

In Vitro* Evaluation of Cytotoxicity and Oxidative Stress Induced by Multiwalled Carbon Nanotubes in Murine RAW 264.7 Macrophages and Human A549 Lung Cells

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Abstract

Objective To investigate *in vitro* cytotoxicity and oxidative stress response induced by multiwalled carbon nanotubes (MWCNTs).

Methods Cultured macrophages (murine RAW264.7 cells) and alveolar epithelium cells type II (human A549 lung cells) were exposed to the blank control, DNA salt control, and the MWCNTs suspensions at 2.5, 10, 25, and 100 µg/mL for 24 h. Each treatment was evaluated by cell viability, cytotoxicity and oxidative stress.

Results Overall, both cell lines had similar patterns in response to the cytotoxicity and oxidative stress of MWCNTs. DNA salt treatment showed no change compared to the blank control. In both cell lines, significant changes at the doses of 25 and 100 µg/mL treatments were found in cell viabilities, cytotoxicity, and oxidative stress indexes. The reactive oxygen species (ROS) generation was also found to be significantly higher at the dose of 10 µg/mL treatment, whereas no change was seen in most of the indexes. The ROS generation in both cell lines went up in minutes, reached the climax within an hour and faded down after several hours.

Conclusion Exposure to MWCNTs resulted in a dose-dependent cytotoxicity in cultured RAW264.7 cells and A549 cells, that was closely correlated to the increased oxidative stress.

Key words: Multi-wall carbon nanotubes; Cytotoxicity; Oxidative stress; RAW 264.7 cells; A549 cells

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INTRODUCTION

Carbon nanotubes (CNTs) are cylinders of one or several (up to 20) graphite layers (single- or multiwall carbon nanotubes, respectively: SWCNT or MWCNT). CNTs are considered to be one of the most promising materials in nanotechnology, with attractive properties for many

technological applications covering composite materials, medical applications, and electronics to energy storage. It is estimated that the worldwide market for products with nanotechnology components (including CNTs) will reach \$1 trillion by 2015 (Roco, 2005). Due to the widespread projected use of CNTs, people are increasingly exposed to various kinds of manufactured nanoparticles, which makes it

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important to understand their potential harmful effects^[1].

One of the first target organs of nanoparticles exposure is the lung, which is directly exposed after inhalation of contaminated air. Due to their size, nanoparticles are distributed in the whole respiratory tract and can reach pulmonary alveoli^[2]. With regard to CNTs, both SWCNTs, and MWCNTs could pose potential health problems as described by an extensive literature. Pulmonary effects of CNTs have been evaluated by a number of *in vivo* and *in vitro* studies. Once taken up by experimental animals, CNTs may cause oxidative stress, inflammation, cell damage, adverse effects on cell performance, and, in a long-term perspective, pathological effects like granulomas and fibrosis. These effects have been observed at a time and dose dependent manner in most *in vivo* studies.

In vitro studies demonstrated that CNTs were taken up by different cell types and evoked effects in the cells. These effects were cytotoxicity, inflammatory responses and oxidative stress. Interestingly, Pulskamp et al.^[3] found that SWCNTs treatment in rat macrophages (NR8383) and human alveolar type II cells (A549) showed no acute toxicity on cell viability or the inflammatory mediators nitric oxide (NO), tumor necrosis factor- alpha (TNF-alpha) or interleukin 8 (IL-8), but rather induced a dose- and time-dependent increase of intracellular reactive oxygen species. *In vitro* evidences on cytotoxicity of CNTs have been controversial. It is only recently that the formation of free radicals (oxidative stress) has been suggested as a key factor in further cell reactions^[4]. Reactive oxygen species (ROS) generation after exposure to CNTs has been directly associated with lipid peroxidation, oxidative stress, inflammation responses and changes in cell morphology^[5-6]. Other consequences of oxidative

injury following CNTs exposure included effects on nuclear factor activation, gene transcription and protein expression^[7-8].

This study aimed at investigating the *in vitro* cytotoxicity and oxidative injury capacity of MWCNTs (Nanocyl® 3150, commercially available from Nanocyl SA, Belgium). Two cell lines, which were murine alveolar macrophages (RAW 264.7) and human lung alveolar type II epithelial cells (A549), were chosen since pulmonary toxicity was the major health concern on MWCNTs. Macrophages are the primary responders to different particles that initiate and propagate inflammatory reactions^[9-10]. Epithelium cells are the major functional cells in the lung and A549 cell line is classically used for lung toxicity. Meanwhile, the oxidative stress effects of MWCNTs on these two cell lines have not been thoroughly examined.

