INHALATION TOXICOLOGY, 2017
VOL. 29, NO. 12-14, 567–576
https://doi.org/10.1080/08958378.2018.1426661

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Subacute inhalation toxicity study of synthetic amorphous silica nanoparticles in Sprague-Dawley rats

Jae Hoon Shin[a], KiSoo Jeon[b], Jin Kwon Kim[c], Younghun Kim[c], Mi Seong Jo[c], Jong Seong Lee[a], Jin Ee Baek[a], Hye Seon Park[c], Hyo Jin An[c], Jung Duck Park[d], Kangho Ahn[e], Seung Min Oh[f] and Il Je Yu[b]

[a]Occupational Lung Diseases Research Institute, KCOMWEL, Incheon, Korea; [b]HCTm Co. LTD, Icheon, Korea; [c]Institute of Nanoproduct Safety Research, Hoseo University, Asan, Korea; [d]College of Medicine, Chung-Ang University, Seoul, Korea; [e]Department of mechanical Engineering, Hanyang University, Ansan, Korea; [f]Department of Nanofusion Technology, Hoseo University, Asan, Korea

ABSTRACT

Synthetic amorphous silica nanoparticles (SiNPs) are one of the most applied nanomaterials and are widely used in a broad variety of industrial and biomedical fields. However, no recent long-term inhalation studies evaluating the toxicity of SiNPs are available and results of acute studies are limited. Thus, we conducted a subacute inhalation toxicity study of SiNPs in Sprague-Dawley rats using a nose-only inhalation system. Rats were separated into four groups and target concentrations selected in this study were as follows: control (fresh air), low- ($0.407 \pm 0.066\ mg/m^3$), middle- ($1.439 \pm 0.177\ mg/m^3$) and high-concentration group ($5.386 \pm 0.729\ mg/m^3$), respectively. The rats were exposed to SiNPs for four consecutive weeks (6 hr/day, 5 days/week) except for control group of rats which received filtered fresh air. After 28-days of inhalation exposure to SiNPs, rats were sacrificed after recovery periods of one, seven and 28 days. Although there were minimal toxic changes such as temporary decrease of body weight after exposure, increased levels of red blood cells (RBCs) and hemoglobin (Hb) concentration, the lung histopathological findings and inflammatory markers in bronchoalveolar lavage (BAL) fluid including polymorphonuclear (PMN) leukocyte, lactate dehydrogenase (LDH), albumin and protein did not show significant changes at any recovery period. The results of this study suggest that the subacute inhalation of SiNPs had no toxic effects on the lung of rats at the concentrations and selected time points used in this study.

ARTICLE HISTORY
Received 17 October 2017
Revised 6 December 2017
Accepted 8 January 2018

KEYWORDS
Amorphous silica; inhalation toxicity; subacute; nanosilica

Introduction

Silica (silicon dioxide, SiO_2) is the most common nonmetal material on earth, which exists as crystalline and amorphous forms in nature. Amorphous silica can be divided into two categories according to the occurrence as naturally occurring (i.e. diatomaceous earth) and micron- or nano-sized synthetic amorphous silica such as silica gel, precipitated, pyrogenic, mesoporous and colloidal silica (Fruijtier-Pölloth, 2012). Typically, chronic occupational inhalation of micron-sized crystalline silica has been well documented as risk factor for pulmonary diseases including silicosis, chronic obstructive pulmonary disease (COPD), pulmonary tuberculosis, as well as lung cancer (IARC, 2012; Leung et al., 2012). In contrast to crystalline silica, amorphous silica has been considered less harmful and was classified Group 3 (not classifiable as to its carcinogenicity to humans) by the International Agency for Research on Cancer (IARC, 1997). However, there have been a few reports on the inhalation of synthetic amorphous silica particles showing that it might result in reversible pulmonary inflammation (Arts et al., 2007; Johnston et al., 2000), granuloma formation, emphysema (Lee & Kelly, 1992) and even more significant pulmonary toxic responses in comparison to crystalline silica forms (Kaewamatawong et al., 2005; Yazdi et al., 2010). Furthermore, since the synthetic amorphous silica nanoparticles (SiNPs) have quite different characteristics with regard to relative small particle size (<100 nm), high surface area ($>60\ m^2/g$), specific surface chemistry, bio-soluble and short retention half-life in the lung (Roelofs & Vogelsberger, 2004) unlike same materials in bulk forms, it is essential to carefully evaluate the hazard assessment and human adverse health effects of SiNPs (Karlsson et al., 2009).

SiNPs are one of the most attractive nanomaterials in many industrial and biochemical fields due to their unique physicochemical properties, ease of synthesis, surface modification and relative low toxicity to humans (Cheng et al., 2010; Tsai et al., 2009). Hence, they are widely used in a broad variety of industrial fields as additives to printing toners, varnishes, cosmetics and food over the past decades (Napierska et al., 2010). Also, manufactured SiNPs are used in biomedical technologies, such as biosensor, biomarker, gene transfection, drug delivery, cancer therapy and enzyme immobilization (Barik et al., 2008; Wang et al., 2015). With the growing applications and commercialization of synthetic amorphous SiNPs, increasing safety concerns about their

CONTACT Il Je Yu u1670916@chol.com HCTm CO., LTD, 74, Seoicheon-ro 578 Beon-gil Majang-myeon, Icheon-si, Gyeonggi-do 17383, Korea
Supplemental data for this article can be accessed here.

potential adverse effects on human health and environment have been raised (Holsapple et al., 2005; Nel et al., 2006). In addition, large production and use of SiNPs might lead to undesirable environmental, occupational and consumer exposure. Nevertheless, the lung is the main route of exposure to SiNPs (Card et al., 2008); the pulmonary toxicity of synthetic amorphous SiNPs is not fully understood and requires more information.

Previous pulmonary toxicity studies of synthetic amorphous SiNPs have demonstrated effects in *in vitro* and *in vivo* experimental settings. For instance, SiNPs have been shown to decrease cell viability, increase the levels of malondialdehyde (MDA) and lactate dehydrogenase (LDH) in a dose-dependent manner and are more cytotoxic than crystalline silica in cultured human bronchoalveolar carcinoma-derived cells (Lin et al., 2006). SiNPs were found to cause increased dose-dependent changes in lung inflammation as demonstrated by bronchoalveolar lavage (BAL) fluid neutrophilia after three consecutive intratracheal instillation in male Sprague-Dawley (SD) rats (Guichard et al., 2015). In addition, Du et al. (2013) reported that blood levels of proinflammatory cytokines such as interleukin (IL)-1beta, IL-6 and tumor necrosis factor (TNF)-alpha were increased after intratracheal instillation of SiNPs for a total of 16 times in Wistar rats. However, the above mentioned studies could not reflect on the toxicity assessment of actual inhalation exposure scenarios in occupational or environmental situations. Therefore, it is necessary to conduct the inhalation toxicity study of SiNPs in a similar manner.

