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Induction of oxidative stress and cytotoxicity by carbon nanomaterials is dependent on physical properties

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Abstract

In this study, we investigated the mechanisms involved in multi-wall carbon particles/nanomaterials (MWCNM) induced cytotoxicity using human embryonic kidney (HEK293) cells and to assess the effect of physico-chemical properties on the cytotoxicity and oxidative stress induced by the carbon nanomaterials (CNM). To elucidate the possible mechanisms of CNM-induced cytotoxicity, cell viability (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT assay]), cell membrane damage (lactate dehydrogenase enzyme [LDH] assay), reduced glutathione (GSH), interleukin-8 (IL-8) and lipid peroxidation levels were quantitatively assessed under carbon nanomaterials exposed (48 h) conditions. Exposure of different sizes of four CNM at dosage levels between 3 and 300 μg/mL decreased cell viability in a concentration- and size-dependent manner. Exposure of CNM (10−100 μg/mL) to HEK cells resulted in size-, surface area- and concentration-dependent cell membrane damage, increased production of IL-8, increased thiobarbituric acid reactive substances (TBARS) and decreased intracellular glutathione levels. In summary, the physical properties of carbon nanoparticles may alter the CNM-induced concentration-dependent cytotoxicity and oxidative stress.

Keywords

carbon nanoparticles, HEK293 cells, oxidative stress, cytotoxicity, in vitro, LDH, MTT, IL-8, MDA, glutathione, TBARS

Introduction

Recently, 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 nanoparticles or carbon nanomaterials (CNM) have attracted a great deal of attention. CNM are cylindrical molecules composed solely of carbon atoms. They can be obtained as single-wall carbon nanotubes (SWCNT) or multi-wall carbon nanotubes (MWCNT). These CNM have more attractive structural, mechanical, electrical and optical properties. In occupational settings, these CNM 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).

In vitro assays are often used to screen potential therapeutic compounds for adverse biological effects before in vivo testing, thereby, leading to a reduction

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in the use of research animals. A number of classical in vitro toxicity assays have been utilized to determine the viability and/or cytotoxicity in cultured cells. These relatively simple assays use colorimetric or fluorescent dyes as markers to determine cell viability assessing membrane integrity or cell metabolism. A number of in vitro studies have also been performed on CNM and have evaluated different mechanistic endpoints. Shvedova et al. (2003) tested SWCNT on human epidermal keratinocytes (HaCaT) and following 18 hours exposure reported oxidative stress and loss of cell viability.

The present in vitro study was aimed at investigating the general mechanisms involved in multi-wall carbon nanoparticles-induced cytotoxicity and also to assess the effect of physico-chemical properties on the cytotoxicity and oxidative stress induced by the carbon nanomaterials. To elucidate the possible mechanisms of cytotoxicity, a variety of surrogate parameters including cell viability, mitochondrial function (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay), cell membrane damage (lactate dehydrogenase enzyme [LDH] assay), reduced glutathione (GSH) and lipid peroxidation levels were quantitatively assessed.

Materials and methods

Chemicals

MTT, 2-thiobarbituric acid (TBA), bovine serum albumin and tetra ethoxy propane (TEP) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Phosphate buffer saline (PBS), fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), penicillin, amphotericin B and streptomycin were purchase from Himedia (Mumbai, India). The glutathione, interleukin-8 (IL-8) and LDH assay kits were purchased from Ray Biotech, Inc (New Delhi, India).

Multi-wall carbon nanoparticles characterization

Four different multi-walled carbon nanomaterials (coded as CNM1, CNM2, CNM3 and CNM4) were obtained from Centre for Environment, Institute of Science and Technology, JNTU, Hyderabad. These carbon nanoparticles were coded according to their increasing order of size (CNM 1-CNM 4). 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) methods, respectively. The surface area of the carbon nanoparticles were measured by the Brunauer-Emmett-Teller method (BET). All the nanoparticles were suspended in phosphate buffer saline (PBS) to get the stock concentration of 10 mg/mL. The final concentrations were made in the cultured media (DMEM) without serum for the uniform dispersion of carbon nanoparticles and a brief sonication was done before the exposure to the cells.

