Comparative study of pulmonary responses to nano- and submicron-sized ferric oxide in rats

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Abstract

Ferric oxide (Fe\textsubscript{2}O\textsubscript{3}) nanoparticles are of considerable interest for application in nanotechnology related fields. However, as iron being a highly redox-active transition metal, the safety of iron nanomaterials need to be further studied. In this study, the size, dose and time dependent of Fe\textsubscript{2}O\textsubscript{3} nanoparticle on pulmonary and coagulation system have been studied after intratracheal instillation. The Fe\textsubscript{2}O\textsubscript{3} nanoparticles with mean diameters of 22 and 280 nm, respectively, were intratracheally instilled to male Sprague Dawley rats at low (0.8 mg/kg bw) and high (20 mg/kg bw) doses. The toxic effects were monitored in the post-instilled 1, 7 and 30 days. Our results showed that the Fe\textsubscript{2}O\textsubscript{3} nanoparticle exposure could induce oxidative stress in lung. Alveolar macrophage (AM) over-loading of phagocytosed nanoparticle by high dose treatment had occurred, while the non-phagocytosed particles were found entering into alveolar epithelial in day 1 after exposure. Several inflammatory reactions including inflammatory and immune cells increase, clinical pathological changes: follicular hyperplasia, protein effusion, pulmonary capillary vessel hyperaemia and alveolar lipoproteinosis in lung were observed. The sustain burden of particles in AM and epithelium cells has caused lung emphysema and pro-sign of lung fibrosis. At the post-instilled day 30, the typical coagulation parameters, prothrombin time (PT) and activated partial thromboplastin time (APTT) in blood of low dose 22 nm-Fe\textsubscript{2}O\textsubscript{3} treated rats were significantly longer than the controls. We concluded that both of the two-sized Fe\textsubscript{2}O\textsubscript{3} particle intratracheal exposure could induce lung injury. Comparing with the submicron-sized Fe\textsubscript{2}O\textsubscript{3} particle, the nano-sized Fe\textsubscript{2}O\textsubscript{3} particle may increase microvascular permeability and cell lysis in lung epitheliums and disturb blood coagulation parameters significantly.

1. Introduction

Engineered nanoparticles can result in unique electronic, photonic and catalytic properties which display great differences from their bulk ones of the same materials. The highly biological and environmental activity and catalysis of nanoparticles have been widely and considerably used in disease treatment, pollutant degradation and so forth (Hanes et al., 1997; Hubbell and Langer, 1995; Torres-Martinez et al., 2001). However, such activities of nanoparticles in organisms might produce extremely high and long-term toxicity. A large amount of epidemiological and experimental studies indicate that ultrafine particles have close association with many respiratory and cardiovascular diseases, such as pneumonia, lung cancer, arteriosclerosis and myocardial infarction (Gamble and Lewis, 1996; Atkinson et al., 1999; Pope et al., 1995; Oberdörster et al., 2005). Particles in the nano-sized range have large specific surface area and high biological activity that may affect great epithelial surface area in lung and are thus more likely to deposit in the lower airways and the alveolar region (Donaldson et al., 1998). Increasing evidence demonstrated that nanoparticles could easily transfer through cell membrane, escape from macrophage phagocytosis and enter into lung interstitial (Renwick et al., 2001; Hoet et al., 2004). It is hypothesized that nanoparticles because of their long retention in the host tissues might repeat their highly catalytic activity with the host in cascade (Limbach et al., 2007).

Iron nanomaterials because of their super-paramagnetic characteristics and high catalytic abilities are of considerable interest for application in nanotechnology related fields including environmental catalysis, magnetic storage, biomedical imaging, magnetic...
target drug delivering, etc. (Hood, 2004). However, as a transition metal, iron is considered to play a pivotal role in modulating oxidative stress and other biological responses in particulate matter (PM), which is speculated to be the critical mechanism in eliciting the adverse effects of PM exposure. Zhou et al. (2003) reported that inhalation of iron particles in rats caused oxidative stress associating with a proinflammatory response in lung. The research of Simeonova and Luster (1995) demonstrated that iron on asbestos fibers catalyzed hydroxyl radical generation and thus stimulated TNF-α in alveolar macrophages expression. Possessing the transition metallic and nanoparticle properties, iron nanomaterials are hypothesized to display toxicity by exposure. However, up to now, the pulmonary responses of engineered iron nanomaterials by inhalation exposure in vivo remain to be adequately elucidated.

