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Cytogenetic Evaluation of Functionalized Single – Walled Carbon Nanotube in Mice Bone Marrow Cells

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Abstract

With their unique structure and physico-chemical properties, single walled carbon nanotubes (SWCNTs) have many potential new applications in medicine and industry. However, there is lack of detailed information concerning their impact on human health and the environment. The aim of this study was to assess the effects, after intra peritoneal injection of functionalized SWCNTs (f-SWCNT) on the induction of reactive oxygen species (ROS), frequency of structural chromosomal aberrations (SCA), frequency of micronuclei (MN) induction, mitotic index (MI) and DNA damage in Swiss-Webster mice. Three doses of f-SWCNTs (0.25, 0.5 and 0.75 mg/Kg) and two controls (negative and positive) were administered to mice, once a day for five days. Bone marrow and peripheral blood samples were collected 24 hours after the last treatment following standard protocols. F-SWCNT exposure significantly enhanced ROS, increased ($p < 0.05$) the number of SCA and the frequency of micro-nucleated cells, increased DNA damage, and decreased the mitotic index in exposed groups compared to negative control. The scientific findings reported here suggest that purified f-SWCNT have the potential to induce oxidative stress mediated genotoxicity in Swiss-Webster mice at higher level of exposure. Further characterization of their systemic toxicity, genotoxicity, and carcinogenicity is also essential.

Keywords

Single walled carbon nanotube; reactive oxygen species; structural chromosomal aberrations; micronucleus; DNA damage; mitotic index Swiss-Webster mice

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CONFLICT OF INTEREST

None

INTRODUCTION

Single-walled carbon nanotubes (SWCNT) are attractive nanomaterials (NMs) for their unique physicochemical properties and usefulness in many technological applications (Endo et al., 2008; Naya et al., 2012). These SWCNTs are becoming increasingly studied, not only for their possible applications in the electronics, optics, and mechanical materials, but also in biological applications, such as imaging, and drug delivery (Cherukuri et al., 2004; Lacerda et al., 2006), bone cell growth (Saito et al., 2008) and cancer treatment (Gannon et al., 2007). With some estimates indicating a 19% predicted growth of the global nanotechnology industry between 2011 and 2013 (RNCOS, 2011) it is likely that increasing human exposure has occurred. With the rapid advances in SWCNT-based new materials and technologies, there is a growing recognition that a fundamental understanding of their toxicological is imperative (Warheit 2006). The biological and toxicological responses to SWCNTs can vary, depending on form, manufacturing process, route of exposure and dosage. There is little information about the possible human health and environmental impacts of SWCNT.

Previous studies have reported the genotoxicity, cytotoxicity, pulmonary and skin toxicity of SWCNTs (Chatterjee et al., 2014; Xu et al., 2013; Yu et al., 2013; Reddy et al., 2012; Farombi et al., 2014; Naya et al., 2012; Lindberg et al., 2013; Smart et al., 2006). Lindberg et al (2009) in their genotoxicity study with human bronchial epithelial cells, observed an increase in DNA damage when exposed to a mixture of SWCNT and other carbon nanotubes. SWCNTs have been proven to be cytotoxic, including the cell viability loss (Jia et al., 2005), oxidative damage (Pulskamp et al., 2007), inflammation (Brown et al., 2007) and apoptosis (Cui et al., 2005; Alazzam et al., 2010). The cytotoxicity depends on the aggregation degree and pretreatment of SWCNTs samples (Wick et al., 2007). It is also suggested that the cytotoxicity of pristine SWCNTs could be reduced via chemical functionalization (Doyle et al., 2006). In the pulmonary and skin toxicity studies, pristine SWCNTs also show considerable toxicity, including animal death, inflammation and other clinical signals (Smart et al., 2006). Most of the exposure studies focus on the biodistribution and pharmacokinetics of SWCNTs (Lacerda et al., 2006). However, the available peer-reviewed toxicological data for CNTs is rather divergent and sparse to assess their toxic effects to humans and laboratory animals. The reason for these discrepancies is not immediately evident but may depend on experimental protocols and/or interferences with test system used.

