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Multi wall carbon nanotubes induce oxidative stress and cytotoxicity in human embryonic kidney (HEK293) cells

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ABSTRACT

The present study was aimed at evaluating the potential toxicity and the general mechanism involved in multi wall carbon nanotubes (MWCNT)-induced cytotoxicity using human embryonic kidney cell line (HEK293) cells. Two multi wall carbon nanotubes (coded as MWCNT1, size: 90–150 nm and MWCNT2, size: 60–80 nm) used in this study are MWCNT1 (produced by the electric arc method and size of the nanotubes was 90–150 nm) and MWCNT2 (produced by the chemical vapor deposition method with size of 60–80 nm). To elucidate the possible mechanisms of MWCNT induced cytotoxicity, cell viability, mitochondrial function (MTT assay), cell membrane damage (LDH assay), reduced glutathione (GSH), interleukin-8 (IL-8) and lipid peroxidation levels were quantitatively assessed under carbon nanotubes exposed (48 h) conditions. Exposure of different sizes of two carbon nanotubes at dosage levels between 3 and 300 μg/ml decreased cell viability in a concentration dependent manner. The IC_{50} values (concentration of nanoparticles to induce 50% cell mortality) of two (MWCNT1, MWCNT2) nanoparticles were found as 42.10 and 36.95 μg/ml. Exposure of MWCNT (10–100 μg/ml) to HEK cells resulted in concentration dependent cell membrane damage (as indicated by the increased levels of LDH), increased production of IL-8, increased TBARS and decreased intracellular glutathione levels. The cytotoxicity and oxidative stress was significantly more in MWCNT2 exposed cells than MWCNT1. In summary, exposure of carbon nanotubes resulted in a concentration dependent cytotoxicity in cultured HEK293 cells that was associated with increased oxidative stress.

1. Introduction

Nanotechnology is widely perceived as one of the key technologies of the 21st century and accordingly there have been huge advances and increased funding in global technological research on nanomaterials. A vast variety of nanomaterials have been developed and nanotechnology has emerged as rewarding key research area in the modern scientific set-up. It is the science of nanoparticles that show new and different properties compared to what they exhibit on a macroscale, enabling unique applications. In this nanotechnological development, carbon nanotubes (CNT) have attracted a great deal of attention. CNTs are cylindrical molecules composed solely of carbon atoms. They can be obtained as single wall carbon nanotubes (SWCNT) or multi wall carbon nanotubes (MWCNT).

Due to their attractive structural, mechanical, electrical, and optical properties, multi-walled carbon nanotubes are candidates for many applications, including fillers in composites for anti-static applications, catalysis, components within rechargeable battery electrodes, and composite materials with improved structural and electrical properties (Gass et al., 2006; Ball, 2001; Popov, 2004; Miaudet et al., 2005). As the production and applications of nanotubes expand, potential human exposures will also increase. In occupational settings, these CNT may release into the surroundings in aerosol form (Maynard et al., 2004).

Nanoparticles have been shown to reach the systemic circulation after inhalation, ingestion or intravenous injection, with further distribution and accumulation in several organs such as lung, liver, spleen, kidneys, brain or heart (Nemmar et al., 2002; Shimida et al., 2006). Oberdorster et al., 2002 reported the extra pulmonary translocation of ultrafine carbon nanoparticles in rats. Rats exposed by inhalation exposure to 13C-labeled ultrafine carbon particles demonstrated that liver accumulated a significant

Abbreviations: MWCNT, multi wall carbon nanotubes; HEK293, human embryonic kidney cells; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GSH, glutathione; LDH, lactate dehydrogenase enzyme; IL-8, interleukin-8.

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amount of these carbon particles by 24 post-exposure, with little detectable deposition in other extra-pulmonary organs. Our preliminary results also showed the translocation of MWCNT in to the kidney and liver and thus produce extra pulmonary toxicity after intra-tracheal instillation in rats.

A number of *in vitro* studies have also been performed on CNT and have evaluated different mechanistic endpoints. Shvedova et al. (2003) tested SWCNT on human epidermal keratinocytes (HaCaT) and following 18 h exposure reported oxidative stress and loss of cell viability. They also observed that exposure resulted in ultra-structural and morphological changes in these skin cells. Recently, 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. But the authors did not elaborate his study towards the evaluation of the parameters which measures the oxidative stress (Massimo et al., 2006). Kagan et al. (2006) also demonstrated that SWCNT resulted in a significant loss of intracellular low molecular weight thiols (GSH) and accumulation of lipid hydroperoxides in murine macrophages. The present study was aimed at evaluating the potential toxicity and the general mechanism involved in multi wall carbon nanotubes-induced cytotoxicity using human embryonic kidney cell line (HEK293) cells.

