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PULMONARY TOXICITY ASSESSMENT AND COMPARISON OF SOME CARBON NANO PARTICLES FOLLOWING INTRA-TRACHEAL INSTILLATION IN RATS

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ABSTRACT

***Introduction**: Carbon nanotubes are major building blocks of this new technology. These are considered to represent the fraction of airborne material that can enter the human respiratory tract. Much attention has been paid on carbon nano materials, due to their small size and extraordinary physicochemical properties. In this study, we have used intratracheal instillation, an accepted route of exposure commonly used to screen nanotubes for potential pulmonary toxicity. **Method**: Groups of male Wistar albino rats of 6-9 weeks old were selected and housed in polypropylene cages in a room where the congenial temperature was 26°C and 12 hrs light and dark cycles were maintained. Fine particle suspension of CNP was prepared with a nontoxic dispersion vehicle for instillation into rat lungs. The BAL fluid was collected from all the above untreated and particle exposed rats at 24 h, 1 week, 1 month and 3 months post instillation periods. **Results**: Transient increase ($p<0.05$) in BAL fluid micro protein levels was also observed in lungs exposed to carbon nanoparticles and quartz exposed rats at 24 h period, which gradually decreased in 1 week, 1 month and 3 months periods. Exposures of carbon nanoparticles to rats resulted in a transient dose dependant increase ($p<0.001$) in BAL fluid MDA values at 24 h post exposure period and were gradually decreased with other time periods. **Conclusion**: Carbon nanotubes produced significantly greater toxicity compared to quartz particles. This is very specifically dangerous for the chronic exposures of workers making and using nanoparticles in manufacturing plants.*

KEY WORDS

Nanotubes, Wistar albino rats, intra tracheal and toxicity.

INTRODUCTION

Nanotechnology has emerged at the front position of science research and Technology development. Carbon nanotubes (CNTs) are major building blocks of this new technology. They possess unique electrical, mechanical, and thermal properties, with potential wide applications in the electronics, computer, aerospace, and other Industries. CNTs exist in two forms, single-wall (SWCNTs) and multi wall (MWCNTs). They are manufactured predominately by electrical arc discharge, laser ablation and chemical vapor deposition processes; these processes involve thermally stripping carbon atoms off from carbon-bearing compounds. An individual CNT molecule is about 1 nm in diameter and several microns long (*Ajayan et al 1997*). The two biologically relevant size fractions currently used are the inhalable (< 10-lm aerodynamic diameter) and respirable fractions (< 4.5-lm aerodynamic diameter). These are considered to represent the fraction of airborne material that can enter the human respiratory tract (inhalable) and can penetrate

beyond the ciliated airways (respirable). Carbon nanofibers could be defined as sp2-based linear filaments with diameter of ca. 100 nm that are characterized by flexibility and their aspect ratio (above 100). Materials in a form of fiber are of great practical and scientific importance. The combination of high specific area, flexibility, and high mechanical strength allow nanofibers to be used in our daily life as well as in fabricating tough composites for vehicles and aerospace. As the production and applications of carbon nanomaterials expand, potential human exposures will also increase. In occupational settings, these CNT may release into the surroundings in aerosol form (*Maynard et al., 2004)*.

Inhaled MWCNT, which deposit in the lungs, are transported to the parietal pleura, the respiratory musculature, liver, kidney, heart and brain in a singlet form and accumulate with time following exposure. The tracheobronchial lymph nodes contain high levels of MWCNT following exposure and further accumulate over nearly a year to levels that are a significant fraction of the lung burden 1 day post exposure. (*Robert R Mercer et al 2013*)**.** Inhalation of pathogenic fibers is associated with fibrosis of the lung, i.e., asbestosis, lung cancer, and mesothelioma (i.e., cancer of the mesothelium lining the pleural and peritoneal cavities). The proposed mechanisms of lung disease caused by fibers are numerous and include oxidative stress, inflammation, and both direct and indirect genotoxicity (*Bernstein et al., 2005; Kane, 1996*). If they are sufficiently thin, even very long fibers can readily penetrate to the distal lung, traveling into the lung by aligning their longitudinal axis with the airstream. The potential hazards related to inhalation of these carbon nanotubes are unknown. Recent experimental studies indicate that inhaled CNT may produce significant lung toxicity in rats and toxicity potential increases with decreasing particle size. (*Shvedova et al 2008*).

There is also incomplete toxicological information on carbon fibers (CNF) and on graphite particles(MWCNT and CNR). The consequences of several epidemiological studies indicated an greater than before incidence of pneumoconiosis among workers exposed to graphite-containing dusts. These include coal miners and millers, carbon electrode manufacturers, and molders at industries . The lung related disorders are reported in both synthetic and natural graphite workers resembles coal workers. Correspondingly, there is a scantiness of data on the health effects related to carbon nanomaterials exposures. As a consequence, no exposure strategy have yet been proposed. So this study was designed as a evaluate preliminary screening to determine whether the MWCNT,CNF and CNR particles impart significant toxicity in the lungs of rats and more importantly, how the activity of the carbon-derived particulates compares with in them and other reference particulate materials like Quartz. Thus, the aim was to assess in rats, using a well-developed, short-term pulmonary bioassay, the acute pulmonary toxicity effects of intratracheally instilled MWCNT,CNF and CNR samples and to compare the lung toxicity of these samples with a low-toxicity particulate (carbonyl Iron as negative control) and a cytotoxic particulate (Quartz as positive control) sample, and to connection the results of these instillation studies with data up to that time generated from various inhalation studies with quartz particles in the form of crystalline silica and with carbonyl iron particles as the inhalation/instillation bridge material.In this study, intratracheal instillation exposure was used as a surrogate for inhalation exposure. In this study, we have used intratracheal instillation, an accepted route of exposure

commonly used to screen nanotubes for potential pulmonary toxicity (*Driscoll et al., 2000; Leong et al., 1998*).

MATERIALS AND METHODS

Experimental Animals

Groups of male Wistar albino rats (Mahavir enterprises, Hyderabad, India) of 6-9 weeks old at study start (mean weights in the range of 220–275 grams) were selected and housed in polypropylene cages in a room where the congenial temperature was 26°C±2°C and 12 hrs light and dark cycles were maintained. The animals were allowed to acclimatize to the environment for 7 days and supplied with a standard pellet diet and water *ad libitum.* All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee of Kakatiya University.

