Toxicity of single-walled carbon nanotube: How we were wrong?

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The first issue that we address and justify in this paper is the pejorative and provocative tone of the title; the contradictory data on the toxic effects of single-walled carbon nanotubes (SWCNT) make us believe that it is appropriate and necessary. Two of the first studies in toxicity of carbon nanotube were by Chiu Wing Lam *et al.*[1] and David Warheit *et al.*[2], who reported that carbon nanotube can damage lung tissue in mice. Their works were subsequently introduced in *Science* in 2003. Since then, toxicity of carbon nanotube has become a fresh research topic and a number of papers have been dedicated to this field. Fig. 1 plots the citations versus calendar year for the past five years to these two papers. A linear increasing number of publications citing Chiu Wing Lam *et al.* and David Warheit *et al.* have appeared. In 2001, Huczko A. *et al.*[3,4] first discussed the fact that fullerene soot with a high content of single-walled carbon nanotubes did not show any signs of health hazard related to skin irritation and allergic risks and did not induce any abnormalities of pulmonary function or measurable inflammation in guinea pigs. Unfortunately, his publications have essentially been ignored (Sum of citations, Fig. 1). Does carbon nanotube really cause toxic effects? We attempt here to briefly demonstrate why we should rationally understand this conception by taking HiPco® single-walled carbon nanotube (Carbon Nanotechnologies, Inc. Houston, TX) as an example. Though biocompatible behavior of functionalized HiPco® carbon nanotube has been researched by a number of authors, we intend to exclude them from the scope of this work.

Table 1 highlights some brief description (details can be found from the literatures) about the key points of investigation of toxicity of single-walled carbon nanotube. The similar experimental process includes:

1. Pretreatment of SWCNT (acid treatment to remove metal contaminant; suspension preparation in PBS, ethanol, DMF, serum, cell culture medium or any other medium, and sequential problems).
2. Cell culture (medium, concentration, time).
3. Exposure conditions (In vitro: mixed with SWCNT; In vivo: pharyngeal aspiration; food paste; intrapharyngeal instillation).

However, the conflicting results can be easily found from the comparison. So what is toxicity anyway? From Wikipedia, Toxicity is the degree to which a substance is able to damage an exposed cell (cytotoxicity) or a whole organism, such as an animal, bacterium, or plant, as well as organs (organotoxicity). Firstly, we want to emphasize that the important point is 'Damage'. Some changes within cells, organs, or whole organisms cannot be referred to as such, namely, it is not real toxicity. This is because cells, organs, or whole organisms will make a natural response (some changes) to stimulation when carbon nanotube attaching or entering. Even flour powder can lead to pulmonary changes when taken in. Secondly, a central concept of toxicology is that effects are dose-dependent. Considering the micro/nano- properties, the toxicology of carbon nanotube is more complicated than that of common chemicals like CO gas. Besides dose-dependence, many factors should be considered in the study, such as impurities (catalyst, graphite, carbon powder, etc.), dimension

