MULTI-WALLED CARBON NANOTUBES INDUCE CYTOTOXICITY, GENOTOXICITY AND APOPTOSIS IN NORMAL HUMAN DERMAL FIBROBLAST CELLS

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Multi-walled carbon nanotubes (MWCNT) have won enormous popularity in nanotechnology. Due to their unusual, one dimensional, hollow nanostructure and unique physicochemical properties they are highly desirable for use within the commercial, environmental and medical sectors. Despite their wide application, little information is known concerning their impact on human health and the environment. While nanotechnology looms large with commercial promise and potential benefit, an equally large issue is the evaluation of potential effects on humans and other biological systems. Our research is focused on cellular response to purified MWCNT in normal human dermal fibroblast cells (NHDF). Three doses (40, 200, 400 µg/mL) of MWCNT and control (tween-80+0.9% saline) were used in this study. Following exposure to MWCNT, cytotoxicity, genotoxicity and apoptosis assays were performed using standard protocols. Our results demonstrated a dose-dependent toxicity with MWCNT. It was found to be toxic and induced massive loss of cell viability through DNA damage and programmed cell-death of all doses compared to control. Our results demonstrate that carbon nanotubes indeed can be very toxic at sufficiently high concentrations and that careful monitoring of toxicity studies is essential for risk assessment. (*Ethn Dis*. 2010;20[Suppl 1]:S1-65–S1-72)

Key Words: Multi-walled Carbon Nanotubes, Human Dermal Fibroblast, DNA, Annexin V, Cytotoxicity

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INTRODUCTION

Nanotechnology involves the development and manipulation of materials at the nanometer scale to create unique products that exploit novel properties. Many nanomaterials have novel biological and chemical properties and most of them are not naturally occurring;[1] one example of nanomaterials is the man-made carbon nanotubes (CNTs). They have splendid potential applications in modern science and technology due to their unique chemical and physical characteristics endowed by their novel nanostructures.[2] However, in view of some similar aspects to fibers, such as structural characteristics, extreme aspect ratio, low specific density, and low solubility, CNTs might exhibit toxicity similar to those observed with other fibrous particles such as asbestos.[3] Thus, the determination of whether CNTs have negative or positive impact on human health and the environment is of interest to the worldwide public.[4–5] Therefore, one hopes that CNTs would be one of the nanomaterials whose toxicity has been identified and recognized well before their industrial uses on a large scale. Recently, nanomaterials, such as nanotubes, nanowires and fullerene derivatives, have received enormous national attention in the creation of new types of analytical tools for biotechnology and life sciences. Despite the wide application of nanomaterials, little information is known concerning their impact on human health and the environment.[6] Multi-walled carbon nanotubes not only are produced by high-technology laboratories but also are found in particulate matter from ordinary combustion of fuel gases.[7] According to Richard Smalley, ''They're also made in every candle flame and in forest fires.''[8] Therefore, MWCNTs are probably ubiquitous in our environment.

To date, only a few studies report on toxic affects of CNTs either *in vivo*[9–11] or *in vitro* [12–20] and the results are often divergent. The bio-persistence, large aspect ratio and fibrogenic character of CNTs are important features that are linked to adverse health effects. However, the available peer-reviewed toxicological data for CNT is rather sparse to assess their toxic effects to humans and laboratory animals. Therefore, evaluation and characterization of their toxic potential is necessary.[21,22]

Many different forms of CNTs are found and they can be chemically modified and/or functionalized with either a hydroxyl or carboxyl or another nanomaterial. Pristine single-walled CNT's can be visualized as a single sheet of graphite rolled-up in the form of a cylinder with seamless ends. Its diameter ranges from 0.4 nm to micrometer. A multi-walled carbon nanotube (MWCNT) consists of several single-walled CNTs stacked one inside another. Its diameter ranges up to 100 nm.[23] These pristine CNTs are chemically inert and insoluble in aqueous solutions and therefore of little use in biological or medical applications. For many applications, CNTs are oxidized in strong acids to create hydroxyl groups and carboxyl groups[24] particularly in their ends to which biomolecules or other nanomaterials can be coupled.[25] These oxidized CNTs are much more readily dispersed in aqueous solutions and have been coupled to oligonucleotides, proteins or peptides and can be applicable in biological or medical applications.[26]

Since very few reports are known about the toxicity of CNTs, particularly of purified MWCNT form, we have studied them in a number of functional

assays with normal human dermal fibroblast cells (NHDF). Fibroblast cells are important as *in vitro* models because one way in which these engineered nanomaterials can enter the human vascular system is through open wounds. Moreover, dermis fibroblasts cells play an important role in the cell renewing system and in maintaining the skin integrity.