Particle types- Characterization

Carbonyl iron particles (The particle sizes range from 0.8 µm to 3.0 µm.; >98% purity) and Quartz particles in the form of crystalline silica (>230 mesh; 58-68µm; 99.94% purity) were obtained from Sigma, USA and SD fine chemicals, Mumbai, India respectively. Graphitized iron free composed of conical platelets 99.9%(D*L100nm*20-200 µm) (coded as CNF),Carbon nanotubes multiwalled >90% carbon basis (D*L 110-170 nm *5-9 µm of size) (coded as MWCNT) and Carbon nano rods (coded as CNR)

Preparation of fine-dust suspensions

Fine particle suspension of CNP was prepared with a nontoxic dispersion vehicle for instillation into rat lungs (*Driscoll et al., 2000; Leong et al., 1998*). The products are extremely difficult to disperse even in the presence of a dispersing agent. All the nanoparticles suspensions were prepared in phosphate buffer saline (PBS) + 1% Tween 80 at a concentration of 10 mg/ml by briefly shearing (2 min in a small glass homogenizing tube) and subsequently sonicating (1-2 min) CNP samples. All the samples with different concentrations were re-sonicated on the day of dosing before the instillation. Each sample was mixed well before an aliquot was drawn for instillation.

General experimental design: The fundamental features of this pulmonary bioassay are (1) dose response evaluation and (2) time course assessments to determine the sustainability of any observed effect. Thus, the major endpoints of this study were the following: (1) time course and dose/response intensity of pulmonary inflammation and cytotoxicity, (2) alveolar macrophage function at 24-hour, 1-week, 1-month, and 3-month recovery periods postexposure (pe), and (3) histopathological evaluation of lung tissue at 24-hour, 1-week, 1-month, and 3-month recovery periods postexposure (pe) .

Intratracheal instillation:

The rats were anesthetized with 3 to 5% Lignocaine in a small chamber and individual rats were secured on an inclined plastic platform and anesthetization continued via a small nose cone. The trachea was exposed by a 1-cm incision on the ventral neck skin for instillation of the dust suspension (*Lam et al., 2002*). The intratracheal fast instillation/nebulization procedure for rats used by *Leong et al 1998* was modified to ensure that instilled material was delivered into the lungs of rats with a good distribution (*Lam et al., 2004*). A small hole was made in the trachea close to the larynx and a 24-gauge plastic catheter was inserted through the hole to the distal end of the trachea; the blunted needle was then inserted inside the plastic catheter. A 1-ml syringe prefilled with 200 µl of air and 50 µl of saline was then connected to the free end of silicone tubing to rapidly propel the test sample from the tubing and needle into the lungs. The neck incision was then sutured,

swabbed with Povidone iodine, and anesthetized with a drop of lidocaine. The rats recovered and were active within 10-15 min after removal from the inhalation anesthetic. The incision healed within 3-4 days, and the animals were observed daily until their scheduled termination

Study design (*Warheit et al., 2004*).

Groups of rats were instilled intra-tracheally with single dose of 0.2 mg/kg, or 1 mg/kg or 5 mg/kg of CNF, MWCNT and CNR, carbonyl Iron (CI) and quartz-crystalline silica particles (Q). All the particles were prepared in a volume of 1.0% Tween 80 and phosphate-buffered saline (PBS) and subjected to Polytron dispersion. Group of PBS-Tween, Carbonyl Iron and Quartz instilled rats were served as control respectively.

Group 1: Received PBS+ 1% Tween 80 [solvent control];
Group 2: Received carbonyl Iron (1mg/kg); [negative control]
Group 3: Received carbonyl Iron (5mg/kg); [negative control]
Group4: Received CNF (1mg/kg);
Group 5: Received CNF (5mg/kg);
Group 6: Received MWCNT (1mg/kg);
Group 7: Received MWCNT (5mg/kg);
Group8: Received CNR (1mg/kg);
Group9: Received CNR (5mg/kg);
Group 10: Received Quartz (1mg/kg); [positive control]
Group 11: Received Quartz (5mg/kg) [positive control]

Bronchoalveolar lavage studies :

Collection of bronchoalveolar lavage (BAL) fluid and biochemical analyses:

The BAL fluid (15-20ml) was collected from all the above untreated and particle exposed rats at 24 h, 1 week, 1 month and 3 months post instillation periods (*Warheit et al., 2004*). The lungs of sham and particulate-exposed rats were lavaged with a warmed PBS solution as described previously (*Warheit et al 1991*). Briefly, the lungs were removed from the thoracic cavity and lavaged with a PBS solution that had been heated to 37°C. A 10-ml syringe was used to fill the lungs with 8 ml of PBS per wash. The lungs were gently manipulated after insertion of the PBS and during the withdrawal of lavage fluid. The first recovered 12 ml of lavaged fluids was used for BAL fluid analyses, and an additional 30 ml was collected for cell counts and differentials. The number of animals used in each group at each post exposure period were six (n=6).

All biochemical assays were performed on BAL fluids to estimate Lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and lavage fluid protein and extent of lipid peroxidation products (malondialdehyde, MDA).

Lactate dehydrogenase is a cytoplasmic enzyme and is used as an indicator of cell injury. Alkaline phosphatase activity is a measure of Type II alveolar epithelial cell secretory activity, and increased ALP activity in BAL fluids is considered to be an indicator of Type II cell toxicity. Increases in BAL fluid protein concentrations generally are consistent with enhanced permeability of vascular proteins into alveolar regions.

Collection of lungs from particle exposed rats

The lungs of particles exposed rats were collected from all the above groups at 24 h, 1 week, 1 month and 3 months post instillation periods. Additional groups of animals were instilled with the particle types listed above as well as PBS-Tween. These studies were dedicated for lung tissue analysis and histopathological evaluations of the lower respiratory tract. Similar to the BAL fluid studies, the histopathological evaluations of lungs were carried out at 24-hour, 1-week, 1 month and 3 months recovery periods

Methods of analysis

All biochemical assays were performed on BAL fluids for the estimation of Lactate dehydrogenase (Ecoline, Merck, Mumbai, India), alkaline phosphatase (ALP;Autospan, Span diagnostics, Ltd, India) using respective diagnostic kit method and total proteins **(***Lowry et al., 1951*) and extent of lipid peroxidation product (Malondialdehyde, MDA) using (*Ohkawaka et al 1979*) method.

The detailed procedures of estimation of various biochemical parameters were as follows

Estimation of Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) is zinc containing intracellular enzyme concerned with reversible oxidation of pyruvate to lactate, involves in glycolytic cycle. The rate of the decrease in NADH concentration is determined photometrically and is directly proportional to the LDH activity in the sample material. The reaction velocity is determined by a decrease in absorbance at 340 nm resulting from oxidation of NADH (*Varley et al., 1980*).

