DOI 10.1007/s10517-015-2835-7

BIOTECHNOLOGIES

Evaluation of Fibrogenic Potential of Industrial Multi-Walled Carbon Nanotubes in Acute Aspiration Experiment

T. O. Khaliullin, A. A. Shvedova*, E. R. Kisin*, R. R. Zalyalov, and L. M. Fatkhutdinova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 158, No. 11, pp. 635-639, November, 2015
Original article submitted September 16, 2013

Local inflammatory response in the lungs and fibrogenic potential of multi-walled carbon nanotubes were studied in an acute aspiration experiment in mice. The doses were chosen based on the concentration of nanotubes in the air at a workplace of the company-producer. ELISA, flow cytometry, enhanced darkfield microscopy, and histological examination showed that multi-walled carbon nanotubes induced local inflammation, oxidative stress, and connective tissue growth (fibrosis). Serum levels of TGF-β1 and osteopontin proteins can serve as potential exposure biomarkers.

Key Words: *carbon nanotubes; in vivo; fibrosis; bronchoalveolar lavage*

The number of plants producing and utilizing multi-walled carbon nanotubes (MCNT) progressively increases. In contrast to single-wall carbon nanotubes, MCNT are more attractive in applications. The number of people contacting with MCNT aerosol at their workplaces constantly grows; the inhalation exposure is more common than percutaneous and oral exposures. The study of the toxic effects of MCNT was launched in the beginning of the 21st century. Soon afterwards, the data were obtained suggesting that fibrosis could be one of the major pathological processes in the lung tissue triggered by exposure to MCNT. After intratracheal instillation of 0.5, 2, or 5 mg MCNT to rats, fibrotic changes and granulomas were observed in the lungs within 60 days (end of experiment); MCNT and asbestos produced similar effects [9]. In a similar comparative study of MCNT, asbestos (amosite), and ultradispersed soot particles (all intraperitoneally injected to mice in a dose of 50 μg), long MCNT and amosite in the form of long fibers caused collagen deposition, formation of granulomas containing visible particles and foreign body giant cells with MCNT/asbestos fibers [11]. The authors pointed out that asbestos-like pathogenicity attributed to carbon nanotubes has a "structure–activity" mechanism typical of fibrogenic fibers. Aspiration experiments with doses of 10-80 μg [8,12] and technically more complicated inhalation experiments with calculated deposited doses of 5-56 μg [7,13] confirmed fibrosis development in the lung tissue. Translocation of individual fibers from the alveolar lumen into the interstitium leading to thickening of the interalveolar septa was described [8,13].

However, fibrosis and related effects were not so evident in other studies. A 3-month study in guinea pigs with intratracheal instillation (3 types of MCNT, cumulative dose 12.5 mg/animal) revealed only epithelium desquamation and interstitial pneumonia [4]. In an inhalation experiment on rats (0.1, 0.5, or 2.5 mg/m^3, 13 weeks, 5 days a week, 6 h a day) granulomatous inflammation and alveolar lipoproteinosis, but not pulmonary fibrosis, were observed [6].

In most studies, laboratory purified MCNT, but not production samples that affect workers in real pro-

Kazan State Medical University, Russia; *National Institute for Occupational Safety and Health, Morgantown, USA. ***Address for correspondence:*** liliya.fatkhutdinova@gmail.com. L. M. Fatkhutdinova

0007-4888/15/1585-0684

duction conditions were used. In addition, the doses selected for *in vivo* studies did not reflect actual exposure conditions in the working area. In some animal experiments, fibrosis markers were assayed in the bronchoalveolar lavage fluid, but not in the blood serum, which would be more informative when extrapolating the results to humans.

Here we studied local inflammatory response in the lungs and fibrogenic potential (including serum fibrosis biomarkers) of MCNT collected at the working areas, in acute aspiration experiment in animals.