The aim of present study was to evaluate the pulmonary toxic effects of SiNPs based on OECD Test Guideline No. 412 (Subacute Inhalation Toxicity: 28-Day Study, OECD, 2009) with consideration of lung burden measurement and BAL fluid analysis that will be included on mandatory test items in the revised new test guideline using a nose-only inhalation system. Thus, the animals were exposed to SiNPs aerosols for four consecutive weeks (6 hr/day, 5 days/week) except for the control group that received high efficiency particulate air (HEPA)-filtered fresh air and then allowed to recover for one, seven or 28 days. This study intended to provide the fundamental data for the assessment of potential adverse health effects to human.

Materials and methods

Aerosol generation

The SiO_2 aerosol nanoparticle generator is very similar to the furnace generator as described by Ostraat et al. (2008). Tetraethyl orthosilicate (TEOS, Sigma-Aldrich CO.LLC., St. Louis, MO) was used for the production of synthetic amorphous SiNPs aerosol in this study. Briefly, SiNPs aerosols were produced by hydrolysis and condensation of TEOS at 600 °C using a metal oxide generator equipped with furnace and dilution system for SiNPs (HCT Co., Ltd., Icheon, Korea; Stöber et al., 1968). Generated ethanol during the generation of SiNPs aerosols was eliminated by activated charcoal and only used SiNPs aerosols were employed in this study. HEPA-filtered fresh air was used as the carrier gas. The dilution rates of SiNPs with HEPA-filtered fresh air were as follows; SiNPs aerosols 3 L/min: fresh air 27 L/min for the low-, SiNPs aerosols 11 L/min: fresh air 19 L/min for the middle- and only SiNPs aerosols 30 L/min for the high-concentration group, respectively. The gas flow was maintained at 30 L/min using a mass flow controller (MFC, FC-7810CD-4V, AREA, Tokyo, Japan) and the flow rate in each chamber was maintained at 1 L/min.

Monitoring of nose-only inhalation chamber

The number of concentrations and size distributions of SiNPs aerosols in each chamber were measured using a condensation particle counter (CPC, Model 3775, TSI Inc., Shoreview, MN) and electrical particle sizer (EPS, HCT Co., Ltd., Icheon, Korea) equipped with differential mobility analyzer (DMA-20, HCT Co., Ltd., Icheon, Korea) and soft X-ray charger (HCT Co., Ltd., Icheon, Korea). The mass concentrations of SiNPs aerosols in each chambers were determined by sampling on a polyvinyl chloride (PVC) filter (size, 37 mm; pore size, 5 µm, SKC, Inc., Eighty Four, PA) based on gravimetric method between pre- and post-weight of filter and referred National Institute for Occupational Safety and Health (NIOSH) manual of analytical method 0500 (NIOSH, 1994a,b). The count median diameters (CMD) measured by EPS were also measured using a 13-stage (size range, 10 nm–10 µm) micro-orifice uniform deposit impactor (MOUDI 125 NR, MSP Co., MN) and 47 mm foil filter. The geometric standard deviation (GSD) of mass median aerodynamic diameter (MMAD) were derived from the cumulative mass distribution of filters.

Characterization of SiNPs

Generated SiNPs aerosols in a nose-only inhalation chamber (NIST 30, HCT Co., Ltd., Icheon, Korea) were collected on a transmission electron microscope (TEM) grid (copper grid, Formavar/Carbon 200 mesh, Tedpella, Inc., Redding, CA) using a nanocollector (HCT Co., Ltd., Icheon, Korea). Morphology of SiNPs was characterized using a field emission (FE)-TEM (JEM2100F, Tokyo, Japan) and elemental analysis was conducted by energy dispersive X-ray spectrometer (EDS, TM200, Oxford Instrument plc., Oxfordshire, UK) based on NIOSH analytical method 7402 (NIOSH, 1994a).

Total deposited doses per rat using multiple-path particle dosimetry without considering clearance

The daily lung burdens per rat in each concentration groups was estimated by multiple-path particle dosimetry model (MPPD v 2.11, 2002; Whalan et al., 2006) and characteristics of SiNPs aerosols in each chamber. The exposed groups of rats were exposed to SiNPs for 6 h per day and 20 days totally. The characteristics of SiNPs aerosol were a minute ventilation of 0.19 L/min in rat, MMAD of 76 nm, GSD 2.29 and lung deposition efficiency of 23.64%. The mass concentrations of SiNPs were 0.407, 1.439 and 5.386 mg/m^3 for the

low-, middle- and high-concentration group, respectively. The following calculations were made (Alexander et al., 2008):

Daily deposited dose (mg/day) per rat = average SiNPs concentration (mg/m^3) × minute ventilation volume (L/min = 0.06 m^3/h) × exposure duration (h/day) × particle lung deposition efficiency.

Animals and conditions

Six-week old male specific-pathogen free (SPF) Sprague-Dawley (SD) rats were obtained from OrientBio (Seongnam, Korea) and acclimatized for two weeks before commencement of nose-only inhalation exposure. During the acclimation, inhalation exposure and recovery periods, the rats were accommodated in polycarbonate cages (2–3 rats/cage) with wooden chip bedding and kept in individually ventilated cage racks. The housing conditions were maintained on a controlled temperature (22 ± 2 °C), humidity (40 ± 5%) and 12-hour light/dark cycle. The rats were received rodent diet (Woojung BSC, CO. Ltd., Suwon, Korea) and filtered water *ad libitum*. The rats were separated into four groups and target concentrations selected in this study were as follows: control (unexposed $n = 15$), low- (0.30 mg/m^3 SiNPs, $n = 15$), middle- (1.25 mg/m^3 SiNPs, $n = 15$) and high-concentration group (5.00 mg/m^3 SiNPs, $n = 15$), respectively. The rats were exposed to the SiNPs aerosols for four consecutive weeks (6 hr/day, 5 days/week) in each nose-only inhalation chamber. On the other hand, the rats of control group in the nose-only holders were exposed to HEPA-filtered fresh air with same times as exposed groups in a nose-only inhalation chamber. After four weeks-repeated inhalation exposure of SiNPs, the rats were followed by recovery periods for 1, 7 and 28 days (five rats/each recovery time period). The rats were observed daily for any significant clinical symptoms relevant to the exposure-related toxic effects. The body weights of rats were measured at the time point of obtainment, grouping, before necropsy and once a week until last recovery day. Also, the food intake of all rats during the exposure and recovery periods was also measured once a week. All animal procedures were carried out in accordance with the Hanyang University's Institutional Animal Care and Use Committee.

Hematology and biochemistry

At necropsy, the rats were anesthetized via intraperitoneal injection of anesthetic agent (Pentobarbital, Entobar®, Hanlim Pharm Co. Ltd., Seoul, Korea) in a dose of 1 mg/kg. Then, blood samples were immediately collected from aorta abdominalis. Differential counts of blood samples were performed using a blood cell counter (Hemavet 0950, CDC Tech., Dayton, OH) and levels of serum biochemical markers such as albumin were analyzed using biochemistry analyzer (7180 Automatic Analyzer, Hitachi High-Technologies Co., Tokyo, Japan). Blood coagulation times for the purpose of evaluation for the cardiovascular effects also were analyzed using coagulation analyzer (ACL7000, Diamond Diagnostics Inc., Holliston, MA).

