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**Review**

# Pleural translocation and lesions by pulmonary exposed multi-walled carbon nanotubes

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**Abstract:** Carbon nanotubes (CNTs) are recently developed tubular nanomaterials, with diameters ranging from a few nanometers to tens of nanometers, and the length reaching up to several micrometers. They can be either single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs). Due to their nano-scaled structure, CNTs have a unique set of mechanical, electrical, and chemical properties that make them useful in information technologies, optoelectronics, energy technologies, material sciences, medical technologies, and other fields. However, with the wide application and increasing production of CNTs, their potential risks have led to concerns regarding their impact on environment and health. The shape of some types of CNTs is similar to asbestos fibers, which suggests that these CNTs may cause characteristic pleural diseases similar to those found in asbestos-exposed humans, such as pleural plaques and malignant mesothelioma. Experimental data indicate that CNTs can induce lung and pleural lesions, inflammation, pleural fibrosis, lung tumors, and malignant mesothelioma upon inhalation in the experimental animals. In this review, we focus on the potential of MWCNTs to induce diseases similar to those by asbestos, molecular and cellular mechanisms associated with these diseases, and we discuss a method for evaluating the pleural toxicity of MWCNTs. (DOI: 10.1293/tox.2019-0075; J Toxicol Pathol 2020; 33: 145–151)

**Key words:** multi-walled carbon nanotubes, pleural translocation, pleural lesions, asbestos

## Introduction

Carbon nanotubes (CNTs) are hollow carbon fibers with diameters ranging from a few nanometers to tens of nanometers and lengths of up to several micrometers. According to their structures, CNTs are divided into two classes: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs)[1]. There are two types of MWCNTs, the Russian Doll model and the Parchment model[2]. In the Russian Doll model, the MWCNTs are formed by multiple coaxially arranged graphene cylinders, in which smaller-diameter graphene tubes are encased by the larger-diameter tubes. In the Parchment model, the MWCNTs are formed by a single graphene sheet that is wrapped around itself, resulting in a CNT with more than one layer, resembling the rolled up scroll of paper. In contrast to MWCNTs, SWCNTs are composed of a single graphene cylinder. CNTs have many unique mechanical, electrical, and chemical properties, and have a broad range of applications in many fields, such as electronic devices, composite materials, and biomedicine[3].

As compared to the wide application of CNTs, and the increasing scale of their production, their safety assessment is lagging behind, and there is an increasing concern regarding their harmful effects on the environment and human health. People can be exposed to carbon nanotubes through various routes in their work and daily life. Exposure to CNTs takes place mainly through the smoke generated by combustion of CNT-containing materials. In addition, workers can be exposed to particles released during the production of CNTs. In medical practice, CNTs may be used as drug carriers and for other purposes.

Nowadays, the global production of carbon nanomaterials each year is several hundred tons, increasing the risk of exposure to CNTs. Long-term exposure triggers a series of toxic reactions in animal models. Due to their small size and low density, CNTs can accumulate in the lungs of the workers, when inhaled through aerosols, at their occupational

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sites. Song *et al.*[4] investigated the pathological changes in a group of patients in Chaoyang Hospital, Beijing, China, after 5 to 13 month-long exposure to polyacrylic acid paint in a poorly ventilated workplace, and found the nanoparticles in the lungs and pleural effusions of these patients. Although similar respiratory toxicity of CNTs in human beings has not been reported yet, it is likely that long-term exposure to CNTs could cause respiratory lesions. Many studies have shown that CNTs induce oxidative stress, significant inflammation, and apoptosis in a variety of cells, and eventually lead to genetic damage and mutations, resulting in lung cancer and mesothelioma[5]. Pleural plaques and malignant mesothelioma are characteristic lesions in asbestos-exposed humans[6]. The fibrous structure of asbestos impedes the clearance of these fibers after translocation from the lung into the pleural cavity. Some CNTs have structures similar to asbestos fibers, suggesting that these CNTs, like asbestos, can induce lesions in the pleura.

