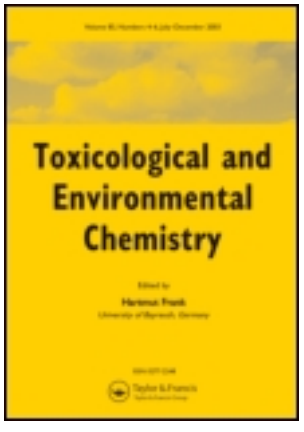

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Yousef Mohammadian[a], Seyed Jamaleddin Shahtaheri[b], Ali Akbar Saboor Yaraghi[c], Hossein Kakooei[b] & Mohammad Hajaghazadeh[a]

[a] Department of Occupational Health, Health Faculty, Urmia University of Medical Sciences, Urmia, Iran

[b] Department of Occupational Health, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

[c] Department of Nutrition and Biochemistry, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

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Cytotoxicity of single-walled carbon nanotubes, multi-walled carbon nanotubes, and chrysotile to human lung epithelial cells

Yousef Mohammadian[a], Seyed Jamaleddin Shahtaheri[b]*, Ali Akbar Saboor Yaraghi[c], Hossein Kakooei[b] and Mohammad Hajaghazadeh[a]

[a]*Department of Occupational Health, Health Faculty, Urmia University of Medical Sciences, Urmia, Iran;* [b]*Department of Occupational Health, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran;* [c]*Department of Nutrition and Biochemistry, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*

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Carbon nanotubes (CNTs) have found numerous applications in various industries. Recently, adverse effects of these materials on human and animal cells *in vitro* have been reported. In the present study, the cytotoxicity of single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), and chrysotile asbestos in human lung epithelial cells has been studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cells were exposed for 6 h and 24 h to between 0.97 and 1500 μg mL^{-1} of CNTs and chrysotile fibers prepared in two culture media containing 5% serum and 0.5% dimethylsulfoxide. Dose–response curves were obtained to determine the nonobservable adverse effect concentration and the half-maximum inhibitory concentration (IC_{50}). The way of dispersion affects the cytotoxicity of CNTs. For MWCNT, the toxicological indexes were lower than for SWCNT. Chrysotile fibers were even less cytotoxic than CNTs. Therefore, workplace control measures are recommended as priority for occupational and environmental conditions.

Keywords: carbon nanotubes; chrysotile; cytotoxicity; MTT assay; human lung cells

Introduction

Carbon nanotubes (CNTs), a group of nanoparticles, are found in two types: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). CNTs have some prominent physicochemical and mechanical properties such as high elasticity, extraordinarily low weight, thermal and chemical stability, and the trait of being semiconductors (Ebbesen et al. 1996). Therefore, they have found applications in a multitude of areas and products in computer industries, for construction, in aeronautics, as chemical sensors, in textile and athletic goods industry, and for medical purposes (Blaise et al. 2008; Donaldson et al. 2006; Gwinn and Vallyathan 2006). Holman and Lackner (2006) estimated that by 2014 the value of CNTs-manufactured goods will be $2.6 trillion. Thus, with growing industrial production of CNTs and their potential pervasive distribution in consumer products, concerns have been raised regarding their environmental and human health effects (Maynard et al. 2006; Oberdörster, Oberdorster, and Oberdorster 2005; Papp et al. 2008).

*Corresponding author. Email: shahtaheri@tums.ac.ir

The effects of exposure to CNTs on both human and animal health have been investigated using *in vivo* and *in vitro* techniques, but there is no definitive agreement on the effects of CNTs for human health (Pacurari, Castranova, and Vallyathan 2010; Tejral, Panyala, and Havel 2009; Ursini et al. 2012) Recent toxicological studies have suggested that the adverse effects of CNTs are similar to those of asbestos fibers (Poland et al. 2008; Sakamoto et al. 2009; Soto, Garza, and Murr 2007; Tabet et al. 2008). Some similarities between CNTs and asbestos fibers in both physical dimensions and biopersistance support this idea. Consequently, in *in vitro* studies asbestos fibers are tested in parallel with CNTs to benchmark the toxicity of such particles.