Histopathology

As nose-only inhalation exposure might result in some skin contamination at head, neck and thorax area of rats, we conducted decontamination processes as follows: After euthanasia of the rats, they were cleaned with running water, then skinned completely and washed again because of the clean dissection and excision of the organs to be analyzed. The organs of rats, including brain, lungs, spleen, kidneys, thymus, testes, heart and liver were immediately removed and weighed. The right lungs were fixed with a 10% formalin solution under 25 water pressure, embedded in paraffin and stained with hematoxylin (H) and eosin (E) solution (BBC biochemical, Mount Vermon, WA). The stained right lungs were then examined with a light microscope (AX10, XEISS, Oberkochen, Germany) for the histopathological evaluation.

Analysis of BAL cells and fluids

After anesthesia of the rat, the chest cavity was opened for the collection of BAL fluid of rat. 3 mL of chilled magnesium- and calcium-free 0.9% NaCl solutions were injected and aspirated carefully and lavage was conducted for four times. Collected BAL fluids were then centrifuged at 500 g for 7 min at 4 °C. BAL cells were suspended in 1 mL of 0.9% sodium chloride solution and then stained with Wright-Giemsa solution. BAL cells were counted using a hemocytometer. The supernatants of centrifuged BAL fluids were further analyzed for the levels of hydrogen peroxide (H_2O_2) by high performance liquid chromatography (HPLC, Agilent 1200, Agilent Technologies, Santa Clara, CA) and malondialdehyde (MDA) by liquid chromatography and atmospheric chemical ionization tandem mass spectrometer (LC-APCI-MS/MS, API 3200 QTRAP, Applied Biosystems, San Mateo, CA) for the evaluation of pulmonary oxidative stress. Also, we measured lactate dehydrogenase (LDH), albumin and protein using biochemistry analyzer (7080 Automatic Analyzer, Hitachi High-Technologies Co., Tokyo, Japan) for the inflammatory responses of the lung.

Statistical analysis

Data from this study were represented as the arithmetic mean ± standard error (SE) and analyzed using SPSS 19.0 software (IBM Statistics. Chicago, IL). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's comparison between control group and exposed groups. The level of statistical significance was set at $p < .05$.

Results

Characteristics of SiNPs

The generated SiNPs aerosols were characterized as spherical shape, ranging from 60–70 nm by FE-TEM (Figure 1(A,B)).

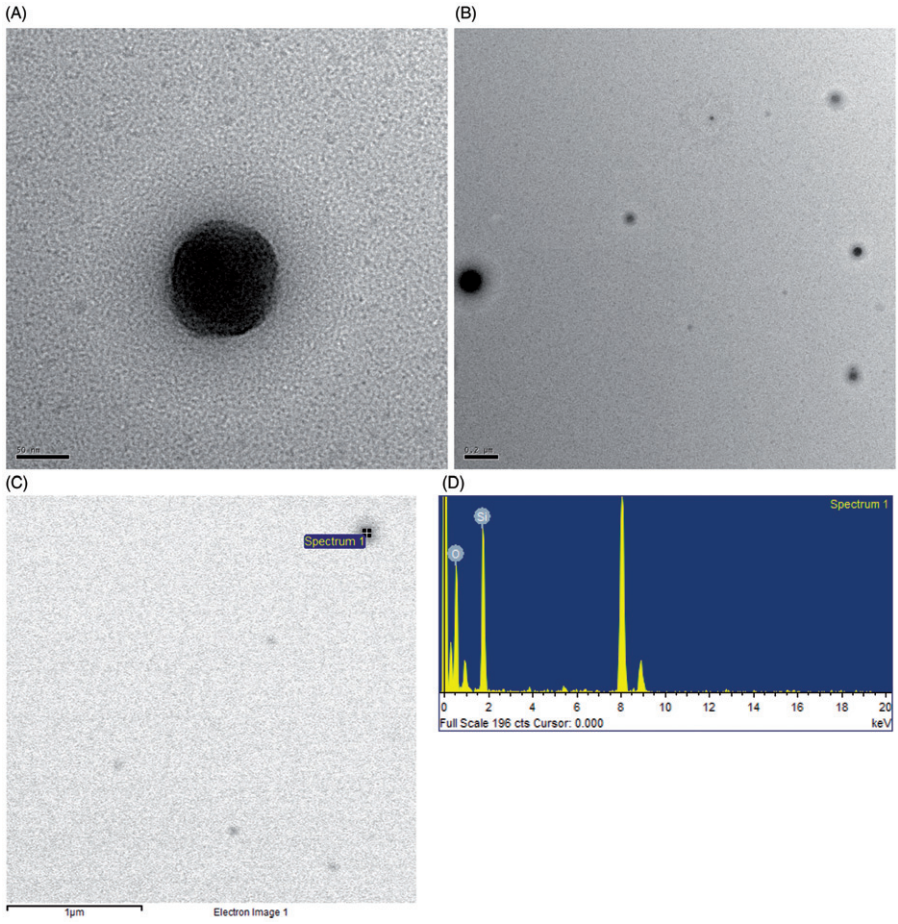

Figure 1. FE-TEM (A (scale bar: 50 nm) and B (scale bar: 200 nm)) and EDS (C and D) analysis of SiNPs in the nose-only inhalation chamber (100,000×).

Table 1. EDS analysis of SiNPs.

Element	Weight %	Atomic %
O	51.65	65.22
Si	48.35	34.78
Total	100.00	100.00

O: Oxygen; Si: Silicon.

The EDS analysis indicated the presence of two elements in atomic percentage (Table 1): oxygen (65.22%) and silicon (34.78%) (Figure 1(C,D)).

Concentration, size distribution and MMAD of SiNPs

The measured mean mass concentrations (mg/m^3) of SiNPs based on weighing the PVC filters in each nose-only inhalation chambers were as follows: 0.407 ± 0.066 for the low-, 1.439 ± 0.177 for the middle, 5.386 ± 0.729 mg/m^3 for the high-concentration group, respectively (Table 2). The measured mean number concentrations (particles/cm^3) using CPC and switching-mode power supply(SMPS) models were $4.254 \times 10^5 \pm 3.031 \times 10^4$, $1.395 \times 10^6 \pm 1.080 \times 10^5$ and $4.468 \times 10^6 \pm 3.532 \times 10^5$ for the low-, middle and high-concentration of SiNPs, respectively (Table 2, Figure 2). The count median diameter and GSD in each nose-only inhalation chamber measured by SMPS were 53 (2.56), 76 (1.53) and 79 (1.52) for the low-, middle and high-concentration of SiNPs, respectively (Table 2, Figure 2). Also, the MMAD and GSD for the SiNPs in the aerosols were 76 nm and 2.29, respectively.

Table 2. Mass concentrations and distribution of SiNPs exposed in each nose-only inhalation chamber.

