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Pulmonary Toxicity in Mice Exposed to Low and Medium Doses of Water-Soluble Multi-Walled Carbon Nanotubes

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Carbon nanotubes (CNTs) are a class of new allotrope of carbon. Different functionalized CNTs may vary from their physical and chemical properties to the biological property. In this study, the toxicity of water-soluble taurine multi-walled CNTs (tau-MWNTs), raw MWNTs and positive control crystalline silicon dioxide particles on mouse lungs via intratracheal instillation (*i.t.*) was investigated. The dosages we used were 0.125, 0.25, 0.5 or 1 mg/kg of tau-MWNTs and raw MWNTs, and 1 mg/kg of silicon dioxide particles; Serum and lungs were collected at 1, 7, 14 or 28 days postexposure. The biochemical and cellular parameters were assessed, which include the ratio of the lung weight and body weight (lung indices), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and angiotensin converting enzyme (ACE) in serum, and malondialdehyde (MDA), reduced glutathione (GSH), total sulfhydryl group (TSH) in lung tissue homogenates as well as the hydroxyproline in lungs. The characteristic recovery of the lung injury at 28 days postexposure was examined by the assessment of LDH, ALP, lung indices, and histopathology. ACE, MDA, GSH, TSH and histopathological changes showed that tau-MWNTs were less toxic than the raw MWNTs. Histopathological and ultrastructural investigation indicated that the acute pulmonary inflammation in lungs alleviated after 7d postexposure, and were greatly recovered within 28d. Meanwhile, the entrapment of tau-MWNTs reduced greatly by the 28d postexposure. Whereas the heavier pathologic changes induced by raw MWNTs lasted 7 days more than that of tau-MWNTs. Notably, no occurrence of granulomas and fibrosis were found in this study both in the two CNTs samples through 28d postexposure. Silicon dioxide particles, on the contrary, produced more severe damage to lungs than CNTs did in lung index, as well as other biochemical and cellular parameters. These findings indicate that water-soluble tau-MWNTs in low and medium doses induce slight and recoverable pulmonary inflammation in mice, and are less toxic than the insoluble raw MWNTs.

Keywords: Taurine Multi-Walled Carbon Nanotubes (Tau-MWNTs), Mouse Lungs, Pulmonary Toxicity, Inflammation, Recovery Character.

1. INTRODUCTION

Rapid developments in nanotechnology lead to a great number of nanomaterials entering our human life and environment or the ecosystem. Carbon nanotube (CNT) is a distinguished member of the nanomaterial family. It has been predicted that hundreds of tons of CNTs will be produced worldwide every year.[1–4] That undoubtedly increases the concern of the adverse effects of CNTs to environment and humans.

During the manufacture and application processes, one of the inevitable exposure routes for the people is respiratory system. Literatures that focus on respiratory toxic effects of CNTs, including single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs), are still limited. In 2001, Huczko et al. firstly studied

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the effects of raw CNTs on the pulmonary function of guinea pigs, and found no evident pulmonary inflammatory lesion after intratracheal instillation (*i.t.*) of the raw CNTs for 4 weeks.[5] But in their following work, they found that the raw CNTs brought on evident granulomas at 90 days postinstillation, and considered that exposure time is the key factor in pulmonary toxic effects.[6] Lam et al.[7] Warheit et al.[8] Shevdova et al.[9] and Muller et al.[10] published their in-depth studies on the pulmonary toxicity of SWNTs in mice or rats given by intratracheal or intrapharyngeal instillation; these authors showed that SWNTs induced inflammation and granulomas. Shvedova also found SWNTs cause progressive interstitial fibrosis. Lam et al. and Shevdova et al. concluded that SWNTs were more toxic than quartz and much more toxic than carbon black. Their reports have largely alerted the estimated risk of human exposure to CNTs. Muller et al. also found MWNTs could induce pulmonary inflammatory lesion and local fibrosis, and the lesion became more evident with the prolonged exposure. Mangum and the colleagues found SWNTs did not cause pulmonary inflammation on rats but induced small interstitial fibrotic lesions in the lung alveolar regions.[11] Recently, Mitchell et al. reported that inhalation exposure to MWNTs did not result in significant lung inflammation but caused systemic immune function alterations.[12]

However, it should be noted that most the mentioned pulmonary toxicity studies were only conducted with the insoluble raw or pristine CNTs. It is well-known that functionalization may change the solubilization and chemical properties, and then the biological properties of CNTs. It is therefore very necessary to investigate the pulmonary toxicity of the functionalized water-soluble CNTs.

Very recently we reported the lung burden and translocation of MWNTs in mice *i.t.* exposed to ^{14}C labeled taurine modified MWNTs (^{14}C-tau-MWNTs). Taurine ($NH_2CH_2CH_2SO_3H$) is a sulfur-containing amino-acid. We found that about 80% of the intratracheally instilled ^{14}C-tau-MWNTs dose was accumulated in lungs when assessed in 1d or 2d postexposure, and was gradually eliminated from the lung 3d postexposure, and further declined to 20% at 28d postexposure. No ^{14}C-activity was detectable in the other tissues after instillation, even when the activity in the lung largely reduced after 28d postexposure.[13] We inferred that tau-MWNTs did not pass through the alveolar basement membrane to the blood stream and distant organs.

Herein, we present the pulmonary toxicological data of mice *i.t.* exposed to low and medium doses of the water-soluble tau-MWNTs, the raw MWNTs and positive control silicon dioxide particles. The data measured include the biological and biochemical parameters of lung indices, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), angiotensin converting enzyme (ACE) in serum, malondialdehyde (MDA), reduced glutathione (GSH), total sulfhydryl group (TSH) and the hydroxyproline in lung tissue homogenates as well as the histopathological and ultrastructural observations of lung tissues. We found that tau-MWNTs brought on the slight and reversible toxic effects on mouse lungs.