According to a classic pathogenesis paradigm for fibrous materials suggested by Standon *et al.*[7], a high length to diameter ratio is an important parameter in assessment of the safety of the CNTs. Donaldson *et al.*[8] have proposed that a fraction of fibers in the lung are routinely transported into the pleural cavity, and cannot be cleared effectively through pleural stomata, resulting in trapping and deposition of the fibers in the pleura, leading to subsequent pro-inflammatory, genotoxic, and mitogenic responses at the sites of deposition. Therefore, pleural pathogenicity of CNTs is length-dependent. It has been reported that intra-pleural injection of long CNT elicits a substantial inflammatory response in the pleural space and granuloma formation on the diaphragm and parietal mesothelium, although the length threshold has not been determined[6].

In this review we focus on pleural toxicology of MWCNTs based on recent research, and discuss translocation of MWCNTs from the lung to the pleural cavity, the pleural lesions induced by respiratory exposure to MWCNTs, and mechanisms associated with these lesions.

## Translocation of MWCNTs from the Lung to the Pleural Cavity

There are several reports[9–12] that investigated the distribution of MWCNTs in test animals, following respiratory exposure. It was found that the MWCNTs were mainly retained in the lung, but over time they were also transported to extra-pulmonary organs and tissues, including lymph nodes, liver, spleen, kidney, and bone marrow. To assess the extent of translocation of MWCNTs into the pleural cavity, we developed a simple and effective method using pleural cavity lavage (PCL)[6, 11]. After intratracheal spraying of MWCNTs into the lungs, they were found in cell pellets from PCL samples and were observed in the parietal and visceral pleura[5, 13] (Fig. 1). Similar results were also reported by other researchers[14]. These results indicate that MWCNTs are able to translocate form the lung to the pleural cavity, although the proportion of translocated MWCNTs is small.

It is still not clear how MWCNTs from the lungs migrate to the pleura and pleural cavity. Several studies[5, 13–15] have observed the penetration of MWCNTs from the lung into the visceral pleura, suggesting that needle-like MWCNTs directly pierce the lining, and when the pierced cells