METHODS

Carbon Nanotubes

Multi-walled carbon nanotubes (MWCNTs) were synthesized by Nano-Lab Inc. (Newton, Mass., USA) by catalytic chemical vapor deposition (outer diameter of 15–30 nm, lengths of 15–20 μm, purity >95%). After synthesis, MWCNTs were heated under argon (2L/min) at 2000°C with 10°C/min temperature increase in order to extract catalyst (Fe-impurities). We started with our purified MWCNTs (purity >95% by TGA) and performed a reflux in sulfuric/nitric acid to functionalize the surfaces of these nanotubes. This process resulted in a large concentration of carboxyl (COOH) groups on the nanotube surface, and might also generate other groups (eg, –OH) as well. After functionalization, these carboxylated nanotubes have 2%–7% COOH by weight.

MWCNT morphology and size were determined by transmission electron microscopy (TEM). MWCNT were directly deposited on a TEM grid and allowed to dry. Samples were directly observed with a TEM. Figure 1 represents the TEM structures of COOH functionalized carbon nanotubes. Surface areas were determined by the isothermal gas adsorption method BET (27) using a Micromeritics Flowsorb 2300 (Norcross, Ga., USA).

To characterize our system, we processed TEM observation of the carbon nanotubes. MWCNT suspension was correctly dispersed with tween 80+0.9% sterile saline as surfactant during sonication. The length of the carbon nanotubes was up to 12 μm for the longer ones (60 mins of sonication). The diameter was 15 nm. Specific surface of carbon nanotube was measured by the classical BET method.[27] The specific surface of long carbon nanotubes for non-purified form was 41 m^2/g and 42 m^2/g for purified form. Figure 2A represents the Raman spectrum and Figure 2B illustrates FTIR spectrum data of the COOH functionalized nanotubes.

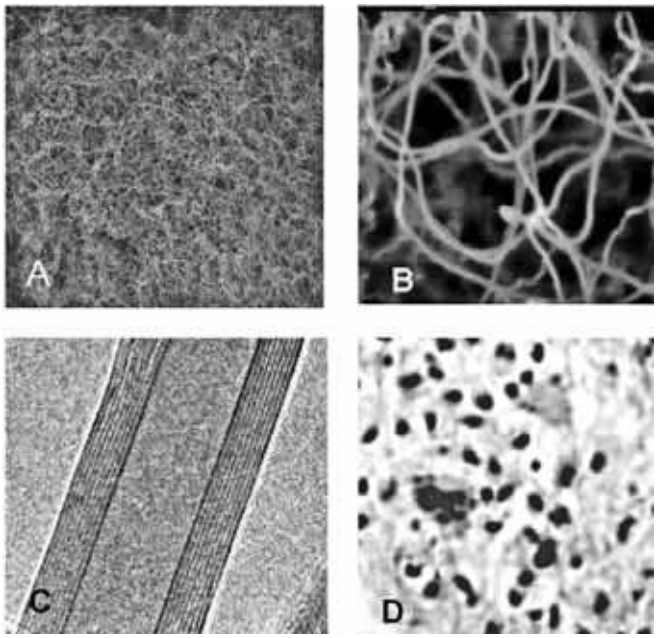


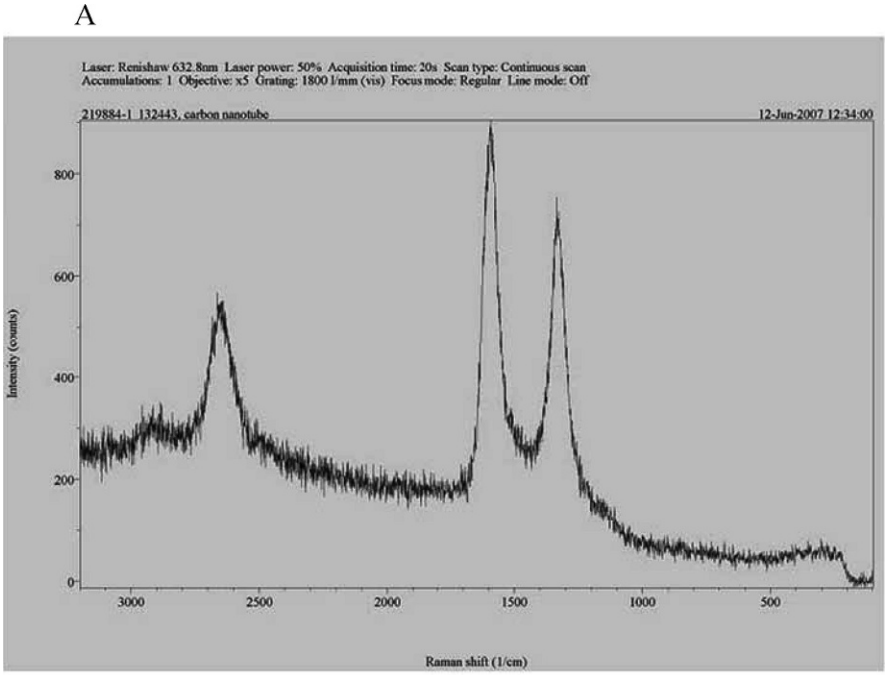
Fig 1. TEM structures of COOH functionalized carbon nanotubes. A) low magnification; B) high magnification; C) internal diameter; and D) photograph showing uptake of carbon nanotube by the cells

