

Age-Related Differences in Pulmonary and Cardiovascular Responses to SiO_2 Nanoparticle Inhalation: Nanotoxicity Has Susceptible Population

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Epidemiologic studies have revealed that pollution by ambient particulates is associated with respiratory and cardiovascular diseases, particularly in older people. Toxicologic sensitivity of nanoparticles in different ages was investigated for the first time to demonstrate and explain an age-related difference in response to manufactured nanoparticles. Young, adult, and old rats physiologically inhaled air containing aerosol of manufactured SiO_2 nanoparticles (24.1 mg/m^3 ; 40 min/day) for four weeks. Changes in serum biomarkers, hemorheologic, pulmonary inflammation, heart injury, and pathology in rats of different ages and their corresponding controls were compared. Inhalation of SiO_2 nanoparticles under identical conditions caused pulmonary and cardiovascular alterations in old rats, yet less change in young and adult rats, including pulmonary inflammation, myocardial ischemic damage, atrio-ventricular blockage, and increase in fibrinogen concentration and blood viscosity. Old individuals were more sensitive to nanoparticle exposure than the young and adult rats. The risk of causing pulmonary damages was: old > young > adult. The risk of cardiovascular disorder was observed only in old age. Our results suggest that different ages may require different biomarkers for identifying pulmonary toxicity during inhalation of nanoparticles.

Introduction

Contamination of ambient air is an important public health issue. Epidemiologic studies revealed that increased levels of ambient nanoparticles are associated with an increase in morbidity, mortality, and incidence of cardiovascular diseases in humans (1–3). Inhalation of ambient particulate matter can adversely affect normal lung function (4) and trigger various cardiovascular effects (e.g., myocardial infarction (5), arrhythmia (6), thrombosis (7)) and elevation of blood viscosity (8). Epidemiologic investigations have found that inhalation of particulate matter in air could affect lung development in children (4) and is associated with lowering of cardiac automatic control in the older population (9, 10). The components and surfaces of these nanoparticles are too complicated to be identified exactly. Several pioneering studies have been devoted to the health effects of ambient nanoparticles (2, 11–13), but it is particularly difficult to identify direct linkage between a specific human disease and exposure to ambient particulates because the composition, size, and other parameters of the particulates are so complicated and variable. This kind of correlation is of great interest because it may provide key information about the origin of certain human diseases. Manufactured nanoparticles enable exploration of the underlying correlations between human diseases and nanoparticle exposure. The establishment of theoretical models based on epidemiologic data is possible because nanoparticles have known parameters (e.g., composition, particle size, concentration, dosage).

Nanoparticles of silicon dioxide (SiO_2) are industrially manufactured and available commercially. The amorphous SiO_2 nanoparticle is widely used in silicon rubber, varnishes, papermaking, and as an additive in drugs and cosmetics. SiO_2 nanoparticles can be readily mixed in air by slight airflow because of their very low density, so workers can readily inhale the aerosol polluted by SiO_2 nanoparticles (i.e., they have an occupational exposure). Inhalation is a main route of human exposure to manufactured SiO_2 nanoparticles during production, storage, transportation, and consumer use. To our knowledge, no studies have investigated the health effects of occupational exposure of manufactured nanoparticles by physiologic inhalation. Such a study may provide information about the correlation between the epidemiologic findings reported in refs 1–8 and the effects of nanoparticles on health.

We chose manufactured SiO_2 nanoparticles as compounds of known chemical composition and parameters to evaluate their effects on health. We focused particularly on pulmonary and cardiovascular effects and differences in health outcomes in different age groups.

Materials and Methods

Nanoparticles. Amorphous SiO_2 nanoparticles (purity >99.9%) were purchased from Jiangsu Haitai Nano Material Company Limited (Jiangsu, China). Before animal experiments, aerosol concentration in the particle exposure system was measured by a real-time monitor (Model 8520; DustTrak Aerosol Monitor, Trust Science Innovation Company Limited (TSI), U.S.A.). Initially, the distribution of particle size was measured by high-resolution atomic force microscopy (AFM; Nano III a SPM, Digital Instruments Incorporated, U.S.A.). The SiO_2 nanoparticle sample was scanned by AFM. The diameter in three directions was measured for each nanoparticle, and mean values of the geometric diameter for each of the 100 SiO_2 nanoparticles selected randomly calculated (Figure S1 in Supporting Information). Their arithmetic mean

