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Biochemical and histopathological evaluation of functionalized single-walled carbon nanotube in Swiss-Webster mice

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Abstract

With their unique physicochemical properties, single walled carbon nanotubes (SWCNTs) have many potential new applications in medicine and industry. A biomedical application of single-wall carbon nanotubes such as drug delivery requires a fundamental understanding of their fate and toxicological profile after administration. However, the toxicity of SWCNT is barely known when they are introduced into the blood circulation, which is especially vital for their biomedical applications. The aim of this study was to assess the effects, after intra peritoneal injection, of functionalized SWCNTs (carboxyl groups) on reactive oxygen species (ROS) induction and various hepatotoxicity markers (ALT, AST, ALP, LPO and morphology of liver) in the mouse model. We exposed mice to three different concentrations of SWCNTs (0.25, 0.5 & 0.75 mg/kg BW) and two controls (negative and positive). Samples were collected 24 hours after the last treatment following standard protocols. Exposure to carboxylated functionalized SWCNT induced ROS, enhanced the activities of serum amino-transferases (ALT/AST), alkaline phosphatases (ALP) and concentration of lipid hydro peroxide compared to control. Histopathology of exposed liver showed a statistically significant effect in the morphological alterations of the tissue compared to controls. The cellular findings reported here do suggest that purified carboxylated functionalized SWCNT has the potential to induce hepatotoxicity in Swiss-Webster mice through activation of the mechanisms of oxidative stress, which is of sufficient significance to warrant *in vivo* animal exposure studies. However, more studies to clarify the role of functionalization in the *in vivo* toxicity of SWCNTs, are required and parallel comparison is preferred.

Keywords

Single walled carbon nanotube; reactive oxygen species; lipid hydroperoxide; serum aminotransferases; alkaline phosphatases; Swiss-Webster mice; histopathology; hepatotoxicity

INTRODUCTION

Nanotechnology is the manufacture and science of materials with at least one dimension in nanometer scale. Among these nanomaterials, single-walled carbon nanotube (SWCNT) is one of most important, for its unique physicochemical properties and promises in technological applications (Endo *et al.*, 2008). 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, drug delivery (Cherukuri *et al.*, 2004 Lacerda *et al.*, 2006), bone cell growth (Saito *et al.*, 2008) and cancer treatment

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(Gannon *et al.*, 2007). The forecasted increase in manufacturing makes it likely that increasing human exposure will occur. It is recently recognized that the use of nanomaterials may raise new challenges in the safety, regulatory and ethical domains that will require scientific debate. With the rapid advances in SWCNT-based new materials and technologies, there is a growing recognition that a fundamental understanding of the toxicological properties of SWCNTs is imperative (Warheit, 2006). However, the toxicity of SWCNTs is barely known when they are introduced into the blood circulation, which is especially vital for their biomedical applications.

Previous studies have reported the cytotoxicity, pulmonary and skin toxicity of SWCNTs (Smart *et al.*, 2006). Pristine 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). 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 (Sayes *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). In fact, most of the exposure studies just 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.

Many different forms of CNTs are found. They can be chemically modified and/or functionalized with either a hydroxyl or carboxyl or another nanomaterial (Dresselhaus *et al.*, 2001). The 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 (Liu *et al.*, 1998) particularly in their ends, to which biomolecules or other nanomaterials can be, coupled (Bottini *et al.*, 2005), this modify their properties for easy handling (Tasis *et al.*, 2006). These oxidized CNTs are much more readily dispersed in aqueous solutions and have been coupled to oligonucleotides, proteins, or peptides (Bottini *et al.*, 2006)

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

Lipid peroxidation (LPO), the oxidative catabolism of polyunsaturated fatty acids, is widely accepted as a general mechanism for cellular injury and death (Gutteridge and Quinlan, 1983; Halliwell, 1984). LPO and free radical generation are complex and deleterious processes that are closely related to toxicity (Murray *et al.*, 1988]. LOP has been implicated in diverse pathological conditions. The extension of the oxidative catabolism of lipid membranes can be evaluated by several endpoints, but the most widely used method is the quantification of lipid hydroperoxide (LHP), one of the stable aldehydic products of lipoperoxidation, present in biological samples (DeZwart *et al.*, 1999).

