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ORIGINAL ARTICLE

Cellular Toxicity of Multi-walled Carbon Nanotubes on Human Lung Cells

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KEYWORDS

Cytotoxicity;
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ABSTRACT: Nowadays, multi-walled carbon nanotubes (MWCNTs) are used in various industries. Considering the exposure probability of these nanomaterials to humans, the purpose of the present study is to assess the effect of MWCNTs on cellular toxicity of human alveolar epithelial. The A549 cells were cultured and treated to various doses of MWCNTs at three different times. Finally, the Tetrazolium colorimetric (MTT) assay was implemented for evaluating the cellular viability. The results indicated that the cytotoxicity for MWCNTs on the human alveolar epithelial cells is related to dose and time of exposure. The inhibitory concentration of 50% (IC50) and non-observed adverse effect concentration (NOAEC) are calculated to be 103.6 as well as 0.65µg/mL, respectively. The findings of this present study could contribute to a better understanding of MWCNTs substances and might be useful as a basis for the future risk evaluation studies of exposed population in industries.

INTRODUCTION

Nanotechnology is a relatively new discipline and key technology in the 21st century [1]. The tubular structure of carbon nanotubes (CNTs) such as single-walled nanotubes (SWNTs) and multi-walled nanotubes (MWNTs) have unique physicochemical characteristics[2]. Currently, CNTs are wildly used in healthcare sectors, semiconductor technology, agriculture, construction, mechanical, chemical products, communication, and military fields[3]. In many workplaces, exposures to nanomaterials occur for a number of individuals[4]. Canu et al. in a review paper expressed that academic and private laboratory workers, primary and secondary manufacturers, and purchasing CNTs lead to occupational exposure with carbon nanomaterials [5].

Despite the high attention paid to physicochemical properties and cytogenotoxic potentiality of CNTs, the toxicity effects of these substances on the in-vitro and in- vivo systems have remained largely unknown. Several studies support that MWCNTs have the most applications among the CNTs compounds [6, 7]. Recently, the functionalized MWCNTs are widely used [8]. Because of the significant mechanical and electrical properties of MWCNTs, these nanomaterials are currently used in the medical and different workplaces[9]. In this regard, Kuijpers et al. reported that the workers and operators exposed to MWCNTs in a commercial company show increased inflammatory and cardiovascular effects [10]. There are many safety and health challenges about the MWCNTs [11, 12]. Inhalation has been identified as the most common pathway for nanomaterials to enter the body [13]. Furthermore, workers may be occasionally exposed to nanomaterials through ingestion or dermal absorption in workplaces[13]. Recently, several studies have reported that phagocytes can be reactivated with nanomaterials and create inflammatory responses [14]. The toxicity effects of the pulmonary epithelial are a significant adverse consequence of

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exposure to MWCNT[14]. Mercer et al. reported that MWCNTs can penetrate into the pulmonary cells and cause alveolar fibrosis[15]. A few studies have detailed the increase in allergic reactions due to exposure to the functionalization of MWCNTs (F-MWCNTs) [17]. MWCNTs can threaten the immune system and increase cardiopulmonary diseases [18]. Zhang et al. reported that pristine-MWCNTs induce a remarkable decline in the lymphatic cells[16]. In a study on the cytotoxicity of MWCNTs, the increased apoptosis followed by decreased cell proliferation[17]. One study demonstrated that MWCNT-COOH did not involve phenotypical ripening of Dendritic Cells (DCs) with concentrations up to 100μg/mL[18]. Mohammadian et al. reported that MWCNTs are more toxic than SWCNT and its toxicology indicators for MWCNTs are less than SWCNT[19].

Iran NanoSafety Network (INSN)[20] has recommended studying the CNT toxicity on human lung cells as a research priority in the country. Although the NIOSH [21] have proposed Occupational Exposure Limits (OELs) for MWCNTs, some uncertainties have been reported about the OELs for CNTs[22]. Hence, the purpose of the present research paper is to investigate the cytotoxicity of MWCNTs on epithelial cells from the human lung (A549).

