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In vitro Toxicity of Multi Wall Carbon Nanoparticles on Hep G 32 Liver Cell Lines

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Abstract: The aim of the present study was to evaluate the *in vitro* toxicity of two Multi Wall Carbon Nanotubes (MWCNT) on human hepatocytes (Hep G 32 cell lines). The toxic effects of carbon nanoparticles were analyzed after 24 h of incubation with different cell lines using MTT assay and also estimated the levels of Lactate Dehydrogenase (LDH) (that is leakaged into the media). The results of the LDH estimation demonstrated that exposure of multi wall carbon nanotubes to hepatocytes (Hep G 32) for 24 h resulted in concentration-dependent increase in LDH leakage and exhibited a significant ($p<0.05$) cytotoxicity at a concentration range of 3-50 μg mL^{-1}. The TC_{50} or IC_{50} values (toxic concentration 50 i.e., concentration of particles inducing 50% cell mortality) of two nanoparticles (1) were found in the range of 36.99-37.15 μg mL^{-1}, (2) were less than that of quartz (known toxic agent, 39.85 μg mL^{-1}), indicating their cytotoxicity of carbon nanoparticles.

Key words: Carbon nanotubes, MWCNT, Hep G 32, cytotoxicity, *in vitro*

INTRODUCTION

Nanotechnology involves the creation and manipulation of materials at nanoscale levels to create products that exhibit novel properties. Carbon Nanotubes (CNT) are considered as one of the most promising materials in nanotechnology, with attractive properties for many technologic applications. Nanomaterials, which range in size from 1 to 100 nm, have been used to create unique devices at the nanoscale level possessing novel physical and chemical functional properties (Colvin, 2003; Oberdörster, 2004). As the production and applications of Multi Wall Carbon Nanotubes (MWCNT) expand, potential human exposures will also increase. In occupational settings, these MWCNT may release into the surroundings in aerosol form (Hussain *et al*., 2005). The potential hazards related to inhalation of these carbon nanotubes are unknown.

Hussain and Frazier (2001, 2002) have reported the cytotoxicity testing of series of high energy chemicals in an *in vitro* model and these data were used to derive a baseline for

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extrapolation to a human health risk assessment (Trohalaki *et al*., 2002). 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.

Although, limited studies have been conducted on the toxicity of nanoparticles, there are few reports on the use of *in vitro* models to evaluate potential toxicity screening of nanomaterials (Hussain and Frazier, 2002). Braydich-Stolle *et al*. (2005) reported the *in vitro* cytotoxicity of nanoparticles in mammalian germline stem cells. Cui *et al*. (2005) also reported the *in vitro* cytotoxic effects of single wall carbon nanotubes on human HEK 293 cells. Recently, Lazou *et al*. (2008) also reported the *in vitro* cytotoxicity of nanoparticles on renal cells. Therefore, the present study was designed to assess the *in vitro* toxicity of Multi Wall Carbon Nanotubes (MWCNT) on human hepatocytes (Hep G 32 cells) cell lines using MTT assay. The present study was conducted on two MWCNT of each one manufactured by electric arc (graphite as a source) and chemical vapor deposition (using methane as hydrocarbon) method. They are coded as MWCNT 1 (electric arc product, 90-150 nm) and MWCNT 2 (CVD product, 60-90 nm).

MATERIALS AND METHODS

Particle Types

Multi Walled Carbon Nanotubes (MWCNT) produced by chemical vapor deposition (60-90 nm of size) using methane as hydrocarbon and electric arc process (90-150 nm) using graphite as a source, were obtained from Centre for Environment, Institute of Science and Technology, JNTU, Hyderabad in the year of 2008. However, these nanotubes exist primarily as agglomerated ropes of nanotubes. Quartz (Min-U-Sil) was purchased from U.S. Silica Co., (Berkeley Springs, West Virginia) at >99% purity. The present study was done in the month of June, 2009.

Experimental Set-Up

To generate a generic experimental set-up, the toxicity of 2 different carbon nanoparticles (produced by different methods and sources) was assessed on human Hep G 32 cell lines using MTT assay. The toxic effects of nanoparticles were analyzed after 24 h of incubation with cells.