CNTs can be chemically modified and/or functionalized with either a hydroxyl or carboxyl or another nanomaterial. Their diameter ranges up to 100 nm (Dresselhans et al., 2001). These pristine CNTs are chemically inert and insoluble in aqueous solutions and therefore appropriate functionalization with green surfactants is important for their use in biological or medical applications. For many applications, CNTs are oxidized in strong acids to create hydroxyl groups and carboxyl groups particularly in their ends, to which biomolecules or other nanomaterials can be coupled. These oxidized CNTs are much more readily dispersed in aqueous solutions and have been coupled to oligonucleotides, proteins or peptides (Bottini et al., 2006).

Interactions of unintentional anthropogenic NMs with cells have been shown to modulate the expression of several cellular macromolecules. The most frequently affected macromolecules are the genes or proteins, which play a role in oxidative stress and DNA damage or injury to the immune system (Schins et al., 2002). Genotoxicity is expressed as varying types of DNA damage (DNA adduct, alkali labile sites, strand breaks) and mutations, ranging from gene to structural or numerical chromosome changes (aneuploidy and polyploidy) Kirsch-Volders et al., 2002; Mateuca et al., 2006). The survival of the damaged cell will depend on the balance between the efficiency of the cellular protection and repair system (antioxidant defenses, base/nucleotide excision repair, mismatch or double strand break repair) and the processes leading to cell death (apoptosis or necrosis) (Muller et al., 2008).

The ability of engineered NMs to interact with biological tissues and generate reactive oxygen species (ROS) has been proposed as possible mechanisms involved in the toxicity (Nei et al., 2006; Pacurari et al., 2008). ROS are well known to play both a deleterious and a beneficial role in biological interactions. Generally, harmful effects on the cell are most often damage of DNA, oxidations of fatty acids in lipids (lipid peroxidation), oxidations of amino acids in proteins and oxidatively inactivate specific enzymes by oxidation of co-factors.

This study assesses the effects, after intra peritoneal (ip) injection, of functionalized SWCNTs (carboxyl groups) on ROS induction and various genotoxicity markers in the mouse model. The question of the health effects of f-SWCNTs is quite acute. The few studies that do observe the effect of ip delivery of CNT are focused on long-term effects of distribution, elimination, and inflammatory responses such as possible mesothelioma induction (Takagi et al., 2008, 2012); lung granulomas (Poland et al., 2008) or pharmacokinetics (Cherukuri et al., 2006). Thus, the acute effects of f-SWCNT are not well understood. This study is the first step towards understanding the genotoxic effects of f-SWCNT in this acute phase. Therefore, the results presented here are of importance for health risk assessment.

MATERIALS AND METHODS

Carbon nanotubes characteristics

Single-walled carbon nanotubes (SWCNTs) were synthesized by NanoLab Inc. (Newton MA, USA) by catalytic chemical vapor deposition (outer diameter of 1.5-3.0 nm, lengths of 15-20 μ m, purity > 95%). After synthesis, SWCNTs were heated under argon (2L/min) at 2000° C with 10° C/min temperature up in order to extract catalyst (Fe-impurities). We started with our purified SWCNTs (purity >95% by TGA) and performed a reflux in sulfuric/nitric (3:1) acid to functionalize the surfaces of these nanotubes. This process resulted in a large concentration of carboxyl (COOH) groups on the nanotube surface. After functionalization, these carboxylated nanotubes have 2-7 wt% COOH by titration (Boehm et al., 1966).

SWCNT morphology and size were determined by scanning electron microscope (SEM) and transmission electron microscopy (TEM). SWCNT dispersions were directly deposited on a

TEM grid and allowed to dry. Samples were directly observed with a TEM component. Surface areas were determined by the isothermal gas adsorption method BET (Brunauer et al., 1938) (Bruising a Micromeritics Flowsorb 2300 (Norcross, USA).

To characterize our system, we performed TEM, SEM examinations of the carbon nanotubes and raman spectrum (Figure. 1: A: TEM, B: SEM, Patlolla et al. (2011) and C: Raman spectra). SWCNTs suspension was correctly dispersed with 1% tween 80 + 0.9% sterile saline as surfactant during sonication. The length of the carbon nanotubes was up to 10 μm for the longer ones (60 mins of sonication) and the diameter was 1.0 nm. Specific surface of carbon nanotube was measured by the classical BET method (Brunauer et al., 1938). The specific surface of long carbon nanotubes for non-purified form was 61 m^2/g and 72 m^2/g for purified form.