MATERIALS AND METHODS

The MWCNTs were bought from the Research Institute of Petroleum Industry (RIPI), Tehran, Iran. Ethanol and bovine serum albumin (BSA) were used for obtaining a good dispersion of MWCANTs for cell exposure. DMEM F-12 with Glutamax (DMEM), Penicillin–streptomycin solution, Trypsin-EDTA (1X0.05), Fetal bovine serum (FBS), and Dimethyl sulfoxide (DMSO) were applied to the cells culture. MTT and trypan blue powder were used for assessment cytotoxicity.

Characterization of MWCNTs

The average diameter of MWCNTs was measured by the TEM (CM30-Philip, Japan) and the SEM (S4160-Hitach, Japan). The specific surface area was calculated using the BET method. Infrared spectroscopy absorption has been measured by FTIR. The hydrodynamic was determined using the dynamic light scattering (DLS) procedure (Malvern Instruments Ltd., Zetasizer Ver. 6.01).

Dispersion of MWCNTs

The nano-genotoxic dispersion procedure was applied to scattering MWCNTs[23]. In brief, standard solution was readied to concentration of 2.56 mg/ml. in this way, 15.36 mg MWCNTs added to 5.94 ml of ethanol and 0.06 ml of BSA.

Cell culture

The A549 cells were purchased from the National Cell Bank of Iran (NCBI). Dulbecco's Modified Eagle's Medium (DMEM) was used to incubate cells. The incubation was accomplished at a temperature of 37℃ and with 5% CO2.

The cells were planted 24 h in the microplates (1×104 cells/mL). The ultrasonic (160 W, 20 kHz, 5 min) was used to scatter solution of the MWCNTs and then the solution was diluted with DMEM to various concentrations (0.1, 1, 10, 50, 100, 200, 300, 500, 600, and 1000 μg/ml). The cell viability percentages after exposure to MWCNTs were evaluated for 24, 48, and 72 h. The untreated cells with the MWCNTs were considered as the control group.

Cellular morphology

The cells were seeded in the microplates (10^4cells per well) and considered an incubation time of 24 h. The cells were treated with the specific doses of the MWCNTs. The untreated cells with the MWCNTs were considered as the control groups. The cellular morphology was determined by an optical microscope (Olympus 1x71, with Olympus DP72 Camera 12.8 megapixel) after twenty-four hours.

Cell viability

The tetrazolium colorimetric (MTT) assay was implemented for evaluating the cellular viability [23]. With respect to this assay, phosphate-buffered saline (PBS) was added to the A549 alveolar cells for washing cells. A 5-mg/mL solution of MTT was prepared,

followed by injecting 10 µl of this solution and 150 µl of complete culture medium on cells. The cells were incubated for 3 h and then the supernatants were expelled and replaced with 150 µl of dimethyl sulfoxide (DMSO). In this way, formazan as an insoluble purple dye turned into soluble products. The plates were covered with aluminum foil and shaken for 20 min. Finally, ELISA plate readers (ELX800, BioTek show, the US) was used to read the absorbance at 570 nm. Because a few nanoparticles such as MWCNT can adsorb the MTT dye, the results may be unreliable. Hence, the cells were washed twice in PBS before using ELISA.

Data analysis and calculation of toxicological indices

The SPSS Ver. 22 program was used for statistical analyses. Analysis of variance (ANOVA) was utilized to describe the association between cell death with the time of exposure and concentration of MWCNTs. The probit model by Minitab 18.1 program was applied to determine the toxicological indices including the total lethal concentration (TLC), the inhibitory concentration of 50% (IC50), and non-observable adverse effect concentration (NOAEC). The NOAEC was defined as a concentration of nanomaterials when cell death occurred in the amount of 10% for nanomaterials [28, 29].

RESULTS

The obtained findings of the results were categorized into four parts: characteristics of MWCNTs, cellular morphology, cell viability and toxicological indices.

characteristics of MWCNTs

Figure 1 indicates a photograph of MWCNTs with the higher and lower resolution by TEM. As it shows, the MWCNTs have the length of 1–3µm and the diameter of 10-17 nm. The MWCNTs had carboxylic groupings (F-MWCNTs) and the surface energy achieved for carboxyl-MWCNT was 25.9 mJ/m^2. Figure 2 shows the FTIR of F-MWCNTs, in this graph, spectra of 1704cm^{-1} is related to C=O, 1206 cm^{-1} for asymmetric bending of C-O-C and 1079 cm^{-1} for C.