The human embryonic kidney cell line (HEK293) was selected first time in the present study as an *in vitro* model to assess cytotoxicity of MWCNT and the eventuality of kidney toxicity. This cell line has been well characterized for its relevance to the toxicity models in human (Fen et al., 2009; Ji et al., 2008). To elucidate the possible mechanisms of cytotoxicity, a variety of surrogate parameters including cell viability, mitochondrial function (MTT assay), cell membrane damage (LDH assay), reduced glutathione (GSH), and lipid peroxidation levels were quantitatively assessed and compared with controls and quartz (positive control) treated groups.

2. Materials and methods

2.1. Chemicals

Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), penicillin, amphotericin B, and streptomycin were purchase from Himedia (Mumbai, India). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-thiobarbituric acid (TBA), bovine serum albumin and tetraethoxypropane were purchased from Sigma Chemical Company (St. Louis, MO, USA). The glutathione, interleukin-8 (IL-8), lactate dehydrogenase (LDH) assay kits were purchased from Ray Biotech, Inc. (New Delhi, India).

2.2. Multi wall carbon nanoparticles-characterization

Multi walled carbon nanotubes produced by electric arc process (coded as MWCNT1) (Journet et al., 1997; Li et al., 2004a) using graphite as a source and chemical vapor deposition (coded as MWCNT2) (Bronikowski et al., 2001; Li et al., 2004b) using methane as hydrocarbon were obtained from Centre for Environment, Institute of Science and Technology, JNTU, Hyderabad. However, these nanotubes exist primarily as agglomerated "ropes" of nanotubes. In dry powder form, each particle system was analyzed for various physico-chemical properties. Size and crystallinity were determined by dynamic light scattering spectroscopy (DLS) (Berne and Pecora, 1975) and X-ray diffraction (XRD) (Otwinowski and Minor, 1997), respectively. The surface area of the carbon nanoparticles were measured by the Brunauer-Emmett-Teller method (BET). All the nanoparticles were suspending in phosphate buffer saline (PBS) to get the stock concentrations of these MWCNT were 10.0 mg/ml. The final concentrations were made in the cultured media (DMEM) without serum for the uniform dispersion of carbon nanotubes and a brief sonication was done before the exposure to the cells.

2.3. Cell culture

The human embryonic kidney cell line (HEK 293) was purchased from cell bank of national centre for cell sciences (Pune, India). Cells were cultured in a full DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37 °C in with 5% CO2.

2.4. Exposure of MWCNT

The stock suspensions of carbon nanoparticles were freshly diluted to different concentrations in the cell culture medium without serum. After cells had attached for 12 h in the full medium, the medium was replaced with low serum DMEM (containing 0.1% FBS) to prevent particle agglomeration. Freshly dispersed particle suspensions were immediately applied to the cells and allowed to incubate for 48 h. Cells free of carbon nanoparticles were used as control cells throughout each assay.

Tests for LDH release, cytotoxicity and cell viability assay (MTT), cytokine production (IL-8), lipid peroxidation products (thiobarbituric acid reactive substances; TBARS) and quantification of intracellular glutathione levels were performed on HEK cell culture system and done in triplicate.

2.5. Assessment of cytotoxicity

Mitochondrial function and cell viability were measured by the MTT assay (Denizot and Lang, 1986). HEK293 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 48 h of treatment with different concentrations of nanoparticles, the cells were incubated with MTT (2.5 mg/ml) for 2 h. The medium was then removed and 100 μl of DMSO was added into each well to dissolve formazan crystals, the metabolite of MTT. After thoroughly mixing, the plate was read at 570 nm for optical density that is directly correlated with cell quantity. Cell death rate was calculated from the relative absorbance at 570 nm and expressed as the percentage of cell death. The concentrations of carbon nanotubes used in this cytotoxicity assay were 3–300 μg/ml.

2.6. LDH release

Cells were seeded in 24-well plates, exposed to increasing concentrations of particle suspensions (10–100 μg/ml). After 48 h of incubation, the plate was centrifuged at 1900 rpm for 4 min. The media were transferred into a fresh 24-well plate and analyzed for LDH release as described in Hussain and Frazier (2002). Each experiment was done in triplicate. Cytotoxicity is expressed relative to the basal LDH release by untreated control cells.