Since most chemicals are metabolized in the liver, the hepatocyte is the cell where a free radical attack results in lipid peroxidation, which can be linked to the electron transport chain of chemical metabolism. The methods normally employed for the detection of hepatotoxicity vary with the circumstances of their use. *In vivo* studies are essential to demonstrate a toxic agent that has in fact a demonstrable adverse effect on the liver in a setting of physiological significance. Biochemically, serum enzyme analyses have become the standard measure of hepatotoxicity during the past 25 years (Zimmerman & Seeff, 1970). Measurement of enzyme activities of serum permit detection of hepatotoxicity with far less labor than that required for other tests. The rationale for the use of serum transaminases and other enzymes is that these enzymes, normally contained in the liver cells, gain entry into the general circulation when liver cells are injured (Zimmerman & Seeff, 1970).

This study assesses the effects, after intra peritoneal (ip) injection, of functionalized SWCNTs (carboxyl groups) on ROS induction and various hepatotoxicity markers in the mouse model. The question of the health effects of SWCNTs is quite acute and this study brings new data in a field where the largest proportion of publications have been conducted with pulmonary models. The few studies involving ip delivery of CNTs focus on the possible mesothelioma induction, lung granulomas (Takagi *et al.*, 2008; Poland *et al.*, 2008) or on pharmacokinetics (Cherukuri *et al.*, 2006). The liver is one of the recurrent target organ found after intra venous (iv) or ip injection of SWCNTs in diverse publications and even sometimes the dominant site of accumulation (Cherukuri *et al.*, 2006; Yang ST *et al.*, and Yang *et al.*, 2007). 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 15-30 nm, lengths of 15-20 μm , 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.

SWCNT morphology and size were determined by scanning electron microscope (SEM) and transmission electron microscopy (TEM). SWCNT were directly deposited on a TEM grid and allowed to dry. Samples were directly observed with a TEM. Surface areas were determined by the isothermal gas adsorption method BET (Brunauer *et al.*, 1938) using a Micromeritics Flowsorb 2300 (Norcross, USA).

To characterize our system, we processed TEM observation of the carbon nanotubes (Figure. 1). 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 10 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 m²/g and 72m²/g for purified form.

Chemicals

Methanol, glacial acetic acid and superfrost microscope slides were purchased from Fischer-Scientific Houston TX, USA. Xylene, ethyl alcohol, paraffin wax, hematoxylin-eosin stain, Diagnostic enzyme assay kits were obtained from Sigma, (St. Louis MO, USA) and Lipid Peroxidation kit (Calbiochem).

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—The 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. Thirty five mice were randomly divided into seven groups, five for each group. One group was chosen as positive control (Carbon black, CB, 0.75 mg/kg), the other three were used as the tween-saline control groups, and the last three were used as experimental groups. 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, a suspension of 0.9% saline and 1% tween-80 was administered to the five animals each of control groups in the same manner as in the treatment groups.

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, 1987).

Preparation of Homogenates—At the end of the 5 days exposure to oxidized SWCNT, liver was excised under anesthesia. The organs were washed thoroughly in ice-cold physiological saline and weighed. 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°C . The supernatant was decanted and centrifuged at $2000 \times g$ for 60 min at 4°C . The cellular fraction obtained was called 'homogenate' and used for the assays.