In this study, the nano- (22 nm) and submicron-sized (280 nm) Fe2O3 particles were purchased from Haoyun Industrial and Trade Co. Ltd., Beijing, China. The nano-sized Fe2O3 particles with a primary particle diameter of 22 nm were obtained from Zunye Nanomaterials Co. Ltd., Shenzhen, China. The average size of the particle was carefully determined by transmission electron microscopy (TEM, JEM 2010CX) (Fig. 1). The purity of the particles was determined by ICP-AES. The crystal form of the two-sized Fe2O3 particles was determined by X-ray Diffraction (XRD, PANalytical’s XPert PRO). The specific surface area was measured by BET method using a surface area analyzer (Autosorb-1 model, Quantochrome, USA). The characterizations of the two-sized Fe2O3 particles were shown in Table 1. The particles were ultrasonic dispersed for 10 min in saline solution before every use.

2. Materials and methods

2.1. Fe2O3 particles

Two kinds of engineered, nano- and submicron-sized Fe2O3 particles were used in the experiments. The nano-sized Fe2O3 particles with a primary particle diameter of 22 nm were purchased from Haoyun Industrial and Trade Co. Ltd., Beijing, China. The submicron-sized Fe2O3 particles with a primary particle diameter of 280 nm were used in the experiments. The nano-sized Fe2O3 particles with a primary particle diameter of 22 nm were purchased from Haoyun Industrial and Trade Co. Ltd., Beijing, China. The size of the particle was carefully determined by transmission electron microscopy (TEM, JEM 2010CX) (Fig. 1). The purity of the particles was determined by ICP-AES. The crystal form of the two-sized Fe2O3 particles was determined by X-ray Diffraction (XRD, PANalytical’s XPert PRO). The specific surface area was measured by BET method using a surface area analyzer (Autosorb-1 model, Quantochrome, USA). The characterizations of the two-sized Fe2O3 particles were shown in Table 1.

The particles were ultrasonic dispersed for 10 min in saline solution before every use.

2.2. Animals

Male Sprague Dawley rats weighing of 240–260 g were purchased from the Experimental Animal Center, Peking University. The animals were housed in plastic cages in a air-condition controlled room (20 ± 2 °C, 50–70% relative humidity, with a 12-h light/dark cycle). Commercial pellet diet and deionized water were available ad libitum. After a week acclimation, rats were divided into experimental and control groups randomly. Six rats were used in every treatment groups. All the animal studies were approved according to the Ethics Committee of Animal Care and Experimentation of the National Institute for Environmental Studies, China.

2.3. Intratracheal instillation

According to previous works of PM and nanomaterials study (Oberdörster et al., 2005; Hughes et al., 1998; Lam et al., 2004), two dosages were used in this experiment: 0.8 mg/kg bw was chosen as a low dose exposure and 20 mg/kg bw represented a high one. The animals were anesthetized by 40 mg/kg bw i.p. sodium pentobarbital (Solarbio, Germany) and then intratracheal instillation exposure. We tested the hypothesis that exposure to Fe2O3 nanoparticle could result in inflammatory and oxidative stress response in lung, and simultaneously might cause blood coagulation alteration.

2.4. Bronchoalveolar lavage (BAL)

After 1, 7 and 30 days of Fe2O3 particle instillation, the rats were anesthetized and immediately, 5 mL calcium and magnesium-free phosphate buffer saline (PBS) (0.1 mol/L, pH 7.3) was instilled into the lungs and lavaged for four times. The first two lavages of BAL fluid (BALF) were collected separately for biochemical assay and cell differential count. The BALF was centrifuged at 50 × g for 10 min at 4 °C. Free cell pellets were resuspended and the number of viable cells was recorded by a hemocytometer using the Wright’s exclusion dye. The supernatant of the primary BALF was aliquoted and stored at −80 °C till use. All the BAL protocols were performed with a standard technique outlined by Gilmour et al. (2004) and Henderson (2005). The lung tissues were taken and frozen in liquid nitrogen immediately.