Chemicals

Methanol, glacial acetic acid, microscope slides, sodium citrate, sodium chloride, potassium chloride (0.05 M KCl), Giesma stain, May-Grunwald stain, and heparin were purchased from Sigma-Aldrich (St. Louis, MO, USA). They were of analytical grade or highest grade available. Comet assay kit was purchased from Trevigen and DCFH-DA Kit from Cayman Chemical (Ann Harbor, Michigan).

Animal Maintenance

Healthy adult male Swiss-Webster mice (6-8 weeks of age, with average body weight (BW) of 30 ± 2 g) were used in this study. They were obtained from Charles River Laboratories in Wilmington MA, USA. The animals were randomly selected and housed in polycarbonate cages (five mice per cage) with steel wire tops and corn-cob bedding. They were maintained in a controlled atmosphere with a 12h:12h dark/light cycle, a temperature of $22 \pm 2^\circ\text{C}$ and 50-70% humidity with free access to pelleted feed and fresh tap water. The animals were supplied with dry food pellets commercially available from PMI Feeds Inc. (St. Louis MO, USA). They were allowed to acclimate for 10 days before treatment.

Dosing of the mice

The f-SWCNT were suspended and sonicated in a sterile 0.9% saline solution containing 1% Tween-80 (Muller et al., 2005) and were dispersed by ultrasonic liquid processor (Misonix, Long Island NY) at 4°C and 30% amplitude with pulse 1 sec on and 1 sec off during 30 mins for long SWCNT. This suspension showed a majority of SWCNT aggregates with a hydrodynamic diameter of $\sim 1 \mu\text{m}$. The concentration of the suspension was 0.5 mg/ml. Twenty five mice were randomly divided into five groups, five for each group. One group was chosen as positive control (Carbon black, CB, 0.75 mg/Kg), one as the tween-saline control group, and the last three were used as experimental groups. F-SWCNT suspension was administered intraperitoneally to animals at the doses of 0.25, 0.5, and 0.75 mg/Kg BW, one dose per 24 h given for 5 days. Each mouse received a total of five doses at 24 h intervals. Saline (0.9 %) +1% tween-80 was administered to the five animals each of control group in the same manner as in the treatment groups.

Intraperitoneal administration is not the natural route of exposure, however; it is the most commonly used method to study the toxicity of chemicals in bone marrow cells as it tends to maximize chemical exposure to target cells. Treatment by multiple injections was done for two reasons; firstly from pharmacological evidence it indicates the necessity for multiple injections to obtain required doses to the bone marrow (Preston et al., 1987) and secondly in order to induce toxic effects in rodents very high doses are required.

The local Ethics committee for animal experiments [Institutional Animal Care and Use Committee] at Jackson State University, Jackson MS, (USA) approved this study. Procedures involving the animals and their care conformed to the institutional guidelines, in compliance with national and international laws and guidelines for the use of animals in biomedical research (Giles et al., 1987).

Preparation of Homogenate

At the end of the 5-day exposure to f-SWCNTs, bone marrow was excised under anesthesia. The bone marrow was flushed in ice-cold physiological saline. A 10% homogenate of each tissue was prepared separately in 0.05 M phosphate buffer (pH 7.4) containing 0.1 mM EDTA using a motor driven Teflon-pestle homogenizer (Fischer), followed by sonication (Branson Sonifer), and centrifugation at $500 \times g$ for 10 min at $4^{\circ}C$. The supernatant was aspirated and centrifuged at $2000 \times g$ for 60 min at $4^{\circ}C$. The cellular fraction obtained after centrifugation was called 'homogenate' and used for the assays.