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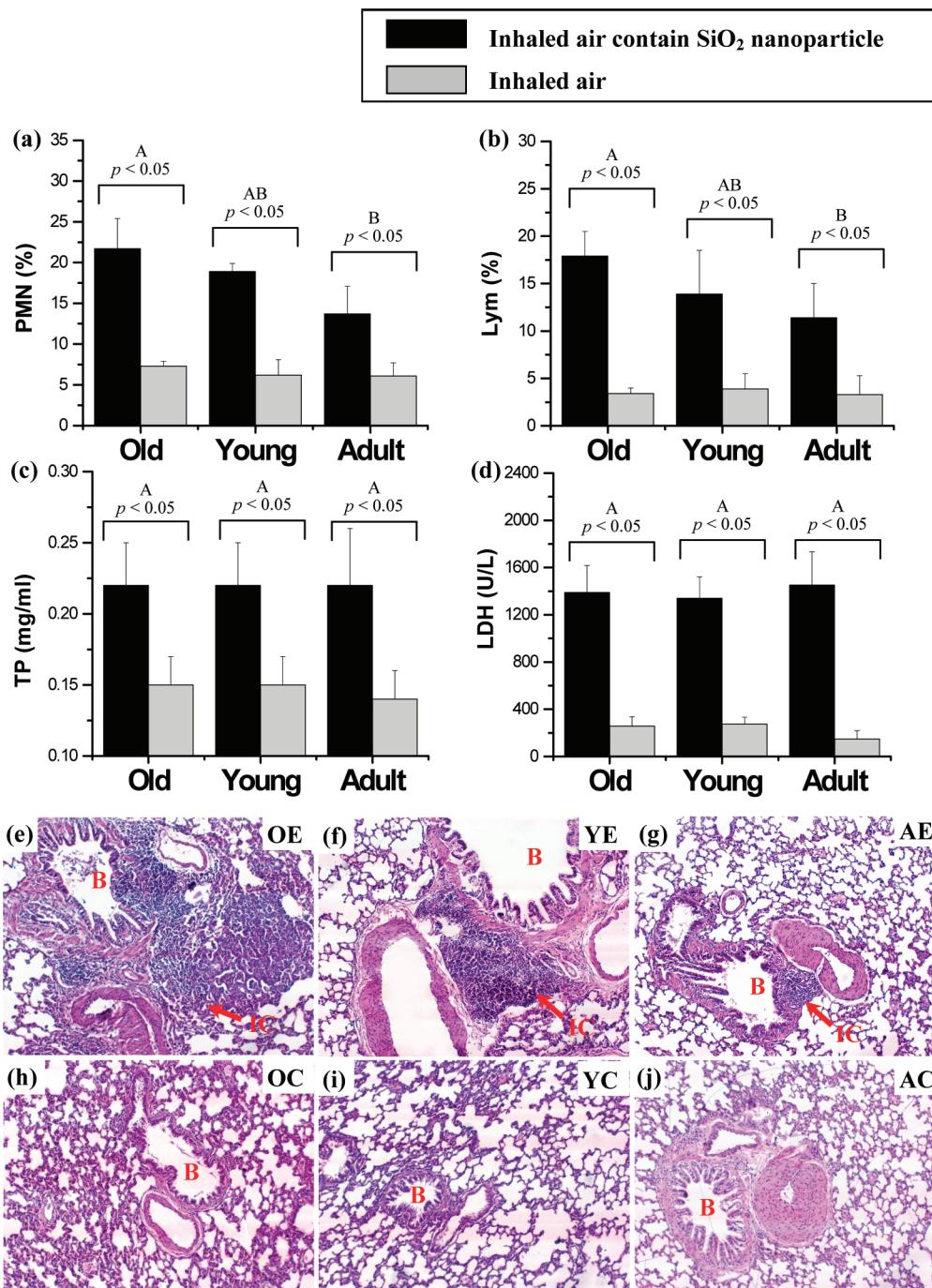


FIGURE 1. Pulmonary damages in different-aged rats who inhaled air containing manufactured SiO₂ nanoparticles. Panels a, b, c, and d display changes in bronchoalveolar lavage parameters as a function of age. *p* is the significance of the *t*-test between different ages of rats exposed to manufactured SiO₂ nanoparticles. A and B denote the Duncan class of Duncan's multiple-range test. Panels e–j show the pathologic changes in lung tissues of exposed rats in the old (e), young (f), and adult (g) groups. Panels h–j are their corresponding controls (magnification, $\times 100$). B indicates bronchia, and IC denotes inflammatory cells. *n* = 6 in each group, identical for all data below.

value represented the average size and the size distribution (Table S1, Supporting Information). The average size of the SiO₂ nanoparticle was 37.9 ± 3.3 nm; the specific surface area was 6.83×10^5 cm²/g, and the particle number was $1.52 \times 10^{10} \mu\text{g}^{-1}$.

SiO₂ nanoparticles were dispersed into medium by ultrasonic waves in sample preparation for AFM measurement to obtain a good dispersed state of nanoparticles in solution; a diluted concentration of particle numbers in medium was prepared and used for the measurement. Thus, results obtained from AFM measurement (Table S1, Supporting Information) are close to the original size of mono-

dispersed SiO₂ nanoparticles but do not reflect the actual size of SiO₂ nanoparticles as an aerosol in the particle exposure system at the time of inhalation. Nanoparticles present as aerosol in the exposure chamber may change their size from their original values because of aggregation.

