A STUDY ON EXPOSURE ASSESSMENT AND EVALUATION OF OCCUPATIONAL HEALTH HAZARDS OF CARBON NANOTUBES

BY

AKBAR ZIAUDDIN

Submitted

in partial fulfillment of the requirement of the degree of

DOCTOR OF PHILOSOPHY

To

University of Petroleum & Energy Studies, Dehradun

November 2014

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DEDICATION

This thesis is dedicated to my parents

who have always stood by me and dealt with all of my absence from many personal occasions

with a smile.

DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Akbar Ziauddin
Date: 25^{h} Nov 2014

THESIS COMPLETION CERTIFICATE

This is to certify that the thesis entitled ***"A Study On Exposure Assessment and Evaluation of Occupational Health Hazards of Carbon Nanotubes"*** submitted by ***Akbar Ziauddin*** to ***University of Petroleum and Energy Studies*** for the award of the degree of ***Doctor of Philosophy*** is a bona fide record of the research work carried out by him under our supervision and guidance. The content of the thesis, in full or parts have not been submitted to any other Institute or University for the award of any other degree or diploma.

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ABSTRACT

Multiwalled Carbon nanotubes (MWCNTs) were discovered some twenty five years ago by Sumio Lijima and are considered to be miracle materials of 21st century due to their wide spread industrial applications. The MWCNT production is expected to grow tremendously over the next ten years with an estimated capacity of 2000 tons per year by 2015 as per the projections given by National Science Foundation. Approximately 6 million workers will be employed by CNT industry by 2020. Currently, all over the world a large number of workers are involved in the production of carbon nanotubes at various companies. Occupational and public exposure to MWCNTs is increasing with growing production and use of MWCNTs in various products. Since workers are involved in handling and production of MWCNTs, occupational safety and health has to given priority while dealing with these materials. In the present investigation, the potential health risks associated with MWCNT exposure in the working environment are estimated.

Initially, a survey was conducted to get an idea about CNT manufacturing industries. A questionnaire proforma was prepared to collect data related to health effects of MWCNTs. MWCNTs can enter into the human body through inhalation and may be more biologically active because of their large surface area per unit mass compared with that of large particles. MWCNTs and A549 epithelial lung cells were procured from Sisco Laboratories, Mumbai and NCCS, Pune respectively. The MWCNTs at different concentrations were dispersed in phosphate buffer solution, ethanol and sterile water using a homogenizer, as they are insoluble in water. The dispersed MWCNTs were treated to lung cells for different exposure periods keeping a control as reference sample. After defined exposure periods, the cells were subjected to cytotoxicity assays: MTT, XTT and Apoptosis. The treated cells were also

observed under microscope to note the morphological changes. This allowed us to understand the hazards due to toxicity of MWCNTs.

The exposure of workers to MWCNTs in the environment was measured by selecting a manufacturing industry situated at Noida. The mass concentration of MWCNTs was measured at a height of 1.2m by selecting different locations. The samples were collected to represent the spatial and temporal variations. The results obtained were used to estimate the amount of MWCNTs that are likely deposited in the human lungs. This will give the risk posed by the workers working in the manufacturing industry.

The questionnaire responses were consistent. The study concluded that the MWCNTs cause cytotoxicity due to their effect on mitochondrial activity of cells. The exposure to MWCNTs was affecting cell viability and cell proliferation. The presence of serum was influencing inhibitory concentrations of MWCNTs. The dispersed MWCNTs are clumping as the concentration increases and these clumps are affecting the viability of cells. The measured field concentrations were ranging from 1.2 micrograms per cubic meter to 2.2 micrograms per cubic meter. This exposure concentration is not resulting in any health hazards due to the highest safety standards being followed by the industry.

---End of Abstract---

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ABBREVIATIONS

Acronym	Full Form
^0C	Centigrade
a/cm^2	Ampere per square centimeter
AB	Alamar Blue
AF-SWCNTs	Acid Functionalized Single – Walled Carbon Nanotubes
ALP	Alkaline Phosphatase
atm	Atmospheric
BAL	Bronchoalveolar Lavage
BALF	Bronchial Alveolar Lavage Fluid
BrdU	Bromodeoxy Uridine
BSC	Biological Safety Cabinet
CCVD	Catalytic chemical vapour deposition
CNTs	Carbon Nanotubes
CoMoCat	Cobalt molybdenum catalyst
CVD	Chemical vapour deposition
DAPI	4^I, 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl suphoxide
DNA	Deoxyribonucleic acid
DPL	Dipalmitoyl Lecithilm
DWNT	Double – Wall Nanotubes
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular Signal Regulated Kinases
ESR	Electron Spin Resonance

Acronym	Full Form
EtoH	Ethanol
FBS	Foetal Bovine Serum
FD	Functional Disorder
FRC	Faculty Research Council
gm	Grams
GPa	Gigapascals
HDF	Human Dermal Fibroblasts
HEK	Human Embryonic Kidney
HiPCO	High-pressure carbon monoxide
IC_{50}	Inhibitory Concentration
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharides
m	Meter
MAPK	Mitogen – activated Protein Kinases
mbar	Millibars
Mg/L	Milligrams per Litre
MH-S	Murine Alveolar Macrophage
ml	Milliliters
mm	Millimeters
mRNA	Messenger RNA
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MWCNO	Multiwalled Carbon Nano – Onions
MWCNTs	Multi-walled carbon nanotubes
NAD(P)H	Reduce form of Nicotinamide Adenine Dinucleated Phosphate
NCCS	National Centre for Cell Science

Acronym	Full Form
nm	Nanometer
NO	Nitric Oxide
OMWCNT	Oxide Multi – Walled Carbon Nanotubes
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
PI	Propidium Iodide
RLE	Rat Liver Epithelial Cells
rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl Sulfate Polyacrylamide gel Electrophoresis
SP	Surfactant Proteins
SW	Sterile Water
SWCNTs	Single-walled carbon nanotubes
TEM	Transmission Electron Microscopy
TGA	Thermo Gravimetric Analyzer
TPa	Terrapascal
TPVG	Trypsin Phosphate Versene Glucose
V	Volts
W/m K	Watts per meter Kelvin
XTT	Sodium 3- (Phenyl amino-carbonlyl-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulphonic acid hydrate
µg	Micrograms
$\mu g/m^3$	Micrograms per cubic meter
µl	Microliters
µm	Micrometer

Chapter-1

1. Introduction

1.1 Problem Statement

CNTs are a form of carbon with cylindrical shape, belonging to fullerene structural family and are made up of thick sheets of carbon called graphene, which were rolled up to form a seamless cylinder. These tubes first observed by Endo (1975), and later by Lijima (1991) in the soot produced by the arc-discharge synthesis of fullerenes. This observation has initiated a new path in carbon research. CNTs are physically strong and stiff and have unique and useful electrical, mechanical, chemical, thermal and optical properties, making them suitable for various applications in different industrial sectors as shown in figure 1.

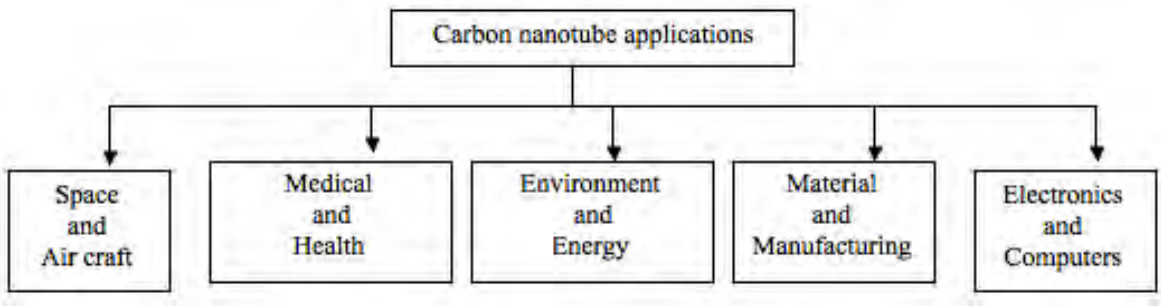

Figure 1: Applications of CNTs in Different Industrial Sectors

Due to wide range of applications, usage of CNT based products is increasing and their manufacture is also being increased proportionately. It is estimated that the market demand

of CNTs has increased by 175 folds from the year 2004 to 2014 as shown in Table 1 and is going to increase further and further.

Table 1: Market Growth of CNTs During Past 10 Years

$ MILLIONS	2004	2009	2014
TOTAL DEMAND	**$6**	**$215**	**$1,070**
BY TYPE			
Single-walled nanotubes	0	95	600
Multi-walled nanotubes	6	120	470
BY END USE			
Electronics	0	90	395
Automotive	1	31	165
Aerospace/Defense	0	10	65
Other	5	84	445
BY REGION			
U.S.	2	57	290

Source: *Freedonia Group*

Many scientists are concerned about the toxicity of the CNTs because of their structural resemblance to asbestos. Laboratory studies have indicated that the increased exposure to CNTs may cause deleterious health effects as summarised in Table 2 and these effects were found to be dose dependent. Particularly biological applications like drug delivery are getting much attention, as the concern for its potential hazards related to CNT exposure is still being debated.

Table 2: Health Effects of Carbon Nanotubes

	Health Effects
Carcinogenicity	Lung irritation, Chronic Lung inflammation Exacerbation of asthma, Formation of Granulomas
Genotoxicity	Formation of Oxygen radicals DNA damage – Fibrosis, Lung cancer
Cytotoxicity	Oxidative stress, Inflammation, Cell damage, Granulomas and Apoptosis

Source: Regina Ma et. al., (2010) "Carbon Nanotubes Risk Assessment", Nano hazard, Dimitris Deligiannis, Greece.

These health effects are neither observed nor proved in any manufacturing industry producing carbon nanotubes. Hence, in the present study, we are trying to correlate the laboratory experimental results with field observations and are trying to understand the need for formulation of exposure standards for Multiwalled Carbon nanotubes.

1.2 Background

Nanotechnology is the branch of science, which deals with making and use of materials, devices and systems in nanoscale, which possess 0.1 to 100 nm dimensions ($1nm=10^{-9}$ m). This branch is now-a-days gaining lot of importance as nanomaterials are being used widely in many industrial sectors for manufacturing different types of nanobased products. Nanomaterials can be synthesized by two different approaches i.e. "Bottom-up" and "Top-down". In bottom-up approach, materials and devices are constructed from molecular components, which were put together by chemical bonds whereas in top-down approach nano entities are built from hefty bodies. (Rodgers 2006).

CNTs are finding wide range of applications there by their the manufacturing companies are also increasing production. More than 100 companies in the world today are manufacturing CNTs and this number may increase to more than 200 in the next five years. Currently, CNTs account for 28% market share of overall nanomaterials demand. The annual production capacity of CNTs for the year 2010 is presented in table 3. In the next five years, the production capacity may increase enormously in some manufacturing units (2011 Nanotechnology research review).

Table 3: Annual Production Capacity and Production Method of CNTs in Global Manufacturing Companies (2010) - (Global CNTs Market-Industry Beckons, 2011).

S.No	Types of CNTs	Manufacturing Company	Annual Production Capacity (metric Tonnes)	Production Method
1.	SWCNTs	Mitsubishi Rayon Co.Ltd	1.2	Chemical vapour deposition (CVD)
2.	SWCNTs	Kleancarbon Inc.	1.0	CVD
3.	SWCNTs	Unidym, Inc	1.5	High-pressure carbon monoxide (HiPCO)
4.	SWCNTs	Toray Industries, Inc	1.5	Catalytic chemical vapour deposition (CCVD)
5.	SWCNTs	SouthWest Nano Technologies Inc	1.0	Cobalt molybdenum catalyst (CoMoCat)
6.	MWCNTs	Showa Denko K.K	500	CCVD
7.	MWCNTs	CNano Technology Limited	500	CCVD
8.	MWCNTs	Nanocyl S.A	400	CCVD
9.	MWCNTs	Bayer Material Science AG	260	CCVD
10.	MWCNTs	Arkema Inc	50	CCVD
11.	MWCNTs	Hyperion Catalysis International, Inc	50	CVD

CNTs are synthesized by arc discharge, laser ablation, HiPCO, CoMoCat and chemical vapour deposition. Each method differs in usage of energy source, purity and yield of CNTs.

The percentage yield and cost of SWCNT production by various production methods is represented in Table 4.

Table 4: Yield Of CNTs Obtained By Different Production Methods (Shifrina (2011) Synthesis Of Carbon Nanotubes).

S.No.	Production Method	% Yield	Cost of Production of SWCNTs (per gram) in 2009
1.	Electric arc discharge	60	$ 1,906
2.	Laser ablation	70	-
3.	HiPCO	90	$ 485
4.	CoMoCat	80-90	-
5.	Chemical vapour deposition	90 %	$ 1,706

CNTs produced by different methods are not pure, and must be purified to remove residual impurities and metal catalysts and support material used for their synthesis, which can be done by rinsing or ultrasonicating with dilute acids. The length of nanotubes can range from several hundred nanometers to several micrometers and have a diameter of 0.2 to 2 nm for SWCNTs and 2 to 100 nm for MWCNTs (Yang et al., 2007).

CNTs are having wide range of applications due to their unique properties such as

- Strength (100 times stronger than steel).
- Light weight (six times lighter than steel).
- Very high surface area.
- Very good thermal conductors.
- High chemical and thermal stability.
- Disperse heat better than any other known material.

Due to their outstanding properties, the potential applications of CNTs are increasing day by day.

1.3 Exposure to CNTs

Humans get exposed to CNTs by two ways: Occupational exposure and environmental mediated exposure.

1.3.1 Occupational Exposure

In CNT manufacturing industries, the workers get exposed to CNT aerosols released into atmosphere during their handling, processing and purification. The occupational exposure of CNTs is shown in figure 2. These aerosols contain CNTs having diameter and length ranging from 20-200 nm and 10^3-10^6 nm respectively. On the other hand CNTs could deposit on the gloves, which were used for safe handling. Maynard et al. 2004 estimated a deposition of 0.2 and 6 mg SWCNTs per each hand and can remain in hands for longer periods. Such clumps might be a reason for dermal exposure and may cause health effects (Tejral 2009).

Figure 2: Occupational Exposure to CNTs

1.3.2 Environmental Mediated Exposure

The unique properties of CNTs are making them to find application in many industrial sectors for formulating a variety of CNT based products and devices. This results in the emission of huge quantities of CNTs into the air and soil environment. The washing of production chambers may also release CNTs into water bodies and thereby severe threat to living organisms. CNTs are one of the least biodegradable materials, insoluble in water and are lipophilic in nature.

1.4 Motivation to do Research

CNTs are a new from of crystalline carbon and can have single or multiple concentric walls as shown in figure 3. These are one of the most important products of nanotechnology possessing high strength, ultralight weight and excellent thermal and chemical stability. Due to their unique metallic and semiconductive properties these tubes found application in high strength materials, biomedical and electronic fields. These CNTs are structurally similar to asbestos. Inhalation of asbestos fibers is known to induce asbestosis, lung cancer and malignant mesothelioma. The size, aspect ratio and surface charges are influencing the toxicity of asbestos and these are similar to CNTs.

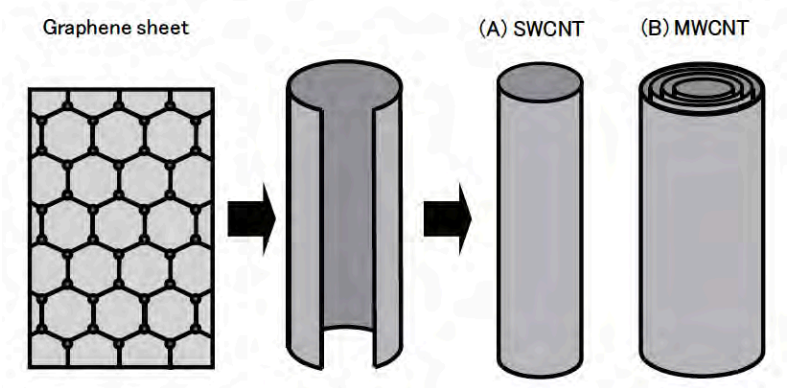

Figure 3: Carbon Nanotubes

The fibrous structure of CNTs has led to the concern that they might cause asbestos like pathology in the lung and mesothelium. Careful evaluation of health effects is required before the revolution of nanotechnology. Proper assessment of potential hazards of CNTs to humans is the need of the day as they are finding vast biomedical applications. Hence there is an urgent need for toxicological studies on CNTs including the associated toxicity and biocompatibility. Therefore cytoxicity of CNTs has been one of the most pressing questions in nanotechnology. Cytoxicity of carbon nanotubes can be attributed to a rage of issues such as length, size distribution, surface area, dispersion and aggregation. The relation between toxicity and exposure is shown in figure 4. The primary route for the entry of CNTs is inhalation and lung is the target organ for the deposition and accumulation of CNTs.

