

Original Article

In vivo Genotoxicity of Silver Nanoparticles after 90-day Silver Nanoparticle Inhalation Exposure

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Objectives: The antimicrobial activity of silver nanoparticles has resulted in their widespread use in many consumer products. Yet, despite their many advantages, it is also important to determine whether silver nanoparticles may represent a hazard to the environment and human health.

Methods: Thus, to evaluate the genotoxic potential of silver nanoparticles, *in vivo* genotoxicity testing (OECD 474, *in vivo* micronuclei test) was conducted after exposing male and female Sprague-Dawley rats to silver nanoparticles by inhalation for 90 days according to OECD test guideline 413 (Subchronic Inhalation Toxicity: 90 Day Study) with a good laboratory practice system. The rats were exposed to silver nanoparticles (18 nm diameter) at concentrations of 0.7×10^6 particles/cm³ (low dose), 1.4×10^6 particles/cm³ (middle dose), and 2.9×10^6 particles/cm³ (high dose) for 6 hr/day in an inhalation chamber for 90 days. The rats were killed 24 hr after the last administration, then the femurs were removed and the bone marrow collected and evaluated for micronucleus induction.

Results: There were no statistically significant differences in the micronucleated polychromatic erythrocytes or in the ratio of polychromatic erythrocytes among the total erythrocytes after silver nanoparticle exposure when compared with the control.

Conclusion: The present results suggest that exposure to silver nanoparticles by inhalation for 90 days does not induce genetic toxicity in male and female rat bone marrow *in vivo*.

Key Words: Silver nanoparticles, Genotoxicity, OECD test guidelines, *In vivo* micronuclei test, Good laboratory practice, Inhalation toxicity

Introduction

The antimicrobial activity of silver nanoparticles has resulted in their widespread use in many consumer products, such as disinfectants, deodorants, antimicrobial sprays and powders, bedding, washers, water purification, toothpaste, shampoo and

rinses, nipples and nursing bottles, fabrics, deodorants, filters, kitchen utensils, toys, and humidifiers. Yet, while the population exposed to silver nanoparticles continues to increase with ever-new applications, silver nanoparticles remain a controversial research area with respect to their toxicity to biological systems. The toxicity of silver nanoparticles has been studied extensively. The acute inhalation toxicity, LC50, of silver nanoparticles is suggested to be higher than 3.1×10^6 particles/cm³ (750 mg/m³) [1]. A toxicity study that exposed rats to twenty-eight days of silver nanoparticle inhalation did not show any significant toxicity up to $(1.32 \times 10^6$ particles/cm³, 61 µg/m³) [2]. In contrast, a study on the oral toxicity of silver

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nanoparticles that exposed rats to silver nanoparticles for 28 days indicated that some significant dose-dependent changes were found in the alkaline phosphatase and cholesterol values in both the male or female rats, which seemed to indicate that exposure to over more than 300 mg/kg of silver nanoparticles may result in slight liver damage [3]. Consistent with these findings, silver nanoparticles were found to be toxic to the liver in both male and female rats. A NOAEL (no observable adverse effect level) of 30 mg/kg and LOAEL (lowest observable adverse effect level) of 125 mg/kg are suggested based on the 90-day subchronic oral toxicity study in reference [4]. Target organs for silver nanoparticles in another 90-day subchronic inhalation toxicity study were considered to be the lungs and liver in male and female rats [5]. Lung function changes were observed when animals were subchronically exposed to silver nanoparticles over a period of 90 days [6]. A no observable adverse effect level of 100 $\mu\text{g}/\text{m}^3$ is suggested from the experiment in reference [5]. Many researchers have studied the genotoxicity of silver nanoparticles. However, the majority of studies could not reflect the practical genotoxicity effect because they performed experiments with *in vitro* systems on microorganisms and cell lines. In contrast, we conducted a study for an *in vivo* system with rats. Notably, we carried out the genotoxicity studies for animals subchronically exposed *via* oral and inhalation routes. The genotoxicity of silver nanoparticles after 28 days of oral administration was negative for the *in vivo* micronucleus test [3]. Accordingly, we have investigated the genotoxicity of silver nanoparticles after subchronic inhalation exposure.