2.5. Biochemical and cytological analysis of BALF

The concentration of total protein in BALF supernatant was determined by a modified Bradford assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) with bovine serum albumin (BSA) as the standard. The activities of lactate dehydrogenase (LDH) and acid phosphatase (ACP) in BALF were measured by colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The resulting cells from the primary and the secondary lavages were combined and resuspended for cytological examination. At least 5 × 104 cells were stained with Wright’s dye (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) on a glass slide. Macrophages, lymphocytes and neutrophils were counted to determine cell differentials under an oil immersion microscope with ×1000 magnification. At least 500 cells of each slide were counted.

2.6. Biochemical assay of lung homogenates

Lung tissues were homogenized in 0.1 mol/L PBS (pH 7.3) at 4 °C. The homogenate was centrifuged with 10,000 × g for 10 min at 4 °C and the resulting supernatant was aliquoted and stored at −80 °C.

Protein levels of lung homogenate were determined by the same method of BALF. The concentrations of total glutathione (T-GSH), reduced glutathione (GSH) and oxidized disulfide (GSSG) were measured by an enzymatic method according to the commercial assay kit procedure (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, T-GSH was assayed using the 5,5-dithio-bis (2-nitrobenzoic)

**Table 1** Characterization of Fe2O3 particles

<table>
<thead>
<tr>
<th></th>
<th>Nano-Fe2O3</th>
<th>Submicron-Fe2O3</th>
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</thead>
<tbody>
<tr>
<td><strong>Particle size</strong></td>
<td>22 nm</td>
<td>280 nm</td>
</tr>
<tr>
<td><strong>Crystal form</strong></td>
<td>Rhombohedral crystal</td>
<td>Rhombohedral crystal</td>
</tr>
<tr>
<td><strong>Specific surface area</strong></td>
<td>53.27 m²/g</td>
<td>4.25 m²/g</td>
</tr>
<tr>
<td><strong>Purity</strong></td>
<td>95.46%</td>
<td>99.77%</td>
</tr>
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</table>
Table 2
Percentage of macrophages, lymphocytes and neutrophils in bronchoalveolar lavage fluid (BALF) after Fe₃O₃ particle intratracheal instillation

<table>
<thead>
<tr>
<th>Post-days</th>
<th>Control</th>
<th>0.8 mg/kg bw</th>
<th>20 mg/kg bw</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>22 nm</td>
<td>280 nm</td>
</tr>
<tr>
<td>Macrophage %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>89.9 ± 1.5</td>
<td>56.7 ± 1.7**</td>
<td>57.3 ± 8.3**</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>73.5 ± 4.2**</td>
<td>73.7 ± 6.0**</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>89.8 ± 2.0</td>
<td>87.9 ± 1.1</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.0 ± 0.9</td>
<td>24.2 ± 4.8**</td>
<td>24.7 ± 4.3**</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>23.8 ± 5.9**</td>
<td>12.7 ± 2.1</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>5.8 ± 1.8</td>
<td>7.2 ± 1.2</td>
</tr>
<tr>
<td>Lymphocyte %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.2 ± 1.2</td>
<td>20.4 ± 6.5**</td>
<td>171 ± 12.2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>6.3 ± 4.1</td>
<td>12.3 ± 6.0**</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>4.6 ± 1.4</td>
<td>4.8 ± 1.0**</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D. (n = 6/group).
* Significant difference from control, p < 0.05.
** Significant difference from control, p < 0.01.

3. Results

3.1. Biochemical and cytological assessments of BALF

In order to elucidate whether the intratracheal instillation of Fe₃O₃ nanoparticles would induce lung inflammation, the biochemical and cytological changes in BALF were analyzed in the time course after instillation. In the control group, no significant changes in the cell number or cell differentials in BALF were observed after instillation of saline (Table 2). Comparatively, a significant increase in the relative number of neutrophils, lymphocytes and macrophages in BALF was counted with a peak at day 1 post-instillation in all the Fe₃O₃ particle-treated rats (p < 0.01), including the 22 nm- and 280 nm-Fe₃O₃ instilled rats at doses of 0.8 and 20 mg/kg bw (Table 2).