Group	Mass concentration[a] (mg/m^3)	Particle concentration[b] (particles/cm^3)	Particle size[c] (nm)
Control	0.05 ± 0.02	1.92 ± 0.79	0
Low	0.407 ± 0.066	$4.254 \times 10^5 \pm 3.031 \times 10^4$	53 (2.56)
Middle	1.439 ± 0.177	$1.395 \times 10^6 \pm 1.080 \times 10^5$	76 (1.53)
High	5.386 ± 0.729	$4.468 \times 10^6 \pm 3.532 \times 10^5$	79 (1.52)

[a,b]Arithmetic mean ± arithmetic standard deviation.
[c]Geometric mean (geometric standard deviation).

Calculated total deposited doses per rat using MPPD without considering clearance

The calculated total deposited doses per rat of exposed groups based on the characteristics of SiNPs and MPPD model were as follows: 0.132 for the low-, 0.466 for the middle and 1.742 mg/rat for the high-concentration group, respectively.

Animal observation, food consumption and effect on body and organ weights

There were no significant clinical signs in all rats during the exposure and recovery periods. Also, there were no

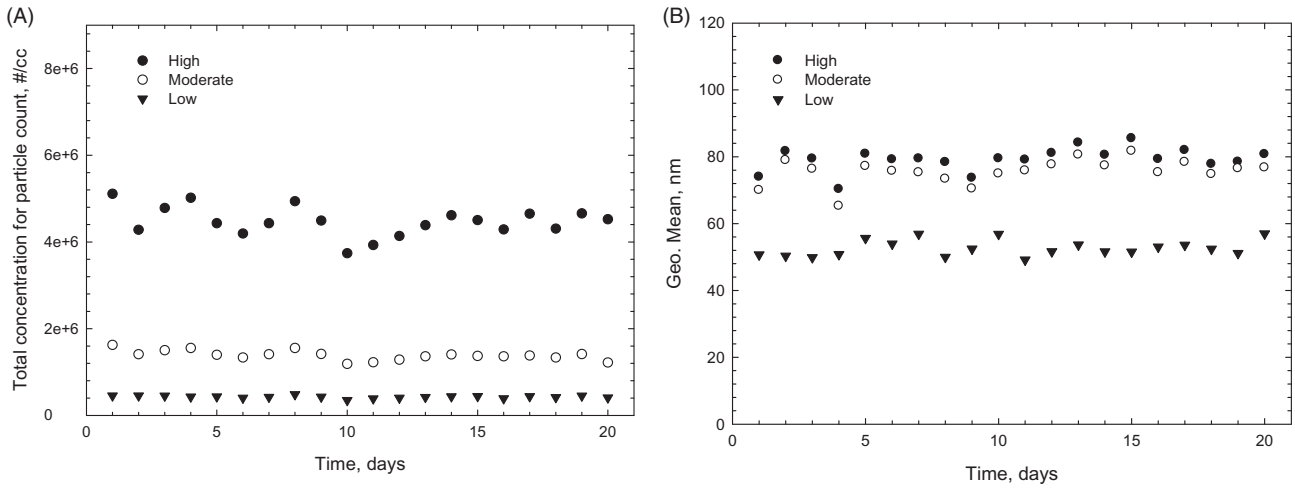

Figure 2. Mass concentration of SiNPs in each nose-only inhalation chamber during exposure periods.

significant changes in food intake between control and SiNPs-exposed groups (Supplementary Figure S1). Significant body weight loss was recorded for the high-concentration group at five weeks from the start of exposure when compared with control group (i.e. control, 408.81 ± 6.30 g versus high-concentration, 381.82 ± 6.16 g; $p < .05$). However, there were no significant body weight changes between control and SiNPs-exposed groups at the rest of observation time points (Supplementary Figure S2). There were no significant gross findings of rat organs including the brain, lungs, spleen, kidneys, thymus, testes, heart and liver at any of the necropsy time. Significant changes of relative weights of organs were noted in the left kidney (i.e. control, 0.31 ± 0.02 g versus high-concentration, 0.37 ± 0.02 g) and liver (i.e. control, 2.43 ± 0.05 g versus high-concentration, 3.17 ± 0.33 g) at recovery day 1 when compared with control group ($p < .05$). No other significant differences of relative weights of organs were recorded at the remaining necropsy times (Supplementary Figure S3–5).

Hematology and blood coagulation

The red blood cell (RBC) counts in the low-, middle- and high-concentration group ($p < .05$) and hemoglobin (Hb) concentration in the low- ($p < .05$) and middle-concentration group ($p < .01$) were significantly higher than those of control group at recovery day 1 (Supplementary Figure S6). The RBC count in the middle-concentration group ($p < .05$), mean corpuscular hemoglobin concentration (MCHC, $p < .05$) and relative percentage of lymphocyte (% LYM, $p < .05$) in the high-concentration group were significantly higher than those of control group at recovery day 7 (Supplementary Figure S6). On the other hand, relative percentage of neutrophil (% NEUT) in the high-concentration group was lower than that of control group at recovery day 7 ($p < .05$) (Supplementary Figure S7). At recovery day 28, the relative percentage and absolute count of monocyte in high-concentration group were significantly higher than those of control group ($p < .05$) and relative percentage of eosinophil (% EO) in low-concentration group were significantly lower than that of control group ($p < .05$; Supplementary Figure S8). There were no significant differences in the blood coagulation time such as prothrombin time (PT) and activated partial thromboplastin time (aPTT) between control and SiNPs-exposed groups during all recovery periods (Supplementary Figure S9).

Serum biochemistry

The levels of serum aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in the middle- ($p < .05$) and high-concentration group ($p < .01$), blood urea nitrogen (BUN), and creatine kinase (CK) in the high-concentration group ($p < .01$) were significantly decreased at recovery day 1 when compared with control group (Table 3). The levels of serum AST in the middle- ($p < .05$) and high-concentration group ($p < .01$), LDH in the low-, middle- and high-concentration group ($p < .01$), alanine aminotransferase (ALT; $p < .05$), total protein (TP; $p < .01$) and CK ($p < .01$) in the high-concentration group were significantly decreased at recovery day 7 (Table 3). At recovery day 28, the levels of serum CK in the high-concentration group were significantly decreased when compared with control group ($p < .05$) (Table 3, Supplementary Figure S10–12). All significant serum biochemical indices in this study except for ALT at recovery day 7 were decreased in a dose-dependent manner.

BAL cells and fluids

BAL cell counts (i.e. total cells, macrophages, polymorphonuclear (PMN) leukocytes and lymphocytes; Table 4), oxidative stress (i.e. H_2O_2) and pulmonary inflammatory markers (i.e. LDH, albumin and protein) in BAL fluid of rats showed no significant changes in exposed groups as compared with control group at all the recovery time points except the levels of MDA in the middle- and high-concentration groups were lower than those of control group ($p < .05$) (Table 5).

Table 3. Levels of serum biochemical markers after four weeks of inhalation exposure to SiNPs followed by 1, 7 and 28 days of recovery ($n = 5$ for each groups).

