

CYTOTOXICITY EVALUATION OF CARBON NANOMATERIALS ON HUMAN CELL LINES USING MTT ASSAY

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ABSTRACT

Objective: The aim of this study was to evaluate and compare the *in vitro* toxicity of three carbon nano particles on five different cell lines.

Methods: Human alveolar epithelial (A549) cells, hepatocytes (Hep G2 cells), human embryonic kidney cells, HCT 116, and intestinal (P407 cells) cells were exposed to multi walled carbon nanotubes, carbon nano fibres and carbon nano rods. The adverse effects of carbon nano particles were analyzed after 48 h incubation with different cell lines using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.

Results: Incubation of carbon nano particles with different cells produced a concentration-dependent inhibition of growth of the cells. The TC₅₀ values (toxic concentration 50, i. e., concentration of particles inducing 50% cell mortality) of three nano particles was found to be in the range 28.29–46.35 µg/mL, and less than that of quartz (known toxic agent, 30.24-54.95 µg/mL).

Conclusion: The results indicating the greater cytotoxic effect of carbon nano particles than quartz particles.

Keywords: MWCNTs, CNFs, CNRs, Cytotoxicity, Cell lines.

INTRODUCTION

The Nano Technology prefix “nano” is derived from the Greek word “nanos” meaning “dwarf”. Nanotechnology involves the manipulation and application of engineered particles or systems that have at least one dimension less than 100 nanometers (nm) in length [1]. Nanotoxicity, a term coined in 2004, refers to the study of the potential toxic impacts of nano particles on biological and ecological systems. Nanotoxicology was proposed as a new branch of toxicology to address the gaps in knowledge and to specifically address the adverse health effects likely to be caused by nanomaterials. As per Donaldson et al. quoted, “discipline of nanotoxicology would make an important contribution to the development of a sustainable and safe nanotechnology” [2]. Although this size definition is no longer explicitly followed in the categorization of nanomaterials, these unique properties make nanoparticles the subject of intense study and commercial/industrial interest. During an average day, people may be exposed to commercially available nanoparticles in many settings, including silver (Ag) nanoparticles in sheets and clothing, titanium dioxide (TiO₂) nanoparticles in cosmetics and sunscreens, carbon nanoparticles in bikes, and even clay nanoparticles in beer bottles. Over the past eight years, the field of nanotoxicity has grown significantly in response to and in hopes of addressing concerns (both public and regulatory) regarding the boom in nanoparticle technology and the subsequently increased possibility of exposure through consumer and medical applications [3]. In particular, biological applications that employ CBNs for DNA, proteins, and drug delivery [4, 5] have attracted much attention. Unfortunately, the information concerning the potential hazards related to CNM exposure is rare and still under debate [6, 7].

The pulmonary toxicity of carbon nanotubes (CNT) has been well described. Findings from CNT inhalation exposures included cytotoxicity, inflammatory cell influx, and interstitial fibrosis in the lung [8-10]. Some more recent studies also suggest the potential of CNM to promote lung tumorigenesis [11]. Several studies also have shown systemic effects such as immunosuppression, systemic inflammation, and changes in molecular signaling in extra pulmonary tissues [12-13]. Reduced vascular responsiveness and increased susceptibility to ischemia / reperfusion injury in cardiac tissue was also a product of CNM exposure [14]. Multi-wall carbon

nanotubes (MWCNTs), Carbon nanofibres (CNFs) and Carbon nanorods (CNRs), unlike graphite, possess highly desirable electrical, mechanical, magnetic, and thermal properties [15]. The peculiar toxicity associated with nanomaterials that are different from bulk materials of the same chemical composition has been a concern. In particular, tubular materials with a high aspect ratio, e. g., CNTs, are suspected of showing asbestos-like toxicity because of their similarity in shape [16].

CNM toxicity is dependent on their shape, size, purity, charge, dose, entry route into the body, concentration in the field of body-target, duration of influence and other factors. Engineered carbon nanomaterials such as multi-walled carbon nanotubes (MWCNT), Carbon nanofibres (CNFs) and Carbon nanorods (CNRs) have applications in structural and electronic devices due to their extraordinary thermal conductivity, mechanical and electrical properties, which creates a potential occupational exposure situation [17]. Potential bioactivity (in vitro toxicity and increased production of inflammatory mediators, and/or in vivo increased inflammation and pathology) of CNM has been attributed to length [18], diameter [19], aggregation state [20], contaminants [21], aspect ratio/rigidity [22], and release of reactive oxygen species [23]. The present study was aimed to evaluate and compare the *in vitro* cytotoxicity of three nanomaterials MWCNTs, CNFs and CNRs using MTT assay on five different human cell lines namely human alveolar epithelial (A549), human hepatocytes (Hep G2 Liver cells), human embryonic kidney (HEK 293) cells, intestinal (P407) cells and HCT 116 Colon Cancer cells.