MATERIALS AND METHODS

Non-purified MCNTs produced industrially by catalytic vapor deposition were used. According to the provided technical documentation, MCNT had outer diameter 8-15 nm, inner diameter 4-8 nm, and length 2-15 μm; the total amount of metal catalyst impurities did not exceed 5%, specific geometrical surface was 300-320 m^2/g. In dipalmitoylphosphatidylcholine (DPPC) solution, MCNT looked like tangles up to 5 μ in width and individual fibers (Fig. 1).

The study was carried out on 2-month-old C57Bl/6J male mice (Jackson Laboratories) weighing 18±2 g. The animals were treated in compliance with the World Medical Association's Declaration of Helsinki. The mice were divided into one control and three experimental groups (40 mice per group). Experimental groups received 20, 40, and 80 μg MCNT in 0.001% DPPC solution via pharyngeal aspiration; the control animals received PBS via the same route. Before administration, all solutions were subjected to ultrasonic dispersion in order to increase particle dispersion.

MCNT dose was selected based on our own data on MCNT level in the air at the working place of the production department. There are no specific models of carbon nanotube depositing in human lungs, therefore we used MPPD model to establish approximate deposited doses [3]. The mean concentration of inhaled MCNT aerosol during a shift reached 29 $\mu g/m^3$, which corresponded to the dose accumulated over 25 years of job tenure per 980 $\mu g/m^2$ of lung epithelium surface. The following input data were used in calculation: MCNT aerosol concentration of 29 $\mu g/m^3$, MCNT air agglomerates size 1.5 μ, respiratory minute volume of 20 liter/min for light exercise, the Yeh–Schum model of aerosol particle distribution, lung epithelium surface area of 102 m^2 [2]. Experimental doses of 20, 40, and 80 μg/mouse corresponded to deposited doses of 400, 800, and 1600 $\mu g/m^2$ of alveolar epithelium respectively (alveolar epithelial surface area in mice is 0.05 m^2 [2]).

In each group, the mice were divided into 4 subgroups and were sacrificed on days 1, 7, 28, and 56 after exposure by injection of a lethal dose of sodium pentobarbital. After euthanasia, bronchoalveolar lavage was performed. The supernatant of the first portion was used for biochemical studies, precipitated cells from the first and second portions were analyzed using enhanced darkfield microscopy (CytoViva) and subjected to Romanovsky–Giemsa staining for subsequent lung cell counting and evaluation of cellular composition. In the lavage fluid, LDH and total protein were measured colorimetrically; IL-6, MCP-1, and TNF-α levels were assessed by flow cytometry. Blood samples were taken from the inferior vena cava after euthanasia and dissection of the abdominal cavity. To assess fibrogenic effects of MCNTs, blood serum TGF-β (main marker) and osteopontin levels were measured by ELISA. The latter was chosen as the reference marker for TGF-β. The levels of reduced glutathione and myeloperoxidase (MPO) were measured in lung homogenates.

In a half of mice in each subgroup, the lungs were removed, fixed, histological sections were stained with hematoxylin, eosin and Masson's trichrome.

Statistical data processing was performed using paired and unpaired Student's *t* test.

RESULTS

Single MCNT aspiration induced local inflammatory response in mice, followed by the development of fibrotic changes in the lung tissues.

TABLE 1. Lavage Fluid Composition 24 h after Aspiration of MCNT or PBS (Control) (*M*±*m*)

Group		Neutrophils, % total cell count	LDH, pg/ml	Total protein, mg/ml	IL-6, pg/ml	MCP-1, pg/ml	TNF-α, pg/ml
Control		0.60±0.38	23.02±0.93	0.31±0.02*	2.3±0.6	9.6±4	4.1±0.5
MCNTs	20 μg	24.35±4.10*	50.40±2.52*	0.70±0.16*	120.9±30.7*	160.5±8.1*	33.2±2.7*
	40 μg	21.7±5.7*	65.9±3.2*	0.63±0.18*	44.5±8.8*	81.6±15.8*	12.9±3.1*
	80 μg	28.7±3.3*	84.40±7.45*	0.76±0.01*	96.7±21.5*	135.5±15.7*	15.2±1.7*

Note. Here and in Table 2: *$p<0.05$ in comparison with the control.