The total protein concentration in BALF showed significant dose- and time-dependent increase. At the day 30 post-instillation, the concentrations of protein in BALF of all the particle-treated rats significantly increased by 24.4–62.7% of the controls. The 280 nm-Fe₃O₃ induced higher BALF protein content in most of the observed dose and time points than 22 nm-Fe₃O₃ did, but no statistical difference were observed between them (Fig. 2). In contrast, significant LDH release was found in the 22 nm-Fe₃O₃ instilled rat at the dose of 0.8 mg/kg bw on all the observed 1, 7, and 30 post-days (p < 0.05), but in 280 nm-Fe₃O₃ treated rat, LDH significant increase was only observed at day 1 post-instillation (Fig. 3). In the meantime, the activities of ACP significantly elevated at day 1 of both 22 nm- and 280 nm-Fe₃O₃ particle instillation (p < 0.01, Fig. 4), but no obvious
Fig. 3. Levels of LDH in BALF. Values are mean ± S.D.; n = 6/group. *Significant difference from control (p < 0.05); "significant difference between 22 and 280-nm group (p < 0.01); **significant difference between 22 and 280-nm group (p < 0.01).

Fig. 4. Levels of ACP in BALF. Values are mean ± S.D.; n = 6/group. *Significant difference from control (p < 0.05); **significant difference from control (p < 0.01).

Fig. 5. Activity of GSH-Px in lung homogenates. Values are mean ± S.D.; n = 6/group. *Significant difference from control (p < 0.05); **significant difference from control (p < 0.01).

Fig. 6. Activity of GST in lung homogenates. Values are mean ± S.D.; n = 6/group. *Significant difference from control (p < 0.05); **significant difference from control (p < 0.01).

changes of alkaline phosphatase (AKP) were found (data not shown here).

3.2. Oxidative stress

The activity of GSH-Px significantly increased at day 1 after 0.8 mg/kg bw of 22 nm- and 280 nm-Fe2O3 instillation (Fig. 5). As for the high dose of 20 mg/kg instillation, the GSH-Px activities in the 22-nm group rat showed time-dependent increase, while in the 280-nm group rats, the GSH-Px activities increased rapidly at the day 1 post-instillation and then gradually reduced in the following days, which presented different responses in lung between the two-sized Fe2O3 particle exposure (Fig. 5).

GST activities showed significant decrease in the lung of both the 22 nm- and 280 nm-Fe2O3 instilled rats at low dose in day 1 post-instillation, but then significantly increased at post 7 and 30 days. However, in the 22-nm high dose group, GST significantly elevated at post-instilled day 1, then decreased in the subsequent days, whereas, at the same dose of 280-nm group rats, no statistic changes of GST in the time course have been detected (Fig. 6).

The contents of T-GSH decreased in both 22 nm- and 280 nm-Fe2O3 instilled rats at 1–7 post-instilled days and then recovered to the control level at the day 30 (Fig. 7). The ratio changes of GSSG/GSH were analyzed at the post-instilled day 7 and 30,
which remained around 0.11, indicating no significant changes have occurred.

MDA was measured to estimate lipid peroxidation extent in lung. As shown in Fig. 8, the contents of MDA in 22-nm (20 mg/kg bw) and 280-nm (0.8 and 20 mg/kg bw) group rats presented a time-dependent increase in lung. At the day 30, significant increase of MDA was found in all the particle-treated rats \((p < 0.05)\), except for the 22 nm-Fe\(_2\)O\(_3\) instilled rats at low dose.

The levels of NO showed significant decrease in lung of the particle-treated rats at the day 1 post-instillation, except for the 280 nm-Fe\(_2\)O\(_3\) instilled rat at high dose, then the content of NO gradually elevated with the post-instillation time, at the day 30, NO in all the particle-treated rats recovered to the normal as control (Fig. 9).

### 3.3. Blood coagulation test

The typical coagulation parameters in blood were observed to estimate the effect of Fe\(_2\)O\(_3\) particle intratracheal exposure on animal haemal system. The prolongation of PT and APTT were found in the low and high dose particle-treated rats. At the post-instilled day 30, PT and APTT in low dose of 22 nm-Fe\(_2\)O\(_3\) rats were significantly longer than the controls. In the 280 nm-Fe\(_2\)O\(_3\) instilled rats, slightly elevated average concentration of FIB was observed (Table 3).