Reagents:

1. Phosphate buffer pH 7.4 - 50 mmol/l and Pyruvate- 0.6mmol/l
2. Goods buffer (NADH) 0.18mmol/l with pH 9.6

Working reagent: Mix reagent 1 and reagent 2 in the ratio of 4+1.

Procedure: To 1 ml of working reagent, 0.1 ml of serum / BAL fluid was added and mixed. After 1 minute, read the decrease in absorbance every minute for 3 minutes using UV spectrophotometer at 340 nm. Calculate the LDH activity in the test sample using the following equation,

LDH activity (IU/L) = ΔA/min * 8095.

One International Unit (IU) of LDH is the enzyme that 346xidize one micromole of NADH per minute at 25°C in 0.1 M phosphate buffer at pH 7.4 (*Wroblewski et al, 1955)*.

Estimation of Alkaline Phosphatase (ALP) Activity

At pH 10.3, ALP catalyses the hydrolysis of colourless p-Nitrophenyl Phosphate (pNPP) to yellow coloured p-Nitrophenol and phosphate. Change in absorbance due to yellow color formation is measured kinetically at 405nm and is proportional to ALP activity in the sample.

P-Nitrophenyl Phosphate + water -----^{ALP}---→ p-Nitro phenol + phosphate

Reagents:

1. AMP (2-amino-2-methyl-1 propanol) buffer comprising AMP (300 mM), Magnesium acetate (2mM), Zinc sulphate (0.8mM) and Chelator (qs).
2. pNPP substrate (pNPP + stabiliser) 10mM

Working reagent:

Mix 1 vial of reagent 2+ 5.5 ml of reagent 1.

Procedure:

To 1 ml of working reagent, 0.1 ml of serum / BAL fluid was added and mixed. After 1 minute, read the decrease in absorbance every minute for 3 minutes using UV spectrophotometer at 405 nm. Calculate the ALP activity in the test sample using the following equation

ALP activity (IU/L) = ΔA/min * 2712.

Estimation of Total Proteins

BAL fluid total protein content was estimated by the method of (*Lowry et al. 1951*). Proteins form chromophoric complex with phenol reagent, which was measured at 610 nm.

Reagents:

1. Alkaline Reagent: 2g of sodium carbonate was added to 100 ml of 0.1N sodium hydroxide solution.
2. Alkaline Mixture: To 100 ml of alkaline reagent 1 ml of 4% aqueous copper sulphate solution was added, this was prepared freshly.

3. Phenol reagent (Folin and Ciocalteu's Reagent) : Diluted by dissolving 0.5 ml of phenol reagent in 4 ml of distilled water before use and stored in refrigerator.

Procedure:

To 0.1 ml of BAL fluid 1 ml of alkaline mixture reagent was added and kept for 10 minutes, then 4 ml of phenol reagent was added, heated at 55°C for 5 minutes for color development and then cooled for 1 minute. Reading was taken against blank at 610 nm using spectrophotometer. The protein content was calculated from standard curve prepared with bovine serum albumin and expressed in terms of gm/ml.

Estimation of Lipid Peroxides

The amount of lipid peroxidation products present in the BAL fluid was estimated by the thiobarbituric acid reactive substances (TBARS) method (*Ohkawaka et al 1979*), which measures the malondialdehyde (MDA) reactive products by using UV-Visible spectroscopy.

Procedure: To 0.5 ml of serum / BAL fluid 0.5 ml of 30% trichloro acetic acid (TCA) was added to precipitate the proteins and vortexed for 30 sec. Clear supernatant was taken after centrifuging at 3000 rpm for 10 min. To the supernatant, 500µl of 1%TBA solution and 500µl of

water was added and this solution was heated for 1hr at 98°C. Cool the solutions to room temperature and kept them in ice for 5 minutes. Then read the pink color at 532 nm using spectrophotometer. Standard graph was plotted using TEP (1, 1, 3, 3-tetra ethoxy propane).

Statistical analysis

For analysis, each of the experimental values was compared to its corresponding sham control value for each time point. A one-way analysis of variance (ANOVA) and Bartlett's test were calculated for each sampling time. When the *F* test from ANOVA was significant, the Dunnett test was used to compare means from the control group and each of the groups exposed to particulates. Significance was judged at the 0.05 probability level.

All the experimental values were expressed as mean ± SD and were compared with sham control value at each time point. One-way analysis of variance (ANOVA) and Dunnett test were used to compare means from the control group and each of the groups exposed to particulates and the statistical significance was judged at the 0.05 probability level. Significance was indicated by: *$p<0.05$, $^{a}p<0.01$,$^{\#}p<0.001$ versus control (n=6).

RESULTS

The intra-tracheal (IT) instillation of all the Carbon nanoparticles does not produce any mortality in rats. The Carbon nanoparticles were well dispersed in the medium and sonication of suspension prevents the agglomeration of necrosis.

Pulmonary Injury and Inflammation-LDH levels

Similar to the quartz, exposures of CNF,MWCNT and CNR to rats resulted in a transient dose dependant increase in BAL fluid lactate dehydrogenase (LDH) values at 24 h post exposure period and were gradually decreased with other (1week, 1 month & 3month) time periods (Fig 4.1 & Table 4.1). The increase in BAL fluid lactate dehydrogenase values at 24 h, 1 week, 1moth and 3 month of post exposure of CNF,MWCNT and CNR were significant with 0.2mg/kg ($p<0.05$), 1mg/kg ($p<0.01$) and 5mg/kg ($p<0.001$) than control. Exposure of carbonyl iron at different doses (0.2, 1, 5mg/kg) to rats resulted insignificant change ($p>0.05$) in BAL fluid LDH levels at different post exposure intervals

Table 1: Mean ± SD BAL fluid LDH levels (IU/L) in carbon nanoparticles instilled rats

