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Acute Pulmonary Toxicity and Body Distribution of Inhaled Metallic Silver Nanoparticles

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The purpose of this study was to determine the acute pulmonary toxicity of metallic silver nanoparticles (MSNPs, 20.30 nm in diameter). Acute pulmonary toxicity and body distribution of inhaled MSNPs in mice were evaluated using a nose-only exposure chamber (NOEC) system. Bronchoalveolar lavage (BAL) fluid analysis, Western blotting, histopathological changes, and silver burdens in various organs were determined in mice. Mice were exposed to MSNPs for 6 hrs. The mean concentration, total surface area, volume and mass concentrations in the NOEC were maintained at 1.93×10^7 particles/cm^3, 1.09×10^{10} nm^2/cm^3, 2.72×10^{11} nm^3/cm^3, and 2854.62 μg/m^3, respectively. Inhalation of MSPNs caused mild pulmonary toxicity with distribution of silver in various organs but the silver burdens decreased rapidly at 24-hrs post-exposure in the lung. Furthermore, inhaled MSNPs induced activation of mitogen-activated protein kinase (MAPK) signaling in the lung. In summary, single inhaled MSNPs caused mild pulmonary toxicity, which was associated with activated MAPK signaling. Taken together, our results suggest that the inhalation toxicity of MSNPs should be carefully considered at the molecular level.

***Key words*:** Inhalation, Silver nanoparticles, Pulmonary toxicity, Distribution, Mitogen-activated protein kinase

INTRODUCTION

Recent developments in nanotechnology have been widely used in medical applications, such as drug/gene delivery systems (Kim *et al*., 2004), high-throughput screening (Yu *et al*., 2007) and body imaging (Perez *et al*., 2003). Silver nanoparticles have been investigated for their antibacterial function (Kim *et al*., 2007). Due to these characteristics, many studies have investigated medical materials (Tian *et al*., 2007), fabrics (Lee *et al*., 2007) and water filters (Jain and Pradeep, 2005). However, exposures of some silver compounds cause adverse health effects in the workplace (Bleehen *et al*., 1981; Rosenman *et al*., 1987).

Several previous studies have shown that inhaled insoluble silver compounds have adverse health effects on exposed workers (DiVincenzo *et al*., 1985) whereas exposure to soluble silver compounds causes argyria, argyrosis and irritation of the respiratory system (Moss *et al*., 1979; Rosenman *et al*., 1979; Rosenman *et al*., 1987), suggesting that soluble silver compounds may be toxic at lower levels than metallic/insoluble silver compounds in the workplace

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(Drake and Hazelwood, 2005). Therfore, the American Conference of Governmental Industrial Hygienists (ACGIH) recommends a threshold limit value-time weighted average (TLV-TWA) for silver aerosols of 0.1 mg/m^3 (metallic) and 0.01 mg/m^3 (soluble) due to the above reasons (ACGIH, 2001). Other studies have reported that low solubility nanoparticles such as TiO_2 cause a greater pro-inflammatory response than the same mass dose of fine particles in a lung epithelial cell line (Duffin *et al*., 2007; Monteiller *et al*., 2007). Thus, it appears that solubility, component, size and structure of nanoparticles may be an important factor in respiratory system toxicity.

The purpose of this study was to determine the acute pulmonary toxicity and body distribution of silver nanoparticles by inhalation of metallic silver nanoparticles (MSNPs) through our nose-only exposure chamber (NOEC) system. Thus, bronchoalveolar lavage (BAL) fluid analysis, Western blotting and silver burdens of organs were determined in mice.

MATERIALS AND METHODS

Animals and experimental design. Specific-pathogen-free male C57BL/6 mice (5-weeks-old), purchased from SLC (Hamamatsu, Japan) were maintained in our laboratory animal facility (23 ± 2°C, 50 ± 20% relative humidity and 12-hr light/dark cycle). The animals were acclimatized for at least 1 week prior to beginning the study. The mice were divided into two groups (unexposed and exposed to MSNPs) and each group consisted of 10 mice. The animals were exposed to MSNPs for 6 hrs in the NOEC system. Controls were exposed to air filtered with a high efficiency particulate air (HEPA) filter. Five mice in each group were sacrificed at the end of exposure at 0 and 24 hrs. All methods used in this study were approved by the Animal Care and Use Committee of Seoul National University (SNU-070109-2).