We therefore did an online measurement of SiO₂ aerosol generated in the exposure chamber, which was directly coupled to Scanning Mobility Particle Sizers (TSI, U.S.A.) with size range of 3–800 nm and a differential mobility analyzer. Generation conditions for SiO₂ aerosol were identical to those in the animal experiments. We measured the background inside the exposure chamber before generation

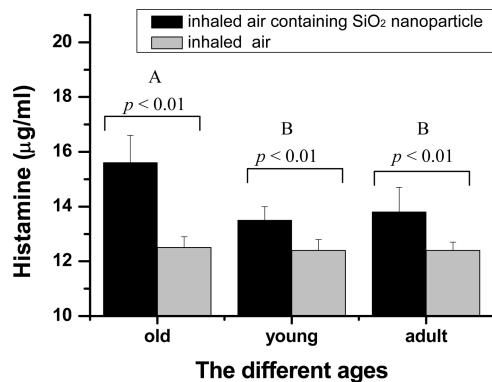


FIGURE 2. Changes in histamine level in serum of different-aged rats who inhaled air containing manufactured SiO₂ nanoparticles. *p* represents the significance of the *t*-test between exposed rats and the corresponding control. A and B are the Duncan class of Duncan's multiple-range test.

of SiO₂ aerosol. SiO₂ aerosol was generated and particle sizes (sample data) were measured online. Final results were obtained from subtracting background data from sample data (Figure S2, Supporting Information). Online measurement by Scanning Mobility Particle Sizers gave a mean size of SiO₂ aerosol in the exposure system of 42.9 nm, which was larger than the AFM measurement (37.9 nm). This was attributed to self-aggregation of SiO₂ nanoparticles in the aerosol state and was also demonstrated from the size distribution of SiO₂ particles: in the aerosol state, the size of SiO₂ nanoparticles was 20–80 nm (even being 7% larger than 200 nm (Table S2, Supporting Information)), but their original range was 20–50 nm (Table S1, Supporting Information). More details for the size distribution measurement of SiO₂ aerosol in the exposure chamber are given in the Supporting Information.

Methods. Animal experiments were carried out in compliance with guidelines set by the local ethics committee.

Details regarding animals, the particle exposure system, and online measurement of aerosol concentration during nanoparticle exposure by a real-time monitor are described in the Supporting Information. In brief, male Sprague–Dawley rats aged 3 weeks (65 g), 8 weeks (265 g), and 20 months (670 g) were models of young, adult, and old age groups, respectively. They inhaled air containing SiO₂ nanoparticles for four weeks. Grouping of the experimental animals was based on a two-factor cross-classification model: age and administration. The age factor involved three levels (young/adult/old) and the factor of administration involved two levels (exposure/control). Rats in groups labeled YE (young exposure), AE (adult exposure), and OE (old exposure) inhaled air containing SiO₂ nanoparticles for 40 min per day. Rats in corresponding control groups labeled YC (young control), AE (adult control), and OE (old control) inhaled room air. A concentration of SiO₂ nanoparticles of 24.1 mg/m³ was chosen. This concentration is approximately equivalent to 10× the particulate concentration in sand-dust; the higher concentration was chosen because rats have stronger endurance than humans (14–16). In the workplace or for users of nanoparticle-related products, the concentration in a local area (exposure) is usually much higher than that naturally generated in air. The conversion quantity of animal-inhaled SiO₂ nanoparticles was designed to be approximately equal to the daily quantity of SiO₂ nanoparticles inhaled by humans in sandy weather. At the end of administration, analysis of bronchoalveolar lavage (BAL) fluid, electrocardiography (ECG), hemorheology, serum biomarkers, and pathology was undertaken (see Supporting Information).

Mimicking natural (physiologic) inhalation is important for risk evaluation of respirable nanoparticles because

intratracheal instillation generates very different toxicological effects *in vivo* from that produced with natural inhalation (17, 18). There are few nanotoxicity studies with natural inhalation exposure in animals because of the lack of a quantification method for nanoparticles during inhalation processes. We therefore designed a novel nanoparticles exposure system: system of inhalation exposure for manufactured nanoparticles (SIEMN).

SIEMN is a sealed Plexiglas exposure chamber specifically for nanoparticle inhalation in animals. A detailed description of SIEMN is given in Supporting Information (Figures S3 and S2 and Table S2). SIEMN is not only a novel facility in which animals can inhale manufactured nanoparticles under physiologic conditions, but it also enables online measurement of nanoparticle concentration and particle-size distribution during exposure. Using SIEMN, young, adult, and old rats inhaled SiO₂ nanoparticles under identical conditions.