Materials and Methods

Generation of silver nanoparticles

The silver nanoparticles were generated as described in previous reports [2,6], and the rats were exposed to the silver nanoparticles in a whole-body-type exposure chamber (1.3 m^3 , Dusturbo, Seoul), consisting of a small ceramic heater connected to an AC power (91.6 V) supply and housed within a quartz tube case [7]. The heater dimensions were $50 \times 5 \times 1.5 \text{ mm}^3$, and a surface temperature of about $1,500^\circ\text{C}$ within a local heating area of $5 \times 10 \text{ mm}^2$ could be achieved within about 10 s. For long-term testing, the source material (about 160 mg, Daedeok Science, Daejeon) was positioned at the highest temperature point. The quartz case was 70 mm in diameter and 140 mm long [8]. Clean (dry and filtered) air was used as the carrier gas, and the gas flow was maintained at 30.0 L/min ($\text{Re} = 572$, laminar flow regime) using a mass flow controller (MFC, AERA, FC-7810CD-4V, Japan). In this study, the system produced different concentrations of nanoparticles (high, middle,

and low) in three separate chambers. The nanoparticle generator was operated at 30 L/min and this was mixed with the 200 L/min flow rate of the main flow through the high-concentration chamber. Using the MFC for the first particle sampler, a portion of the high nanoparticle concentration was diverted to the middle-concentration chamber and diluted by the MFC flow rate. In the same way, a portion of the middle nanoparticle concentration was also diverted to the low-concentration chamber and diluted by the MFC flow rate. The flow rates for the high, middle, and low doses were $47.02 \pm 0.14 \text{ lpm}$, $6.76 \pm 0.16 \text{ lpm}$, and $5.42 \pm 0.18 \text{ lpm}$ (mean \pm S.E.), respectively.

Monitoring the inhalation chamber and analysis of silver nanoparticles

In the individual chambers containing the different nanoparticle concentrations, the nanoparticle distribution with respect to size was measured directly using a differential mobility analyzer (nano-DMA, 4220, HCT Co., Ltd. Korea, range 2.5-150 nm) and ultra-condensation particle counter (UCPC, 4312, HCT Co., Ltd. Korea, 3025, 0-108/ cm^3 detection range). Nanoparticles from 1.98 to 64.9 nm were measured using sheath air at 5 L/min and polydispersed aerosol air at 1 L/min, with these values being the operational conditions for nano-DMA and UCPC, respectively. The particle numbers per cm^3 in the fresh-air control chamber were measured using a particle sensor (4103, HCT Co., Ltd. Korea) that consisted of channel 1 (below 300 nm) and channel 2 (over 300 nm).

Animals and conditions

Six-week-old male and female, specific-pathogen-free (SPF) Sprague-Dawley rats were purchased from SLC (Tokyo, Japan) and acclimated for 2 wks before starting the experiments. During the acclimation and experimental periods, the rats were housed in polycarbonate cages (5 rats per cage) in a room with controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 7\%$) with a 12-h light/dark cycle. The rats were fed a rodent diet (Harlan Teklab, Plaster International Co., Seoul) and filtered water *ad libitum*. The 8-week-old rats, weighing about 253 g for the males and 162 g for the females, were then divided into 4 groups (10 rats in each group): fresh-air control, low-dose group (target dose, 0.6×10^6 particles/ cm^3 , $1.0 \times 10^9 \text{ nm}^2/\text{cm}^2$), middle-dose group (target dose, 1.4×10^6 particles/ cm^3 , $2.5 \times 10^9 \text{ nm}^2/\text{cm}^2$), and high-dose group (target dose, 3.0×10^6 particles/ cm^3 , $5.0 \times 10^9 \text{ nm}^2/\text{cm}^2$), and exposed to silver nanoparticles for 6 hr/day, 5 days/wk, for 13 weeks [5]. 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

evaluated at the time of purchase, at the time of grouping, once a week during the inhalation exposure, and before necropsy.