Chemicals

Methanol, glacial acetic acid, superfrost microscope slides, sterile tissue culture flasks, petri dishes, and sterile glass pipettes were purchased from Fischer-Scientific (Houston, Tex., USA). Hanks Balanced Salt Solution was purchased from GIBCO (Grand Island, N.Y., USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, Utah, USA). Comet assay kit was purchased from Trevigen, Inc. (Gaithersburg Md., USA). FITC-Annexin-V was purchased from Bio Vision (Mountain View, Calif., USA).

Cell Culture

Normal human dermal fibroblast cells (NHDF) were purchased from Lonza, Inc. (Rockland, Maine, USA). Parental NHDF cells, stored in liquid nitrogen, were thawed by gentle agitation of their containers (vials) for 2 min in a water bath at 37°C. After thawing, the content of each vial was transferred to a 75 cm^2 tissue culture flask, diluted with FBM (Lonza Inc.) media supplemented with 2% FBS, 1ng/mL basic human fibroblast growth factor, antibiotics (gentamycin 50μg/mL, amphotericin-B 50 ng/mL) and 5μg/mL insulin (Lonza Inc.) and incubated for 24 h at 37°C in 5% CO_2 atmosphere to allow cells to grow and to form a monolayer in the flask. Cells grown to 80%–95% confluence were washed with phosphate buffer saline (PBS), trypsinized with 3 mL of 0.25% (v) trypsin-0.03%) EDTA, diluted, counted and seeded in either 96-well plate or 35mm petri dishes, depending upon the assay. Treatment of cells was done by adding 0.5 mL of nanomaterial (CNTs) dispersion in water, or 0.5 mL water alone,



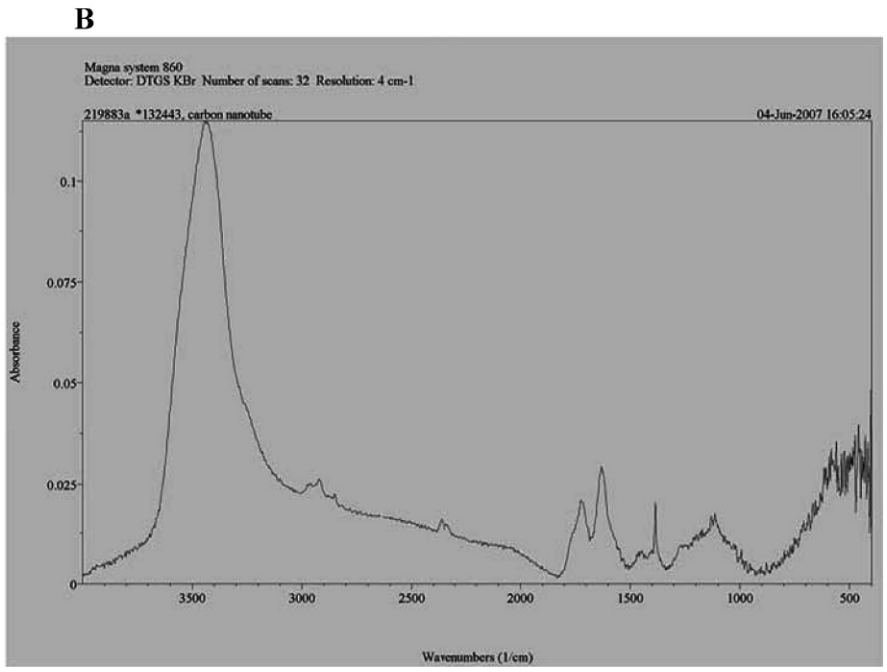


Fig 2A. Raman spectrum of COOH functionalized carbon nanotubes

Fig 2B. FTIR spectrum data of COOH functionalized carbon nanotubes

to 4.5 mL of cell suspension containing 2×10^5 cells at 37°C in 5% CO_2 atmosphere. The final concentrations of the added nanomaterial (NM) were $c_1=1$ ng NM/cell, $c_2=5$ ng NM/cell and $c_3=10$ ng NM/cell.