Cell culture

The human embryonic kidney cell line (HEK 293) was purchased from cell bank of national centre for cell sciences (Pune, India). The HEK293 was selected first time in the present study as an in vitro model to assess cytotoxicity of MWCNM and the eventuality of kidney toxicity. This cell line has been well characterized for its relevance to the toxicity models in human (Cui et al., 2005; Fen et al., 2009; Ji et al., 2008). Cells were cultured in a full DMEM containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, and incubated at 37°C in with 5% CO_2.

Exposure of carbon nanoparticles

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 hours 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 hours. Cells free of carbon nanoparticles were used as control cells throughout each assay.

Assessment of cytotoxicity and oxidative stress

After 48 hours of incubation of HEK293 cells with different concentrations of carbon nanoparticles, different mechanistic end points were evaluated to assess the cytotoxicity and induction of oxidative stress. Tests for cytotoxicity and cell viability assay (MTT), LDH release, cytokine production (IL-8), lipid peroxidation products (Malondialdehyde [MDA] and other thiobarbituric acid reactive substances [TBARS]) and quantification of intracellular glutathione levels were

performed on HEK cell culture system to assess the cytotoxicity and induction of oxidative stress and done in triplicate.

Assessment of cytotoxicity

Cytotoxicity and cell viability assay on HEK cells was performed by the MTT method (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 hours of treatment with different concentrations of nanoparticles, the cells were incubated with MTT (2.5 mg/ mL) for 2 hours. The medium was then removed and 100 μL of DMSO were 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 nanoparticles used in this cytotoxicity assay were 3−300 μg/mL.

LDH release

Cells were seeded in 24-well plates, exposed to increasing concentrations of particle suspensions (10−100 μg/mL). After 48 hours 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.

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 hours. 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.

Quantification of intracellular GSH levels

Cellular levels of reduced GSH were determined using the glutathione 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-hour 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.

Estimation of lipid peroxidation products

The MDA content, a measure of lipid peroxidation, was assayed in the form of 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-hour exposure to MWCNM (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 (Butylated hydroxytoluene in acetonitrile). 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 tetra ethoxy propane as a reference standard. The quantities of TBARS were presented as the percentage of TBARS production over the control.

Protein assay

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

Table 1. Characterization of multi-wall carbon nanoparticles

Sample code	Sample	Particle size (nm)	Surface area (m^2/g)	Crystallinity
CNM1	Fe catalyzed carbon nanorods	100–800	24.0	Orthorhombic
CNM2	Fe catalyzed carbon nanorods	200–500	16.7	Hexagonal
CNM3	Fe catalyzed carbon nanorods	150–750	13.1	Hexagonal
CNM4	Fe catalyzed carbon nanorods	230–1700	11.3	Cubic / rhombohedral

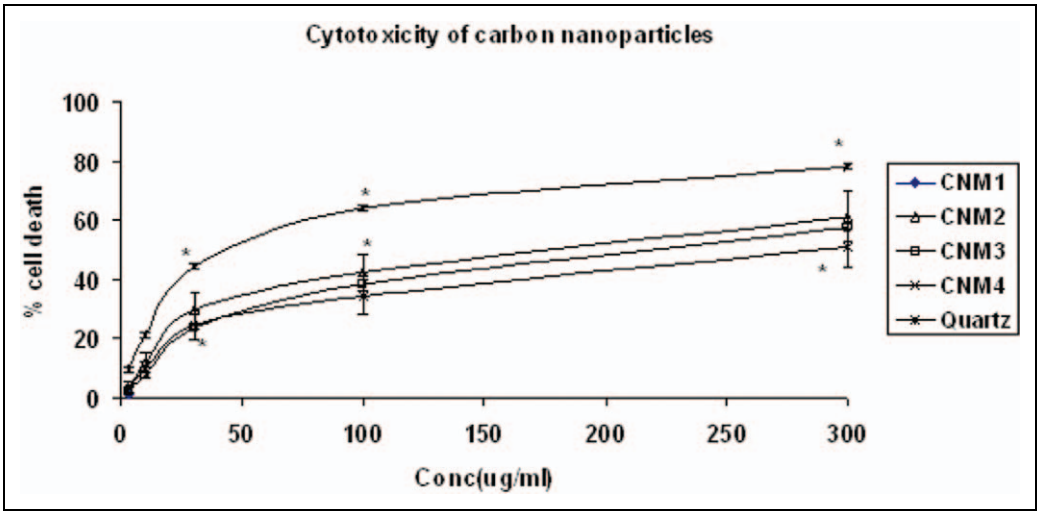

Figure 1. Cytotoxicity of two carbon nanoparticles in human embryonic kidney (HEK293) cells. Significance was indicated by $^*p < 0.05$ versus control cells.