2. MATERIALS AND METHODS

2.1. Materials and Characterization

The raw MWNTs, produced by chemical vapor deposition method (CVD) with a diameter of 10–20 nm and a purity of >95% were purchased from Shenzhen Nanoharbor, China. The morphological characterization of length and diameter of the purified MWNTs were made by transmission electron microscopy (TEM, JEM-200CX) in our lab. MWNTs were further characterized in our laboratory by thermogravimetric analysis (TGA) and ICP-MS. The metal content of MWNTs was determined by ICP-MS (Thermo Elemental X7) following Lam's method.[7] Tau-MWNTs were synthesized in our laboratory and further characterized by a variety of methods, including micro-infrared spectrometry (IR), TEM (JEM-200CX), X-ray photoelectron spectroscopy (XPS, Kratos Axis Ultra, UK). Information on synthesis and more detailed characterization were stated in our previous work.[13]

Silicon dioxide was used as a positive control. It was obtained from the National Institute for Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention (Beijing, China). Silicon dioxide has a purity of 99%. More than 95% of the crystalline silica particles were less than 5 μm in diameter. The surface area for silicon dioxide is 21.26 m^2/g according to the BET measurement.

2.2. Preparation of MWNTs Suspension

MWNTs and tau-MWNTs were freshly suspended in phosphate buffered saline (PBS) with a Dounce homogenizer (about 20 strokes) and sonicated for 20 min at 40 kHz in a short break every 2 min for vortex. A stable suspension of MWNTs and tau-MWNTs were obtained in this way and used immediately.

2.3. Animals

Healthy adult male CD-1 (*ICR*) mice weighing 18∼22 g were purchased from Department of Laboratory Animal Science, Peking University Health Science Center. They were randomly grouped (5 mice/group) and kept in a conventional animal facility and housed in air-conditioned units (25 °C, 50% relative humidity) on a 12:12 h light/dark cycle. They were cared for and used humanely according to Animal Care and Use Program Guidelines of Peking University. The experimental protocol was approved by the local ethical committee for animals in research.

2.4. Intratracheal Instillation (i.t.) Exposure

After being anesthetized with ether for 20∼30 sec in a small chamber, individual mouse was secured on an inclined ligneous platform. The trachea was exposed by a 0.5 cm incision on the ventral neck skin, and a small hole was made in the trachea close to the larynx, then the blunted needle was inserted inside trachea and the prefilled 0.125, 0.25, 0.5 or 1 mg/kg tau-MWNTs, raw MWNTs, 1 mg/kg silicon dioxide and PBS (negative control) were rapidly propelled into the lungs. The neck incision was then sutured, swabbed with iodine. The mice were recovered active within 3 min after removal from anesthetic. The incision healed within one day, and the mice were observed daily until their scheduled termination. There were another 10 mice taken as blank control that was instilled nothing. Mice were sacrificed respectively after exposure to CNTs for 1, 7, 14 and 28 days. The blood samples and the organs including lungs, liver, kidneys, heart and spleen were collected for the following analysis.

2.5. Biochemical Assay

The blood samples were collected by picking off the eyeballs. Serum was obtained after centrifugation of the collected blood samples at 4000 rpm for 10 min. All biochemical assays were performed using a Hitachi 7170A clinical automatic chemistry analyzer. Activity of LDH, ALP and ACE were measured using the commercial kits (Bühlmann Laboratories, Switzerland).

2.6. Histopathological Study

The portions of lungs, liver, kidneys, heart and spleen samples for microscopic examination were formalin-fixed, embedded in paraffin, thin-sections were mounted on glass microscope slides using standard histopathological techniques. Sections were stained with hematoxylin-eosin and examined by light microscopy.

2.7. Ultrastructural Analysis of the Lungs

Representative portions of the lungs were prefixed with 1% glutaraldehyde at 4 °C overnight. After being washed, they were postfixed with 1% osmium tetraoxide at 4 °C for 3 hours and washed with 0.1 M cacodylate buffer. Following dehydration, resin embedding, sectioning to 70 nm thick and staining with uranyl acetate and lead citrate, the lung samples were examined with a Hitachi H-600 transmission electron microscope (TEM).

2.8. Preparation of Lung Homogenates

A piece of each lung sample (0.5–1 g) was taken for tests. The tissues were minced and washed several times by cold physiological saline (0.9% NaCl), and homogenized in 4 °C PBS for 3 times (10 s/time, intermittent for 30 sec). At last, the homogenates were centrifuged at 2000 rpm for 10 min to obtain the lung supernatant. Protein concentrations of lung supernatant were determined according to the Bradford's method, using bovine serum albumin (BSA) as the standard.[14] BSA and coomassie brilliant blue G-250 was purchased from Sigma.

2.9. Determination of GSH, TSH and MDA

The levels of GSH and TSH in lung supernatant were examined using the modified Ellman method by Sedlak et al.[15] The levels of the TSH were measured at 412 nm based on the reduction of 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) to 2-nitro-5-thiobenzoate anion (NTP).

The levels of lipid peroxidation were reflected by the value of MDA, which was determined using the method of thiobarbituric acid reactive species with 1,1,3,3-tetraethoxypropane as the standard.[16]

2.10. Determination of Hydroxyproline

The levels of tissue hydroxyproline (Hyp) were examined using the modified method of Edwards and O'Brien.[17]

2.11. Statistics

Each of the experimental data was presented as mean ± S.D. and compared to the blank control for each time point and dose level. A one-way analysis of variance (ANOVA) was calculated. When the F test from ANOVA was significant, least significant difference (LSD) t test was used to compare means of each group. Statistical significance was considered at $p < 0.05$.

3. RESULTS

3.1. Characterization of MWNTs and Tau-MWNTs

After oxidation cutting, the length of MWNTs was shortened to 300–600 nm. TGA displayed the purity of nanotube is around 97% by weight. The TEM image (Fig. 1)

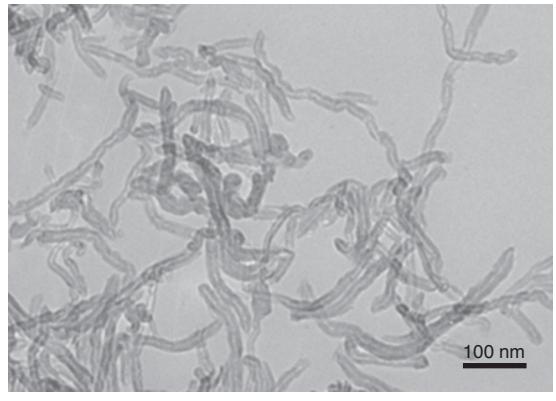


Fig. 1. Representative TEM image of the tau-MWNTs.

of tau-MWNTs clearly depicted that the skeleton structure of tau-MWNTs remained intact and well dispersed in the solution without bundling. ICP-MS data show that MWNTs contain around 1% metals by weight, such as Ni (0.85%), Fe (0.62%) and Co (0.38%). BET (Brunauer-Emmett-Teller analysis) analysis showed that the surface area is estimated to be 133.70 m^2/g. More detailed characterization information was presented in our previous work.[13]

3.2. Symptoms Observation and Body Weight Gaining

The mice of all groups did not show any symptoms of abnormality, such as lethargy, anorexia, vomiting, and diarrhea etc. during the whole experimental period. There was no difference on body weight gaining between all exposed groups and the blank control during the experimental period ($p > 0.05$).

