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Article

Genotoxicity of Silver Nanoparticles in Lung Cells of Sprague Dawley Rats after 12 Weeks of Inhalation Exposure

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Abstract: Due to the widespread use of silver nanoparticles in consumer products, the toxicity of silver nanoparticles has also been studied in relation to their application. However, most genotoxicity studies of silver nanoparticles have been performed *in vitro*. Therefore, this study evaluated the DNA damage to lung cells caused by repeated inhalation of silver nanoparticles. Male Sprague Dawley rats were exposed to silver nanoparticles for 12 weeks in a whole-body inhalation chamber. The animals were divided into one control group and three dose groups that were exposed to silver nanoparticles (14–15 nm diameter) at concentrations of 0.66 × 10^6 particles/cm^3 (49 μg/m^3, low dose), 1.41 × 10^6 particles/cm^3 (117 μg/m^3, middle dose), and 3.24 × 10^6 particles /cm^3 (381 μg/m^3, high dose), respectively, for six hours/day over 12 weeks. The rats were sacrificed after the 12-week exposure period and the DNA damage assessed using a Comet assay of cells obtained from the right lungs. The olive tail moment values were 2.93 ± 0.19, 3.81 ± 0.23, 3.40 ± 0.22, and 5.16 ± 0.32 for the control, low-, middle-, and high-dose groups, respectively. Although no

dose-dependent results were observed, a significant increase in the level of DNA damage was noted for the high-dose group.

Keywords: silver nanoparticle; lung cells; genotoxicity; comet assay; single cell gel electrophoresis; inhalation exposure

1. Introduction

The expanding widespread use of silver nanoparticles in consumer products covers bedding, washers, water purification, toothpaste, shampoo and rinse, nipples and nursing bottles, fabrics, deodorants, filters, kitchen utensils, toys, humidifiers, printed electronics, and biocides [1]. Airborne silver nanoparticle exposure covers the whole life-cycle of a consumer product: its production, use, and disposal. Thus, the size, shape, and surface coatings of silver nanoparticles released in relation to the production and use of consumer products can be important toxicity determinants.

According to previous toxicity studies, the acute inhalation toxicity LC_{50} for silver nanoparticles has been suggested at higher than 3.1×10^6 particles/cm^3 (750 μg/m^3) [2], although a 28-day silver nanoparticle inhalation toxicity study showed no significant toxicity up to 1.32×10^6 particles/cm^3 (61 μg/m^3) [3]. In previous studies, using male and female rats, 90-day subchronic inhalation exposure to silver nanoparticles was found to induce infiltrate mixed cells and chronic alveolar inflammation, along with thickened alveolar walls, small granulomatous lesions in the lungs, and a dose-dependent increase in bile-duct hyperplasia [4,5]. These studies also showed that the target organs for the silver nanoparticles were the lungs and liver [5], and lung function changes were also observed [4]. However, the experimental results indicated a no observed adverse effect level of 100 μg/m^3 [5]. Meanwhile, another recent study investigating the recovery from silver-nanoparticle-exposure-induced lung inflammation and lung function changes, and conducted in parallel with the present study, reported a potential persistence of lung function changes and inflammation induced by silver nanoparticle exposure above the no observed adverse effect level (117 μg/m^3) [6].

Despite the range of genotoxicity data on silver nanoparticles, the *in vitro* genotoxicity of silver nanoparticles does not necessarily reflect the *in vivo* genotoxicity. For example, the silver nanoparticle genotoxicity results were all negative in an *in vivo* micronucleus test of rat bone marrow cells after 28 days of oral administration [7] and after 90 days of inhalation exposure [8]. However, in the case of inhalation exposure, the genotoxicity in the lung cells is clearly more relevant. Accordingly, this study exposed rats to silver nanoparticles for six hours/day in an inhalation chamber over 12 weeks. The rat lung cells were then isolated and the genotoxicity evaluated using a Comet assay.

2. Results and Discussion

2.1. Animal Observations: Effect on Body Weight and Lung Weight

The rats exhibited no significant toxicity signs during the exposure period, and none of the rats died. However, while the exposure period produced no significant changes in the body weights (Table 1), there were significant changes ($p < 0.01$–$p < 0.05$) in the weights of the right and left lungs for

the high-dose group when compared with the fresh-air control and low-dose group as previously published [6] (Table S1).

Table 1. Body weight changes for male rats during subchronic exposure to silver nanoparticles.