The toxicity of CNTs depends on the physical and chemical properties, manufacturing process, impurities, and the method of preparing suspension solutions (Donaldson et al. 2006; Herzog et al. 2007; Shvedova et al. 2008; Tejral, Panyala, and Havel 2009; Wick et al. 2007). The first and most important route of exposure to nanoparticles is inhalation from occupational environments (Smart et al. 2006). In an exposure assessment study, the workers' exposure to MWCNTs was 0.33 mg m^{-3} (Han et al. 2008). In the present study, human lung epithelial cells (A549) were chosen to evaluate the cytotoxicity of CNTs and chrysotile asbestos. For comparing the cytotoxicity of SWCNTs, MWCNTs, and chrysotile fibers in human lung cells, toxicological indexes such as nonobservable adverse effect concentration (NOAEC) and the half-maximal inhibitory concentration (IC_{50}) were derived.

Materials and methods

Test materials

SWCNTs and MWCNTs were obtained from the Research Institute of Petroleum Industry (RIPI, Tehran, Iran). CNTs were produced by chemical vapor deposition (CVD) (Rashidi et al. 2007). This material contained 5 wt% cobalt. Chrysotile asbestos was donated by the Department of Occupational Health, Tehran University of Medical Sciences (TUMS, Tehran, Iran).

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and phosphate-buffered saline (PBS) were purchased from Sigma Aldrich (St. Louis, USA). The cell culture medium of Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), 10% penicillin/streptomycin, and trypsin–EDTA were obtained from PAA Ltd. (Sydney, Australia). Dimethylsulfoxide (DMSO) was bought from Merck (Darmstadt, Germany).

Characterization of CNTs and chrysotile

A high-resolution transmission electron microscope (HRTEM, LEO-912-AB; Carl Zeiss SMT AG, Oberkochen, Germany) operated at 120 KV was used to determine the diameter and length of CNTs. The specific surface area of CNTs was measured by nitrogen adsorption isothermally at 77 K using a Micrometrics ASAP 2010 Accelerated Surface Area Analyzer and the BET method. A scanning electron microscope (SEM) (VEGA-TESCAN, Brno, Czech Republic) operated at 16 KV was used to determine the length and width of chrysotile fibers.

In order to characterize the chrysotile fibers using SEM-EDS, chrysotile fibers were deposited on a membrane filter. To evenly distribute the fibers on the surface of filter, the fibers were milled using the ball milling method (Koch and Whittenberger 1996), and were suspended in distilled water. The solution was shaken for 5 min before filtration to enhance the homogeneity of suspension. Then, the solution was vacuum-filtered using a mixed cellulose esters filter with a pore size and diameter of 0.45 μm and 37 mm, respectively (Sartorius, Gottingen, Germany). The surface of the filter was coated with gold using a sputter coater (JFC-1100E, JEOL, Akishima, Japan). The mean diameter and length of 50 chrysotile fibers were measured using SEM.

Preparation of solutions of CNTs and chrysotile

Chrysotile fibers were milled using a ball to obtain small size fibers, which are shown in the results. For sterilization, 1.5 mg the powdered CNTs and chrysotile fibers were heated in an oven at 160 °C for 15 min in a glass covered with aluminum foil. For the stock suspension of 1.5 mg L^{-1}, 1.5 mg CNTs or chrysotile fibers were suspended in 1000 mL culture medium containing 5% serum and ultrasonicated for 10 min according to Davoren et al. (2007). Since the CNT particles did not disperse properly, a mixture of culture medium and 0.5% DMSO was used (Asakura et al. 2010). Before the main assay, the toxicity of culture medium containing 0.5% DMSO in lung epithelial cells was assessed; no cytotoxicity was observed.

An ultrasonic tip (VP-30S, 20 KHz, 300 W, TAITEC Co. Ltd., Tokyo, Japan) was used to homogenize the stock suspensions before preparation of test solutions. A 20-min sonication process was carried out with the sequence of 5-min sonication and 30-s shaking. Before and after sonication, the suspension was shaken using vortex shaker (IKA – Werke GmbH and co. KG, Staufen, Germany) for 1 min for homogenization. The stock solution was diluted with both culture medium containing 5% serum and culture medium containing 0.5% DMSO to prepare the test concentrations of 0.97, 1.94, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1000, 1100, 1200, 1300, and 1400 μg mL^{-1}.

Cell culture medium

A549 cells (ATCC, CCL-185), a human lung epithelial cell line, were employed for testing. The cells were cultured in DMEM with 2 m mol L^{-1} L-glutamine supplemented with 10% FBS, and 45 IU mL^{-1} penicillin and 45 μg mL^{-1} streptomycin at 37 °C in a 5% CO_2 humidified incubator.

