The Acute Pulmonary Toxicity in Mice Induced by Multiwall Carbon Nanotubes, Benzene, and Their Combination

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ABSTRACT: Carbon nanotubes (CNTs) have been synthesized and produced on large scale for their wide application. They have high absorption ability to organic contaminants (such as benzene) and can form CNTs-benzene combination with benzene. In this article, the acute pulmonary toxicity, induced by multiwall carbon nanotubes (MWCNTs), benzene, and their combination, was studied by administrating the three test materials into mice lungs via intratracheal instillation. The biochemical parameters in bronchoalveolar lavage fluid (BALF) and pathological lesions in lungs were used as endpoints to evaluate the pulmonary toxicity of the three test materials at 3-day and 7-day postexposure, respectively. After the mice were intratracheally instilled with MWCNTs, benzene and MWCNTs-benzene combination at doses of 6.67 mg/kg, 2.67 mg/kg, and 9.34 mg/kg (containing 6.67 mg/kg MWCNTs and 2.67 mg/kg benzene), the total protein, alkaline phosphatase (ALP), acid phosphatase (ACP), and lactate dehydrogenase (LDH) in BALF and pathological lesions in lungs were examined. At 3-day postexposure, MWCNTs induced obvious pulmonary toxicity and benzene only induced slight pulmonary toxicity, whereas their combination induced very severe pulmonary toxicity. At 7-day postexposure, MWCNTs and benzene did not induce pulmonary toxicity individually, whereas their combination still induced severe pulmonary toxicity. These data indicated that, at the instilled doses in this experiment, the MWCNTs can alone induce acute pulmonary toxicity in mice and the benzene does not induce pulmonary toxicity, but the pulmonary toxicity of MWCNTs is enhanced after they form MWCNTs-benzene combination with low dose of benzene. The enhanced pulmonary toxicity may be due to the change of MWCNTs aggregation ability after benzene is adsorbed on them. © 2009 Wiley Periodicals, Inc. Environ Toxicol 25: 409–417, 2010.

Keywords: multi-wall carbon nanotubes; benzene; combination; adsorption; intratracheal instillation; pulmonary toxicity

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INTRODUCTION

Carbon nanotubes (CNTs) are typical of nanoparticles. As widely applicable nanomaterials, they have been synthesized and produced on large scale. But they are extremely small and buoyant and can enter into human respiratory system (Maynard et al., 2004), thus their potential pulmonary toxicity evoked concerns by worldwide public (Ball, 2001; Colvin, 2003; Nel et al., 2006). In recent years,

increasing effort has been made to identify their respiratory toxicity (Lam et al., 2004; Warheit et al., 2004; Muller et al., 2005). These data suggested that if people are exposed to respirable CNTs, they might be at risk of developing serious lung diseases. Up to now, the concerning reports predominately focused on respiratory toxicity of pure CNTs rather than their combination. However, it is possible that people contract CNTs-organic-contaminant combination other than pure CNTs in environmental and occupational settings. As well known, CNTs have high adsorption ability to organic molecules, for example, they were used as sorbent of dibenzo-*p*-dioxin and 1,2-dichlorobenzene (Long and Yang, 2001; Peng et al., 2003). Thus it is easy that they can adsorb organic contaminants (such as benzene) to form CNTs-organic-contaminant combination. In addition, a certain dose of CNTs vapor can be ''synthesized'' in ambient air (such as natural gas combustion) (Bang et al., 2004; Esquivel and Murr, 2004; Murr et al., 2004a,b). This provides a chance for them to form combination with the volatile organic contaminants in environment.

Benzene, a ubiquitous contaminant in the ambient air, is a constituent of gasoline, auto exhaust, and cigarette smoke (Yardley-Jones et al., 1991; Snyder et al., 1993; Snyder, 2000; Madl and Paustenbach, 2002). It is a volatile solvent, which can enter into human or animal body by the exposure routes of inhalation, oral, and dermal. Among the three exposure routes, inhalation exposure is usually of primary concern in occupational and environmental scenarios. The animal studies have shown that the lung is a target organ of benzene tumorigenicity (Mullin et al., 1998). Human would also take the risk of adenocarcinoma and squamous cell carcinoma of lung when occupationally exposed to benzene (Aksoy, 1989; Yin et al., 1989, 1996) as well as to cigarette smoke (Darrall et al., 1998).

Although the pulmonary toxicity of pure CNTs or benzene vapor has been studied by some research groups, to our knowledge, few studies are performed on the pulmonary toxicity of CNTs-benzene combination. In consideration of the existence of CNTs-benzene combination in the ambient air and little know about their potential pulmonary toxicity, in this article, low dose of MWCNTs and benzene, and MWCNTs-benzene combination were intratracheally instilled into lungs of mice, and their pulmonary toxicity was examined at 3-day and 7-day postexposure, respectively.

MATERIALS AND METHODS

Physicochemical Characterizations of MWCNTs

The MWCNTs were obtained from Shenzhen Nanotech Port, Shenzhen, China. They were synthesized by chemical vapor deposition (CVD) with nickel and lanthanum catalysts. Their characterizations have been provided in manufacturer's specification, as shown in Table I. Their size, structure, specific surface area, and metal impurity are important factors affecting their toxicity (Dick et al., 2003; Nel et al., 2006; Koyama et al., 2009), which were independently characterized in this experiment. (1) Their size and structure were characterized by scanning electron microscopy (SEM, LEO 1590VP) and transmission electron microscopy (TEM, JEM-2000CX). (2) Their specific surface area was determined by BET surface area analyzer (ASAP 2100). (3) Their metal impurity was determined by inductively coupled plasma mass spectroscopic (ICP-MS, X-7, Thermo Elemental) analysis. The pristine MWCNTs were used directly in this experiment without any physicochemical processes before hand.