3.3. Lung Indices

Lung indices, which means the ratio of the lung weight and body weight, was calculated using the weight of lung divided by the body weight. Shown in Table I, lung indices of tau-MWNTs of all dose levels at 1d post-exposure were significantly higher compared to the PBS control and blank control groups ($p < 0.05$). Of all treated groups, lung indices exhibited descending trend with the increasing exposure time and returned to the blank control level at 28d. The trends of lung indices of raw MWNTs were similar to those of tau-MWNTs. But, that of silicon dioxide group went up in the last two weeks postexposure.

3.4. Mouse Serum Biochemical Indices

We choose three indices to evaluate the pulmonary bioeffects by the MWNTs, including ALP, LDH and ACE. The increase of ALP activity in serum is considered to be a toxicity indicator of type II alveolar epithelial cell. LDH is a cytoplasmic enzyme and is often used as an indicator of AM injury.[18] Serum ACE activity represents an index of toxicity of pulmonary capillary endothelial cells.[19]

The ALP activity of all dose groups went up significantly (higher than the blank control) within 7d postexposure and most of the tau-MWNT groups (except for 1 mg/kg) peaked at 7d, while those of the raw MWNTs groups peaked at 14d. Then all serum ALP activities recovered to the blank control level at 28d. (Data are shown in Table II). The LDH activity changes of tau-MWNTs and raw MWNTs were very similar to ALP activity changes. However, that of silicon dioxide group went up at 28d postexposure. (Table III)

The serum ACE activity of the tau-MWNTs groups did not change significantly, compared with that of PBS and the blank controls, however that of the raw MWNTs groups went up within 1d postexposure and quickly dropped to the blank control level at 7d. The ACE activity of the silicon dioxide group experienced a trend of descending first then ascending. (Table IV)

3.5. Histopathological Changes

Histopathological evaluation of lung tissues revealed that pulmonary exposure to tau-MWNTs in mice induced a dose-dependent lung inflammatory response characterized by neutrophils and alveolar macrophage (AM) accumulation (Fig. 2). Compared to the PBS and blank controls, there were evident inflammatory response and proliferation changes in lungs of every dose and time point. Lungs of high dose level (0.5 mg/kg and 1 mg/kg) presented severer pathologic changes. After 1d and 7d (Figs. 2(C and D)) postexposure, alveolar walls thicken, parenchymal cells proliferate, many neutrophils and alveolar macrophages accumulate, some alveolar cavities are squeezed to forfeit ventilation function. And moreover, some others became compensationally emphysema and some AM and the pulmonary interstitial were filled with many tau-MWNTs. Pathological changes were gradually relieving from porta

Table I. Lung indexes of different doses and time points after exposure (mean ± SD).

Dose groups (mg/kg)		Lung indexes 1d	7d	14d	28d
Control		0.0054 ± 0.0002	0.0055 ± 0.0005	0.0051 ± 0.0006	0.0052 ± 0.0003[b]
PBS Control		0.0061 ± 0.0008	0.0066 ± 0.0004	0.0058 ± 0.0007	0.0057 ± 0.0011[b]
tau-MWNTs	0.1250	0.0078 ± 0.0003[a]	0.0065 ± 0.0001	0.0058 ± 0.0003	0.0050 ± 0.0006[b]
	0.2500	0.0087 ± 0.0001[a]	0.0072 ± 0.0006	0.0060 ± 0.0003	0.0053 ± 0.0001[b]
	0.5000	0.0083 ± 0.0007[a]	0.0076 ± 0.0008	0.0062 ± 0.0003	0.0055 ± 0.0007[b]
	1.0000	0.0115 ± 0.0004[a]	0.0091 ± 0.0006	0.0087 ± 0.0002	0.0054 ± 0.0001[b]
raw MWNTs	0.1250	0.0082 ± 0.0003[a]	0.0067 ± 0.0008	0.0065 ± 0.0006	0.0054 ± 0.0004[b]
	0.2500	0.0085 ± 0.0012[a]	0.0073 ± 0.0001	0.0064 ± 0.0003	0.0060 ± 0.0006
	0.5000	0.0089 ± 0.0013[a]	0.0075 ± 0.0011	0.0067 ± 0.0012	0.0060 ± 0.0008
	1.0000	0.0115 ± 0.0014[a]	0.0073 ± 0.0005	0.0064 ± 0.0005	0.0064 ± 0.0003
Quartz	1.0000	0.0074 ± 0.0005[a]	0.0079 ± 0.0005	0.0074 ± 0.0004	0.0073 ± 0.0022

[a]Groups statistically different from the PBS control, $P < 0.05$, [b]Groups of 1d postexposure statistically different from Groups of 28d postexposure, $P < 0.05$.

Table II. ALP activity of different doses and time points after exposure.

		ALP activity after i.t. exposure (μ mol·s^{-1}/L)			
Dose groups (mg/kg)		1d	7d	14d	28d
Control		2.3800 ± 0.3600	2.4100 ± 0.5100	2.3500 ± 0.5600	2.3900 ± 0.6700
PBS Control		2.0600 ± 0.4500	2.1100 ± 0.6400	1.9900 ± 0.3500	2.0700 ± 0.4100
tau-MWNTs	0.1250	2.6600 ± 0.4600	3.5000 ± 1.4100[a, b]	2.2200 ± 0.2000	1.8000 ± 0.2500
	0.2500	3.0000 ± 0.5600[a, b]	3.7700 ± 1.1100[a, b]	2.5200 ± 0.4200	1.8500 ± 0.1000
	0.5000	3.2000 ± 0.5800[a, b]	4.1500 ± 0.7000[a, b]	2.5600 ± 0.5800	1.7700 ± 0.2600
	1.0000	4.5800 ± 0.7000[a, b]	4.7200 ± 0.8900[a, b]	4.1100 ± 0.7400[a, b]	2.0800 ± 0.2200
raw MWNTs	0.1250	2.2400 ± 0.4500	3.8600 ± 0.7000[a, b]	4.4500 ± 0.5800[a, b]	2.5900 ± 0.2900
	0.2500	2.2500 ± 0.4400	3.8900 ± 0.5300[a, b]	4.2900 ± 0.2300[a, b]	2.5400 ± 0.3200
	0.5000	2.3100 ± 0.5600	3.8800 ± 0.6700[a, b]	4.8600 ± 0.7300[a, b]	2.6100 ± 0.4100
	1.0000	3.2600 ± 0.4900[a, b]	3.9500 ± 0.3800[a, b]	4.3700 ± 0.6600[a, b]	2.7100 ± 0.1200
Quartz	1.0000	4.4200 ± 0.8600[a, b]	3.7900 ± 0.4400[a, b]	4.1400 ± 0.7100[a, b]	5.9500 ± 0.5400[a, b]

[a]Groups statistically different from the PBS control, $P < 0.05$, [b]Groups statistically different from the blank control, $P < 0.05$.

