Comparative Study of Pathological Lesions Induced by Multiwalled Carbon Nanotubes in Lungs of Mice by Intratracheal Instillation and Inhalation

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ABSTRACT: The pathological lesions induced by multiwalled carbon nanotubes (MWCNTs) in bronchi and alveoli of mice were studied by intratracheal instillation and inhalation. In instillation groups, the dose was 0.05 mg MWCNTs/mouse. Similar size clumps of MWCNTs were distributed in bronchi and alveoli. The clumps led to inflammation to the lining wall of bronchi and severe destruction to alveolar netted structure around them. In the inhalation groups, the mice were exposed to aerosolized MWCNTs with mean concentration of 32.61 mg/m^3, the intralung deposition dose were roughly 0.07, 0.14, and 0.21 mg in the 8-day group, 16-day group, and 24-day group, respectively. Most of aggregations of MWCNTs in the alveoli were smaller than that in bronchi. The aggregations induced proliferation and thickening of alveolar walls. With the exception of these moderate pathological lesions, the general alveolar structure was still remained. The preliminary study demonstrated a difference in lung pathological lesions induced by instilled MWCNTs and inhaled ones, which may be due to the different size and distribution of aggregations of MWCNTs in lung. Environ Toxicol 22: 415–421, 2007.

Keywords: multiwalled carbon nanotubes; aerosol; intratracheal instillation; inhalation; pathological lesions; toxicity

INTRODUCTION

Carbon nanotubes (CNTs) have splendid potential applications in modern science and technology due to their unique chemical and physical characteristics endowed by their novel nanostructures. However, in view of some similar aspects to fibers, such as structural characteristics, extreme aspect ratio, low specific density, and low solubility, CNTs might exhibit toxicity similar to those observed in other fibrous particles such as asbestos (Maynard et al., 2004). Thus the suspense whether CNTs have negative impact on human health and the environment evokes concern by worldwide public (Ball, 2001; Colvin, 2003; Nel et al., 2006). Some researchers have reviewed potential toxicity of CNTs in detail, and concluded the strategies of their workplace safety evaluation, trends and perspectives in this field (Donaldson et al., 2006; Hurt et al., 2006; Tsuji et al., 2006). One hopes that CNTs would be one of nanomaterials, whose toxicity has been identified and recognized well before their industrial uses on a large scale. In recent years,

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increasing effort has been made to identify the respiratory toxicity of CNTs. Lam et al. (2004) found that single-walled nanotubes (SWCNTs), after intratracheal instillation, induced dose-dependent epithelioid granulomas and, in some cases, interstitial inflammation in the mice. At almost the same time, Warheit et al. (2004) studied pulmonary toxicity of SWCNTs in rats also with intratracheal instillation and found that SWCNTs produced transient inflammation, cell injury, and a series of nondose-dependent multifocal granulomas. In investigation of the respiratory toxicity of multiwalled carbon nanotubes (MWCNTs) in rats, Muller et al. (2005) drew a conclusion that the MWCNTs induced inflammatory and fibrotic reactions and still presented in the lung even at 60 days after intratracheal administration. These data suggested that if workers were exposed to respirable CNTs, they might be at risk of developing serious lung diseases. However, in all these reports, the experimental studies on the pulmonary toxicity of CNTs were carried out by intratracheal instillation of a single dose CNTs to mouse or rat. This route of exposure is obviously the nonphysiologic rapid delivery of the nanoparticles, which is less consistent with inhalation (Driscoll et al., 2000). Very recently, Warheit (2006) summarized and evaluated the pulmonary toxicity of intratracheally instilled SWCNTs in rats. The author indicated that inhalation toxicity study in rats must be conducted with aerosolized CNTs to evaluate the realistic risks. Other authors also pointed out the need for natural exposure method, namely inhalation rather than instillation (Hurt et al., 2006; Muller et al., 2006). To our knowledge, few reports thus far have been given on pulmonary toxicity responsible for exposure to aerosolized CNTs. In this work, instillation exposure to MCWNTs in suspension and inhalation exposure to aerosolized MCWNTs were conducted. The experiments showed how the MCWNTs interact with and affect bronchi and alveoli of mice. By comparing the difference in the pulmonary pathological lesions, this preliminary investigation may be able to pave the way for verifying and evaluating true pulmonary toxicity of exposure to carbon nanomaterials.