NOEC system. The nanoparticle exposure system was adopted from the methods of Kwon *et al.* (Kwon *et al*., 2008). Flow rate (10 *l*/minute) in the NOEC system was controlled by a mass-flow controller (Brooks Instruments, Hatfield, PA, USA).

MSNP generation and monitoring. We used a small-cartridge heater generator system modified from a previous method for metal nanoparticle generation (Jung *et al*., 2006). Briefly, silver evaporation by a small cartridge heater (6Ø, Kyungdo Electrol, Seoul, Korea) with a coiled silver wire (diameter 1.0mm, Sigma Aldrich, St Louis, MO, USA) was used. HEPA filtered air was passed through the generator and silver nanoparticles were formed by nucleation and growth processes in the generator (Granqvist and Buhrman, 1976; Lee, 1983). The silver nanoparticle number and size distribution were measured using a scanning nano particle spectrometer (SNPS, HCT 4450, Icheon, Korea) with a differential mobility analyzer (DMA, HCT 4220, Icheon, Korea). The measurement was performed in 25 independent measurements with 15 min intervals. Additionally, the silver nanoparticles were sampled with a nanoparticle sampler (HCT, Icheon, Korea) to further validate the actual size and analyzed under scanning electron microscopy (SEM, JEOL, Tokyo, Japan).

BAL analysis. BAL fluid from mice was obtained by repeated whole-lung lavage, The mice lungs were lavaged five times with 1 m*l* each of sterile saline (recovery of BAL fluid, > 85%). The BAL fluid was centrifuged at 400 ×g for 10 min. The volume recovered from the cell free supernatant of the first two lavages was kept separate from the other samples for biochemical analyses. Recovered cells from all lavages were resuspended in saline. Total cells (TC) and polymorphonuclear leukocytes (PMN) in the BAL fluid were counted with a hemacytometer and a Cyto-Spin analysis, respectively. Total protein (TP) in the BAL fluid supernatant was quantified using a Pierce BCA protein assay kit (Rockford. IL, USA), according to the manufacturer's protocol. The amount of lactate acid dehydrogenase (LDH) in the BAL fluid was measured using an automated biochemical analyzer (Vitalab, Merck, The Netherlands).

Western blot analysis of the lung. Lungs were collected, and the protein concentrations of the homogenized lysates were measured using a Bradford kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein sample were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 hr in Tris-buffered saline + Tween20 (TBST) containing 5% skim milk; immunoblotting was performed by incubating the membranes overnight with their corresponding primary antibodies at 4°C. The extracellular signal-regulated kinase (ERK1/2), c-Jun amino-terminal kinase (JNK), p38 and actin antibodies for Western blots were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After washing in TBST, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody (Zymed, San Francisco, CA, USA) and the bands-of-interest were detected using a luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan). Results were quantified using Multi Gauge version 2.02 program of the LAS-3000.

Silver burdens in the body. Mice were sacrificed at the endpoint of the study. Organs were collected and frozen at -20°C overnight in a glass vial. Then, the sample was moved to a vacuum freeze-drier and freeze-dried. The samples were weighed, and the silver burdens in the organs and tissues were quantified with an atomic absorption-graphite furnace (AA-GFA, Varian Spectr AA 880, Australia) after

microwave-assisted digestion of samples with HNO_3 using a microwave digestion system (MARS5, CEMS, Oxford, UK).

Statistical analysis. The Student's *t*-test (Graphpad Software, San Diego, CA, USA) and two-way ANOVA (IBM SPSS statics 19, Chicago, IL, USA) analysis was used to compare exposed groups with results obtained from the unexposed control group.

RESULTS

Characteristics and analysis of MSNPs generated in the NOEC exposure chamber. A non-aggregated and spherical morphology of the MSNPs was observed on SEM images (Fig. 1A). The SNPS study clearly showed that our generated MSNPs in the NOEC system narrowed the size distribution (Fig. 1B). Also, the concentrations of generated MSNPs were maintained constantly and uniformly during the exposure period (Fig. 1C). The geometric mean diameter of the generated MSNPs was 20.30 nm. The mean values of concentration, total surface area, volume, and mass concentrations in the NOEC system were maintained at 1.93×10^7 particles number/cm^3, 1.09×10^{10} nm^2/cm^3, 2.72×10^{11} nm^3/cm^3 and 2854.62 μg/m^3, respectively.