### 3.4. Histopathology

The pathological changes in lung were evaluated with HE staining in Figs. 10 and 11. The follicular lymphoid hyperplasia with inflammatory cells aggregated around bronchia were observed in both the 22 nm- and the 280 nm-Fe\(_2\)O\(_3\) instilled rats from days 7 to 30 post-instillation, and the injury was more severe with the treated dose increase (Figs. 10 and 11). The thickened alveolar walls were found in all the particle-treatment rats, indicating the earlier fibrosis formation (Fig. 10). Particles phagocytosed by macrophage were observed in the pulmonary alveoli (Fig. 11). The iron particles appeared over-loading in alveolar macrophage by the high dose of 20 mg/kg bw instillation (Fig. 11). Inside the pulmonary alveolus, the inflammatory cells, such as neutrophils, lymphocytes and eosinophils could be found (Fig. 12). At the day 30 post-instillation, the inflammatory responses still extended, presenting as the severe follicular hyperplasia of the lymph node, capillary hyperaemia, alveolar lipoproteinosis and emphysema (Figs. 11 and 12).

### Table 3

The levels of PT, APPT and FIB in blood

<table>
<thead>
<tr>
<th></th>
<th>Post-days</th>
<th>0.8 mg/kg bw</th>
<th>0.8 mg/kg bw</th>
<th>20 mg/kg bw</th>
<th>20 mg/kg bw</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>22 nm</td>
<td>280 nm</td>
<td>22 nm</td>
<td>280 nm</td>
</tr>
<tr>
<td><strong>PT (s)</strong></td>
<td>7</td>
<td>14.34</td>
<td>14.78 ± 0.90</td>
<td>14.93 ± 0.86</td>
<td>14.64 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>± 0.90</td>
<td>15.44 ± 0.95</td>
<td>15.62 ± 0.85</td>
<td>14.48 ± 0.96</td>
</tr>
<tr>
<td><strong>APPT (s)</strong></td>
<td>7</td>
<td>14.34</td>
<td>14.78 ± 0.90</td>
<td>14.93 ± 0.86</td>
<td>14.64 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>± 0.90</td>
<td>15.44 ± 0.95</td>
<td>15.62 ± 0.85</td>
<td>14.48 ± 0.96</td>
</tr>
<tr>
<td><strong>FIB (mg/dL)</strong></td>
<td>7</td>
<td>14.34</td>
<td>14.78 ± 0.90</td>
<td>14.93 ± 0.86</td>
<td>14.64 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>± 0.90</td>
<td>15.44 ± 0.95</td>
<td>15.62 ± 0.85</td>
<td>14.48 ± 0.96</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D. \((n = 6)/group\).

\* Significant difference from control, \(p < 0.05\).

** Significant difference from control, \(p < 0.01\).
Fig. 10. Histopathology of lung at days 7 and 30 after intratracheal instillation of particles or saline (hematoxylin–eosin stain, magnification = 50). Follicular hyperplasia of the lymph node was formed at trachea forks (dark arrow). (A) Control group; (B) days 7 after instillation of 0.8 mg/kg bw 22 nm-Fe$_2$O$_3$; (C) days 30 after instillation of 0.8 mg/kg bw 22 nm-Fe$_2$O$_3$; (D) days 7 after instillation of 0.8 mg/kg bw 280 nm-Fe$_2$O$_3$; (E) days 30 after instillation of 0.8 mg/kg bw 280 nm-Fe$_2$O$_3$; (F) days 7 after instillation of 20 mg/kg bw 22 nm-Fe$_2$O$_3$; (G) days 30 after instillation of 20 mg/kg bw 22 nm-Fe$_2$O$_3$; (H) days 7 after instillation of 20 mg/kg bw 280 nm-Fe$_2$O$_3$; (I) days 30 after instillation of 20 mg/kg bw 280 nm-Fe$_2$O$_3$.

3.5. TEM inspection

The images of TEM presented the increase of lysosome in the lung. On the post-instilled day 30, mitochondria tumefaction and organelles dissolution indicating the damage of alveolar pathological changes were summarized in Table 4. To summarize, the pathological examination indicates that both the 22 nm- and 280 nm-Fe$_2$O$_3$ intratracheal instillation could induce lung injury and the injury would more severe with the treated dose increase.
epithelium occurred. The increased amount of collagen formation was observed, showing a tendency sign of lung fibrosis induction. In the meantime, particles translocated into the lung epithelium in the days 1 and 7 can be observed as well (Fig. 13).