Rat bone marrow micronucleus test

The micronucleus assay was conducted using a method based on OECD guideline 474 [9,10]. Briefly, the rats were killed 24 hr after the last administration, then the femurs were removed and the bone marrow collected in 1.5 ml tubes containing 1 ml of fetal bovine serum, and these tubes were then centrifuged for 5 min at 1,000 rpm. Two smears were prepared, which were allowed to dry in air prior to fixation with methanol and staining with an acridine orange solution. One drop of a 0.04 mM acridine orange solution in a phosphate buffer was placed on the fixed cells and covered with a coverslip. The number of micronucleated polychromatic erythrocytes (MNPCEs) among every 2000 polychromatic erythrocytes (PCEs) per animal was examined within a day using a fluorescent microscope (Leica, Germany), and the slides were coded and scored blindly by one expert scorer. Plus, since normochromatic erythrocytes (NCE) become opaque when using a fluorescence stain, one additional slide per animal was stained with May-Grünwald and Giemsa solutions. To evaluate the bone marrow toxicity, the ratio $PCE / (PCE + NCE)$ was calculated based on a total of 200 erythrocytes using these slides [11].

Statistics

The statistical analyses were performed using SPSS 12.1, and the data was expressed as the mean \pm S.D. An X^2 test and a one-way analysis of variance (ANOVA) were applied to test all the data. A value of $p < 0.05$ indicated statistical significance.

Results

The *in vivo* genotoxic effect of the silver nanoparticles was

examined using a micronucleus test, as a mammalian *in vivo* micronucleus test is widely used for the detection of cytogenetic damage with test substances. The results of the micronucleus assay were determined after the 90-day silver nanoparticles inhalation exposure at various doses to the Sprague-Dawley male and female rats. All the animals appeared normal and remained healthy until the bone marrow was harvested. There were no significant changes in the body weights of the male rats. Although the female rats exhibited a significant body weight difference between the high and middle dose groups, there were no significant dose-related changes [5]. No significant organ weight changes were observed in either the male or female rats after the 90 days of silver nanoparticle exposure [5]. However, a dose-dependent deposition of silver nanoparticles was found in the blood, stomach, brain, liver, kidneys, lungs, and testes, indicating that the silver nanoparticles were systemically distributed in the mammalian tissues.

The frequency of micronucleated polychromatic erythrocytes (MN PCEs) in every 2000 PCEs for the male rats was 0.13, 0.21, and 0.18 percent for the groups exposed to low, middle, and high concentrations of silver nanoparticles, respectively, while that for the control was 0.14 percent. Plus, the frequency of MN PCEs in every 2000 PCEs for the female rats was 0.09, 0.08, and 0.13 for the groups exposed to low, middle, and high concentrations of silver nanoparticles, respectively, while that for the control was 0.14 percent. Thus, although a dose-related increase was found in the number of MN PCEs in the male rats, no significant treatment-related increase of MN PCEs was detected in the male and female rats when compared to the corresponding negative controls (Table 1, 2). No statistically significant difference in the $PCE / (PCE + NCE)$ ratio, representing the absence of bone marrow cytotoxicity, was observed in the male and female rats after silver nanoparticle exposure when compared with the control (Table 1, 2).

Table 1. Frequency of MN PCEs and $PCE / (PCE + NCE)$ ratio in bone marrow of male rats

Dose	No. of rats (Male)	Frequency of MN PCEs in every 2000 PCEs (Mean \pm SE, %)	$PCE / (PCE + NCE)$ (Mean \pm SE, %)
0	10	0.14 \pm 0.10	0.36 \pm 0.10
Low	10	0.13 \pm 0.09	0.39 \pm 0.07
Middle	10	0.21 \pm 0.09	0.31 \pm 0.05
High	10	0.18 \pm 0.13	0.30 \pm 0.08

MN PCE: micronucleated polychromatic erythrocytes, PCE: polychromatic erythrocytes, NCE: normochromatic erythrocytes.