Cell Culture and Treatment

We used human hepatocytes (Hep G 32 cells), were purchased from National Centre for Cell Service (NCCS, Pune), India. These cell lines were grown and maintained using suitable media (Ham's Nutrient Mixture F-12, 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, India). Cells were seeded in 25 cm^2 tissue culture flasks (tarsons, India), at 250 000 cells $flask^{-1}$ 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 a rate of 30,000 cells/0.1 mL. Twenty four hours after seeding, cells were washed 3 times with culture medium without any additive (FBS or antibiotics) and 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 1000 μg mL^{-1} 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 (100-1 μg mL^{-1}). Preliminary experiments demonstrated the necessity to add 0.1% Tween 80 to the culture medium to obtain a homogenous suspension for two nanoparticles. Cells were exposed for 24 h to medium alone or in presence of nanomaterials. At that time, MTT was performed to evaluate the toxicity of nanoparticles on different cell types.

Cytotoxicity Endpoints

Estimation of Lactate Dehydrogenase Levels (LDH)

The LDH leakage due to membrane damage was assessed by measuring the activity of LDH in the cells and media as described elsewhere with some modifications (Liu *et al*., 2008).

MTT Assay

At the end of exposure, cell culture medium was discarded and each well washed with 200 μL Phosphate Buffer Solution (PBS, Himedia, India). Cells were then incubated at 37°C, under 5% CO_2 with 20 μL of 2.5 mg mL^{-1} MTT solution (Sigma, USA) in PBS. After 2 h, 80 μL of lysis buffer (sodium lauryl sulphate in 1:1 mixture of distilled water and N, N-dimethylsulfoxide, pH 2.5) was added to each well and kept in shaker for overnight and read optical density at 520 nm using ELISA plate reader (ELx800, Biotech). Viability was calculated as the ratio of the mean of OD obtained for each condition to that of control (no particle) condition. In order to evaluate if any modification of OD due to particles can be measured, some OD measurement were performed again on 150 μL of the supernatant of each well that has been transferred to a new 96-well plate. No modification of OD was observed (data not shown). Therefore, all OD measurements have been performed on the original 96-wells plates.

Statistical Analysis

The data were expressed as Mean±SD of four independent experiments. Wherever appropriate, the data were subjected to statistical analysis by One-Way Analysis of Variance (ANOVA) followed by Dunnett's method for multiple comparisons. A value of $p<0.05$ was considered as significant.

RESULTS

The results of the LDH estimation demonstrated that exposure of multi wall carbon nanotubes to hepatocytes (Hep G 32) for 24 h resulted in concentration-dependent increase in LDH leakage and exhibited a significant ($p<0.05$) cytotoxicity at a concentrations of 3-50 μg mL^{-1} (Fig. 1, 2). The MWCNT exposure produced a significant concentration dependent LDH leakage into the media. Based on LDH results, nanomaterials that produced by chemical vapor deposition method (MWCNT 2, 60-90 nm) have slightly more cytotoxic than arc produced (MWCNT 1, 90-150 nm) nanoparticles. In the present study, quartz was used only as a positive control for significant toxicity.

The cytotoxicity data of two test multi wall carbon nanoparticles on Hep G 32 cells with MTT assay was presented in Fig. 2. Both nanotubes produced a significant concentration dependent inhibition of growth of the cells. For both nanoparticles TC_{50}, TC_{25} and TC_{75} values (respectively concentration corresponding to 50, 75 and 25% viability) were calculated and were shown in Table 1. The TC_{50} values of two nanomaterials were found in the range of 36.99-37.15 μg mL^{-1}.

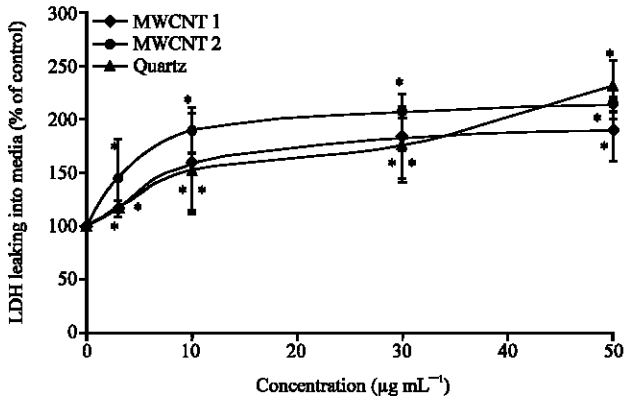

Fig. 1: Effect of multi wall carbon nanotubes on LDH leakage in Hep 3B cell lines. Data are Mean±SD (n = 4), *p<0.05