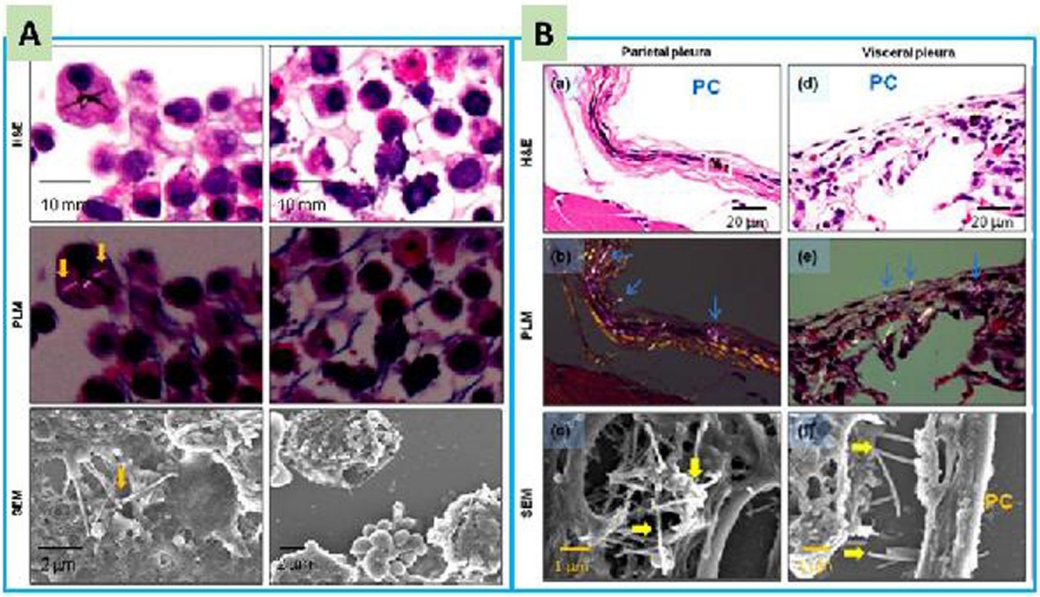


**Fig. 1.** Evidence of multi-walled carbon nanotube (MWCNT) fibers in the cell pellets of pleural cavity lavage (PCL) (A) and in the pleura (B): H&E, hematoxylin and eosin staining; PLM, polarized light microscopy; SEM; scanning electronic microscopy; and PC, pleural cavity. Arrows indicate MWCNT fibers. Adapted from Xu, J. *et al.* Cancer Science 2014, 105, 763–769. MWCNT fibers were observed in the PCL and pleura by using PLM and SEM.

die, the fibers are released into the pleural cavity. Another possibility is that MWCNT-burdened macrophages carry the fibers into the pleural cavity during the process of inflammatory infiltration. The second scenario is supported by the observation that macrophages, some with phagocytosed MWCNTs, accumulated in the pleural side of visceral pleura[16]. It is unlikely that MWCNTs are transported into the pleural cavity through blood or lymphatic fluids.

## Induction of Pleural Lesions in Animal Models

One of the physical characteristics of MWCNTs is a high aspect ratio and some MWCNTs have a needle-like shape, similar to that of asbestos. Thus, like asbestos, some MWCNTs are suspected to be able to cause lesions such as pleural plaque and mesothelioma[14, 17]. In the initial studies, injection of MWCNTs into the peritoneal cavity or scrotum, a surrogate method for observation of pleural toxicity especially for mesothelial lesions and malignant mesothelioma[17–20], as both these tissues are lined by mesothelium. Intraperitoneal exposure to different types of MWCNT at different doses resulted in the development of malignant mesothelioma in all cases[21]. These results suggest that MWCNTs have the potential to cause pleural lesions, if deposited in the pleura.

Short-term toxicity studies[5, 13, 15] show that intratracheal spraying of MWCNTs causes acute inflammation in the pleural cavity, fibrosis, and mesothelial cell proliferation in rodents. Fibrosis is observed in both parietal and visceral pleura, and is often associated with deposition of fibers (Fig. 2A). Although neoplastic lesions were not found in these short-term studies, proliferative cell nuclear antigen (PCNA) staining showed increased mesothelial cell proliferation, especially in the inflammatory and fibrotic sites, suggesting that cell proliferation is involved in an acute phase responsive lesion (Fig. 2B). A 2-year long study[22] showed that, when the MWCNTs were intratracheally sprayed for 2 weeks at the beginning of the experiment, 15.8% of the Fisher rats developed malignant mesothelioma in the pleural cavity (Fig. 2C). Importantly, MWCNTs were also deposited in the pleura, suggesting that these depositions were the cause of the mesothelioma.

It should be noted that pleural lesions induced by MWCNTs are dependent on their length and rigidity[23]. Short tangled MWCNTs do not cause pleural fibrosis and mesothelial proliferation[13]. Long and thick MWCNTs are more likely to cause DNA damage and inflammation than the short MWCNTs[24]. Also, tumorigenicity can occur at very low concentrations of MWCNT fibers (single peritoneal injection, 3 μg/mouse, median length of 2 μm)[20], very similar