Reactive Oxygen Species (ROS) Detection

ROS production was quantified by the 2', 7'- dichlorofluorescein diacetate (DCFH-DA) method (Lawler et al., 2003). DCFH-DA, a redox-sensitive fluorescent probe that emits light in the green spectrum when oxidized, was purchased from Cell Biolabs, Inc (San Diego, CA). DCFH-DA passes through cell membranes where it is cleaved by esterases to DCFH and becomes activated by oxidation. The bone marrow was isolated as described above and loaded one-half of the samples with DCFH-DA ($50 \mu M$) in phosphate buffer saline (PBS), and placed the other half of the samples in PBS alone as a control. Samples were then incubated on a shaker at $37^{\circ}C$ for 30 min. Following incubation, the bone marrow specimen were homogenized (10:1 w/v) in potassium phosphate buffer (pH=7.4). Total protein was measured using the Bradford technique. Fluorescence of the samples was measured using Fluorescence Plate Reader (Turner Biosystems, Sunnyvale, CA, USA). Peak excitation wavelength for oxidized DCFH was 488 nm and emission was 525 nm. Calibration of the fluorometry procedure was accomplished by running standard curves for serial dilutions of fluorescein. Standards for the samples were conducted using serial dilutions of metal contaminant-free hydrogen peroxide (Merck, Darmstadt, Germany) incubated in DCFH-DA with esterase (20 U/ml) for 30 min at $37^{\circ}C$. DCFH oxidation was then calculated per μM H_2O_2 . Samples loaded with DCFH-DA were subtracted by their respective controls to determine the true DCFH oxidation levels in the bone marrow samples.

Aliquot of homogenates were centrifuged at $1000 \times g$ for 10 min ($4^{\circ}C$). The supernatants were re-centrifuged at $1000 \times g$ for 20 min at $4^{\circ}C$, and then the pellets were re-suspended. The DCFH-DA solution with the final concentration of $50 \mu M$ and re-suspension were

incubated for 30 min at 37° C. Fluorescence of the samples was monitored at an excitation wavelength of 485 nm and an emission wavelength of 538 nm using Fluorescence plate reader (Turner Biosystems, Sunnyvale, CA, USA).

Chromosome Aberration Assay

The mice were sacrificed by cervical dislocation 24h after administration of the last dose for chromosome aberration assay. Cytogenetic analysis was performed in bone marrow cells following the protocol of Preston et al (1987), with slight modifications. Experimental animals were injected (i.p.) with colchicine (2mg/kg) 1.5 h prior to sacrifice. Both femora were dissected out and cleaned of any adhering muscle. Bone-marrow cells were collected from both femora by flushing in KCL (0.075 M, at 37 ° C) and incubated at 37 ° C for 25 min. Collected cells were centrifuged at 2000 × g for 10 min, and fixed in aceto-methanol (acetic acid:methanol, 1:3, v/v). Centrifugation and fixation were repeated five times at an interval of 20 min. The cells were resuspended in a small volume of fixative, dropped onto chilled slides, flame-dried and stained the following day with freshly prepared 2% Giemsa stain for 3-5 min, and washed in distilled water to remove excess stain.

Mitotic Index Determination

The mitotic index (number of dividing cells/total number of cells × 100) was used to determine the rate of cell division. The slides prepared for the assessment of chromosomal aberrations were also used for calculating the mitotic index. Randomly selected views on the slides were monitored to determine the number of dividing cells (metaphase stage) and the total number of cells. At least 1000 cells were examined in each preparation.

Micronucleus Test

Mice were sacrificed by cervical dislocation 24 h after the last treatment. The frequency of micronucleated cells in femoral bone marrow was evaluated according to the procedure of Schmid et al., (1976), with slight modifications as reported by Agarwal and Chauhan (1993). The bone marrow was flushed out from both femora using 2ml of Fetal Calf Serum and Hanks Balanced Salt Solution (3:1) and centrifuged at 2000x g for 10 min. The supernatant was discarded. Evenly spread bone marrow smears were stained using the May-Grunwald and Giemsa protocol.

Scoring of Slides

Bone marrow preparations for the analysis of chromosome aberrations in metaphase cells were obtained using the technique by Preston et al (1987). The slides were stained with 2% Giemsa. Well-spread metaphases presenting 40 ± 1 chromosomes were analyzed. One hundred metaphases per animal were screened to a total of 500 metaphases for each treatment and control to obtain the total number of chromosomal aberrations. The mitotic indices were obtained by counting the number of mitotic cells in 1000 cells per animal to a total of 5000 cells per treatment and control. The mitotic index was calculated as the ratio of the number of dividing cells to the total number of cells, multiplied by 100. A total of 5000 cells/treatment were scored, on coded slides to evaluate the frequency of micronucleated cells in bone marrow under an Olympus BX41 microscope.