Statistical analysis

All the experimental values were expressed as mean $\pm$ 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. Results with $p < 0.05$ were considered to be statistically significant.

Results

Characterization of carbon nanoparticles

All the four carbon nanoparticles were well dispersed in the cultured medium without serum. Brief sonication of nanoparticle suspensions before addition to the HEK cells prevents the agglomeration of nanoparticles. The size, surface area and crystallinity of all the carbon nanoparticles are shown in Table 1.

Cytotoxicity of carbon nanoparticles

Exposure of multi-wall carbon nanoparticles to HEK293 cells at different doses (3, 10, 30, 100 and 300 μg/mL) for 48 hours produced cytotoxicity in dose-dependent manner. Cell viability is decreased as a function of dosage levels (Figure 1). The IC_{50} values (concentration of nanoparticles to induce 50% cell mortality) of four nanoparticles were found between 183 and 271 μg/mL, indicating the potency of toxicity of carbon nanoparticles. This cytotoxicity of nanoparticles was inversely proportional to the size and directly proportional to the surface area of the nanoparticles.

LDH leakage

LDH leakage assay is used to assess the cell membrane damage induced by carbon nanoparticles. Since LDH, a stable cytosolic enzyme in normal cells, can leak into the extracellular fluid only after membrane damage. Exposure of CNM (10−100 μg/mL) to HEK293 cells for 48 hours resulted in significant ($p < 0.01$) LDH release into media in a concentration-dependent manner (Figure 2). Exposure of all the carbon nanomaterials results in significant cell death (Figure 1) by causing considerable membrane damage (Figure 2). The cell membrane

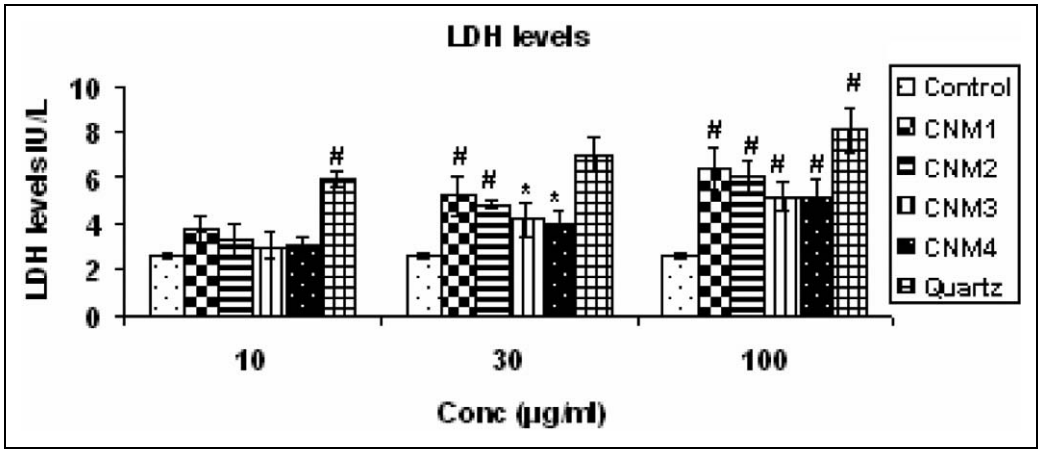

Figure 2. Concentration-dependent membrane damage as determined by lactate dehydrogenase enzyme (LDH) leakage from human embryonic kidney (HEK293) cells incubated with carbon nanomaterials (CNM) for 48 hours. Significance was indicated by $*p < 0.05$, $\#p < 0.01$ versus control cells ($n = 3$).

damage by nanoparticles was inversely proportional to the size and directly proportional to the surface area of the nanoparticles.

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 chemical signal that attracts the neutrophils at the site of inflammation. Exposure of CNM (10−100 µg/mL) for 48 hours caused a significant dose-dependant and size-dependant increase in IL-8 release from HEK 293 cells (Figure 3), indicating an inflammation response of nanoparticles to kidney cells.

GSH estimation

Glutathione is a sulfhydryl-containing molecule in cells that are 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 Figure 4, all the carbon nanoparticles decreased GSH levels in the cells in a concentration- and size-dependent manner. Overall, the data demonstrated a significant depletion of GSH levels were found in carbon nanoparticles-exposed cells.