Summary of body weights								
GROUP: (mean ± S.E)	**Control**	***N***	**Low**	***N***	**Middle**	***N***	**High**	***N***
Day 0	171.06 ± 1.61	17	171.04 ± 1.61	17	171.14 ± 1.61	17	171.26 ± 1.64	17
Day 1	192.76 ± 1.91	17	191.29 ± 1.81	17	190.39 ± 1.64	17	193.09 ± 1.83	17
Week 1	219.44 ± 2.24	17	218.03 ± 2.07	17	214.26 ± 1.88	17	220.14 ± 2.21	17
Week 2	265.39 ± 2.91	17	263.12 ± 2.91	17	258.41 ± 2.66	17	263.82 ± 3.23	17
Week 3	297.72 ± 3.66	17	290.87 ± 4.05	17	288.19 ± 3.31	17	296.90 ± 4.40	17
Week 4	324.52 ± 4.50	17	315.15 ± 5.61	17	310.48 ± 4.33	17	320.94 ± 5.60	17
Week 5	342.62 ± 5.18	17	334.66 ± 6.57	17	330.17 ± 4.71	17	342.92 ± 6.24	17
Week 6	352.05 ± 5.69	17	337.49 ± 6.81	17	339.65 ± 4.60	17	349.72 ± 7.33	17
Week 7	369.36 ± 6.06	17	362.93 ± 7.20	17	358.58 ± 5.20	17	369.94 ± 7.40	17
Week 8	382.38 ± 6.22	17	375.19 ± 8.06	17	371.60 ± 5.57	17	380.96 ± 7.68	17
Week 9	395.49 ± 6.58	17	389.68 ± 8.67	17	383.28 ± 5.51	17	393.93 ± 8.10	17
Week 10	405.93 ± 6.89	17	400.85 ± 8.93	17	394.81 ± 5.88	17	402.02 ± 8.40	17
Week 11	416.25 ± 7.23	17	410.86 ± 9.21	17	404.68 ± 5.87	17	410.87 ± 9.03	17
Week 12	419.33 ± 7.22	17	413.90 ± 9.20	17	407.70 ± 5.86	17	413.95 ± 9.03	17
Sacrifice (12 weeks exposure)	397.05 ± 7.23	9	402.10 ± 13.47	9	397.16 ± 6.69	9	394.96 ± 11.72	9

N, number of animals.

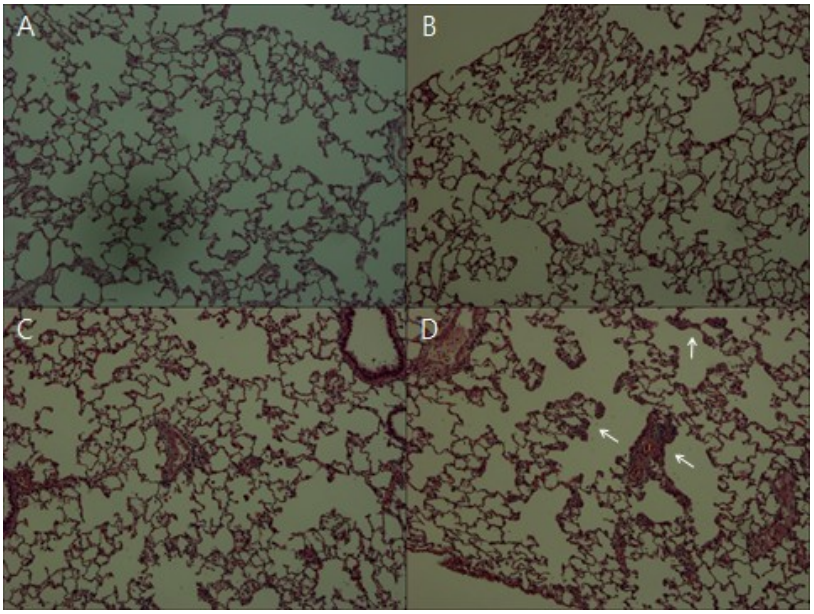
2.2. Silver Nanoparticle Generation and Particle Characterization

The generator was operated at 99.6 V with a carrier airflow rate of 46 L/min. In the high-concentration chamber, the geometric mean diameter (GMD), geometric standard deviation (GSD), total number concentration, and mass concentration and surface area of the silver nanoparticles were 15.00 (1.75) nm, 3.24×10^6 particles/cm^3, 381.43 μg/m^3, and 4.85×10^9 nm^2/cm^3, respectively, in the middle-concentration chamber, these measurements were 14.38 (1.64) nm, 1.41×10^6 particles/cm^3, 117.14 μg/m^3, and 1.70×10^9 nm^2/cm^3, respectively, and in the low-concentration chamber they were 14.54 (1.62) nm, 0.66×10^6 particles/cm^3, 48.76 μg/m^3, and 0.76×10^9 nm^2/cm^3, respectively, as previously published [6] (Table S2). These same silver nanoparticle concentrations in each chamber were maintained throughout the 12-week exposure period. The other physicochemical properties and shapes of the silver nanoparticles are previously published by Song *et al*. [6].