MATERIALS AND METHODS

Particle types

The Carbon Nano Materials MWCNTs (D*L 110-170 nm *5-9 µm), CNFs (D*L 100 nm 20-200 µm), CNRs (D*L 100 nm * 4-5 µm) were purchased from Sigma, St. Louis, USA. Quartz (Min-U-Sil) was purchased from U. S. Silica Company (Berkeley Springs, West Virginia) at >99% purity.

Cell culture and treatment

Human alveolar epithelial (A549), human hepatocytes (Hep G2 Liver cells), human embryonic kidney (HEK 293) cells, intestinal (P407) cells and HCT 116 Colon Cancer cells were purchased from National Centre for Cell Science (NCCS, Pune), India.

These cell lines were grown and maintained using suitable media (DMEM/RPMI 1640, HiMedia, Mumbai, India). All the cell lines were grown in culture medium supplemented with 10% fetal bovine serum (FBS, HiMedia, Mumbai, India), 1% L-glutamine (HiMedia, Mumbai, India), and 1% penicillin-streptomycin-amphotericin B antibiotic solution (HiMedia, Mumbai, India). Cells were seeded at 250,000 cells/flask in a total volume of 9 mL. When confluent, all the cells were trypsinized (using trypsin-EDTA, HiMedia, Mumbai, India), and seeded in 96 well plates (Tarsons, India) at the rate of 2500/0.1 mL. Particle suspension (in phosphate buffer saline (PBS)/0.1% Tween 80) or medium alone was added to each well. For each nanomaterial, a stock solution of 1mg/mL particle in culture medium without any additive was prepared, vortex at maximum speed for 1 min and bath-sonicated for 5 min.

Different concentrations of nanoparticles in culture medium were prepared and used (1–100 µg/mL). Preliminary experiments demonstrated the necessity to add 0.1% Tween 80 to the culture medium to obtain a homogenous suspension for three nanoparticles. Cells were exposed for 72 h to medium alone or in the presence of nanomaterials. At that time, MTT assay was performed to evaluate the toxicity of nanoparticles on different cell types.

MTT assay method

Mitochondrial function and cell viability were measured by the MTT assay [24]. Briefly, the cells were plated into a 96-well plate at a density of 1.0×10^4 cells /well. Cells were grown overnight in the full medium and then switched to the low serum media followed by exposure to carbon nanoparticles. After 48h of treatment with different concentrations of nanoparticles, the cells were incubated with MTT (2.5 mg/ ml) for 2 h. Then, 80 µl of lysis buffer (15% sodium lauryl sulphate in 1:1 mixture of N, N, dimethyl formamide and water) was added into each well to dissolve formazan crystals, the metabolite of MTT.

After thorough mixing for overnight, the plate was read at 490 nm for optical density that is directly correlated with cell quantity using ELISA (Biotech, UK) multiple plate reader. Inhibition of growth of cells was calculated from the relative absorbance of untreated control cells at 490 nm and expressed as the percentage inhibition.

Statistical analysis

When at least 2 viability values were below 50% of control condition, the TC₅₀ (toxic concentration 50, concentration of particles inducing 50% cell mortality) was calculated using GraphPad Prism software (logarithmic transformation of X-values and non linear regression -sigmoidal dose-response analysis with variable slope- with bottom and top constrains set at 0 and 100 respectively). If a TC₅₀ could be calculated, TC₂₅ and TC₇₅ were calculated (respectively concentration corresponding to 75 and 25% viability), using the following equation: $TC_f = [(f / 100-f)^{1/H}] * TC_{50}$ where f: percentage that needs to be calculated, H: hillslope, *: multiply, **: to the power.

RESULTS

The cytotoxicity data of the three tested carbon nanoparticles with the MTT assay method on five different human cell lines are presented in Figures 1–5. Similar to quartz (a known toxic agent), exposure of three tested carbon nanoparticles to the different cells produced a concentration-dependent inhibition of growth of cells, resulting in reduction of viability percentage of the cells in nanoparticles exposed wells. For all the nanoparticles, TC₅₀, TC₂₅, and TC₇₅ values (respectively concentration corresponding to 50%, 75%, and 25% viability) were calculated on all the cell types and are shown in Table 1. The TC₅₀ values of three nanomaterials were found to be in the range 28.29–46.35 µg/mL. Irrespective of the type of cells, three nanomaterials produced greater cytotoxicity in all cell types tested.