MATERIALS AND METHODS

MWCNTs and Animals

The MWCNTs were obtained from Shenzhen Nanotech Port, Shenzhen, China, with average external diameter of 50 nm, mean length of 10 μm, purity $>$ 95%, ash (La, Ni) $<$ 0.2 wt %, special surface area of 280 m^2/g, and amorphous carbon $<$3%. The pristine MWCNTs were used directly in this experiment without any physicochemical processes before hand.

The experimental animals, female Kunming mice with weight of 30 g and 10 weeks of age, were purchased from Experimental Animal Center, Fu Dan University, Shanghai.

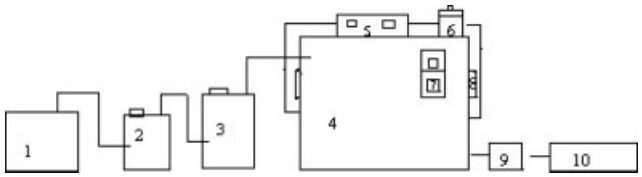

Fig. 1. Inhalation equipment: 1, powder generator; 2, first depositor; 3, second depositor; 4, inhalation chamber; 5, P-5L2C digital dust monitor; 6, exhaust location **I**; 7, sensor for the measurement of temperature and humidity; 8, lid; 9, exhaust location **II**; 10, vacuum pump.

All animals were allowed to acclimate for 1 week prior to the onset of instillation and inhalation exposure. The animal studies were carried out in compliance with the national regulations related to the conduct of experimentation.

Intratracheal Instillation Experiment

The MWCNTs were suspended and sonicated (15 min) in sterile 0.9% saline containing 1% of Tween-80 (Muller et al., 2005). The concentration of the suspension was 0.5 mg/mL. Thirty-five mice were randomly divided into seven groups, five for each group. One group was chosen as control, the other three were used as the tween-saline control groups, and the last three were used as experimental groups. All the mice in the tween-saline control groups and experimental groups were intraperitoneally anesthetized with 0.2 mL 0.5% pentobarbital sodium solution. Then 0.1 mL tween-saline liquid or equivalent volume of suspensions (containing 0.05 mg MWCNTs) was intratracheally instilled into lungs of mice, respectively. After instillation, pathological lesions were examined at 8th, 16th, and 24th day, and experimental groups were denominated 8-day group, 16-day group, and 24-day group, respectively.

Inhalation Equipment

The inhalation equipment (Fig. 1), mainly referred to reports (Pauluhn, 2003, 2005), was made up of powder generator, first depositor, second depositor, inhalation chamber, P-5L2C digital dust monitor, sensor for the measurement of temperature and humidity, exhaust location (100 ppi sponges soaked with 5% SDS solution and HEPA filter), exhaust location **II** (cotton-wool aerosol filter and HEPA filter), and vacuum pump. Volume of the inhalation chamber was 0.07 m^3.

Inhalation Experiment

From dust generator, obtained from Liaoyang Applied Technology Institute, Liaoyang, China the dry dusts of the MWCNTs were pressed out by rapid airflow. As larger particles among dusts deposited orderly in the first and second

TABLE I. The inhalation exposure design of three experimental groups

	Experimental Period (day)		
	1, 2, 3, 4, 5, 6, 7, 8	9, 10, 11, 12, 13, 14, 15, 16	17, 18, 19, 20, 21, 22, 23, 24
8-day group	Six mice were actually exposed for 5 days in 8-day exposure period		
16-day group	Six mice in this group were actually exposed for 10 days in 16-day exposure period		
24-day group	Six mice in this group were actually exposed for 15 days in 24-day exposure period		

n means that the mice were exposed at that day.

depositors, the remained smaller particles were insufflated into the inhalation chamber, and formed MWCNTs aerosol in it. During the inhalation experiment, 18 mice were exposed to MWCNTs aerosol in inhalation chamber. According to the period from exposure to pathological lesions examination, the mice were divided into three groups, denominated 8-day group, 16-day group, and 24-day group, respectively. However, their actual exposure time was 5, 10, and 15 days for 8-day group, 16-day group, and 24-day group, respectively (Table I). The inhalation experiment was conducted in ventilated fume hoods at room temperature (18–22 °C).