Reactive Oxygen Species (ROS) Detection—ROS production was quantified by the DCFH-DA method (Lawler *et al.*, 2003) based on the ROS-dependent oxidation of DCFH-DA to DCF. An aliquot of homogenates from each exposed group and controls were centrifuged at $1000 \times g$ for 10 min (4°C). The supernatants were re-centrifuged at $1000 \times g$ for 20 min at 4°C , and then the pellet was re-suspended. The DCFH-DA solution with the final concentration of $50\ \mu\text{M}$ 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 after 5 days. The positive control, hydrogen peroxide (30% H₂O₂), was used to assess the reactivity of the probe.

Serum Biochemical Analysis—Following anesthetization, blood specimens were immediately collected using heparinized syringes, and transferred into polypropylene tubes. Each sample was allowed to clot for a minimum of 30 min (maximum 60 min). After clotting, the sample was centrifuged at 750 x g for 10 min. The serum then was pipetted from the cellular elements (erythrocytes, platelets, leucocytes) and transferred to an acid-washed polypropylene tube, properly labeled, and stored at 4° C until ready for analysis. The activities of certain liver enzymes such as alanine (GPT) and aspartate (GOT) aminotransferases, alkaline (ALP) phosphatase in the serum samples were determined using colorimetric assay kits from Sigma (St. Louis MO, USA).

Enzyme Analysis

Serum Aminotransferases—A method by Reitman and Frankel (1957) was followed to determine the activities of alanine or glutamate pyruvate transaminase (ALT/GPT) and aspartate or glutamate oxaloacetate transaminase (AST/GOT) in serum. Human serum contains many different transaminases. The two most commonly determined are ALT/GPT and AST/GOT. These enzymes catalyze transfer of alpha amino groups from specific amino acids to alpha-ketoglutaric acid [AKG] to yield glutamic acid and oxaloacetic or pyruvic acid. The keto acids are then determined colorimetrically after their reaction 2, 4-dinitrophenyl hydrazine [DNP]. The absorbance of the resulting color is then measured at wavelength of approximately 505 nm to take advantage in the absorption that exists between the hydrazones of AKG and the hydrazones of oxaloacetic acid or pyruvic acid.

The reaction for GOT is as follows:

The reaction for GPT is as follows:

Alkaline Phosphatases—To determine the activity of alkaline phosphatase in serum a method by Kay *et al.*, (1930) was followed; it was measured using a Diagnostic kit from Sigma (St. Louis MO, USA). Alkaline phosphatase is also known as orthophosphoric monoester phosphohydrolase, ALP. It is a prototype of those enzymes that reflect pathological reductions in bile flow. This enzyme has been extensively employed in experimentally induced hepatic dysfunction. Alkaline phosphatase refers, not to a single enzyme, but to a family of enzymes with different physico-chemical properties and broad overlapping substrate specificities.

The procedure for alkaline phosphatase depends upon the hydrolysis of p-nitrophenyl phosphate by the enzyme, yielding p-nitrophenol and inorganic phosphate. When made alkaline, p-nitrophenol is converted to a yellow complex readily measured at 400-420 nm. The intensity of color formed is proportional to phosphatase activity.

The reaction for ALP is as follows:

ALP

Lipid hydro peroxides (LPO) assay

The tissues were homogenized (1:8, w/v) in cold HPLC-grade water. Five hundred microliter (500 µl) of the each tissue homogenate was taken in a glass test tube and equal

volume of Calbiochem supplied Extract R saturated methanol was added. The mixture was vortexed for few minutes and 1 ml of cold deoxygenated chloroform was added to the sample mixture, vortexed it thoroughly. The mixture was centrifuged at 1500 x g for 5 min at 0°C (Beckman XL-100K, USA) and bottom chloroform layer was collected. Five hundred µl of the bottom chloroform was mixed with 450µl of chloroform:methanol (2:1) mixture and 50µl of Calbiochem supplied chromogen (thiocyanate ion). Then the mixture was incubated for 5 min and the absorbance of each sample was recorded at 500 nm wavelength using spectrophotometer (2800 Unico spectrophotometer USA). This method directly measures the lipid hydro-peroxides utilizing redox reactions with ferrous ions, the produced hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The produced ferric ions were detected using thiocyanate ion as chromogen. Calbiochem supplied lipid hydroperoxides solution was used as reference standard.