Group/Post instillation period	1 Day	1 Week	1 Month	3 Months
PBS+1%Tween 80	4.52 ± 1.65	3.98± 1.76	3.46 ± 1.13	3.18 ± 1.26
C Iron (1 mg/kg)	4.87 ± 1.12	4.42 ± 2.04	3.96± 1.31	3.41 ± 1.39
C Iron (5 mg/kg)	5.21 ± 1.48	4.98 ± 1.14	4.24 ± 1.42	3.88 ± 0.93
CNF 1mg/kg	29.76 ± 4.08	22.59 ± 5.80	11.41± 3.09	9.14 ± 2.54
CNF 5mg/kg	98.34± 13.94	75.43± 11.40	38.98± 8.14	15.58± 3.51
MWCNT 1mg/kg	19.56 ± 3.08	16.59 ± 3.80	9.41± 2.09	7.14 ± 1.54
MWCNT 5mg/kg	88.34± 11.94	63.43± 9.40	31.68± 6.14	12.52± 2.51
CNR 1mg/kg	14.39 ± 1.41	10.95 ± 1.26	8.02 ±2.08	6.77 ± 1.31
CNR 5mg/kg	61.54 ± 4.99	46.05 ± 4.34	24.32 ± 3.72	9.58 ± 2.23
Quartz 1mg/kg	10.71± 2.52	8.25± 2.26	7.11±3.30	5.76± 2.47
Quartz 5mg/kg	83.12± 25.12	68.14± 22.82	19.74± 4.41	8.58± 2.98

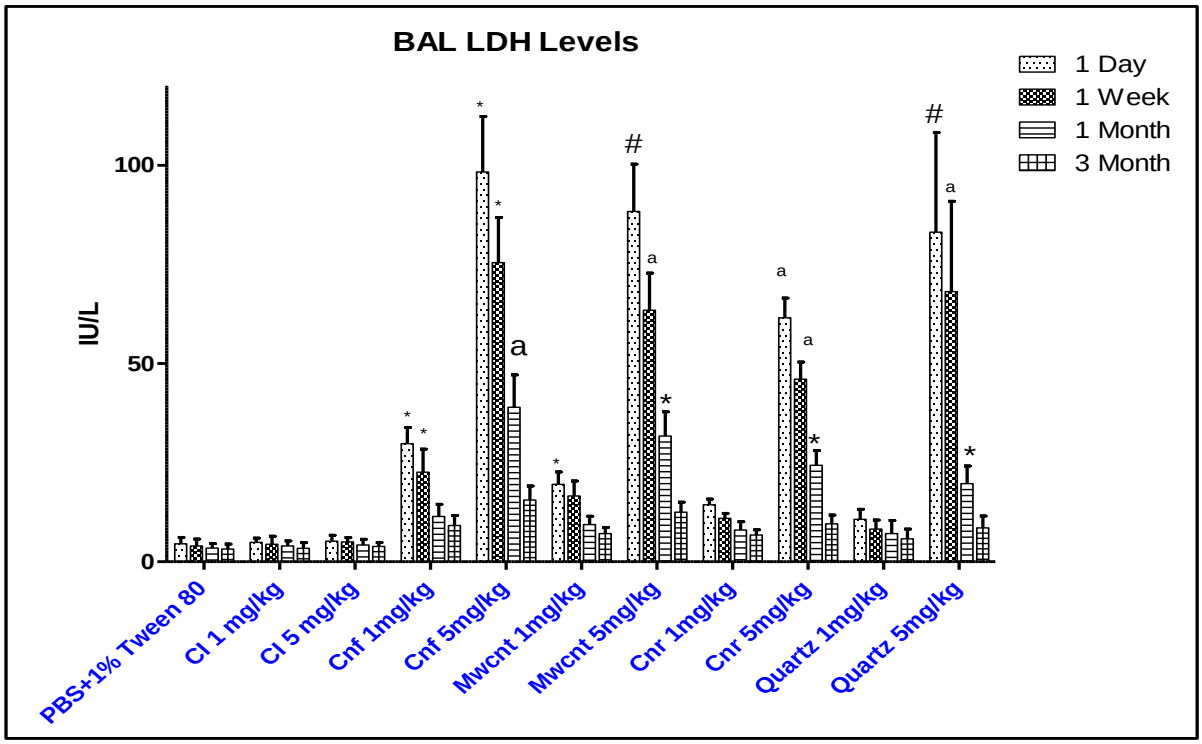

Figure 1: BAL fluid LDH levels (IU/L) in carbon nanoparticles instilled rats

Alkaline Phosphatase (ALP) levels

Exposures of different doses of CNF, MWCNTand CNR to rats produced a transient dose dependant increase in BAL fluid ALP levels at 24 h post exposure period and were gradually decreased with other time periods (Fig 4.2 & Table 4.2). Similar to the quartz, the BAL fluid ALP values at 24 h, 1 week, 1 month and 3 months of post exposure of MWCNT were significantly higher with 0.2mg/kg ($p<0.05$), 1mg/kg ($p<0.01$) and 5mg/kg ($p<0.001$) doses than sham control rats. No significant change ($p>0.05$) in BAL ALP levels were observed with exposure of carbonyl iron at different doses (0.2, 1, 5mg/kg). These above results suggest that intratracheal instillation exposures of these particle types produced a short-term, pulmonary inflammatory response.

Table 2: Mean ± SD BAL fluid ALP levels (IU/L) in carbon nanoparticles instilled rats

Group/Post instillation period	1 Day	1 Week	1 Month	3 Months
PBS+1%Tween 80	4.79±0.47	3.91 ±0.46	3.83 ±0.26	3.62 ±1.23
C Iron (1 mg/kg)	4.83±0.36	4.13 ±0.13	3.79±0.45	3.65±0.56
C Iron (5 mg/kg)	4.97±0.47	4.36 ±0.36	4.04 ±0.36	3.81 ±0.16
CNF 1mg/kg	9.19±0.94	8.76 ±0.84	8.65 ±0.47	8.43 ±0.17
CNF 5mg/kg	16.52±1.32	13.96 ±0.78	12.89 ±0.79	11.13 ±1.69
MWCNT 1mg/kg	8.86±0.87	8.64 ±0.78	8.45 ±0.79	7.93 ±0.69
MWCNT 5mg/kg	14.88±0.56	13.26 ±0.25	12.74 ±0.15	10.83 ±.48
CNR 1mg/kg	8.35±0.45	8.28±1.23	7.72±1.12	7.36±0.47
CNR 5mg/kg	13.01±0.14	12.82±0.48	11.56±0.46	9.42±0.13
Quartz 1mg/kg	9.56±0.13	7.53 ±0.46	6.92 ±1.45	6.67 ±0.89
Quartz 5mg/kg	13.45±1.05	10.68 ±1.02	9.88 ±0.16	9.14 ±79