Comet Assay

Single Cell gel electrophoresis (SCGE) or comet assay was performed using Singh et al. (1988) method with slight modifications. Mice leukocytes (T-lymphocytes) were isolated and re-suspended in phosphate buffer saline. Following isolation the cells were mixed with 0.4% Trypan blue solution. After 15-20 min cells were counted and checked for viability. The remaining cells were immediately used for single-cell gel electrophoresis. In a 2 ml centrifuge tube, 50 μ l of the lymphocyte suspension and 500 μ l of low melting agarose were mixed and 75 μ l of the suspension 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 hr. Slides were removed from lysing 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 epifluorescence microscope and computer based DNA damage analysis software from Loats & Associates (Westminster, MD). The data were based on 100 randomly selected cells per sample, i.e., 50 cells were from each of the two replicate slides. Percent DNA was selected as an indicator of DNA damage.

Statistical Analysis

All data were expressed as means $\pm$ S.Ds. Statistical significance of differences among different groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett multiple-comparisons as a post hoc test. A p-value $\leq$ 0.05 was considered statistically significant.

RESULTS

ROS Detection

The administration of purified f-SWCNT to mice significantly enhanced the ROS level at three tested doses as compared to the negative control animals. Figure 2 summarizes the detection of intracellular production of ROS in Swiss-Webster mice exposed to purified f-SWCNT and controls. The results yielded fluorescence of 20.83 ± 5.89 , 33.3 ± 0.11 , 41.6 ± 11.8 , 66.6 ± 23.5 and 83.3 ± 23.5 for control, carbon black, and 0.25, 0.5 and 0.75 mg/Kg of purified f-SWCNT respectively.

Mitotic Index

The mitotic index was used to determine the rate of cell division. The slides prepared for the assessment of chromosomal aberrations were used for calculating the mitotic index. It was found that the mitotic index significantly decreased as the f-SWCNT doses increased.

Mitotic indices of $11.98 \pm 1.5\%$, $11.5 \pm 0.56\%$, $10.06 \pm 1.65\%$, $7.12 \pm 0.79\%$, 4.55 ± 0.83 , 2.88 ± 0.24 and $2.12 \pm 0.26\%$ were recorded for 0.9% saline + 1% tween-80, positive control carbon black (CB) and f-SWCNT doses of 0.25, 0.5 and 0.75 mg/Kg BW, respectively. Each experiment was done in triplicate. Data represents mean $\pm$ SD. Statistical significance ($p < 0.05$) is depicted as (*). The results of mitotic index are depicted in Figure 3.

Chromosome Aberrations

The metaphase analysis of bone marrow cells revealed various types of chromosomal aberrations (CA), which consisted of chromatid and isochromatid types of gaps, breaks, unions, and fragments. Chromatid gaps and breaks were noted to be more frequent than others Figure 4. Relatively higher frequencies of gaps were observed for all the doses of f-SWCNTs tested. A quantitative assessment of the distribution of breaks and gaps revealed that the distal regions of the chromosomes were more vulnerable to the effects of f-SWCNT.

The results of the chromosomal aberration assay in bone marrow cells after intraperitoneal treatment with f-SWCNT are summarized in Figure 4. The frequency of CA also increased with increasing doses of f-SWCNT, and statistically significant differences ($p < 0.05$) from the negative control were observed. The mean percentages of the induced CAs were $2.3 \pm 0.01\%$, $3.0 \pm 0.03\%$, $8.0 \pm 0.01\%$, $11.7 \pm 0.05\%$, $16.3 \pm 0.03\%$, saline (0.9%) + tween-80 (0.25%) positive control carbon black (CB), and f-SWCNT doses of 0.25, 0.5 and 0.75 mg/Kg BW, respectively.

Micronuclei Induction

The micronuclei (MN) frequencies in bone marrow cells after intraperitoneal treatment with f-SWCNT are summarized in Figure 4. F-SWCNT induced a dose-related increase in micronuclei frequency, and significant differences ($p < 0.05$) from the negative control were observed. The mean percentages of micro-nucleated cells were $2.4 \pm 0.02\%$, $5.2 \pm 0.04\%$, $7.2 \pm 0.032\%$, $8.0 \pm 0.03\%$, 15.6 ± 0.013 , for 0.9% saline + 1% tween-80 (0.25%), positive control carbon black (CB), and 0.25, 0.5, 0.75 mg/Kg BW of f-SWCNT, respectively.