Table 2. Frequency of MNPCEs and $PCE / (PCE + NCE)$ ratio in bone marrow of female rats

Dose (mg/kg/day)	No. of rats (Female)	Frequency of MN PCEs in every 2000 PCEs (Mean \pm SE, %)	$PCE / (PCE + NCE)$ (Mean \pm SE, %)
0	10	0.14 \pm 0.08	0.29 \pm 0.08
30	10	0.09 \pm 0.06	0.30 \pm 0.09
300	10	0.08 \pm 0.06	0.35 \pm 0.08
1,000	10	0.13 \pm 0.10	0.31 \pm 0.08

MN PCE: micronucleated polychromatic erythrocytes, PCE: polychromatic erythrocytes, NCE: normochromatic erythrocytes.

Discussion

At present, silver nanoparticles are the most commonly applied nanomaterial to clothing, foot wear, textiles, medical devices, home appliances, and cosmetics [12-15]. Despite their enormous benefits, their health and environmental effects are not obvious and many researchers endeavor to find what they are. It is known that silver nanoparticles reduce the mitochondrial function and glutathione level and increase the intercellular ROS level in various cells, which induces oxidative stress, DNA damage, and apoptosis [16-18]. In addition, many genotoxicity data have recently been reported. According to Wise et al. [19], they investigated the cytotoxicity and genotoxicity using silver nanospheres (30 nm) which were exposed to a medaka (*Oryzias latipes*) cell line [19]. They reported that silver nanoparticles induced cell death at 0.05-5 $\mu\text{g}/\text{cm}^2$ and chromosomal aberration and aneuploidy at 0.05-0.3 $\mu\text{g}/\text{cm}^2$. [19]. On the contrary, Lu et al. [20] assessed the cytotoxicity and genotoxicity for colloidal silver nanoparticles (30 nm) using the MTT assay and Comet assay at 100 $\mu\text{g}/\text{ml}$. The result showed that silver nanoparticles did not induce toxic effects for human keratinocytes [20]. There have been several *in vitro* genotoxicity experiments for silver nanoparticles. Yet the results conflicted with each other due to the use of various test methods, test materials, and other conditions, such as capping agents and particle aggregation/agglomeration. In a previous *in vivo* micronucleus study, after 28 days of silver nanoparticle oral administration, the present authors found that silver nanoparticles did not affect either the frequency of micronucleated polychromatic erythrocytes, taken as an indicator of DNA damage, or the PCE / (PCE + NCE) ratio, an indicator of toxicity to bone marrow cells, in male and female rats [3]. The current study used a different route of administration and a longer term of exposure duration; the male and female rats did not exhibit any effect on the frequency of their micronucleated polychromatic erythrocyte level or PCE / (PCE + NCE) ratio. Inhalation exposure of silver nanoparticles, which is known to be better dosimetry than any other exposure type, such as oral, injection, or intratracheal instillation, is an effective method for delivering silver nanoparticles systemically. The 90-day subchronic inhalation toxicity study conducted simultaneously with this genotoxic study showed that the lungs and liver were the major target tissues for prolonged silver nanoparticle exposure [5]. Furthermore, silver nanoparticle exposure-related bile-duct hyperplasia was noted in both the male and female animals [5]. 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 [2,3]. Thus the inhaled silver nanoparticle

could be exposed to bone marrow cells. Current other genotoxicity tests, such as the *in vitro* bacterial reverse mutation test (so called Ames test) and *in vitro* chromosomal aberration test also indicated negative results for the genotoxicity tests (unpublished data). Thus silver nanoparticle or silver ions generated from the surface of silver nanoparticles may not act as a direct or indirect mutagen.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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