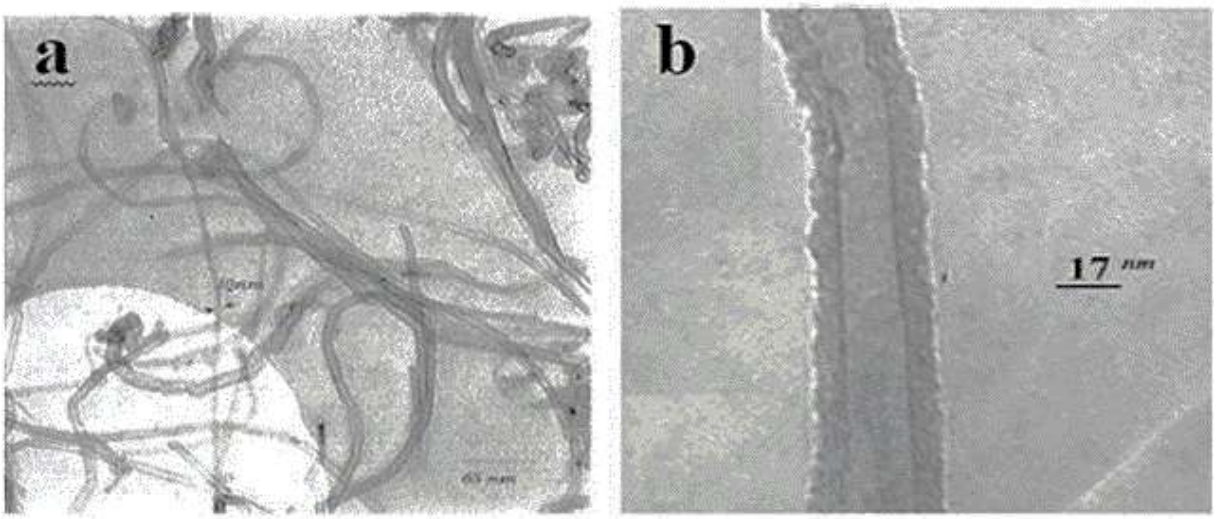

Figure 1. The photograph of MWCNTs with the higher and lower resolution by TEM

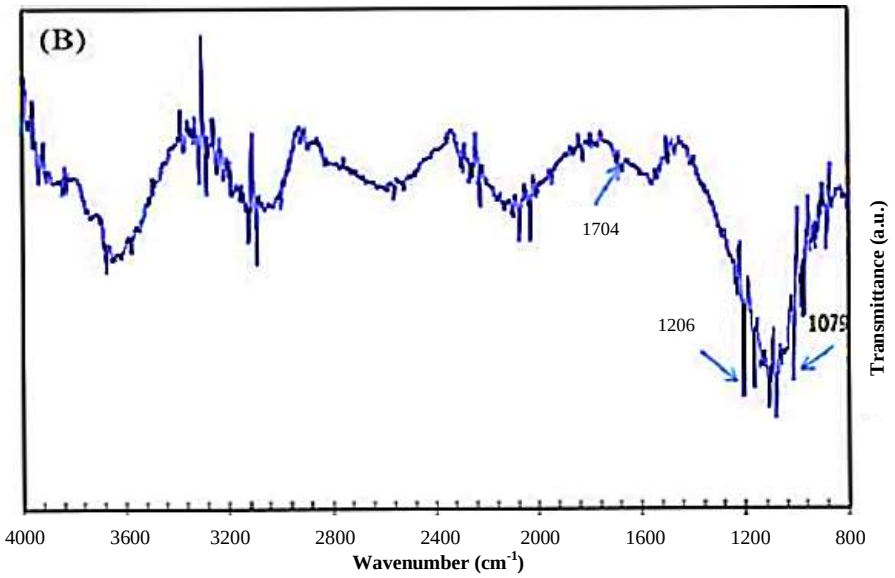

Figure 2. The FTIR of carbonylated MWCNTs.

