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One time nose-only inhalation of MWCNTs: Exploring the mechanism of toxicity by intermittent sacrifice in Wistar rats

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ABSTRACT

We have investigated the time-dependent effect of multi-walled carbon nanotubes (MWCNTs) in rats upon single inhalation exposure followed by intermittent sacrifice. The effects were monitored by analyzing the bronchoalveolar lavage fluid (BALF) and histopathological analysis. Cell count, neutrophils, lymphocytes, lactate dehydrogenase, alkaline phosphatase, protein and cytokines (tumor necrosis factor-alpha (TNF-α) and interleukin 4 (IL-4)) were significantly increased, while cell viability and alveolar macrophage count significantly decreased in the BALF of MWCNT-treated rats on day 1, day 7 and day 14 post-exposure, when compared to control rats. Histopathological analysis revealed inflammation, fibrosis and granuloma in the lungs of MWCNTs-treated rats on day 7 and day 14 post-exposure. We interpret that MWCNT induces inflammation, fibrosis and granuloma characterized by progressive elevation of TNF-α and IL-4. Histopathological studies further support our view and reveal the distribution of MWCNT in lungs and tracheobronchial lymph nodes (TBLN). We conclude that MWCNT-induced pulmonary toxicity is considerable even on single exposure.

1. Introduction

Carbon nanotubes (CNTs) due to their unique properties are making a breakthrough in industries and biomedicine [1]. As a result, the production rate of CNTs is rising considerably. Recent reports reveal that the global production of CNTs exceeds several thousand tons per year [2]. In this scenario, the exposure of the environment including human beings and the ecosystem to CNTs and the threat of CNT toxicity are also increasing. However, the information about the possible human health and environmental impacts produced by CNTs is still scanty. CNTs exhibit a toxic potential, similar to those observed with fibrous materials due to their high aspect ratio [3]. CNTs induced dose-dependent severity of the lesions like persistent epitheloid granulomas and interstitial inflammation in mice after single intra-tracheal treatment was observed [4]. In mice and rats, pulmonary inflammation characterized by alterations in cellularity and enzyme activities of bronchoalveolar lavage fluid (BALF) and microscopic findings like infiltration of macrophage, granulomas, and fibrosis were observed after intra-tracheal administration of SWCNTs or MWCNTs [5–7]. Long MWCNTs with needle shaped structure, similar

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to asbestos, produce asbestos-like pathological changes after intraperitoneal injection [8]. Moreover, histopathological lesions were observed in the in the upper and lower parts of respiratory tract after inhalation exposure of rats to MWCNTs. Previous studies reported the inflammatory changes by examining the BALF, which revealed the variation in polymorphonuclear neutrophils, macrophages, lymphocytes and expression of inflammatory cytokines [9,10]. However, the time dependent studies on effects of CNTs in lung after one time nose-only inhalation and the mechanism of toxicity are limited.

We investigated the time dependent pulmonary toxicity of MWCNTs after one time nose-only inhalation of MWCNTs followed by intermittent sacrifice on day 1, 7 and 14 post-exposure in rat model. The concentrations of MWCNTs were selected based on permissible exposure limit of carbon materials such as graphite as reported by National Institute for Occupational Safety and Health, USA [34]. The inflammatory and fibrotic responses in the lung of MWCNTs-inhaled rats were examined by the changes in the lung histopathology, alveolar macrophages, neutrophils, lymphocytes and the expression of TNF-α and IL-4 in the broncho-alveolar lavage fluid.

2. Experimental details

2.1. Materials

MWCNTs (purity > 95%), obtained as gift sample from Department of Physics, IIT, Madras, were used for the current study. Cytokine kits (Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-4 (IL-4)) were purchased from Ray Biotech USA. All other chemicals and reagents used for the study were procured from Sigma–Aldrich Corporation, India.

2.2. Animals

Twenty-four male Wistar rats with body weight 180–200 g, free from clinical signs of disease, were obtained from the Animal house, International Institute of Biotechnology and Toxicology (IIBAT), Padappai, India. The protocol of animal study was approved by Institutional Animal Ethics Committee (6/106/IAEC/2013). The rats were divided into four groups and housed in polypropylene cages in a room on a 12:12-h light/dark photoperiod at a temperature of 23 ± 2 °C and relative humidity of 53 ± 5%. Rats were provided with gamma-irradiated rodent pellet feed (Tetragon Chemie Pvt. Ltd., Bangalore, India) and reverse osmosis water *ad libitum* except during the exposure period. Animals were permitted to acclimatize to the animal room conditions for one week before the initiation of the study. All animals were acclimatized for 6 h to the inhalation system on 2 consecutive days before start of the exposure period and the experimental conditions were similar to those used in pilot study.

2.3. Characterization of MWCNTs

The structural alignment of MWCNTs and purity were determined from X-ray diffraction analysis using Rigaku diffract meter with Cu-Kα radiation λ = 1.54060 Å. The diffraction pattern was obtained in the range of $2\theta = 20–80^{\circ}$. The obtained diffraction pattern was compared with standard JCPDS values to determine their crystal system and its related parameter. Moreover, the surface morphology of MWCNTs was determined using high-resolution scanning electron microscope HRSEM (FEI Quanta FEG 200, Japan) and FEI Tencai 30 G2 S-TWIN high-resolution transmission electron microscopy (HRTEM). The elemental analysis (EDAX) was done by RTEM2 EDAX, AMETEK operating at 250 kV.

2.4. MWCNTs exposure

Male Wistar rats were exposed to MWCNTs using nose-only inhalation exposure chambers (CH. Technologies, USA) for 4 h, to an aerosol concentration of 5 mg/m^3. The concentrations of MWCNTs were selected based on permissible exposure limit of carbon materials such as graphite as reported by National Institute for Occupational Safety and Health (NIOSH) [34]. The inhalation systems contain three major modules: an aerosol generator – Wright Dust Feeder (BGI, Inc.), a rodent nose-only inhalation exposure chamber and an aerosol concentration measurement device – gravimetric single filter (BGI, Inc.). The rats were kept in glass restraint tubes (attached to the wall of the cylinder) with their snouts projecting into the inhalation chamber. The dilution of the aerosol in the breathing zone with external air was avoided by generating positive pressure inside the inhalation chamber. Moreover, the whole exposure system was kept under exhaust hoods in an air-conditioned room. MWCNTs aerosols were generated using the Wright Dust feeder, which was connected to the inlets of each inhalation chamber. The actual concentrations of the MWCNTs in the inhalation chambers were determined gravimetrically 3 times during each exposure day. In addition, samples were taken twice from the exposure chambers to determine the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) using a Mercer seven-stage cascade impactor (CH Technologies, USA). A pilot study was performed in advance to the animal exposure by generating MWCNT aerosols continuously for 4 h in inhalation chamber to realize the particle size distribution of the generated aerosol.

To explore the exacerbation of toxic effects of MWCNTs exposure due to the biopersistance of nanotubes, the rats were sacrificed on day 1, day 7 and day 14 post-exposure. The toxicity potential was determined by observing body weight, food consumption parameters, and the clinical signs of toxicity throughout the study.

2.5. Broncho-alveolar lavage fluid analysis

Rats were euthanized with an overdose of Thiopentone I.P. (Thiosol^{TM} sodium) via intraperitoneal injection. The right portion of the lungs of rats was immediately lavaged twice with 5 mL of Ca^{2+}, Mg^{2+} free phosphate buffered saline (Himedia, India). About 90% of the total volume instilled was retrieved and the volume is similar in all groups. The lavage collections were centrifuged