Cytotoxicity assessment test

A549 cells were seeded using glass well plates in 100 μL DMEM (10,000 cell/well) and the plates were placed in an incubator for 24 h at 37 °C in a 5% CO_2 for stabilization. After 24-h incubation, the wells were rinsed with PBS. Cells were exposed to 100 μL suspensions of CNTs and chrysotile fibers at the concentrations mentioned above. Three wells served as control in the study: to minimize the field absorbance of black color caused by CNTs and chrysotile, which can cause a positive error in the results, the wells were rinsed with PBS before measuring the absorbance. However, this method could not completely omit the colors. Therefore, a well containing CNTs and chrysotile fibers

served as a control well at the concentrations mentioned above. Regarding the other two control wells, one well contained culture medium and the other well contained culture medium plus cells.

The cells were exposed to suspensions of CNTs and chrysotile at the concentrations mentioned above for 6 and 24 h. Then, the supernatant was removed from the wells, and the cells were rinsed with 100 μL PBS, and 100 μL serum-free DMEM was added. An aliquot of 10 μL of 5 mg mL^{-1} MTT was added to each well, and the well plates were placed in an incubator. After 3 h, the supernatant was removed, 200 μL DMSO were added to each well, and the plate shaken at 240 rpm for 20 min and the absorbance was measured at 750 nm. Three independent experiments were carried out for all test concentrations and the control wells. The cytotoxicity assessment was performed for both dispersions, the one with culture medium containing 5% serum and the other with culture medium containing 0.5% DMSO.

Dose–response curves

Dose–response curves were plotted for CNTs and chrysotile fibers after subtracting the absorbance of controls. NOAEC and IC_{50} indexes were extrapolated graphically from the plotted absorbance data.

Statistics

All experimental values were expressed as mean ± standard deviation (SD). Data were analyzed by the independent sample *t*-test using SPSS software version 16. The independent sample *t*-test was conducted to determine whether or not the mean values of toxicological indexes obtained from the two dispersion solutions were significantly different from one another. Significant levels were confirmed at $P > 0.05$.

Results

Characterizations of CNTs and chrysotile

SWCNTs and MWCNTs had the same length (10 μm), while the diameter of SWCNTs and MWCNTs were 10 and 16 nm, respectively. The BET surface areas of SWCNTs and MWCNTs were determined as 303 and 133 $m^2\ g^{-1}$, respectively. The average length and diameter of chrysotile fibers were 27.4 and 2.9 μm, respectively.

Cell toxicity

Dose–response curves and mortality of MWCNs, SWCNTs, and chrysotile fibers are shown in Figures 1–3.

NOAEC and IC_{50} indexes were extrapolated graphically from the plotted absorbance data. The mean (±SD) values of NOAEC and IC_{50} indexes are summarized in Table 1. For SWCNTs, the IC_{50} was not determined in the culture medium containing 5% serum after the exposure period of 6 h because the exposure time was not enough to cause 50% cell death.

The results of the independent samples *t*-test were summarized in Table 2 for two different dispersion solutions after both exposure periods. There is no statistical difference