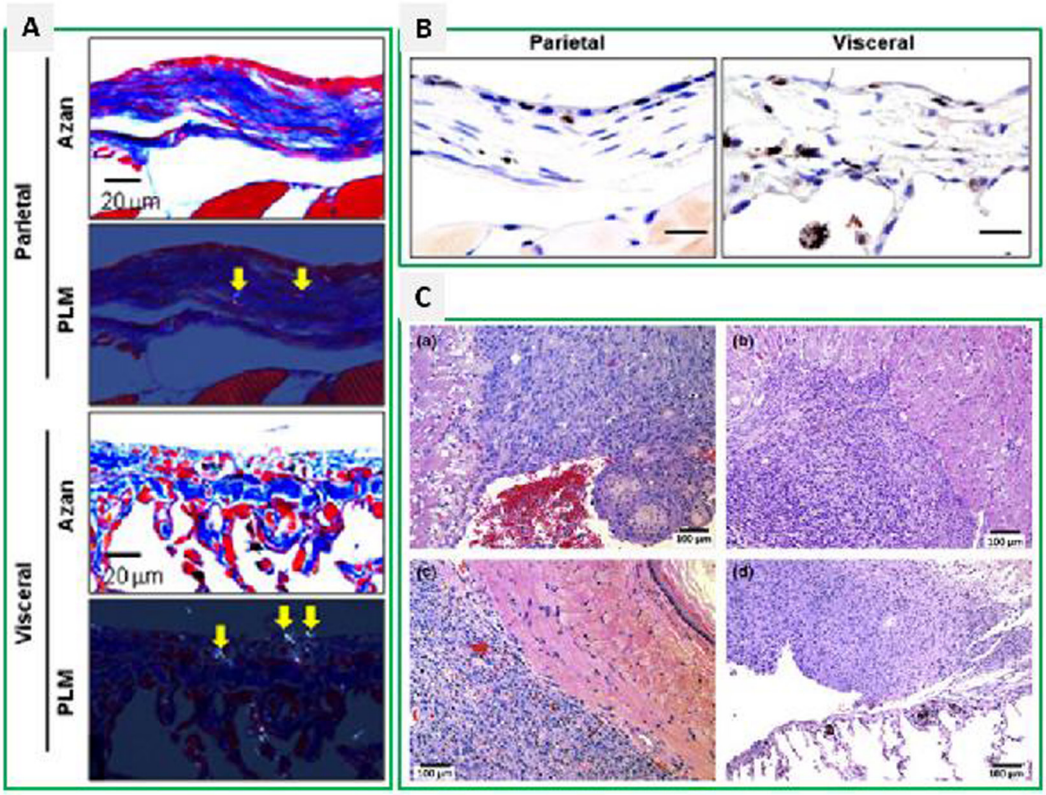


**Fig. 2.** Pleural lesions induced by multi-walled carbon nanotubes (MWCNTs) in rats: A, pleural fibrosis by Azan-Mallory staining; B, mesothelial cell proliferation by PCNA staining; and C, malignant mesothelioma. PLM, polarized light microscopy. Arrows indicate MWCNT fibers. Adapted from Xu, J. *et al.* Cancer Science 2014, 105, 763–769, and from Suzui, M. *et al.* Cancer Science 2016, 107, 924-935.

to the asbestos-induced malignant mesothelioma in humans.

## The Mechanisms of Pleural Toxicity

A major factor determining the pleural toxicity of engineered materials is whether they translocate and persist in the pleural cavity. According to the fiber pathogenicity paradigm described by Donaldson[8], fibrous materials with diameters small enough to allow the deposition beyond the ciliated airways, and with a length and rigidity sufficient to impede clearing by macrophages, and are biopersistent, are potential lung carcinogens. If such fibers are translocated from the lungs into the pleural cavity and if they cannot be cleared through the parietal pleura stomata, they pose the risk of pleural diseases[6]. As these fibers cannot be effectively cleared, their deposition results in cellular and molecular changes that can eventually progress into pleural lesions at the deposition sites. In rats, intratracheal instillation of MWCNTs for 2 weeks leads to persistent pleural inflammation, fibrosis, and mesothelial cell proliferation, and the administered fibers can be detected in the pleura 3 months after instillation[15].