Group (mean ± SE)	Control	Low	Middle	High
Recovery day 1				
AST (IU/L)	211.40 ± 15.77	184.40 ± 4.46	160.00 ± 15.12[a]	91.40 ± 3.84[b]
LDH (IU/L)	3032.20 ± 312.63	2438.00 ± 73.93	2041.60 ± 365.26[a]	404.40 ± 76.01[b]
BUN (mg/dL)	20.10 ± 0.68	19.98 ± 0.87	19.96 ± 0.80	16.94 ± 0.60[a]
CK (IU/L)	1876.40 ± 171.38	1928.80 ± 92.57	1964.60 ± 257.80	561.60 ± 75.38[b]
Recovery day 7				
AST (IU/L)	220.80 ± 10.98	177.20 ± 22.51	166.80 ± 7.38[a]	104.40 ± 7.61[b]
LDH (IU/L)	3654.00 ± 230.13	2417.80 ± 507.86[b]	2209.60 ± 107.55[b]	1024.00 ± 153.38[b]
CK (IU/L)	2503.80 ± 194.02	2390.80 ± 404.78	1796.40 ± 162.60	970.00 ± 71.56[b]
ALT (IU/L)	36.20 ± 1.11	38.00 ± 0.55	34.20 ± 3.62	28.00 ± 1.87[a]
TP (g/dL)	5.82 ± 0.07	5.76 ± 0.09	5.70 ± 0.07	5.34 ± 0.12[b]
Recovery day 28				
CK (IU/L)	1839.00 ± 388.94	1645.20 ± 134.40	1115.80 ± 148.35	933.80 ± 83.52[a]

[a]$p < .05$ comparison with control group.
[b]$p < .01$ comparison with control group.
AST: aspartate aminotransferase; LDH: lactate dehydrogenase; CK: creatine kinase; ALT: alanine aminotransferase; TP: total protein.

Table 4. BAL cell counts of rats after four weeks inhalation of SiNPs followed by 1, 7 and 28 days of recovery ($n = 5$ for each groups).

Group (mean ± SE, ×10^6/mL)	Control	Low	Middle	High
Recovery day 1				
Total cell	0.24 ± 0.04	0.28 ± 0.05	0.28 ± 0.08	0.21 ± 0.05
Macrophage	0.21 ± 0.04	0.24 ± 0.04	0.24 ± 0.08	0.19 ± 0.05
PMN leukocyte	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocyte	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Recovery day 7				
Total cell	0.22 ± 0.04	0.25 ± 0.03	0.22 ± 0.09	0.27 ± 0.03
Macrophage	0.19 ± 0.04	0.20 ± 0.02	0.19 ± 0.07	0.21 ± 0.02
PMN leukocyte	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocyte	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.02	0.06 ± 0.01
Recovery Day 28				
Total cell	0.52 ± 0.06	0.44 ± 0.04	0.57 ± 0.07	0.55 ± 0.05
Macrophage	0.43 ± 0.05	0.37 ± 0.02	0.45 ± 0.06	0.45 ± 0.05
PMN leukocyte	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocyte	0.09 ± 0.02	0.07 ± 0.02	0.12 ± 0.03	0.10 ± 0.01

PMN: polymorphonuclear.

Table 5. Levels of pulmonary oxidative stress and inflammatory indicators in BAL fluid of rats after four weeks of inhalation exposure to SiNPs followed by 1, 7 and 28 days of recovery ($n = 5$ for each groups).

Group (mean ± SE)	Control	Low	Middle	High
Recovery day 1				
H_2O_2 (μmol/L)	0.98 ± 0.28	0.59 ± 0.30	1.27 ± 0.79	1.75 ± 0.53
MDA (nmol/L)	13.04 ± 3.28	10.23 ± 3.12	25.35 ± 11.60	8.44 ± 1.28
LDH (IU/L)	42.40 ± 7.39	38.60 ± 4.85	67.00 ± 30.52	61.80 ± 8.72
Albumin (μg/mL)	14.98 ± 2.65	13.71 ± 0.98	17.21 ± 4.12	22.97 ± 5.66
Protein (μg/mL)	8.45 ± 0.87	8.64 ± 0.43	10.11 ± 1.43	12.23 ± 4.81
Recovery day 7				
H_2O_2 (μmol/L)	0.54 ± 0.24	1.77 ± 0.93	5.05 ± 3.14	0.50 ± 0.28
MDA (nmol/L)	36.15 ± 20.72	6.35 ± 1.88	8.98 ± 2.23	7.10 ± 1.15
LDH (IU/L)	39.00 ± 6.59	47.00 ± 5.11	56.00 ± 24.36	29.00 ± 2.21
Albumin (μg/mL)	13.33 ± 2.16	24.52 ± 6.57	39.50 ± 17.43	13.38 ± 3.05
Protein (μg/mL)	7.81 ± 0.16	12.18 ± 2.31	19.40 ± 8.35	8.71 ± 0.73
Recovery day 28				
H_2O_2 (μmol/L)	0.94 ± 0.11	1.18 ± 0.24	1.31 ± 0.15	1.22 ± 0.26
MDA (nmol/L)	18.70 ± 4.64	27.68 ± 3.17	7.75 ± 0.75[a]	7.51 ± 0.77[a]
LDH (IU/L)	30.00 ± 1.02	25.00 ± 2.31	26.00 ± 1.52	25.00 ± 1.70
Albumin (μg/mL)	13.52 ± 2.29	16.01 ± 2.16	11.94 ± 1.67	11.90 ± 1.53
Protein (μg/mL)	8.32 ± 0.72	9.81 ± 0.76	8.58 ± 0.51	8.12 ± 0.45

[a]$p < .05$ comparison with control group.
H_2O_2: hydrogen peroxide; MDA: malondialdehyde; LDH: lactate dehydrogenase.

Histopathology

There were no significant toxicological and inflammatory changes in the lungs of exposed groups during the all recovery days (Figure 3).

Discussion

The aim of this study was to evaluate the inhalation toxicity of synthetic amorphous SiNPs which were characterized as spherical structure with a diameter less than 100 nm by TEM. As the utilization of SiNPs is increasing in various industrial and biomedical fields, the potential exposure to SiNPs by inhalation would be augmented in occupational and environmental settings. Since the respiratory system is a unique target for the potential toxicity of SiNPs, it is important to study the inhalation toxicity, which is more relevant than intratracheal instillation, oral administration or intravenous injection of the nanomaterials into the body cavity (Poland et al., 2008; Ryman-Rasmussen et al., 2009). To investigate the inhalation toxicity of SiNPs used in this study (i.e. low, 0.3 mg/m^3; middle, 1.25 mg/m^{3}and high, 5.0 mg/m^3), we developed new metal oxide generator equipped with furnace by the modification of Ostraat et al. (2008) for the generation of SiNPs aerosols. This pyrolysis generation method is very similar to the pyrolysis method used in the fumed silica manufacturing plant (Oh et al., 2014). The geometric mean diameter measured in the plant was 64 nm (40–90 nm), while the diameter of generated SiNPs aerosols in our study ranged from 60–70 nm. The elemental analysis by EDS of both fumed silica particles from manufacturing plant and our furnace generator showed silicon and oxygen as major component (Oh et al., 2014). These results support our notion that the physicochemical properties of generated SiNPs aerosols in this study were similar to those in the working environment, although detailed characterizations of fumed silica particles were studied in our as well as workplace monitoring study. Synthesized amorphous SiNPs aerosol particles in this study were consistently generated and maintained mass and particle number concentrations within ±20% during the all exposure periods. Target concentrations of subacute