4. Discussion

Generally, the oxidative stress caused by the particle is considered to be one of the important mechanisms of nanoparticle toxicity, especially for those particles containing transition metals.
Based on increasing experimental results (Simeonova and Luster, 1995; Chao et al., 1996), it is hypothesized that the respiratory and cardiovascular diseases caused by transition metal or its oxide particle exposure may be due to the induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS). We demonstrated that the nano- and submicron-sized Fe$_2$O$_3$ particles could generate inflammation and mediate oxidative stress in lung by either low or high dose intratracheal exposure. In our study, T-GSH, GSH-Px and GST were simultaneously measured to evaluate the oxidative stress in lung tissues after ferric oxide particle exposure. Increase of both GSH-Px and GST and decrease of T-GSH levels in Fe$_2$O$_3$-particle instilled rat indicated that the antioxidant defense system in lung tissues might over-load. Decrease of NO content in lung tissues after exposure at the first post-instilled day might be explained by free radical reactions. It is known that superfluous ROS may react with NO in the process of producing highly reactive and cytotoxic nitrogen products, such as peroxynitrite (ONOO-) and peroxynitrous acid (ONOOH). Peroxynitrite in turn reacts with and modifies various molecules, such as lipids, DNA, and proteins, resulting in functional NO deficiency. The significant increase of MDA at post-instillation day 30 demonstrated that lipid peroxidation had occurred. Another possibility of NO decrease might result from the synthesis of NO reduction, which may probably predict the injuries of cell functions.

It is hypothesized that oxidative stress could exert the macrophages on nanoparticles phagocytosis (Donaldson et al., 1998). Nanoparticles were found to deposit in the most distal parts of the lungs and had direct contact with the membrane of airways and alveoli (Brown et al., 2000). Depending on particle size, surface chemistry and possible charge, nanoparticles were reported to across epithelial and endothelial cell layers and translocated into the circulatory system within minutes after inhalation (Mehta et al., 2004; Heckel et al., 2004). We have observed in our pathological and TEM images that particles were phagocytosed by AM and entered alveolar epithelium, which supported the hypothesis that ultrafine particles were hard to be cleared from lungs. Moreover, the above results also suggested that Fe$_2$O$_3$ nanoparticle exposure may probably stimulate both the AM and epithelium cells. In the histopathological observation, the activated AMs presented in alveolus as a clustered form (Fig. 12A) and some were in foamy condition (Fig. 12B). The inflammatory cells of neutrophils, lymphocytes and eosinophils were observed as well. The increasing numbers of neutrophils and lymphocytes cells in BALF at days 7 post-instillation demonstrated that the inflammation of lung had occurred. Furthermore, phagocytosis of particles may stimulate AM releasing proinflammatory cytokines such as interleukin 1 (IL-1), interleukin 8 (IL-8), and tumor necrosis factor (TNF), which may activate neutrophils and eosinophils marginating and egressing into alveolar space, as similar results showed in our pathological results (Fig. 12C and D). The activated neutrophils and eosinophils are the rich source of leukotrienes, protease, platelet activating factor (PAF), etc., which contribute to local tissue damage, thus, inflammatory response can be amplified and sustained. In our study, the inflammation of lung has been found from days 7 till 30 of post-instillation (Figs. 12 and 13). In the TEM images, the significant acute inflammatory reaction in lung after irritant exposure of Fe$_2$O$_3$ particle has been presented as follicular hyperplasia of the lymph node, protein effusion, pulmonary capillary vessel hyperaemia and alveolar lipoproteinosis (Fig. 13B). Consistently, the increases in microvascular permeability and cell lysis in lung epitheliums were found by the determination of total protein, LDH and ACP activities, suggesting the injury of lung epitheliums after Fe$_2$O$_3$ nanoparticle instillation as well. Similar results have been reported by Hetland et al. (2004) that exposure to ultrafine ambient PM induced cell toxicity and apoptosis in lung epithelial cells.