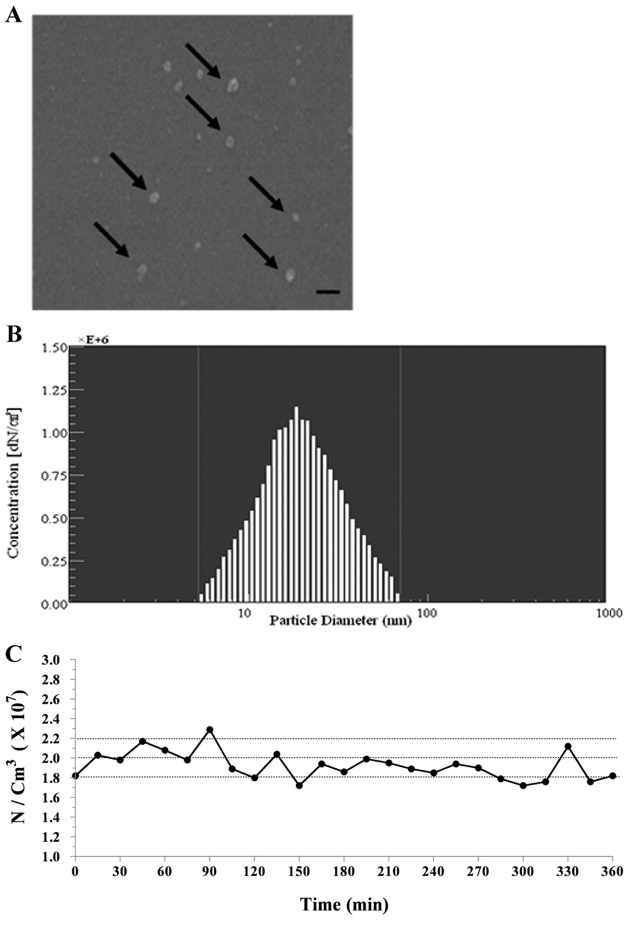

Fig. 1. Characteristics of the metallic silver nanoparticles were averaged over the exposure period. A). Scanning electron micrograph image of the metallic silver nanoparticles sampled with the nanoparticle collector, arrow: metallic silver nanoparticles; scale bar: 100 nm. B). nanoparticle size distribution of the metallic silver nanoparticles. C) Total particle number distribution of the generated metallic silver nanoparticles during the exposure period.

Pulmonary toxicity of MSNPs. Total protein (TP), LDH, and cytology assays were performed on BAL fluid to determine the level of lung injury. The TP level increased at the end of exposure (0 hr) but recovered by 24 hrs post-exposure (Fig. 2A). No significant changes in the LDH, TC or PMN values were observed in either MSNP-inhaled group (Fig. 2B). Furthermore, inhaling the MSNPs did not cause abnormal histopathological changes in the lung (data not shown).

Body distribution of inhaled MSNPs. The AA-GFA study was performed to evaluate the body distribution of silver in mice exposed to MSNPs. As shown in Table 1, silver was distributed in various organs and tissues, particularly the lungs, heart, spleen and testis. In contrast, silver burdens were not significantly different from those in the control brain, kidneys and liver. Silver burdens decreased rapidly in the heart, lungs and spleen at 24 hrs post-exposure, whereas they increased in testis. In particular, the silver burdens in the lungs increased an average of 13.18 times at the end of exposure compared with those in the control.

Activation of MAPK signaling. A previous study showed that nanoparticles induced immunotoxcity through MAPK pathway (Wang *et al*., 2007). Thus, we investigated effect of MAPK pathway by inhalation of MSNPs in lung. As shown in Fig. 3, analysis of the MAPK signal in the lungs of mice exposed to MSNPs showed a significant increase in ERK1/2, JNK and p38 protein levels compared with those in the control at the end of exposure, whereas ERK1/2, JNK and p38 decreased significantly at 24 hrs post-exposure.