MATERIALS AND METHODS

Nanomaterials and Dispersion

Commercially available MWCNTs (Nanocyl® 3150, Nanocyl S.A. Belgium) were used in the experiment. According to product datasheet, MWCNTs were produced via the catalytic carbon vapor deposition (CCVD) process and purified to over 95%, the rest being metal oxides with the major impurities of Ferrum (0.19%), Cobalt (0.07%), and Sulfur (0.14%), and the minor impurities of Aluminium (0.03%)^[11]. The average diameter was 9.5 nm and the length was less than 1 μm . The surface was not carboxyl, amino, hydroxyl, or sulphhydryl-functionalized. Figure 1 shows the scanning electron microscopic (SEM) and transmission electron microscopic (TEM) images of the dispersed MWCNTs.

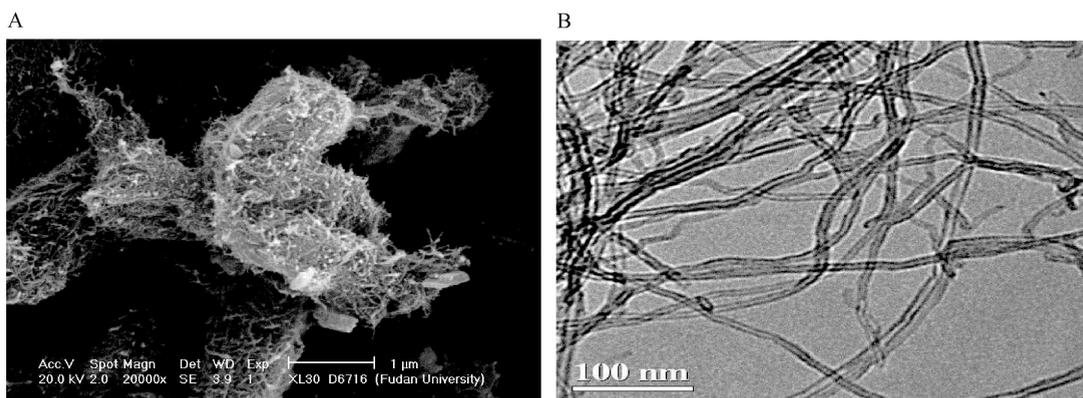


Figure 1. (A) Scanning electron microscopic (SEM) and (B) transmission electron microscopic (TEM) images of dispersed MWCNTs (Nanocyl® 3150).

DNA sodium salt solution (DNA salt) was used to improve the dispersion of MWCNTs in cell culture medium. Salmon sperm DNA (Sigma-Aldrich, U.S.) and tested MWCNTs were mixed and dissolved in saline solution (NaCl-0.9%). The MWCNTs-DNA salt solution was sonicated at cold temperature (5 °C) for 2 h and then left for 24 h to allow any impurities to precipitate to form sediments. The MWCNTs-DNA salt solution was sterilized and kept in dark at 4 °C, and sonicated again for 10 min before being added into the culture medium.

Cell Culture and Exposure to MWCNTs

Murine RAW 264.7 macrophages and human A549 lung cells (both from the Shanghai Cell Line Bank, China) were grown in DMEM (Gibco, U.S.) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco, U.S.), 100 units/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere (5% CO₂ plus 95% air) at 37 °C. RAW 264.7 and A549 were exposed to tested MWCNTs at a concentration of 2.5, 10, 25, or 100 µg/mL. Salmon sperm DNA in all these experiment settings had a concentration equal to the content of MWCNTs (1:1 match). Each experiment had two parallel control groups treated with the equal volume of saline solution (NaCl-0.9%) or DNA salt solution containing 100 µg/mL Salmon sperm DNA. In all experiment settings, cells were treated in sextuplicate in each dose group.

Cell Viability

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Chaoyan Biotech Co. LTD, Shanghai, China) according to the manufacturer's instructions. RAW 264.7 cells were planted into 96-well plates at a density of 2.0×10^4 cells per well in 200 µL culture medium and allowed to attach for 24 h before exposure to MWCNTs-DNA salt solutions for another 24 h. After treatment, 20 µL MTT (0.5 mg/mL) was added to each well and incubated at 37 °C for 4 h. The culture medium was aspirated cautiously, after which 100 µL dimethyl sulfoxide (DMSO) was added and mixed thoroughly for 10 min. Optical density (OD) was read on an ELISA reader (ELX-800, BioTex, U.S.) at absorption wavelength of 570 nm. Viability of A549 cells was measured in the similar procedure described above with minor change in which the cells was planted at a density of 3×10^5 cells per well in a 100 µL culture medium.