Histopathological Analysis

Liver was surgically removed from mice under diethyl ether anesthesia. Portions of liver were taken and washed with ice-cold normal saline (0.9% NaCl) and 20 mM EDTA to remove blood, cut into small pieces, and fixed immediately in 10 percent phosphate-buffered formalin for 48 hrs. The tissues were then transferred to 70% ethyl alcohol and stored until processed. The tissue specimens (liver) were processed, embedded in paraffin, sectioned at 0.1 µm, and stained with hematoxylin and eosin (H & E) for histological examination under a light microscope. The extent of tissue injury was estimated semi-quantitatively and lesions scored as multi-focal fibrosis/necrosis. At least 10 slides of each sample were scored for liver histology. The liver morphology scored as follows: 0= normal, 1 = mild cellular disruption in less than 25% of field area, 2 = moderate cellular disruption and hepato cellular vacuolation greater than 50% of field area, 3 = extensive cell disruption, hepato cellular vacuolation and condensed nuclei (pycknotic) of hepatocytes in greater than 50% of field area, 4 = extensive cell disruption, hepato cellular vacuolation, pycknotic and occasional central vein injury and 5= extensive cell disruption, multi central vein necrosis and degenerating of liver in more than 50% of field area.

Statistical Analysis—Statistical analysis was performed with SAS 9.1 software for Windows XP. Data was presented as Means ± SDs. One-way analysis of variance (ANOVA) with p-values less than 0.05 were considered as statistically significant.

Results

ROS Detection

The administration of purified/functionalized SWCNT to mice significantly enhanced the ROS level at three tested doses as compared to the control animals. Figure 2 summarizes the detection of intracellular production of ROS in Swiss-Webster mice exposed to purified/functionalized SWCNT and controls. The results yielded fluorescence of 40.56 ± 7.52 , 60.6 ± 6.4 , 160.7 ± 4.6 , 239.71 ± 14.48 and 465 ± 87.7 for control, carbon black, and 0.25, 0.5 and 0.75 mg/kg of purified/functionalized SWCNT respectively.

Lipid Hydro Peroxides (LPO)

Lipid hydro peroxides assay was performed to determine the hydroperoxides levels in liver homogenates of mice exposed to purified/functionalized SWCNT and controls. The LPO standard curve is presented in Figure 3. The LPO levels of the liver were significantly increased in all the treatment groups compared to the control groups. The LPO levels in liver were 16.6 ± 1.84 , 18.63 ± 2.34 , 25.6 ± 2.8 , 29.4 ± 1.99 and 34.23 ± 3.2 µM for saline + tween (control), carbon black (CB), 0.25, 0.5 and 0.75 mg/kg of purified/functionalized SWCNT respectively. Figure 4 represents the experimental data of LPO.

Alanine aminotransferase

Figure 5 presents the experimental data obtained from the analysis of alanine aminotransferases (ALT/GPT). The results yielded optical density readings of 0.565 ± 0.01 , 0.572 ± 0.003 , 0.585 ± 0.01 , 0.610 ± 0.005 , 0.668 ± 0.003 for saline + tween, carbon black, 0.25, 0.5 and 0.75 mg/kg purified/functionalized single-walled CNT's respectively. As shown in this figure there was an increase in the activity of alanine (ALT/GPT) in the serum of Swiss-Webster mice. However, the highest doses 0.5 and 0.75 mg/kg were found to show statistically significant effect in elevating the activity of ALT/GPT when compared to control.

Aspartate Aminotransferase

Figure 6 presents the experimental data obtained from the analysis of aspartate aminotransferases. Functionalized SWCNT exposure resulted in elevating the activity of AST/GOT in dose-dependent manner. However, the increase was not statistically significant when compared to control. Optical density readings of 0.56 ± 0.011 , 0.653 ± 0.004 , 0.693 ± 0.011 , 0.719 ± 0.013 , 0.797 ± 0.04 for saline + tween, carbon black, 0.25, 0.5 and 0.75 mg/kg purified/functionalized SWCNT respectively were obtained.