Cytotoxicity Assay

The effect of purified MWCNT on cell viability was assessed using the dimethylthiazole-tetrazolium (MTT) and lactate dehydrogenase (LDH) release assays. Briefly, 10^5 NHDF cells were seeded in a volume of 100 µl into a 96-well plate (Fischer-Scientific, Pittsburg, Penn.) and incubated overnight. Cells were then treated in triplicate with different CNT particles in suspensions for 24, 48, 72, 96 hours. 20 µl MTT (3-(4,5 dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide) was added to each well at 5 mg/mL and further incubated for 2h at 37°C and 5% CO_2. After which, 130 µl of 10% sodium dodecyl sulphate in 0.01 M HCL was added to each well and mixed thoroughly. The plate was then incubated at 37°C in the dark overnight. Optical density was read with a Microplate Reader at 550 nm.

As a measure of cell membrane damage, lactate dehyrogenase (LDH) activity was also measured in the supernatant of cells from treated and control groups following the reduction of NAD^+. The optical density was read at 340 nm.

Comet Assay

To determine the DNA damaging effect, 1×10^6 NHDF cells were seeded into 35 mm petri dishes and incubated overnight. Cells with 80% confluency were treated with three doses of MWCNT (40, 200 and 400 µg/mL), and control respectively, in 5% CO_2 at 37° for 48 hr. After incubation, the cells were washed with PBS, scrapped, centrifuged and re-suspended in 100 µl PBS. In a 2 mL centrifuge tube, 50 µl of the cell suspension and 500 µl of low melting agarose were mixed and 75 µl pipetted onto a pre-warmed comet-slide. The slides were placed flat in the dark at 4°C for 10 min for the mixture to solidify. The slides were then placed in pre-chilled lysing solution at 4°C for 1 h. Slides were removed from lysine solution, tapped on a paper towel to remove any excess lysis solution and immersed in alkaline solution (pH=13) for 45 min at room temperature in the dark. The slides were washed twice for 5 min with tris-borate (TBE) buffer. Next the slides were electrophoresed at

low voltage (300 mA, 25V) for 20 min. Slides were removed from the electrophoresis unit after the designated time, tapped to remove excess tris-borate buffer and immediately placed in 70% ethanol for 5 min and air-dried overnight at room temperature. After overnight drying, the slides were stained with SYBR-Green designed for comet assay and allowed to dry overnight. All the steps of the comet assay were conducted under yellow lamp in the dark to prevent additional DNA damage. The slides were read using an automated epiflourescent microscope and computer-based DNA damage analysis software from Loats & Associates (Westminster, Md., USA). The data were based on 100 randomly selected cells per sample, ie, 50 cells were from each of the two replicate slides. The percent tail DNA was considered the best indicator for DNA damage.

Annexin V Staining

Apoptosis was evaluated using annexin V assay (Bio Vision Research Products, Mountain View, Calif., USA). This assay is based on the observation that soon after initiating apoptosis, cells translocate the membrane phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a high affinity for PS. To determine cellular apoptosis, 1×10^6 NHDF cells were seeded into 3.5 cm petri dishes (Fischer-Scientific, Pittsburgh, Penn., USA) and incubated overnight. Cells were treated in duplicate with three different particle suspension (without tween) of MWCNTs for 48 h. After treatment, the cells were harvested by trypsinization. The pellets were resuspended and incubated in the dark for 15 min in 100 μL annexin labeling solution consisting of 2% annexin-V FLUOS and 0.1 μg/mL PI in HEPES buffer (10 mM HEPES, 140 mM NaCl, 2mM $CaCl_2$, 5 mM KCL and 1 mM $MgCl_2$, pH 7.4). Then the cells were dropped onto microscope slides and 1000 cells per culture were scored with Olympus BX60 fluorescence microscope at a magnification of ×400. Cells positive for annexin V-FITC were determined to be apoptotic. The results were expressed as the number of apoptotic cells per thousand cells counted.

DNA Ladder Analysis by Gel Elctrophoresis

Qualitative analysis of the DNA damage for MWCNT (0–400 μg/mL) treated cells were assessed by the DNA ladder technique using agarose gel electrophoresis. The effect of various doses of MWCNT after 48 h was assessed using calcium activated endonuclease-dependent ladder like DNA fragmentation by gel electrophoresis. Briefly, the MWCNT-treated cells were washed with chilled Tris-EDTA sarkosyl buffer and digested with proteinase K at 50°C for several hours. After digestion, DNA was extracted three times with phenol/chloroform (1:1) and precipitated with absolute ethanol/2.5M sodium acetate (pH 4.0). DNA was quantified spectrophotometrically at 260 nm and loaded onto an agarose gel (10 μg DNA/lane), and electrophoresed at a voltage of 60 volts. A Hind III digest of DNA served as a molecular marker. Gels were illuminated with 300 nm UV light and a photographic record of laddering was made and later the bands were quantified using densitometry software (Molecular Dynamics, Sunnyvale, Calif, USA).