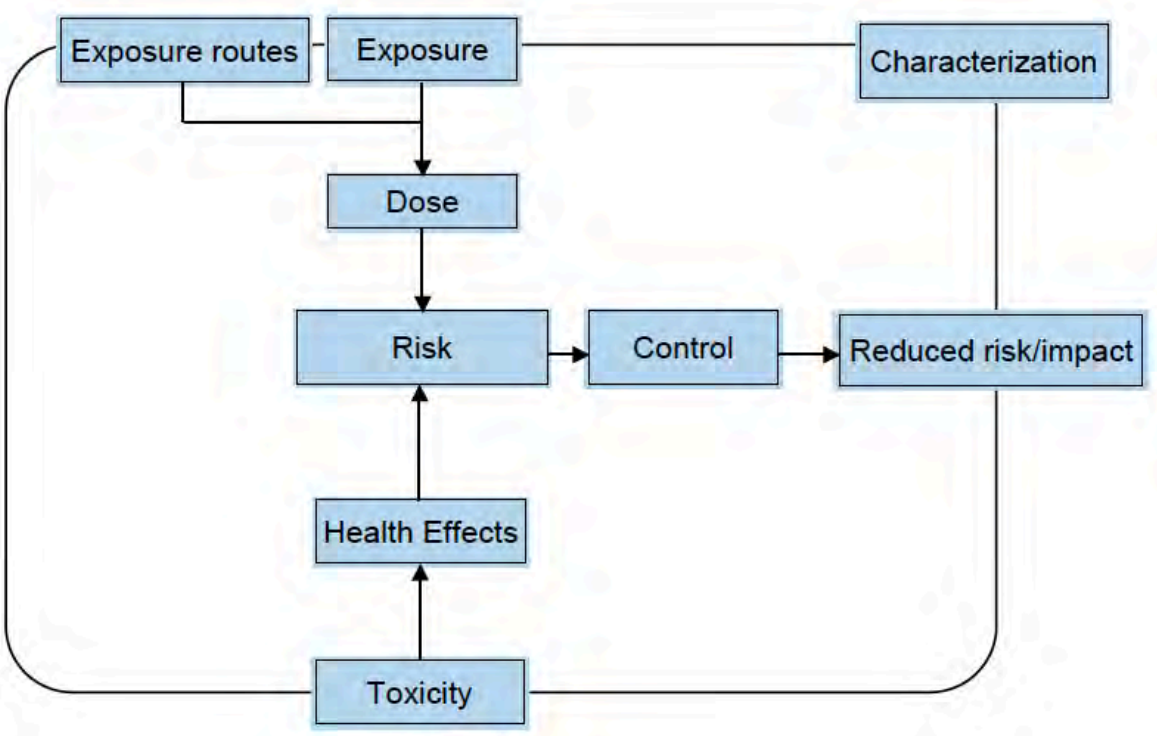

Figure 4: Relation Between Toxicity and Exposure

1.5 Scope

The scope of the present study is to understand the pulmonary toxicity of MWCNTs. Pulmonary toxicity is of major concern as nano materials are very small and when airborne, a large fraction of these particles will be deposited within the alveolar regions of the lungs. Hence the present study mainly focused on MWCNTs toxicity on lung cells.

1.6 Objectives

The present study would be designed at every stage keeping in view the following objectives.

- To understand and evaluate the potential effects of MWCNTs on human respiratory system mainly on lungs that may result from inhalation of airborne MWCNTs.
- To analyze the effects of MWCNTs on human lung cells by exposing them to various concentrations.
- To study the concentrations of MWCNTs in the industrial environments that may possibly inhaled by workers.
- To generate data that will help in development and formulation of safety standards for the exposure to MWCNTs at various manufacturing units in order to protect the health of workers.

1.7 Research Framework

MWCNTs are procured from Hi-Media laboratories, A549 lung cell-line has been procured from National Cell Culture Center, Pune., and all the necessary chemicals and glassware has been procured from Lotus Enterprises, Visakhapatnam. The laboratory equipment available in the Biotechnology Department of GITAM University was utilized for carrying out the

experimental work. The Laboratory tests carried out on lung cells are MTT assay, XTT assay and Apoptosis. A list of carbon manufacturing units was prepared and a questionnaire was formulated and circulated to the employees of manufacturing units. The survey forms were analyzed using Cronbach Analysis. One CNT manufacturing industry located in Noida was selected for the field study. The air samples at different locations in the industry were collected and analyzed for CNT concentrations.

---End of Chapter 1---

Chapter-2

2. CNT: Production, Properties, Applications & Exposure

2.1 Carbon Nanotubes

Sumio lijima synthesized MWCNTs in the year 1991 by arc discharge method. He was able to synthesize SWCNTs after two years. Since then CNTs have captured the attention of worldwide researchers. They are allotropes of carbon with a cylindrical structure. They exhibit extraordinary strength and are the stiffest materials found on the earth.

CNTs can be categorized as SWCNTs and MWCNTs. MWCNTs exist in single tubes and have van der Waals forces between the molecules, which causes them to aggregate into microscopic bundles. The major limitation for the use of CNTs is insolubility and difficulty in manipulation in any solvents. These materials can be dispersed in some solvents by sonication, but precipitation occurs immediately when this process is interrupted. These CNTs are produced from carbon atoms found in graphite. In this chapter we will present an overview of the CNTs production methods, properties, applications and exposure sources.

2.2 Production Methods of CNT's

CNTs are synthesized by various methods like arc- discharge, laser ablation, chemical vapour deposition (CVD), High pressure carbon monoxide (HiPCO), CoMoCat etc. An energy source (electricity, heat from a furnace or high light intensity) is added to carbon source for the synthesis of CNTs, which may vary depending on the synthesis method (Donaldson et al. 2006). Though the CNTs are synthesized by different methods (Table 5), CVD, HiPCO and CoMoCat are the most widely used methods for production of CNTs due to their high yield, purity and low cost of production and the reason for fewer yields of CNTs in arc discharge and laser ablation is due to the evaporation of carbon source at high temperatures. Every year the production capacity of CNTs is growing in an exponential manner. The details of production methods are given below.

2.2.1 Electrical Arc Discharge

It is the oldest method used for the production of CNTs. Pure and metal doped graphite electrodes are used for the synthesis of MWCNTs and SWCNTs respectively (Popov 2004). The electrodes are temporarily brought into contact and an arc is struck. Low pressure (between 50 and 700 mbar) and controlled atmosphere composed of inert (like helium or argon) gases are maintained for the production. In the inter electrode zone, high temperature is maintained, so that the carbon sublimes from positive electrode (anode) and is consumed. A constant gap (1mm) is maintained between the two electrodes which can be done by adjusting the position of anode. Plasma formed between the electrodes, can be maintained constant for prolonged periods by controlling the distance between the two electrodes and the voltage (25-40 V). After de-pressurization and cooling the reaction chamber, nanotubes together with by-products can be collected (Shifrina 2011; Szabo et al. 2010).

2.2.2 Laser Ablation

In the year 1995, Richard E Smalley and his group used laser ablation for the first time to produce high quality CNTs. In this process, a graphite target gets vaporized by a pulsed laser in a tube furnace which is heated to 1200°C. Inert gases like helium or argon were sent into the chamber to carry the grown nanotubes to the copper collector. Nanotubes develop on the cooler surfaces of the reactor as the vaporized carbon condenses (Shifrina 2011). Pure electrodes can be used for the production of MWCNTs whereas metal-doped electrodes are used for the production of SWCNTs (Dai 2002).

2.2.3 HiPCO

In the year 1999, Richard E Smalley and his co-workers developed a high pressure carbon monoxide method (HiPCO) for the synthesis of CNTs. In this process a continuous gas phase carbon monoxide acts as feedstock and iron carbon monoxide ($Fe(CO)_5$) acts as a catalyst. Thinner SWCNTs with high purity, less structural defects and high intrinsic selectivity was obtained (Shifrina 2011).

2.2.4 CoMoCat

In 2000, Kitiyanan suggested another method in which a mixture of cobalt and molybdenum was used as a catalyst and hence, this process was named after the unique catalyst. At high temperatures (between 700°C-950°C) carbon monoxide decomposes into simple carbon and carbon dioxide (Shifrina 2011). The advantage of this method is that it reduces the formation of by-products as compared to arc discharge and laser ablation methods.

2.2.5 Chemical Vapour Deposition

The catalyst material (most commonly nickel, cobalt, iron or a combination) is heated to high temperature in a tube furnace and a hydrocarbon gas is passed through the reactor in controlled manner for a definite period of time. The hydrocarbon gas dissociates into individual components in the furnace and supplies the necessary carbon atoms for the CNT growth. Low temperatures (500-800°C) yield MWCNTs and high temperatures (600-1200°C) yield SWCNTs. This process often synthesizes commercially available CNTs.

Table 5: % Yield Of CNTs Obtained By Different Production Methods (Shifrina (2011) Synthesis Of Carbon Nanotubes).

S.No.	Production Methods	Energy Source	Substrate Used	Catalyst Used	Conditions Maintained	% Yield
1.	Electric arc discharge	Electric arc	Pure or metal (Fe, Co, Ni, Mo, Y) doped graphite electrode	-	Low pressure (between 50-700 mbar)	60
2.	Laser ablation	Pulsed laser	Graphite	Co, Cu, Nb, Ni, Pt	High temperature (1200°C)	70
3.	HiPCO	Heat	Carbon monoxide	$Fe(CO)_5$	High temperature (1000-1100°C), high pressure	90
4.	CoMoCat	High temperature	Carbon monoxide	Co-Mo	700-950°C, 1-10 atm	80-90

					pressure	
5.	Chemical vapour deposition	Heat from furnace	Carbon monoxide	Fe, Co, Ni, Mo	Temperature (500-1100°C), atmospheric pressure	90

2.3 Properties of CNT's

CNTs, due to their tiny size exhibit many interesting and unique properties.

2.3.1 Mechanical

CNTs are made up of sheets of graphene and the C-C bond in a graphene layer is probably the strongest chemical bond known in nature. CNTs are the strongest and stiffest materials yet discovered in terms of tensile strength and elastic modulus.

2.3.1.1 Tensile Strength

The tensile strength of CNTs is due to the covalent sp^2 bonds formed between the individual carbon atoms. The CNTs can sustain extremely high-tension force of about 130 GPa (gigapascals) where as the steel can withstand <5 GPa. Yu et al. (2000) tested the tensile strength of SWCNTs and MWCNTs and is found to be 13-52 GPa and 63 GPa respectively. The tensile strength of a single layer of MWCNTs is 100 times stronger than that of steel.

2.3.1.2 Elasticity

The CNTs are elastic and they can withstand stress. The elasticity can be measured experimentally by calculating the Young's modulus. Lourie and Wagner (1998) reported Young's modulus of 2.8-3.6 TPa (terra pascal) for SWCNTs and 1.7-2.4 TPa for MWCNTs.

2.3.2 Electronic

CNTs possess unusual electronic properties and act as conductors of energy. The diameter and helicity (n, m) of carbon atoms in the nanotube shell are believed to determine their conductivity (metallic or semiconductor). Theoretical calculations for electronic properties by Hamada et al. 1992; Mintmire et al. 1992; Saito et al. 1992 showed that CNTs are very sensitive due to their geometrical structure. Theoretically it was determined that metallic nanotubes (where the energy gap between the valence and conducting states is zero) can carry an electric current density of 4×10^{9} A/cm^{2} (ampere per square centimeter) which is 1000 times higher than copper (Hong et al. 2007).

2.3.3 Thermal

CNTs are very good thermal conductors due to their geometrical structure. The thermal conductivity of CNTs was evaluated both theoretically and experimentally at room temperature. Theoretically it was predicted that CNTs exhibit a thermal conductivity of 6600 W/m K, which is larger than graphite (> 2000 W/m K) or diamond (3320 W/m K) (Berber et al. 2000). The measured value of thermal conductivity for bulk samples of SWCNTs is over 200 W/m K (Watts per meter Kelvin) and for individual MWCNTs is over 3000 W/m K (Hone 2004).

2.3.4 Optical

CNTs possess unique optical properties and can be studied using a variety of theoretical tools. The calculated optic and nonlinear properties are important for various applications. Light absorption, photoluminescence and Raman spectroscopy measurements are needed to observe the optical properties. The optical properties can be detected by spectroscopic studies. The optical properties of CNTs can be derived from electronic transitions within one-dimensional density of states. Optical responses of semiconducting species are greater than the metallic nanotubes. CNTs have light emitting capacity and vary between metallic and semiconducting CNTs.

2.4 Applications of CNT's

The combination of structure, dimensions and topology creates an unique physical properties in CNTs that are not found in any known material. CNTs have grown from a material of dreams to a real world material that has found its application in various fields. CNTs are the new class of materials for biomedical and industrial applications. The major application will be presented in the broad areas of energy, electronics, composites, field emission, sensors, biological and other minor fields.

2.4.1 CNTs in Energy Applications

Carbon nanotubes are the preferred electrode material because of unique electronic & electrical properties, a broad electro-chemical stability window and accessible surface area. Nanotubes found application in super capacitors, li-ion batteries fuel cells & solar cells. The energy domain is the largest in the bulk of nanotubes.

2.4.2 CNTs in Electronics

Nanotubes are promising electronic materials due to their nanoscale dimensions and ballistic electronic conduction and not sensitive to electro migration. Nanotubes are the best electronic materials that are available presently. These tubes are used in the manufacture of semiconductors and transistors. Nanotubes can also perform the function of interconnects because of their metallic characteristics. CNTs can function as nano electromechanical devices due to mechanical resilence and electrical conductivity. High electrical conductivity and high aspect ratio, good dispersion in polymer matrices, good electrical percolation of nanotubes leads to electrostatic discharge and electrical shielding applications.

2.4.3 CNTs for Mechanical Applications

Nanotubes are fibers with superior mechanical properties. Nanotubes under compression can sustain large strains. Flexibility depends on the geometric parameters of nanotubes. The flexibility of nanotubes under mechanical loading is making them suitable for nano probes and in electromechanical applications. Nanotubes can also improve the properties of 3D composite. The addition of nanotubes to polymer matrices has application in the area of vibration damping.

2.4.4 CNT Sensors

Electrical, Electrochemical and optical properties of CNTs making them effective sensing elements detection of low concentrations of toxic gases is the promising application of CNTs in gas sensors. The CNTs excellent materials for using them as electrodes in electrochemical sensors due to fast electron transfer kinetics.

2.4.5 CNTs in Field Emission & Lighting Applications

Low threshold voltage, high emission stability and long emitter stability makes CNTs preferred field emitters. The nanotubes are used in the fabrication of cathode ray lighting elements and flat panel displays due to electron relaxation process of CNTs.

2.4.6 CNTs for Biological Applications

CNTs are used in imaging application within live cells and tissues due to optical properties of CNTs. Nanotubes have large resonance enhanced Raman scattering profile, which is available for the detection of cells. New drug delivery vehicles are being developed in-order to increase the therapeutic effect of drugs. Due to high surface area to volume ratio, CNTs can accommodate high loading of therapeutic agents. The cells can internalize CNTs and surface modification reduces cytotoxicity. For these reasons CNTs are potential vehicles for drug delivery.

2.5 Exposure to CNT's

For decades industry intentionally producing different kinds of nano particles for various applications. In addition nanotechnology is generating new materials and products that are based on nano particles. These nano particles are the greatest risk to the occupational health of the workers involved in research and manufacture. Exposure to nanoparticles is mainly related to workers since synthesis reactions take place in working environments. The diagrammatic representation of nanoparticle synthesis reactions and possible ways of exposure to nanoparticles was depicted in figure 5. Uptake of nanoparticles may occur by inhalation, transdermal or by ingestion. Mostly discussed exposure route is via inhalation, as

this route has to be treated differently to transdermal process and ingestion. It's very difficult to assess personal exposure to nanoparticles, as there is no personal sampler that exists to measure the concentrations of CNT's below 100 nm diameter. Current standard occupational exposure measurements are based on mass concentrations of the inhalable particle size fraction. The measurements of number size distribution of particles at working places to assess the potential exposure have to be conducted at working places. The exposure concentrations can be calculated based on conducted measurements.

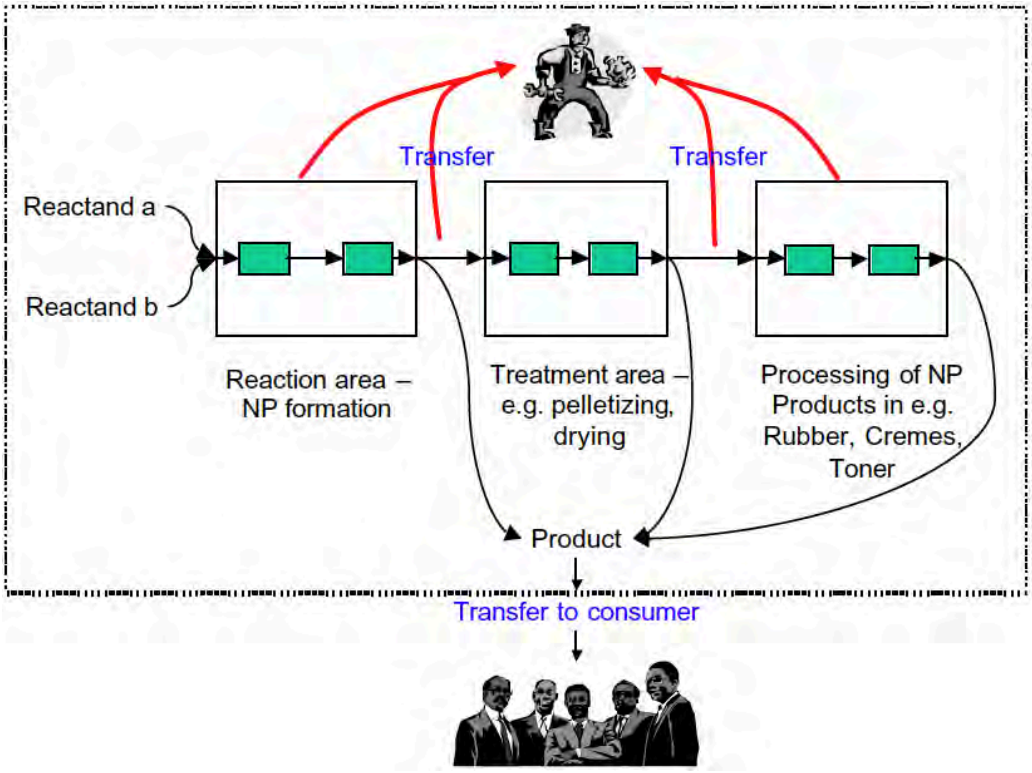

Figure 5: Production and Possible Ways Exposure to Nanoparticles

Source: Paul JA Borm et. al., (2006) "The potential risks of nanomaterials: a review carried out for ECETOC", Particle and Fibre Toxicology.