Pathological Examination

For examination of pathological lesions, the mice, after exposure to MWCNTs with both intratracheal instillation and inhalation, were intraperitoneally anesthetized with 0.3 mL 0.5% pentobarbital sodium solution, and their lungs were excised and fixed by 4% formalin. The formalin-fixed lungs were embedded in paraffin, thin-sectioned, and mounted on glass microscope slides. Sections were stained with hematoxylin and eosin, and then examined by light microscopy.

RESULTS

Characteristics of Aerosolized MWCNTs and Its Concentration Descending

In this work a piece of silicon slice was placed horizontally on the bottom of the inhalation chamber filled with MWCNTs aerosol for 90 min, then the sample was subjected to observe the aspect of MWCNTs with scanning electron microscopy (SEM; LEO 1530VP). SEM micrograph showed that the small size clusters formed on the silicon slice from aggregation of various numbers nanotubes, implying the aerosolized MWCNTs in inhalation chamber were almost respirable particles (Fig. 2).

During mice inhalation exposure, the concentration of MWCNTs aerosol in the inhalation chamber decreased regularly from about 80 to 13 mg/m^3 per 90 min (Fig. 3). Similar exposure was continually repeated for four times in an exposure day, namely, the cumulative exposure time was 6 h in the exposure day.

Except for the process of aerosol generation, the HEPA filter was removed from the exhaust location **I**. In this case, fresh air out of the chamber was allowed to exchange with that in the chamber without substantial

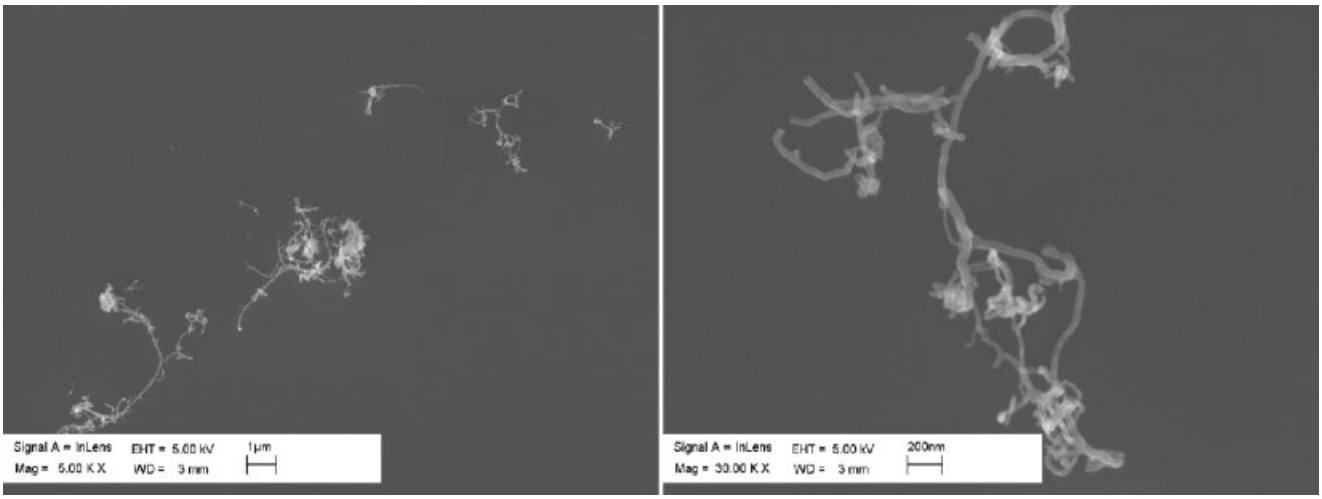

Fig. 2. The aerosolized MWCNTs dispersed on the silicon slice after a 90-min deposition.