The purity of the MWCNT was 99.98%. The average hydrodynamic diameter (HD) of MWCNTs in the aqueous suspension was 313.9 nm. The PDI shows no agglomeration of the MWCNTs in the DMEM culture medium (PdI=0.608). Figure 3 display the size distribution of F-MWCNT in culture medium.

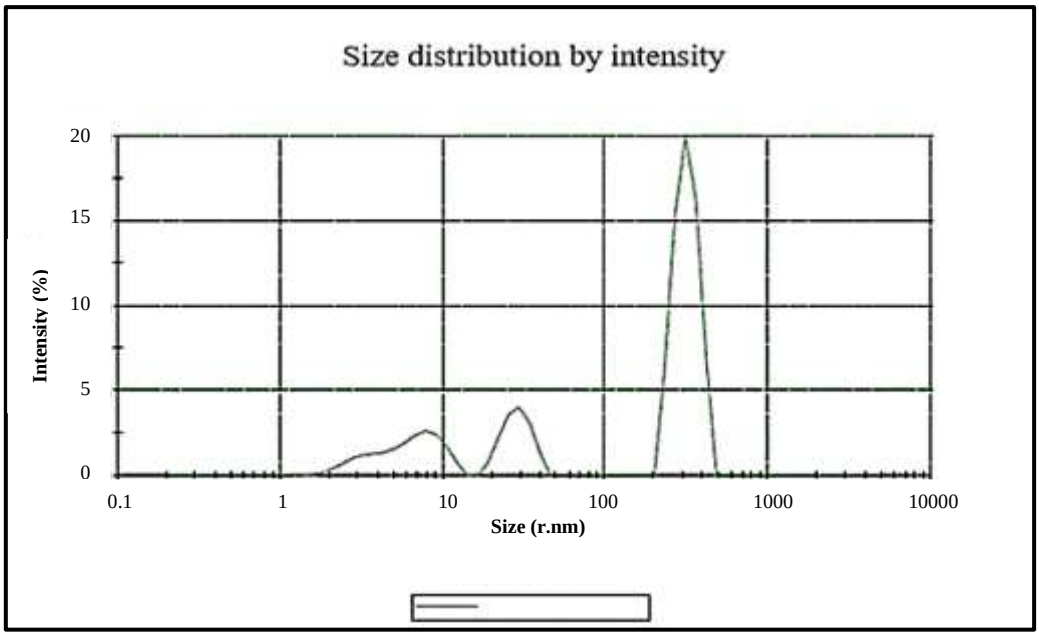

Figure 3. Size distribution of F-MWCNTs in a basal medium containing DMEM.

Cellular morphology

The cellular morphology is an important factor in recognizing the shape, building, and size of cells. In this study, no morphology change was observed in cells treated with F-MWCNTs and control groups after the exposure to F-MWCNTs. Both treated and untreated cells, which were adhered to the culture media plates, had a spindle form (Figure 4).