TABLE 2. Level of Reduced Glutathione and MPO in the Lung Tissue of Mice after Exposure to MCNT (*M*±*m*)

Term	Dose, μg	Glutathione, % of control level	MPO, % of control level
24 h	Control	100.00±2.84	100.0±7.2
	20	82.16±7.26	112.06±7.38
	40	55.60±3.25*	114.11±7.71
	80	55.35±3.22*	117.15±3.16*
Day 7	Control	100.00±6.05	100.00±5.64
	20	116.59±1.43*	124.49±2.88*
	40	110.85±5.26	107.39±3.44
	80	116.41±3.53*	122.80±5.11*
Day 28	Control	100.00±4.21	100.00±2.57
	20	93.64±3.58	108.72±5.16
	40	106.56±1.46	111.71±2.84*
	80	110.57±4.06	114.71±2.31*
Day 56	Control	100.00±3.23	100.00±3.01
	20	103.43±1.88	110.31±2.22*
	40	107.71±0.78	112.40±3.50*
	80	103.78±1.8	116.10±2.58*

A significant increase in neutrophil count in the lavage fluid (compared to control) along with elevated levels of LDH, total protein, and inflammatory cytokines IL-6, MCP-1, and TNF-α (Table 1) were observed 24 h after exposure, which is indicative of early local inflammatory response, increased membrane permeability and cell damage.

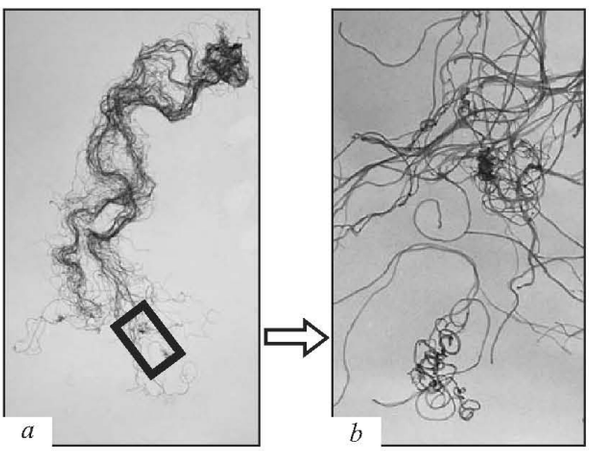

Fig. 1. Transmission electron microscopy of MCNT sample in DPPC solution, ×3000 (*a*), ×15,000 (*b*).

The inflammatory response decreased with time, but residual effects were observed until sacrifice. On day 7 and at all subsequent terms, the total protein, IL-6, and MCP-1 only slightly surpassed the control levels. Neutrophil count decreased throughout the experiment and reached 2-4% of total cell count in the lavage fluid in the exposed groups on day 56. The concentrations of LDH and TNF-α were slightly decreased, but still surpassed the control values (data not shown).

In all exposed mice, the serum TGF-β level increased in 24 h after exposure and remained elevated until the end of the experiment, and a direct dose-dependent effect was observed: the higher was the MCNT dose, the higher was serum TGF-β concentration (Fig. 2, *a*). Serum osteopontin level significantly