TABLE I. The physicochemical characterizations of MWCNTs in manufacturer's specifications and independently measured in this experiment

Characterizations	Manufacturer's Specifications	Measured Characterizations
Purity	>95%	
Diameter	40–60 nn	50 nm
Length	5–15 μm	
Amorphous carbon	<3%	
Ash (catalyst residue)	<0.2%	
Surface area	40–300 m^2/g	83.8 m^2/g
Thermal conductivity	~2000 W/m k	
Metal impurities		
Cobalt		0.01%
Copper		0.005%
Nickel		1.25%
Iron		0.07%

Benzene

Benzene was commercially available from Sinopharm Chemical Reagent Co., Ltd, China. It is analytical reagent (AR) with density of 0.88 g/cm^3. Its solubility in water is 1.8 g/L (at 298 K).

Experimental Animals

The study was conducted using 7-week-old male Kunming mice that were obtained from Shanghai Slack Experimental Animal Center, China. The mice were housed in clean cages by five in a ventilated animal room. Room temperature was maintained at 18–20°C, relative humidity at 55 ± 10%, and a 12-hour light–dark cycle. All mice were supplied with sterilized food and pure water. They were allowed to acclimate for 1 week before onset of instillation. Their average weight at the time of study was 30 ± 3 g. The animal study was carried out in compliance with the national regulations related to the conduct of experimentation.

Preparation of MWCNTs-Benzene Combination

MWCNTs were washed with deioned water for four times. Then they were placed into oven for being dried with temperature of 105°C. After the MWCNTs were dried, 0.2 g of them was weighed out and put into 280-mL glass bottle full of PBS (experimental bottle) for 15-minute ultrasonication. Furthermore, 0.176 g benzene was also added into this bottle, and this bottle's faucet was turned on tightly for 5-minute ultrasonication. After 16-hour agitation on the magnetic agitator at room temperature, the suspension in this bottle was filtered for further analysis. On the other hand, another 280-mL glass bottle with same volume of PBS and benzene was used as control bottle, which was also agitated for 16 hours at the same condition of the experimental bottle. After the 16 hours agitation, the benzene concentration in the control bottle was used as the before adsorption concentration (C_0), while the benzene concentration in the experimental bottle, from which MWCNTs-benzene combination has been filtered off, was used as the postadsorption concentration (C_e). C_e could be calculated with equation ($C = 1.0624\ X - 0.0534$, correlation coefficient $R = 0.9987$, $n = 6$) between concentration of benzene suspension (C, mg/mL) and its corresponding absorbency (X). The equation was fitted with the relationship between the different concentrations of benzene suspension and their corresponding absorbencies (Fig. 1).

Based on the before and after adsorption concentrations of benzene suspension, the benzene mass (q_e) that adsorbed on per mg MWCNTs can be calculated with the following formula (Hindarso et al., 2001; Wibowo et al., 2007).

$$q_e = (C_0 - C_e)V/m$$

Where V is total volume of suspension, m is total mass of MWCNTs used as sorbent.

After three reproducible experiments, benzene mass (q_e) that adsorbed on per mg MWCNTs is 0.407 + 0.078 mg ($n = 3$), namely, on this experimental condition, the adsorption ratio of benzene on MWCNTs is 40.5%. The MWCNTs-benzene combination (including 0.2 g MWCNTs and 0.08 g benzene) was dried at room temperature, and then put into 100 mL PBS to prepare MWCNTs-benzene combination suspension. The suspension can be used for intratracheal instillation of mice.

Intratracheal Instillation of MWCNTs, Benzene, and MWCNTs-Benzene Combination

Besides the preparation of MWCNTs-benzene combination suspension, the MWCNTs were put into PBS for 15-minute ultrasonication to prepare MWCNTs suspension. In addition, benzene was added into PBS to prepare benzene suspension. Before instillation, MWCNTs suspension, benzene suspension, and MWCNTs-benzene combination suspension were ultrasonicated for 10 seconds, respectively.

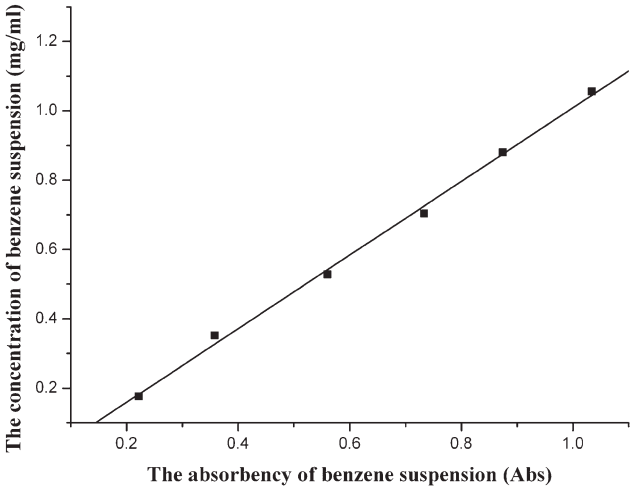

Fig. 1. The relationship between the concentrations of benzene suspension and their corresponding absorbencies.