The pathogenic mechanism is quite complex and involves cytotoxicity, generation of reactive oxygen species (ROS), genetic toxicity, and inflammatory responses. Many *in vitro* studies have demonstrated that MWCNTs are toxic to several cell types, including lung epithelial cells, airway epithelial cells, mesothelial cells, and macrophages[11, 25–29], although lethal doses vary, depending on the type of the cell and the MWCNT used. One reason for the cytotoxicity of MWCNTs is high ROS generation. Normally, antioxidant systems and ROS production are in equilibrium in the cell. When the ROS generation exceeds the capacity of the antioxidant system to neutralize them, the cell faces oxidative stress[30]. Elevated levels of ROS cause oxidation of membrane lipids, cellular proteins, and DNA[31, 32]. Metal residues in MWCNTs, usually from the catalytic substances used in their production, also contribute to ROS production, probably by the Fenton reaction, in which transition elements like iron, catalyze the generation of oxygen free radicals, which enhance the levels of reduced coenzyme II and xanthine oxidase in macrophages and neutrophils[33, 34]. Exposure to MWCNTs can cause genetic alterations, like DNA damages in rat bone marrow cells, chromosome aberration, and micronucleus formation in alveolar type II epithelial cells[35, 36].

The respiratory tract is a major exposure route for air-borne particles. Inhaled nanomaterials, including MWCNTs, can induce pulmonary inflammation. Their intrinsic cytotoxicity and induction of elevated ROS are responsible for triggering an inflammatory response in the lung[37, 38]. Excessive ROS causes NF-κB to transfer from the cytoplasm to the nucleus, activating the NF-κB signal transduction pathway, which in turn initiates transcription of a variety of pro-inflammatory cytokines such as TNF-α and IL-8[33]. It is worth noting that MWCNT-mediated oxidative damage may have a synergistic effect on the inflammatory responses, associated with formation and activation of NLRP3 inflammasomes[39]. *In vitro* and animal experiments have shown that, in addition to phagocytosis by macrophages and mesothelial cells, MWCNTs can also penetrate and kill non-phagocytic cells[34]. Induction of cell damage and death by MWCNTs stimulate the release of inflammatory mediators (TNF-α, IL-6, IL-8, IL-33, etc.) and promote the formation of inflammation and fibrosis[34, 40]. Many MWCNTs are characterized by high surface reactivity that causes inflammation and damage at the sites of deposition[41]. In the pleural cavity and the pleural tissue, MWCNTs elicit similar inflammatory reactions at the deposit sites. Intratracheally instilled MWCNT are found to induce increased number of inflammatory cells and higher levels of cytokines in the PCL, promoting inflammation, fibrosis, and proliferation of mesothelial cells[5, 13]. If the translocated MWCNTs have a shape that impedes clearance from the pleural cavity, these reactions will be persistent[15].

Macrophages play an important role in the MWCNT-induced lung toxicity and development of pleural lesions. Numerous studies have shown that macrophages are major infiltrating cells after MWCNTs enter the lung tissue. Alveolar macrophages, together with ciliary epithelial cells, are the first line of defense against the inhaled dust and invading microbes. Interaction of macrophages with MWCNTs triggers inflammation, fibrosis, and other reactions by releasing IL-33, CCL2/7, IL-12, and other chemokines. These chemokines recruit other immune cells to the site of inflammation[42–44]. Typical inflammatory lesions induced by MWCNTs are granulomas[45]. Production of PDGF-AA by MWCNT-burdened alveolar macrophages[46] and interaction of macrophages with MWCNTs[47] also promote fibrosis. Approximately 80% of the infiltrating cells in pleural cavity of MWCNT-administered test animals are CD68 positive cells[5], indicating that macrophages are the principal infiltrating cell-type, and most MWCNT fibers in the pleural cavity of these test animals are within the macrophages[5]. Interaction of macrophages with MWCNTs also induces pleural inflammation, enhances fibrosis, and stimulates cytokine release by mesothelial cells[47], promotes the production of a variety of cytokines[5, 13, 15]. Accumulation of macrophages loaded with phagocytosed MWCNTs are observed on the surface of the visceral pleura[16]. As the long MWCNTs cannot be completely engulfed by the macrophages, leading to frustrated phagocytosis, there is constant interaction of macrophages in the pleural cavity with these MWCNTs and this results in persistent inflammatory reactions, fibrosis, and stimulation of mesothelial cells. Thus, macrophages in the pleural cavity promote pleural lesions.