Results and Discussion

1. Effect of Inhalation of Air Containing Manufactured SiO₂ Nanoparticles on Pulmonary Inflammation of Rats of Different Ages. During natural inhalation of nanoparticles, the very small size of particulates enables them to readily reach the lungs with less turbulence in the nose, larynx, or branch points of the airways. The lung is therefore the first target organ of nanoparticle inhalation. A comprehensive study of pulmonary inflammation and lung injury in YE versus YC (young rats), AE versus AC (adult rats), and OE versus OC (old rats) groups was done. BAL parameters, that is, percentage of polymorphonuclear neutrophils (%PMN) and lymphocytes (%Lym) and levels of total protein (TP) and lactate dehydrogenase (LDH), are sensitive biomarkers of pulmonary inflammation caused by particulate inhalation (19). We initially measured changes in BAL parameters, and further investigated the pathology associated with pulmonary inflammation in young, adult, and old rats exposed to SiO₂ nanoparticles (Figure 1). Figure 1a–d shows that the four inflammation-related indices in SiO₂ nanoparticles-exposed rats of all ages became higher compared with corresponding control rats who inhaled room air (*p* < 0.01 in two-factor analysis of variance ANOVA). We analyzed data using Duncan's multiple-range test to quantitatively evaluate the sensitivity of nanoparticle-induced inflammation at different ages. Relative increment of %PMN in OE was much higher than that in AE and YE for the same exposure of SiO₂ nanoparticles (Figure 1a). An identical tendency was observed for lymphocyte release: old > young > adult (Figure 1b). Lymphocyte release is a sensitive indicator of an immune-based inflammatory response. The results imply that old rats suffered a more severe inflammatory response than young and adult rats under identical exposure conditions. This age-dependent tendency was not observed in the changes of TP and LDH, which denote permeability of the alveolar/capillary barrier and cell death, respectively.

We studied the lung tissues of all rat groups to examine if pulmonary inflammation caused pathologic changes. Results were consistent with the findings with BAL parameters. Lymphocytes, neutrophils, and monocytes were in the interstitial tissue around bronchia (Figure 1e–g), suggesting a physiologic response for clearance of inhaled SiO₂ nanoparticles in the process of acute pulmonary inflammation. Massive infiltration of inflammatory cells was observed in the lung tissue of old rats (Figure 1e) but only sporadically in young (Figure 1f) and adult rats (Figure 1g). This clearly demonstrated that, under identical exposure conditions, manufactured SiO₂ nanoparticles induced more severe pulmonary inflammation in old rats than in young or adult rats.