As regards experimental design, 90 mice were randomly divided evenly into five groups (18 mice/group), which were called control group, PBS group, MWCNTs group, benzene group, and MWCNTs-benzene group, respectively. All the mice were intraperitoneally anesthetized with 0.2 mL 0.5% pentobarbital sodium solution. The mice in PBS group, MWCNTs group, benzene group, and MWCNTs-benzene group were intratracheally instilled with same volume (0.1 mL) of PBS, MWCNTs suspension (containing 0.2 mg MWCNTs), benzene suspension (concentration 0.8 mg/mL), MWCNTs-benzene combination suspension (containing 0.2 mg MWCNTs and 0.08 mg), respectively. The corresponding instilled doses of MWCNTs, benzene, and MWCNTs-benzene combination were 6.67 mg/kg, 2.67 mg/kg, and 9.34 mg/kg.

At 3-day postexposure, the biochemical parameters in bronchoalveolar lavage fluid (BALF) were analyzed with six mice of each group, while the pathological observation of lungs was carried out with three mice of each group. At 7-day postexposure, another nine mice of each group were treated with the same method as mentioned above.

Biochemical Parameters Analysis in BALF

Nice mice in each group were anesthetized by intraperitoneal injection of 0.3 mL 0.5% pentobarbital sodium solution and exsanguinated via abdominalis aorta at 3-day postexposure. Six mice were randomly chosen from nine mice in each group, and bronchoalveolar lavage was performed on them by cannulating the trachea and infusing lungs two times with 2 mL physiological saline (37°C). The BALF were centrifuged (400 × g, 10 minutes, 4°C), and

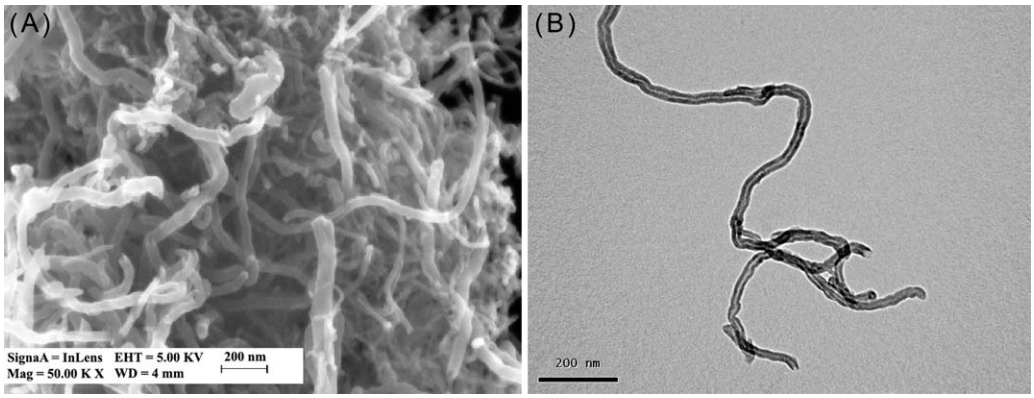

Fig. 2. Images of MWCNTs by SEM (A) and TEM (B). (A) was achieved from the agglomerated powders of MWCNTs by SEM. Most of the pristine MWCNTs are bended and agglomerated together and their external diameter is about 50 nm. The MWCNTs were dispersed into PBS by 5-minute ultrasonication and then dried for TEM. As shown in (B), their tube-shaped structure can be observed.

biochemical parameters [total protein, alkaline phosphatase (ALP), acid phosphatase (ACP), and lactate dehydrogenase (LDH)] in their supernatant were analyzed. The remained three mice were used for pathological examination. At 7-day postexposure, the biochemical parameters analysis of another nine mice in each group was carried out using above mentioned method. All the biochemical parameters in supernatant of BALF were analyzed with commercial reagent kits that were obtained from Nanjing Jiancheng Bioengineering Institute, China.

Pathological Examination

At 3-day and 7-day postexposure, the lungs of remained three mice in each group were excised and fixed with 4% formalin, then embedded in paraffin, sectioned coronally, and mounted on glass microscope slides. Sections were stained with hematoxylin-eosin and examined by light microscopy.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). For analysis, each of the experimental values was compared with the control group value. Data were expressed as means ± SD. Significance was judged at the $P < 0.05$ and $P < 0.01$ probability level.

RESULTS

MWCNTs Characterizations

The electron micrographs of MWCNTs demonstrated that the MWCNTs external diameter is about 50 nm, as shown in Figure 2. They are bended and agglomerated together. The BET analysis result indicated that their specific surface area is 83.8 m^2/g, which is in range of 40–300 m^2/g as provided by manufacturer. The metal impurity analysis results with ICP-MS demonstrated that cobalt is 0.01%, copper 0.005%, nickel 1.25% and iron 0.07%. A higher catalyst residue (nickel, 1.25%) was detected compared to the declared value of <0.2% in manufacturer's specifications.

The Changes of Biochemical Parameters in BALF

At 3-day postexpoure, the MWCNTs led to significant concentration increment of total protein ($P < 0.01$) and LDH ($P < 0.01$) in BALF, and slight concentration increment of ALP and ACP in BALF. The benzene led to significant concentration increment of LDH at $P < 0.05$ level and slight concentration increment of total protein, ALP, and ACP. The MWCNTs-benzene combination led to significant concentration increment of ALP ($P < 0.05$), total protein ($P < 0.01$), ACP ($P < 0.01$), and LDH ($P < 0.01$). The PBS did not lead to any obvious changes of any biochemical parameters in BALF. These results were shown in Figure 3.