Although CNTs are being used in numerous industrial sectors due to their beneficial applications, they are also denoting a danger to human health as materials behave differently at the nanoscale level from their original form. CNTs enter into the body through various

routes like skin, lungs and digestive tract. After gaining entry, they can accumulate in different body parts and bring out changes. The biokinetics of CNTs is shown in figure 6. Many CNT toxicity studies have been conducted both *in vivo* and *in vitro* to determine the fate and effect of CNTs in the body. All the studies represented that CNTs are toxic to humans causing both cytotoxic and genotoxic effects. The toxicity of CNTs is due to the dimensions like large surface area, small size (length to diameter ratio) and presence of metal catalyst impurities, which make the CNTs to interact well with cells and thereby causing health effects.

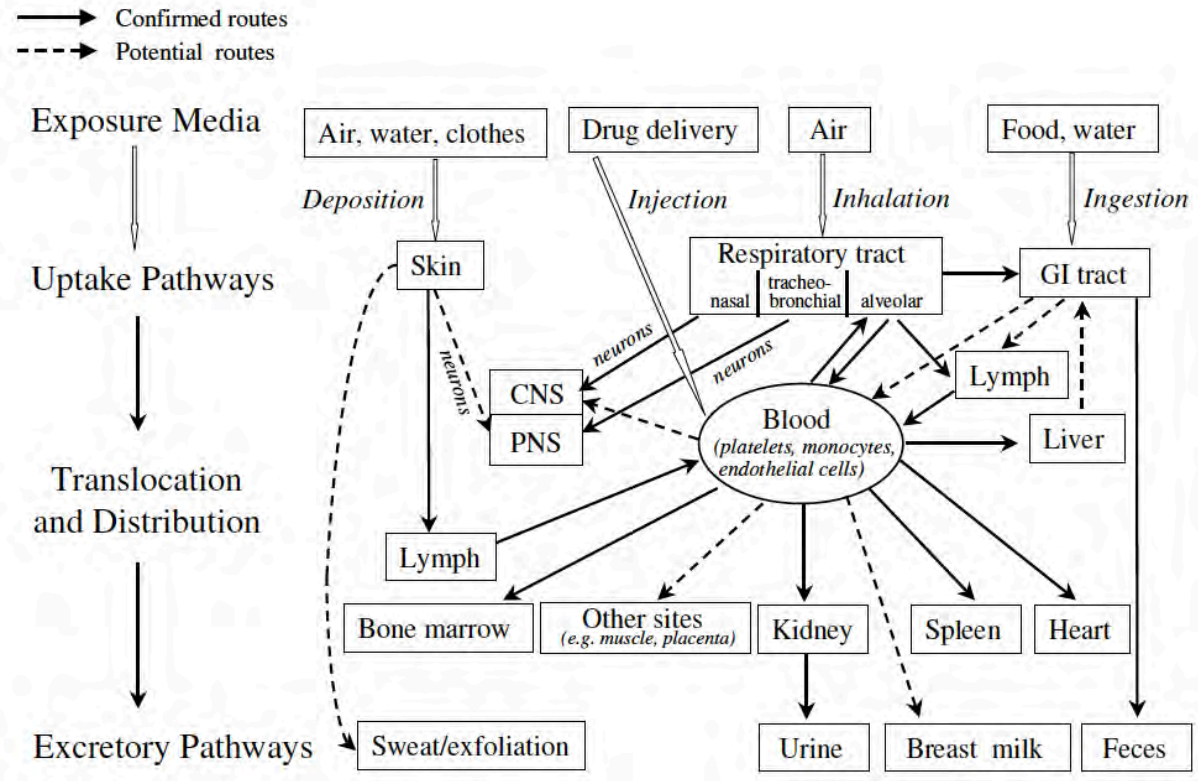

Figure 6: Biokinetics of CNTs

(**Source:** Gunter Oberdorster et. al., (2005) "Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles", Environmental Health Perspectives.

---End of Chapter 2---

Chapter-3

3. Review of Literature

Carbon nanotubes are important nano-materials due to their unique properties. These tubes are manufactured with single or multiple walls for their potential applications in various industries. The nanotube manufacturing industrial workers and users of nanotube-based products are exposed continuously to CNT environments. The nanotubes can enter into human body through gastrointestinal tract, skin and lungs. Both in-vitro & in-vivo studies were conducted to understand the health effects of CNT's. The literature available for health effects of CNT's was classified as in-vitro studies and/or in-vivo studies and presented here.

3.1 Health Effects of CNT's

Easily accessible organs in a given CNT polluted environment are skin, lungs and blood borne cells. Therefore, these exposure routes and specific cell lines have been studied for toxicity assessment of these organs and these investigations were reviewed.

3.1.1 In-vitro Studies

The toxicity of CNTs depends strongly on how CNTs are administered to the cells i.e. homogenously dispersed or not, with or without surfactant, type and concentration of the surfactant. Further, CNTs may contain contaminants that may be bioactive. If the exact

methodology and CNT characterization are not given then this makes it difficult to compare the data. However an attempt was made to compare and review the existing literature of Invitro studies on health effects of CNTs on dermal, lung and other types of cells.

3.1.1.1 Studies with Dermal Cells

Dermal exposure to carbon nanotubes is especially because of the increased use of various dermal creams, lotions and cosmetics containing nanoparticles and also during handling of manufactured CNTs. But there is no concrete evidence to support this scenario. There are indications that such particles can accumulate around the hair roots. They reach the deeper layers during the hair growth, as the hair follicles get open. The uptake through healthy skin has not yet demonstrated (**Simko *et al.,* 2010)**. The toxicity studies carried out using skin cells were reviewed and presented.

Huczko and Lange (2001) evaluated skin hazards of CNTs by selecting two methods of skin testing. The authors tested the biochemical activity potential of SWCNTs using two dermatological testing methods. These two methods were generally applied to determine the skin sensitivity. In the first method forty volunteers were subjected to a patch test with fullerene soot. In the second method four rabbits were tested using modified Draize rabbit eye test. The test results indicated that skin irritation and allergy are not associated due to working with CNT soot. Both the tests showed negative results indicating that the soot containing SWCNT will not cause any skin irritation and allergy. The study inferred that special precautions were not required during handling of CNTs.

Shvedova *et al.,* (2003) investigated effects of single walled carbon nanotubes on immortalized human epithelial keratinocytes. These cells were exposed to various SWCNT

concentrations for varying durations. The results of their study revealed that SWCNTs can induce oxidative stress by forming free radicals and accumulating peroxidative products. Their study further indicated that cytotoxicity of CNTs is mainly associated with iron catalyst particles.

Maynard *et al.,* (2004) undertaken a laboratory study to evaluate the aerosol nature during mechanical agitation of two production processes. The laboratory study was complemented by field studies. Airborne and dermal exposure to SWCNTs was investigated in the field study. Release of unrefined SWCNTs into air is insufficient concentrations during lab studies, but during field studies material handling has released low concentrations. Nanotube materials generated in all cases are estimated as lower then 53 $\mu g/m^3$.

Cui *et al.,* (2005) investigated the influence of SWCNT on human HEK 293 cells. The results indicated that SWCNTs can inhibit cell proliferation and decreases the adhesive ability in a dose and time dependent manner. The cells treated with SWCNT have showed active responses such as secretion of proteins to wrap SWCNTs, cell aggregation and nodular structure formation within the cells. The inhibition of cell growth is due to the induction of cell apoptosis and decrease in cell adhesion ability.

Ding *et al.,* (2005) performed a study to determine the effect of MWCNTs and MWCNO (multiwalled carbon nano-onions) on human skin fibroblasts and human embryonic lung fibroblasts. Cells were exposed to doses of 0.6 and 6 mg/L of MWCNO and 0.06 and 0.6 mg/L of MWCNTs. After 24 or 48 h of exposure, cells were counted by staining and evaluated for cytotoxicity, proliferation, gene expression, transcriptional changes and promoter analysis. The results indicated that MWCNTs activated the genes involved in

cellular transport, metabolism, cell cycle regulation and stress response. Nanotubes also induced genes responsible for inflammatory and immune reaction in skin fibroblasts. MWCNO exposure brought changes in genes responsible to induce external stimuli. Promoter analysis demonstrated that MWCNTs exert more harmful effects than MWCNO.

Sayes *et al.,* (2006) performed SWCNT cytotoxicity screens using cultured human dermal fibroblasts. Investigated the cytotoxic response of human dermal fibroblasts (HDF) to four water–dispersible SWCNTs by exposing the cells to the CNT samples at varying concentrations (3 μg/mL-30 mg/mL) and to 1% pluronic acid (control) for 48 hr later cytotoxicity tests were conducted using calcein AM, ethidium homodimer stains and MTT assay, followed by microscopic analysis. The results demonstrated that as the degree of sidewall functionalization increases, the SWCNT sample becomes less cytotoxic and sidewall functionalized SWCNTs are less cytotoxic than surfactant stabilized SWNTs. The cell viability decreased with increase in concentration of SWCNT suspension but cell death did not exceeded 50%. Microscopic studies revealed that after 36 hr of exposure SWCNTs are able to interact with biomembranes by forming aggregates.

Tian *et al.,* (2006) performed a study to correlate the toxic effects of five engineered carbon nanomaterials (1.SWCNTs, 2.Active carbon, 3.Carbon Black, 4.MWCNTs, and 5.Carbon Graphite) to their physico-chemical characteristics by exposing the human fibroblasts cells to a wide range of concentrations for different time intervals depending on the assay. Cell survival, cell adhesion, cell death assays were performed and cell morphology was assessed by microscopic studies. Immunocytochemical and western blot analysis was also carried out. From this study, the authors reported that SWCNTs induced strongest adverse effects like apoptosis and necrosis of all carbon nanomaterials used in the experiment. The refined

SWCNTs are more toxic than unrefined SWCNTs. Finally, the study concluded that the surface area is one of the important factors, which can predict the toxic effects.

Zhang *et al*., (2007) Studies the interactions of cells with derivatised soluble SWCNTs being used for therapeutic drug delivery. In their study human epidermal keratinocytes were exposed to functionalized SWCNTs of concentrations ranging from 0.00005 to 0.05 mg/ml. The MTT assay reveals that the cell viability decreased from 0.00005 to 0.05 mg/ml after 24 hr. The proinflammatory mediators, cytokines, TNF -α, IL-10, IL- 1 beta have not increased where as significant increase was observed in interleukin. TEM observations revealed intra cytoplasmic vacuoles in SWCNT treated cells.

Patlolla *et al*., (2010) tested the toxic effects of purified MWCNTs on normal human dermal fibroblasts cells. Three different doses and control were used in the study. After exposure to CNTs cytotoxicity, genotoxicity and apoptosis assays were performed as per standard protocols. The results showed a dose dependent toxicity of MWCNTs. Exposure to MWCNTs damages DNA thereby loss of cell viability. The results further demonstrated that CNTs are toxic at high concentrations.

3.1.1.2 Studies with Lung Cells

The common entry of airborne nanoparticles in humans is through the respiratory tract. It has been shown that the inhaled nanotubes are deposited by diffusion mechanism in all regions of the lungs.

Murr *et al.*, (2005) assessed the cytotoxicity of commercial SWCNTs and two different MWCNTs by performing viability assays on murine lung macrophage cell lines. The authors

compared the toxicity of CNTs with the toxocity of Chrysolite asbestos nanotubes and black carbon nanoaggregates. The size of the nano particles is 1 to 2μm in diameters. For all the carbon nanotube materials there is a strong concentration toxicity relationship relative to the black carbon and asbestos nanotubes. The studied MWCNTs are similar to the MWCNT's released from the cooking stoves. Their data also indicated a correlation between asthma incidences and exposure to gas stoves.

Lanone *et al.*, (2006) investigated the toxicological effects of MWCNT's on human alveolar epithelial cells with reference to their degree of dispersion. The cells were exposed to 0.1 to 100μg/ml MWCNT concentrations for 6 – 72 hr. The cytotoxicity was evaluated by neutral red and MTT assays. Optical and electron microscopy was used to assess the dispersion of MWCNT's. There is no change in neutral red irrespective of the exposure time and concentration. But MTT test indicated decrease in cell viability at 24 hr exposure time for 50 and 100μg/ml exposure concentrations. The effect was higher when nanotubes are suspended in ethanol rather than PBS due to the formation of bigger aggregates.

Magrez *et al.*, (2006) studied cellular toxicity of carbon based nano-materials including MWCNTs, carbon nanofibers and carbon nano particles as function of their aspect ratio and surface chemistry. The toxicity of these materials was tested on H596, H446 and Calu-1 lung tumor cells. The results demonstrated that these materials inhibit cell proliferation and promotes cell death. The carbon nanotubes are less toxic as compared to carbon fibers and nano particles. When carbonyl and hydroxyl groups are situated on the surfaces of SWCNTs their toxicity increases.

Davoren *et al.*, (2007) assessed the cytotoxicity of single walled carbon nanotubes on A549 cells, a human lung cell line. The cells were exposed to varied concentrations of SWCNT's (1 – 800μg/ml) and cell viability was determined using neutral red, alamar blue and MTT assays. These tests evaluated lysosomal, metabolic and mitochondrial activities. The other performed assays AK release and interleukin 8 indicated the loss of cell membrane integrity and inflammation response respectively. Exposures were conducted in 5% serum containing and serum free media. Cytotoxicity test results revealed that the SWCNT's will have very low acute toxicity to the A549 cells. TEM studies confirmed that there is no intracellular localization of SWCNT's in A549 cells.

Salvador-Morales *et al.*, (2007) investigated the interaction between double walled carbon nanotubes and lung surfactant proteins. Bronchoalveolar lavage fluid was collected from alveolar proteinosis patients as they have much higher concentration of lung surfactant proteins than healthy humans. SP-A & SP-D were isolated and estimated the protein content. The selective binding of proteins to CNT's was tested using affinity chromatography. The SDS-PAGE and western blotting showed that the surfactant proteins selectively bind to carbon nanotubes. The study revealed that CNT's can cause potential damage to lung immune defense mechanisms.

Simon-Deckers *et al.*, (2008) studied the toxicity of aluminium oxide, titanium oxide nano particles and MWCNT's to human A549 Pneumocytes with reference to size, crystal structure and chemical composition. The cell viability and intracellular accumulation assays were performed for the exposed cells. The study revealed that carbon nanotubes are more toxic than metal oxide nanoparticles. Both nanotubes and nanoparticles were able to enter into the cells rapidly and distributed in the cytoplasm and intracellular vesicles. The study

further demonstrated differences in biological response as a function of nanoparticle size, crystalline structure and chemical composition. The length of the nanotubes is not influencing the cytotoxicity.

Tabet *et al.,* (2009) evaluated the effects of MWCNTs on human lung epithelial cell line, A549 and mesothelial cell line. MWCNTs were dispersed in a pulmonary surfactant component, dipalmitoyl lecithin (DPL). The effects of dispersion in DPL was compared to the dispersion media, ethanol and phosphate buffer saline. MWCNT effects were also compared with asbestos fibers and carbon black nanoparticles. Large and more numerous agglomerates of MWCNTs were formed in PBS than in DPL and ethanol. The metabolic activity has decreased with 100µg/ml MWCNT concentration irrespective of the dispersion media. The study demonstrated that MWCNTs can cause adverse health effects without internalization by human epithelial and mesothelial cell lines.

Cesta *et al.,* (2010) postulated that lipopolysaccharide that causes lung inflammation would enhance the fibrosis caused by MWCNT's. After 21 days of exposure to MWCNT's, pulmonary fibrosis was observed but LPS alone will not cause any fibrosis. LPS plus MWCNT's exposure will enhance the fibrosis. Persons with pre existing pulmonary inflammation are at greater risk for the adverse effects of MWCNT's.

Thurnherra *et al.,* (2011) compared the acute and long-term effects of MWCNTs produced for industrial purposes on human lung epithelial cell line A549 and immune cells, Jurkat T-lymphocytes in-vitro. Pristine MWCNTs did not result in cell death in these cells even after acute exposure to 30µg/ml. But observed metabolic activity decreased and high levels of reactive oxygen species might affect the long-term viability of the tested cells. Exposure to

0.5µg/ml MWCNTs for six months did not result in adverse effects. Large amounts of nanotubes were accumulated in A549 cells.

Fenoglio *et al.,* (2012) studied the in-vitro toxicity of MWCNTs on murine alveolar macrophages, MH-5 using different diameter MWCNT's. The exposed cells were tested for cytotoxicity, uptake and oxidative stress induction. In-vivo toxicity was evaluated after intratracheal instillation in rats by measuring LDH activity and total proteins in bronchoalveolar lavage fluid. The MWCNTs of both diameters (9.4 and 70 nm) were internalized in MH-5 cells. The results of in-vitro and in-vivo assays reveal that the toxicity of thin MWCNTs was higher than thicker ones. This data indicates that the diameter is an important factor in the toxicological assessment of MWCNTs.