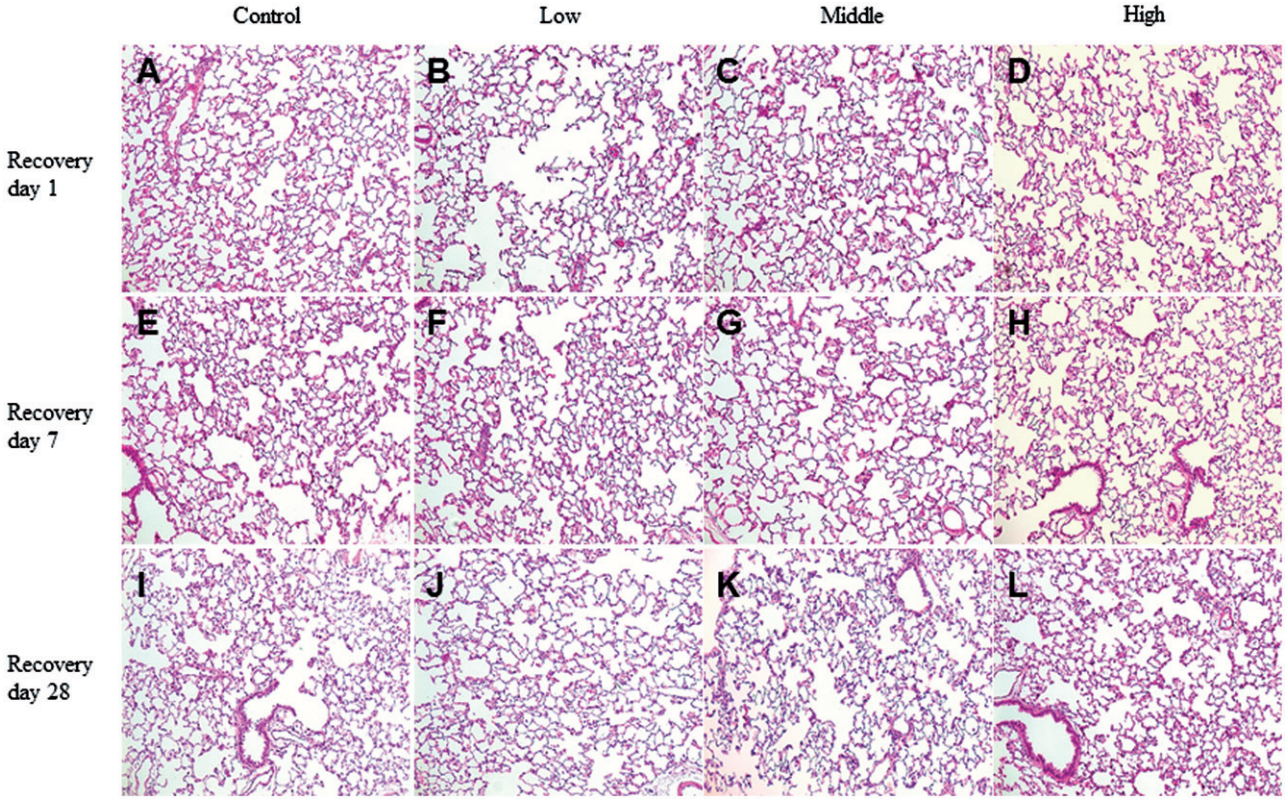

Figure 3. Lung histopathology after four weeks repeated inhalation of SiNPs followed by 1,7 and 28 days recovery. Control: A, E and I; Low: B, F and J; Middle: C, G and F; High: D, H and L.

inhalation exposure to SiNPs in this study were based on OECD (2009) guidelines for the testing of chemicals (Test No. 412: Subacute Inhalation Toxicity: 28-Day Study). One thing to be noted was the diameter of SiNPs in the low exposure group was much smaller than those in middle and high exposure groups in this study. The reason for the difference of diameters among concentration groups was resulted from the exposure of SiNPs aerosols at different exposure concentrations using a single generator. This gave rise to the decrease of mean free path of particles as the increase of exposure concentrations. The decreased mean free path of higher exposure group could induce growth of particles and eventually the diameter of SiNPs in the high concentration groups was larger than the low concentration group (Hinds, 1999). In fact, there were no statistical differences in particle size distribution among concentration groups. Thus differences in particle size distribution would not affect results of hazard assessment.

There were no significant changes of clinical signs, food intakes and gross findings of extracted organs such as liver during the 28-days inhalation of SiNPs and recovery periods. A body weight loss was observed for the high-concentration group in a dose-dependent manner at point fifth weeks from the start of exposure. However the food intakes were not significant between control and exposed groups, this decreased body weight was due to the dose-dependent decrease of food intake at fifth week and nose-only inhalation exposure-related stress. The rest of body weights and food intakes at measurement time points until last recovery day did not show any significant changes comparing to the control group. There were also no significant changes in the relative weights of most organs, although minor changes were noted in the left kidney and liver in the high-concentration group, which were significantly higher than those of control group at recovery day 1 without any dose-dependent fashion. Consequently, these results obtained from this study indicate that the subacute inhalation exposure to SiNPs only had a minimal toxic effect on the exposed groups. Similar results about clinical signs, measurement of body and organ weights related to the exposure of SiNPs have been reported, where the continuous intraperitoneal injection of mesoporous hollow SiNPs into ICR mice twice a week for six weeks at 10–50 mg/kg dosage showed no unusual behavior and obvious difference in body and organ weights changes of all the groups (Liu et al., 2012). We measured the hematological indices in the blood to evaluate toxic responses of SiNPs selected concentration in this study. Most of the hematological data were analyzed to be no significant differences when comparing to the control group, although some indices were significant in RBC counts and Hb concentrations at recovery day 1, RBC counts, MCHC, NE and LY at recovery day 7 and MO and EO at recovery day 28. Also, there were no significant differences in the blood coagulation time between control and exposed groups during all recovery periods. When comparing the hematological data from 180 normal SD rats which were not exposed to any toxic substances, most of significant hematological data were within a normal and healthy range (mean ±2 standard deviation; Giknis & Clifford, 2008) and had no dose-dependency. Thus, there was not relevant toxicity for selected high-concentration

(5.386 mg/m^3) group of exposed rats in this study. The toxicity of SiNPs related to hematological analysis has been demonstrated in intranasal exposure or intratracheal instillation of animal model, where intranasal exposure to SiNPs with diameters of 30, 70 or 100 nm at concentrations of 500 μg/mouse for seven days showed white blood cells (WBC), LY, MO and PT unchanged in all groups, although platelets (PLT) counts decreased and prolonged aPTT in the SiNPs-treated groups (Yoshida et al., 2013). On the other hand, intratracheal instillation of SiNPs with diameters of 30, 60 or 90 nm for a total of 16 times at concentrations of 10 mg/kg in Wistar rats produced elevated WBC and PLT counts in all SiNPs-treated groups (Du et al., 2013). These concentrations were unrealistic and high when comparing with realistic exposure situation in the workplace.