## How to Evaluate the Chest Toxicity of MWCNTs

Current research data indicate that, like asbestos, MWCNTs have the potential to induce pleural diseases. However, there is no uniform standard method to evaluate the pleural toxicity of inhaled material. Observation of pleural lesions in patients with pleural effusion, usually involves imaging and cytological techniques, while in animal experi-

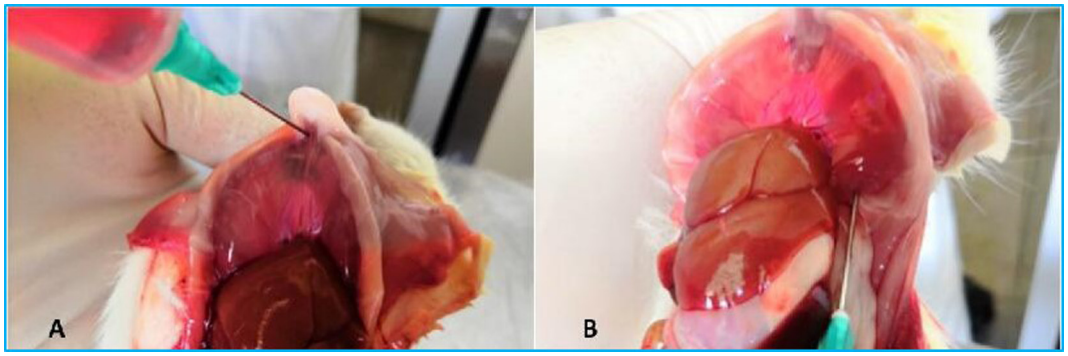


**Fig. 3.** Pleural cavity lavage in rats: A, injection of 10 ml RPMI 1640 medium into the pleural cavity at the diaphragm near the sternum; B, collection the injected medium from the left bottom of the diaphragm. Approximately 9 ml or more of the medium can be collected (not published data).

ments, pleural histopathology is the most common method. Although these methods have their own advantages, they cannot evaluate the elimination and retention of nanomaterials in the thoracic cavity, the exudation of inflammatory cells, and the production of cytokines. Therefore, an effective method for evaluating toxicity in the chest and pleura is urgently needed.

Previously we developed a new method using the PCL and collection of cells and materials from it. Collection of the PLC from rats is shown in Fig. 3. Histological examination of cell pellets obtained from the PLC allows the observation of the infiltration of inflammatory cells and MWCNT fiber retention in the pleural cavity[5, 13]. Examination of the PCL and the PCL cell pellet is a simple and effective method for evaluating pleural toxicity. This method has the following advantages: 1) it allows observation of the degree of infiltration of various inflammatory cells into the pleural cavity; 2) it is also possible to observe the nanomaterials in the cell pellets under polarized light microscope and electron microscope, which reflects the removal and retention of nanomaterials in the pleural cavity; 3) it allows the analysis of the types and levels of cytokines in the PCL supernatant by methods such as ELISA; 4) combined with pathological examination, it can effectively evaluate the presence and retention of nanomaterials in the pleural cavity and the relationship between inflammatory responses and pleural lesions. This method is economical and effective in the assessment of pleural toxicity.

## Conclusion

Studies *in vitro* and in animal models suggest that MWCNTs, like asbestos fibers, have the potential to induce pleural lesions, such as chronic inflammation, fibrosis, and malignant mesothelioma. Production and application of MWCNTs and other fibrous materials should be strictly controlled to prevent another asbestos disaster.

**Disclosure of Potential Conflicts of Interest:** The authors have no conflict of interest.

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