Statistical Analysis

Data were compared by ANOVA. Statistical analysis was performed using SAS for Windows (2003, SAS, Cary, N.C., USA). Using Dunnett test, multiple comparisons were performed. All values were reported as means±SD for all the experiments. The significance level was set at $P<.05$.

RESULTS

Cytotoxic Response

LDH release, a marker of cell membrane damage, increased in a time- and dose-dependent manner after exposure to MWCNT ($P<.05$) (Figure 3A). Moreover, exposure to MWCNT caused a significant ($P<.05$) time and dose-dependent decrease in cells viability. The reduction of cell viability reached almost (70%) at the highest dose of MWCNT (400 μg/mL) tested (Figure 3B). Altogether, we concluded that MWCNT caused a clear cytotoxicity from dose 40 μg/mL.

Measurement of DNA Damage by Comet Assay

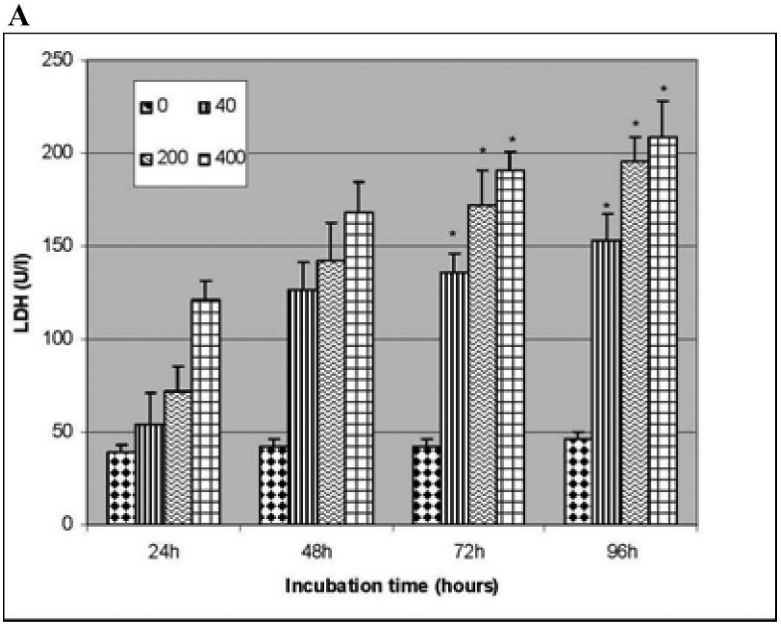
Percent tail DNA is an important parameter in evaluating DNA damage in the cells. The assay was repeated three times and the results were reproducible. All the doses of MWCNT induced a statistically significant increase in percent tail DNA [3.96%–20.39%] when compared with control [2.02%]. Maximum increase in tail DNA was observed in MWCNTs (400 μg/mL) at 48 h post-treatment when compared with control. Results of DNA damage are illustrated in Figure 4 (A and B).

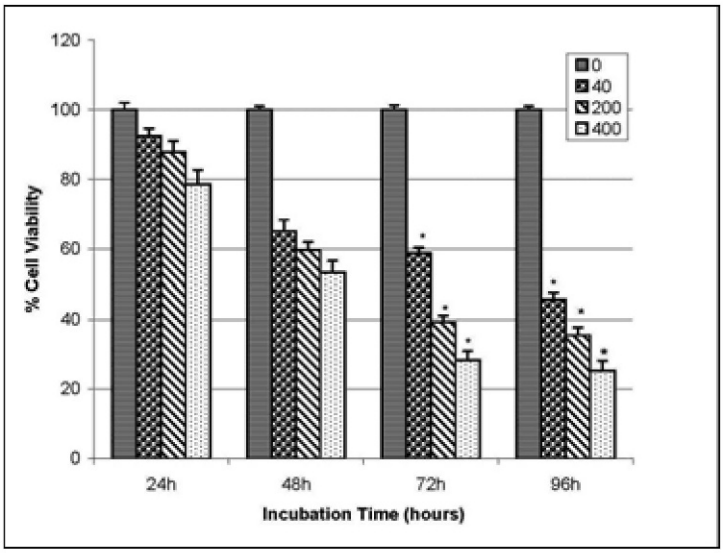
Induction of DNA Laddering

We tested whether MWCNTs would affect a ladder-like formation of genomic DNA damage. Following 48 h incubation of the NHDF cells with 40, 200 & 400 μg/mL dose of MWCNTs (functionalized), we observed there was dose-dependent increase in genomic DNA damage (Figure 5). Lanes 3–5 demonstrate the dose-dependent fragmentation of genomic DNA by CNTs. As shown in Figure 5 all doses of MWCNT were found to show statistically significant increase in genomic DNA damage when compared to control.