Hussain *et al.,* (2014) investigated the mechanisms involved in the injury of primary human bronchial epithelial cells due to exposure to MWCNTs and injury's contribution in induction of profibrotic response. Primary human bronchial epithelial cells were exposed to MWCNTs and lung fibroblast cells were exposed to fourteen different diluted conditioned mediums from HBE cells. MWCNTs induced pyroptosis in human bronchial cells in time and dose dependent manner. Conditioned medium has increased mRNA expression of profibrotic markers in lung fibroblast cells. The results indicated that the toxicity of MWCNTs shares common mechanisms with well known respiratory pathologies.

3.1.1.3 Studies with Other Cells

Bottini *et al.,* (2005) compared the toxicity of pristine MWCNTs with oxides MWCNT on human T-lymphocytes. The study revealed that oxidised MWCNTs were more toxic and can

induce significant loss of cell viability because of programmed cell death at a concentration of 400 μg/ml.

Zhang *et al.*, (2007) investigated the effects of SWCNTs, DWNT's & MWCNT on primary osteoblasts proliferation, differentiation and mineralization. Nanotubes with various lengths and diameters reduced the viability of osteoblasts. During final stages of differentiation, formation of mineralized nodules was inhibited in a dose dependent manner. Protein expression in osteoblasts was inhibited due to interaction with CNTs. CNTs may affect the cellular behavior from inside and outside the cells.

Zeni *et al.*, (2008) investigated the possibility for the induction of toxicity by SWCNTs in human blood cells. Cell viability, growth, metabolic activity and apoptosis were evaluated in human blood lymphocytes. In untreated cells DNA damage was evaluated. The studied SWCNT concentrations were 1 to 50μg/ml for durations carrying from 6 to 72 hr. Decrease in cell growth was observed at highest studied concentrations but this decrease was not associated to apoptosis. The decrease in cell growth is due to decrease in metabolic activity.

Bang *et.al.*, (2011) studied the effects of multi-walled carbon nanotubes on the cell viability of neuroscreen cells. The study results indicated that MWCNTs reduce cell viability and long exposure is needed to induce cell death. Apoptosis results in cell death with the depolarization of mitochondrial membranes and fragmentation of DNA.

Sun *et al.*, (2011) evaluated the effect of CNTs on cytotoxicity mediated by lymphocytes in-vitro. The results obtained revealed that CNTs at low concentrations would not cause direct cell death or apoptosis. CNTs have enhanced lymphocyte mediated cell toxicity against

human cell lines. The study suggested that CNTs at low concentrations may initiate cytotoxicity indirectly by affecting lymphocyte function.

3.1.2 In-vivo Studies

Animals like mice, rats, guinea pigs have been utilized as experimental animals for studying health effects of CNTs, these animals have been exposed to various dosages of CNTs. Exposure through environmental media is highly relevant as there is interaction between environmental compartments and properties of CNTs. Consequently this may change the behavior of CNTs in environment and may influence impacts of CNTs. CNTs after entering into the body get deposited into various other organs of body and show their deleterious effects. The studies carried out using experimental animals and investigated effects on lungs and other animal subjects are presented.

3.1.2.1 Studies with Lung Cells

Huczko *et al.,* (2001) performed a study to detect whether CNTs impose health effects similar to asbestos fibers, as they possess similar aspect ratio to that of asbestos fibers. Similar tests were used to detect health hazards associated with asbestos fibers were performed in this study. Pathogen free guinea pigs were taken and exposed to 25 mg of CNTs containing soot given suspended in 0.5 ml of sterile saline intratracheally under anesthesia. CNTs-free soot suspension samples were employed as control. After four weeks, pulmonary function tests were performed by non-invasive respiratory analysis. Later the animals were sacrificed and BALF was collected, subjected to total protein and differential cell count estimations. The results of pulmonary tests showed that the test and control

samples did not differed in tidal volume, frequency of breath and lung resistance. No momentous differences in cell distribution and protein concentration were observed. The study inferred that intratracheal instillation of fibrous CNTs has not changed pulmonary function and has not induced inflammation. Thus CNT contained soot did not have any health hazards.

Lam. C *et al.,* (2003) intratracheally instilled mice with a single dose of 0.1 or 0.5 mg/kg single walled carbon nanotubes and tested for toxicity after 7 days or 90 days. Dose – dependent epithelioid granulomas were observed in all exposed mice. In some cases interstitial inflammation was observed. These lesions were more pronounced in mice observed after 90 days. The obtained experimental results revealed that the carbon nanotubes are much more toxic if they are able to reach the lung and this has to be considered as a serious occupational hazard in chronic exposures.

Warheit *et al.,* (2004) evaluated the acute lung toxicity of SWCNT in intratracheally-instilled rats. The rats were instilled with 1 or 5 mg/kg of SWCNT, quartz carbonyl iron particles, PBS + tween, and graphite particles. The lungs of exposed were assessed using biomarkers, cell proliferation methods and histopathological examination at 24 hr, 1 week, 1 month and 3 months after instillation. High dose of SWCNTs resulted in 15% mortality due to mechanical blockage of upper airways. Inflammation and injury was produced in other SWCNT exposed rats.

Shvedova *et al.,* (2005) studied the pulmonary response of mice exposed to single walled carbon nanotubes. The study demonstrated that the aspiration of SWCNTs elicited serious

pulmonary effects in combination with acute inflammation. These symptoms will lead to early onset of progressive fibrosis and granulomas.

Mangum *et al.,* (2006) investigated the fibrogenic potential of SWCNTs, which synthesized by chemical vapor deposition using molybdenum and cobalt as catalysts. A single dose of SWCNT's was given by oropharyngeal aspiration and histopathology; cells proliferation and growth factor mRNAs were evaluated at 1 and 21 days of post exposure. No inflammatory response was observed in any of the exposed rats. Focal interstitial fibrotic lesions were induced within the alveolar region of the cell. Intracellular carbon structures composed of SWCNTs were observed and these structure bridged lung macrophages. These carbon bridges might be a identification mark of CNT exposure.

Li *et al.,* (2007) evaluated alterations of aortic mitochondrial activity by performing oxidative stress assays. These assays also include quantitative polymerase chain reaction of mitochondrial DNA. Mice were exposed to SWCNTs were analyzed morphometrically for plague formation by intrapharyngeal installation and the results indicated that exposure to SWCNTs induced hemeoxygenase activation in lung's aorta and heart tissues. The authors also found that mice exposed SWCNTs for 7, 28 & 60 days has developed mitochondrial DNA damage in aorta. This damage was accompanied by changes in mitochondrial glutathione and protein carbonyl levels. Exposure SWCNTs have not modified lipid profiles in mice, but this resulted in acceleration of plague formation in the aortas and in the brachiocephalic arteries. This study indicates the possibility for systemic effects of SWCNT under workplace.

Mitchell *et al.,* (2007) assessed the pulmonary and immune response as a function of time and dose in mice exposed to MWCNTs. The mice were exposed to 0.3, 1 and 5 mg/m^3 of MWCNTs for 7 or 14 days. (6hr/days). The exposed mice histopathology has revealed that there was no tissue damage or inflammation and alveolar macrophages are having black particles. Systemic immunosuppression was observed in mice exposed to higher concentration for 14 days duration.

Mercer *et al.,* (2008) tested the changes in pulmonary distribution and response that will result from the exposure to more dispersed SWCNTs. Single walled CNTs with a mean diameter 0.69μm were dispersed and given to mice by pharyngeal aspiration. More dispersion of SWCNTs results in aspiration of small particles and thereby entering into the alveolar walls. Dispersed SWCNT's response is interstitial fibrotic reaction is more potent than granuloma formation.

Shvedova *et al.,* (2008) conducted inhalation and pharyngeal aspiration studies using stable and uniform SWCNT dispersions, which were obtained by a new aerosolization technique. The inhalation of impure SWCNT at 5 mg/m^3 for 5 hr a day for four days was compared with pharyngeal aspiration of same SWCNTs with doses varying from 5 - 20μg per mouse. Inhalation of SWNCT's was more effective in causing inflammatory response, collagen deposition, oxidation stress, fibrosis and genetic mutations than aspiration. Inhalation exposure was more potent than aspiration of an equivalent amount of SWCNTs.

Ma-Hock *et al.,* (2009) performed MWCNT inhalation toxicity study for a period of 90 days by exposing Winstar rats for 6 h/day, 5 days/week for 13 weeks. The MWCNT concentrations used were 0.1, 0.5 or 2.5 mg/m^3. Inhalation of MWCNT has not produced any

systemic toxicity. At 0.5 & 2.5 mg/m^3 concentrations, lung weight has increased, more pronounced inflammation and intra alveolar lipo-proteinosis were observed in lungs and lung associated tissues. The dust forming potential of tested MWCNTs was measured by standardized drop method and was found to be low.

Nygaard *et al.,* (2009) examined the capacity of SWCNTs and MWCNTs in promoting allergic responses in mice, in injection model as well as intranasal model. Mice were exposed to three doses of SWCNT, MWCNT and ultrafine particles during ovalbumin allergen sensitization. After 5 days of exposure, serum antibodies, number of inflammatory cells and cytokine levels were determined. After a single intranasal exposure, differential cell counts were determined. Serum levels have increased in both SWCNT and MWCNT exposed cells together with ovalbumin demonstrating that CNT's promote allergic responses in mice.

Crouzier *et al.,* (2010) conducted in-vivo toxicity assays for understanding the increased oxidative stress. A concentration of 1.5mg/kg doubled walled CNTs were intra-nasally instilled to mice and after 6, 24 or 48 hr of administration, inflammation and localization in lungs was observed microscopically. Electron Spin Resonance (ESR) and spin trapping experiments were used to investigate local oxidative perturbations and plasma concentration of cytokines were measured for assessing systemic inflammation. The results confirmed the induction of inflammatory reaction by MWCNT's and this was accompanied by decrease in local oxidative stress.

Pauluhn et.al., (2010) exposed Winstar rats to MWCNTs for 13 weeks by inhalation. His study focused on respiratory tract and systemic toxicity, which includes biokinetics of

MWCNTs in the lungs and associated lymph nodes. The pulmonary effects were examined up to a post exposure period of six months by observing changes in bronchoalveolar lavage and histopathology. Animals tolerated respirable solid MWCNT aerosols without any systemic toxicity. The biokinetic study demonstrated a delayed lung clearance of MWCNTs at overload conditions. MWCNTs were translocated into lymph nodes at high concentrations. At high exposure levels the weights of lungs and lymph nodes has increased significantly. His study demonstrated that MWCNTs induce pathological changes due to overload phenomena. The inflammatory response of MWCNTs can be related to the high volume displacement of low density MWCNTs rather than their intrinsic toxic properties.

Meng *et al.*, (2013) investigated the effect of MWCNTs with varying concentrations of iron impurities on rat pheochromocytona cells. These types of MWCNTs are generated during the large-scale production of MWCNTs by chemical vapor deposition. Exposure to MWCNTs with high iron content can reduce the cell viability; cytoskeletal disruption can be increased and reduced their ability to form mature neural cells. The study found the critical role of iron impurities in the adverse effects of MWCNTs on neural cells.

Rydman *et al.*, (2014) exposed mice to rigid and flexible carbon nanotubes for four hours a day, for four consecutive days by means of inhalation. The subsequent changes were monitored immediately, after 24 hr and after four days of post exposure period and this is mimicking the occupational workweek. The same study was conducted with mast cell deficient mice in order to evaluate the role of mast cells in causing inflammation. The results showed that short-term inhalation of rod like CNTs can induce airway inflammation in healthy mice. Mast cells can partially regulate the inflammation but alveolar macrophages plays an important role in the early stages.

Upadhay *et al.*, (2014) investigated the cardiovascular effects of ultrafine carbon particles on aged hypersensitive rats and the results were compared with earlier studies in order to identify the effects of age. The rats were exposed to ultrafine carbon particles via inhalation route and blood pressure and heart rate were assessed and Interleukin 6 and proinflammatory cytokines, haptoglobin, serum C reactive protein and plasma fibrinogen were measured in the bronchoalveolar fluid. Transcript levels in the lung and heart were measured to assess oxidative stress and conjugation cascade. Fine carbon particles can induce pulmonary and systemic inflammation in aged rats due to oxidative stress and disturbed coagulatory hemostasis.

3.1.2.2 Studies with Other Cells

Li *et al.*, (2010) conducted a study to know the effect of oxide MWCNTs in mice stomach. Female Kongming white mice were used for the study. Mice were fed with hydrated diet 24-36hr before starting the experiment. Then four groups of mice were injected with L-arginine intraperitoneally for 5 days to prepare gastric functional disorder (FD) mice. Normal group was intraperitoneally injected with moral saline to be used as control. On fifth day of formulating of FD mice one group out of four was injected with Oxide MWCNTs (500 μg/mouse) for 3 days another one group of FD was continued to inject with L-arginine for 3 days. The mice were sacrificed and tested for Nitric Oxide (NO), acetylcholine, pepsin activity and kinetic studies. The results indicated that NO content in OMWCNTs treated FD mice was decreased and acetylcholine was increased. Gastric motility factor *k* was obtained by kinetic studies and it was higher in OMWCNTs treated FD mice. At certain dosage, MWCNTs can improve gastric emptying and motility in mice.

Liu *et al.,* (2014) presented a new model the Drosophila embryo to assess toxicity of MWCNTs during embryonic development and on cells. This model offers rapid inexpensive and complete analysis of CNT toxicity. The injected MWCNTs become incorporated into Drosophila embryo cells and consequences of CNTs uptake on cell communication and organ formation can be studied. The injected cells are having normal division capacity and differentiation patterns. The survival of ectodermal cells has decreased but not neural stem cells. The study indicated that Drosophila can be used as tool to study toxicity of MWCNTs.

Wei *et al.,* (2014) investigated the effects of oxidised MWCNTs on hepatotoxicity of cadmium in mice. Cadmium was injected into mice and then they were exposed to various dosages of oxidised MWCNTs. The results showed that oxidised MWCNTs can release cadmium from the accumulated cadmium metallothione mice that were injected oxidised MWCNTs and cadmium together showed reduced hepatotoxicity of co-exposure than that of single exposure. The study deduced that metallothione might be connected with oxidised MWCNTs in order to reduce their hepatotoxicity.

3.1.3 In-Vitro and In-vivo Studies

Sato *et al.,* (2005) investigated the effect of CNT length on cytotoxicity of MWCNTs using in-*vitro* and *in-vivo* studies. Human acute monocytic leukemia cell line was used for in-vitro studies and response in subcutaneous rats was used for in-vivo studies. The results indicated that the inflammation was higher for longer CNTs than shorter one. This is due to faster enclosure by macrophages. The observed responses were not severe.

Saxena *et al.*, (2007) compared the in-vivo and in-vitro toxic effects of pristine and acid functionalized single walled carbon nanotubes on mouse lung cells. Acid functionalized SWCNTs exerted strong cytotoxic effects on cultured mouse lung epithelial cells. These AF-SWCNTs also inhibited cell cycling of lung epithelial cells. AF-SWCNT's oropharyngeal aspiration induced acute inflammatory response in the lungs of mice. Neutralizing their surface charges can reverse these toxic effects.

Muller *et al.*, (2008) addressed the genotoxic potential of MWCNT in rats. For in-vivo experiments rats were administered with a single dose of 0.5 or 2 mg of MWCNTs. After 3 days, micronuclei were assessed in-vivo in type II Pneumocytes. For Invitro experiments rat lung epithelial cells were exposed to 10, 25 & 50μg/ml MWCNTs. Micronucleus assay was performed for exposed cells. Clastogenic and aneugenic mechanisms were differentiated by pancentromeric fluorescent probe. The observations revealed that MWCNTs induced centromere positive and negative multinuclei in cells indicating their ability to induce clastogenic and aneugenic effects.

Osmond-Mclead *et al.*, (2011) determined the durability of four different types of CNTs in simulated biological fluids and their in-vivo pathogenicity using a mouse model. The in-vivo and in-vitro results were compared with the durability of glass wool and asbestos fibers. After 24 weeks of incubation in simulated biological fluid, the durability and persistence of glass wool and asbestos is similar to their known, whereas three types of CNTs out of four tested showed no loss of mass or change in length or morphological characteristics when examined by electron microscopy. The fourth type of MWCNT's showed 30% loss in mass within first three weeks of incubation. These results indicate that carbon nanotubes are durable but may be subjected to modification in specific samples.

Hamilton *et al.,* (2013) examined the effects of MWCNT properties – diameter, length, purification and carboxylation on in-vitro in-vivo bioactivity. MWCNTs with varied properties were tested for in-vitro cytotoxicity and inflammation activity using THP-I cells and primary alveolar macrophages. In-vivo bioactivity of MWCNTs was tested in mice by oropharyngeal aspiration administration. The results demonstrated that increased size of MWCNTs increased their bioactivity as indicated by inflammation activity completely. Purification had very less effect on the cells. In-vivo studies revealed that increase in size has resulted in severe inflammation. Both in-vivo and in-vitro studies have given similar results.

3.2 Summary of Literature Review

To investigate the effects of CNTs, researchers exposed various cell lines and animal subjects to CNTs, produced by different methods possessing diverse lengths, diameters and aspect ratios. The parameters, specific surface area and size of CNTs were measured using Brunauer-Emmet-Teller method and Transmission electron microscopy respectively (Zhang et. al, 2005).

3.2.1 Cytotoxicity

The above mentioned studies used different bioassays to investigate the effects of CNTs on cells. Cytotoxicity of CNTs (SWCNTs & MWCNTs) can be assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH), sodium 3'-(phenyl amino-carbonyl-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene-sulphonic acid hydrate (XTT), Alamar Blue (AB), neutral red, Alkaline Phosphatase (ALP) assays and staining techniques such as trypan blue exclusion, Hoechst, propidium iodide, YO-PRO1, Diamino-2-Phenyl Indole dihydrochloride (DAPI), annexin V , and

Bromodeoxy uridine (BrdU) antibody stains and methods like DNA fragmentation, caspase-3 and 3/7 activity measurement etc.