Table III. LDH activity of different doses and time points after exposure.

		LDH activity after i.t. exposure (μ mol·s^{-1}/L)			
Dose groups (mg/kg)		1d	7d	14d	28d
Control		10.8500 ± 2.6800	11.3100 ± 3.1100	11.0700 ± 5.0100	10.8700 ± 4.2900
PBS Control		11.1500 ± 6.3700	11.2000 ± 3.9100	10.9800 ± 1.3300	11.2100 ± 6.6100
tau-MWNTs	0.1250	15.3500 ± 3.4300[a, b]	13.4100 ± 3.3000	11.8400 ± 2.9200	11.6900 ± 3.9300
	0.2500	10.7500 ± 1.6400	13.2000 ± 3.7100	11.6700 ± 2.1900	10.0200 ± 2.8000
	0.5000	16.6800 ± 3.1500[a, b]	17.0300 ± 1.4000[a, b]	9.6700 ± 6.3700	10.4900 ± 3.8200
	1.0000	14.4300 ± 3.2300	13.4000 ± 2.9200	12.5700 ± 4.6200	14.1800 ± 1.7000
raw MWNTs	0.1250	11.5100 ± 1.4500	13.5200 ± 2.7000	11.7300 ± 1.3400	12.4800 ± 2.1900
	0.2500	11.5800 ± 2.3400	13.5600 ± 1.5200	12.7600 ± 2.3100	12.1100 ± 1.2100
	0.5000	11.7400 ± 1.5600	14.0600 ± 1.1100	13.2600 ± 1.1900	12.4800 ± 3.8100
	1.0000	11.9800 ± 3.1100	14.6900 ± 1.2500[a, b]	13.7200 ± 2.6300	12.7600 ± 2.0300
Quartz	1.0000	14.9100 ± 2.3600[a, b]	16.7600 ± 1.5900[a, b]	15.0800 ± 3.3700[a, b]	13.2200 ± 1.9200

[a]Groups statistically different from the PBS control, $P < 0.05$, [b]Groups statistically different from the blank control, $P < 0.05$.

of lung to the distance. But in lungs of 14d postexposure (Fig. 2), pulmonary inflammation recovered, and alveolar walls became thinner than before. Also, the amount of tau-MWNTs entrapped in alveolar space and interstitial tissues decreased. At 28d postexposure (Fig. 2), injuries in lungs were greatly recovered just like as that in PBS group. In contrast, the lung tissue responses to raw MWNTs clearly were more serious compared with tau-MWNTs (Fig. 3). No granulomas and fibrosis was observed in both of CNTs exposed lungs.

Table IV. ACE activity of different doses and time points after exposure.

		ACE activity after i.t. exposure (μ mol·s^{-1}/L)			
Dose groups (mg/kg)		1d	7d	14d	28d
Control		5.5800 ± 0.3400	5.6900 ± 0.5200	5.6100 ± 0.3100	5.4900 ± 0.2300
PBS Control		5.5100 ± 0.3400	5.7000 ± 0.1300	5.6100 ± 0.2700	5.4400 ± 0.2900
tau-MWNTs	0.1250	5.1700 ± 0.4100	5.7800 ± 0.4000	5.5600 ± 0.4300	5.3000 ± 0.2000
	0.2500	4.7400 ± 0.4600	5.8800 ± 0.2600	5.5400 ± 0.3600	5.4400 ± 0.3300
	0.5000	5.2200 ± 0.3300	5.9300 ± 0.3200	5.6000 ± 0.3300	5.6500 ± 0.4100
	1.0000	4.8700 ± 0.6800	5.8100 ± 0.1100	5.2500 ± 0.1700	5.6600 ± 0.4900
raw MWNTs	0.1250	7.8700 ± 0.4500[a, b]	5.4500 ± 0.7000	5.7400 ± 0.1900	5.6100 ± 0.2400
	0.2500	8.1700 ± 0.3900[a, b]	5.3300 ± 0.3200	5.5900 ± 0.3100	5.5800 ± 0.2100
	0.5000	8.5400 ± 0.2100[a, b]	5.5300 ± 0.2600	5.4200 ± 0.2700	5.7100 ± 0.8100
	1.0000	8.4000 ± 0.1500[a, b]	5.2400 ± 0.2500	5.3500 ± 0.2600	5.7100 ± 0.2800
Quartz	1.0000	8.7000 ± 0.2800[a, b]	5.4400 ± 0.5300	5.7400 ± 0.1200	7.7100 ± 0.6200[a, b]

[a]Groups statistically different from the PBS control, $P < 0.05$, [b]Groups statistically different from the blank control, $P < 0.05$.