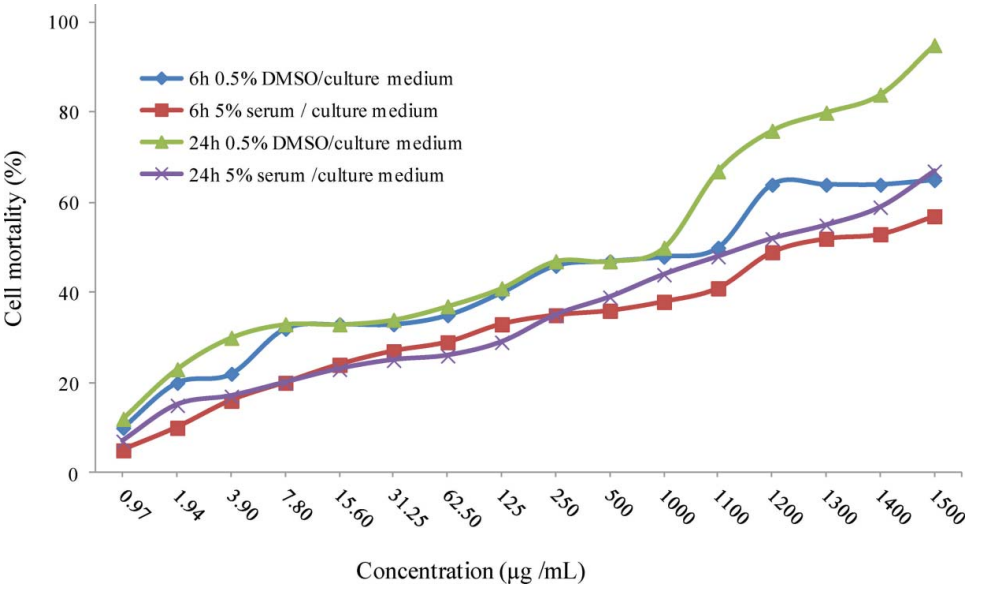

Figure 1. Concentration–cell mortality curves of MWCNTs following 6-h and 24-h exposure periods of lung cells (A549) in two dispersion solutions.

between the NOAEC of all test materials obtained from the two dispersion solutions after both exposure periods ($P > 0.05$). For the IC_{50} of MWCNTs, only the mean of index showed a significant difference between the two solutions after both exposure periods ($P < 0.05$).

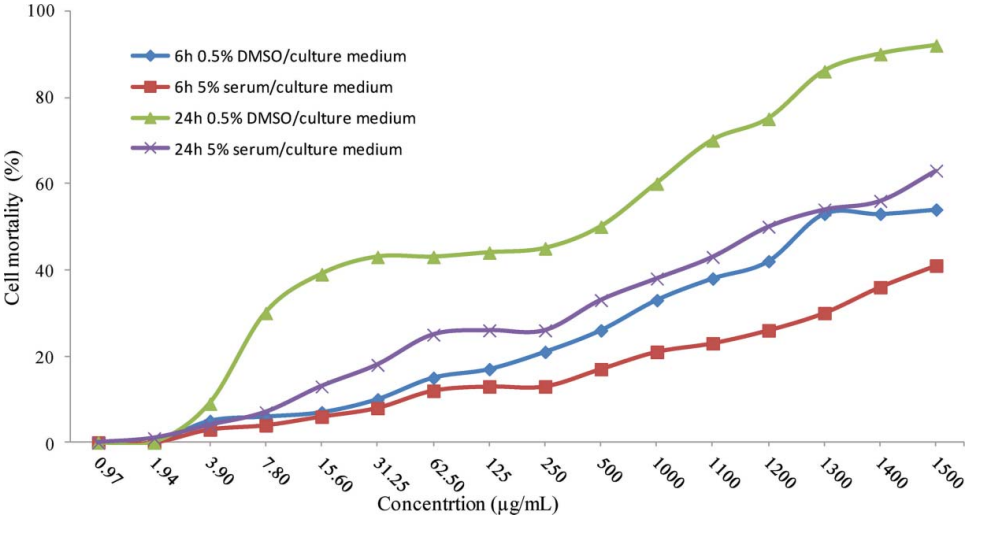

Figure 2. Concentration–cell mortality curves of SWCNTs following 6-h and 24-h exposure periods of lung cells (A549) in two dispersion solutions.

