles between the inhalation studies, which were 15–19 nm in the 28- and 90-day inhalation studies and 60 nm in the oral-dose study, did not appear to influence the silver nanoparticle tissue distribution or the gender difference in the accumulation of silver nanoparticles in the kidneys. The only exception was the higher accumulation of silver particles in the lungs in the inhalation studies compared with the minimal accumulation in the lungs in the oral-dose study, indicating that lung distribution was not the major route in the case of oral ingestion.

One limitation to the data on organ distribution of silver is that we were unable to correct for tissue content of silver contained in blood. We feel that this is unlikely to significantly effect our conclusions because blood concentrations of silver were substantially less than nearly all tissue concentrations. A second limitation is that we did not differentiate among ionic forms of silver in tissues or test for the presence of insoluble salts (particularly in kidneys). Thus, we are unable to delineate a mechanism for the observed distribution of silver in liver and kidneys.

As there is no current data on workplace air concentrations of silver nanoparticles or silver nanoparticle concentrations released from consumer products, the concentrations used in this study are difficult to relate human exposures. Concentrations used in this study were based on the current ACGIH silver dust threshold limit value (TLV) of 0.1 mg/m^3 (ACGIH, 2001). As such, the low, middle, and high doses were 1/2, 1, and 5 times the ACGIH silver dust TLV, respectively, in terms of mass dose. In addition, the high dose used in this study is nearly 500-fold higher than the ACGIH silver dust TLV in terms of surface area and fivefold higher than the high dose used in the previous 28-day inhalation study.

Based on the test article–related effects (minimal bile-duct hyperplasia in males and females, chronic alveolar inflammation

and macrophage accumulation in the lungs of males and females, and erythrocyte aggregation in females) reported in this study, we found a NOAEL of 100 μg/m^3. This NOAEL is consistent with the current ACGIH silver dust TLV of 0.1 mg/m^3. However, lung function changes previously reported from this study (Sung *et al*., 2008) indicate significant physiological decreases in tidal volume for all dose levels in males and minute volume decreases for all dose levels in females. The origin of the difference in effects measurements remains to be resolved.

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