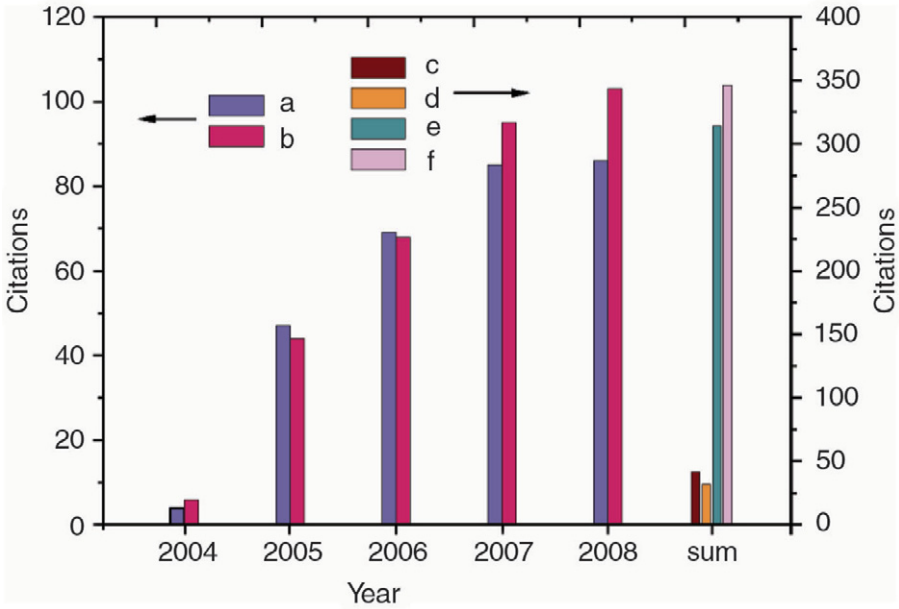

Figure 1 Citations versus publication year. (a, e) Warheit, D. B. et al. Toxicol. Sci. 77, 117-125 (2004); (b, f) Lam, C. W. et al. Toxicol. Sci. 77, 126-134 (2004); (c-f) Sum of citations. (c) Huczko, A. et al. Fullerene Sci. Tech. 9, 251-254 (2001); (d) Huczko, A. Fullerene Sci. Tech. 9, 247-250 (2001).

and its distribution, crystal structure; aggregation degree, effect of cell culture medium, many other secondary chemicals, pH values, etc. After reviewing the literature, we noted that different pretreatment processes of SWCNT were used. No uniform criterion can be found in the studies. The same problems apply to exposure conditions (different cell culture medium, different amount of micronutrients, and different ratio between SWCNT and medium). The assay method can also be problematic (see Table 1 for different assay methods), as there were no comparisons and references for the same instruments of different companies, as well as for the test results by different techniques.

In view of the issues concerned above and based on the current developing status of carbon nanotube, we should rationally understand this conception of toxicity of SWCNT, as well as other nanomaterials. We suggest that the assessment of effect of carbon nanotube on the cells, organ, or whole organism should also be standardized.

Table 1 Toxicity studies on HiPco® SWCNT

Cell line	CNT treatment	Exposure conditions	Test	Conclusions	Ref.
A549 cells (ATCC, CCL-185) a human lung carcinoma epithelial cell line	No acid treatment; SWCNT mix with serum medium.	In vitro Cells were exposed to the SWCNT in 5%F12K medium	AB, NR, CB, and MTT assay	Very low acute toxicity; Greater toxicity in the absence of serum; Adsorption of SWCNT in medium resulted in an adverse effect on cellular proliferative capacity	5-8
Human hepatoma (HepG2, ATCC HB 8065) cell line	Functionalized with aryl sulfonate groups; Dispersed in RPMI1640-11875 cell culture medium	In vitro The cells were trypsinized with 1% trypsin/EDTA, and incubated with CNT/RPMI; folate;	MTS assay	Adsorption of essential micronutrients from cell culture medium results in the toxicity (Cell viability, DNA damage and apoptosis).	9
Human osteoblast -like (SAOS-2) cell line	No acid treatment; Suspended in ethanol	In vitro Cells were covered with SWCNT films	Fluorescence Metabolic activity	SWCNTs films are not toxic for human osteoblasts and could be used for biomedical applications.	10
Mouse peritoneal macrophage-like (J774.1) cell lines	80 μg/mL of SWCNT suspended in 1 wt.% Pluronic F108	In vitro Cell cultured and then treated with SWCNT	Near-IR fluorescence	Macrophage cells can actively ingest significant quantities of SWCNT without showing toxic effects	11
Drosophila melanogaster (fruit flies)	No acid treatment SWCNT suspensions: raw SWCNT/bovine serum albumin/PBS	In vivo Mix SWCNT solution with dry Baker's yeast powder (CNT food)	Near-IR fluorescence	No short-term toxicity or impaired growth or viability or fertility; Negligible physiological impact.	12
Rat heart cell line (H9c2 (2-1), Cardiac muscle cells)	Highly purified SWCNTs were suspended in Dulbecco's modified Eagle's medium	In vitro Cells were cultured in medium and treated with SWCNT suspension	Fluorescence	No evident short-term toxicity; Long-term negative effects are probably due to physical interactions.	13
Human embryo kidney cells (HEK293 cells)	No pretreatment description	In vitro HEK293 cells were treated with 25 μg/ml of SWCNTs for 1-5 days.	MTT; Spectrophotometry; Flow cytometry; SDS-PAGE and Western blot	Inhibit cells growth; death of cells within 24h (250 μg/ml) only slight influence(less than 1 μg/ml SWCNTs in the medium)	14
Rat aortic smooth muscle cells (SMC)	Acid-treatment SWCNT mixed with DMEM/F-12, FBS, L-glutamine, penicillin, streptomycin	In vitro	Fluorescence image	Inhibit cells growth	15