Alkaline Phosphatases

The activity of alkaline phosphatase exposed to purified/functionalized SWCNT is represented in Figure 7. As shown in the figure there was an increase in the activity of alkaline phosphatases in mice treated with CNT's compared to control. However, the highest concentration 0.75 mg/kg was alone found to show statistically significant effect in elevating the activity of alkaline phosphatases in serum of Swiss-Webster mice. Optical density readings of 0.169 ± 0.019 , 0.188 ± 0.032 , 0.238 ± 0.019 , 0.252 ± 0.013 and 0.403 ± 0.027 for saline + tween, carbon black, 0.25, 0.5 and 0.75 mg/kg functionalized SWCNT respectively were obtained.

Histopathological Evaluation of Liver

Figure 8 and 9 summarizes the histological score for each group. Indices of liver (liver wet weight/body weight) were apparently increased in mice after intraperitoneal injection of purified functionalized SWCNT compared with normal animals. Microscopic examination of the control liver had normal structure and compactly arranged hepatocytes. Sinusoids were scattered randomly all over the hepatocytes and they had uniform morphology along with central vein. However, the mice exposed to 0.25, 0.5 and 0.75 mg/kg bwt. of purified functionalized SWCNT had remarkable morphological alterations. Hepatocytes disruption and hepatocellular vacuolation was observed in microscopic examination of 0.25 mg/kg SWCNT exposed mice liver. In addition to the 0.25 mg/kg SWCNT alterations, pyknotic or karyomegaly (condensed nuclei) of hepatocytes and partial disruption of central vein was observed in 0.5 mg/kg SWCNT exposed mice liver. In addition to the above alterations, degeneration of liver (atrophy) and central vein injury was observed in 0.75 mg/kg exposed mice liver. The results indicated that hepatic injury was successfully induced in mice treated with purified functionalized SWCNT.

DISCUSSION

Nanomaterials and nanoparticles have received considerable attention recently due to their unique properties and applications in diverse biotechnology and life science assays. Despite the rapid progress and early acceptance of nanobiotechnology, the potential for adverse health effects due to prolonged exposure at various concentration levels, in humans and the environment has not yet been established. However, the environmental impact of

nanomaterial is expected to increase substantially in the future. In particular, the behavior of nanotubes and nanoparticles inside the cells is still an enigma, and no cellular responses induced by these nanotubes or particles are understood so far. Nanotoxicology takes the challenge to study the molecular events that regulate bioaccumulation and toxicity of nanotubes and nanoparticles.

In the present study oxidative stress and hepatotoxicity biomarkers were investigated using ROS induction, measurement of lipid hydroperoxide, activities of certain liver enzymes such as ALT/GPT, AST/GOT, ALP and histopathological characterization of liver tissue in mice, exposed to purified/functionalized SWCNT. In this study, we observed that there was a significant increase in the level of ROS in liver homogenate of mice exposed to functionalized SWCNT compared to controls. ROS has been implicated in the toxicity of carbon nanotubes by several authors (Muller *et al.*, 2008; Inoue *et al.*, 2010; Shvedone *et al.*, 2008). Their formation with subsequent cellular damage is considered as the molecular mechanism of carbon nanotube-induced toxicity. Living organisms have developed elaborate systems to defend themselves against toxic agents. Metabolism, distribution, and excretion are linked aspects that are essential in predicting the adverse effects of an agent and thus determining the risk of exposure to it. Although, most cells in the body are capable of metabolism, the primary organ for detoxification is the liver. The liver has a variety of specialized cells that produce enzymes to aid in the metabolism of toxic agents. Liver is often important in tests of oxidative stress because of LPO is a major cause of liver lesions. According to our results, the dominant toxicological mechanism of the intraperitoneally exposed SWCNTs would be oxidative stress. It is a broadly existent phenomenon when cells are exposed to SWCNTs (Lewinski *et al.*, 2008). Although the cytotoxicity of SWCNTs is not always observed in the cell culture studies, oxidative stress is regarded as the cause of the cytotoxicity (Pulskamp *et al.*, 2007). In fact, oxidative stress is taken as an important pathway of toxicity of SWCNTs and other nanomaterials (Stern and McNeil, 2008).