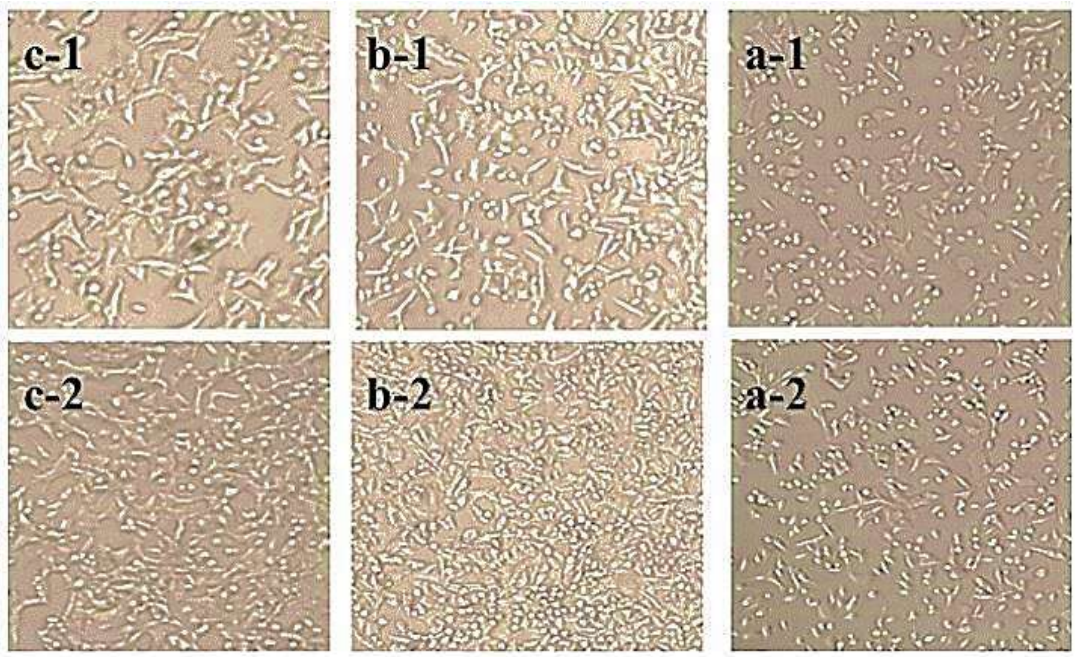

Figure 4. The photographs of A549 cells by the optical microscope: (a-1) Cells treated with F-MWCNTs at 24 h; (a-2) the control at 24 h; (b-1) Cells treated with F-MWCNTs at 48 h; (b-2) the control at 48h; (c-1) Cells treated with F-MWCNTs at 72 h; and (c-2) the control at 48 h

Cell viability

MTT assay was used to obtain the percentage of live cells. In analogous laboratory conditions, the absorbance spectra of F-MWCNTs itself (0.01) was estimated and considered in calculations of the percentage of live cells. At concentrations above 100 µg/mL of F-MWCNTs, the number of live cells had a trend of shifting to lower IC50 after 24 and 48 h of exposure. However, at 71.41 µg/mL concentration, the cell viability was 50% following the exposure to F-MWCNTs at 72 h. Cell death was 42.31±27.50, 47.21±27.58, and 51.64±27.85 in 24, 48, and 72 h, respectively. ANOVA results showed a statistically significant association of cell death with exposure period to F-MWCNTs (p-value=0.026) and exposed concentration (p-value=0.00) (Figure 5).

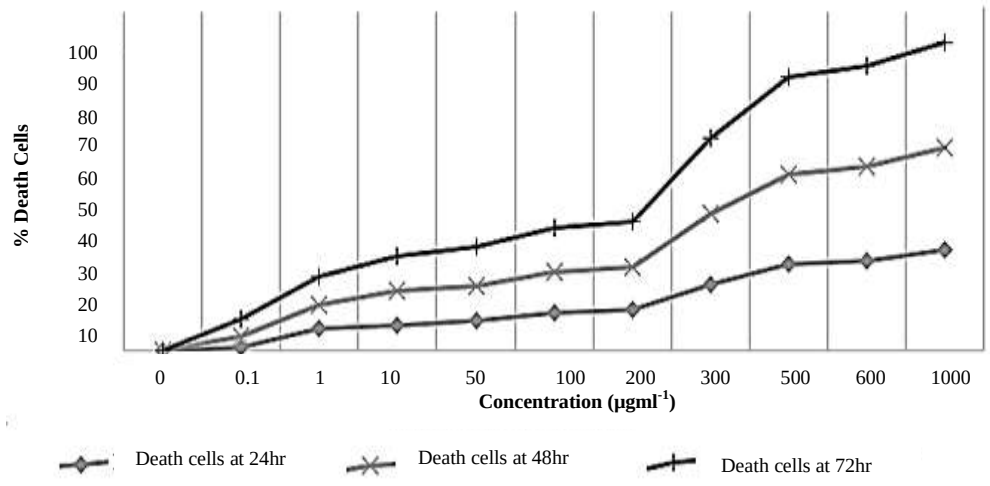

Figure 5. Dose-response curve for cells treated with F-MWCNTs at various times

Toxicology indices

The probit regression was considered to achieve the toxicological indices including TLC, IC50, and NAOEC [24, 25] (Figure 6). Also, Table 1 presents the toxicological indices for F-MWCNTs.