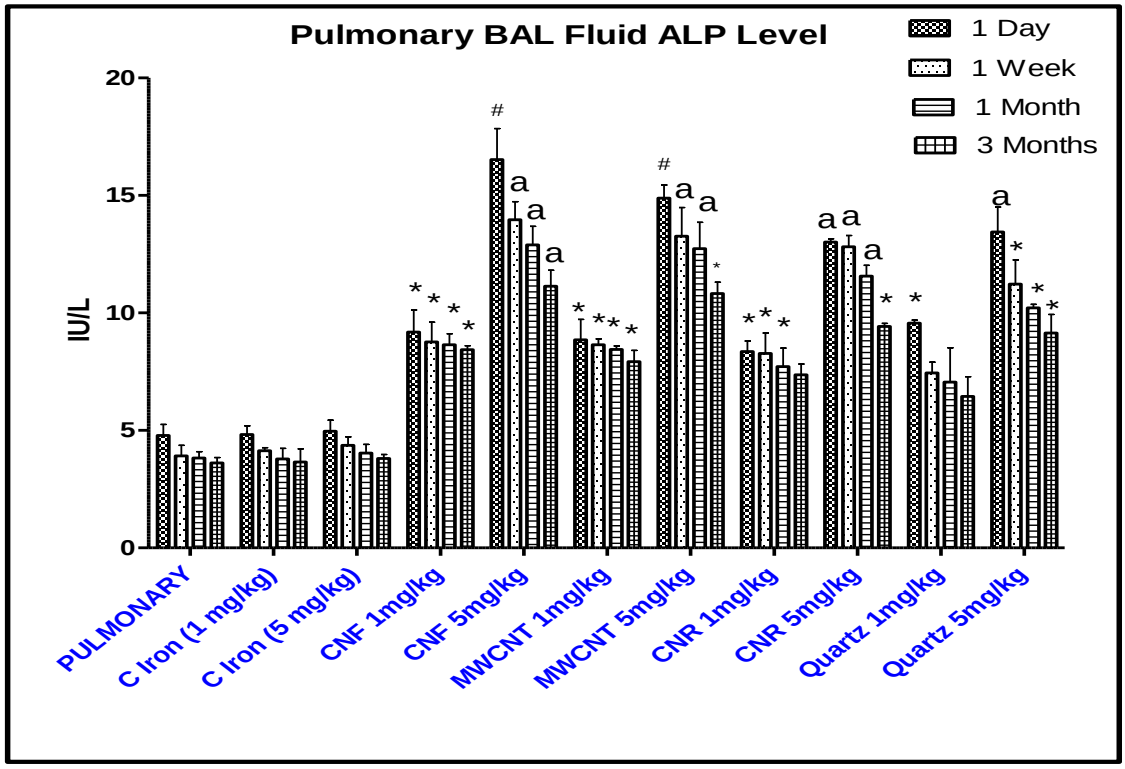

Figure 2: BAL fluid ALP levels (IU/L) in carbon nanoparticles instilled rats

Total protein levels

Transient increase ($p<0.05$) in BAL fluid microprotein (MTP) levels was also observed in lungs exposed to carbon nanoparticles and quartz exposed rats at 24 h period, which gradually decreased in 1 week, 1 month and 3 months periods (Fig 4.3 & Table 4.3). The increase in MTP levels was more significant with 5mg/kg of these carbon nanoparticles exposed rats. Similar to the quartz, lower doses of MWCNT (0.2mg/kg) does not produce any significant change in ($p>0.05$) in BAL MTP levels.

Table3: Mean ± SD BAL fluid Poteine levels (IU/L) in carbon nanoparticles instilled rats

Group/Post instillation period	1 Day		1 Week		1Month		3Month	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PBS+1%Tween 80	595.12	25.13	572.35	21.14	547.86	15.36	521.27	13.96
C Iron (1 mg/kg)	606.41	15.23	589.28	24.96	562.92	14.39	543.33	17.23
C Iron (5 mg/kg)	611.29	18.64	602.37	36.00	587.12	16.98	551.00	15.64
CNF 1mg/kg	889.36	19.12	757.42	17.26	683.34	25.36	643.33	22.95
CNF 5mg/kg	1436.29	40.16	1288.58	35.12	891.52	27.48	833.49	26.34
MWCNT 1mg/kg	845.69	24.12	732.38	23.45	661.44	22.96	625.13	21.82
MWCNT 5mg/kg	1387.35	37.46	1255.19	36.48	863.39	24.95	806.26	26.34
CNR 1mg/kg	718.32	25.12	706.15	25.36	637.42	21.36	605.51	25.34
CNR 5mg/kg	1237.42	39.46	1159.83	32.45	780.55	28.36	751.69	26.83
Quartz 1mg/kg	691.42	32.15	673.18	16.41	641.54	23.45	623.87	19.36
Quartz 5mg/kg	1294.19	38.15	1216.67	36.25	878.46	19.68	802.53	27.28