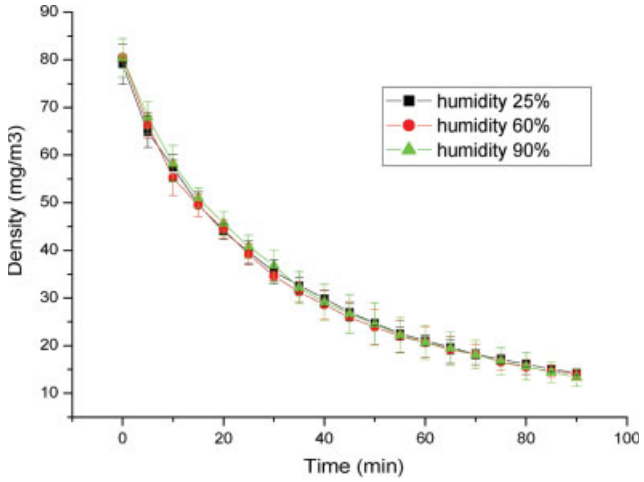

Fig. 3. Variation of the concentration of MWCNTs aerosol in the inhalation chamber with time at relative humidity of 25–90% (values are mean ± SD; $n = 5$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

release of aerosolized MWCNTs from the inhalation chamber. The pretest showed that when the MWCNTs aerosol was insufflated into the inhalation chamber in this manner at interval of every 90 min, the mice (<20) were able to live normally in the inhalation chamber. At the end of exposure day, the exhaust location **II** and the vacuum pump were operated until the aerosol concentration in chamber was reduced to be lower than 0.229 mg/m^3, a minimum level of MWCNTs concentration the P-5L2C digital dust monitor could detect. Then the mice were transferred from the inhalation chamber to raising cages, and waiting for next exposure at following days.

Mice Responses to MWCNTs by Intratracheal Instillation

After the mice were administrated by MWCNTs (0.05 mg/mouse) in the intratracheal instillation groups, the mice were anesthetized and then sacrificed at 8th, 16th, and 24th day, respectively. The light micrographs for lung tissue were shown in Figure 4. Black clumps with different size and morphology, agglomerated from MWCNTs, distributed in bronchi, but not blocked them up completely. For the mice in the 8-day group and 16-day group, the clumps of MWCNTs were deposited on lining wall of bronchi without obvious inflammatory cells around them [Fig. 4(b,c)]. In further time course, the clumps led to inflammation to the lining wall of bronchi in the 24-day group [Fig. 4(d)]. On the other hand, the similar clumps, also deposited into the alveoli, induced more and more severe destruction to alveolar netted structure around them [Fig. 4(f–h)].

Mice Responses to MWCNTs by Inhalation

After the inhalation exposure, the pathological examinations of three inhalation experimental groups were showed in Figure 5. The aggregations of MWCNTs in the bronchi were adhered on the lining wall of bronchi, and no inflammatory cells covered or accumulated around them [Fig. 5(b–d), as shown by arrow]. Most of the aggregations in the alveoli were smaller than those in bronchi and usually entered into alveolar walls [Fig. 5(f–h), as shown by arrow]. The main pathological lesions of alveoli induced by these aggregations were proliferation and thickening of alveolar walls [Fig. 5(f–h)]. With the exception of these moderate pathological lesions, the general alveolar netted structure was still remained.

DISCUSSION

In this experiment, we demonstrated the large differences of the pulmonary response to MWCNTs by instillation and inhalation exposure routes. In intratrachaeal instillation groups, 0.05 mg MWCNTs were intratrachaeally instilled into per experimental mouse. Unlike intratrachaeal instillation experiments, in inhalation groups the intralung deposition dose of aerosolized MWCNTs was calculated according to the following formula:

$$D_{\text{intra}} = F \cdot V \cdot \overline{\rho} \cdot T \tag{1}$$

where D_{intra} was intralung deposition dose, F was a fractional deposition ratio of aerosolized MWCNTs, V was volume of aerosol inhaled by the mouse in unit time, $\overline{\rho}$ was weighted mean concentration of MWCNTs in the aerosol, and T was exposure time. In present work, the weighted mean concentration in 90-min exposure was calculated from the following formula:

$$\overline{\rho} = \sum \Delta T_{\text{i}} \overline{\rho}_{\text{i}} / \sum \Delta T_{\text{i}} \tag{2}$$

where ΔT_{i} was time interval (around 5 min) and $\overline{\rho}_{\text{i}}$ was arithmetic mean concentration in that time interval.