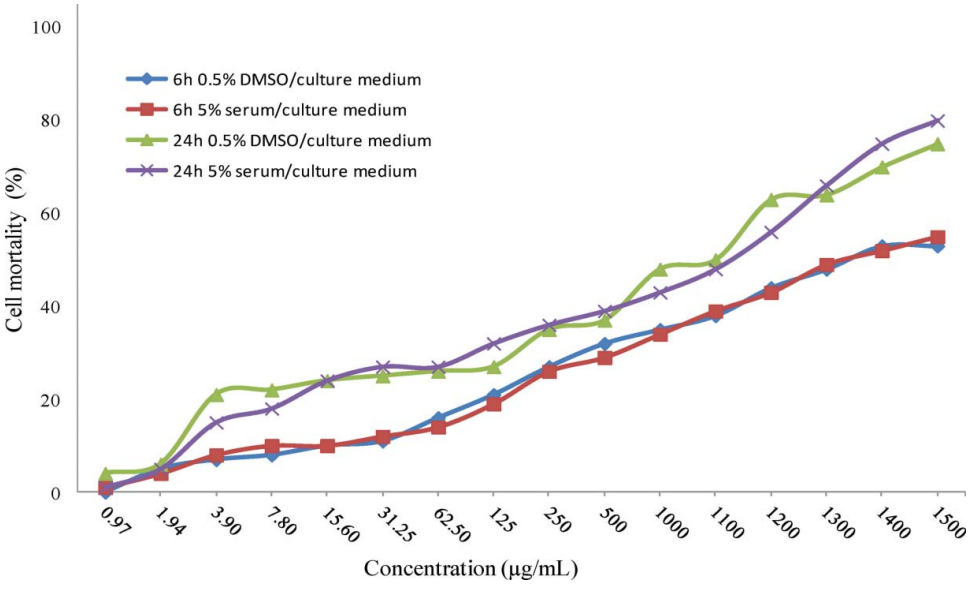

Figure 3. Concentration–cell mortality curves of chrysotile fibers MWCNTs following 6-h and 24-h exposure periods of lung cells (A549) in two dispersion solutions.

Discussion

The mode of dispersion of nanomaterials for test exposures is decisive for the outcome of *in vitro* studies. In the current study, DMEM containing 5% serum was chosen for dispersion (Davoren et al. 2007), but agglomeration of CNTs was observed. When DMEM culture medium containing 0.5% DMSO was chosen (Asakura et al. 2010), no agglomeration of CNTs was visually observed. The agglomeration of CNTs might be related to their size. In the current investigation, the diameters of MWCNTs and SWCNTs were 16 and 10 nm, respectively, whereas in the study of Davoren et al. (2007) the diameter of SWCNT was 1 nm.

Casey et al. (2008) showed the ability of SWCNTs to induce an indirect cytotoxic effect in A549 lung cells by medium depletion. They dispersed nanotubes in the cell culture medium and then removed it by centrifugation and filtration. The result of the experiments revealed that SWCNTs can induce an indirect cytotoxicity by alteration of cell culture medium, which potentially results in a false positive toxic effect. Indirect induction of CNTs cytotoxicity by altered composition of cell culture medium was not assessed in the current study.

The cytotoxicity of MWCNTs and SWCNTs in the dispersion solution of culture medium containing 0.5% DMSO was on average higher than in the culture medium containing 5% serum, perhaps due to better dispersion of CNTs in the former medium. Although the internalization of CNTs into the cells has not been studied, the higher cytotoxicity of suspensions in medium containing DMSO might be associated with higher bioavailability. Smart et al. (2006) have studied the biocompatibility of CNTs and have proposed that inconsistency in biocompatibility data may be associated with different dispersion methods. The cytotoxicity of chrysotile fibers in both culture media was roughly the same at all concentrations, perhaps due to better dispersion of chrysotile fibers.

Table 1. The values of toxicity indexes for CNTs and chrysotile fibers in two dispersion solutions.

Dispersion solution	Toxicity index (μg/mL)	MWCNT		SWCNT		Chrysotile	
		6 h	24 h	6 h	24 h	6 h	24 h
Culture medium containing 0.5% DMSO	NOAEC	1 ± 0.35	0.9 ± 0.2	31.25 ± 11.2	4 ± 1.34	15.6 ± 3.95	2.5 ± 0.87
	IC_{50}	1011 ± 221	1104.6 ± 125	1294 ± 98.5	512 ± 56.45	1395 ± 254	1121 ± 246
Culture medium containing 5% serum	NOAEC	1.5 ± 1.5	2.3 ± 1.11	49 ± 31.2	13 ± 3.21	16 ± 9.13	2.6 ± 2.43
	IC_{50}	1180 ± 201	1360 ± 313	–	1199 ± 101.3	1396 ± 235	1118± 256

Note: Values are expressed as mean ± SD.

Table 2. Results of independent samples *t*-test on two different dispersion solutions in both exposure periods.

Toxicity index	Nanoparticles	Exposure period	
		6 h	24 h
NOAEC	MWCNT	$P = 0.482$	$P = 0.42$
	SWCNT	$P = 0.40$	$P = 0.415$
	Chrysotile	$P = 0.422$	$P = 0.433$
IC_{50}	MWCNT	$^{*}P = 0.02$	$^{*}P = 0.001$
	SWCNT	–	$P = 0.537$
	Chrysotile	$P = 0.184$	$P = 0.419$

Note: An asterisk denotes a significant difference ($P < 0.05$) of the index value obtained from the two dispersion solutions.