At 7-day postexpoure, the MWCNTs and benzene did not individually lead to any obvious changes of any biochemical parameters in BALF. The MWCNTs-benzene combination led to significant concentration increment of total protein ($P < 0.05$), ACP ($P < 0.05$), LDH ($P < 0.05$), and ALP ($P < 0.01$). The PBS did not lead to any obvious changes of any biochemical parameters in BALF. These results were shown in Figure 4.

Pathologic Lesions in Lungs Tissue

Compared with the pathologic examination in the control group, PBS did not cause obvious pathologic lesions in

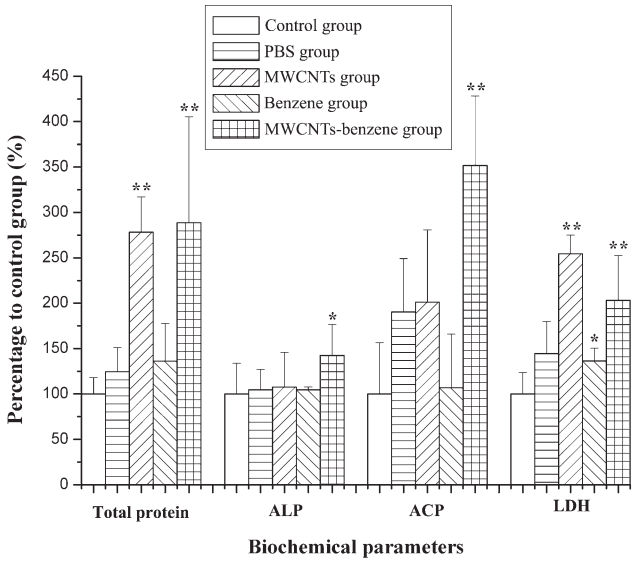

Fig. 3. The changes of biochemical parameters in BALF of mice in each group at 3-day postexposure. The data are expressed as percentage of PBS group and experimental groups to control group, whose values are shown as means ± SD ($n = 6$). *presents significant difference at $P < 0.05$ level ($n = 6$), **presents significant difference at $P < 0.01$ level ($n = 6$).

lungs tissue at 3-day postexposure, as were shown in Figure 5(A–C).

At 3-day postexposure, in the MWCNTs group, it could be observed that some MWCNTs aggregations adsorbed to inner wall of bronchi in lungs tissue [Fig. 5(D)], and some thinner bronchi were almost blocked by these MWCNTs aggregations [Fig. 5(E)]. In alveolar area, many inflammatory cells got together around the MWCNTs aggregations, and alveolar netted structure was destroyed at different degree [Fig. 5(D–F)], especially the alveolar netted structure around the MWCNTs aggregations was destroyed severely [Fig. 5(F)]. In the benzene group, no severe pathologic lesions were observed in bronchi of the lungs tissue. Many alveolar walls were hyperplastic and became thicker than that in the control group [Fig. 5(G)], but the basic alveolar netted structure still remained [Fig. 5(H,I)]. In the MWCNTs-benzene group, many bronchi were blocked by the aggregations of MWCNTs-benzene combination [Fig. 5(J)]. The alveolar netted structure was destroyed more severely than that in the MWCNTs group [Fig. 5(K,L)], and the alveolar structure around the aggregations of MWCNTs-benzene combination could not be almost discriminated.

At 7-day postexposure, in the MWCNTs group, quantity of MWCNTs aggregations adsorbed to the inner wall of bronchi decreased significantly [Fig. 6(A,B)]. It could not be observed that the bronchi were blocked by MWCNTs aggregations. The alveolar netted structure around MWCNTs aggregations was almost kept integrated [Fig. 6(B,C)]. In the benzene group, no severe pathologic lesions were observed in the bronchi. Some alveolar walls were slightly hyperplastic [Fig. 6(D–F)], but the basic alveolar netted structure was still kept integrated [Fig. 6(E,F)]. In the MWCNTs-benzene group, smaller-sized aggregations of MWCNTs-benzene combination adsorbed to the inner wall of some bronchi [Fig. 6(G–I)]. It could not be observed that the bronchi were blocked by these smaller-sized aggregations. Although the general alveolar netted structure was recovered at certain degree, the alveolar structure around the MWCNTs aggregations still sustained severe injury [Fig. 6(G–I)].

DISCUSSION

In this experiment, in order to calculate benzene mass that adsorbed on MWCNTs, ultraviolet spectrometer was used to measure benzene concentration before their adsorption on the MWCNTs and their postadsorption concentration. This method has been used to measure the benzene concentration in water solution, its veracity is about ± 0.1 mg/L (Hindarso et al., 2001; Wibowo et al., 2007), and accurate enough to satisfy our experiment.