Measurement of TP, LDH, and NO in Cell Culture Supernatant Fluids

RAW 264.7 or A549 cells were planted into 6-well plates at a density of 2.0×10^5 per mL in 2.5 mL culture medium and allowed to attach and cover 80% surface area of the plates before treatment with MWCNTs for 24 h exposure. After treatment, culture supernatant fluids were collected to measure the levels of total proteins (TP), lactate dehydrogenase (LDH), and nitric oxide (NO) by commercial kits (Jiancheng Bioengineering Co. Ltd, Nanjing, China). The TP content was determined using an assay kit of Coomassie Brilliant Blue protein. The activity of LDH was assayed spectrophotometrically by monitoring the reduction of NAD⁺ at 340 nm in the presence of lactate. The NO production was assessed using an assay kit of nitric acid reductase according to the manufacturer's instructions.

Measurement of Intracellular GSH, SOD, and MDA

RAW 264.7 or A549 cells were planted into 6-well plates at a density of 2.0×10^5 per mL in 2.5 mL culture medium and allowed to attach and cover 80% surface area of the plates before treatment with MWCNTs for 24 h exposure. After treatment, the cells were rinsed with ice-cold PBS, trypsinized and immediately disrupted by a repeated frozen-thaw process (three times). The cell lysates were centrifuged and froze at minus 20 °C for subsequent determination. glutathione (GSH), malondialdehyde (MDA), and superoxide dismutase (SOD) were respectively measured using commercial kits (Jiancheng Bioengineering Co. Ltd, Nanjing, China).

Measurement of Hydrogen Peroxide (H₂O₂) Generation

The intracellular H₂O₂ were measured by the chemiluminescence (CL) method based on the horseradish peroxidase (HRP) catalyzed oxidation of luminol by radicals in alkaline solution. Briefly, RAW 264.7 or A549 cells were planted into 6-well plates at a density of 2.0×10^5 per mL in 2.5 mL culture medium and allowed to attach and cover 80% surface area of the plates before treatment with MWCNTs for 1 h exposure. After treatment, the cells were rinsed with ice-cold PBS, harvested by trypsinization. The cell lysates were centrifuged, rinsed and suspended in two epindorf (EP) of 400 µL PBS for radical measurement. After 4 µL luminol and 8 µL horseradish peroxidase (HRP) were added into the 400 µL aliquots, chemiluminescence was measured at each second from 0 to 9th second at 25 °C with a

Sirius Luminometer apparatus (Zylux, U.S.), and the results were shown as the mean of all measurements from total seconds.

Measurement of Intracellular Reactive Oxygen Species (ROS)

The intracellular ROS was determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). Briefly, RAW 264.7 or A549 cells were planted into 6-well plates at a density of 1.0×10^5 per mL in 2.5 mL culture medium and allowed to attach and cover 80% surface area of the plates before treatment. The cells then underwent time-dependent and dose dependent experiments. In the time-dependent setting, the cells were rinsed with PBS for three times, and incubated with DCFH (10 $\mu\text{mol/L}$) and MWCNTs (25 $\mu\text{g/mL}$) containing serum for 0, 0.5, 1, 1.5, 2, 3, or 4 h, respectively. Then the cells were washed three times with cell culture medium without serum to eliminate DCFH-DA that did not enter the cells. Cells were harvested by trypsinization, centrifuged and suspended in PBS. Cells were observed under an inverted fluorescence microscope (Nikon, Eclipse, TE2000-U, Japan). The fluorescence was then determined at 503 nm excitation and 525 nm emission using a fluorospectrophotometer (Shimadzu RF-510, Japan). In dose-dependent settings, cells were treated with DCFH (10 $\mu\text{mol/L}$) and MWCNTs at concentrations of 2.5, 10, 25, or 100 $\mu\text{g/mL}$, respectively. The cells then underwent similar procedure as mentioned above.

Statistical Analysis

In all experiment settings, cells were treated in sextuplicate in each dose group and the experiments were replicated three independent times. The data are presented as mean \pm SD (standard deviation). Statistical analysis of the data was carried out using one way analysis of variance (ANOVA), followed by the least significant difference test (equal variances) or Dunnett's T3 test (unequal variances). All statistics were performed using SPSS 11.0 and the tests were two-sided ($\alpha=0.05$)

RESULTS

Cytotoxicity

Cytotoxicity of MWCNTs was assessed by cell viability and biochemical index disturbance of total protein, LDH and nitric oxide in cell culture supernatant fluids. Generally, both RAW 264.7

macrophages and A549 cells had similar patterns in response to the cytotoxicity of MWCNTs. DNA salt treatment showed no change in cell viability or the increase of total protein content, LDH activity and nitric oxide generation.