2.3. Histopathogical Evaluation

The histopathological examination of the lungs from the rats exposed to the silver nanoparticles for 12 weeks showed increases in the incidence of perivascular and chronic alveolar inflammation, accompanied by alveolaritis, granulomatous lesions, and alveolar wall thickening and alveolar macrophage accumulation (Figure 1).

Figure 1. Histopathology of lungs from male rats exposed to silver nanoparticles for 12 weeks: (**A**) control; (**B**) low dose; (**C**) middle dose; and (**D**) high dose. Arrows indicate lesions with mixed cell infiltrate perivascular and chronic alveolar inflammation, including alveolaritis, granulomatous lesions, and alveolar wall thickening and alveolar macrophage accumulation.



2.4. Silver Concentration in Lungs

A statistically significant ($p < 0.01$) dose-dependent increase in the silver concentration was observed in the lung tissue from the groups exposed to the silver nanoparticles for 12 weeks, as previously published [6] (Table S3).

2.5. Comet Assay

The comet assay was conducted on cells from the right lungs of the male rats exposed to the silver nanoparticles for 12 weeks. Although no exposure-response relationship was observed in the olive tail moment (OTM) values, an increasing trend in the OTM values was observed among the dose groups, where the high-dose group showed a statistically significant increase ($p < 0.01$) when compared to the control, low-, and medium-dose groups (Table 2).

Table 2. Quantitative analysis of Sprague Dawley rat lung cell DNA damage after subchronic exposure to silver nanoparticles using single cell gel electrophoresis (Comet assay).

Concentration	*N*	OTM † (Mean ± S.E)
Control	5	2.93 ± 0.19
Low	5	3.81 ± 0.23
Middle	4	3.40 ± 0.22
High	4	5.16 ± 0.32 **

N, number of animals; †, Olive Tail Moment; **, $p < 0.01$, high *vs*. control.

2.6. Other Assays

The results of the blood biochemistry, hematology, lung function test, and silver concentrations in other tissues have been published elsewhere [6]. Silver nanoparticles have already been shown to have an antibacterial effect and induce cytotoxicity and genotoxicity in certain cell lines [9–14]. For example, cytotoxicity and genotoxicity tests using silver nanoparticles (nm) with a medaka (Oryzias latipes) cell line showed cell death at 0.05–5 μg/cm^2 and chromosomal aberration and aneuploidy at 0.05–0.3 μg/cm^2 [11]. Yet, cytotoxicity and genotoxicity tests using colloidal silver nanoparticles (30 nm) based on an MTT assay and Comet assay at 100 μg/mL showed no induced toxic effects on human keratinocytes [15]. Thus, despite various *in vitro* genotoxicity studies using silver nanoparticles, the results are conflicting due to the use of various test methods, test materials, and other conditions, such as the capping agents and particle aggregation/agglomeration.

In our previous *in vivo* genotoxiciy studies of silver nanoparticles based on 28 days of oral administration [7] and 90 days of inhalation exposure [8], the *in vivo* micronucleus test results were negative. Plus, another *in vitro* bacterial reverse mutation test and chromosomal aberration test using silver nanoparticles were also negative, although some cytotoxicity was observed [16]. However, the present *in vivo* Comet assay of lung cells exposed to silver nanoparticles for 12 weeks showed a positive response for the high dose that was higher than the proposed No Observed Adverse Effect level (NOAEL) (117 μg/m^3). A parallel study also reported an exposure-related lung function decrease in the male rats after the 12-week exposure period and 12 weeks after the exposure cessation [6]. Interestingly, the female rats in the parallel study did not show a consistent lung function decrease, either during the exposure period or following the exposure cessation. Furthermore, the histopathological observations after the 12-week exposure and during the 12-week recovery indicated that exposure to the high dose of silver nanoparticles (381 μg/m^3) induced lung inflammation with a lung function decrease during the 12-week exposure period [6]. In the present study, the rats exposed to the silver nanoparticles for 12 weeks exhibited significant increases in the incidence of perivascular and chronic alveolar inflammation, accompanied by alveolaritis, granulomatous lesions, and alveolar wall thickening and alveolar macrophage accumulation. This inflammatory response also corresponded with a statistically significant ($p < 0.01$) dose-dependent increase in the silver concentration in the lung tissue. These responses could induce reactive oxygen species (ROS) generation or some other mechanism, such as a decrease in the intracellular glutathione level [17]. Silver nanoparticles are already known to cause oxidative stress, DNA damage, and cytokine induction. Thus, the ability of silver nanoparticles to generate ROS plays a key role in inducing toxicity. Increased ROS production decreases the cellular antioxidant defenses and disrupts the mitochondrial function. The increased oxidative stress then induces cellular damage, such as mitochondrial apoptosis and necrosis [18]. ROS can also react with critical cellular molecules, such as lipids, proteins, nucleic acids, and carbohydrates, and generate additional radicals. Asharani *et al*. [10] previously measured the DNA damage caused by ROS due to silver nanoparticle exposure, and observed G2/M cell-cycle arrest, possibly due to DNA damage repair, following exposure to starch-capped globular particles of nano-Ag 6–20 nm in size. Thus, the increased ROS generated by silver nanoparticle exposure can interact with and damage proteins or DNA [10]. Silver ions can also bind directly with DNA and RNA [19]. As such, in the present study, the persistent inflammation caused by the high-dose exposure that was