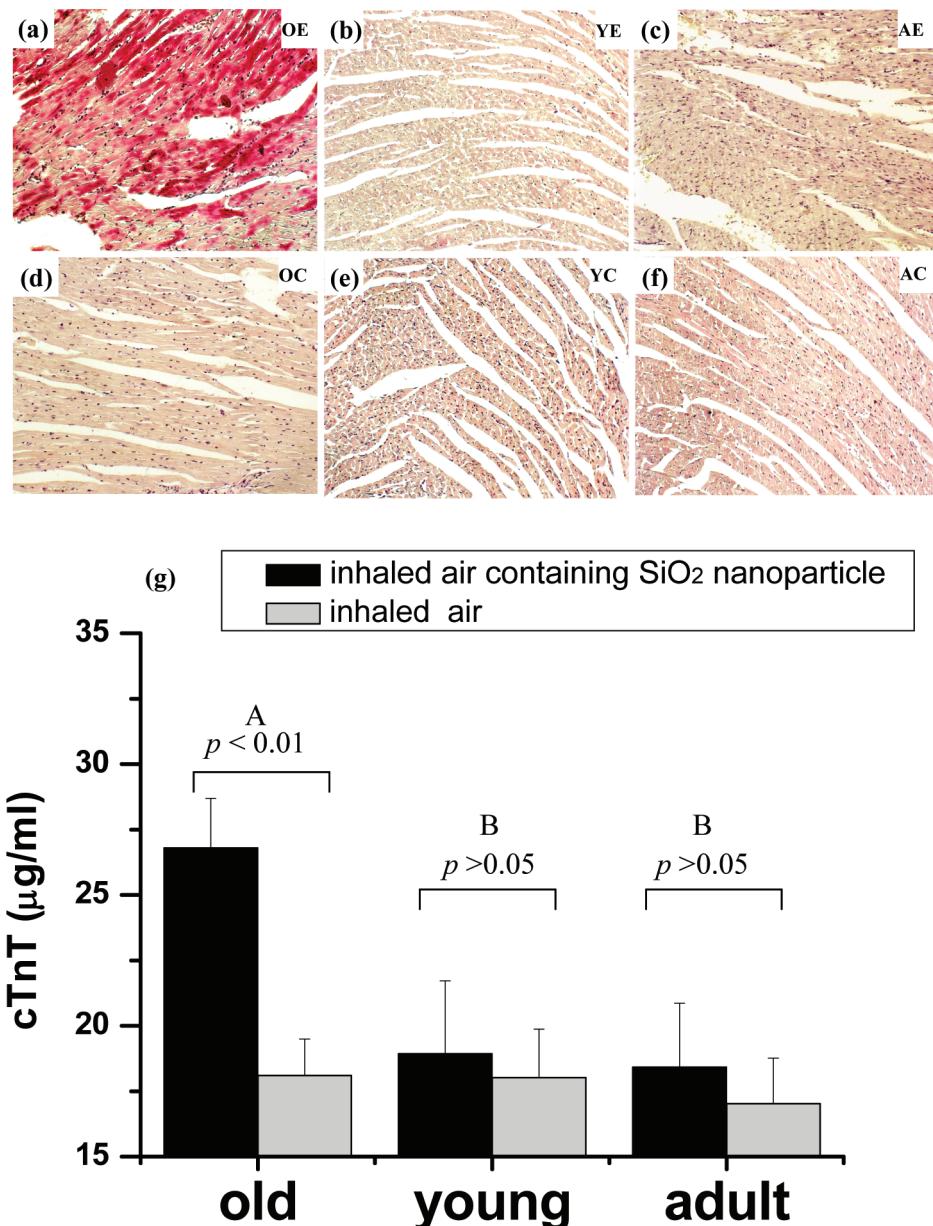


FIGURE 3. Nagar-Olsen staining shows the pathologic changes in the heart tissues of exposed rats in the old (a), young (b), and adult (c) groups. Panels d–f are corresponding controls (magnification, $\times 200$). Red color denotes anoxic myocardial cells and the fulvous is normal cells. Panel g shows changes in serum cTnT level as a function of age. p , A and B have the same meanings as in Figure 2. With identical inhalation of nanoparticles, myocardial ischemic damage was seen only in older rats.

Histamine plays a major part in the pathophysiology of inflammation; serum histamine is a sensitive indicator of inflammatory response (20, 21). Changes in serum histamine in exposed rats of different ages were investigated (Figure 2). Under the same exposure, the increment of serum histamine versus corresponding controls in old, adult, and young rats was 24.8%, 11.2%, and 8.9%, respectively. Duncan's multiple-range test showed that old rats were much more sensitive than young or adult rats. These data also unveiled the age-dependent sensitivity of nanoparticle exposure: old > adult \approx young. Old rats were more susceptible to nanoparticle exposure. Histamine level is a biochemical index of assessing potential risk of myocardial ischemia (21, 22), and its release usually results in coronary vasoconstriction (23) and platelet activation (24).

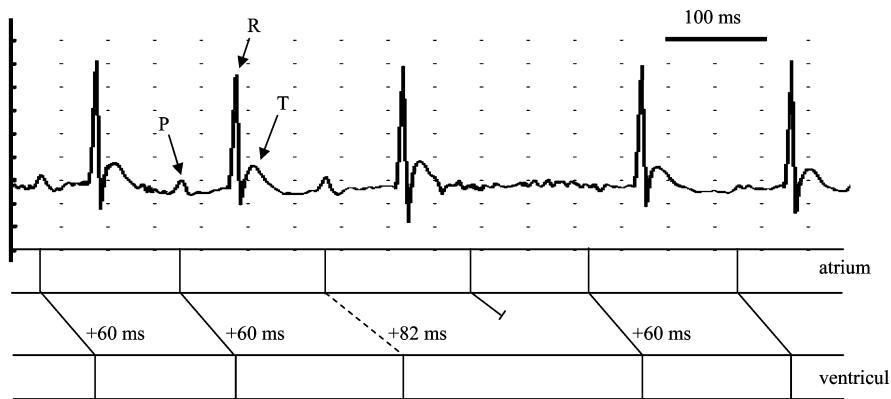
This is the first time that the sensitivity of nanotoxicity at different ages has been revealed: the most susceptible age for pulmonary damage because of nanoparticle inhalation is old age, followed by young age. This implies that nanosized

fraction of particles in air particulates may have more important roles in causing pulmonary diseases in older people than in adults. The potential risk of pulmonary diseases induced by inhalation of SiO₂ nanoparticles was old > young > adult.

As a different age implies a different lung volume and perhaps a different respiration rate, the dose of deposited particles in the lung may differ. The difference in toxicologic sensitivity between old, adult, and young rats may be partially caused by age because the old rat may have a higher respiration volume which means a higher uptake of SiO₂ nanoparticles than adult or young rats. This factor may partially reflect the observed tendency (old > young > adult) in pulmonary responses to exposure to SiO₂ nanoparticles.

2. Effect of Inhaling Air Containing Manufactured SiO₂ Nanoparticles on Cardiovascular Responses in Rats of Different Ages. The results stated above indicate that inhalation of SiO₂ nanoparticles cause pulmonary inflammation more readily in old rats than in young or adult rats.