Cell viability was analyzed with the MTT method. Compared with the DNA salt control group, MTT values of RAW 264.7 macrophages decreased significantly from 10 $\mu\text{g/mL}$ to 25 $\mu\text{g/mL}$ and to 100 $\mu\text{g/mL}$ incubation, reaching 73% of the control values for 25 $\mu\text{g/mL}$ incubation at 24 h postexposure and 62% for 100 $\mu\text{g/mL}$ incubation (Figure 2). Similar results were obtained after exposure of A549 cells to DNA-mixed MWCNTs, reaching 66% of the control values for 25 $\mu\text{g/mL}$ incubation at 24 h postexposure and 51% for 100 $\mu\text{g/mL}$ incubation (Figure 2).

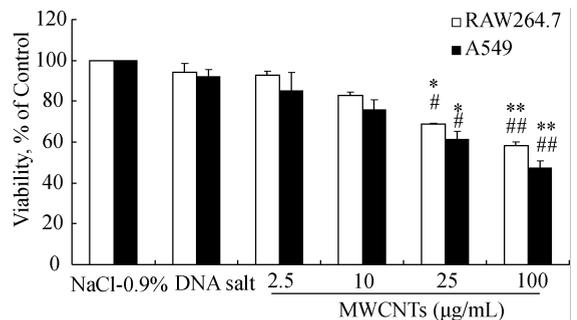


Figure 2. Viability of MWCNTs to murine RAW 264.7 macrophages (o) and human A549 lung cells (v) after 24 h exposure determined by the MTT assay. Data are expressed as percentage of the control (Saline solution, NaCl-0.9%) mean \pm SD of three repeated MWCNTs experiments at the concentration of 2.5, 10, 25, and 100 $\mu\text{g/mL}$, respectively. *Denotes a significant difference from the NaCl-0.9% control ($P < 0.05$; ** $P < 0.01$). #Denotes a significant difference from the DNA salt controls ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$).

Total protein content in culture supernatant fluids was assessed by Coomassie Blue assay. MWCNTs incubation increased the total protein generation in both cell lines, but the significant difference was only found in 25 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ treatment as compared to the NaCl-0.9% control group in RAW 264.7 macrophages and to both control groups (NaCl-0.9% and DNA salt) in A549 cells (Figure 3A). Compared to the DNA salt controls, the total protein content in supernatant fluids was 1.59 and 1.82 times higher than that for 25 and 100 $\mu\text{g/mL}$ incubation in A549 cells, respectively.

Increased activity of LDH is a sign of membrane leakage. MWCNTs incubation in RAW 264.7 macrophages resulted in significant increase in LDH

release in 25 and 100 $\mu\text{g}/\text{mL}$ treatments compared to both controls (NaCl-0.9% and DNA salt), which was 1.15-1.20 times as high as the control values for 25 $\mu\text{g}/\text{mL}$ incubation and was 1.23-1.28 times for 100 $\mu\text{g}/\text{mL}$ incubation (Figure 3B). Similar results were obtained after exposure of A549 cells to DNA-mixed MWCNTs, but significant difference compared to DNA salt control group was only found in 100 $\mu\text{g}/\text{mL}$ treatment, reaching 1.27 times as high as the control values.