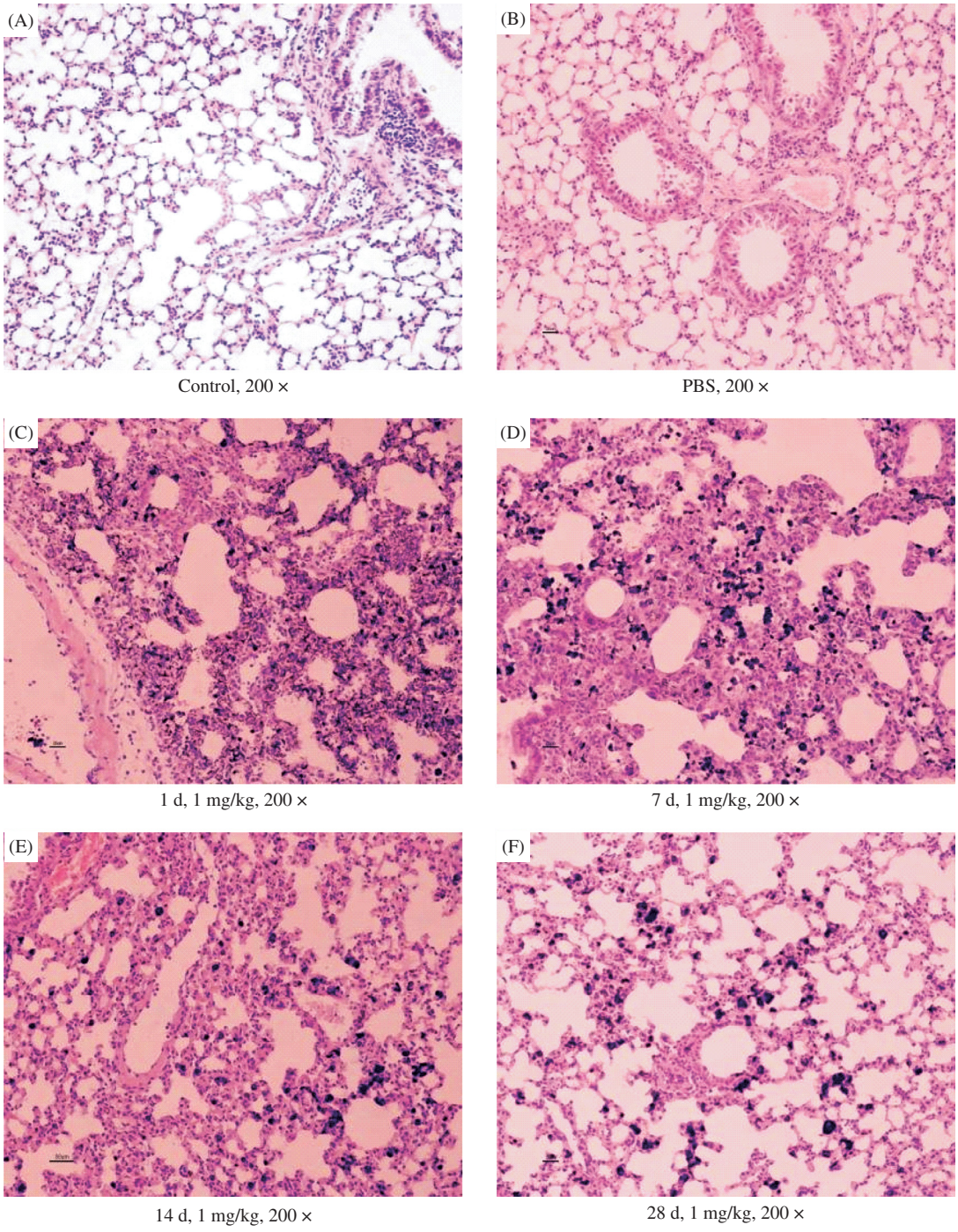


Fig. 2. Representative histopathologic light micrographs of lung tissue, (A) the blank control group; (B) PBS group; (C) exposed to tau-MWNTs (1 mg/kg), 1 day post-exposure; (D) exposed to tau-MWNTs (1 mg/kg), 7 days post-exposure; (E) exposed to tau-MWNTs (1 mg/kg), 14 days post-exposure; (F) exposed to tau-MWNTs (1 mg/kg), 28 days post-exposure. Magnification = 200×.

3.6. Ultrastructural Evaluation of Lungs

Ultrastructural TEM image (Fig. 4) shows that lungs present significant changes indicating pulmonary inflammation and lung cell proliferation. Many tau-MWNTs were clearly found in phagosomes of the AM, some bronchial epithelia, and also epithelia of alveolar sac and ductuli alveolar. There was no sign of necrosis in mouse lungs. Similar ultrastructural alterations were observed in raw MWNTs instilled lungs.

3.7. Tissue GSH, TSH and MDA Levels

There was no significant change in GSH, TSH and MDA levels in lung tissues of tau-MWNTs exposed groups when compared with the blank control. However, changes in raw