Based on the earlier formulas, the weighted mean concentration ($\overline{\rho}$) of MWCNTs in the aerosol, in a typical test, was calculated to be 32.61 mg/m^3. Following the recent article (Lam et al., 2006), the fractional deposition ratio (F) of aerosolized CNTs into lung was about 4%. It was also assumed that a 30-g mouse breathes in 30 mL air per min (Parent, 1992). Thus intralung deposition dose of mice in the inhalation experimental groups were roughly 0.07, 0.14, and 0.21 mg in the 8-day group, 16-day group, and 24-day group, respectively.

In intratrachaeal instillation groups, although the sonication and nonionic dispersant Tween-80 have been taken before instillation, the MWCNTs still aggregated into

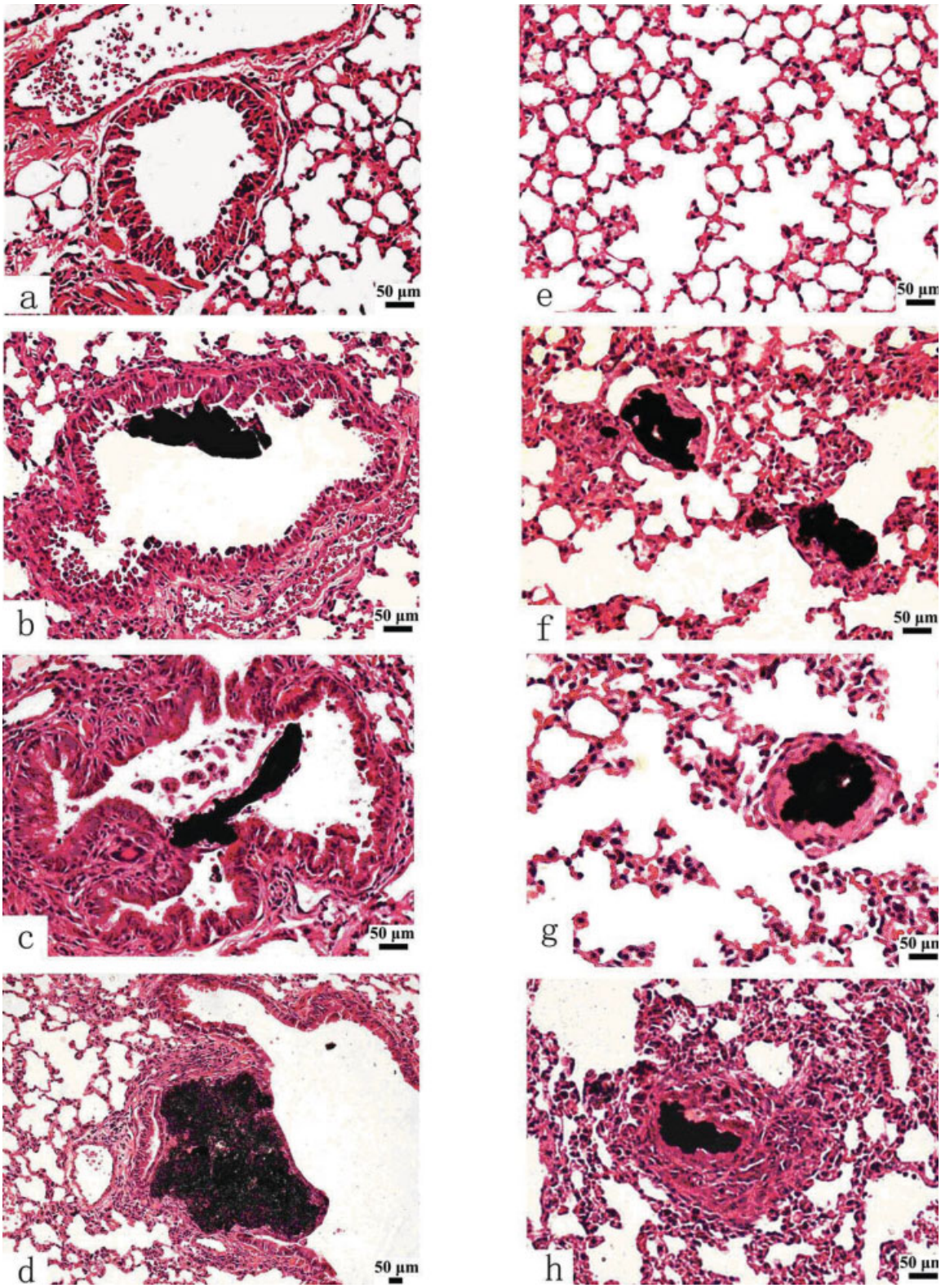

Fig. 4. The panels present the light micrograph of lung tissue from mice administrated by MWCNTs (0.05 mg/mouse) in the intratracheal instillation groups. The bronchus of tween-saline control group is in image (a). The bronchial lesions in 8-day group, 16-day group, and 24-day group are presented from images (b) to (d). The alveoli of tween-saline control group are in image (e). The alveolar lesions in 8-day group, 16-day group, and 24-day group are presented from images (f) to (h). Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