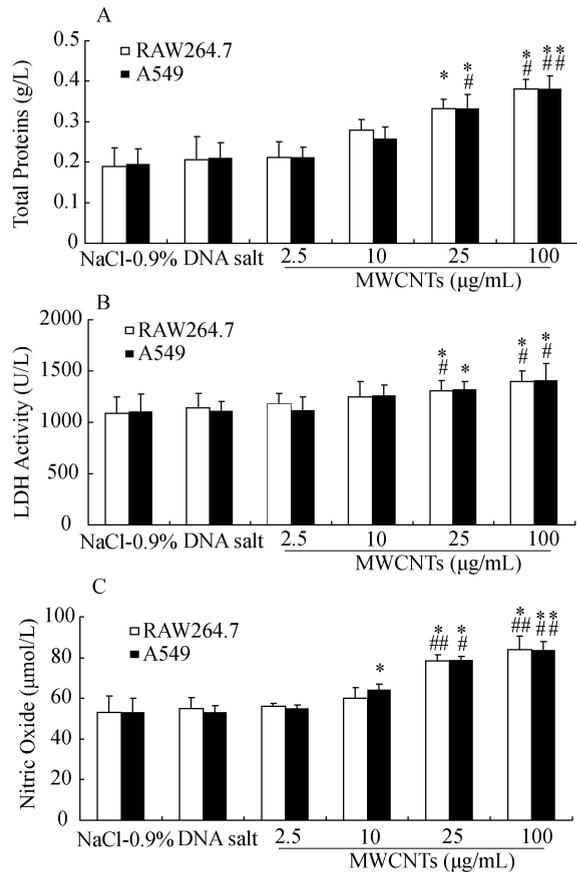


Figure 3. Cytotoxicity of MWCNTs to murine RAW 264.7 macrophages (o) and human A549 lung cells (v) after 24 h exposure by measurement of levels of (A) total proteins, (B) lactate dehydrogenase (LDH), and (C) nitric oxide in culture supernatant fluids. Data are expressed as mean \pm SD of three repeated MWCNTs experiments at the concentration of 2.5, 10, 25, and 100 $\mu\text{g}/\text{mL}$, respectively. *Denotes a significant difference from the NaCl-0.9% control ($^*P<0.05$; $^{**}P<0.01$). #Denotes a significant difference from the DNA salt controls ($^{\#}P<0.05$; $^{\#\#}P<0.01$).

The effect of MWCNTs incubation on nitric oxide secretion in culture supernatant fluids was examined.

As shown in Figure 3C, compared to DNA salt control, significant increase was only found in 100 $\mu\text{g}/\text{mL}$ treatment in RAW 264.7 macrophages, which was 1.53 times higher than the control values, but in both 25 and 100 $\mu\text{g}/\text{mL}$ treatment in A549 cells, that was 1.49 and 1.58 times higher than the control values, respectively. Compared to NaCl-0.9% control, significant nitric oxide secretion was also found in 25 $\mu\text{g}/\text{mL}$ treatment in RAW 264.7 macrophages and in 10 $\mu\text{g}/\text{mL}$ in A549 cells.

Oxidative Stress

The effects of MWCNTs incubation on oxidative stress were thoroughly investigated by examining intracellular GSH, SOD, MDA, H_2O_2 , and ROS. Generally, both RAW 264.7 macrophages and A549 cells had similar patterns in oxidative stress in response to MWCNTs treatment. There was no difference in oxidative stress in cell lines treated with either NaCl-0.9% or DNA salt solutions.

Dose-dependent effects were found in all of the intracellular GSH, SOD, MDA, H_2O_2 , and ROS. Compared to the DNA salt controls, significant GSH reductions of 78% and 66% in RAW 264.7 macrophages as well as 77% and 66% in A549 cells were observed after 24 h incubation of 25 and 100 $\mu\text{g}/\text{mL}$ MWCNTs (Figure 4A). Similar results were also found in SOD after MWCNTs incubation, resulting in 73% and 61% reductions in RAW 264.7 macrophages and 69% and 59% reductions in A549 cells, although the 25 $\mu\text{g}/\text{mL}$ treatment in RAW 264.7 macrophages was not significant compared to DNA salt control (Figure 4B).

The intracellular MDA as a sign of lipid peroxidation was also measured (Figure 4C). Significant MDA elevations were found in both cell lines after 24 h incubation of 25 and 100 $\mu\text{g}/\text{mL}$ MWCNTs when comparing to both controls (NaCl-0.9% and DNA salt). Elevation was also found in A549 cells after 10 $\mu\text{g}/\text{mL}$ treatment. In RAW 264.7 macrophages, significant elevation was found to be 1.93 times and 2.39 times higher than the DNA salt control level after 25 and 100 $\mu\text{g}/\text{mL}$ incubation, respectively. In A549 cells, the elevation was 1.96, 2.43 and 3.61 times higher than of the DNA control level after 10, 25, and 100 $\mu\text{g}/\text{mL}$ incubation.

MWCNTs effects on radical formation were assessed by measuring intracellular H_2O_2 and ROS. Comparing to the DNA salt control, H_2O_2 generation achieved a less than 2-fold elevation in 25 and 100 $\mu\text{g}/\text{mL}$ incubation in both RAW 264.7 macrophages and A549 cells (Figure 4D), where ROS generation was much higher than the controls in both cell lines