Due to the better dispersion of CNTs in the culture medium containing 0.5% DMSO, the obtained results are discussed in more detail. In the current study, the NOAEC and IC_{50} of MWCNTs obtained after both exposure periods were less than that of SWCNTs. This result means that MWCNTs are more toxic than SWCNTs. Furthermore, at lower concentrations, the toxicity of MWCNTs is higher than that of SWCNTs (Table 1). According to the NOAECs, SWCNTs at concentrations lower than 4 μg mL^{-1} and MWCNTs at all concentrations exhibit no remarkable cytotoxicity in lung cells during either the 6-h or the 24-h exposure period. Davoren et al. (2007) studied the cytotoxicity of SWCNTs in human A549 lung cells. They reported that at concentrations lower than 3 μg mL^{-1}, the cytotoxicity of SWCNTs following a 24-h exposure period is unremarkable. The results of the current study are in agreement with this study.

Tabet et al. (2008) investigated the cellular toxicity of MWCNT in human lung epithelial cells. They found that MWCNT causes a 15% cellular death rate at a concentration of 0.1 μg mL^{-1}, which is similar to the results obtained in the current study. This agreement in results emphasizes the cytotoxicity of MWCNTs at lower concentrations. The results of some studies might show poor agreement. Tejral, Panyala, and Havel (2009) have concluded that differences in the toxicity of CNTs are due to discrepancies in physical and chemical specifications of CNTs, production processes, impurities and methods of suspension solution preparation.

The NOAEC and IC_{50} indexes of MWCNTs obtained after both exposure periods were lower than those of chrysotile. This result indicates that MWCNTs are more toxic than chrysotile fibers. As mentioned above, MWCNTs showed remarkable cytotoxicity at all test concentrations after both exposure periods, whereas the noticeable cytotoxicity of chrysotile fibers was observed at concentrations higher than 15.6 and 3.9 μg mL^{-1}, respectively, after exposure periods of 6 and 24 h. Asakura et al. (2010) investigated the cytotoxicity of MWCNTs and chrysotile fibers in hamster lung cells. They concluded that the cytotoxicity of chrysotile was higher than that of MWCNTs. The difference between the results of current study and that by Asakura et al. (2010) can be attributed to the dimensions and impurities of MWCNTs. They used MWCNTs with a smaller length (5 μm) and a larger diameter (88 nm) than the current study. They also reported nickel, iron, and chromium as the impurities MWCNT compared to cobalt as the sole impurity of CNTs used in the present study. Using a different cell line might be another potential cause of discrepancy in results.

Nilsen (2011) studied the cytotoxic and inflammatory responses of human lung cells exposed to two different MWCNTs produced in Japan and Norway by two distinct production methods. The results of his study showed that the MWCNT produced in Japan is more cytotoxic than the MWCNTs produced in Norway and crocidolite asbestos. The same method, CVD, was used for the production of the MWCNTs produced in Japan and used in the current study. Comparing the NOAEC of SWCNTs and chrysotile fibers revealed that during both exposure periods, chrysotile fibers were more cytotoxic than SWCNTs at lower concentrations. By contrast, at higher concentrations, SWCNTs are more cytotoxic than chrysotile according to IC_{50} values.

Han et al. (2008) showed that, in product factories, workers are exposed to MWCNT respiratory particles at a low concentration of 0.33 mg m^{-3}. Based on the result of current study, exposure to a low concentration of MWCNTs is toxic for A549 lung cells (Table 1). Therefore, CNTs can be toxic for humans at low concentrations, and compared to SWCNTs and chrysotile, MWCNTs have more cytotoxicity in A549 lung cells (Table 1).

In this study, we showed that cytotoxicity depends on the time of exposure. MWCNTs were more cytotoxic after a 24-h exposure period compared to a 6-h exposure period. Previous studies also proved that the cytotoxicity of MWCNTs and SWCNTs depend on exposure time (Tejral, Panyala, and Havel 2009). The cytotoxicity of SWCNTs is more dependent on exposure time compared to MWCNTs and chrysotile (Table 1 and Figures 1–3).

Conclusion

The results of this study showed that CNTs might have cytotoxic effects on lung cells that are comparable to the cytotoxic effects of asbestos fibers. The complete dispersion of CNTs in the test solutions and avoiding the agglomeration of particles is a vital factor and affects the toxicity data. The dimensions of particles might be an influencing parameter on the dispersion and, hence, on the degree of cellular toxicity. Other properties such as specific surface area and metal impurities might yield different dose–response data. The fiber-like similarities of CNTs to asbestos fibers besides the cytotoxicity of CNTs in human lung cells raise the concerns of health effects induced by CNTs. Therefore, safety and health measures should be considered during the production, application, and handling of CNTs.

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