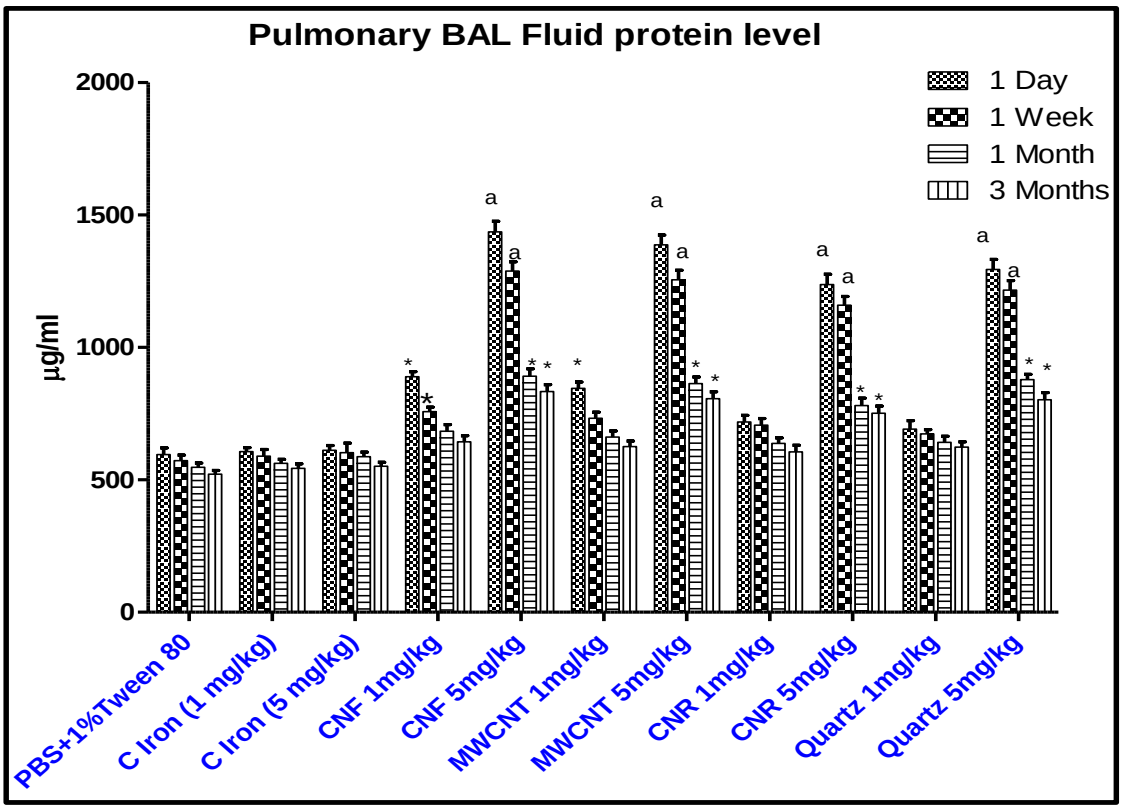

Figure 3: BAL fluid Proteine levels (IU/L) in carbon nanoparticles instilled rats

Extent of lipid peroxidation-MDA estimation

The extent of lipid peroxidation (MDA; Malondialdehyde) in BAL fluid was estimated in all post exposure periods. Exposures of carbon nanoparticles to rats resulted in a transient dose dependant increase ($p<0.001$) in BAL fluid MDA values at 24 h post exposure period and were gradually decreased with other (1week, 1 month & 3month) time periods (Fig 4& Table 4) post exposure periods.

Table 4: Mean ± SD BAL fluid MDA levels (IU/L) in carbon nanoparticles instilled rats

Group/Post instillation period	1 Day		1 Week		1Month		3Month	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
PBS+1%Tween 80	0.91	0.25	0.74	0.450	0.56	0.12	0.52	0.75
C Iron (1 mg/kg)	1.01	0.23	0.97	0.120	0.81	0.23	0.58	0.74
C Iron (5 mg/kg)	1.29	0.54	1.42	0.230	0.97	0.65	0.72	0.16
CNF 1mg/kg	3.15	0.64	3.54	0.120	3.29	0.12	2.96	0.47
CNF 5mg/kg	3.91	0.34	4.23	0.230	3.74	1.02	3.36	0.45
MWCNT 1mg/kg	2.99	0.25	3.35	0.650	3.11	0.98	2.65	0.25
MWCNT 5mg/kg	3.78	0.98	3.92	0.450	3.48	0.78	3.31	0.65
CNR 1mg/kg	2.73	0.47	2.91	1.020	2.66	0.56	2.31	0.34
CNR 5mg/kg	3.45	0.56	3.51	0.470	3.01	0.36	2.75	0.14
Quartz 1mg/kg	2.79	0.47	2.88	0.780	2.63	0.98	2.19	1.34
Quartz 5mg/kg	3.19	1.23	3.42	0.460	3.23	0.46	3.02	0.65

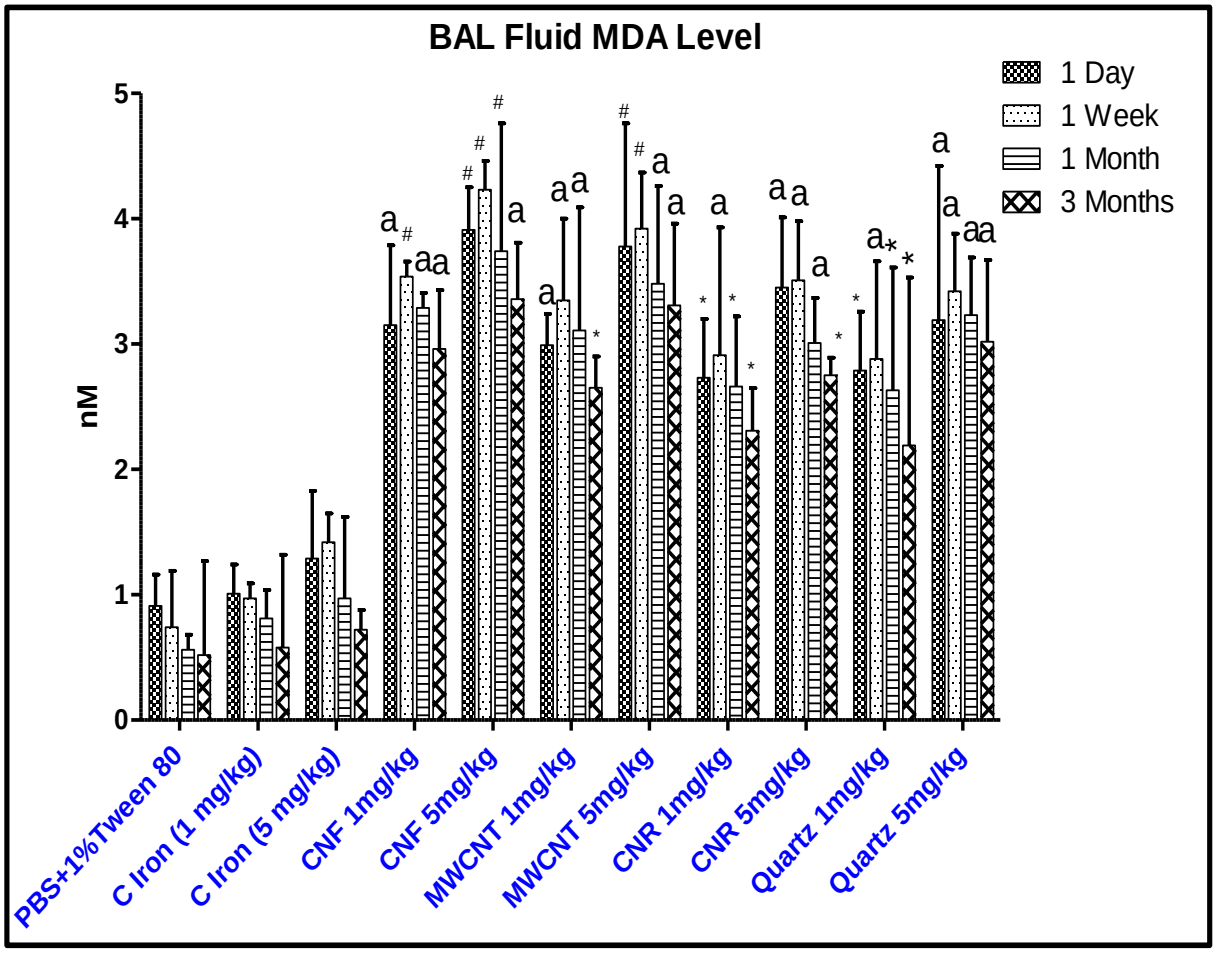

Figure 4: BAL fluid MDA levels (IU/L) in carbon nanoparticles instilled rats

Histopathological examination of tissues:

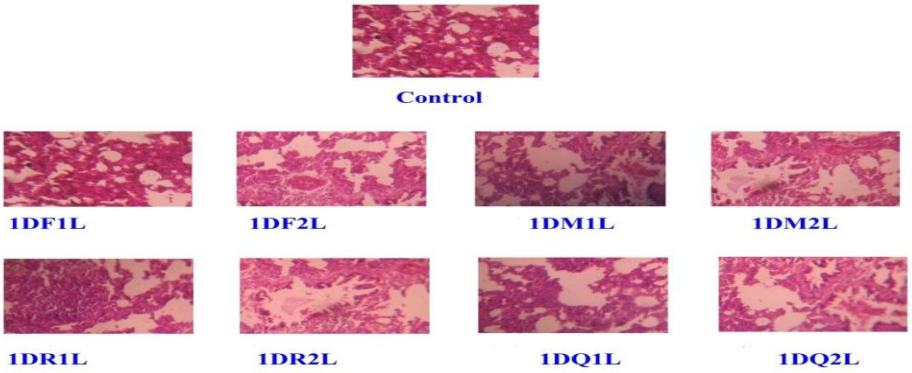

Figure 5: Light micrograph of Rat lungs tissue at 1 Day post instillation of carbon nanoparticles exposure :

Control: PBS+1% Tween 80 control; 1DF1L: CNF (1mg/kg);1DF2L: CNF (5mg/kg) 1DM1L: MWCNT(1mg/kg); 1DM2L: MWCNT (5mg/kg); 1DR1L: CNR(1mg/kg); 1DR2L: CNR (5mg/kg) ;1DQ1L: Quartz (1mg/kg); 1DQ2L:Quartz (5mg/kg);

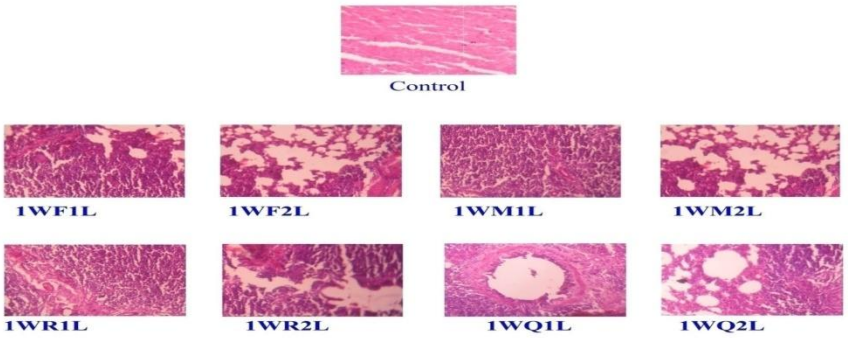

Figure 6: Light micrograph of Rat lungs tissue at 1 Week post instillation of carbon nanoparticles exposure: Control: PBS+1% Tween 80 control; 1WF1L: CNF (1mg/kg);1WF2L: CNF (5mg/kg) 1WM1L: MWCNT(1mg/kg); 1WM2L: MWCNT (5mg/kg); 1WR1L: CNR(1mg/kg); 1WR2L: CNR (5mg/kg) ;1WQ1L: Quartz (1mg/kg); 1WQ2L:Quartz (5mg/kg);

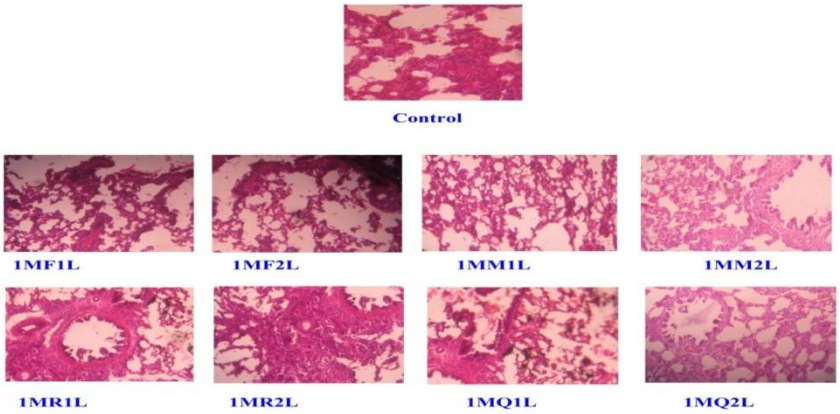

Figure 7: Light micrograph of Rat lungs tissue at 1 Month post instillation of carbon nanoparticles exposure :

Control: PBS+1% Tween 80 control; 1MF1L: CNF (1mg/kg);1MF2L: CNF (5mg/kg) 1MM1L: MWCNT(1mg/kg); 1MM2L: MWCNT (5mg/kg); 1MR1L: CNR(1mg/kg); 1MR2L: CNR (5mg/kg) ;1MQ1L: Quartz (1mg/kg); 1MQ2L:Quartz (5mg/kg);

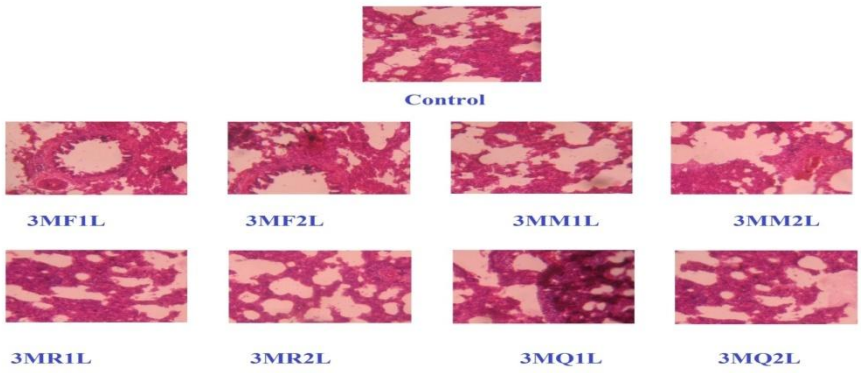

Figure 8: Light micrograph of Rat lungs tissue at 1 Month post instillation exposure :