DISCUSSION

The efficient and constant generation of nanoparticles by an inhalation exposure system is very important to evaluate pulmonary toxicity. The current study demonstrated that our MSNP generator is an efficient and simple system; thus, producing constant and uniform MSNPs during the exposure period (Fig. 1).

Biomarkers in the BAL fluid such as TP and LDH are related to pulmonary toxicity. LDH in BAL fluids is a susceptive marker of cytotoxicity (Warheit *et al*., 1991).

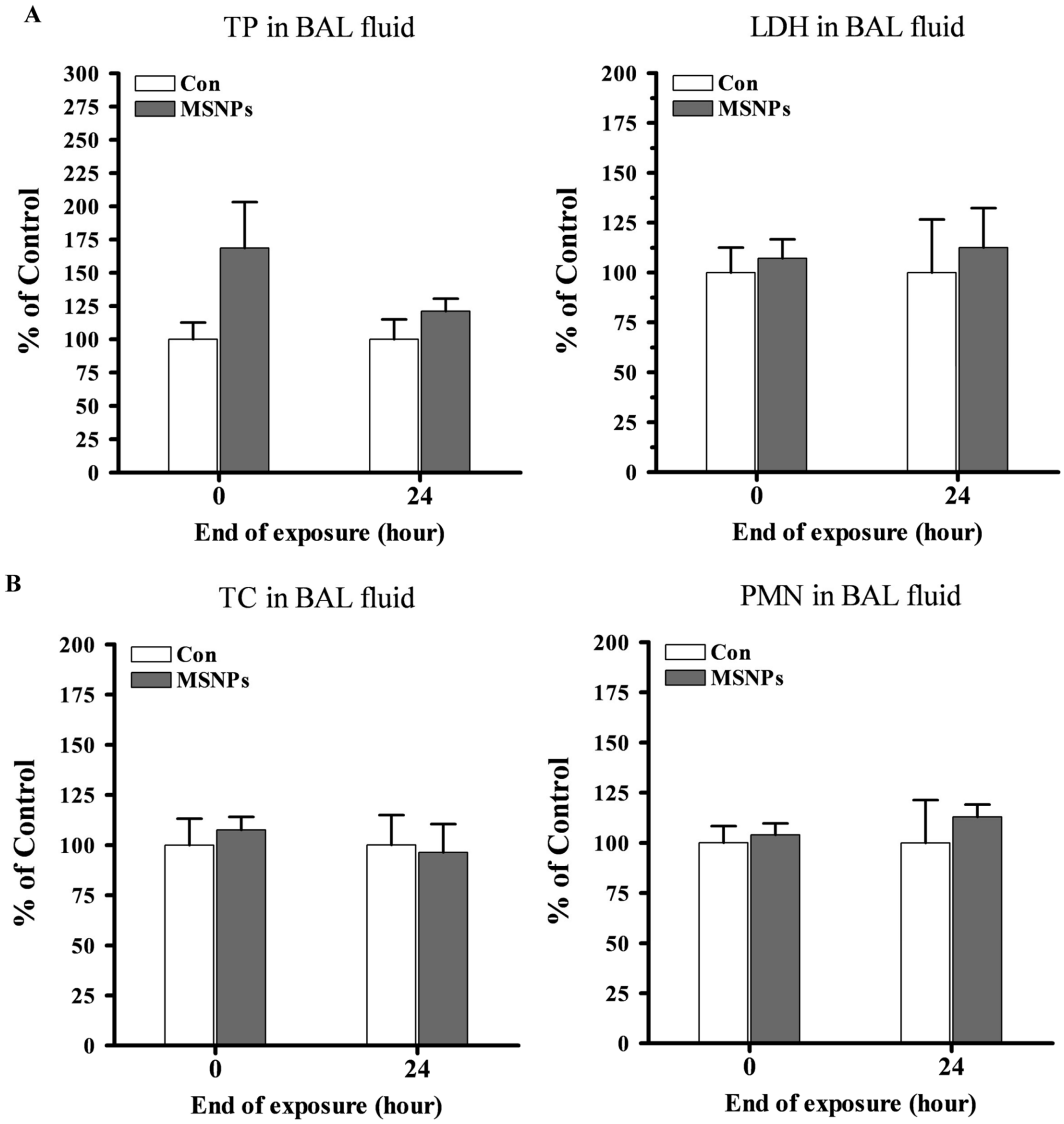

Fig. 2. Results of the biochemical and cytology analyses for mice exposed to metallic silver nanoparticles. A). Bronchoalveolar lavage (BAL) fluid biochemical values: total protein (TP), lactic acid dehydrogenase (LDH). B) Cytology of cells recovered by BAL fluid: total cells (TC) and polymorphonuclear leukocyte (PMN). Mean ± SEM.