DNA Damage

Percent DNA damage is an important parameter in evaluating the genotoxicity. All the doses of f-SWCNT induced increase in percent DNA damage indicating genotoxicity when compared with negative controls. However, the highest two doses (0.5 mg/Kg and 0.75 mg/Kg BWt) showed statistically significant increase in DNA damage compared negative control. The mean percentages of DNA damage of leukocytes were 4.6 ± 0.1 , 13.3 ± 0.01 , 20.4 ± 0.03 , 39.4 ± 0.3 , 42.4 ± 0.4 for 0.9% saline + 1% tween-80 (0.25%), positive control carbon black (CB), and 0.25, 0.5, 0.75 mg/Kg BW of f-SWCNT, respectively. The results of comet assay on percent DNA damage are illustrated in Figures 5A and 5B.

DISCUSSION

In the present study, we have evaluated the clastogenic/genotoxic potential of functionalized single-walled carbon nanotubes (f-SWCNTs) in mice using CA, MN and comet assays. The results clearly indicated a significant increase of cytogenetic damage in the bone-marrow

cells, due to exposure to f-SWCNTs through intraperitoneal administration. The percentages of aberrant cells in the bone-marrow of Swiss-Webster mice exposed to f-SWCNT showed statistically significant increases as compared to the negative controls. Out of all types of aberrations, chromatid breaks, acentric fragments, and gaps were the predominant forms of CA observed. Chromosome type aberrations such as dicentrics were also observed. We observed a decrease in the mean mitotic index values in f-SWCNT-exposed groups as compared to the negative controls. This could be due to a slower progression of cells from S (DNA synthesis) phase to M (mitosis) phase of the cell cycle as a result of f-SWCNT exposure. Although it is most likely that this impairment in cell cycle progression is associated with f-SWCNT, further experiments are needed to elucidate the biochemical mechanisms involved. Several reports (Siegrist et al., 2014; Cortez et al., 2008; Cortez et al., 2011; Sargent et al., 2012) are in accordance with our study, showing that interaction between carbon nanotubes and DNA produces a conformational change at G-C rich areas on chromosomal and telomeric DNA. DNA intercalation and telomeric binding have been shown to induce damage to chromosomes, the carbon nanotubes at and above certain doses may possibly cause damage. Data from immortalized and primary epithelial lung cells has shown multipolar mitotic spindles, centrosome fragmentation, and errors in chromosome number. Disruption of the mitotic spindle and aneuploidy, in particular, raise concern over the potential carcinogenic effects of carbon nanotubes because the same problems were shown to result from exposure to asbestos (Siegrist et al., 2014). An *in vitro* study of chrysotile asbestos demonstrated that exposure can result in multipolar mitotic spindles due to amplification of the centrosome and a G2/M block. These observations, which were also found in experiments involving SWCNTs, strongly correlate with *in vivo* carcinogenesis and point to the importance of both genotoxicity and culture models in studies of carcinogenesis. The results from our study showed that there was a statistically significant difference in the frequencies of CA, MN induction and percent DNA damage for f-SWCNT exposed animals.

While investigating the mechanisms of NM-induced genotoxicity, several studies (Xu et al., 2013; Schins et al., 2002; Knaapen et al., 2004; Cicchetti et al., 2011) have reported the theory of primary versus secondary genotoxicity. When it is directly related to the exposure of the substance, genotoxicity is referred to as primary. However, secondary genotoxicity is the result of the substance interacting with cells or tissues and releasing factors which cause the adverse effects, such as inflammation and oxidative stress. To investigate the genotoxic potential of f-SWCNT, an intraperitoneal injection (i.p.) method was employed since it was extensively used in the 1970s and 1980s for the elucidation of key dimensions of the fiber (e.g. length and diameter) and for toxicity assessment of various man-made fibers. Although the route of exposure is not realistic for humans however; it is the most commonly used method to study the toxicity of chemicals in bone marrow cells as it tends to maximize chemical exposure to target cells. Takagi et al (2008, 2012) used similar route of MWCNT exposure in p53 heterozygous mice. Their study reported dose-dependent peritoneal mesotheliomas that were shown by an increase in the cumulative incidence of tumors in the mice. Study by Nagai et al (2011) reported that the deleterious effects of nonfunctionalized MWCNTs on human mesothelial cells were associated with their diameter-dependent piercing of the cell membrane that are critical factors in mesothelial injury and carcinogenesis. Above all, for any adverse effect to occur, the number of such fibres must

reach a sufficient level to cause activation of inflammatory cells, genotoxicity, fibrosis and cancer in the target tissue.