A few serum biochemical markers revealed significant differences between control and exposed groups. The levels of serum AST, LDH, BUN and CK at recovery 1 day, AST, ALT, LDH, CK and TP at recovery day 7, CK at recovery day 28 in exposed groups were lower than control group. Although significant serum biochemical markers showed a dose-dependent decrease except for ALT, it would appear that nose-only inhalation of SiNPs at the selected concentration in this study is not toxicologically relevant. Notwithstanding, a previous study showed that the single intravenous injection of SiNPs in ICR mice increased the serum levels of LDH in all treated groups and ALT in high-concentration group (Yu et al., 2013). In another study, intranasal exposure to SiNPs in BALB/c mice revealed that serum levels of ALT were in a normal range and BUN, ALB had no significant difference between control and exposed groups (Yoshida et al., 2013). These discordant results between previous studies and our report may be due to many differences in exposure routes, methods and target concentrations. Accordingly, our present results indicate that we need to more accurately evaluate the biological responses of SiNPs by nose-only inhalation on all tissues in the body such as liver histopathology and inflammatory cells such as polymorphonuclear (PMN) leukocytes, macrophages and lymphocytes.

The BAL cell count and toxicity indicators from the BAL fluid, including the levels of H_2O_2, MDA, LDH, albumin and protein failed to show any apparent toxic effects linked to the nose-only inhalation of SiNPs in this study. Also, the lung histopathological findings did not showed significant changes during all recovery periods. The absence of inflammatory changes even at high-concentration of SiNPs was due to the relatively high solubility of SiNPs in physiological solution (Vogelsberger & Schmidt, 2009) and this may indicate that the inhaled SiNPs were removed by the mechanism of mucociliary clearance of the lung. Similar reports about nose-only inhalation toxicity of amorphous SiNPs have been previously reported by Sayes et al. (2008) and Sutunkova et al. (2017). Sayes et al. (2008) demonstrated that inhalation of 37 nm SiNPs at concentration of 1.8 mg/m^3 or 83 nm at 86 mg/m^3 in rats for one or three days showed no significant differences in the levels of LDH, protein and alkaline phosphatase in BAL fluid of rats at any post exposure time period. In another previous study, Sutunkova et al. (2017) reported that inhalation exposure to nano-SiO2 collected from the flue-gas ducts for three or six months at concentration of 2.6 or 10.6 mg/m^3 had very low systemic toxicity and negligible pulmonary fibrogenicity due to the low SiO_2 retention in the lungs and relatively high solubility of SiNPs. On the other hand, Coccini et al. (2013) reported that intratracheal instillation of SiNPs at dosage of 600 μg/rat induced granuloma formation, pulmonary inflammatory responses and alveolar wall thickness in the lung parenchyma. Our findings on no increase of inflammatory cells, especially, PMN leukocyte may indicate that inflammatory cells infiltration could be observed in the early exposure period were recovered during 28-day exposure and recovery periods. The substantially lower of inflammatory effects of amorphous silica demonstrated in several other inhalation studies (Hemenway et al., 1986; Lee & Kelly, 1992, 1993; Warheìt et al., 1995) even at high-concentration of amorphous silica were due to the relatively higher solubility of amorphous silica than crystalline silica in physiological solution and may indicate that the inhaled SnNPs were removed by the mechanism of mucociliary clearance and dissolution which are the major mechanisms of clearance (IARC, 1997).

Importance of lung clearance particle should not be disregarded. Current modeling tool to estimate particle lung deposition and clearance such as MPPD is applicable to poorly soluble particles. Crystalline silica forms are completely different from amorphous forms. Crystalline forms are more toxic and may not be cleared well, while amorphous forms are more soluble, less toxic and cleared well (IARC, 1997; Sayes et al., 2008; Sutunkova et al., 2017). Thus we did not estimate clearance of amorphous silica by MPPD. We attempted to quantify the SiNPs particles in the lung for the lung burden analysis using inductively coupled plasma-optical emission spectrometry (ICP-OES), but we could not detect the SiNPs particles in the lungs because our analyzed results were lower than detection limit. Therefore, there is a necessity to analyze the SiNPs particles quantitatively using more sensitive methods.

In summary, although there were minimal toxic changes at the concentrations and selected time points used in this study such as temporary decrease of body weight after exposure, increased levels of RBC and Hb concentration, the lung histopathological findings and toxicological indicator in BAL fluid did not showed any significant change after exposure and during recovery periods. When taken together, the results of this study suggest that the subacute inhalation of SiNPs had a no toxic effect on the lung of rats. It was necessary to study the long-term inhalation toxicity for the evaluation of bio persistence and transference of SiNPs from the lung to other organs in the body.

Disclosure statement

The authors alone are responsible for the content and writing of this paper.

Funding

This research was supported by the Industrial Technology Innovation Program (10052901), Development of highly usable nanomaterial

inhalation toxicity testing system in commerce through the Korea Evaluation Institute of Industrial Technology by the Korean Ministry of Trade, Industry & Energy.

References

Alexander DJ, Collins CJ, Coombs DW, et al. (2008). Association of Inhalation Toxicologists (AIT) working party recommendation for standard delivered dose calculation and expression in non-clinical aerosol inhalation toxicology studies with pharmaceuticals. Inhal Toxicol 20:1179–89.

Arts JH, Muijser H, Duistermaat E, et al. (2007). Five-day inhalation toxicity study of three types of synthetic amorphous silicas in Wistar rats and post-exposure evaluations for up to 3 months. Food Chem Toxicol 45:1856–67.

Barik TK, Sahu B, Swain V. (2008). Nanosilica-from medicine to pest control. Parasitol Res 103:253–8.

Card JW, Zeldin DC, Bonner JC, Nestmann ER. (2008). Pulmonary applications and toxicity of engineered nanoparticles. Am J Physiol Lung Cell Mol Physiol 295:L400–11.

Cheng SH, Lee CH, Chen MC, et al. (2010). Tri-functionalization of mesoporous silica nanoparticles for comprehensive cancer theranostics: the trio of imaging, targeting and therapy. J Mater Chem 20:6149–57.

Coccini T, Barni S, Vaccarone R, et al. (2013). Pulmonary toxicity of instilled cadmium-doped silica nanoparticles during acute and subacute stages in rats. Histol Histopathol 28:195–209.

Du Z, Zhao D, Jing L, et al. (2013). Cardiovascular toxicity of different sizes amorphous silica nanoparticles in rats after intratracheal instillation. Cardiovasc Toxicol 13:194–207.

Fruijtier-Pölloth C. (2012). The toxicological mode of action and the safety of synthetic amorphous silica-a nanostructured material. Toxicology 294:61–79.