3.2.1.1 Cell Viability

Scientists (Cui et al. 2005; Magrez et al. 2006; Davoren et al. 2007; Zhang et al 2007; Muller et al. 2008; Simon-Deckers et al. 2008; Tabet et al. 2009; Patlolla et al 2010; Thurnherr et al. 2011) have performed MTT assay in order to measure the mitochondrial activity of cells exposed to CNTs with varying concentrations.

HEK 293 cell viability decreased in time and dose dependent manner after exposing to 0.78125-200 μg/mL of SWCNTs (Cui et al 2005). The cell viability was inversely proportional to the dosage of MWCNTs in H596 human lung tumor cells (Magrez et al. 2006). Maximum cytotoxicity of A549 cells was observed at 800 μg/mL of SWCNTs after 24hr, however the percentage inhibition was more in the absence of serum (45%) when compared to its presence (32%) (Davoren et al. 2007). Reduction in cell viability was 60 % at a dose of 100 μg/ml of MWCNTs after one day in Rat Lung Epithelial cells (Muller et al. 2008). Cell death is 50 % at a concentration of 0.5 μg/mL MWCNTs after two days in A549 cells (Simon-Deckers et al. 2008). Decrease in cell viability is 60 % at a concentration of 100 μg/mL of MWCNTs in both A549 & MeT5A cells (Tabet et al. 2009). Patlolla et al 2010 observed time and dose dependent decrease of human dermal fibroblast viability after 24, 48, 72 and 96hr exposure to 40, 200 and 400 μg/mL MWCNTs and the reduction in cell viability is 50 % at 400 μg/mL. Dose dependent decrease in viability of A549 cells is due to acute exposure (3.2, 6.25, 12.5, 25 and 30 μg/mL) of MWCNTs (Thurnherr et al. 2011). The

decrease in cell viability in all the studies might be due to the mitochondrial damage and interruption of NAD(P)H flux.

Cell death was 10 % after two days of exposure to MWCNTs in A549 cells at a concentration of 50 µg/mL and this was quantified by XTT assay (Simon-Deckers et al. 2008). Tabet et al. (2009) did not observe any significant alteration in cell viability through neutral red assay. Shvedova et al. (2003) used Alamar blue bio assay to determine cell viability of human epidermal keratinocytes by exposing them to 60, 120 and 240 µg/mL SWCNTs for 18 hr and observed a decrease in cell viability of 11.3 %, 24.5 % and 37.2 % respectively indicating the concentration dependent decrease in cell viability. Bottini et al. 2006 exposed T lymphocytes to MWCNTs (oxidized and pristine) at a concentration of 0.001 and 0.01µg/ml up to five days and assessed the cell viability by trypan blue staining. Oxidized MWCNTs were found to be more toxic than pristine MWCNTs.

Zhang et al. (2007) used ALP assay to investigate the potential effects of CNTs on osteoblast's differentiation and the study indicated the inhibition of ALP activity at all concentrations of CNTs in a time dependent manner without any dose dependence. Zeni et al (2008) exposed Human peripheral blood lymphocytes to 5, 10, 25, 50 µg/mL concentrations of SWCNTs for one, two and three days and tested the cell viability with trypan blue staining. Dose-dependent decrease in cell viability was observed, which was significant at 25 and 50 µg/mL concentrations. Bang et al. (2011) studied the effect of soluble MWCNTs on neural cells at two concentrations i.e., 190 and 295 µg/mL. 40 % decrease of resazurin conversion capacity was observed after one-day exposure to CNTs, and was more pronounced after two days. Less than half of the treated cells had intact plasma membrane by second day. The non

toxicity of functional CNTs is due to the specific groups which reduce the availability of reactive sites on the surface of CNTs (Sayes et. al., (2006)

3.2.1.2 Cell Damage

The extent of damage to the cell can be determined by LDH release assay. The cell damage was dose-dependent in RLE cells and was significant at 100 and 150 µg/mL of MWCNTs (Muller et al. 2008). Exposure to 100 µg/mL MWCNTs for two days has resulted in 35-40 % cell damage in A549 cells (Simon-Deckers et al. 2008). Zein et al 2008 observed a slight increase in LDH in human peripheral blood lymphocytes after two days exposure to 5, 10, 25 and 50 µg/mL SWCNTs. Patlolla et al. (2010) exposed human dermal fibroblasts to MWCNTs and the release of LDH increased in time and in dose-dependent manner. The extent of cell damage is also dependent on the thickness of CNTs as evident from the study performed by Fenoglio et al. (2012) in which thin $MWCNTs_{9.4}$ were found to be more toxic than thicker ones ($MWCNT_{70}$).

3.2.1.3 Apoptosis

Apoptotic cells can be identified using annexin V staining. Six hours exposure to MWCNTs induced a significant apoptotic response at 50 µg/mL concentration in RLE cells (Muller et al. 2008). Exposure of A549 cells to 30 µg/mL MWCNTs for six days has increased the number of apoptotic cells (Thurnherr et al. 2011). In addition to annexin V, Thurnherr et al. (2011) used propidium iodide stain to detect late apoptotic/ necrotic cells. Incubation of A549 with 100 µg/mL MWCNTs for one, two and three days have resulted in 15-20 % reduction in cell number (Tabet et al. 2009).

Apoptosis and necrosis were induced in MWCNT treated skin fibroblast cells in a dose dependent manner (Ding et al. 2005). Induction of apoptosis was started from a dosage of 40 μg/mL. A significant dose dependent increase in the percentage of apoptotic cells was observed after exposure to MWCNTs (40, 200, 400 μg/mL) for two days (Patlolla et al. 2010).

MWCNTs stimulated apoptosis/necrosis was tested using a double-stain flow cytometric assay. In neural cells CNTs induced cell death in an apoptotic manner and not by necrosis and moreover apoptosis occurs more slowly. Measurement of active Caspase 3/7 levels in CNT treated cells will indicate the extent of cell damage and apoptosis. The observation of Bang et al. (2011) suggests that the Caspase 3/7 activity is 20-30 % higher in CNT treated cells than controls and is maximum after three days. SWCNT treated HEK 293 cells displayed morphological changes and became apoptotic at 25μg/mL concentration after one day.

Methods like DNA fragmentation and annexinV-FITC (fluorescein isothiocyanate) staining, Caspase-3 and 3/7 activity were used to identify apoptotic cells. Cui et al. (2005) after exposing HEK 293 cells to various concentrations of MWCNTs for 6 days observed apoptosis after one day at 25 μg/mL concentration which was increased gradually with increase in time. Dose dependent stimulation of apoptotic cells were monitored by Bottini et al. (2006) after five days exposure to MWCNTs exposure. Zeni et al. (2008) did not observe any increase in Caspase-3 activity after treating with SWCNTs at 25 and 50 μg/mL concentrations for seven hours a day.

3.2.1.4 Cell Proliferation

Proliferation of skin fibroblasts was measured by incorporating BrdU and was reduced by ~50 % in MWCNT treated fibroblasts after two days at 0.06 µg /mL (Ding et al. 2005). Davoren et al. 2007 used Alamar blue assay to test the inhibition of cell proliferation by SWCNTs in A549 lung cells both in the presence and absence of serum.

3.2.1.5 Oxidative Stress

Oxidative stress was induced when the human epidermal keratinocytes were exposed to SWCNTs for 18hr at 37^0C. This was confirmed by the formation of free radical species, decrease in vitamin E, total antioxidant reserves, accumulation of peroxidative products and reduction of protein thiols in the CNT treated cells (Shvedova et al. 2003).

3.2.2 Genotoxic Effects

Exposure to CNTs may effect the genetic material of the cells, and were evaluated by MN assay, Comet assay and DNA ladder analysis.

A dose dependent increase in multinucleated pneumocytes and micronucleus were observed *in vivo* and *in vitro* studies respectively Muller et al. (2008). Neither DNA damage nor chromosomal damage was observed in A549 lung epithelial cells and Jurkat T Lymphocytes (Thurnherr et. al, 2011).

MWCNT treatment causes changes in gene expression, which are involved in down regulation and up regulation. CNT treatment will alter the promoters of genes (Ding, et. al,

2005) Cells treated with high dose of CNTs caused more changes in gene expression than lower dosage. The cellular response to CNTs treatment mimics to the response of viral infection because the dimensions of the CNTs are similar to that of the virus (Ding, et. al, 2005). At high doses CNTs induces innate immune response and the carbon atoms released may participate in cell's metabolic pathways (Ding et al. 2005). The genomic DNA damage was increased with the increase of CNT dosage, which was measured by Comet assay and DNA ladder analysis. Patlolla et al. (2010) monitored CNT treated human dermal fibroblasts and the cells have shown significant increase in percent tail DNA (2.96 %-16.39 %) and DNA damage with increase in dosage.

3.2.3 Inflammation and Fibrosis of Lung Cells

Exposure to CNTs developed inflammation and fibrosis in experimental animals used for the study.

Post instillation of SWCNTs has increased the LDH levels and short-term pulmonary inflammatory response was due to increase in neutrophils after 1 day. Dose independent multifocal granulomas were also observed. BAL cell counting and Sirius red staining indicated increased accumulation of neutrophils followed by sequential appearance of lymphocytes and macrophages (Shvedova et al. 2005). Magnum et al. (2006) counted cells, analyzed total protein and LDH levels in BALF (broncho alveolar lavage fluid) and observed the formation of fibrotic lesions in rat lungs after 21 days of exposure to 200 μg/kg of SWCNTs. Increased PDGF levels suggest their role in the formation of SWCNT induced fibrotic lesions. The dispersion of SWCNTs will increase the thickness of the alveolar wall as the number of alveolar macrophages was elevated (Mercer et al. 2008).

Muller et al. (2008) observed inflammatory response in rats exposed to MWCNTs (500, 2000 μg/rat) due to dose-dependent increase in levels of LDH, protein, macrophages and neutrophils. Inhalation of 500 and 2500 $\mu g/m^3$ concentration of MWCNTs resulted in increase in lung weight, inflammation, neutrophilic and intra-alveolar lipoproteinosis in the lung and lung associated lymph nodes and slight blood neutrophilia at 25 $\mu g/m^3$ (Ma-Hock et al. 2009). Cesta et al. (2010) exposed rats to MWCNTs (4000 μg/kg) for a period of 21 days and observed significant fibrosis & lesions in lungs. Prior exposure to 2500 μg/kg lipopolysaccharide (LPS) of E.*coli* enhanced the effect of MWCNTs by inducing fibrosis, increasing total protein and platelet derived growth factor by two-fold and three to four folds respectively after one day. Fenoglio et al. (2012) measured LDH activity, protein content in BAL and inflammatory cell count in alveoli of rats treated with 2000 μg of MWCNT and the effect has decreased with increase in diameter.

3.2.4 Cellular Internalization of CNTs

Transmission electron microscopy (TEM) observations of MWCNT exposed A549 cells have revealed the presence of two to three micrometer or smaller MWCNTs in the cytoplasm, in an isolated manner, and altered the morphology of cells (Simon- Deckers et al. 2008). In contrast, agglomerates of MWCNTs with different sizes were found in the cytoplasm of murine alveolar macrophage (MH-S) cells (Fenoglio et al. 2012).

Although many toxicity studies were conducted for assessing the health effects of CNTs, only few studies have indicated the presence of CNTs within the lung cells which may be due to the membrane permeability of a particular cell line and high dosage of CNTs used in that study. The formation of agglomerates within the cell depends on surface chemistry of CNTs.

The effect of CNTs on cell viability varies due to the use of different cell lines, varied concentrations & type of CNTs and presence of catalyst impurities (Simon Deckers et al. 2008; Tabet et al. 2009).

The literature review reveals that CNT may cause acute effects such as cell damage, inflammation, inhibition of cell proliferation, oxidative stress and chronic effects such as granuloma formation, fibrosis etc. All the effects have been observed as dose dependent and time dependent. At this juncture, it is crucial that an investigation into cytotoxicity of CNTs is undertaken as soon as possible. In the present study an attempt was made to study the effects of MWCNTs on cultured human lung cells and exposure assessment of MWCNT production unit.

3.3 Guidelines and Standards

At present, the scale of industries handling CNTs is small, but in future it is expected to increase extensively due to unique properties. The present exposure assessment of CNTs is considerably different from those studies conducted previously for other materials. This difference is due to changing scenario and limited data availability in case of CNTs. With limited available data, it is not possible to develop exposure assessment procedure applicable to all kinds of scenarios. It is essential to develop a procedure for establishment of a provisional value for an acceptable exposure concentration in the occupational environment. Assuming certain emission and exposure scenarios, CNT inhalation exposure of workers can be estimated for the worker who handles CNT powder directly. For proper practical formulation of standards, extensive industry data is needed which is applicable to all industrial environments.

---End of Chapter 3---

Chapter-4

4. Research Methodology

Although human beings have been exposed to nanoparticles since ages, such exposure has increased enormously over the last century due to manmade sources. Safety and potential hazards of nanotubes is the information needed to solve the issue of biocompatibility as these are effectively deposited by diffusion in all regions of the respiratory tract.

Methodology adapted to evaluate the effects of MWCNTs respiratory exposure on lung cells and assessment of risk occurring via inhalation at the workplace in a CNT manufacturing industry is presented in this chapter.

4.1 Survey

A preliminary survey was conducted using the proform given in table 6 for a sample population of workers working in multi-walled carbon nanotube manufacturing industries, to collect information regarding amounts of carbon nanotubes produced, population of workers working in different units, number of working hours per day/week in those industries etc.

4.1.1 Preliminary Survey Pro-forma

In order to build contact with CNT manufacturing industries preliminary information was sought and this proforma was mailed to all the contacts collected through internet. The sample proforma is given below.

Table 6: Preliminary Survey Proforma

	Department of HSE **University of Petroleum and Energy Studies** **Dehradun**	**Department of Biotechnology** **GITAM Institute of Technology,** **GITAM University, Visakhapatnam**
	Proforma	
1	Name of the company:	
2	Address:	
3	Contact number:	
4	E-mail:	
5	Quantity of CNTs produced:	
6	Method used for CNT production:	
7	No of Employees working:	
8	No of Working hours per day:	

Thank you for your co-operation. Kindly email this filled-in form to the e-mail id khasimb@gitam.edu. Or akbarziauddin@gmail.com

4.1.2 List of CNT Industries

The above proforma was sent to the following industries as given in table 7.

Table 7: List of CNT Producing Industries

S.No	Name of the Company	Products Manufactured	Address of the Company	Contact Number	Email
1.	Cheap Tubes inc.	Carbon Nanotubes (CNTs)	112 Mercury drive, Brattleboro, VT 05301 USA	Tel: 802.254.6969 Fax: 802.254.7070	mike@cheaptubes.com sales@cheaptubes.com
2.	Unidyam, INC.	Development, manufacture and application of CNTs	Unidym, Inc., 1244 Reamwood Avenue, Sunnyvale, CA 94089	Ph: (408) 934 – 6850. Fax: (408) 701 – 5001.	Gen Inq: info@unidym.net Sales Inq: sales@unidym.net
3.	Nano Mix	CNTs based sensors for detecting chemical vapours	Nanomix, Inc. 5980 Horton street suite 600	Tel: 510.428.5300 Fax 510.658.0425	info@nano.com
4.	Nano-proprietary, Inc	Sensor based up on enzyme coated CNTs for analyzing chemicals in liquid samples	3006 Longhorn Blvd., Ste. 107, Austin, TX 78758, U.S.	Ph: 512 – 339 – 5020. Fax: 512 – 339 – 5021.	
5.	Xin Ray Systems	Nano tubes based X-ray systems	Xin ray systems Inc P.o. box 12848 Research Triangle Park North Carolina 27709 USA	Tel: 919.313.9685 Fax: 919.313.9686	sinraysystems@xinraysystems.com
6.	Hyperion	Nano tube based plastic mold compounds	Head quarters & Main sales office, 38	Tel: 617.354.9678 Fax: 617.354.9691	info@hyperioncatalysis.com

			smith place, Cambridge, MA 02138, USA		
7.	Nano Lab	Functionalized nanotubes and nono arrays	Nano Lab, inc 179 bear hill road, Waltham, M A 02451	Tel: 781.609.2722	info@nano-lab.com sales@nano-lab.com
8.	Nano Ledge	Nanotube based resins	Corporate headquarters, Americas,75 Boulevard de mortagne, suite 121, boucherville, quebec, j4b6y4 Canada	Tel: 450.641.5475 Fax: 450.641.5915	
9.	Nanocs	Functionalized nanotubes	244 fifth avenue, #2949, Newyork, NY 10001	Tel: 917.400.4863 Fax: 917.591.2212	sales@nanocs.com
10.	Nanocyl	Nanotube based epoxy resins, coatings and conductive plastics	Belgium Nanocyl S.A (head quarters), Rue de 1 Essor, 4,B-5060 sambreville Belgium	Tel: +3271750380 Fax: +3271750390	sales@nanocyl.com
11	Catalytic materials	CNTs and graphite nanofibres	325 heart land drive, pittsboro, NC 27312	Tel: 919.918.7638 Mbl: 919.704.5736	info@catalyticmaterials.com
12	MER corporation	CNTs and bucky balls	Materials and Electro-chemical Research, MER corporation, 7690 South kolb road, Tucson,	Tel: 520.574.1980 Fax: 520.574.1983	mercorp@mercorp.com www.mercorp.com