Induction of Apoptosis

Forty eight hours after exposure to MWCNTs, we observed a significant





3B

Fig 3A. Dose and time-dependent increase in normal human fibroblast lactate dehydrogenase (LDH) by MWCNTs
Fig 3B. Dose and time-dependent reduction in normal human fibroblast cell viability by MWCNTs. The values were calculated as percent of control (untreated cells) and represent the mean±SD for three independent determinations. Statistically significance is indicated in the figure as (*) for $P<.05$

dose-dependent increase of the percentage of apoptotic cells [6.24–18.65] when compared with control [3.1]. The assay was repeated three times and results were reproducible. Figure 6 (A and B) represent the results of apoptotic cells induced by oxidized MWCNTs. These results indicated that MWCNT induced early apoptosis from a dose of 40 μg/mL.

DISCUSSION

There is a clear gap in our current knowledge about the potential health effects of carbon nanotubes. They are seen as having a huge potential in many areas of research and application.[16] With the inclusion of CNTs to improve the quality and performance of many widely used products, as well as potentially in medicine, it is likely that occupational and public exposure to CNT-based nanomaterials will increase in the near future.[26] With the rapid advances in carbon nanotube-based new materials and technologies, there is a growing recognition that a fundamental understanding of the toxicological properties of carbon nanotubes is imperative.[11] It is recently recognized that the use of nanotechnology may raise new challenges in the safety, regulatory and ethical domains that will require scientific debate.

In our study, we have investigated the toxicity of purified MWCNTs in normal human dermal fibroblast cells using cell viability, DNA damage and apoptosis as the toxicological endpoints. The results clearly indicated a significant increase in cytotoxicity, genotoxicity and apoptosis in the normal human dermal fibroblast cell line, due to exposure to purified MWCNT. The percentages of viable cells in the NHDF cell line exposed to purified MWCNT showed a statistically significant decrease and LDH release increase as compared to the controls. These results are in accordance with those obtained by Cui et al.[12] Monteiro-Riviere et al,[28] Muller et al[20] and Ye et al,[29] who reported similar findings with single-walled MWCNTs on the proliferation of HEK293 kidney epithelial cells, MWCNTs on skin epithelial cells, rat