Table 1: Cytotoxicity of carbon nanomaterials on different human cell lines using MTT assay

Nanoparticles	Cell Type	TC ₅₀ (µg / ml)	TC ₂₅ (µg / ml)	TC ₇₅ (µg / ml)
CNF	A 549 cells	28.29	4.90	163.10
CNR	A 549 cells	43.02	7.32	252.62
MWCNT	A 549 cells	35.77	6.17	207.04
Quartz	A 549 cells	38.74	5.67	264.68
CNF	Hep G2 cells	29.22	5.08	168.04
CNR	Hep G2 cells	41.18	6.96	243.55
MWCNT	Hep G2 cells	33.53	5.70	197.00
Quartz	Hep G2 cells	30.24	5.36	170.54
CNF	HEK cells	30.51	5.13	181.28
CNR	HEK cells	42.17	6.75	263.31
MWCNT	HEK cells	35.03	5.33	229.91
Quartz	HEK cells	39.46	6.63	234.73
CNF	P407 cells	33.38	5.73	194.40
CNR	P407 cells	46.35	7.39	290.65
MWCNT	P407 cells	39.19	6.50	236.13
Quartz	P407 cells	52.75	8.51	326.78
CNF	HCT 116 cells	34.65	6.15	195.20
CNR	HCT 116 cells	44.32	7.21	272.41
MWCNT	HCT 116 cells	38.27	6.34	230.93
Quartz	HCT 116 cells	54.95	8.98	336.03

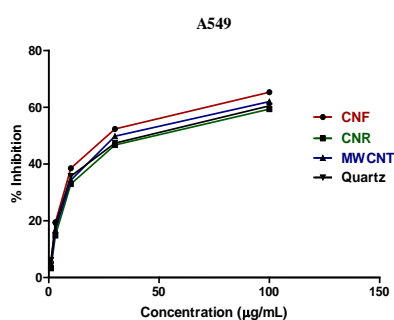


Fig. 1: Cytotoxicity of carbon nanomaterials on A549 cell lines

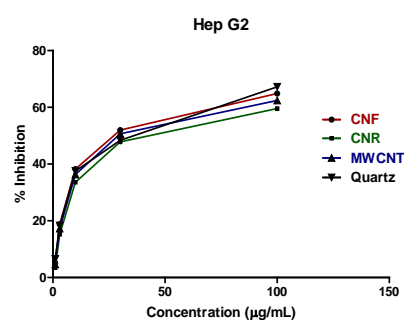


Fig. 2: Cytotoxicity of carbon nanomaterials on HepG2 cell lines

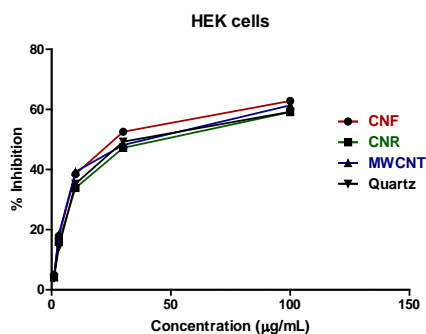


Fig. 3: Cytotoxicity of carbon nanomaterials on HEK cell lines

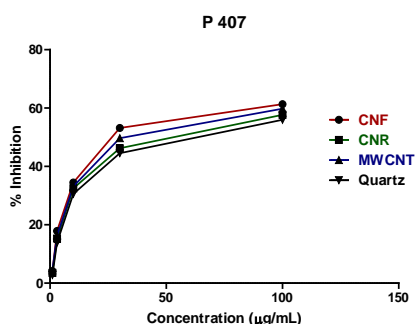


Fig. 4: Cytotoxicity of carbon nanomaterials on P402 cell lines

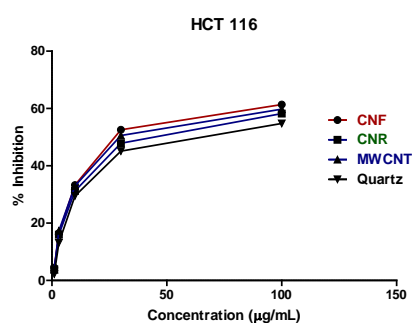


Fig. 5: Cytotoxicity of carbon nanomaterials on HCT116 cell lines

DISCUSSION

The present study investigate and compare the *in vitro* cytotoxicity of three carbon nanomaterials MWCNTs, CNFs and CNRs using MTT assay on five different human cell lines namely human alveolar epithelial (A549), human hepatocytes (Hep G2 Liver cells), human embryonic kidney (HEK 293) cells, intestinal (P407) cells and HCT 116 Colon Cancer cells. To date there are very few studies directly or indirectly investigating the toxic effects of nanomaterials and no clear guidelines are presently available to quantify these effects.