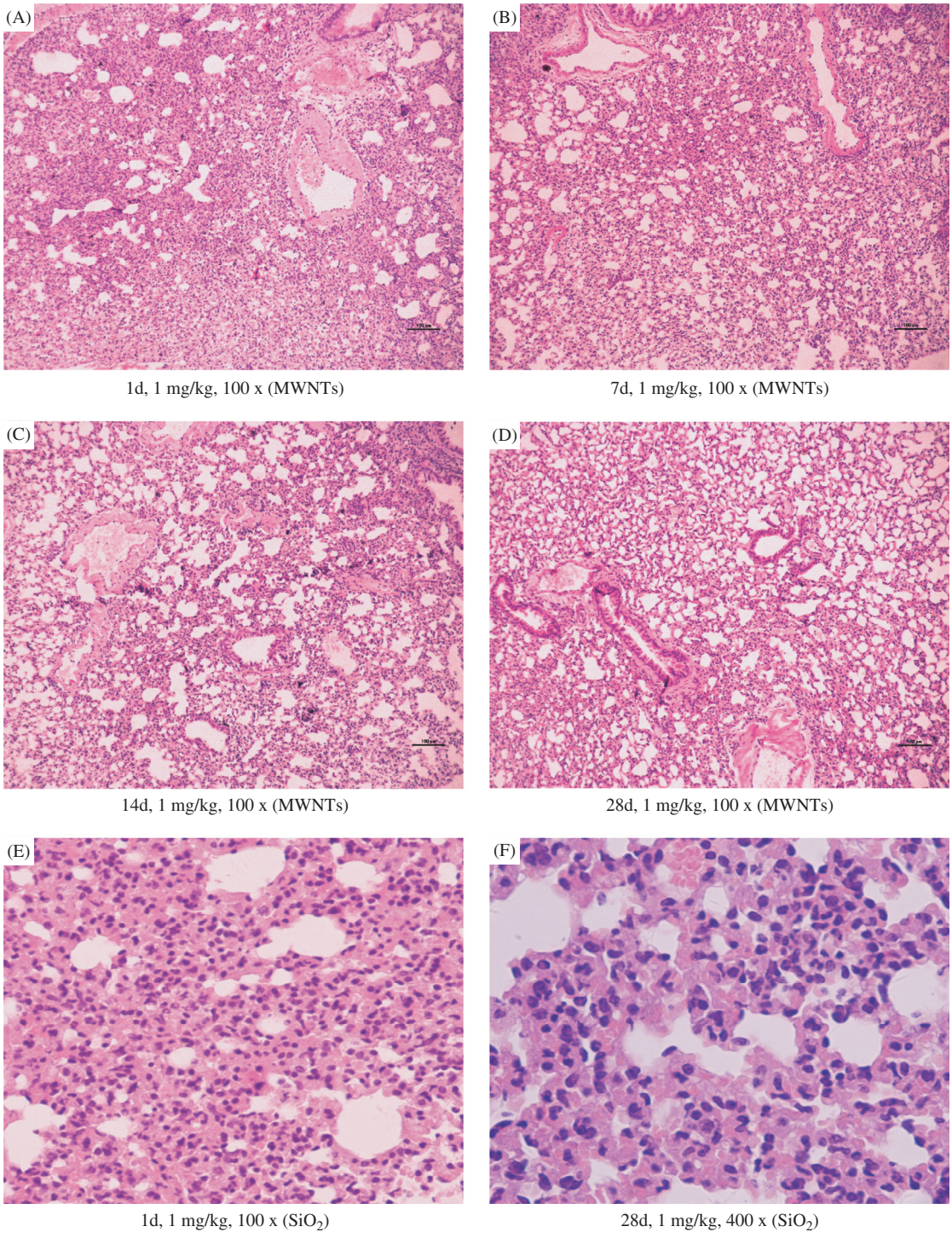


Fig. 3. Representative histopathologic light micrographs of lung tissue, (A) exposed to MWNTs (1 mg/kg), 1 days post-exposure; (B) exposed to MWNTs (1 mg/kg), 7 days post-exposure; (C) exposed to MWNTs (1 mg/kg), 14 days post-exposure; (D) exposed to MWNTs (1 mg/kg), 28 days post-exposure; (E) exposed to SiO_2 (1 mg/kg), 1 days post-exposure; (F) exposed to SiO_2 (1 mg/kg), 28 days post-exposure. Magnification = 100×. (F:400×).

MWNTs exposed groups were statistically significant. 1d after the instillation of raw MWNTs the GSH of all groups went up to the highest levels and then dropped to the blank control level at 7d in 0.125, 0.25 and 0.5 mg/kg groups, but 1 mg/kg went up again from 14d to the 28d postexposure. Dose-dependent effect was explicitly seen at 1d, 1 mg/kg group presented the highest level, 10 times of the blank control ($p < 0.05$). (Fig. 5) The silicon dioxide induced much higher production of GSH compared with the two CNTs samples.

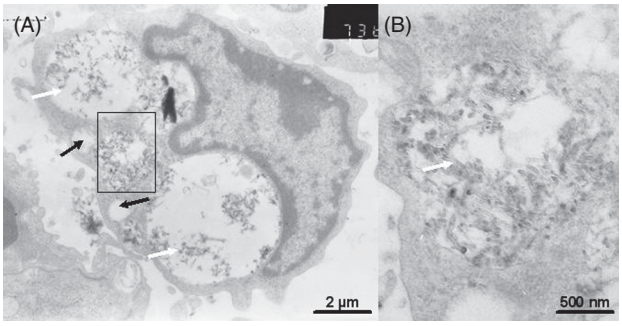


Fig. 4. (A) Representative TEM image of an AM. Exposed to 0.125 mg/kg, 28d post-exposure, (B) Magnification of the black rectangle in A. The black arrows indicate the mitochondria; the white arrows indicate the phagosomes filled with CNTs.

In groups of raw MWNTs, TSH levels of 0.125, 0.5 and 1 mg/kg group went up after 14d postexposure. (Fig. 6) The silicon dioxide induced much higher response of GSH to mouse lungs than the two CNTs samples did ($p < 0.05$).

In groups of raw MWNTs, MDA of all dose groups went up significantly ($p < 0.05$) and peaked at 14d (Fig. 7).

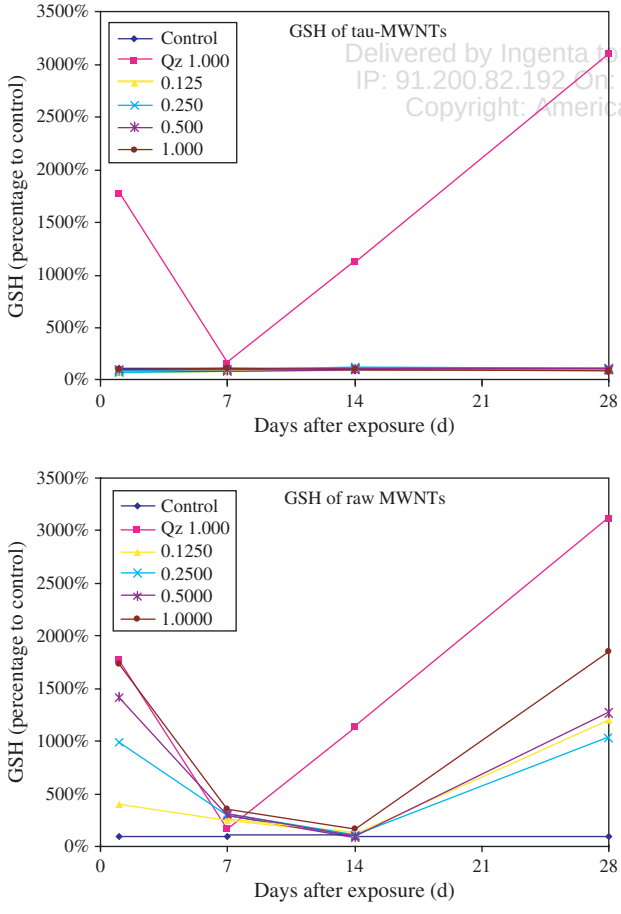


Fig. 5. Changes of GSH content in exposed lungs after exposed to tau-MWNT and raw MWNTs.