(a) Wenckebach's Rhythm



(b) Normal Sinus Rhythm

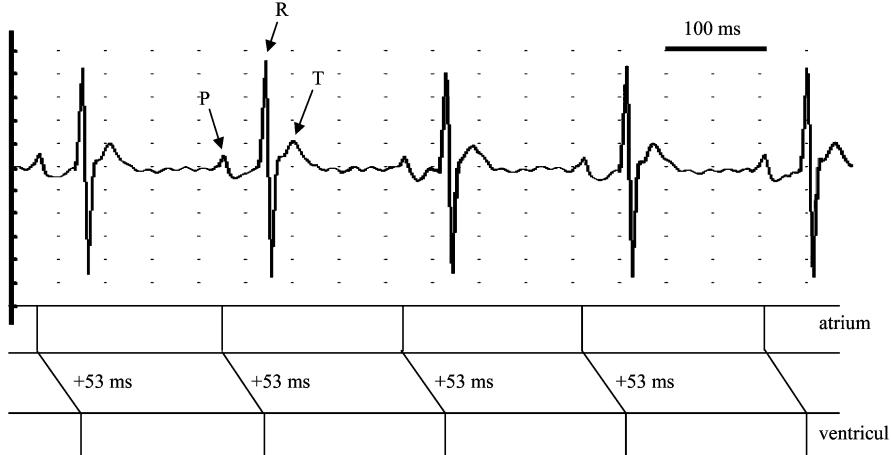


FIGURE 4. ECG shows that cardiovascular function changes occurred only in old rats ages but not in young and adult rats. Panel a represents the ECG of Wenckebach's rhythm from the old rat group, and panel b represents the normal sinus rhythm of the control group of old rats. The P-wave represents atria depolarization (excitation) and R-waves show ventricle depolarization. Only the older rats exhibited atrioventricular block. The auriculo-ventricular block can lead to progressive lengthening of P–R interval. In old rats, a lack of ventricular beat occurred until excitation conducted to the absolute refractory period of ventricular cells.

Researchers found that the pulmonary inflammation induced by airborne particulates can be linked with cardiovascular diseases (1, 8, 25). We investigated cardiovascular damage of rats of different ages exposed to SiO_2 nanoparticles. We measured myocardial damage by specific histopathologic diagnosis, sensitive serum biomarkers, ECG analyses, and hemorheological assay, parameters widely used to examine cardiovascular damage by inhaled airborne particulates.

Nagari–Olsen staining is a specific histopathologic evaluation for damage caused by myocardial ischemic. Anoxic/ischemic myocardial cells are dyed red versus fulvous for normal cells. The upper panel of Figure 3 shows histopathologic examination of heart tissues: age-dependent myocardial ischemia is clearly observed. The heart tissue of rats in each control group had normal fulvous color (Figure 3d–f). Damage because of myocardial ischemia occurred only in OE rats; many anoxic/ischemic myocardial cells were observed in their heart tissues (Figure 3a). Few changes were observed in young (Figure 3b) and adult (Figure 3c) rats compared with corresponding controls. We then measured cardiac troponin-T (cTnT) in serum, which is a highly sensitive biomarker for acute myocardial ischemia (26) (Figure 3, lower panel). Age-dependent damage was seen: identical inhalation of nanoparticles caused much more severe myocardial injury in old rats than in young or adult rats. The significant increase of cTnT level in the old group was 48.1% compared with control. cTnT increases were 8.2% and 5.0% in the adult and young groups, respectively, versus

the corresponding controls, and were not sufficiently large to cause statistic significance in the one-way ANOVA t -test ($p > 0.05$). Duncan's multiple-range test revealed that old rats were much more sensitive than young and adult rats at an identical exposure of nanoparticles.

Myocardial ischemia affects the normal function of myocardial cells (e.g., conductivity of excitation). We therefore studied the functional alternation of conduction between the atrium and ventricle as reflected in the ECG. Abnormal cardiac rhythms were observed only in the OE group. Five out of six rats in the OE group exhibited Wenckebach type-I periodicity (Figure 4a), which is a form of incomplete atrioventricular heart block (27). Because of progressive prolongation of atrio-ventricular conduction time, P–R interval increased until a dropped ventricular beat because excitation arrives during the absolute refractory period of ventricular cells. This occurred frequently after an inferior myocardial infarction and tended to be self-limiting. In YE and AE groups, rats appeared to have normal sinus rhythm similar to corresponding controls whose P–R intervals represent atrioventricular conduction and were quite stable (Figure 4b). The morbidity of atrio-ventricular block in rats of different ages is summarized in Supporting Information, Table S2.

We investigated hemorheologic changes after young, adult, and old rats inhaled SiO_2 nanoparticles: changes were observed in old rats (Figure 5), but not in young or adult rats. Whole blood is considered a non-Newtonian fluid, its apparent viscosity usually changes with different shear rate.