Control: PBS+1% Tween 80 control; 3MF1L: CNF (1mg/kg);3MF2L: CNF (5mg/kg) 3MM1L: MWCNT(1mg/kg); 3MM2L: MWCNT (5mg/kg); 3MR1L: CNR(1mg/kg); 3MR2L: CNR (5mg/kg) ;3MQ1L: Quartz (1mg/kg); 3MQ2L:Quartz (5mg/kg);

DISCUSSION

The present study investigated the dose dependent acute lung toxicity of intratracheally instilled two MWCNT in rats. Exposure of all the mentioned particles at different doses does not produce any mortality in exposed rats. Analysis of BAL fluid for tissue damage biomarkers (LDH & ALP enzymes) levels revealed that, these enzyme activity/levels in MWCNT exposed rats (1& 5mg/kg b.w) have significantly higher than sham control at all the mentioned post exposure periods (Fig 1&2). It was also observed the dose dependent elevation of BAL total protein levels in groups of rats exposed to 1 & 5mg/kg of these carbon nanoparticles (fig 3). The lipid peroxidation product levels were significantly increased in nanoparticles treated rats in dose dependent manner (figure 4.4). These above all results suggest the dose dependent lung toxicity of intratracheally instilled these carbon nanoparticles in rats, which was supported by the histopathological examination of lungs of particle exposed rats. These above results suggest that intratracheal instillation of these carbon nanopfibers produced a greater pulmonary inflammatory response.

Exposure of quartz particles produced a pulmonary inflammation, necrosis, foamy macrophage accumulation and tissue thickening (i.e fibrosis). Whereas exposed to these carbon nanoparticles produced transient lung inflammation, lymphocytic infiltration, peribronchiolar lymphoid aggregates, mulfifocal granulomas and cytotoxicity or necrosis of alveoli at all post-exposure periods. Exposures of these carbon nanoparticles produced progressive lesions which were confirmed by elevated levels of pulmonary biomarkers of cell injury and inflammation.

For the validation of the present study design, the carbonyl iron and quartz particles were used as negative and positive control respectively. Exposure of carbonyl iron at different doses (1, 5mg/kg) to rat lungs resulted insignificant change ($p>0.05$) in BAL fluid LDH levels at different post exposure intervals and also in histology of lungs. Whereas exposure of

quartz produced a dose dependant pulmonary inflammation characterized by elevated BAL fluid tissue damage markers and also by the histopathological examination of rat lungs.

Multi-wall carbon nanotubes (CNT) or ground CNT were administered intratracheally (0.5, 2 or 5 mg) to Sprague–Dawley rats and we estimated lung persistence, inflammation and fibrosis biochemically and histologically. CNT and ground CNT were still present in the lung after 60 days (80% and 40% of the lowest dose) and both induced inflammatory and fibrotic reactions. At 2 months, pulmonary lesions induced by CNT were characterized by the formation of collagen-rich granulomas protruding in the bronchial lumen, in association with alveolitis in the surrounding tissues. These lesions were caused by the accumulation of large CNT agglomerates in the airways. Ground CNT was better dispersed in the lung parenchyma and also induced inflammatory and fibrotic responses. Both CNT and ground CNT stimulated the production of TNF-a in the lung of treated animals. In vitro, ground CNT induced the overproduction of TNF-a by macrophages. These results suggest that carbon nanotubes are potentially toxic to humans and that strict industrial hygiene measures should to be taken to limit exposure during their manipulation (*Julie Mullera et al 2005*).

Quartz and carbon NP at equal mass dose were used as controls, and the authors concluded that, on an equal mass basis, SWCNT in the lungs were far more toxic than carbon black and even quartz. The relative toxicity compared to quartz was evident from histological images where the nanotubes caused extensive granulomas and fibrous lesions, whereas the controls did not.

Christie M systematically evaluated a number of variables which strongly impact the ability of in vitro screening studies to accurately reflect in vivo pulmonary toxicity of several particle types in rats. The variables tested included particle dose, time course, duration of treatment exposure, and pulmonary cell types. In vivo effects in the lungs of rats were utilized as the barometer for comparison and validation. Under the conditions of this study, the results of in vivo and in vitro cytotoxicity and inflammatory cell measurements demonstrated little correlation (*Christie M et al 2007*).

The high dose showed cytotoxicity and inflammation that persisted through 84 d after exposure. The middle dose had no polymorphonuclear cell influx with transient cytotoxicity. The low dose was associated with a low grade inflammatory response measured by changes in mRNA expression. Increased inflammatory proteins were present in the lavage fluid at the high and middle dose through 28 d post-exposure. Pathology, including epithelial hyperplasia and peribronchiolar inflammation, was only noted at the high dose (*Aaron Erdely1et al 2013*).

D. B. Warheit found that pulmonary bioassay study was to assess the acute lung toxicity of intratracheally instilled SWCNT in rats. SWCNT exposure at 5 mg/kg produced mortality in ≤_15% of the exposed rats. This was due to the impact of agglomerating the major airways in the rat and not due to inherent toxicity of SWCNT. Positive control quartz particle exposures produced pulmonary inflammation, cytotoxicity, enhanced parenchymal cell proliferation, foamy macrophage accumulation, and tissue thickening (i.e., fibrosis). Exposures to SWCNT produced transient lung inflammation and subsequent multifocal granulomas (*D. B. Warheit et al 2004*).

(*Shvedova et al. 2005*) studied mice exposed to SWCNT of 99.7% weight elemental carbon and 0.23% weight iron. The primary nanotubes

were 1–4 nm in diameter, but, as delivered by pharyngeal aspiration, two distinct particle morphologies were observed: compact aggregates and dispersed structures. These two morphologies were linked to two distinct responses with dense SWCNT aggregates being associated with foci of granulomatous inflammation, including discrete granulomas with hypertrophic epithelial cells.

CONCLUSION

We have demonstrated the effects of multi wall carbon nanotubes upon exposure to rats by intratracheal instillation produced a dose dependent multifocal granulomas and cytotoxicity or necrosis of alveoli. These effects are similar effects to those of quartz particles. Carbon nanotubes produced significantly greater toxicity compared to quartz particles. This is very specifically dangerous for the chronic exposures of workers making and using nanoparticles in manufacturing plants.

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