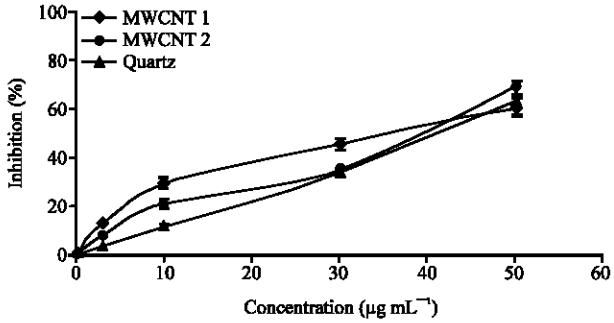

Fig. 2: *In vitro* cytotoxicity of multi wall carbon nanotubes on Hep G 32 cell lines

Table 1: Cytotoxicity of multi wall carbon nanoparticles (MTT assay)

Nanoparticles	Cell Type	TC_{50} (μg mL^{-1})	TC_{25} (μg mL^{-1})	TC_{75} (μg mL^{-1})
MWCNT 1	Hep G 32 liver cell lines	36.99	14.38	59.60
MWCNT 2		37.15	17.54	56.77
Quartz		39.85	19.63	60.07

DISCUSSION

The present study investigated the *in vitro* cytotoxicity of two multi walled carbon nanotubes against the liver Hep G 32 cell lines. 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.

Recently, Liu *et al.* (2008) reported the dose and time dependant pulmonary toxicity of MWCNT in rats using intratracheal instillation method. In this study, pulmonary exposures to MWCNT in rats produced a series of multiple lesions in a dose-dependent and time-dependent manner. It is also reported the extra-pulmonary translocation of carbon nanoparticles after its inhalation/aspiration/injection to various organs like liver, kidney and pancreas (Nemmar *et al.*, 2002; Oberdörster *et al.*, 2002; Shimida *et al.*, 2006) and resulting in tissue/organ damage caused by the generation of reactive oxygen species. So, the present study was used to assess the toxicity of nanoparticles in an *in vitro* model derived from human liver cells Hep G 32.

The results of the present study showed the higher cytotoxicity of two nanoparticles against all cell type tested and was comparable with known cytotoxic agent, quartz. The IC_{50} values of two nanoparticles were found in the range of 36.99-37.15 μg mL^{-1}, were slightly less than that of quartz (39.85 μg mL^{-1}), indicating the similar/equal cytotoxicity of carbon nanoparticles with quartz particles. The analysis of particles exposure media for LDH demonstrate that both carbon nanomaterials increase LDH leakage and reduces MTT reduction in a dose dependent manner over 24 h exposure duration. Based on LDH results, nanomaterials that produced by chemical vapor deposition method (MWCNT 2, 60-90 nm) have slightly more cytotoxic than arc produced (MWCNT 1, 90-150 nm) nanoparticles. This size dependent cytotoxicity was also reported by the Fen *et al.* (2009) and Kipen and Laskin (2005).

The results of the present study were supported by Hussain *et al.* (2005). They reported that exposure of Ag nanoparticles to BRL 3A rat liver cells for 24 h resulted in concentration-dependent increase in LDH leakage and exhibited a significant ($p<0.05$) cytotoxicity at 10-50 μg mL^{-1}. Cui *et al.* (2005) also reported the dose and time dependent inhibition of growth of the human HEK 293 cells *in vitro* by inducing cell apoptosis and decreasing cellular adhesion ability. These results support the dose dependent LDH leakage and growth inhibition of the Hep G 32 cells by the MWCNT in the present study.

Massimo *et al.* (2006) also reported the *in vitro* time and dose dependent cytotoxicity of multi wall carbon nanoparticles against T-lymphocyte and also reported that MWCNT induces apoptosis after their incubation with T-lymphocytes. 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. In the literature, various nanoparticles have been demonstrated to generate ROS and to a greater extent more than larger particles and this mechanism is thought to play a role in their cytotoxic effects (Martin *et al*., 1997; Koike and Kobayashi, 2006). *In vitro* studies, with NR8383 and A549 cells also demonstrated dose- and time-dependent increases in intracellular ROS production after exposure to SWCNT or MWCNT, suggesting that CNT exposure induces cellular oxidative stress (Pulskump *et al*., 2007).

In summary, carbon nanoparticles may lead to cellular morphological modifications, LDH leakage and mitochondrial dysfunction. Our preliminary data also suggested that oxidative stress is likely to contribute to carbon nanoparticles cytotoxicity. Further studies are needed to investigate the correlation of these *in vitro* results with *in vivo* toxic effects of carbon nanoparticles.

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