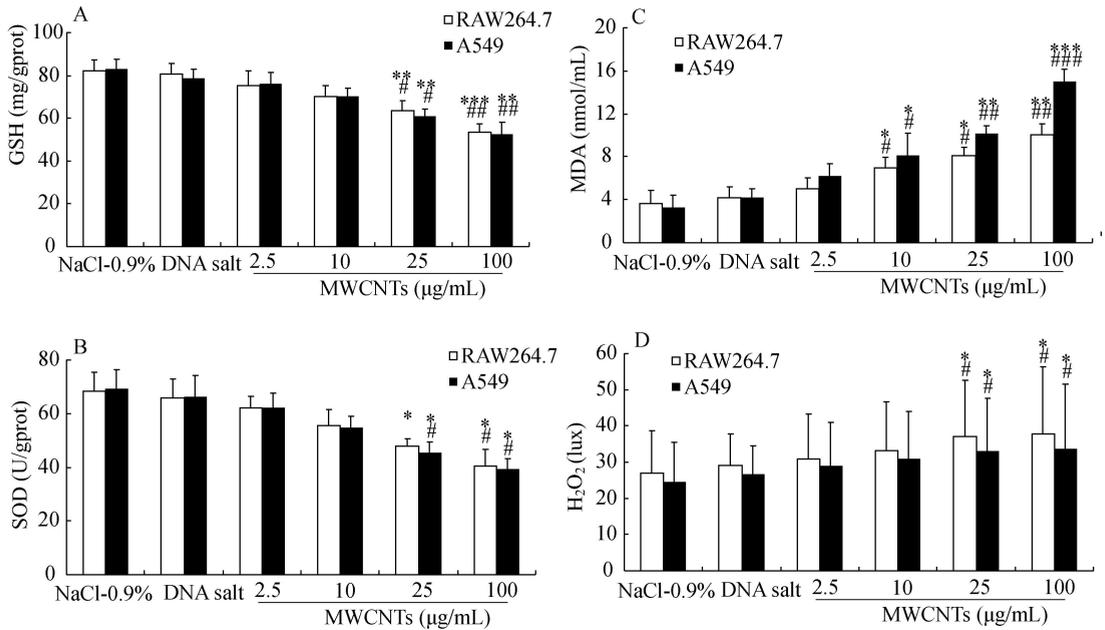


Figure 4. Oxidative stress induced by MWCNTs in murine RAW 264.7 macrophages (o) and human A549 lung cells (v) after 24 h exposure by measurement of levels of (A) glutathione (GSH), (B) superoxide dismutase (SOD), and (C) malondialdehyde (MDA) in cell pellet, or after 1 h exposure by measurement of levels of (D) hydrogen peroxide (H_2O_2). Data are expressed as mean \pm SD of three repeated MWCNTs experiments at the concentration of 2.5, 10, 25, and 100 μ g/mL, respectively. *Denotes a significant difference from the NaCl-0.9% control (* P <0.05; ** P <0.01; *** P <0.001). #Denotes a significant difference from the DNA salt controls (# P <0.05; ## P <0.01; ### P <0.001).

most MWCNTs-treated groups, which was 6.68, 13.87, and 16.03 times as high as the control levels in RAW 264.7 macrophages and was 5.34, 11.00, and 12.94 times as high as in A549 cells following 10, 25, and 100 μ g/mL incubation, respectively (Figure 5A). Figure 5B also shows the fluorescence images of MWCNTs effects on ROS generation. Increased density of green spots representing ROS generation was shown to be related to the increased dosage of MWCNTs incubation. Figure 5C shows the time-effect curve of ROS generation in both cell lines after exposure to MWCNTs. Peak fluorescence was found to start at the 1 h time point after MWCNTs treatment.

DISCUSSION

Cytotoxicity of CNTs has been well documented. However, there are different types of CNTs produced in and applied to products with a variety of physical and chemical properties and potential exposure routes. Postsynthesis treatment alters various properties of CNTs such as length, purity, degree of aggregation, wall structure (doping), and surface functionalization. The toxicity of nanoparticles depends on specific physiochemical and environmental

factors^[4,12]. Thus, as mentioned by Helland et al.^[1], the toxic potential of each type of nanoparticle has to be evaluated individually.

In the present study, we found that purified raw MWCNTs (Nanocyl[®] 3150) induced significant GSH depletion and ROS generation in a dose-dependent manner. The accumulation of ROS, H_2O_2 as an example, depleted cellular GSH and the defense system of antioxidant SOD. Subsequently, redundant free radicals interacted with biomolecules including proteins (e.g. TP), enzymes (e.g. LDH), and membrane lipids (MDA as a marker of lipid peroxidation). We also observed the stimulation of inflammation response by MWCNTs incubation as shown by the increase of NO release, which has been identified as a marker of inflammation. ROS generation coupled with functional disturbance of antioxidant defense system, cell inflammation, membrane damage, the release of proteins and enzymes, and the loss of cell viability, indicated that the oxidative stress was probably a key factor leading to cytotoxicity.