The present study provides the evidence of the clastogenic/genotoxic potential f-SWCNTs in bone-marrow cells of Swiss-Webster mice. *In vivo*, we observed a dose-dependent increase in the frequencies of CA and MN in bone marrow cells, which could be ascribed to primary or secondary genotoxicity. We have demonstrated in our present study that f-SWCNT induced intracellular reactive oxygen species (ROS) in the bone marrow cells of mice. DCF fluorescence intensity statistically increased after 5 days exposure to all examined SWCNT doses compared to the negative controls. Since we had demonstrated in this study that f-SWCNT induced ROS in the bone marrow cells, we could not exclude the implication of secondary genotoxicity mechanisms. The results from our study are in accordance with the reports of (Pacurari et al., 2008; Folkmann et al., 2009; Naya et al., 2011), where SWCNT's have been shown to generate ROS and exert toxic effects in different cell types. The increased generation of ROS caused by exposure to particles has been shown for many different forms of fine, ultrafine, and nanoscale particles, including SWCNTs, to be associated with minimal metal contamination (Shvedova et al 2008).

The main molecular mechanism of genotoxicity of nanomaterials is the induction of oxidative stress by free radical formation and ROS are able to cause the oxidation of DNA, and DNA strand breaks (Muller et al., 2010). Several *in vitro* and *in vivo* studies have shown that CNTs exhibit greater genotoxicity than any other nanoparticles which elicited more oxidative stress (Lindberg et al., 2009; Nel et al., 2006; Pacurari et al., 2008; Singh et al., 2009; Muller et al., 2010; Yang et al., 2009). Due to its fibrogenic nature, CNTs might penetrate into cell nucleus through nucleopores, and then destruct the DNA double helix (Pantarotto et al., 2004). Several hypotheses can be suggested to account for the clastogenic/genotoxic effects of f-SWCNT, including the formation of adduct and/or damage at the level of DNA or chromosomes. Carbon nanotubes interact with DNA at G-C rich regions (Li et al., 2006a; Li et al., 2006b). Carbon nanotubes have been observed to form hybrids with tubulin in acellular systems (Dinu et al., 2009). In addition, carbon nanotubes interact with subcellular structures and disrupt the centrosome resulting in aneuploidy (Siegrist et al., 2014; Sargent et al., 2012; Sargent et al., 2010).

The level of DNA damage assessed by comet assay in the present study reported a significant increase in the highest dose at 5 days post-treatment compared to negative controls. These results are in accordance with those reported by Xu et al (2013); Cicchetti et al (2011) and Migliore et al (2010). Similar results were reported in chinese hamster lung fibroblasts (Kisin et al., 2007) in human mesothelial cells (Pacurari et al., 2008) and in human bronchial epithelial cells (Lindberg et al., 2009). Previously, published report from our laboratory (Patlolla et al., 2010) showed a dose-dependent increase in CA, MN and comet tail length for MWCNT in mouse model. MWCNT exposure caused a significant increase in tail DNA indicative of DNA damage when compared to controls. Our results are in accordance with those of Xu et al., (2013); Muller et al., (2008); Yang et al., (2009), and Zhu et al., (2007) reporting a dose-dependent DNA damage exposed to CNTs in human umbilical vein endothelial cells, epithelial cells, mouse embryonic stem cells and mouse embryo fibroblasts cells. A direct interaction between the particles and the genetic material

is also possible. Li et al., (2005) has reported such possibilities suggesting that CNTs are efficient in interacting with biomolecules with similar dimensions such as DNA. Particles may also activate cells to enhance their intracellular production of ROS, of which the stable and diffusible forms such as hydrogen peroxide or lipid peroxidation intermediates could hit nuclear DNA (Schins et al., 2007; Grabinski et al., 2007). Several potential mechanisms can contribute to explain the aneugenic effect of CNTs, including a physical interaction with components of the mitotic spindle during cell division or the interaction with proteins directly or indirectly involved in chromosome segregation (e.g. tubulin, actin) (Muller et al., 2008).