2.7. Production of IL-8

Cells (2×10^4 cells/ml) were grown in 24-well plates and incubated with different concentrations of (10–100 μg/ml) nanoparticles for 48 h. The supernatants were collected, centrifuged to remove any remaining nanoparticles. Concentrations of the pro-inflammatory cytokine, interleukine-8 (IL-8), were determined by human enzyme-linked immunoabsorbant assay (ELISA) according to the manufacturer's guidelines (Ray Biotech, Inc., India). Cells incubated without nanoparticles were used as a control. The absorbance was measured at 450 nm and quantified with a microplate reader.

2.8. Quantification of intracellular GSH levels

Cellular levels of reduced GSH were determined using the GSH-400 colorimetric assay kit. The method is based on a chemical reaction between GSH and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to generate glutathione disulfide (GSSG) and -nitro-5-thiobenzoic acid, a yellow colored product. Thus GSH concentration in a sample solution can be determined by the measurement at 412 nm absorbance (Akerboom and Sies, 1981). HEK293 cells were plated into a 24-well plate at a density of 2×10^4 cells/ml. After 48 h exposure to carbon nanoparticles, the cells were washed twice in ice-cold PBS and then homogenized in 400 μl of 0.5% Triton X-100. The cell homogenate was centrifuged at 3000×*g* at 4 °C for 10 min. The assay was performed on 200 μl centrifugation supernatants according to manufacturer's protocol, and the absorbance of the supernatant was measured at 400 nm using a UV–visible spectrophotometer (Elico, India). Protein content was determined for the same cell homogenate. GSH level was calculated and expressed as the percentage of control.

2.9. Estimation of lipid peroxidation

The MDA content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reactive substance (TBARS) (Ohkawa et al., 1979). HEK293 cells were plated into a 24-well plate at a density of 1×10^5 cells/well. After 48 h exposure to MWCNT (10–100 μg/ml), the cells were washed with ice-cold PBS and homogenized in 400 μl of 0.5% Triton X-100. The cell homogenates were used in the TBARS assay. Briefly, 100 μl cell homogenates were mixed with 1 ml of 0.67% TBA, 1.5 ml 20% trichloroacetic acid, and 1.5 ml 0.04% BHT in test tubes. The mixtures were incubated in a boiling water bath for 20 min. After cooling to room temperature, the reaction mixture was centrifuged at 4000×*g* for 10 min and the absorbance of the supernatant was measured at 532 nm using the same UV–visible spectrophotometer. The concentrations of TBARS were calculated using tetraethoxypropane as

Table 1
Characterization of multi wall carbon nanotubes.

Carbon nanotubes	Method of production	Size (nm)	Crystallinity	Surface area (m^2/g)
ARC	Electric arc	90–150	Hexagonal	197
CVD	Chemical vapor deposition (CVD)	60–80	Cubic	252

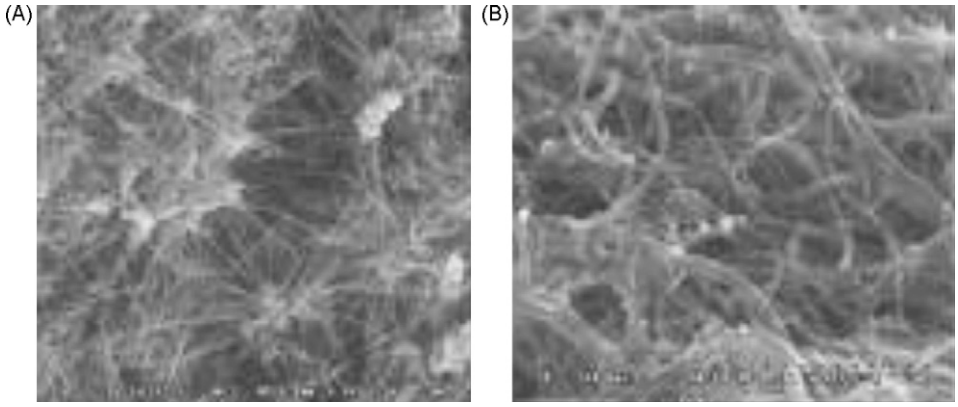

Fig. 1. Scanning electron micrographs (SEM) of 1 (A) MWCNT1 and 1 (B) MWCNT2.

a reference standard. The quantities of TBARS were presented as the percentage of TBARS production over the control.

2.10. Protein assay

The total protein concentration was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as the standards.