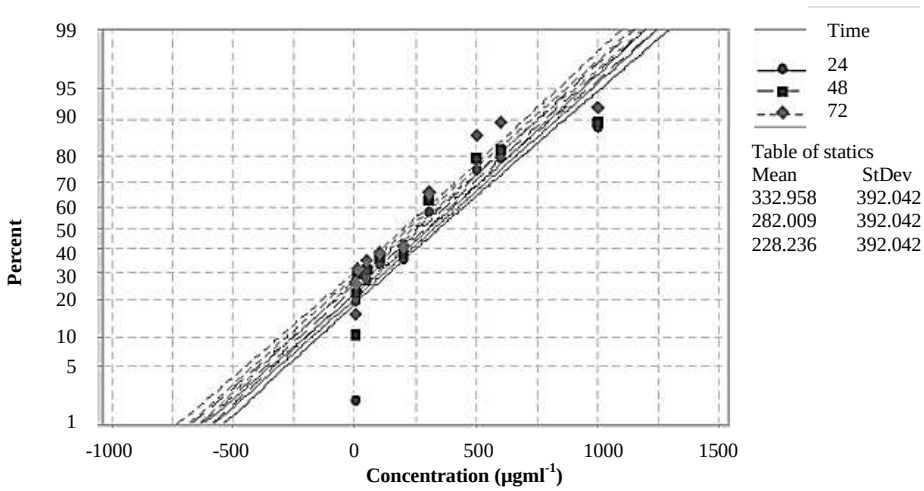

Figure 6. Dose-response curve for F-MWCNTs regarding time, by probit model

Table 1. Toxicology indices for F-MWCNTs

Time exposure (hr.)	Toxicology indicators (µg/ml)		
	TLC	**IC_{50}**	**NAOEC**
24	23729.3	148.72	0.95
48	16875.8	105.77	0.68
72	11394.1	71.41	0.46

According to concentration of F-MWCNTs, the dose-response relationship was achieved without consideration of exposure times, by probit model (Figure 7). In this regard, the TLC, IC_{50}, and NOAEC were calculated equal to 16829.4, 103.6 and 0.65µg/ml, respectively.

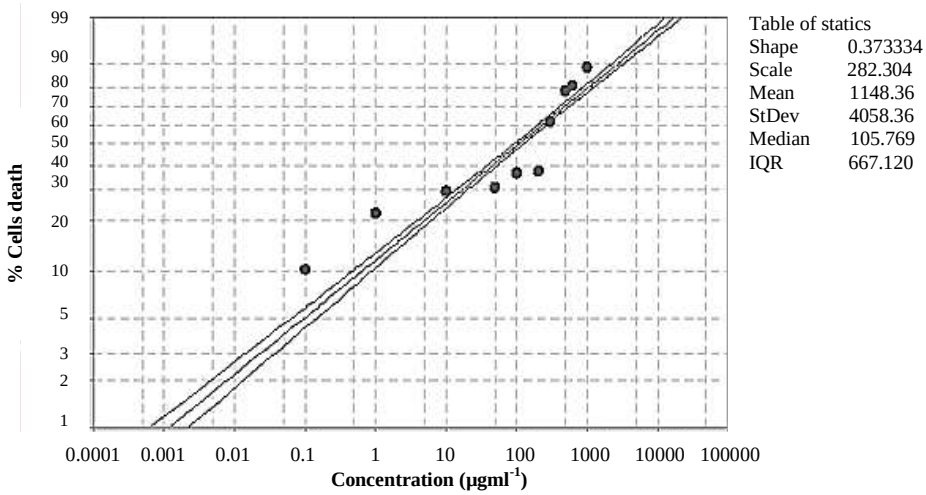

Figure 7. Dose-response curve for F-MWCNTs regarding concentration, by probit model.