Giknis MA, Clifford CB. (2008). Clinical laboratory parameters for Crl: WI(Han). Wiilmington, MA: Charles river.

Guichard Y, Maire MA, Sébillaud S, et al. (2015). Genotoxicity of synthetic amorphous silica nanoparticles in rats following short-term exposure. Part 2: intratracheal instillation and intravenous injection. Environ Mol Mutagen 56:228–44.

Hemenway DR, Absher MP, Landesman M, et al. (1986). Differential lung response following silicon dioxide polymorph aerosol exposure. In: Goldsmith DF, Winn MD, Shy CM (eds.). Silica, silicosis and cancer. Controversy in occupational Medicine. Vol. 2, New York: Prager, 105–116.

Hinds WC. (1999). Coagulation. In: Aerosol technology: properties, behavior, and measurement of airborne particles. 2nd ed. Hoboken, NJ: Wiley-Interscience.

Holsapple MP, Farland WH, Landry TD, et al. (2005). Research strategies for safety evaluation of nanomaterials, part II: toxicological and safety evaluation of nanomaterials, current challenges and data needs. Toxicol Sci 88:12–17.

IARC. (1997). IARC monographs on the evaluation of carcinogenic risks to humans, some silicates, cola dust and para-aramid fibrils. Vol. 68. Lyon, France: IRAC.

IARC. (2012). IARC monographs on the evaluation of carcinogenic risks to humans: Arsenic, metals, fibres, and dusts. Vol. 110C. Lyon, France: IRAC

Johnston CJ, Driscoll KE, Finkelstein JN, et al. (2000). Pulmonary chemokine and mutagenic responses in rats after subchronic inhalation of amorphous and crystalline silica. Toxicol Sci 56:405–13.

Kaewamatawong T, Kawamura N, Okajima M, et al. (2005). Acute pulmonary toxicity caused by exposure to colloidal silica: particle size dependent pathological changes in mice. Toxicol Pathol 33:743–9.

Karlsson HL, Gustafsson J, Cronholm P, Möller L. (2009). Size-dependent toxicity of metal oxide particles-a comparison between nano- and micrometer size. Toxicol Lett 188:112–18.

Lee KP, Kelly DP. (1992). The pulmonary response and clearance of Ludox colloidal silica after a 4-week inhalation exposure in rats. Fundam Appl Toxicol 19:399–410.

Lee KP, Kelly DP. (1993). Translocation of particle-laden alveolar macrophages and intraalveolar granuloma formation in rats exposed to Ludox colloidal amorphous silica by inhalation. Toxicology 77:205–22.

Leung CC, Yu IT, Chen W. (2012). Silicosis. Lancet 379:2008–18.

Lin W, Huang YW, Zhou XD, Ma Y. (2006). *In vitro* toxicity of silica nanoparticles in human lung cancer cells. Toxicol Appl Pharmacol 217:252–9.

Liu T, Li L, Fu C, et al. (2012). Pathological mechanisms of liver injury caused by continuous intraperitoneal injection of silica nanoparticles. Biomaterials 33:2399–407.

MPPD v 2.11. (2002). Multiple-path particle Dosimetry. Model v.2.0. Arlington, VA.

Napierska D, Thomassen LC, Lison D, et al. (2010). The nanosilica hazard: another variable entity. Part Fibre Toxicol 7:39.

National Institute of Occupational Safety and Health (NIOSH). (1994a) Manual of Analytical Method 0500: Particulate not otherwise regulated, Total.

National Institute of Occupational Safety and Health (NIOSH). (1994b). Manual of Analytical Method 7402: Asbestos by TEM.

Nel A, Xia T, Mädler L, Li N. (2006). Toxic potential of materials at the nanolevel. Science 311:622–7.

OECD. (2009). OECD guidelines for the testing of chemicals, Section 4/Test No. 412: subacute inhalation toxicity: 28-day study. Paris: OECD.

Oh S, Kim B, Kim H. (2014). Comparison of nanoparticle exposures between fumed and sol-gel nano-silica manufacturing facilities. Ind Health 52:190–8.

Ostraat ML1, Swain KA, Krajewski JJ. (2008). SiO_2 aerosol nanoparticle reactor for occupational health and safety studies. J Occup Environ Hyg 5:390–8.

Poland CA, Duffin R, Kinloch I, et al. (2008). Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotechnol 3:423–8.

Roelofs F, Vogelsberger W. (2004). Dissolution kinetics of synthetic amorphous silica in biological-like media and its theoretical description. J Phys Chem B 108:11308–16.

Ryman-Rasmussen JP, Cesta MF, Brody AR, et al. (2009). Inhaled carbon nanotubes reach the subpleural tissue in mice. Nat Nanotechnol 4:747–51.

Sayes CM, Reed KL, Glover KP, et al. (2008). Changing the dose metric for inhalation toxicity studies: short-term study in rats with engineered aerosolized amorphous silica nanoparticles. Inhal Toxicol 22:348–54.

Stöber W, Fink A, Bohn E. (1968). Controlled growth of monodisperse silica spheres in the micron size range. J Colloid Interface Sci 26:62–9.

Sutunkova MP, Solovyeva SN, katsnelson BA, et al. (2017). A paradoxical response of the rat organism to long-term inhalation of silica-containing submicron (predominantly nanoscale) particles of a collected industrial aerosol at realistic exposure levels. Toxicology 384:59–68.

Tsai CP, Chen CY, Hung Y, et al. (2009). Monoclonal antibody-functionalized mesoporous silica nanoparticles (MSN) for selective targeting breast cancer cells. J Mater Chem 19:5737–43.

Vogelsberger W, Schmidt J. (2009). Dissolution behavior of a nanoparticle in a microscale volume of solvent: thermodynamic and kinetic considerations. Inhal Toxicol 21:8–16.

Wang Y, Zhao Q, Han N, et al. (2015). Mesoporous silica nanoparticles in drug delivery and biomedical applications. Nanomedicine 11:313–27.

Warheìt DB, McHugh TA, Hartsky MA. (1995). DifferentiaI pulmonary responses in rats inhaling crystalline, colloidal or amorphous silica dusts. Scand J Work Environ Health 21:19–21.

Whalan JE, Foureman GL, Vandenberg JJ. (2006). Inhalation risk assessment at the Environmental Protection Agency. In: Salem H,

Katz SA (eds). Inhalation toxicology. 2nd edn. Boca Raton, FL: CRC Press, 26–8.

Yazdi AS, Guarda G, Riteau N, et al. (2010). Nanoparticles activate the NLR pyrin domain containing 3 (Nlrp3) inflammasome and cause pulmonary inflammation through release of IL-1α and IL-1β. Proc Natl Acad Sci USA 107:19449–54.

Yoshida T, Yoshioka Y, Tochigi S, et al. (2013). Intranasal exposure to amorphous nanosilica particles could activate intrinsic coagulation cascade and platelets in mice. Part Fibre Toxicol 10:41.

Yu Y, Li Y, Wang W, et al. (2013). Acute toxicity of amorphous silica nanoparticles in intravenously exposed ICR mice. PLoS One 8:e61346.