Table 1. Body distribution of silver after inhalation of metallic silver nanoparticles

Organ	Group		
	Con	PE-0 h	PE-24 h
Brain	18.86 ± 6.49	62.22 ± 7.29	42.38 ± 6.45
Heart	ND	512.72 ± 21.77	84.33 ± 2.17$^{\#\#}$
Lung	36.97 ± 7.68	487.45 ± 24.42**	117.07 ± 11.07$^{*\#\#}$
Liver	37.24 ± 4.55	48.97 ± 4.69	52.34 ± 5.67
Spleen	ND	382.52 ± 120.35	186.59 ± 38.38
Testis	ND	126.23 ± 26.47	135.11 ± 15.22

ng/g dry weight, PE (post-exposure); ND (not detected); Mean ± SEM, *$p < 0.05$, **$p < 0.01$, statistically significant compared to control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant difference between end of exposure: 0 h and end of exposure: 24 h.

Enhanced TP concentrations in BAL fluid indicate damage to the alveolar/capillary membrane barrier in the lungs (Warheit *et al*., 2007). The pulmonary toxicity of the MSNPs increased the TP level in the BAL fluid at the end of exposure (0 hr) whereas LDH, TC and PMN did not show such patterns of change in the BAL fluid analysis (Fig. 2). Concentrations of the TP in BAL fluid recovered after 24 hrs post-exposure. Recently, two inhalation silver nanoparticle toxicity studies reported that repeated inhalation of metallic nanoparticles penetrates to other organs and affects lung function (Ji *et al*., 2007; Sung *et al*., 2008). In the 4 weeks study, no significant changes in body weight, lung histopathology, hematology, or blood biochemical values of inhaled silver nanoparticles (high dose group: 15 nm,