The data obtained for the hepatotoxicity biomarker study clearly show that highest dose 0.75 mg/kg of purified/functionalized SWCNT has significantly increased the activity of serum alkaline phosphatases compared to control. Alkaline phosphatase of the liver is produced by the cells lining the small bile ducts (ductules) in the liver. If the liver disease is primarily of an obstructive nature (cholestatic) i.e., involving the biliary drainage system, the alkaline phosphatase will be the first and foremost enzyme that is found to increase. Serum activity of the enzyme has been reported to increase and is indicative of an impaired hepatic clearance (cholestasis). The results with serum aminotransferases were also found to show an increase in the activity of ALT/GPT and AST/GOT with increasing concentration of purified functionalized SWCNT, however only the highest doses 0.5 mg/kg and 0.75 mg/kg were found to show a statistically significant increase in the activity of ALT/GPT compared to control. Aspartate transferases did not show any statistically significant effect in elevating the activity of the enzyme. Our results are in agreement with the study of Lacerda *et al.*, 2008 with serum suspended multiwalled carbon nanotubes showing higher acute toxicity in mice. Since the toxicity of SWCNTs is dependent on their functionalization degree (Sayes *et al.*, 2006), such hepatic toxicity might be reduced when proper chemical functionalization is adopted to obtain a high functionalization degree of SWCNTs with higher *in vivo* stability. To clarify the role of functionalization in the *in vivo* toxicity of SWCNTs, more efforts are required and parallel comparison is preferred.

To establish the role of oxidative stress as a decisive factor in SWCNT-induced toxicity, the level of lipid hydroperoxides in liver homogenates was performed. Lipid hydroperoxides (LOOHs) are prominent non-radical intermediates of lipid peroxidation whose identification can often provide valuable mechanistic information, e.g., whether a primary reaction is mediated by singlet oxygen or oxyradicals. The results in the present investigation

demonstrated that a dose-dependent increase in the level of lipid hydroperoxides was observed, however, the highest doses of 0.5 and 0.75 mg/kg bwt SWCNT were found to be statistically significant in increasing the level of lipid hydroperoxidase. Our data is also in accordance with several other reports indicating the influence of nanotubes in implicating lipid hydroperoxides in SWCNT toxicity.

Kupffer cells are resident macrophages of the liver and play an important role in its normal physiology and homeostasis as well as participating in the acute and chronic responses of the liver to toxic compounds. Activation of Kupffer cells directly or indirectly by toxic agents results in the release of an array of inflammatory mediators, growth factors, and reactive oxygen species. This activation appears to modulate acute hepatocyte injury as well as chronic liver responses including hepatic cancer. Understanding the role Kupffer cells play in these diverse responses is a key in understanding mechanisms of liver injury (Robert *et al.*, 2007). In our study histopathological evaluation of liver exposed to purified/functionalized SWCNT showed remarkable morphological alterations such as hepatocytes disruption, hepatocellular vacuolation, pyknotic or karyomegaly of hepatocytes and atrophy when compared to control. Although it is most likely that this impairment in hepatotoxicity biomarkers is associated with SWCNT toxicity, further experiments are needed to elucidate the biochemical mechanisms involved. At present, there are no published studies assessing the effect of SWCNT on the hepatotoxicity biomarkers in biological systems.