Inhaled MWCNT, which deposit in the lungs, are transported to the parietal pleura, the respiratory musculature, liver, kidney, heart and brain in a single form and accumulate with time following exposure. The tracheobronchial lymph nodes contain high levels of MWCNT following exposure and further accumulate over nearly a year to levels that are a significant fraction of the lung burden 1 day post-exposure [25]. Intraperitoneal injection of long (> 5 µm) MWCNT in mice causes fibrotic lesions and mesothelial cell proliferation [26, 27]. So, the present study was used to assess the *in vitro* systemic effects of three carbon nanomaterials following its exposure.

Low-dose, long-term exposure of bronchial epithelial cells to MWCNT has been shown to induce cell transformation, and these transformed cells induce tumors after injection into nude mice [28, 29]. Although, *in vitro* data is not a substitute for whole animal studies. Use of simple *in vitro* models with end points that reveal a general mechanism of toxicity can be a basis for further assessing the potential risk of chemical/material exposure.

The results of the present study showed the higher cytotoxicity of three nanoparticles against all cell types tested and was comparable with a known cytotoxic agent, quartz. The TC_{50} , TC_{25} , TC_{75} values of three nanoparticles and quartz on five different human cell lines were showed in table 1. The TC_{50} values of three nanoparticles were found in the range of 28.29–46.35 µg/mL, were less than that of quartz (30.24 - 54.95 µg/ml), indicating the greater cytotoxicity of carbon nanoparticles than quartz particles. Among the three tested carbon nano materials, CNFs showed potent cytotoxicity on all cell types. The order of cytotoxicity was CNF>MWCNT>CNR.

Roberta brayner [30], investigate the cytotoxicity using MTT assay of three carbon based nano materials. In this study reported that, the toxicity order is CB>CNF>MWCNT. The tested carbon nano materials have shown more potent cytotoxicity towards lung, liver and kidney cells as compare to all cell lines. Equal or greater potency of SWCNT, MWCNT, and CNF compared with other inhaled particles (ultrafine carbon black, crystalline silica, and asbestos) in causing adverse lung effects including pulmonary inflammation and fibrosis [31-33]. On a mass dose bases, inflammation and lung damage at 1 day post-exposure followed the potency sequence of SWCNT>CNF>asbestos. The same potency sequence was observed for TNF and IL-6 production at 1 day post-exposure. SWCNT agglomerates were associated with the rapid (7 days) development of granulomas, while neither CNF nor asbestos (being more dispersed) caused granulomatous lesions. Interstitial fibrosis (noted as TGF production, lung collagen, and Sirius red staining of the alveolar septa) was observed at 28 days post-exposure with a mass-based potency sequence of SWCNT>CNF=asbestos. The potency sequence for fibrosis was not found to be related to structure number or particle surface area (determined by BET gas absorption method) delivered to the lung [33].

Previous results showed the ability of carbon nanoparticles to induce various effects in function of the cell type considered. For example, incubation of carbon nanotubes with different cells produced a concentration-dependent inhibition of growth of the cells [34]. Gold nanoparticle-induced death response in a human carcinoma lung cell line (A549 cells) whereas no effect was observed in BHK21 (hamster kidney) or HepG2 (human hepatocellular liver carcinoma) cells [35]. In contrast, our results showed that, irrespective of type of cells, all carbon nanoparticles produced a dose dependent inhibition of growth of cells. Therefore, developing such *in vitro* models to assess nanoparticles systemic toxicity would be of particular interest regarding development of routine screening tests and investigation of nanoparticles precise mechanisms of action.

An unidentified radical is generated by two long, needle-like MWCNTs and these two CNTs were more cytotoxic than the other CNTs tested, suggesting that this radical could be related to the adverse effects of MWCNTs [36]. *In vitro* studies also indicate that exposure to CNF can cause genotoxicity (micronuclei) as a result of reactive oxygen species (ROS) production, which in turn reacts with DNA, and by interfering physically with the DNA/chromosomes and/or mitotic apparatus [37].

CONCLUSION

In summary, exposure of carbon nanoparticles to cells produced concentration dependant cytotoxicity. Further studies are needed to investigate the general mechanisms involved in carbon nanotubes induced *in vitro* cytotoxicity and also to correlate these *in vitro* results with those of *in vivo* toxic effects.

CONFLICT OF INTERESTS

Declared None

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