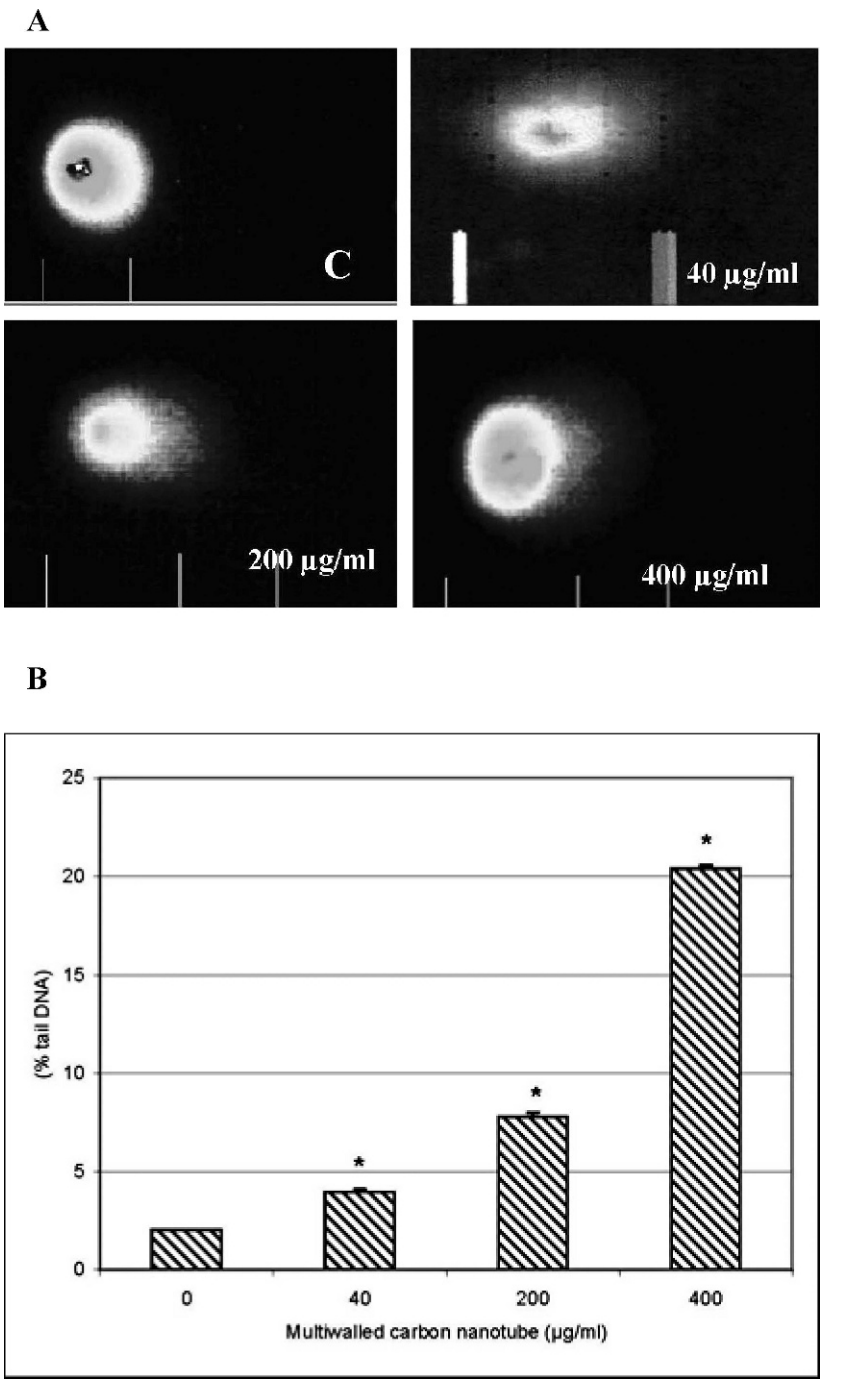


Fig 4A. Comet assay photographs of NHDF cells exposed to MWCNTs. Representative comet images A=control; B=40 µg/mL; C=200 µg/mL; 400 µg/mL MWCNT
Fig 4B. Genotoxicity of MWCNT in NHDF cells

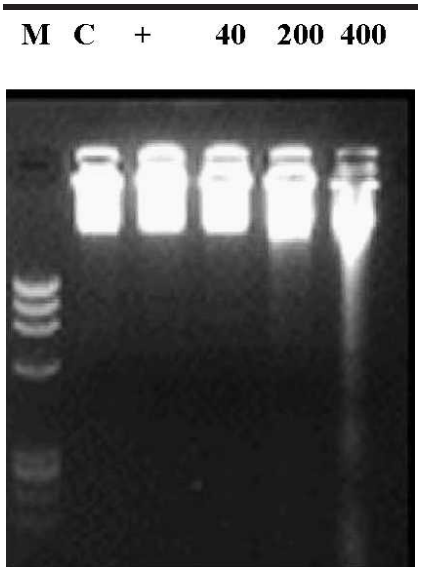


Fig 5. Agarose gel electrophoresis of DNA extracted from NHDF untreated and treated cells with different concentrations of MWCNT for 48 hours. Lane 1=control; Lane 2=40 µg/mL; Lane 3=200 µg/mL and Lane 4=400 µg/mL, M=Molecular marker

lung epithelial cells and A549 cells respectively. These studies significantly demonstrate that the physical form of carbon has a major impact on toxicity. Furthermore, this study determined that molecular structure and topology of a carbon-containing nanomaterial plays an essential role in the characterization and evaluation of its toxicity. The increased toxicity of CNTs, which are highly desirable for use in biological applications, may be due to several reasons: 1) they are better dispersed in aqueous solution and can therefore reach higher concentration of free CNTs at similar weight per volume values[17]; and 2) dispersant agents.[6]

We used two independent test systems to evaluate early cell viability (LDH release and MTT assay); we can exclude a possible interference of MWCNT with the assay. Apoptosis was examined because neoplasia and tumor progression are generally associated with a deregulation of cell proliferation and suppression of apoptosis.[30] A number of DNA-damaging agents are known to induce apoptosis which may be a reflection of severe genotoxic effects triggering the process of programmed cell death.[31] Several hypotheses can be suggested to account for the genotoxic effects of MWCNT, including the formation of adduct and/or damage at the level of DNA or chromosomes. A direct interaction between the particles and the genetic material should be considered.[32] This possibility is sup-