2.11. Statistical analysis

All the experimental values were expressed as mean ± standard deviation (SD). Statistical analysis was performed for the experiments conducted in at least triplicate using one-way analysis of variance (ANOVA) and Dunnett test. Student's *t*-test was used to compare the means of MWCNT1 with MWCNT2. Results with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Characterization of carbon nanotubes

Both MWCNT were well dispersed in the cultured medium without serum. The estimated size, purity and crystallinity of the carbon nanotubes were showed in Table 1. The scanning electron micrographs (SEM) of two multi wall carbon nanotubes were showed in Fig. 1(A) and (B).

3.2. Dose-dependent cytotoxicity of carbon nanoparticles

Multi wall carbon nanoparticles were exposed to HEK293 cells at 3, 10, 30, 100 and 300 μg/ml for 48 h. Cell viability decreased as a function of dosage levels (Fig. 2) Significant cell death (cytotoxicity) was observed with all the MWCNT at and above concentrations of 10 μg/ml. The IC_{50} values (concentration of nanoparticles to induce 50% cell mortality) of two (MWCNT1, MWCNT2) nanoparticles were found as 42.10 and 36.95 μg/ml, which was almost equal to that of quartz (36.19 μg/ml), indicating the potency of toxicity of carbon nanotubes.

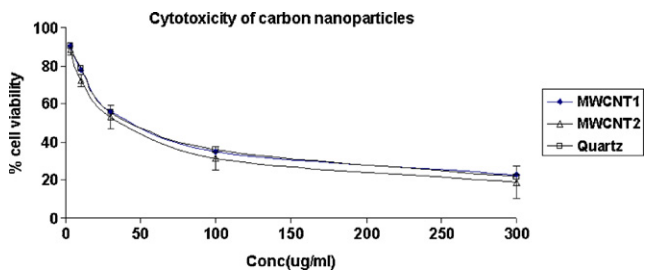

Fig. 2. Dose dependent toxicity of two carbon nanoparticles in HEK293 cells.

3.3. LDH leakage

The cell membrane damage induced by carbon nanoparticles was also monitored by the LDH leakage assay, since LDH, a stable cytosolic enzyme in normal cells can leak into the extracellular fluid only after membrane damage. Exposure of MWCNT (10–100 μg/ml) to HEK 293 cells for 48 h resulted in significant ($p < 0.001$) LDH release into media in a concentration dependent manner (Fig. 3). Since both carbon nanoparticles significantly inhibited cell proliferation leading to cell death (Fig. 2) and caused considerable membrane damage (Fig. 3). Between two tested carbon nanoparticles, MWCNT2 produced a greater ($p > 0.05$) LDH release in to media than others, due to its smaller size.

3.4. IL-8 production

Inflammation is indicated by the release of inflammatory mediators like cytokine IL-8, produced by the HEK cells. IL-8 serves as a

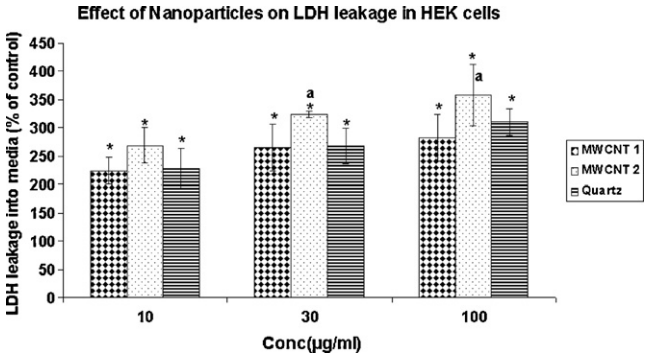

Fig. 3. Concentration dependent membrane damage as determined by LDH leakage from HEK293 cells incubated with MWCNT for 48 h. Significance was indicated by: *$p < 0.001$ versus control cells; [a] $p < 0.05$ versus MWCNT1.

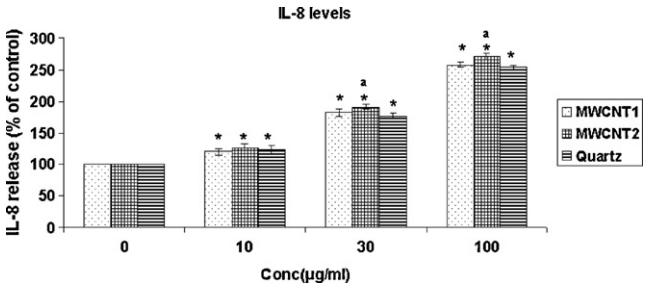

Fig. 4. Effect of MWCNT on IL-8 release from HEK293 cells. Significance was indicated by: $^{*}p < 0.01$ versus control cells; ^{a} $p < 0.05$ versus MWCNT1.