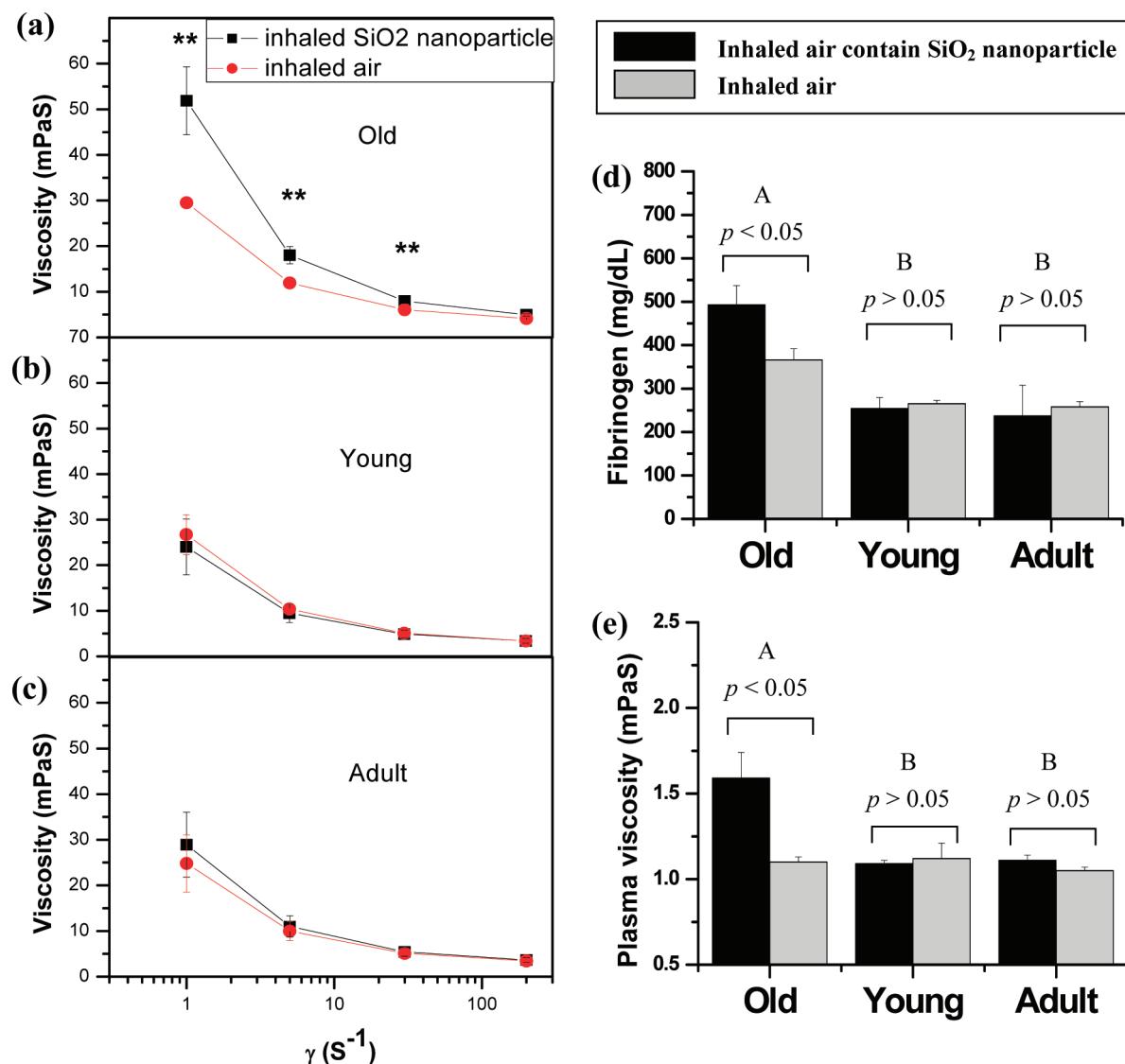


FIGURE 5. Changes in whole blood viscosity (η_b) in different-aged rats who inhaled air containing manufactured SiO₂ nanoparticles. η_b (mean \pm SD) is plotted against shear rate at intervals from 1 s $^{-1}$ to 200 s $^{-1}$ and hematocrit at 41%. η_b was significantly elevated in the exposed group of old rats (a), but no statistical differences were observed between the exposed and control groups in each of the young (b) and adult (c) groups. Two asterisks (*** represents $p < 0.01$ in the one-way ANOVA t -test. Panels d and e show the changes in fibrinogen and plasma viscosity (η_p) as a function of age. p , A, and B are defined in Figure 2.

Compared with the control groups (YC and AC), nanoparticle inhalation did not cause changes in YE and AE rats. In old rats, nanoparticle inhalation caused significant alteration of whole blood viscosity (η_b), particularly at low shear rate: η_b increased from 21.5 ± 1.2 to 51.8 ± 7.4 mPaS at 1 s $^{-1}$, from 11.9 ± 0.4 to 18.0 ± 1.91 mPaS at 5 s $^{-1}$, and from 6.1 ± 0.2 to 8.8 ± 0.51 mPaS at 30 s $^{-1}$ (Figure 5c). Elevation of η_b at low shear rate (1–30 s $^{-1}$) represents aggregation of red blood cells (RBCs). Under identical exposure, nanoparticle inhalation caused aggregation of RBCs more readily in the old-age group compared with the other two groups. We found significant elevation of serum fibrinogen in old rats, but not in adult or young rats (Figure 5d). One-way ANOVA t -test between exposure and control groups showed that fibrinogen in old rats changed from 366 ± 26 mg/dL to 493 ± 44 mg/dL, but without significant changes in young and adult rats. This result is consistent with the results of plasma viscosity (η_p) of nanoparticle-exposed rats (Figure 5(e)) because fibrinogen concentration determines plasma viscosity.