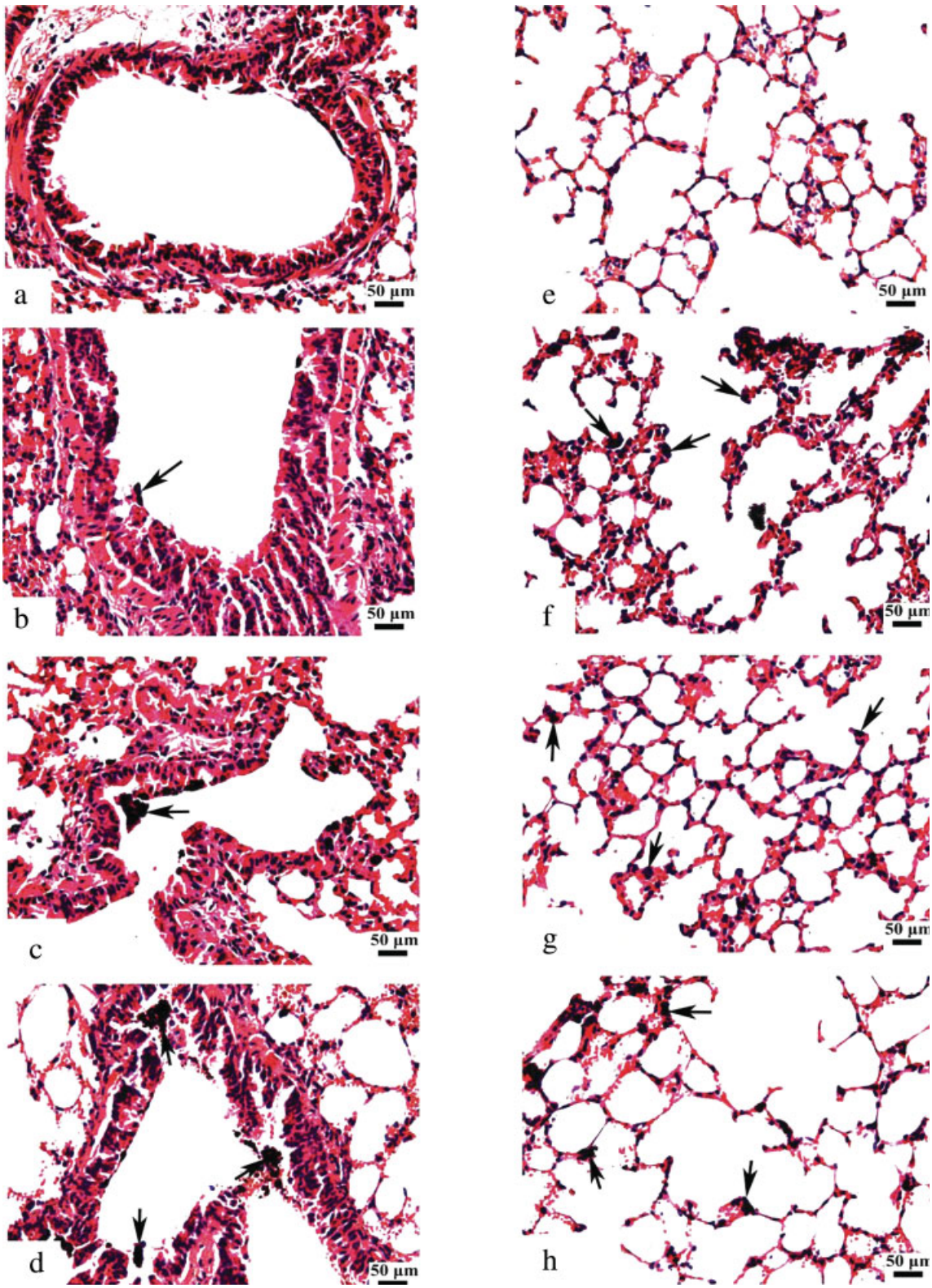

Fig. 5. The panels present the light micrograph of lung tissue from mice administrated by aerosolized MWCNTs in the inhalation experimental groups. The bronchus of control group is in image (a). The bronchial lesions in 8-day group, 16-day group, and 24-day group are presented from images (b) to (d). The alveoli of control group are in image (e). The alveolar lesions in 8-day group, 16-day group, and 24-day group are presented from images (f) to (h). Bar $=$ 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

clumps in bronchi and alveoli. These clumps led to inflammation to the lining wall of bronchi in the 24-day group [Fig. 4(d)] and severe destruction to alveolar netted structure around them [Fig. 4(f–h)], which were more like the mechanical lesions from foreign-body response. These pathological lesions were different from the granumolas around CNTs, observed in previous concerning reports (Warheit et al., 2004; Muller et al., 2005). Perhaps the different physicochemical characteristics of CNTs, such as diameter and length, were the main reason to produce different pathological lesions. The nominal size of SWCNTs, used in report of Warheit et al., is about 1 nm diameter $\times$ 1 μm length, and the characteristics of individual MWCNT and ground MWCNT, used in report of Muller et al., were 9.7 nm outer diameter $\times$ 5.9 μm length and 11.3 nm outer diameter $\times$ 0.7 μm length. However, characteristics of the pristine MWCNT in present study were about 50 nm outer diameter $\times$ 10 μm length, which were likely very difficult to disperse well in sterile saline with Tween-80 by simple sonication. Clumps of these MWCNTs, even far beyond the scope of the respirable particles ($<$3 μm) for small experimental animals, could still bypass the muco-ciliary system, and directly