higher than the NOAEL likely elevated the ROS level, while reducing the level of GSH in the lung cells during the 12-week silver nanoparticle exposure. Consequently, these phenomena seemingly induced the genotoxicity observed in the Comet assay of the lung cells.

3. Materials and Methods

3.1. Generation of Silver Nanoparticles

The silver nanoparticles were generated as described in previous reports [6], and the rats were exposed using a whole-body-type inhalation chamber (1.3 m^3, Dusturbo, Seoul, Korea), consisting of a small ceramic heater connected to an AC power supply that was housed within a quartz tube case. The heater dimensions were 50 × 5 × 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 [20]. For the 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 was maintained at 30 L/min (Re = 572, laminar flow regime) using a mass flow controller (MFC, AERA, FC-7810CD-4V, Tokyo, Japan) [3]. In this study, the system produced different concentrations of nanoparticles (high, middle, and low) in three separate chambers. The nanoparticle generator was operated for 45.99 ± 0.02 L/min (mean ± SE) using the MFC and mixed with 200 L/min at the main flow rate through the high-concentration chamber. Using the MFC for the first dilutor (27.19 ± 0.05 L/min, mean ± SE), a portion of the high nanoparticle concentration was diverted to the middle-concentration chamber. In the same way, a portion of the middle nanoparticle concentration was diverted to the low-concentration chamber using a second MFC (2.33 ± 0.01 L/min) [6].

3.2. Monitoring of Inhalation Chamber and Analysis of Silver Nanoparticles

The monitoring of the inhalation chamber and silver nanoparticle analysis were conducted as previously described by Song *et al*. [6]. In the individual chambers, the nanoparticle size distribution was directly measured in real time using a differential mobility analyzer (Short type DMA, 4220, HCT Co., Ltd., Icheon, Korea, range 5–150 nm) and condensation particle counter (CPC, 4312, HCT Co., Ltd. Korea, 0–10^8/cm^3 detection range), in combination referred to as a DMAS (differential mobility analyzing system) or SMPS (scanning mobility particle sizer). Nanoparticles from 4.23 to 46.95 nm were measured using sheath air at 5 L/min and a polydispersed aerosol air at 1 L/min to meet the operational conditions for the DMA and CPC, respectively. Meanwhile, the particle concentration in the fresh-air control chamber was measured using a particle sensor (4123, HCT Co., Ltd., Icheon, Korea) that consisted of 2 channels: 300–1000 nm and over 1000 nm [6].

3.3. Animals and Conditions

Five-week-old male, specific-pathogen-free Sprague Dawley rats were purchased from KOATECH Co., (Pyeongtaek, Korea) and acclimated for 1 week before starting the experiments. During the acclimation and experimental periods, the rats were housed in polycarbonate cages (3 rats per cage) in a room with controlled temperature (21.6 ± 1.2 °C), humidity (43.7% ± 6.8%), and a 12-h light/dark cycle. The rats were fed a rodent diet (HarlanTeklab, Plaster International Co., Seoul, Korea) and