Effect of carbon nanoparticles on lipid peroxidation

One of the major indicators of oxidative stress is the estimation of lipid peroxidation products like TBARS. As shown in Figure 5, exposure of both nanoparticles to HEK293 cells resulted in significant ($p < 0.05$) increased TBARS levels in a concentration- and size-dependent manner.

Discussion

The present in vitro study was aimed at investigating the general mechanisms involved in multi wall carbon nanoparticles-induced cytotoxicity and also to assess the effect of physico-chemical properties on the cytotoxicity and oxidative stress induced by the carbon nanomaterials. To date, there are very few studies directly or indirectly investigating the toxic effects of MWCNT (Crystal et al., 2009) and no clear guidelines are presently available to quantify these effects. In this study, the cytotoxicity of four carbon nanomaterials of different physical properties were used to assess toxic effects on cultured HEK293 cells.

The adverse health effects of particulate pollutants have been proposed by several mechanisms. There are many evidences showing that nanoparticles increase reactive oxygen species (ROS) production and can cause cell death in different types of cultured cells (Becker et al., 2002; Park et al., 2008; Pulskamp et al., 2007). 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 the present study, it was found that exposure to carbon nanoparticles to HEK cells at dosage levels of

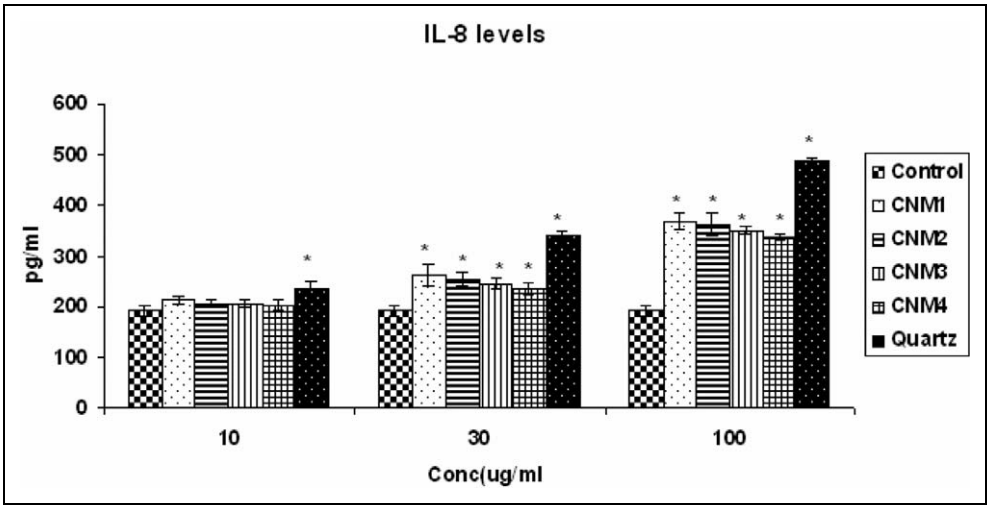

Figure 3. Effect of carbon nanomaterials (CNM) on interleukin-8 (IL-8) release from human embryonic kidney (HEK293) cells. Significance was indicated by $*p < 0.01$ versus control cells $n = 3$.