			Arizona 85706		
13	Nanocarb lab	CNTs	Nanocarb lab (NCL), Medchemlabs inc. (MCL) division,1812 years street 7 apt.6, 121170 Moscow, Russia	Tel: +7-095-7781037	irina.kazakova@nanocarblab.com
14	Nanothinx	CNTs	Nanothinx S.A, Stadious street, Platani, P.O.Box 1414, Riopatras 26504, Greece	Tel: +30-2610-965208	info@nanothinx.com
15	Rosseter holdings ltd	CNTs	4 Nikiforoulytra, P.O.Box 57220, 3310 Limassol, Cyprus	Tel: +35725591600, Fax: +35725591888	contact@e-nanoscience.com
16	Rosseter holdings ltd	CNTs	USA: 55 paul revere road,lexington,MA 02421	Tel: +18136452360	contact@e-nanoscience.com
17	Nanoshel	CNTs, metal and oxide nanoparticles	Nanoshel LLC, 3422 Old Capitol Trail Suite 1305, Wilming Tonde, Delaware-19808, USA	Tel: 302652-3464, Fax: 302996-5818	avena@nanoshel.com, contact-us@nanoshel.com
18	Nanoshel	CNTs, metal and oxide nanoparticles	Intelligent Materials Pvt Ltd, Plot No. 211, Sector 12, Panchkula – 134112,	Tel: +91.9779.880077	sales@nanoshel.com

			Haryana, India.		
19	Xintek	Nanotube based cathodes, AFM probes and X-ray tubes	Xintek Inc. 7020 Kit creek road suite 200, P.O.Box 13788, Research Triangle Park, NC 27709	Tel: 919.313.9638	info@xintek.com
20	Nano Biochemicals	Producer of high quality nanoparticles	Nano Biochemicals India pvt ltd (corporate headquarters-mumbai maharashtra, # 1004, Shivtapi, HG road, Gamdevi, Mumbai-400007	Tel: +91.222.367.6377	info@nanobiochemicals.com website:www.nanobiochemicals.com
21	Quantum Materials Corp.	Manufactures CNTs and Garaphene	Nanowerk LLC, 700 bishop street, 17th floor, suite 1700, honolulu, HI 96813 USA	Ph: (858) 465 - 7300	
22	Reinstei Nano Ventures	A manufacturer of Nanomaterials	A-105, level 3, sector 63, noida, P.C 201301, India	Tel: +91.120.478 1.217, 214	info@reinstei.com, inquiry@reinstei.com

4.2 Data Collection and Analysis

A questionnaire was prepared and sent through mail to all the industries given in table 7. All the workers of the industries were requested to give the details, which are mentioned in the proforma as a reply mail. The questionnaire basically contains questions relating to Human health & safety factors. Cronbach alpha test was used to estimate the reliability of obtained responses to the questionnaire. Data collected was maintained confidentially and analyzed statistically using regression modeling to know the dependence of other health parameters. Certain correlations were done, once they are dependent. Depending on the analyzed data, experiments were planned and designed to study the effect of MWCNTs on human cell cultures in the laboratory.

4.2.1 Questionnaire Pro-forma

Questionnaire was developed which was intended to understand the exposure to CNTs and their health effects. The questionnaire consisted of seven questions related to health and safety aspects of workers. The responses of those 7 questions were used for the calculation of Cronbach Alpha. This Cronbach Alpha analysis will tell us the reliability of the prepared questionnaire. The sample questionnaire is given below in table 8.

Table 8: Preliminary Survey Proforma

	Department of HSE University of Petroleum and Energy Studies, Dehradun	**Department of Biotechnology GITAM Institute of Technology, GITAM University, Visakhapatnam**
	Proforma	
1	Name of the company:	
2.	Address:	
3.	Contact number:	
4.	E-Mail:	
5.	Age:	
6.	Sex (Male/Female):	
7.	Date of birth:	
8.	Name the of organization:	
9.	Department:	
10.	Occupation (Supervisor /Technician/ Engineer/ worker /Administration):	
11.	Work Nature (Physical / Mental):	
12.	Severity of the work (Light/ Moderate/ heavy):	
13.	Duration of the work (hours/day):	
14.	Date of joining (Date/month/year):	
15.	Work experience (in years):	
16.	Nature of Nano particles manufactured or used in the company (carbon/metal/oxide):	
17.	Method of carbon nanotube production:	
18.	In which form carbon nanotubes are produced (raw/pure/extra pure):	
19.	Quantity of Carbon nanotubes produced or utilised:	
20.	Type of carbon nanotubes manufactured or used	

	(Single walled/ multiwalled/ double walled/ side-wall functionalized):	
21.	Use of Carbon nanotubes in the industry (pure form/ along with other metals):	
22.	Approximate size of nano particle (nm):	
23.	Health problems if any before. Please mention (No, Dermal, Allergic Ocular, Pulmonary, Cardiac):	
24.	Does exposure to nanoparticles is causing any skin irritation/allergy? (Not Applicable, No, Negligible, Light, Moderate, Severe):	
25.	Breathing complaints if any (Not Applicable, No, Negligible, Light, Moderate, Severe):	
26.	Heart related complaints. Please mention (Not Applicable, No, Negligible, Light, Moderate, Severe):	
27.	Any incidences of cancer (No, Stage I, Stage II, Stage III):	
28.	Are you practicing any safety practices. Please mention (High, Moderate, Light, Negligible, Not Applicable):	
29.	Health check-ups if any: monthly/yearly? Please mention (Very high (monthly), High (3 months), low (6 months), very low (yearly), No, Not Applicable):	
30.	Any other information which you would like to share:	

Thank you for your co-operation. This information is very valuable to us. We will email you the findings of our research.

Kindly email this filled in form to the e-mail id khasimb@gitam.edu. Or akbarziauddin@gmail.com

4.2.2 Cronbach Alpha Analysis

Cronbach alpha is used to measure the internal consistency of the data and is an estimate of the reliability of the data collected from a sample of examinees. To assess the reliability of one subscale of the collected data was tabulated for all health related questions i.e. from Q23 to Q29 of the main survey proforma. Minitab software was used to calculate Cronbach Alpha value.

4.3 Laboratory Studies

Laboratory studies for toxicity tests were conducted in the department of Biotechnology, GITAM University, Visakhapatnam, Andhra Pradesh. All the experiments were conducted in triplicate and average value was reported. The equipment used were calibrated prior to their usage.

4.3.1 Introduction

Pulmonary toxicity tests of MWCNTs were carried out in the laboratory using lung cancer cell-lines. The study was mainly concerned with exposure to acute concentrations of MWCNTs.

4.3.2 Materials and Methods

The suppliers for MWCNTs, MTT, XTT, PI, Culture media, glassware and chemicals were browsed on the internet and were contacted for supplying them.

The procedures for carrying out the toxicity tests - MTT, XTT and Apoptosis were standardized using known anticancer drug.

4.3.2.1 Materials and Reagents

Multiwalled carbon nanotubes: 1gm of MWCNTs was purchased from Sisco research laboratories, Mumbai, India. The length of MWCNTs is in the range of 10 – 30 μm and the outer diameter is the range of 30 – 50 nm. The purity of procured MWCNTs is 95%.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT): 500mg of MTT dye was procured from Sisco research laboratories, Mumbai.

Sodium 3- (Phenyl amino-carbonlyl-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulphonic acid hydrate (XTT): 25mg of XTT dye was also procured from Sisco laboratories, Mumbai.

Propidium Iodide: 25 mg of Propidium Iodide was procured from Sisco laboratories, Mumbai.

Cell line: A549, a human lung carcinoma epithelial cell line was procured from National Centre for Cell Science (NCCS), Pune.

Culture media and chemicals: Cell culture media and other chemicals were purchased from HIMEDIA labs, Mumbai, India.

4.3.2.2 Cell Culture

Cells were cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 30µg/ml penicillin and 50µg/ml streptomycin at 37°C in 5% CO_2 incubator shown in figure 7.

Materials required for cell culture:

TPVG solution, PBS buffer, Trypsin, Tissue culture flasks (T-25), centrifuge, centrifuge tubes, double distilled water, CO_2 incubator, biological safety cabinet (BSC), sterile lab coat and gloves, sterile pipettes, pipette holder, 70 % ethanol, inverted microscope, Neubar chamber, cell lines and cell culture media.

Initiation of cell culture:

The vial containing cells in frozen medium was taken from the nitrogen tank and placed immediately in 37°C water bath and the cells were thawed rapidly. The vial was wiped with 70% ethanol before opening it. Later on, the vial contents were transferred into a 15 ml centrifuge tube containing culture medium and centrifuged at 1200 rpm for 5 minutes and the supernatant was discarded. New flask was prepared with fresh culture medium and the cell pellet was mixed. The prepared flask was examined under inverted microscope and incubated at 37°C.

Figure 7: CO_2 Incubator

Culture growth and passage of cells:

The mother culture procured from NCCS, Pune was grown in T25 flask with DMEM medium as shown in figure 8. The incubated culture flask was observed for confluency everyday under inverted microscope. Once the culture flask has attained a confluency of 80%, the medium in the flask was removed using Pasteur pipette and the cells were washed with Phosphate Buffer Saline (PBS) in order to remove the residual impurities. Then, trypsin-EDTA solution of 1 to 1.5ml was added and incubated for 2 minutes approximately. The culture flask was observed under microscope for cell detachment which is shown in figure 9. 2 ml of DMEM media was added to minimize further activity of trypsin. The medium was mixed with pipette to disperse the cell clumps. The cell suspension was transferred to 15 ml centrifugation tube and centrifuged at 1200 rpm for 5 minutes. The cell pellet was added to fresh culture medium and transferred to new culture flasks and incubated

in CO_2 incubator at 37 °C. The old medium was replaced with fresh medium periodically i.e. at an interval of 3 to 4 days.

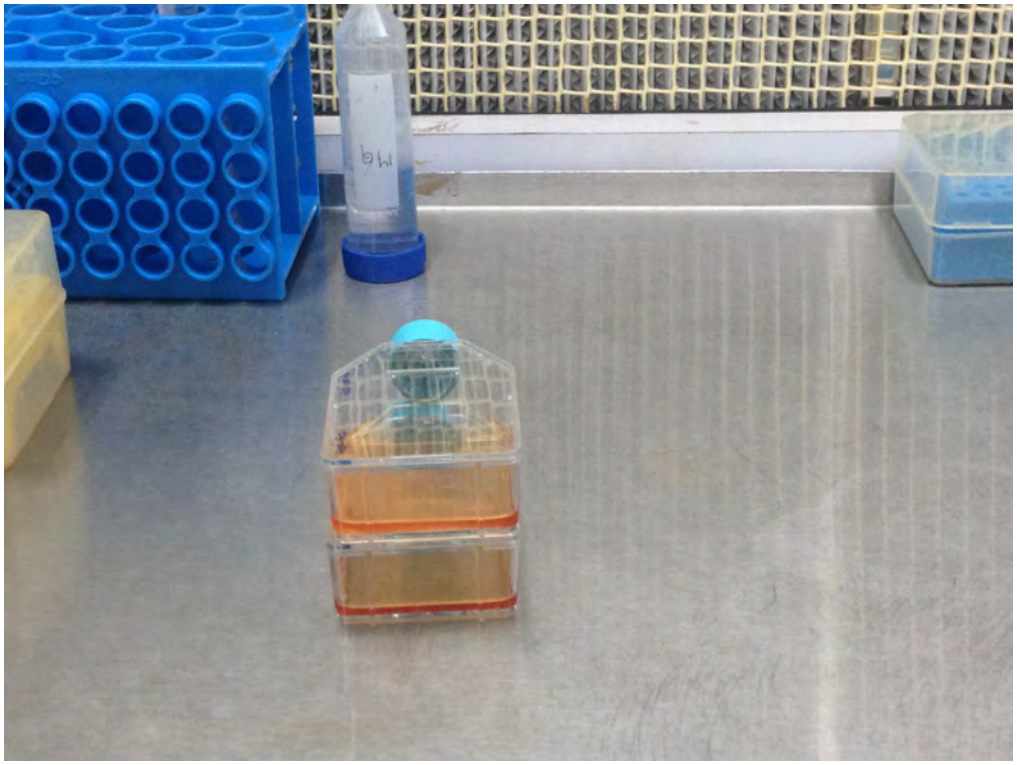

Figure 8: Tissue Culture Flask

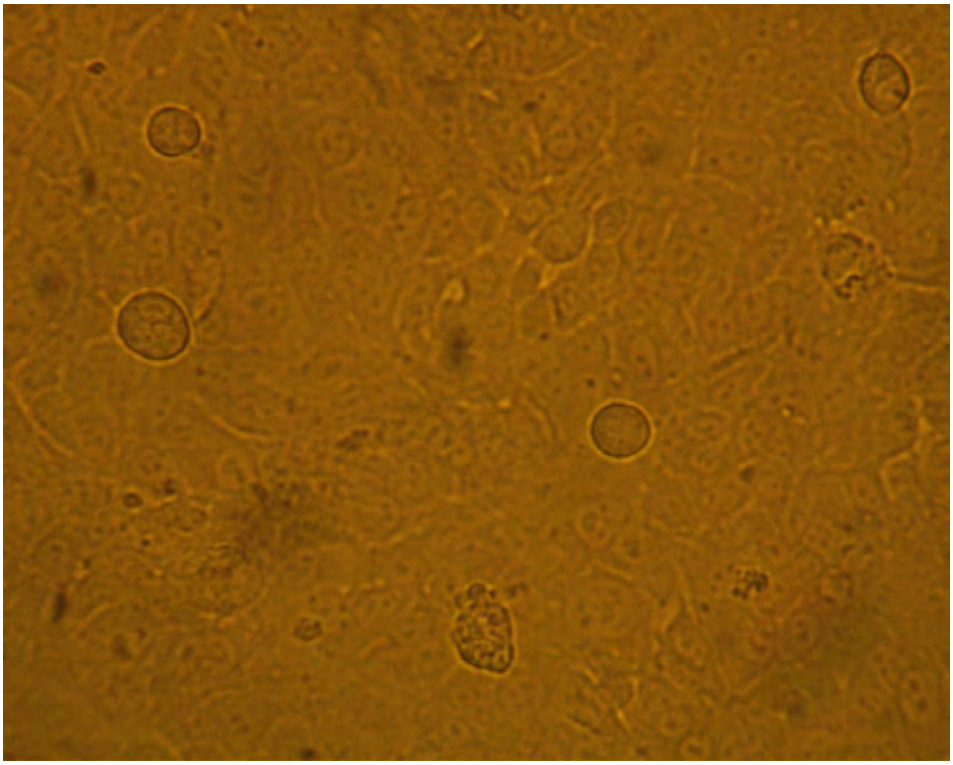

Figure 9: A549 mother culture procured from NCCS, Pune

Counting of cells:

1 ml of dilute cell suspension was taken in an Eppendorf tube after trypsinization. Two drops (10 µl) of trypan blue was placed on a piece of parafilm and a drop of culture was mixed. The mixture was placed on haemocytometer and observed under inverted microscope. The cells on the top and left border of the square and in all the four corner squares were counted.

The number of cells per milliliter is calculated.

$$\text{Concentration (cells/ml)} = \text{The average count per square} \times \text{Dilution factor} \times 10^4$$

$$\text{Total cell number} = \text{concentration (cells/ml)} \times \text{volume of sample (ml)}.$$

Freezing of cells:

The cells growing in log phase (1~6x10^6 cell/ml) were treated with trypsin for detaching the cells from the substratum. The detached cells were centrifuged and cryomedium (10% DMSO+90 % culture medium) was added. The medium in the centrifuge tubes was tapped for proper dispersion of cells. 1ml aliquots were taken into the cryovials and placed in a deep freezer at -80^{0}C. The same cryovials can also be stored in liquid nitrogen storage tank.

4.3.2.3 Preparation of MWCNTs Suspensions

A stock solution of MWCNTs (1mg/ml) was prepared by the addition of MWCNTs to PBS or ethanol or sterile water. The solution is sonicated for uniform dispersion of MWCNTs. This solution is added to medium for obtaining desired concentrations.

4.3.2.4 Cytotoxicity Assays

For cytotoxicity assays cell culture density of 1×10^5 cells/ml was used. 100 µl of A549 cells were seeded in 96-well microplates. The microplate were incubated for 24 hours for the attachment of cells. These wells are treated with 100 µl of MWCNTs dispersed solution was added at varied concentrations as shown in figure 10. For reference wells, only medium was added instead of MWCNTs.

Figure 10: Treatment of MWCNTs in Well plates

Exposure of MWCNTs to cell cultures:

In order to understand the short terms effects of MWCNTs cells, which were in log phase, were exposed to varied concentrations of MWCNTs i.e. 6.25, 12.5, 25, 50, 100, 200, 400 µg/ml for an exposure period of 2, 4, 8, 16, 32 and 48hr. After incubating for a specified exposure period the cytotoxicity test like MTT, XTT and apoptotic assays were performed to

observe the viability of the cells. All these tests were also conducted in serum free media in order to understand the effect of serum on MWCNTs toxicity.