DISCUSSION

The manufacturing of MWCNTs and their expanded applications in new technologies have raised the likelihood of human exposure and worries about their negative effects. The objective of the present study is to assess the cytotoxicity of MWCNTs on epithelial cells from the human lung.

F-MWCNTs remarkably increased water solubility. In addition, pristine MWCNTs had poor solubility in water and settled in the suspension. Several studies also demonstrated that functionalized MWCNTs have low toxicity than Pristine-MWCNTs [24-26], which can be attributed to the increased water solubility and enhanced

biomedical functions[27]. In contrast to these studies, Chatterjee et al. showed that functionalized MWCNTs are more toxic than Pristine-MWCNTs. They reported cytotoxicity in the range of 5-200 mg/L concentration tested on the human cell line (BEAS2B)[28]. In this regard, other factors, such as type of solvent, may affect the solubility and toxicity of MWCNTs-COOH under in-vitro conditions.

The MWCNTs-COOH can decline cell viability. Several studies have affirmed the negative effect of MWCNTs on both the tissues and cells[29]. The results of a study indicated that MWCNTs-COOH with a diameter of 20 nm had an improving cytotoxicity effect on A549 cells [30]. In another research, cell death was less than 40% (32 μm/ml) for MWCNTs-COOH, with a diameter of 21.7 nm and the same length with the current study[24]. In the present study, cell death was observed in less than 30% for 10 and 50 μg/mL concentration of MWCNTs-COOH. This phenomenon was consistent with the findings of other researchers. Therefore, cell death was associated with the size of nanoparticles.

The NOAEC results indicated that MWCNTs-COOH containing IC_{10} can create toxic effects on epithelial cells at 0.95 μg/ml. The lowest NOAEL was determined at 0.1 mg/m^3 in a 13-weeks inhalation study on Wistar rats [31]. However, there are no studies clearly presenting NOAEC. NIOSH recommended an exposure limit (REL) for carbon nanotubes equal to 1 $\mu g/m^3$[21], which is calculated by the ratio of NOAEC/LOAEC. It seems that a higher risk of adverse lung effects might occur at lower levels. Therefore, much attention should be paid to decrease the carbon concentration.

In vitro cytotoxicity assay on MWCNTs-COOH gave IC_{50} of 103.6 μg/ml. In another study, IC_{50} of MWCNT was 400 μg/ml after 48 on MC4L2 cell [32]. Zhou et al. reported that IC_{50} for MWCNTs-COOH with an average diameter of 15 nm occurred at a concentration of ≥ 1 mg/ml [33]. Sanand et al., using correlation-regression analysis, calculated the IC_{50} values of MWCNTs (average size of MWCNTs was 231 nm) to be about 44.91 mg/mL in 30 min on stem cells [34]. Pantarotto et al. indicated that MWCNTs have the toxicity effects for HeLa cells and reported an IC_{50} value of 10 mg/mL [35]. These results are in contrast with current evidence. IC_{50} values calculated from the different cytotoxicity assays as well as the types of cells and functionalization can cause different cytotoxic effects.

TLC was estimated at concentrations higher than 1000 mg/ml. Another study suggested that the TLC of MWCNTs was 3100 μg/ml[19]. TLC was not reported in other studies. Probably, since IC_{50} and NOAECs have the important roles to determine reference value to exposure dose, TLC is less discussed than two described parameters.

Cytotoxicity depends on the exposure period and time-dependent increase in uptake for MWCNTs-COOH. In addition, MWCNTs have more cytotoxic effects after 72 h compared to 24-hour and 48-hour exposure. Several studies have reported that the toxicity effects of MWCNTs rely on the time of exposure[19, 36, 37]. This result is consistent with that determined by the precision of the results. Exposure time-dependent increase in cell death can be attributed to activating the toxicity mechanisms after 24 h

CONCLUSIONS

According to the findings of the current study, the number of live cells decreases with increasing the dose of MWCNTs and the time of exposure. The value of NOAEC was calculated to be 0.65 μg/ml for MWCNT. The results of this study could contribute to more understanding of MWCNTs substances and might be helpful for the future risk evaluation of the exposed working population.

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Conflict of interest

The authors declare that there is no conflict of interest

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