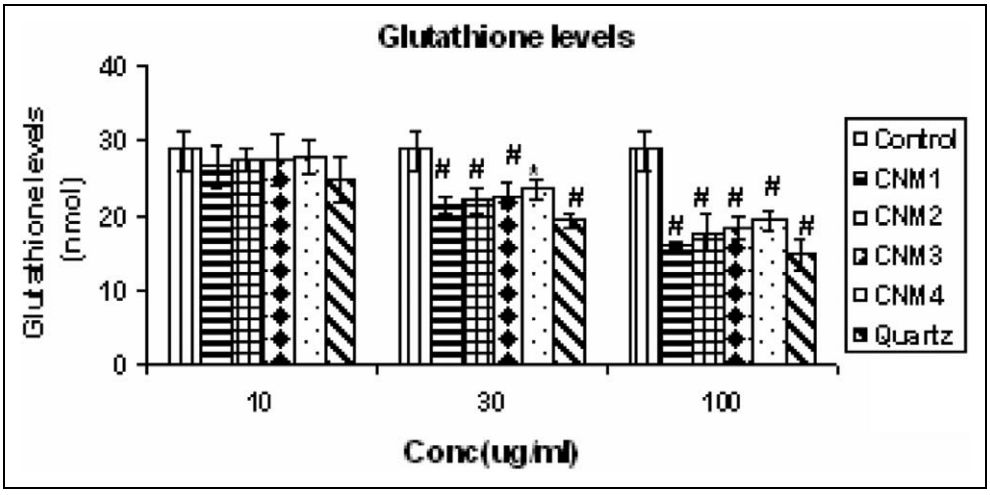

Figure 4. Effect of carbon nanomaterials (CNM) on glutathione (GSH) content from human embryonic kidney (HEK293) cells. Significance was indicated by: $\#p < 0.01$ versus control cells; $n = 3$.

3–300 μg/mL caused a significant cytotoxicity, which are dependent on their size, surface area and exposure doses. Concomitant cellular oxidative stress was manifested by reduced GSH levels and increased lipid peroxidation. Exposure of all CNM (10−100 μg/mL) for 48 hours to HEK cells gave rise to elevated LDH, IL-8 and TBARS levels and reduced glutathione levels, indicating the induction of oxidative stress and cytotoxicity (Figure 2−5).

The aim of the study was to investigate the hypothesis that exposure to carbon nanoparticles induced oxidative stress. The 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 CNM 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 support the studies of Stone et al., 2007.

There was a strong correlation between induction of oxidative stress (decreased cell viability, glutathione and increased IL-8, LDH, TBARS levels) and their physical properties of nanoparticles after

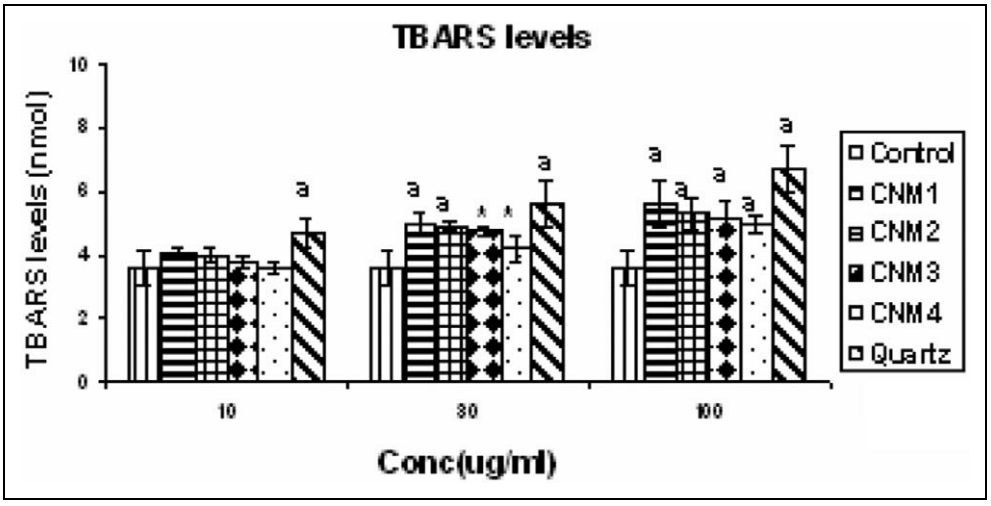

Figure 5. Cellular lipid peroxidation product levels of human embryonic kidney (HEK293) cells after 48 hours exposure to carbon nanomaterials (CNM). Significance was indicated by: *$p < 0.05$, #$p < 0.01$ versus control cells; $n = 3$.

48 hours exposure. The induction of oxidative stress was direct relation with surface area and reverse correlation with size of the nanomaterials suggest the role physical properties on nanomaterials induced toxicity. These indicate the carbon nanoparticles having smaller size or large surface area induce more oxidative stress and cytotoxicity than large-size nanoparticles. These results support the studies conducted by Fen et al., 2009 and Kipen and Laskin, 2005.

In conclusion, in vitro exposure of carbon nanomaterials to human embryonic kidney cells produced a cytotoxicity and oxidative stress in a concentration-dependent manner, which was confirmed by the cell membrane damage, increased production of IL-8, increased lipid peroxidation and decreased intracellular glutathione levels. The physicochemical properties significantly affect the cytotoxicity and oxidative stress induced by the nanoparticles.

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