A wide range of literature has documented oxidative stress as a common mechanism for cell damage induced by CNTs. Shvedova et al.^[5] reported that exposure of human epidermal keratinocytes to

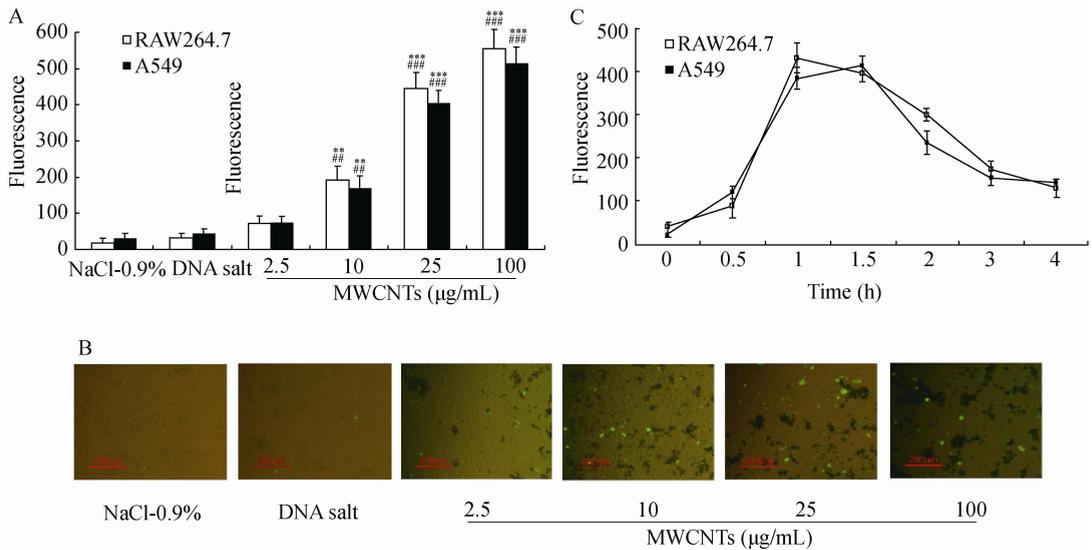


Figure 5. MWCNTs induced reactive oxygen species (ROS) generation in murine RAW 264.7 macrophages and human A549 lung cells. **A:** ROS generation in both cell lines after 1 h exposure to MWCNTs at the concentration of 2.5, 10, 25, and 100 µg/mL, respectively. Data are expressed as mean \pm SD of three repeated MWCNTs experiments. *Denotes a significant difference from the NaCl-0.9% control (** $P < 0.01$; *** $P < 0.001$). #Denotes a significant difference from the control group treated with DNA sodium salt solution (## $P < 0.01$; ### $P < 0.001$). **B:** Images showing ROS generation (green spots) in human A549 lung cells after 1 h exposure to MWCNTs at the concentration of 2.5, 10, 25, and 100 µg/mL, respectively. **C:** Time-effect curve of ROS generation in both cell lines after exposure to MWCNTs.

SWCNT produced oxidative stress and cellular toxicity, as indicated by the formation of free radicals, accumulation of peroxidative products, antioxidant depletion, and loss of cell viability after 18 h of SWCNT exposure. Ding et al.^[13] reported that MWCNTs and multiwall carbon nano-onions (MWCNOs) exposure activated genes involved in cellular transport, metabolism, cell cycle regulation, and stress response. Kagan et al.^[14] found that neither purified (0.23 wt.% of iron) nor non-purified (26 wt.% of iron) SWCNT were able to generate intracellular production of superoxide radicals in RAW 264.7 macrophages. In the presence of zymosan-stimulated RAW 264.7 macrophages, non-purified iron-rich SWCNT were more effective in generating hydroxyl radicals than purified SWCNT. Pulskamp et al.^[3] failed to observe any acute toxicity in cultured rat NR8383 macrophages and A549 cells on viability and inflammation upon incubation with CNTs, but did detect a dose- and time-dependent increase of intracellular ROS. Pacurari et al.^[8] found that exposure to SWCNTs could trigger exposure to SWCNTs induced ROS generation, increased cell death, enhanced DNA damage and H2AX phosphorylation, and activated PARP, AP-1, NF-kappaB, p38, and Akt in a dose-dependent

manner. Ye et al.^[6] suggested that ROS and NF-kappaB were involved in upregulation of IL-8 in A549 cells exposed to MWCNTs. Yang et al.^[15] found CNTs induced significant GSH depletion, SOD resistance and ROS generation in a dose-dependent manner. Herzog et al.^[16] observed that *in vitro* exposure of lung epithelial cells to HiPco derived SWCNT resulted only in moderate or low oxidative stress under the exposure conditions employed (50 µg/mL), whereas the presence of dipalmitoylphosphatidylcholine (DPPC) increased intracellular ROS formation, and foetal calf serum (FCS) seemed to protect the cells from oxidative insult. Murray et al.^[17] reported that exposure of murine epidermal cells (JB6 P+ cells) to unpurified SWCNT (30% iron) resulted in the production of ESR detectable hydroxyl radicals and caused a significant dose-dependent activation of AP-1.