In consideration of the following reasons, the method of intratracheal instillation was used to administrate MWCNTs, benzene and MWCNTs-benzene combination into mice lungs. (1) During intratracheal instillation, the actual dose delivered to the lungs of each animal can be essentially assured (Driscoll et al., 2000). The accurate

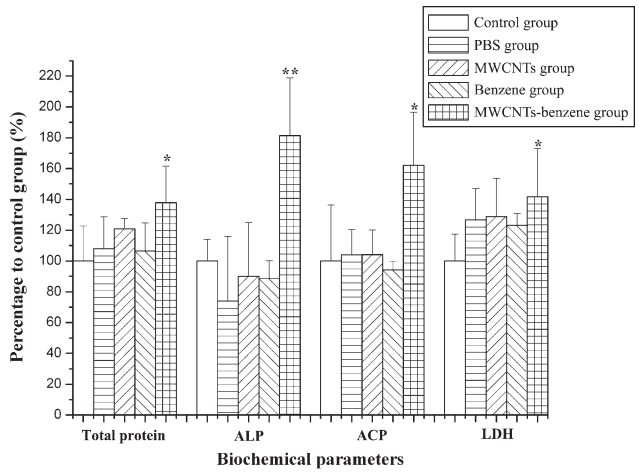

Fig. 4. The changes of biochemical parameters in BALF of mice in each group at 7-day postexposure. The data are expressed as percentage of PBS group and experimental groups to control group, whose values are shown as means ± SD ($n = 6$). *presents significant difference at $P < 0.05$ level ($n = 6$), **presents significant difference at $P < 0.01$ level ($n = 6$).

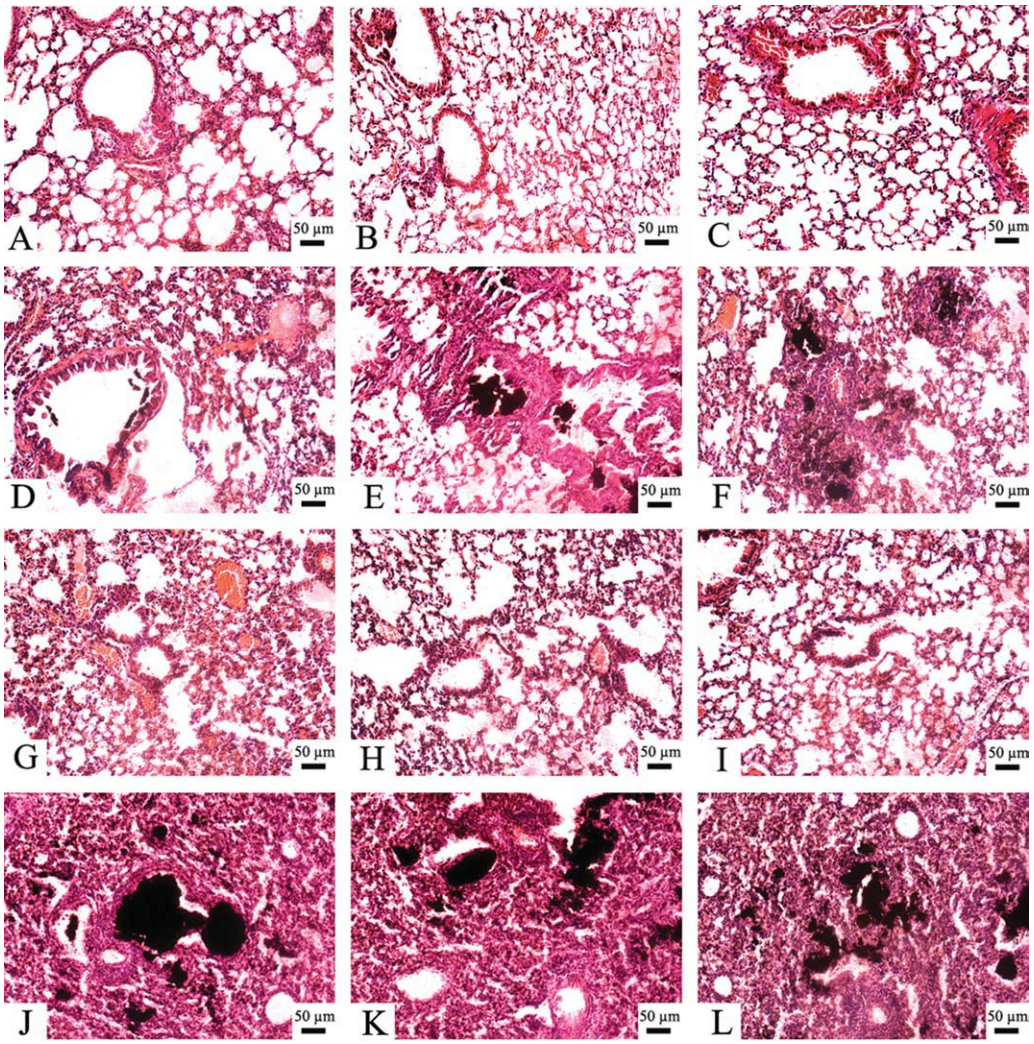

Fig. 5. The panels present the light micrographs of lung tissue from control, PBS, MWCNTs, benzene, and MWCNTs-benzene groups at 3-day postexposure. The light micrograph of lung tissue from control group is in (A). The light micrographs of lung tissue from PBS group are in (B) and (C). The light micrographs of lung tissue from MWCNTs group are in (D)–(F). The light micrographs of lung tissue from benzene group are in (G)–(I). The light micrographs of lung tissue from MWCNTs-benzene group are in (J)–(L). Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

intralung dose is important to determine the ranking of toxicity of MWCNTs, benzene and MWCNTs-benzene combination. (2) For the CNTs aggregation characterization, some of their aggregations could reach the deep lung regions in humans by inhalation but would not reach these regions in rodent lungs for the significant differences of respiratory system between humans and rodents. The pulmonary toxicity of these aggregations can be evaluated in rodents by the intratracheal instillation to a certain extent.