enter into lung by intratrachaeal instillation as shown in Figure 4. In addition, the clumps into lung were too large to be phagocytized by the alveolar macrophages and consequently to be cleared in time, which likely induce persistent pathological lesions in bronchi and alveoli.

In inhalation experiments groups, the MWCNTs could be dispersed well in aerosol, which were almost respirable particles (Fig. 2). Most of the aggregations in the alveoli were smaller than that in bronchi and usually entered into alveolar walls [Fig. 5(f–h)], as shown by arrow]. The main pathological lesions induced by them in the alveoli were moderate proliferation and thickening of alveolar walls. With the exception of the moderate pathological lesions, the general alveolar structure was still remained. Thus the pathological lesions in inhalation groups were very different with that in intratrachaeal instillation groups. The main reasons may due to the following ones. The introduction of aerosolized MWCNTs by inhalation is a physiologic and active process, and nonrespirable MWCNTs particles could not bypass muco-ciliary system. Moreover, aerosolized MWCNTs were inhaled very slowly and regularly into the airways and alveoli in a relatively long period. The larger size aggregations of MWCNTs distributed in bronchi and smaller size ones distributed in alveoli, they did not further aggregated into clumps. In addition, it is possible that some of proper size aggregations in alveoli could be phagocytized sequentially and cleared by the alveolar macrophages in inhalation groups.

In summary, when the mice were exposed to MWCNTs by intratracheal instillation and inhalation, the pathological lesions induced by them were different, which may be due to their aggregations size and distribution in lung.

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