MTT assay:

- **Cells:** cells growing in log phased with recognisable levels of mitochondrial activity were used in this assay.
- **Equipment:** 96-well tissue culture plate, Scanning multi well spectrophotometer, glassware, and automatic plate shaker.
- **Materials Required:** DMEM medium, 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT), Dimethylsulphoxide (DMSO), KCl, KH_2PO_4, NaCl, $NaHCO_3$ and $Na_2PO_4.7H_2O$.
- **Preparation of solutions:**
  - ***Hank's salt solution*** was prepared by mixing 0.4gm of KCl, 0.06gm of KH_2PO_4, 8gm of NaCl, 0.35gm of $NaHCO_3$, 0.09gm of $Na_2PO_4.7H_2O$ was mixed in 1000ml of sterile water.
  - ***MTT solution*** was prepared by dissolving 5mg of MMT in 1ml of Hank's salt solution.
- **Procedure:**

  The cells were diluted to the required concentration (5×10^6 cell/ml) in complete medium and 100 µl of cell suspension was seeded into 95 wells of a 96-well tissue culture plates. In the remaining well only 100 µl of culture medium was added (blank). Cells were diluted in medium and were plated out and incubated for a period of 24 hr. The culture medium was replaced with complete medium containing the MWCNTs and incubated for 2, 4, 8, 16, 32 and 48 hr.

For each exposure period 6.25, 12.5, 25, 50, 100, 200, 400 µg/ml concentrations of MWCNTs suspensions was added into 7 wells and a reference sample in the 8th well. This plate was incubated at 37^{0}C for defined exposure period. 10 µl of MTT solution and 100 µl of DMSO solution was added to each well and agitated for 5 min in a plate shaker. The plates were read at a 550 nm and 620 nm on a scanning multiwell spectrophotometer. The optical density of each was recorded and corrected with reference to the blank. Percentage of cell survival was calculated as: (absorbance of treated cells / absorbance of control cells) × 100. The results recorded were the mean of three experimental determinations.

Dose response relation was calculated for A549 lung cells over the tested range of concentrations. This enabled us to obtain IC_{50} values.

XTT Assay

- **Materials Required:** XTT labelling reagent, Electron coupling reagent
- **Preparation of solutions:**
    - XTT labelling reagent: 1mg of XTT was dissolved in 1ml of tissue culture medium and sterilized by Millipore filtration unit. The solution was stable at -20°C and was stored in dark. The prepared XTT reagent was thawed and stored in 5 ml aliquots.
    - Electron coupling reagent: 0.383 mg of PMS solution was dissolved in 1ml of PBS and sterilised by Millipore filtration. 5 ml of the XTT reagent was mixed with 0.1 ml of the electron coupling reagent just before the usage of XTT reagent.

- **Procedure:**

  A seeding density of 1×10^4 cells were seeded in 200 µl of medium per well of the microplate and the cells were allowed to adhere to the wells for an incubation period of 24 hr. Then the used medium was removed and fresh medium was added. 10 µl solution of MWCNTs with various concentrations i.e. 6.25, 12.5, 25, 50, 100, 200, 400 µg/ml was added and incubated for 2, 4, 8, 16, 32 and 48 hr. After the required time of incubation, 100 µl of XTT solution was added per well and incubated for 12hr. Optical density was measured at 500 nm, with a reference wavelength of 650 nm was plotted against the concentration of MWCNTs.

Apoptosis assay

Staining of Apoptotic Cells with DAPI

- **Materials Required:**

  1. Cells growing in a suitable complete medium, treated with MWCNTs inducing apoptosis.
  2. 0.25% trypsin in saline or any other cell dissociating agent used for the cell line.
  3. PBS
  4. DAPI

- **Procedure:**

  Apoptosis was examined by 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) staining coupled to fluorescence microscope was used to count the cell number. Cells were seeded into microplate reader and were exposed for 2, 4, 8, 16, 32 and 48 hr to 100, 200, 400 µg/ml of MWCNTs or suspension medium alone. At the end of stimulation, the cells were fixed with 4 % paraformaldehyde in PBS for 25 min at

room temperature. 1 µg/ml of DAPI solution was added and incubated for 5 min at 37^0C. The cells were observed under fluorescence microscope.

4.3.2.5 Optical Microscopy

Optical microscopy was performed on cells exposed for 24 and 48 hours to 400 µg/ml MWCNT's. At the end of stimulation, cells were fixed in acetone and strained with Harris haematoxylin.

4.3.2.6 Statistical Analysis

The MTT, XTT and apoptosis results were analyzed using statistica software and the results were given as the means ± S.E.M. The data was analyzed by simple regression. The significance for all statistics was accepted at $p<0.05$.

4.4 Field Studies

4.4.1 Selection of Industry

MWCNTs manufacturing industry, located at Noida was selected to measure the concentrations of MWCNTs in the work environment.

4.4.2 Measurement of Aerosol Concentration

Filter based sampling was employed to assess exposure concentrations in the industrial air environments. An aerosol monitor was used to monitor particle mass concentration of MWCNTs. An impactor with a cutoff of 1µm was used to allow sampling from 0.1 to 1 µm

as the mass measurement is dominated by larger sized particles. A filter testing chamber was designed to determine mask filter particle retention in the workplace during real time operating conditions and exposure situations. Particle laden air during regular production was drawn in with a vacuum pump and controlled by a rotameter. Pressure drop between two compartments was measured with a manometer. The data set included measurements under background (non-production) and production conditions. These are the two diverse environments with respect to concentration levels.

Five sampling locations were chosen to capture the spatial and temporal variation in particle levels. The sampling inlets were positioned at 1.2m above the ground. This level will quantify the breathing concentration between standing and sitting working position. Worker's activity includes reactor maintenance and cleaning, mechanical adjustments to the reactor system and its operation, powder handling and packaging and work place cleaning. Background levels were determined each day prior to the production start. Temporal measurements were also made with the same sampling system by collecting samples during the months of May, June and September. The filters were analyzed gravimetrically for the mass concentration of MWCNTs using Pyris 1 TGA.

4.5 Comparison of Laboratory and Fields Study

The results obtained for the concentrations tested in the lab were compared with the concentrations present in the industrial air environment. The probable effects were extrapolated and discussed.

4.6 Summary of Methodology

The summary of methodology followed to assess health effects of MWCNTs is depicted in the flow chart given in figure 11.

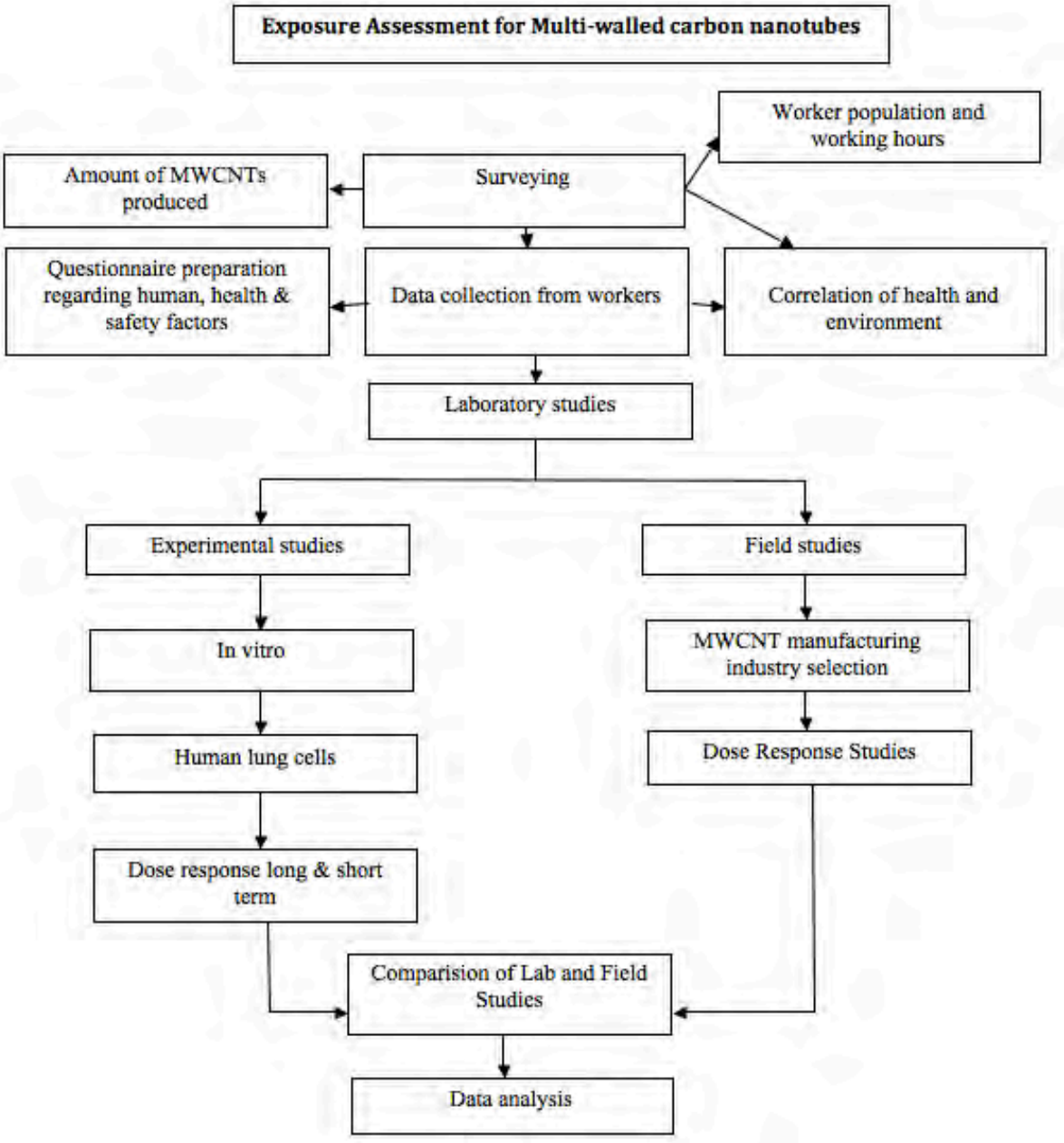

Figure 11: Summary of Methodology

---End of Chapter 4---

Chapter-5

5. Results & Discussion - A

The inhalation toxicity is most important for assessment of hazards caused by inhalation. Inhalation of MWCNTs causes potential health hazards and the results obtained from the toxicity evaluation of MWCNTs on lung cells are presented in this chapter systematically.

5.1 Analysis of Survey Data

A huge debate exists on the possible adverse effects of MWCNTs on human health and environment. An analogy with asbestos has been shown since 1998 based on their solubility and fibrous characteristics. However the chemical composition of MWCNTs is very different from that of Asbestos. Such difference will be reflected in the surface activity of MWCNTs. In order to understand these effects on Occupational health of workers a preliminary survey was carried out to know the quantities of MWCNTs being produced by the industry and the number of people being effected in those industries. The survey results indicated the number of workers working in those industries are few in number, but in future its going to be expanded to a greater magnitude.

A questionnaire (Table 8) was prepared for collecting the information regarding health and safety issues being faced by the workers of MWCNT manufacturing industry. The key questions, which were used for the analysis, are as follows:

- Q 23) Health problems if any before: Please mention (No, Dermal, Allergic Ocular, Pulmonary, Cardiac):
- Q 24) Does exposure to nanoparticles is causing any skin irritation / allergy? (Not Applicable, No, Negligible, Light, Moderate, Severe):
- Q 25) Breathing complaints if any (Not Applicable, No, Negligible, Light, Moderate, Severe):
- Q 26) Heart related complaints. Please mention (Not Applicable, No, Negligible, Light, Moderate, Severe):
- Q 27) Any incidences of cancer (No, Stage I, Stage II, Stage III):
- Q 28) Are you practicing any safety practices. Please mention (High, Moderate, Light, Negligible, Not Applicable):
- Q 29) Health check-ups if any: monthly/yearly? Please mention {Very high (monthly), High (3 months), Low (6 months), Very Low (Yearly), No, Not Applicable):

The filled in questionnaire Annexure was analyzed for Cronbach Alpha value, using Minitab software. The responses of the key questions were assigned scores as shown in table 9. The obtained scores (table 10) were entered into Minitab and resultant utilized for the calculation of Chronbach Alpha analysis. The step-by-step methods followed are captured and shown in figure 12-16. The resultant coefficient value i.e. 0.793 indicates that the responses are relatively consistent.

Table 9: Scores Assigned for Key Questions

Question No.	Response	Score
23	No/NA	1
	Dermal	2
	Allergic	3
	Ocular	4
	Pulmonary	5
	Cardiac	6
24	Not Applicable	1
	No	2
	Negligible	3
	Light	4
	Moderate	5
	Severe	6
25	Not Applicable	1
	No	2
	Negligible	3
	Light	4
	Moderate	5
	Severe	6
26	Not Applicable	1
	No	2
	Negligible	3
	Light	4
	Moderate	5
	Severe	6
27	No	1
	Stage 1	2
	Stage 2	3
	Stage 3	4
28	High	1
	Moderate	2
	Light	3
	Negligible	4
	No	5
	Not Applicable	6
29	Very High (monthly)	1
	High (3 months)	2
	Low (6 months)	3
	Very Low (yearly)	4
	No	5
	Not Applicable	6

Table 10: Data of Filled Questionnaire from Questions No 23 to 29

Employee No	Q 23	Q 24	Q 25	Q 26	Q 27	Q 28	Q 29
1	2	2	3	4	3	3	2
2	3	3	3	3	2	3	4
3	2	4	3	3	3	3	2
4	3	3	3	4	4	2	3
5	2	2	2	2	2	2	2
6	2	3	3	3	2	1	1
7	4	4	3	3	4	2	2
8	1	2	1	1	2	3	1
9	3	3	2	2	3	2	1
10	3	3	2	3	3	2	2
11	4	2	3	3	2	4	3
12	2	3	1	2	1	3	3
13	4	4	2	4	2	3	4
14	1	2	1	2	1	3	1
15	3	4	2	4	4	3	2
16	2	3	3	4	2	2	1
17	4	4	3	3	3	3	2
18	2	3	3	3	1	2	3
19	3	3	3	3	3	3	3
20	3	3	3	3	3	3	2
21	3	4	2	2	2	2	3
22	1	2	1	2	1	4	2
23	4	4	4	4	4	3	4
24	1	4	2	2	2	3	1
25	2	3	2	1	2	3	2
26	3	3	3	3	3	2	3
27	1	3	3	2	1	2	3
28	4	4	4	4	4	3	2
29	4	4	4	3	4	3	3
30	2	2	1	1	1	2	3
31	4	4	4	4	4	4	4
32	2	3	1	1	2	3	3
33	4	4	4	3	1	3	2
34	4	4	4	4	4	4	3
35	4	4	3	2	1	2	3
36	3	3	3	3	3	2	2
37	2	2	1	1	2	2	2
38	2	2	2	2	4	3	2
39	4	4	4	4	4	4	4
40	3	4	3	2	4	3	3
41	3	3	4	4	1	2	2
42	3	2	1	1	3	2	2
43	3	3	2	2	2	2	1
44	1	4	2	4	3	2	2
45	4	2	3	3	3	2	2
46	4	4	3	3	1	3	2

Employee No	Q 23	Q 24	Q 25	Q 26	Q 27	Q 28	Q 29
47	1	3	1	1	3	3	2
48	4	4	3	3	4	2	2
49	4	4	2	3	1	2	3
50	2	3	1	1	3	2	3
51	4	3	2	3	1	2	3
52	2	2	2	2	2	2	3
53	2	2	1	1	1	3	2
54	1	1	2	1	1	2	2
55	1	3	1	2	1	3	3
56	2	3	3	2	2	3	3
57	3	3	3	4	4	3	2
58	4	4	3	4	1	2	3
59	2	2	1	1	1	3	2
60	3	3	4	4	4	3	2
61	2	2	3	3	3	2	3
62	1	3	2	1	3	1	3
63	3	4	4	3	1	3	3
64	3	3	2	3	3	2	2
65	2	3	2	1	1	3	2
66	4	4	4	4	4	4	3
67	1	4	2	1	1	1	3
68	2	4	1	2	1	2	3
69	3	4	3	3	3	3	2
70	4	3	4	4	3	2	3