However, the intratracheal instillation has its own limitations as compared with the inhalation. The obvious limitations are that the test materials are not introduced into lungs physiologically and the lungs are exposed to greater dose and/or dose rate of the test materials within a short time. The deposition and clearance of instilled material within lung tissue are different from that of inhaled material. In addition, the mechanical lesions from instilled vehicle and greater-sized aggregation of material will affect the toxicity evaluation of test material (Driscoll et al., 2000). Our previous work and recent evidence indicated that CNTs deposition patterns within lungs are different when they are introduced into lungs via intratracheal instillation and inhalation (Li et al., 2007; Mitchell et al., 2007; Ryman-Rasmussen et al., 2009).

In order to reduce the influence of intratracheal instillation itself to pulmonary toxicity evaluation, the following measures were taken into account. (1) The instilled dose was strictly controlled to a proper level. (2) Evaluation of

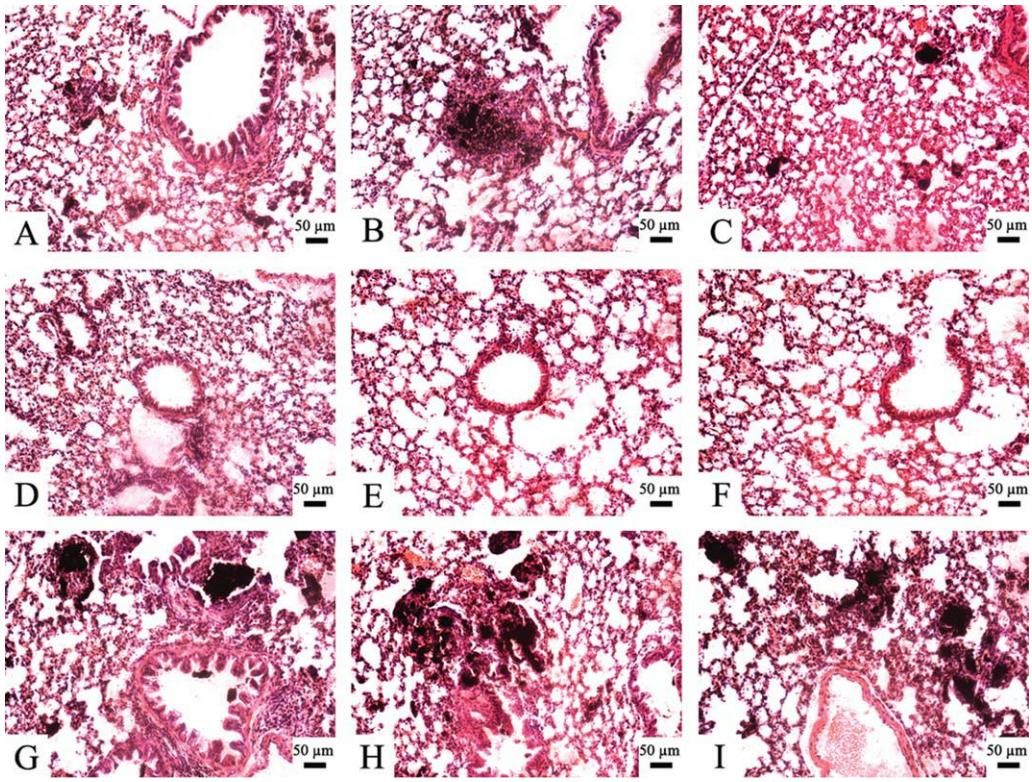

Fig. 6. The panels present light micrographs of lung tissue from MWCNTs, benzene, and MWCNTs-benzene groups at 7-day postexposure. The light micrographs of lung tissue from MWCNTs group are in (A)–(C). The light micrographs of lung tissue from benzene group are in (D)–(F). The light micrographs of lung tissue from MWCNTs-benzene group are in (G)–(I). Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pulmonary toxicity was carried out at 3-day and 7-day postexposure rather than at 1-day postexposure. (3) The deposition and clearance patterns of instilled materials within lung tissue were not used as the endpoints to evaluate pulmonary toxicity of the instilled materials. (4) PBS was used as medium to disperse the instilled materials.

In this work, the intratracheally instilled dose of MWCNTs in mice is 6.67 mg/kg body weight, which is four times of the dose used in Li's report (Li et al., 2007), and the corresponding pathologic lesions in lung are more serious than that in Li's report. When compared with other previous reports (Lam et al., 2004; Warheit et al., 2004; Muller et al., 2005), the instilled dose of MWCNTs in this work is similar to that in these reports, and the corresponding pathologic lesions are similar to that in these reports. In other words, the pulmonary lesions can be caused by the MWCNTs at the instilled dose of 6.67 mg/kg, and the lesions can be diluted with longer time elapsed. When the instilled dose of MWCNTs is too high, the mechanical lesions in lung will become obvious, and these mechanical lesions will cover the real pulmonary toxicity induced by MWCNTs. When the instilled dose of MWCNTs is too low, correspondingly, the benzene mass that adsorbed on the MWCNTs is much lower, and the acute pulmonary toxicity of benzene is too slight to be examined.