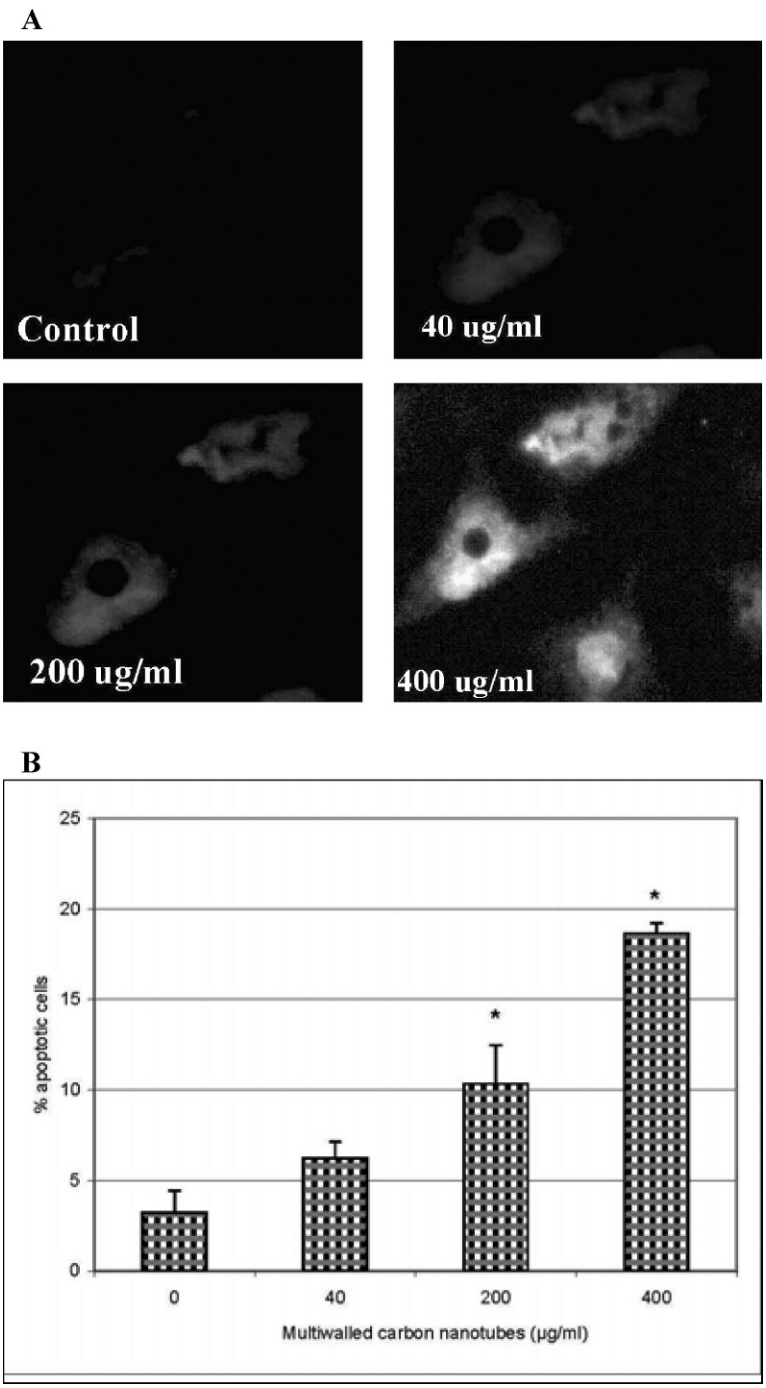


Fig 6A. MWCNTs induction of apoptosis of NHDF cells. Immunofloresence images of NHDF cells treated for 48 hours with MWCNT. Cells treated with oxidized MWCNT stained positive with annexin V-FITC, A=control; B=40 µg/mL; C=200µg/mL; and D: 400µg/mL

Fig 6B. MWCNTs induction of apoptosis of NHDF cells. Graph shows percentage of annexin V positive cells after incubation for 48 hours with 0, 40, 200 and 400 µg/mL of MWCNT. The values represent mean±SD for three independent experiments. Statistical significance is indicated by (*) in the figure for $P<.05$

ported by the data reported by Li et al[31] suggesting that CNT are efficient in interacting with biomolecules with similar dimensions such as DNA.

Our results are in accordance with the studies of Tian et al[16] and Bottini et al[26] which found, in addition to other factors of surface chemistry, functionalization and refinement of carbon-based nanomaterial play a significant role in the induction of cytotoxicity, genotoxicity and apoptosis. Our studies do not imply that CNTs should be abandoned for biological or medical purposes. However, our findings and proposed mechanisms can help to remark the toxicity of carbon nanomaterials while pointing out the importance of surface area. The results of our *in vitro* model could help elucidate the toxicity of engineered nanomaterials, which is more likely dependent on many other factors than concentration. Since nanotechnology is entering into large-scale use, health and safety issues of CNTs should be promptly addressed.[34] However, the biomedical application of carbon nanotubes requires a clear understanding of their fate and toxicological profile after administration. Therefore, further toxicological studies *in vivo* and *in vitro* have to be developed for evaluating its potential toxicity.

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