3. Preliminary Understanding of Age-Dependent Nanotoxicity. The results detailed above suggest that subacute inhalation of manufactured SiO₂ nanoparticles can cause

pulmonary inflammation and cardiac injuries. This effect is age-dependent: old individuals are more sensitive and susceptible than young and adult individuals. This is consistent with the findings in epidemiologic studies: increased levels of natural nanoparticles in air are associated with increasing human morbidity and mortality, mostly from respiratory and cardiovascular diseases (1–3). Analyzing our observations may help identify an underlying linkage between a specific human disease and exposure to ambient particulates or provide clues for understanding the intrinsic relation between human diseases and the environment.

The significant increase in fibrinogen level (from 366 ± 26 to 493 ± 44 mg/dL) was observed in the blood of old rats, but not in adult and young rats. Fibrinogen is one of the acute-phase proteins of pulmonary inflammation. Its concentration in blood increases usually when tissues are damaged. It was known that if the exotic particles caused inflammatory stimulations, the fibrinogen synthesis can be activated in hepatocytes and then be released into blood circulation (28). This indicates that nanoparticles readily disturb the normal physiologic equilibrium between coagulation and fibrinolysis in the blood of old individuals than

in adults and children. An increase in fibrinogen concentration can significantly contribute to blood viscosity, coagulation, blood-cell adhesion, and platelet aggregation; it can directly influence the rheologic characteristics of blood, and its elevation proportionally relates to myocardial infarction and thrombotic stroke (29, 30). Elevation of η_b at low shear rate (1–30 S⁻¹), which represents RBC aggregation, was also observed in old-age groups only. RBC aggregation is a risk factor for ischemic damage and may impair oxygen exchange in tissue or even embolize in capillary vessels.

During a study of the daily concentrations of air pollution and plasma fibrinogen of residents in London, England, a short-term association between air pollution and cardiovascular illness that was mediated through increased fibrinogen concentration related to inflammatory reactions was observed (31). Increased η_p and altered blood rheology of residents were reported during an episode of air pollution in Europe in 1985 (8); this was attributed to pulmonary inflammation caused by inhalation of polluted ambient particles (though data for different ages of residents were not recorded, and how much the contribution from inhalation of nanoparticles in air was unknown). The reported toxicologic phenomena and trends were very similar to nanoparticle inhalation in our study. The present results show that nanoparticle-induced changes in blood rheology are explicitly age-dependent: older individuals are much more susceptible than young individuals. Clinically, the increase in η_b can be perilous for cardiovascular diseases because (1) strengthened peripheral vascular resistance aggravates cardiac load, (2) decreased perfusion of the coronary artery leads to myocardial ischemia, (3) a high viscous bloodstream facilitates the propensity for thrombosis, and (4) increase in fibrinogen concentration usually occurs after acute myocardial infarction, and subsequent rheologic alterations (elevation of η_p and RBC aggregation) increase the risk of thrombosis. These deleterious factors can form a vicious circle that aggravates the potential risk of cardiovascular disease in old age.

The present results indicate that inhaled SiO₂ nanoparticles increased levels of %PMN and %Lym, which have key roles in causing inflammation and destabilization of atherosclerotic plaques *in vivo* (32). This was also an age-sensitive process. Because nanoparticle-induced inflammatory stimulation in old was much larger, it accelerates release of polymorphonuclear neutrophils and lymphocytes from bone marrow. This was supported by the nanoparticle-induced increment of serum histamine: [old]↑ > [adult]↑ > [young]↑. Interactions of inhaled particles with lung epithelial cells or during phagocytosis by alveolar macrophages were found to produce release of cytokines and chemokines that moved into the circulation and provoked systematic inflammation (33, 34). The latter accelerated progression of atherosclerosis and plaque destabilization (35–38). Taking into account the present findings, exploring if the effects on human health observed epidemiologically are dominated by nanosized fractions in air particulates will be very important.

Acknowledgments

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Supporting Information Available

Descriptions of the characterization of SiO₂ nanoparticles, particle exposure system, and experimental details, figures showing AFM image for SiO₂ nanoparticles, size distribution of SiO₂ aerosol in the exposure chamber, particle exposing system for experimental animals, aerosol concentration in

the particle exposure system during experimental periods, and tables listing the size distribution of SiO₂ nanoparticles and Atrio-ventricular block in different-aged rats. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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