filtered water *ad libitum*. The 6-week-old male rats, weighing about 171 g, were then divided into 5 groups (each group consisted of 17 rats: 9 rats were sacrificed after the 12 weeks of exposure, where the lungs of 5 rats were used for the Comet assay and histopathology and the lungs of 4 rats were used for the bronchoalveolar lavage fluid assay after measuring the lung function, 4 rats were sacrificed after 4 weeks of recovery, and 4 rats were sacrificed after 12 weeks of recovery): fresh-air control, low-dose group (target dose, 0.6×10^6 particles/cm^3, 1.0×10^9 nm^2/cm^2), middle-dose group (target dose, 1.4×10^6 particles/cm^3, 2.5×10^9 nm^2/cm^2), and high-dose group (target dose, 3.0×10^6 particles/cm^3, 5.0×10^9 nm^2/cm^2) and exposed to silver nanoparticles for 6 h/day, 5 days/week, for 12 weeks. The animals were examined daily on weekdays for any evidence of exposure-related effects, including respiratory, dermal, behavioral, nasal, or genitourinary changes suggestive of irritancy. The body weights were measured at the time of purchase, at the time of grouping, one day after exposure, once a week during the inhalation exposure and recovery, and before necropsy.

3.4. Histopathological Evaluation

After the 12-week exposure, the rats were anaesthetized with pentobarbitol and blood was collected from the abdominal aorta. The lungs were removed and fixed in a 10% formalin solution containing neutral phosphate buffered saline. The specimens were then embedded in paraffin, stained with hematoxylin and eosin (H & E), and examined using light microscopy.

3.5. Silver Concentration in Lungs

The silver concentrations in the lungs from the male rats exposed to the silver nanoparticles for 12 weeks were analyzed using a Zeeman graphite furnace atomic absorption spectrophotometer (Perkin Elmer 5100 ZL, Zeeman Furnace Module, MA, USA) based on the National Institute for Occupational Safety and Health (NIOSH) 7300 method [21].

3.6. Cell Isolation

The right lungs were removed following the 12-week exposure, minced, suspended in chilled phosphate-buffered saline (PBS), and gently homogenized using a tissue grinder (Kontes, Vineland, NJ, USA) on ice. The cell suspensions were then transferred through a nylon cell strainer (BD Falcon, NC, USA) into sterile tubes. The viable cell counts for the cell suspensions were determined using the trytopan blue dye exclusion method.

3.7. Single Cell Gel Electrophoresis (Comet Assay)

The right lungs were also used for the Comet assay, where the lung tissue was minced into very small pieces and left to stand for 5 min in ice-cold 1× PBS (with 20 mM EDTA, Ca^{++}, and Mg^{++} free). Following centrifugation, the cell resuspensions (1×10^5/10 μL) were then mixed with 85 μL of 0.7% low-melting agarose and rapidly spread as the first layer. Thereafter, 85 μL of 0.7% low-melting agarose was used as the top layer. After lying flat at 4 °C in the dark for 30 min, the slides were immersed for 50 min in an ice-cold lysis solution (2.5 M NaCl, 100 mM Na2-EDTA, 10 mM

Tris-HCl, 1% Triton X-100, and 10% DMSO, pH 10.0). The slides were then removed from the lysis solution, and the DNA allowed to unwind for 50 min in a freshly prepared alkaline unwinding solution (1 mM EDTA, 300 mM NaOH; pH > 13). Next, the electrophoresis was performed in the same buffer for 40 min at 21 V and 300 mA. The alkaline unwinding and electrophoresis were both performed at 4 °C in the dark. Following the electrophoresis, the slides were washed twice for 5 min in distilled water, fixed in absolute ethanol, stained with ethidium bromide (2 μg/mL), and covered with a cover glass. Finally, the slides were coded and analyzed using a fluorescence microscope (LEICA DM2500, Wetzlar, Germany) and automated comet counter (Komet 5.5, Kinetic Imaging Ltd., Nottingham, UK). In this study, the DNA damage was measured based on counting 100 cells per animal (two replicates, 50 cells each), and the Olive Tail Moment (OTM) used as the DNA damage indicator [22]. The OTM was analyzed using a fluorescent microscope (Leica, Wetzlar, Germany) and image program (Kinetic Imaging, Nottingham, UK), and is a commonly used parameter that provides a good correlation with the dose of the genotoxic agent in a Comet assay [22].

3.8. Statistical Analysis

The statistical analysis of the data was performed using SPSS software version 12.0. The results are expressed as the means ± S.E. An ANOVA test and Dunnett T3 multiple range tests were used to compare with the control group. The level of significance was set as $p < 0.05$ and $p < 0.01$.

4. Conclusions

The *in vivo* comet assay of lung cells exposed to silver nanoparticles for 12 weeks showed a positive response for the high dose (381 μg/m^3) that was higher than the proposed NOAEL (117 μg/m^3).

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Conflicts of Interest

The authors declare that they have no competing interests.

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