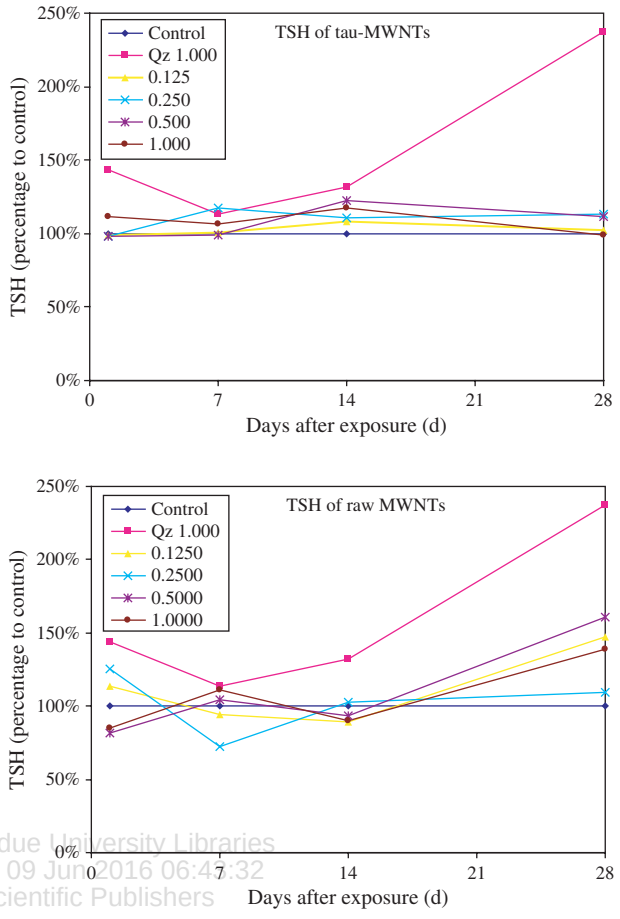


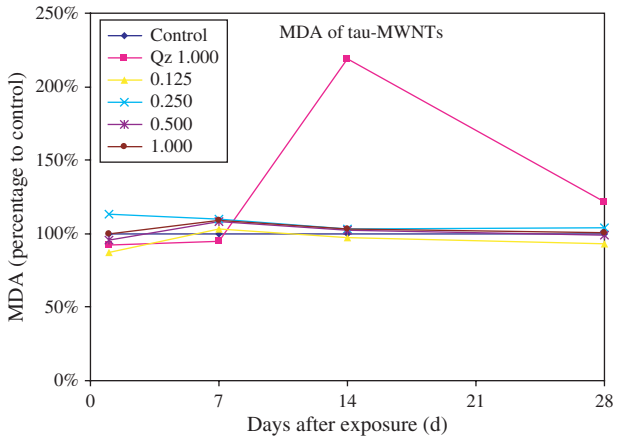
Fig. 6. Changes of TSH content in exposed lungs after exposed to tau-MWNT and raw MWNTs.

3.8. Tissue Hydroxyproline Levels

The hydroxyproline levels of raw MWNTs (except for 0.125 mg/kg) peaked at 7d significantly (higher than that of the blank control, $p < 0.05$) and then dropped to the blank control level at 28d. The changes of hydroxyproline levels in tau-MWNTs groups were not statistically significant. However, the hydroxyproline level in silicon dioxide group went up at 28d postexposure. (Fig. 8)

4. DISCUSSION

Respiratory system is an inescapable system to be exposed to the nanomaterials which can be aerosolized in the air, however, to date the published papers focused on the pulmonary toxicity of CNTs are still insufficient. All the results obtained were based on the insoluble raw or pristine CNTs, and water soluble CNTs have not yet been tackled.[6–11] Tau-MWNTs were synthesized by modifying taurine group in the surface of raw MWNTs and became water soluble CNTs, which may broaden their biological applications. However there is little data about the toxicity of water soluble CNTs. The previous studies indicate that CNTs produce adverse effects even more severely



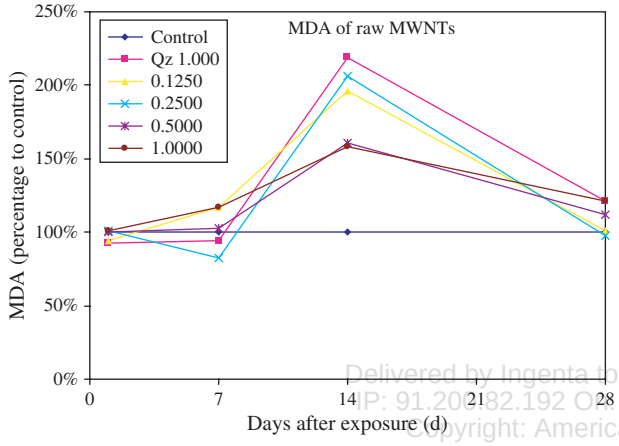
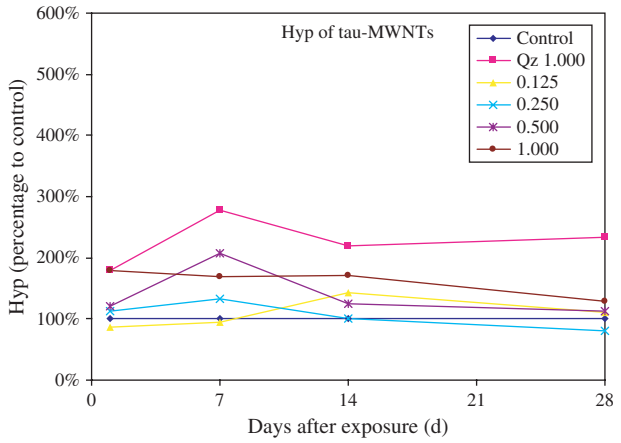


Fig. 7. Changes of MDA content in exposed lungs after exposed to tau-MWNT and raw MWNTs.