The macrophages over-loaded by the phagocytosis of particles were clearly presented in the lung TEM images of days 7 and 30 post-instillation, though the lysosome increase was shown to resist the particles in the early days, the subsequent alveolar epitheliums damage had still occurred, including mitochondria tumefaction and organelles dissolution. More severely, the alveolar walls thickening and collagen formation in lung epitheliums can be observed,

Fig. 12. (A) Phagocytosed alveolar macrophages clustered in alveolus (thin arrow); (B) alveolar lipoproteinosis (thick arrow) and foamy alveolar macrophages were presented; (C) eosinophils egressed (thin arrow) and particle-laden alveolar macrophages (thick arrow); (D) neutrophils margination and egress into alveolar space.
indicating the pro-sign of lung fibrosis induction. Shvedova et al. (2005) reported that pharyngeal aspiration of SWCNTs could lead to fibrosis and granuloma formation. In our study, emphysema that presented as destruction of alveolar walls had formed in the early days after instillation and had changed to exacerbation in latter days, which may be due to the long-term and over-loaded deposition of Fe$_2$O$_3$ particles in alveolar. All those pathological changes combined with the biochemical alteration in BALF indicated an acute aspiration pneumonia symptom. Though some biochemical and oxidative stress markers recovered to the control levels in the later time course, however, pathological and TEM observation indicated that the lung injury could sustain for relatively long time even without repeated particle exposure.

The inflammatory reactivities of alveolar macrophages, neutrophils, eosinophils and epithelial cells can generate cytokines and ROS (Donaldson et al., 1998), which may continuously enhance the content of fibrinogen in blood and influence blood coagulation system. It was reported that the ROS induced by transition metal particle could arouse lipid peroxidation in vivo and subsequently cause atheromatous plaques or inflammation, which might result in fibrinogen formation and vessel viscosity increase (Sorensen et al., 2003). In population exposed to air pollution episodes, the levels of blood viscosity, fibrinogen, and C-reactive protein (CRP) were found higher than normal (Seaton et al., 1995; Berry et al., 1977). In this study, though not all of the chemical markers of blood coagulation exhibited significant differences between the particle-treated and control rats, prolongation of PT, APTT and higher level of FIB in Fe$_2$O$_3$ particle-treated rats were observed. It is noteworthy that PT and APTT in the low dose of 22 nm-Fe$_2$O$_3$ instilled rats were significantly prolonged than in the control, suggesting that the 22 nm-sized particles may be more susceptible to affect coagulation process. Similar observations were found in previous researches as well (Inoue et al., 2006; MacNee and Donaldson, 2000). These previous findings suggested that smaller sized nanoparticles could facilitate coagulatory disturbance because of oxidative stress and inflammation in lung, which induced endothelial-epithelial damage and subsequent infiltrated leukocytes allowed large amounts of smaller nanoparticles to pass easily into circulation (Inoue et al., 2006; MacNee and Donaldson, 2000). Our results supported the previous studies that the prolongation of PT and APTT in 22-nm
groups might result from the 22 nm-Fe₂O₃ nanoparticles diffusion in blood circulation and the generation of local and systemic oxidative stress and inflammation.

In conclusion, we demonstrated that both of the nano- and micron-sized Fe₂O₃ particle intratracheal exposure could initiate acute lung injury. Oxidative stress in lung was induced. The phagocytosis of nanoparticle by AMs in lung was over-loaded after high dose exposure. The non-phagocytosed particles were found to enter into alveolar epithelial, suggesting that nanoparticles may be taken up by epithelial cells and hard to be cleared from lungs. The long term and over-loaded of particles in AM and epithelium cells could even cause pathological symptoms of lung emphysema and the pro-sign of lung fibrosis. More attention should be paid on the toxic effect induced by nano-sized Fe₂O₃ particle. Our results suggest that nano-sized Fe₂O₃ particle intratracheal instillation may increase microvascular permeability and cell lysis in lung epitheliums and effect on blood coagulation system.

Acknowledgments

The authors are grateful to the foundations of MOST 973 program (2006CB705605, 2006CB932505 and 2007CB935604), the Chinese Academy of Sciences (KJCX3.SYW.N3) and the National Natural Science Foundation of China (10490181, 20475055, 10675139, and 10523524).

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