The weighted average permissible concentration of benzene in working settings is 6 mg/m^3 (1.9 ppm) in China (GBZ 2-2002). It is also assumed that a 30-g mouse breathes in 30 mL air per min (Parent, 1992) and fractional deposition rate of benzene into lung is about 50% (Pekari et al., 1992; Yu et al., 1998; Lee et al., 2006). Thus intralung deposition dose at 3-day postexposure is roughly 3.33 mg/kg. The instilled dose of benzene in our experiment is 2.67 mg/kg, which is slightly lower than the theoretically calculated value (3.33 mg/kg) at 3-day postexposure. It is reasonable that this dose of benzene only induced slight pulmonary toxicity at 3-day postexposure.

At 3-day and 7-day postexposure the biochemical parameters in BALF and pathologic lesions in lungs identically indicated that the pulmonary toxicity induced by MWCNTs-benzene combination is more severe than that induced by MWCNTs and benzene individually. The result may be explained by the following two reasons.

When low level benzene is alone inhaled into lung, it can enter blood via passive diffusion from lung and is rapidly distributed throughout the body (Rickert et al., 1979).

Then it is rapidly metabolized into several reactive metabolites (phenol, catechol, and hydroquinone) in liver (Medinsky et al., 1989; Snyder and Hedli, 1996). It is widely accepted that benzene toxicity is dependent upon these metabolites. As benzene is adsorbed on CNTs via π-stacking interactions between benzene and CNTs (Tournus and Charlier, 2005; Gauden et al., 2006; Crespo and Yang, 2006), most of it retain in lung together with the intralung CNTs (part of it will desorb from CNTs into lung liquid, for example, 13% benzene desorbs from CNTs to PBS in this experiment). As a result, the benzene is concentrated in lung by MWCNTs. Some experimental results demonstrated that the lung also plays an important role in benzene metabolism and, therefore, toxicity (Powley and Carlson, 1999).

In addition, it is possible that the MWCNTs-benzene combination aggregations in lungs are more difficult to be dispersed than the pure MWCNTs aggregations. As shown in Figures 4 and 5, the size of MWCNTs aggregations in mice lungs of MWCNTs group at 7-day postexposure is obviously smaller than that at 3-day postexposure, while the size of MWCNTs-benzene combination aggregations in mice lungs of MWCNTs-benzene group at 7-day postexposure is only slightly smaller than that at 3-day postexposure. These larger-sized aggregations of MWCNTs-benzene combination are difficult to be cleaned by alveolar macrophages. Thus the increment in aggregation ability of MWCNTs-benzene combination may be also an important reason to increase the pulmonary toxicity of the combination.

In summary, at the instilled doses in this experiment, the MWCNTs can alone induce acute pulmonary toxicity in mice and the benzene does not induce pulmonary toxicity, but the MWCNTs pulmonary toxicity is enhanced after they form MWCNTs-benzene combination with low dose of benzene. The enhanced pulmonary toxicity may be due to the change of MWCNTs aggregation ability after benzene is adsorbed on them.

REFERENCES

Aksoy M. 1989. Hematotoxicity and carcinogenicity of benzene. Environ Health Perspect 82:193–197.

Ball P. 2001. Roll up for the revolution. Nature 414:142–144.

Bang JJ, Guerrero PA, Lopez DA, Murr LE, Esquivel EV. 2004. Carbon nanotubes and other fullerene nanocrystals in domestic propane and natural gas combustion streams. J Nanosci Nanotechnol 4:716–718.

Colvin VL. 2003. The potential environmental impact of engineered nanomaterials. Nat Biotechnol 21:1166–1170.

Crespo D, Yang RT. 2006. Adsorption of organic vapors on single-walled carbon nanotubes. Ind Eng Chem Res 45:5524–5530.

Darrall K, Figgins J, Brown R, Phillips G. 1998. Determination of benzene and associated volatile compounds in mainstream cigarette smoke. Analyst 123:1095–1101.

Dick CA, Brown DM, Donaldson K, Stone V. 2003. The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types. Inhal Toxicol 15:39–52.

Driscoll KE, Costa DL, Hatch G, Henderson R, Oberdorster G, Salem H, Schlesinger RB. 2000. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: Uses and limitations. Toxicol Sci 55:24–35.

Esquivel EV, Murr LE. 2004. A TEM analysis of nanoparticulates in a Polar ice core. Mat Charact 52:15–25.

Gauden PA, Terzyk A P, Rychlicki G, Kowalczyk P, Lota K, Raymundopinero E, Frackowiak E, Beguin F. 2006. Thermodynamic properties of benzene adsorbed in activated carbons and multi-walled carbon nanotubes. Chem Phy Lett 421:409–414.

Hindarso H, Ismadji S, Wicaksana F, Mudjijati, Indraswati N. 2001. Adsorption of benzene and toluene from aqueous solution onto granular activated carbon. J Chem Eng Data 46:788–791.

Koyama S, Kim YA, Hayashi T, Takeuchi K, Fujii C, Kuroiwa N, Koyama H, Tsukahara T, Endo M. 2009. In vivo immunological toxicity in mice of carbon nanotubes with impurities. Carbon 47:1365–1372.

Lam CW, James JT, McCluskey R, Hunter RL. 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol Sci 77:126–134.

Lee CW, Dai YT, Chien CH, Hsu DJ. 2006. Characteristics and health impacts of volatile organic compounds in photocopy centers. Environ Res 100:139–149.

Li JG, Li WX, Xu JJ, Cai XQ, Liu RL, Li YJ, Zhao QF, Li QN. 2007. Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation. Environ Toxicol 22:415–421.