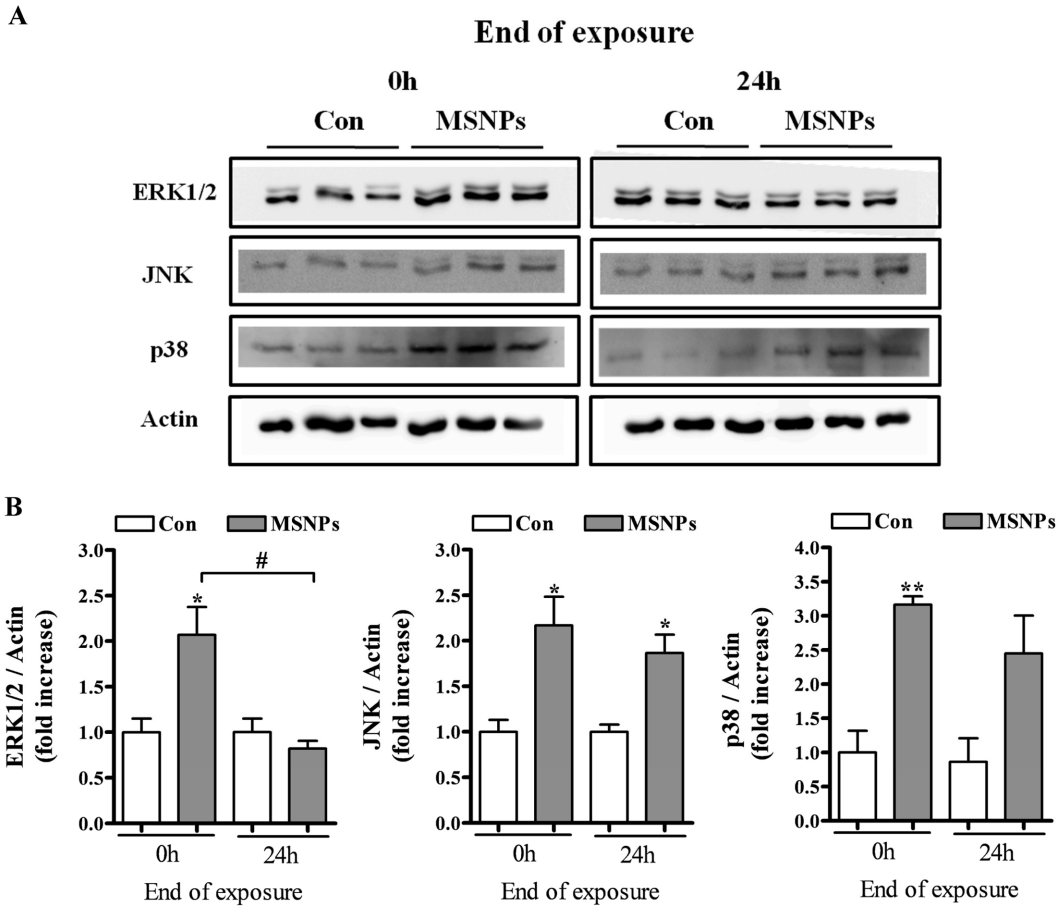

Fig. 3. Western blot analysis of MAPK family activation following inhalation of metallic silver nanoparticles in mice. A). Lysates from the lungs were analyzed for ERK1/2, p38 and JNK protein levels. B). Densitometric analysis. Data were normalized to actin (Mean ± SEM). Con, control; MSNPs, metallic silver nanoparticles, *P < 0.05 and **P < 0.01 significantly different from control. # p > 0.05 compared with the both end of exposure (0 and 24 hr).

1.32×10^6 total particle number/cm^3 and 61 $\mu g/m^3$) were observed. Lung function was suppressed significantly with increased TP concentrations in the BAL fluid in the female high concentration group (18.93 nm, 2.85×10^6 total particle number/cm^3 and 514.78 $\mu g/m^3$) during the 13-week repeated inhalation exposure study. Therefore, temporarily increased TP levels in BAL fluid suggest that MSNPs may cause permeability changes in the alveolar region.

The experiment of Takenaka (2001) showed that inhaled elemental silver nanoparticles (concentration $3 \times 10^6/cm^3$ and 15 nm modal diameter) are distributed in the lungs and other organs of rats, and that silver concentrations in the lungs decrease 24 hrs post-exposure (clearance rate: 1 - silver burden at 24 hr post-exposure/silver burden at end of exposure = 0.62). In contrast, our results demonstrated that MSNPs distributed in the lungs of mice decreased rapidly (clearance rate: 0.75) at 24 hrs post-exposure in mice. These results may be due to differences in circulating blood volume. Circulating blood volume in mice is 78~80 m*l*/kg, whereas rats have 55~70 m*l*/kg. Inhaled nanoparticles were most likely to affect the lungs where rapid translocation through the blood circulation is possible to other organs in the body (Kwon *et al*., 2008). Taken together, these results indicate that inhaled particles spread through the blood circulation, and that mice with a high circulating blood volume are better able to remove particles in the lungs than rats.

MAPKs play an important role in a variety of cell functions (Davis, 1993). Three subfamilies of MAPK have been identified, including ERK, p38 MAPK and JNK. They are regulated by three distinctive signaling pathways, but each family has its own function (Cano and Mahadevan, 1995). Acute exposure to ambient particulate matters (PMs) activates ERK, JNK and p38 MAPK signaling pathways in human bronchial epithelial cell lines (Samet *et al*., 1998; Wu *et al*., 2001). Furthermore, elevated MAPK can potentially induce an immune response in the lungs (Roberts *et al*., 2003). Results obtained in this study showed an increase in ERK1/2, p38 and JNK protein levels in the lungs (Fig. 3). These results showed that inhaled MSNPs

induce the p38-related pathway and the ERK/JNK signaling pathway which may affect immune function through the lungs. However, further studies are needed to clarify this issue. In fact, extensive studies must be conducted on the immune effect of repeated inhalation of MSNPs.

In summary, inhalation of MSNPs caused mild pulmonary toxicity and silver distribution in various organs. The silver burdens decreased rapidly at 24 hrs post-exposure in the lungs. Furthermore, inhaling MSNPs activated the MAPK pathway in the lungs. Taken together, our results suggest that the inhalation toxicity of MSNPs should be carefully considered at the molecular level.

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