Several hypotheses can be suggested to account for the clastogenic/genotoxic effects of CNT, including the formation of adduct and/or damage at the level of DNA or chromosomes. Rahman et al (2002) reported an induction of MN and apoptosis in hamster fibroblasts exposed to ultrafine titanium dioxide. They suggested that clastogenic events are involved in the formation of these MN. Consistent with this mechanism, significant genotoxicity was shown with comet, MN and hypoxanthine phosphoribosyl transferase assays in human lymphoblastoid cells exposed to ultrafine titanium dioxide (Wang et al., 2007a). It was also demonstrated that crystalline silica exerted a genotoxic effect in human lymphoblastoid cells as reflected by the induction of MN and gene mutations (Wang et al., 2007b).

The increased genotoxicity/clastogenicity of functionalized CNTs which are considered better suited for biological applications, may well be because they are better dispersed in aqueous solution and therefore reach higher concentrations of free CNTs at similar weight per volume values. Similar findings were reported in *in vitro* studies using human T-lymphocytes cells where the non-functionalized or pristine form was found to be less toxic than functionalized or oxidized CNTs (Bottini et al., 2006). Significantly, we find that the physical form of carbon has a major impact on toxicity. CNTs are more toxic than similar chemical amounts of carbon in amorphous carbon black, which is quite non-toxic even at the highest concentration (0.75mg/Kg) (Chatterjee et al., 2014). Thus, the molecular structure and topology is essential for the clastogenicity/genotoxicity of carbonaceous nanomaterials. Our results suggest that SWCNTs indeed can cause genetic damage in a dose-dependent manner. These results are accordance with the findings of Tian et al (2006) and Bottini et al (2006) that besides other factors of surface chemistry, functionalization and refinement of carbon-based nanomaterial play a significant role in the induction of clastogenicity in the target system. SWCNT is shown to cause mitotic disruption in human airway epithelial cells at occupationally relevant doses indicating a greater potential to pass the genetic damage to daughter cells. The disruption of centromere is common in many cancers, suggesting it may play a role in both tumorigenesis and tumor progression (Cortez et al., 2011).

Our study does not imply that CNTs should be abandoned for biological or medical purposes. However, our proposed mechanism of toxicity of these nanomaterials based on their surface properties can assist materials scientists to design/synthesize biocompatible materials. It may also assist toxicologists to further characterize clastogenicity/genotoxicity of dispersed carbon nanomaterials in *in vitro* and *in vivo* studies and caution should be used in the handling and processing of carbon nanotubes.

CONCLUSION

From this study, we have demonstrated that f-SWCNT exposure significantly enhanced ROS production, increased the number of SCA and the frequency of micronucleated cells, increased DNA damage, and decreased the mitotic index in exposed mice compared to negative control. The scientific findings suggest that purified carboxylated SWCNTs has the potential to induce genotoxicity in Swiss-Webster mice through activation of oxidative stress at higher level of exposure. Further characterization and parallel comparison of their systemic toxicity, genotoxicity, and carcinogenicity is also essential.

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A**B** **C****Figure 1.**

(A) Scanning electron microscope (SEM) (B) Transmission electron microscope (TEM) photographs of functionalized single walled carbon nanotubes (Patlolla et al 2011) and (C) Characterization graph of single-walled carbon nanotube.

Figure 2. ROS induction in bone marrow homogenate exposed to purified/functionalized SWCNT. Each experiment was done in triplicate. Data represents mean + SD. Statistical significance ($p < 0.05$) is depicted as (*).

Figure 3.

Effect of purified/functionalized SWCNT on the induction of mitotic index in bone marrow cells of Swiss-Webster Mice. Each experiment was done in triplicate. Data represents mean + SD. Statistical significance ($p < 0.05$) is depicted as (*).

Figure 4.

Effect of purified/functionalized SWCNT on the frequency of chromosomal aberrations and micronucleus induction in bone marrow cells of Swiss-Webster Mice. Each experiment was done in triplicate. Data represents mean + SD. Statistical significance ($p < 0.05$) is depicted as (*).

Figure 5A.

Effect of purified/functionalized SWCNT on the percent DNA damage in peripheral blood leukocytes (T-Lymphocytes) Swiss-Webster Mice. Each experiment was done in triplicate. Data represents mean + SD. Statistical significance ($p < 0.05$) is depicted as (*).

Figure 5B.

Comet Assay images of peripheral blood leukocytes (T-Lymphocyte) of Swiss Webster mice exposed to functionalized SWCNTs. Representative photographs of comets from A- Saline (negative control) ; B-Carbon black (positive control); C- 0.25 mg/Kg; D – 0.5 mg/Kg; E-0.75 mg/Kg.