Long RQ, Yang RT. 2001. Carbon nanotubes as superior sorbent for dioxin removal. J Am Chem Soc 123:2058–2059.

Madl AK, Paustenbach DJ. 2002. Airborne concentrations of benzene due to diesel locomotive exhaust in a roundhouse. J Toxicol Environ Health A 65:1945–1964.

Maynard AD, Baron PA, Foley M, Shvedova AA, Kisin ER, Castranova V. 2004. Exposure to carbon nanotube material during the handling of unrefined single walled carbon nanotube material. J Toxicol Environ Health A 67:87–107.

Medinsky MA, Sabourin PJ, Henderson RF, Lucier G, Birnbaum LS. 1989. Differences in the pathways for metabolism of benzene in rats and mice simulated by a physiological model. Environ Health Perspect 82:43–49.

Mitchell LA, Gao J, Wal RV, Gigliotti A, Burchiel SW, McDonald JD. 2007. Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. Toxicol Sci 100:203–214.

Muller J, Huaux F, Moreau N, Misson P, Heilier JF, Delos M, Arras M, Fonseca A, Nagy JB, Lison D. 2005. Respiratory toxicity of multi-wall carbon nanotubes. Toxicol Appl Pharmacol 207:221–231.

Mullin AH, Nataraj D, Ren JJ, Mullin DA. 1998. Inhaled benzene increases the frequency and length of *lacI* deletion mutations in lung tissues of mice. Carcinogenesis 19:1723–1733.

Murr LE, Bang JJ, Esquivel EV, Guerrero PA, Lopez DA. 2004a. Carbon Nanotubes, nanocrystal forms, and complex nanoparticle aggregates in common fuel-gas combustion sources and the ambient air. J Nanopart Res 6:241–251.

Murr LE, Soto KF, Esquivel EV, Bang JJ, Guerrero PA, Lopez DA, Ramirez DA. 2004b. Carbon nanotubes and other fullerene-related nanocrystals in the environment: A TEM study. J Miner Met Mater Soc 56:28–31.

Nel A, Xia T, Madler L, Li N. 2006. Toxic potential of materials at the nanolevel. Science 311:622–627.

Parent RA. 1992. Comparative Biology of the Normal Lung. Boca Raton, FL: CRC Press. pp 217–241.

Pekari K, Vainiotalo S, Heikkila P, Palotie A, Luotamo M, Riihimaki V. 1992. Biological monitoring of occupational exposure to low levels of benzene. Scand J Work Environ Health 18: 317–322.

Peng X, Li Y, Luan Z, Di Z, Wang H, Tian B, Jia Z. 2003. Adsorption of 1, 2-dichlorobenzene from to carbon nanotubes. Chem Phys Lett 376:154–158.

Powley MW, Carlson GP. 1999. Species comparison of hepatic and pulmonary metabolism of benzene. Toxicology 139:207–217.

Rickert DE, Baker TS, Bus JS, Barrow CS, Irons RD. 1979. Benzene disposition in the rat after exposure by inhalation. Toxicol Appl Pharmacol 49:417–423.

Ryman-Rasmussen JP, Tewksbury EW, Moss OR, Cesta MF, Wong BA, Bonner JC. 2009. Inhaled multi-walled carbon nanotubes potentiate airway fibrosis in murine allegic asthma. Am J Respir Cell Mol Bio 40:349–358.

Snyder R. 2000. Overview of the toxicology of benzene. J Toxicol Environ Health 61:339–346.

Snyder R, Hedli CC. 1996. An overview of benzene metabolism. Environ Health Perspect 104:1165–1171.

Snyder R, Witz G, Goldstein BD. 1993. The toxicology of benzene. Environ Health Perspect 100:293–306.

Tournus F, Charlier JC. 2005. Ab initio study of benzene adsorption on carbon nanotubes. Phys Rev B 71:165421–165428.

Warheit BD, Laurence BR, Reed KL, Roach DH, Reynolds GA, Webb TR. 2004. Comparative pulmonary toxicity assessment of single wall carbon nanotubes in rats. Toxicol Sci 77:117–125.

Wibowo N, Setyadhi L, Wibowo D, Setiawan J, Ismadji S. 2007. Adsorption of benzene and toluene from aqueous solutions onto activated carbon and its acid and heat treated forms: Influence of surface chemistry on adsorption. J Hazard Mater 146:237–242.

Yardley-Jones A, Anderson D, Parke DV. 1991. The toxicity of benzene and its metabolism and molecular pathology in human risk assessment. Br J Ind Med 48:437–444.

Yin S, Hayes R, Linet M, Li G, Dosemeci M, Travis L, Li C, Zhang Z, Li D, Chow W, Wacholder S, Wang Y, Jiang Z, Dai T, Zhang W, Chao X, Ye P, Kou Q, Zhang X, Lin X, Meng J, Ding C, Zho J, Blot W. 1996. A cohort study of cancer among benzene-exposed workers in China: Overall results. Am J Ind Med 29:227–235.

Yin S, Li G, Tain F, Fu Z, Jin C, Chen Y, Luo S, Ye P, Zhang J, Wang G, Zhang X, Wu H, Zhong Q. 1989. A retrospective cohort study of leukemia and other cancers in benzene workers. Environ Health Perspect 82:207–213.

Yu R, Weisel CP. 1998. Measurement of benzene in human breath associated with an environmental exposure. J Expos Anal Environ Epidemiol 6:261–277.