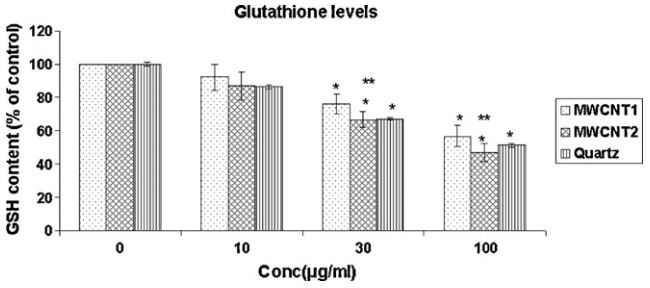

Fig. 5. Effect of MWCNT on GSH content from HEK293 cells. Significance was indicated by: $^{*}p < 0.01$ versus control cells; $^{**}p < 0.05$ versus MWCNT1.

chemical signal that attracts the neutrophils at the site of inflammation. Similar to quartz, exposure of MWCNT (10–100 μg/ml) for 48 h caused a significant dose-dependant increase in IL-8 release from HEK 293 cells (Fig. 4), indicating an inflammation response of nanotubes to kidney cells.

3.5. GSH estimation

GSH is a ubiquitous sulfhydryl-containing molecule in cells that is responsible for maintaining cellular oxidation–reduction homeostasis. Alterations in GSH homeostasis can be considered as an indication of functional-damage to the cells. As shown in Fig. 5, both carbon nanoparticles decreased GSH levels in the cells in a concentration dependent manner. Between two tested carbon nanoparticles, MWCNT2 produced a greater ($p > 0.05$) LDH release in to media than others, due to its smaller size. When the exposure dose of nanoparticles increased to 100 μg/ml, the intracellular GSH levels were almost reduced to 50% when compared to the control (Fig. 5). Overall, the data demonstrated a significant depletion of GSH levels in carbon nanoparticles exposed cells.

3.6. Effect of carbon nanoparticles on lipid peroxidation

The sensitivity of measuring TBARS assay is the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. As showed in Fig. 6, exposure of both nanoparticles to HEK293 cells resulted in significant ($p < 0.05$) increased TBARS levels in a concentration dependent manner.

4. Discussion

The purpose of this investigation was to evaluate potential toxicity and the general mechanism involved in multi wall carbon nanotubes-induced cytotoxicity. To date there are very few studies directly or indirectly investigating the toxic effects of

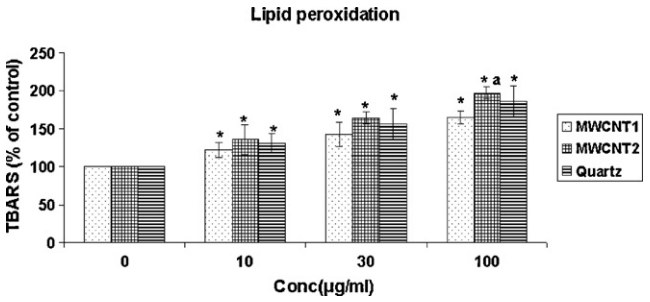

Fig. 6. Cellular lipid peroxidation product levels of HEK293 cells after 48 h exposure to MWCNT. Significance was indicated by: $^{*}p < 0.01$ versus control cells; ^{a} $p < 0.05$ versus MWCNT1.

MWCNT(Crystal et al., 2009) and no clear guidelines are presently available to quantify these effects. In this study, the cytotoxicity of two different sizes of MWCNT was investigated in cultured human embryonic kidney (HEK293) cells.

In the present study, it was found that exposure to carbon nanoparticles at dosage levels of 3–300 μg/ml caused dose- dependent cytotoxicity as revealed by MTT assay. It was found the higher cytotoxicity of both nanoparticles against all cell type tested and was comparable with known cytotoxic agent, quartz. The IC_{50} values of both carbon nanoparticles and quartz were almost equal (39.85 ± 2.58 μg/ml), indicating the comparable cytotoxicity of carbon nanoparticles with quartz particles.

The cell membrane damage induced by carbon nanoparticles was also monitored by the LDH leakage assay, since LDH, a stable cytosolic enzyme in normal cells can leak into the extracellular fluid only after membrane damage. Exposure to MWCNT and quartz particles (10–100 μg/ml) for 48 h gave rise to greater LDH release from HEK cells in a (Fig. 3). The analysis of particles exposure media for LDH demonstrates that carbon nanomaterials increase LDH leakage in a dose dependent manner at 48 h exposure period.