Elimination of metal residues from SWCNT samples is very hard, even though most of the metals can be removed by refluxing SWCNTs sample in H₂O₂ and acid. Majority of metals loaded on the outside and internal surface of tubes can be removed by traditional purification; however this method is in vain for metals packed inside the surrounding carbon fragments. A few studies reported that metal contents in SWCNTs were partly responsible for the serious oxidative stress (Guo *et al.*, 2007; Liu *et al.*, 2007; 2008; Pulskamp *et al.*, 2007). Recently, a report by Liu *et al.*, focused on the bioavailability of metals in SWCNTs suggests that the encapsulated metals are non-bioavailable for at least two months (Liu *et al.*, 2008). Our purification procedure is almost similar to Liu *et al.*, (2008). The metal impurities left in our SWCNTs samples should be inside the SWCNTs or encapsulated in carbon fragments, therefore they are hardly attributed to the toxicity of SWCNTs. In other words, the oxidative stress is mediated completely from SWCNTs *per se*.

In summary, short-term and high toxicity in mice exposed to functionalized SWCNTs are reported. Serum biochemical changes, ROS induction, increase in the level of LOP and damage to the liver tissue were observed. The proposed main toxicological mechanism is oxidative stress aroused in liver. The high toxicity of functionalized SWCNTs does not implicate that they should be banned for biomedical applications, however improving the dispersion and excretion of SWCNTs by further chemical functionalization is required. Therefore, further toxicological studies *in vivo* have to be developed for evaluating hazards of occupational or environmental exposure to nanomaterials.

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Figure 1.
(A) Scanning electron microscope (SEM) (B) Transmission electron microscope (TEM)
photographs of functionalized single walled carbon nanotubes.

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

Figure 3. Standard curve for Lipid hydro peroxides (LPO) assay where y-axis represents absorbance at 500nm whereas x-axis represents the concentrations of reference standard (nmol).

Figure 4. Effect of purified/functionalized SWCNT on the level of lipid hydroperoxides in liver homogenate of Swiss-Webster Mice. Each experiment was done in triplicate. Data represents mean $\pm$ SD. Statistical significance ($p < 0.05$) is depicted as (*).

Figure 5. Effect of purified/functionalized SWCNT on the activity of Alanine Transferases (ALT) in serum of Swiss-Webster Mice. Each experiment was done in triplicate. Data represents mean $\pm$ SD. Statistical significance ($p < 0.05$) is depicted as (*).

Figure 6.

Effect of purified/functionalized SWCNT on the activity of Aspartate Transferases (AST) in serum of Swiss-Webster Mice. Each experiment was done in triplicate. Data represents mean $\pm$ SD. Statistical significance ($p < 0.05$) is depicted as (*).

Figure 7. Effect of purified/functionalized SWCNT on the activity of Alkaline phosphatases (ALP) in serum of Swiss-Webster Mice. Each experiment was done in triplicate. Data represents mean $\pm$ SD. Statistical significance ($p < 0.05$) is depicted as (*).

Figure 8.

Histopathological Characterization (H & E Staining 1000 X) of liver in *Swiss-Webster mice* exposed to purified/functionalized SWCNT. **A**= Negative Control (CV= Central vein, HP=Hepatocytes), **B**= Positive Control (carbon black) exposed liver (CVD= Central vein damage, HPV= Hepato cellular vacuolation, NC=Necrosis), **C**= 0.25 mg/kg (CVD= Central vein damage and NC= Necrosis), **D**= 0.5 mg/kg (CVD= Central vein damage, HPV= Hepato cellular vacuolation and PC=Pycknotic) and **E**= 0.75 mg/kg (C= Central vein damage, HPV=Hepato cellular vacuolation, NC=Necrosis and PC=Pycknotic). Each point represents a mean value and standard deviation of ten values.

Figure 9.

Effect of purified/functionalized SWCNT on morphological alterations in liver tissue. Each point represents a mean value and standard deviation of ten values. Statistical significance ($p < 0.05$) is depicted as (*).