A key finding of our study was that ROS generation in both cell lines (RAW 264.7 and A549) went up in minutes, reached the climax within an hour and faded down after several hours. MWCNTs only had a low effect on peroxide radical generation (a less than two-fold increase after 24 h exposure to relatively high concentrations of nanoparticles: 25 and 100 µg/mL). However, within one hour, ROS

generation was much higher even in a low dose of incubation group (10 µg/mL), which was 5 times as high as the controls. It seems that ROS generation came out much earlier than other phenotypes including oxidative stress, inflammation and cytotoxicity. And this is possibly the reasons why others studies where ROS was measured after more than 4 h exposure to CNTs showed inconsistent results^[3,15,18-20].

It should be noted that most literature did not observe radical generation capacity. Fenoglio et al.^[21] reported that MWCNTs (metal impurity: Co, 0.29%; Fe, 0.47%; and Al 0.05%) in aqueous suspension did not generate oxygen or carbon-centered free radicals in the presence of H₂O₂ or formate, respectively, as detected with the spin-trapping technique. Conversely, the author observed that, when in contact with an external source of hydroxyl or superoxide radicals, MWCNT exhibited a remarkable radical scavenging capacity. In the *in vitro* experiment settings, a significant increase in intracellular ROS generation was not observed in three studies in different cell lines^[18-20]. Two of the studies^[19-20] used purified MWCNTs. In another study, Tabet et al.^[22] found no significant change in mRNA expression of oxidative stress related genes (HO-1, SOD2, GPx, and NOX4) in A549 cells after 6 or 24 h incubation of tested MWCNTs. However, Ye et al.^[6] observed a concentration-dependent increase of ROS production in A549 cells after 24 h incubation of 99.79% purity MWCNTs. Ye et al.^[6] also found that ROS and NF-kappaB were involved in upregulation of IL-8 in A549 cells exposed to MWCNTs. In this study, we found that 95% purity MWCNTs were able to induce intracellular ROS generation in both RAW 264.7 macrophages and A549 cells. This radical generation capacity was associated with the cytotoxicity and inflammation phenotypes in treated cells. The reasons why the results in this study and in Ye et al.'s study are different to others are not immediately evident, but may depend on the experimental protocols and/or interference with the test systems used. This may be supported by the findings in our study that MWCNTs could induce ROS generation within minutes and the effects got weaker after several hours.

The inconsistent results with our study and some literatures may also be derived from the different types of MWCNTs. It seems that the metal impurity of MWCNTs in this study (metal impurity: Fe 0.19%, Co 0.07%, Al 0.03%) were lower than that in some literatures as mentioned above. ROS generation was not found to be significant in

Fenoglio et al.'s study (metal impurity: Co, 0.29%; Fe, 0.47%; and Al 0.05%)^[21] and Tabet et al.'s study (metal impurity: Al 2.4%, Fe, 2%)^[22]. Although metal-containing CNTs are usually more toxic than metal-free nanoparticles, other unknown physical or chemical features that differentiate the tested MWCNTs in our study to other studies may lead to the inconsistent results.

Cannon nanotubes have a strong tendency to aggregate together due to van der Waals forces^[23]. This aggregation in culture media complicates the interpretation of the toxicity test results. Macrophages may well be able to phagocytose a large aggregate of nanotubes as a single entity and clear it when the same amount of singlet CNT would be more difficult to handle^[23]. But it was also found that large aggregates of titanium dioxide nanoparticle had more evident effect on cell viability and gene expression when compared with the small aggregates of the same amount^[24]. To reduce the unexplained effects of aggregation, most literature simply applied sonication to break the agglomerate, while we used Salmon sperm DNA salt solution to disperse tested MWCNTs in this study. Recently, DNA-wrapped MWCNTs have been proved to have good properties of high dispersion^[25]. Most interestingly, Kim et al.^[26] reported that salmon sperm DNA significantly enriched SWCNTs with an enrichment rate of over 86%. The scanning optical microscopic images of DNA-suspended MWCNTs in cultured medium ensured the uniform dispersion of tested nanoparticles in the experiments. The DNA salt controls in Figure 2 to Figure 5 showed slight higher extent of both cytotoxicity and oxidative stress than the saline solution controls, which may be explained by the more difficult clearance of dispersed nanoparticles compared to the aggregates.

Overall, we concluded that purified raw MWCNTs induced significant oxidative stress, together with cell inflammation, membrane leakage, lipid peroxidation, and protein release, all of which may be the causes of the damage in cell viability. Future studies are therefore necessary to understand the mechanisms and the results of the oxidative stress both *in vitro* and *in vivo*, and the associations between stress and the characteristics of MWCNTs, such as metal impurity and surface functionalization.

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