Table 1 continued...

Cell line	CNT treatment	Exposure conditions	Test	Conclusions	Ref.
Male FVB/N -TgN (Ho1-luc) Xen mice; Male C57BL/6J mice; B6.129P2-Apoe^{tm1Unc} (ApoE$^{-/-}$) mice	Acid treatment Suspension of CNT was prepared in PBS by sonification	In vivo Intrapharyngeal instillation	QPCR; Lowry; Bioxytech GSH/GSSG-412 Colorimetric; en face method	Aortic mtDNA damage at 7, 28, and 60 days (C57BL/6 mice,10 and 40 μg/mouse); Stimulates the progression of atherosclerosis in ApoE$^{-/-}$ transgenic mice.	16
Epidermal JB6 P+ cells; SKH-1 Hairless mice (3-4 weeks; 16-18 g body weight)	Acid treatment	In vitro JB6 P+ cells In vivo SKH-1 mice	ESR spin trapping; AB; Luminometer; Flow cytometry; ELISA immunoassay	Unpurified SWCNT can cause dermal toxicity associated with free radical generation, oxidative stress, and inflammation	17
Human dermis fibroblasts cells	Refluxed at 120^{O}C in 4 M HCl for 19h	In vitro	MTT; Immunocytochemical analysis; Western blot	Refined SWCNTs are more toxic than its unrefined counterpart.	18
Human epidermal keratinocytes (HaCaT)	No acid treatment SWCNT mix with KGM basal medium	In vitro HaCaT cells were incubated with SWCNT in KGM basal medium	ESR spin trapping; SEM; TEM; HPLC; Microphotograph; AB; Chemiluminescence; Fluorescence; Bradford	Cytotoxicity is associated with iron catalytic effects; Unrefined SWCNT can result in accelerated oxidative stress and may produce dermal toxicity	19
Specific-pathogen-free adult female C57BL/6 mice (7-8 week)	Acid treatment	In vivo Pharyngeal aspiration	Immunofluorescence Bradford assay Spectrophotometry fluorescence luminescence	SWCNT aggregates induces a robust acute inflammatory reaction and forms granulomas; Pulmonary exposure to SWCNT caused persistent changes in pulmonary functions and decreased bacterial clearance	20

Abbreviation:
AB: Alamar blue
NR: Neutral red
CB: Coomassie blue
MTT: Ttetrazolium salt assay
MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium inner salt
SDS-PAGE: Sodium dodecylsulfatepolyacrylamide gel electrophoresis
QPCR: Quantitative polymerase chain reaction
GSH/GSSG: Mitochondrial reduced glutathione/oxidized glutathione
ELISA: Enzyme linked immunosorbent assay
IR: Infrared
ESR: Electron spin resonance
SEM: Scanning electron microcopy
TEM: Transmission electron microscopy

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