Inflammation is a type of nonspecific immune response and a basic way in which the body reacts to infection or physical and chemical irritation. One indication is the release of inflammatory mediators like cytokine IL-8, produced by HEK cells. IL-8 serves as a chemical signal that attracts the neutrophils at the site of inflammation. Since both MWCNT significantly inhibited cell proliferation leading to cell death (Fig. 2) and caused considerable membrane damage (Fig. 3), we focused on the inflammation response of HEK cells. It is important to note that inflammation is the response of living tissue and these nanoparticles had low effect on cell viability. Exposure to MWCNT for 48 h caused a significant time-dependant increase in IL-8 release from HEK293 cells (Fig. 4), indicating an inflammation response to MWCNT. Quartz produced a similar release of IL-8 from HEK cells.

Concomitant cellular oxidative stress was manifested by reduced GSH levels, and increased lipid peroxidation. The inverse linear relationship between the exposure concentration and the GSH level indicated that exposure to carbon nanoparticles reduced intracellular glutathione levels ($p < 0.01$). Moreover, the increased levels of TBARS content resulted in the production of malondialdehyde, an indication of lipid peroxidation.

There was a strong correlation between decreased cell viability and increased lipid peroxidation after 48 h exposure. The reverse correlation between the decreased cell viability and the increased TBARS suggested that cell death was the primary cause of the membrane damage by lipid peroxidation. Lactate dehydrogenase leakage form cells are another evidence for penetration of particles into the cells and cell membrane damage (Balduzzi et al., 2004; Sayes et al., 2005). It has been well documented that lactate dehydrogenase levels (as a marker of necrosis) in the cell medium

elevated after the cells exposed to nanoparticles (Hussain et al., 2005; Lin et al., 2006).

The results of the present study showed the greater cytotoxicity of MWCNT2 (60–80 nm) than MWCNT1 (90–150 nm). LDH release, IL-8 production, lipid peroxidation were more significantly increased and glutathione levels were decreased in MWCNT2 exposed cells than that of MWCNT1. These indicate the smaller size carbon nanoparticles induce more oxidative stress and cytotoxicity than large size nanoparticles. These results were supported by the studies conducted by the Fen et al. (2009) and Kipen and Laskin (2005).

Several mechanisms have been proposed to explain the adverse health effects of particulate pollutants. ROS production and the generation of oxidative stress have received the most attention. ROS, such as superoxides, hydrogen peroxide, hydroxyl and other oxygen radicals, are capable of directly oxidizing the DNA, proteins, and lipids (Yoshida et al., 2004). There are many evidences showing that nanoparticles increase ROS production and can cause cell death in different types of cultured cells (Becker et al., 2002;Pulskamp et al., 2007; Park et al., 2008). Furthermore, it has been well documented that GSH depletion and ROS production cause mitochondrial dysfunction and changes in expression of distinct genes and pathways related to inflammatory responses and apoptosis including MAPK/ERK kinase, NFjB, MIP-2, caspase- 3, Bcl-2 (Driscoll, 2000; Fubini and Hubbard, 2003).

The aim of the study was to investigate the hypothesis that exposure to carbon nanoparticles induced oxidative stress. The recent study by Oberdorster (2004) indicated that nanomaterials (Fullerenes C_{60}) induced oxidative stress in a fish model, as demonstrated by a significant elevation of lipid peroxidation and marginal GSH depletion. Results of the present study showed that exposure of MWCNT to HEK cells produced a dose dependent cytotoxicity, cell membrane damage, reduced intracellular glutathione levels, increased IL-8 production, and elevated lipid peroxidation products, indicating the oxidative stress contributes to the cytotoxicity induced by the multi wall carbon nanoparticles. These results were supported by Stone et al. (2007). They reported that oxidative stress has been proposed as a common mechanism of cell damage induced by many types of nanoparticles (Stone et al., 2007).

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. 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. 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. *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 (Pulskamp et al., 2007).

In conclusion, multi wall carbon nanotubes exposure to human embryonic kidney cells produced a concentration dependent cytotoxicity. Exposure of MWCNT to HEK cells resulted in cell membrane damage, increased production of IL-8, increased lipid peroxidation and decreased intracellular glutathione levels, indicating that oxidative stress contributes to the MWCNT induced cytotoxicity in HEK kidney cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

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