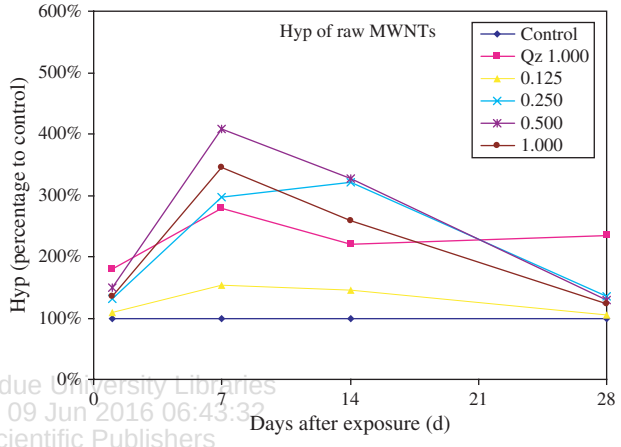


Fig. 8. Changes of Hydroxyproline content in exposed lungs after exposed to tau-MWNT and raw MWNTs.

than quartz did. However we should notice that the dose levels in these experiments are high. In the workplace, the concentration of potential exposure of workers to the CNT aerosols is estimated to be 53 μg/m.[3, 20] Based on the working schedule of 8 hours per workday, 5 days per work week, and on the man's tidal volume of 10 liters per minute, and only 1% air participates to gas exchange, which means 99% air people breathed in is breathed out unchangeable. We estimated that 12.72 μg CNTs might be inhaled into lungs of the workers per week . Therefore, supposing all the CNTs are inhaled into the lungs, we choose the dose level of about 1/4, 1/2, 1 and 2 time(s) of this estimation to investigate the pulmonary bio-effects of our MWNTs on mice lungs. In fact, the human body weight is supposed as 60 kg, and the mouse body weight is about 20 g, here the transfer coefficeint (or extrapolation coefficeint) is about 60000/20 = 3000. Moreover, we adopted intratracheal instillation as the exposure means, which is a convenient, effective and practicable way in the current pulmonary toxicological studies.

Lung index is widely used to evaluate the pulmonary toxicity. After the mice exposed to both CNTs, their lung indices significantly increase, simultaneously the pathological changes demonstrate the inflammatory response. At 28 days postexposure, lung indices go back to the blank control level, indicating that the inflammatory effusion has been absorbed and the lungs gradually recover from the inflammation. This is quite different from that of silicon dioxide. The silicon dioxide exposure might turn to chronic pulmonary inflammation or fibrosis in lungs based on our pathological observation.

The increase of ALP activity in serum is considered to be a toxicity indicator of type II alveolar epithelial cell. LDH is a cytoplasmic enzyme and is often used as an indicator of AM injury.[18] Serum ACE activity represents an index of toxicity of pulmonary capillary endothelial cells . The results of serum ALP and LDH reveal that within our dose range at one week postexposure, tau-MWNTs impair some AM and bronchial epithelial cells. But, this injury markedly recovers in the following three weeks. This is consistent with the reduced lung indices observed. Interestingly, such recovery phenomena are also consistent well with the short-term pulmonary inflammation response and transient increases in the bronchoalveolar lavaged (BAL) fluid biomarkers (including LDH and ALP) at 24 h postexposure reported by Warheit et al.[8] ACE activity illuminates that tau-MWNTs do not impair the pulmonary capillary

endothelial cells nor pass through the alveolar basement membrane into the pulmonary capillary. This is compatible with the toxicokinetics data reported previously.[13] However, it is indicated that the raw MWNTs have impaired the pulmonary capillary endothelia during the first couple of days after instillation, and fortunately this injury recovers in the following 3 weeks. The different patterns of ALP, LDH and ACE activity induced show that the water-soluble tau-MWNTs are less toxic than raw MWNTs to the type II alveolar epithelia and pulmonary capillary endothelia. Of the silicon dioxide group, the type II alveolar epithelia and pulmonary capillary endothelia are impaired and the toxicity patterns vary from the CNTs.

Hydroxyproline as an index of collagen synthesis was used to reflect fibrosis in lungs. Many researches have observed fibrosis in lungs exposed to CNTs,[9–11] however, we also did not observed fibrosis in lungs (as evidenced by pathologic and hydroxyproline examination).

Lung inflammation was pathologically recovering during 28d postexposure, no lung granuloma and fibrosis were found both in tau-MWNTs and raw MWNTs. The probable reasons are inferred as follows. Most importantly, our dose levels were comparatively lower than those used in previous studies (1 and 5 mg/kg).[7,8] Interestingly, our findings still obey the classic rule posed by the Swiss sage Paracelsus (1493–1541), "The dose makes the poison." Moreover, about 80% of the initial total tau-MWNTs eliminated from the lungs by 28d,[13] which definitely weakens the toxic effects of CNTs too.

Nevertheless, there might be some minor factors ascribed to the weaker toxicity of CNTs:

(1) Lengths of previously used CNTs are all in several microns, whereas ours are shortened to 300 nm–600 nm. It was reported that several micron asbestos fibers were long and stiff, and too big to be taken up by one AM, and may be encapsulated by multiple AMs, resulting eventually in the formation of granulomas.[21] Here, the shorter length of CNTs might be another reason of evading formation of granulomas. Our TEM observation depicted clearly that many CNTs were phagocytosed in phagosomes within individual AM.

(2) The impurities of our CNTs sample are obviously tiny compared with that in the raw CNTs used by the other studies. Even if all of these tiny catalysts would fully invade into the lungs, they would not evoke the toxic effects.

(3) Well-dispersed CNTs (CNT-bundles) show less cytotoxicity than non-dispersed CNTs (CNT-agglomerates), suggesting that the degree of dispersion is able to modify CNTs toxicity.[22]

Our tau-MWNTs sample was very well-dispersed, whereas the raw MWNTs used in this study and CNTs samples studied in previous pulmonary toxicity studies were poorly dispersed. From the data of GSH, TSH and MDA, we may infer that the water-soluble tau-MWNTs have no significant influence on the lung's redox state and can inhibit the OH^- free radical production in lungs. Whereas the raw MWNTs present apparent effects on these three parameters, demonstrating that raw MWNTs have influenced the redox state of the mouse lungs. We also found that tau-MWNTs could effectively inhibit the production of OH^- free radical in Fenton-Reaction system detected by the electronic spin resonance (ESR) analysis while the raw MWNTs samples could not (data not shown), which could further explain the phenomena observed in the redox results.

In conclusion, when mice exposed to water-soluble tau-MWNTs via intratracheal instillation in low and medium doses tau-MWNTs can induce acute and recoverable pulmonary inflammatory, which is slighter than the consequence of raw MWNTs and silicon dioxide. And notably, in the histopathologic and biochemical examination neither granulomas nor fibrosis are found in the lung tissues at our dose level. Presumably, this less toxic phenomenon is mainly attributed to the relatively low dose. The water-soluble and well-dispersed feature, the shorter length (300 nm–600 nm) and considerably high purity of the CNTs are all possible minor reasons for the slight toxicity observed.

This work should be a significant supplementary to the current pulmonary toxicity studies of CNTs and is instructive for further research on pulmonary toxicology of nanoparticles, particularly the water-soluble CNTs. The results are also beneficial to the study of CNTs based drug delivery systems in the future.

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