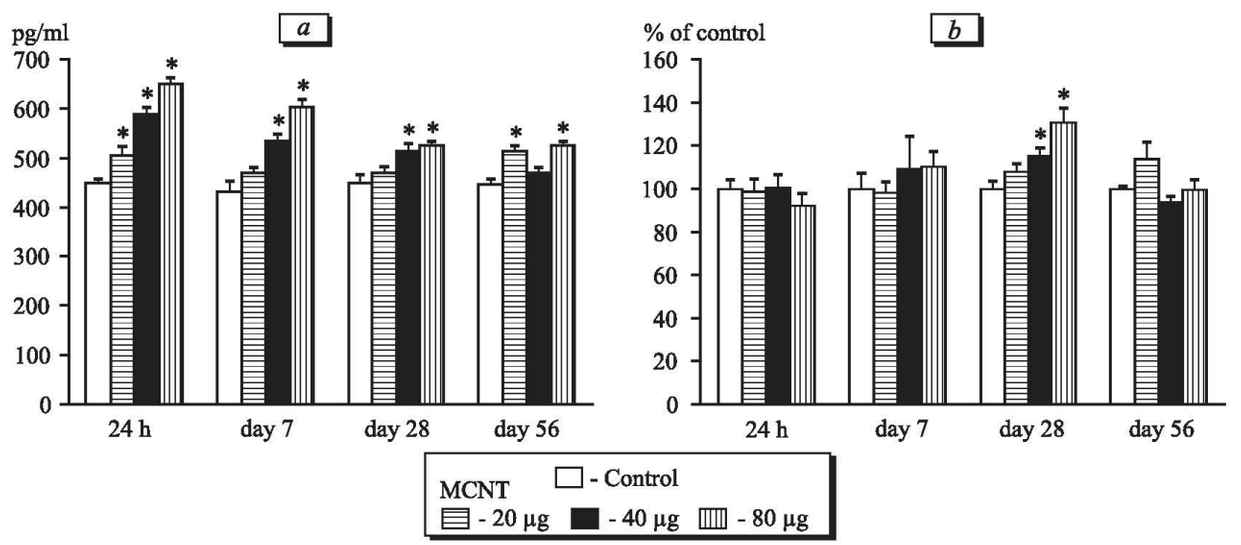

Fig. 2. Serum concentrations of TGF-β (*a*) and osteopontin (*b*) in mice. *$p<0.05$ in comparison with the control.

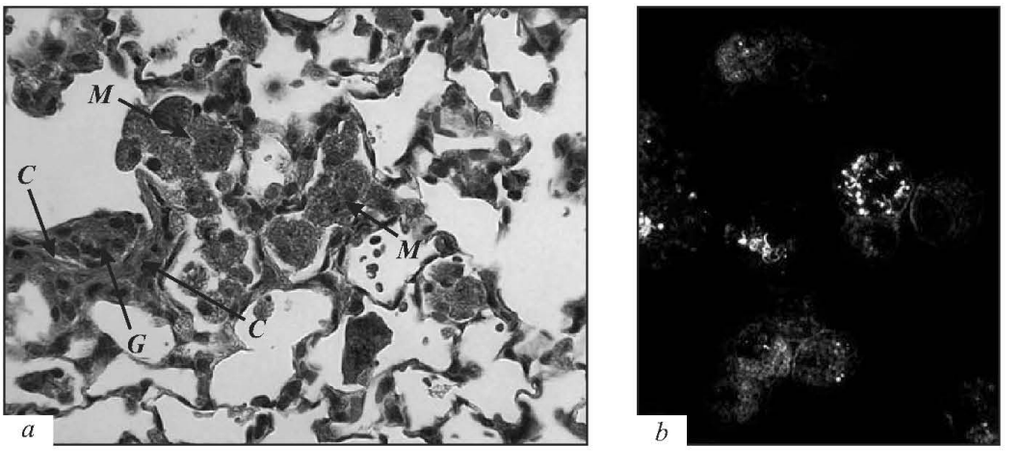

Fig. 3. Histological picture of the lungs. *a*) Lung section, Masson's staining, 20 μg MCNT, day 28, ×40; *b*) bronchoalveolar lavage, enhanced darkfield microscopy, 80 μg MCNT, day 56, ×100. *M*: macrophages and giant cells with MNCT incorporations, *G*: granulema, *C*: collagen fibers.

increased in exposed mice by day 28, but on day 56 returned to the control levels (Fig. 2, *b*).

On days 28 and 56, the histological picture in the lungs was characterized by the presence of granulomas with MCNT agglomerates in the center, numerous alveolar macrophages, giant cells, and fibroblasts (Fig. 3, *a*). Two months after aspiration, macrophages with instantly recognizable inclusions of nanotubes were still observed in the lavage fluid (Fig. 3, *b*). Staining of the lung sections with Masson's trichrome revealed increased number of collagen fibers in all experimental groups.

Therefore, there is every reason to suggest long-term deposition and fibrogenic potential of the studied MCNT. It was found that serum TGF-β level is a sensitive indicator of biological effect of MCNT and markedly increased in 24 h after exposure, whereas the concentration of osteopontin expressed by granulomas of various origin including silicosis [10], was significantly elevated in blood only on day 28.

Intracellular reduced glutathione level in the lung tissue sharply decreased in 24 h after aspiration with a slight overcompensation at subsequent time points (Table 2). MPO level in lung homogenates of exposed mice remained stably high (10-15%), in comparison with the control, throughout the experiment, which is indicative of high phagocytic activity. Both values (glutathione and MPO levels) provide evidence for prolonged oxidative stress, which plays an important role in pathogenesis of dust disease of lungs [1]. The important role of MPO in degradation of single-walled carbon nanotubes is known [14]; the same mechanism probably plays an important role in biodegradation of MCNT in granulomas.

Thus, the obtained results confirm the development of local inflammation, oxidative stress and fibrosis induction, obtained with laboratory MCNT samples [7,8,12,13]. Unlike other *in vivo* toxicity studies of MCNT, in our experiment industrial crude MCNTs were used, and aspiration doses were calculated based on actual working conditions at the manufacturing facility that produces this nanomaterial. This study design renders the data obtained in animal studies more valid for evaluation of occupational risks. Apart from morphological characteristics of the pathological process, serum concentrations of two early fibrosis biomarkers (TGF-β and osteopontin) were measured and their dependence on the aspiration dose and exposure was demonstrated. These pioneer data can be used for planning of further toxicological and epidemiological studies.

REFERENCES

1. B. T. Velichkovskii, *Ecological Pulmonology. Impact of Free-Radical Processes*, Ekaterinburg (2001).
2. *Ann ICRP*, **24**, Nos. 1-3, 1-482 (1994).
3. R. de Winter-Sorkina and F. R. Cassee, *National Institute for Public Health and the Environment (RIVM) Report*, Bilthoven (2002).
4. H. Grubek-Jaworska, P. Nejman, K. Czuminska, *et al.*, *Carbon*, **44**, No. 6, 1057-1063 (2006).
5. H. F. Lakatos, H. A. Burgess, T. H. Thatcher, *et al.*, *Exp. Lung Res.*, **32**, No. 5, 181-199 (2006).
6. L. Ma-Hock, S. Treumann, V. Strauss, *et al.*, *Toxicol. Sci.*, **112**, No. 2, 468-481 (2009).
7. R. R. Mercer, J. F. Scabilloni, A. F. Hubbs, *et al.*, *Part Fibre Toxicol.*, **10**, No. 1, 38 (2013).
8. R. R. Mercer, J. F. Scabilloni, and A. F. Hubbs, *Part Fibre Toxicol.*, **8**, 21 (2011).
9. J. Muller, F. Huaux, N. Moreau, *et al.*, *Toxicol. Appl. Pharmacol.*, **207**, No. 3, 221-231 (2005).
10. G. J. Nau, P. Guilfoile, G. L. Chupp, *et al.*, *Proc. Natl Acad. Sci. USA*, **94**, No. 12, 6414-6419 (1997).
11. C. A. Poland, R. Duffin, I. Kinloch, *et al.*, *Nat. Nanotechnol.*, **3**, No. 7, 423-428 (2008).
12. D. W. Porter, A. F. Hubbs, R. R. Mercer, *et al.*, *Toxicology*, **269**, No. 2-3, 136-147 (2010).
13. J. P. Ryman-Rasmussen, M. F. Cesta, A. R. Brody, *et al.*, *Nat. Nanotechnol.*, **4**, No. 11, 747-751 (2009).
14. A. A. Shvedova, A. A. Kapralov, W. H. Feng, *et al.*, *PLoS One*, **7**, No. 3, doi: 1371/journal.pone.0030923 (2012).