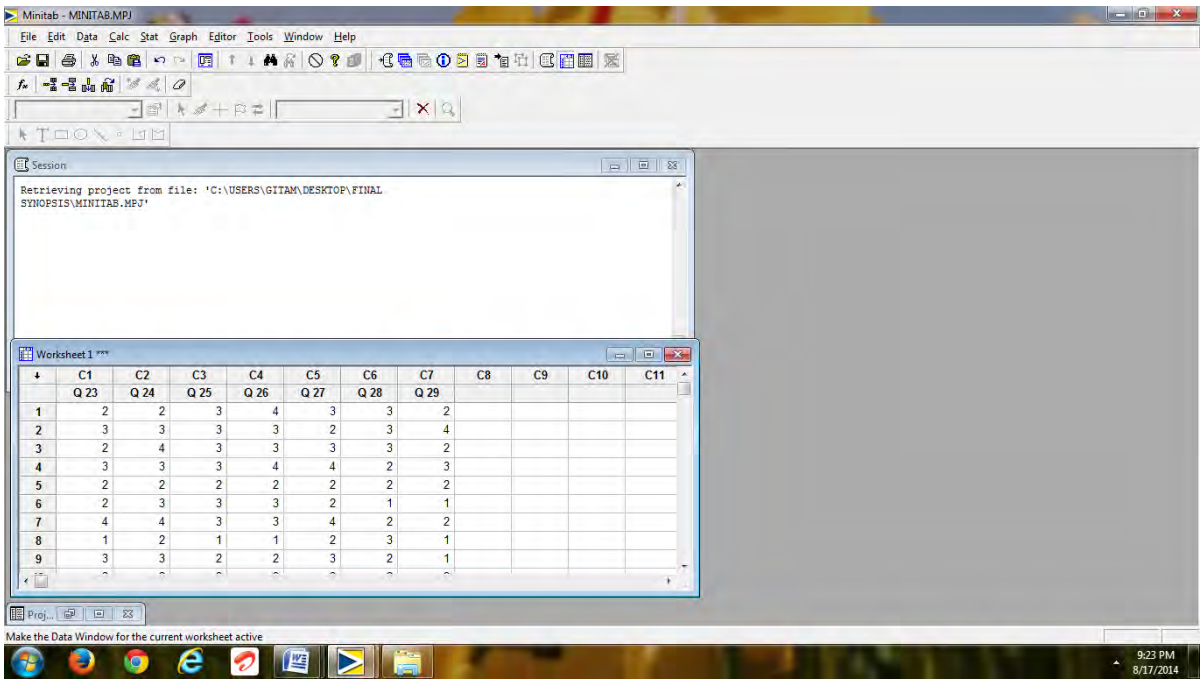

Figure 12: Screen Capture of Cronbach Alpha Analysis - 1

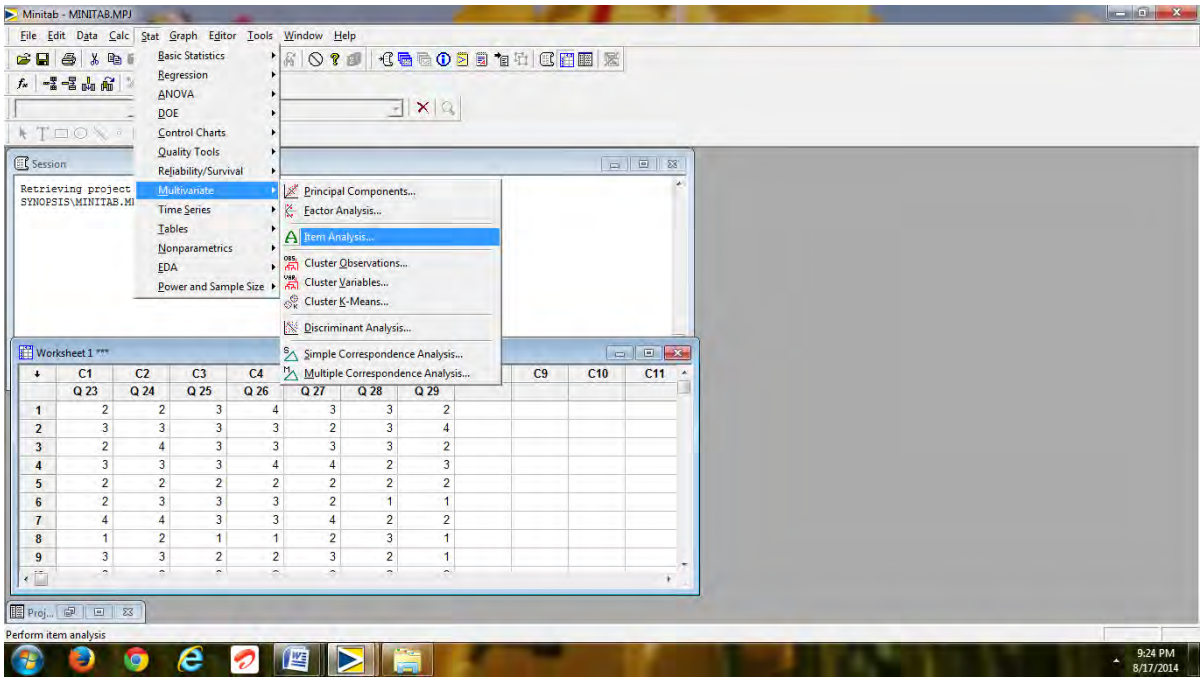

Figure 13: Screen Capture of Cronbach Alpha Analysis - 2

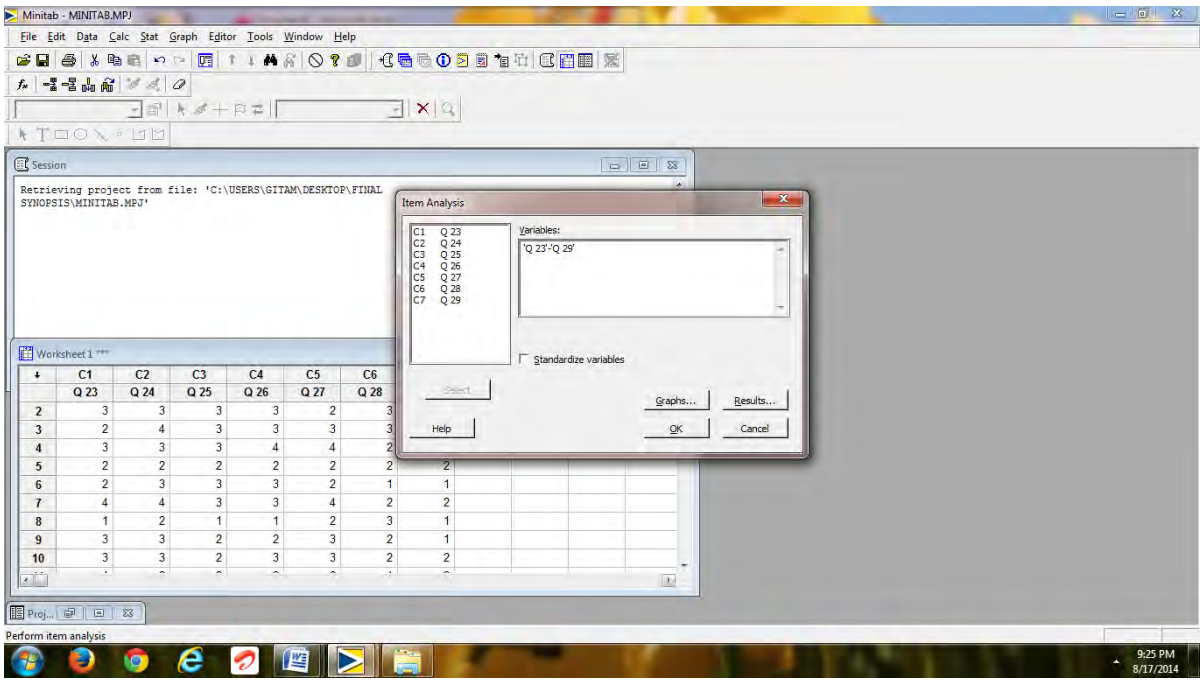

Figure 14: Screen Capture of Cronbach Alpha Analysis - 3

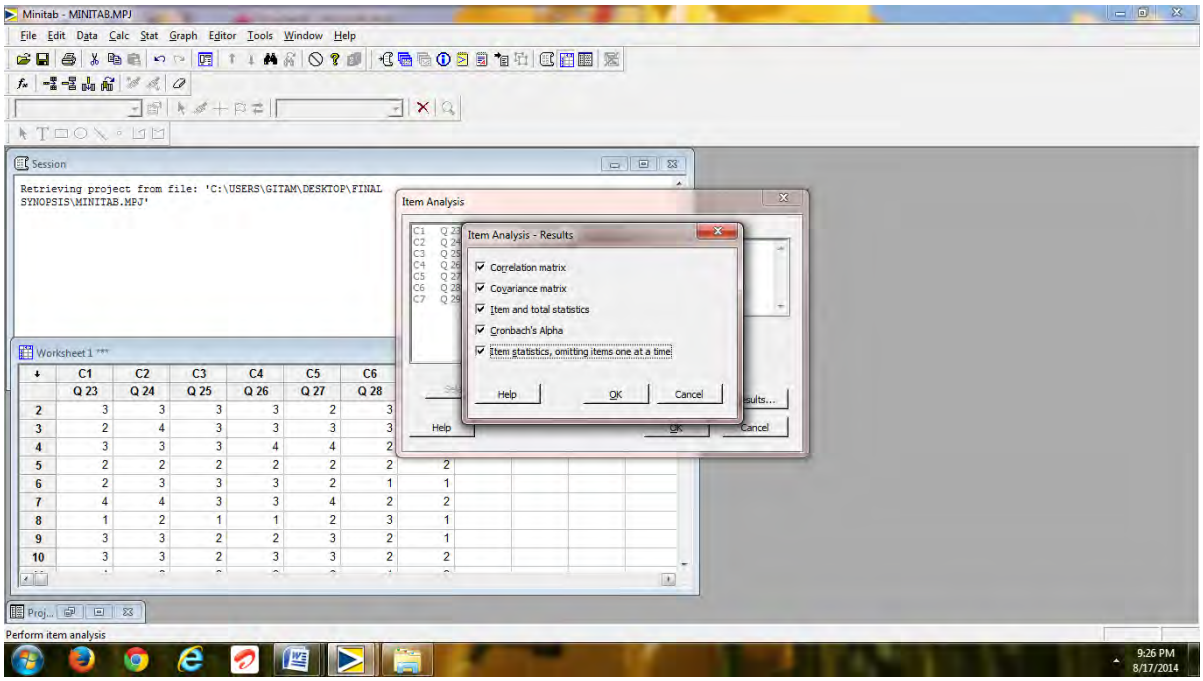

Figure 15: Screen Capture of Cronbach Alpha Analysis - 4

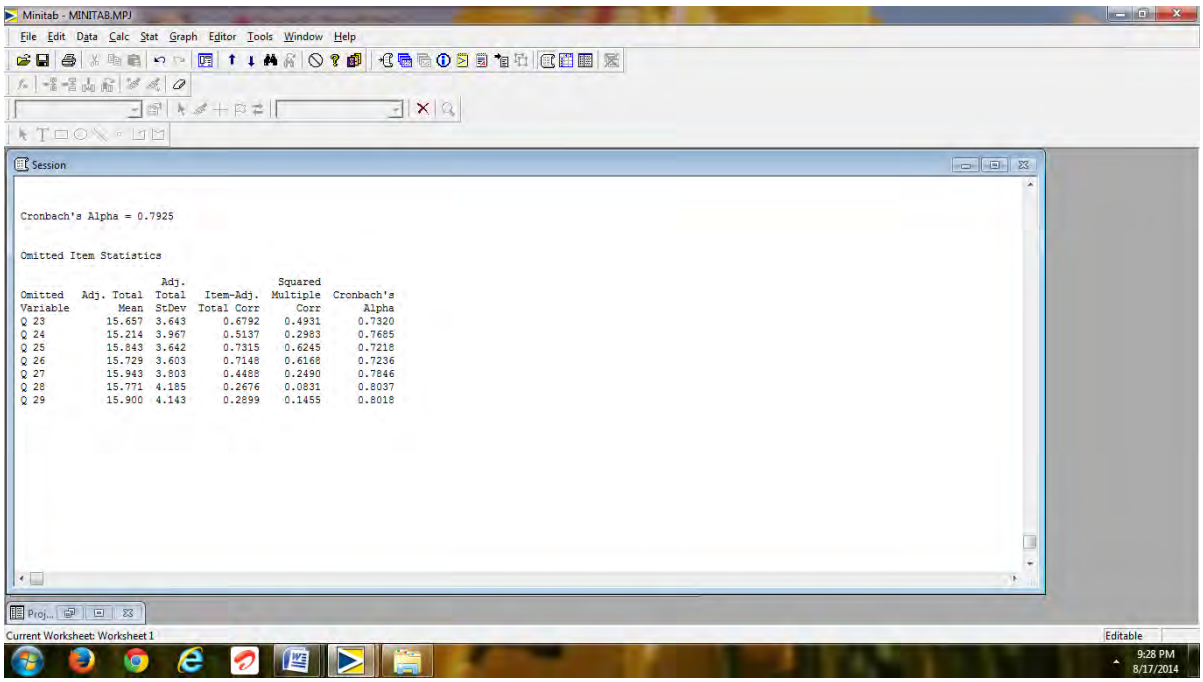

Figure 16: Screen Capture of Cronbach Alpha Analysis – 5

5.2 Laboratory Studies

MWCNTs are biopersistent and have the potential to induce severe inflammatory and fibrotic reactions. The published in-vitro studies have given a diverging data. Several studies demonstrated that MWCNTs are able to induce cytotoxic effects and Apoptosis in different cell types. Other studies indicated that there is very low or no toxic effects. The reason for these discrepancies is not evident probably this may be due to different experimental protocols and interferences with test systems. Hence the present study has standardized the experimental protocol and used the same for all the laboratory experiments.

5.3 MTT Assay Results

MTT assay, the commonly used colorimetric assay for cell viability involves the assessment of mitochondrial activity. Live cells were able to convert yellow soluble tetrazolium dye to purple insoluble formazan with the help of NAD(P)H- dependent oxidoreductases in mitochondrial cytosol. In MTT assay, dark blue formazan crystals will be accumulated inside living cells after their treatment with MTT. Addition of DMSO will destruct the cell membrane, which will result in the liberation and solubilization of the crystals. Cytotoxicity was evaluated by MTT assay performed on MWCNT exposed human lung epithelial cells. MWCNTs were dispersed in PBS, ethanol and sterile water and A549 lung cells were exposed and cell viability was assayed by MTT.

The viable cell number was directly proportional to the quantity of formazan product formed. The formazan was quantified by measuring absorbance at 570 nm. A linear relationship was established between cell numbers and optical density of exposed A549 cells, and control cells. This allowed for an accurate quantification of changes in the rate of cell proliferation.

The results of cell viability was different for all the three dispersion media and was depicted in figures 17, 18, 19 for 400 µg/ml concentration of MWCNTs.

The complete MTT assay results presented in Table 11, 12 & 13 reveals that exposure to MWCNTs, the number of viable cells decreases as a function of MWCNT dosage for all tested concentrations. No change in MTT was observed when cells were incubated upto 50 µg/ml concentration. MTT values decreased significantly from 100 µg/ml to 400 µg/ml concentration of MWCNTs. This decrease is upto 50% for 200 µg/ml and 400 µg/ml at 48 hr post exposure for serum free and serum containing media respectively. The proteins present in fetal bovine serum will probably mask the surface of the MWCNTs making them less toxic. The MTT values indicate that dispersion in PBS media was more toxic as shown in tables. CNTs are known to interfere with the MTT assay at high concentrations but the interference was minimal for the highest studied concentrations.

MWCNTs are one of least biodegradable anthropogenic materials and are totally insoluble in water. These materials can only be dispersed in water. There are many methods for dispersion of MWCNTs like using organic solvents or a series of surfactants. The degree of dispersion is affecting the cytotoxicity of MWCNTs. These are lipophilic in nature and can accumulate along the food chain. In aqueous environments, MWCNTs clump together and formed as aggregates of micrometer range. This aggregation has not affected by changes in temperature or salinity of the medium. But the aggregation was affected with pH changes in water. The figure depicts the formation of stable aggregates of MWCNTs.

Table 11: % of cell viability of MTT Assay of A549 lung cells in serum containing & serum free media after exposure to various concentrations of MWCNTs dispersed in Phosphate Buffer Saline for different durations

Exposure	Duration in hours											
	2		4		8		16		32		48	
Concentration in µg/ml	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media
6.25	99.33	99.55	98.41	99.10	91.62	98.42	85.42	89.92	80.97	82.60	75.35	78.97
12.5	98.21	98.45	96.38	98.20	87.93	97.29	83.16	86.39	74.55	79.53	72.27	78.27
25	95.76	96.46	95.24	95.28	85.22	95.49	79.64	82.36	73.52	76.72	68.72	75.70
50	93.09	93.58	89.59	90.56	81.77	92.79	75.87	78.33	71.97	74.93	61.84	69.15
100	89.53	92.47	84.38	86.74	80.78	91.21	72.86	76.07	68.89	73.14	60.42	66.12
200	83.51	82.30	74.20	84.04	77.09	89.18	67.83	74.30	66.58	69.05	54.26	62.61
400	77.72	78.98	72.17	75.73	73.39	74.56	66.83	68.01	60.92	61.38	50.47	55.60

Table 12: % of cell viability of MTT Assay of A549 lung cells in serum containing & serum free media after exposure to various concentrations of MWCNTs dispersed in Ethanol for different durations

Exposure	Duration in hours											
	2		4		8		16		32		48	
Concentration in µg/ml	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media
6.25	98.40	99.10	94.02	97.05	87.47	95.03	80.60	87.93	73.05	77.43	72.98	77.23
12.5	96.57	98.42	90.76	91.15	82.74	93.38	78.33	85.92	70.00	74.35	69.90	74.41
25	93.60	95.05	87.50	90.24	80.85	90.54	74.81	81.40	68.33	73.33	59.24	70.89
50	89.04	90.78	86.41	87.30	79.66	83.21	65.49	78.39	65.27	67.69	57.58	68.07
100	86.98	88.98	83.96	83.44	78.72	79.19	64.23	75.37	61.92	63.58	56.39	62.67
200	82.42	82.02	74.18	80.72	76.59	73.52	62.21	73.36	56.66	57.94	52.60	59.38
400	75.79	77.52	70.38	76.41	73.28	70.21	57.43	60.30	52.77	53.84	48.57	52.11

Table 13: % of cell viability of MTT Assay of A549 lung cells in serum containing & serum free media after exposure to various concentrations of MWCNTs dispersed in Sterile Water for different durations

Exposure	Duration											
	2		4		8		16		32		48	
Concentration in µg/ml	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media
6.25	98.24	98.70	92.60	93.34	85.32	94.20	78.88	86.38	71.98	75.39	70.67	70.58
12.5	92.77	92.87	87.94	88.24	83.83	85.99	75.06	79.95	68.06	67.27	56.97	66.88
25	85.99	86.82	85.75	86.47	80.59	84.78	67.93	76.98	64.70	65.70	53.60	64.48
50	83.80	86.17	79.17	83.14	77.36	81.15	64.37	75.95	61.62	64.92	51.68	58.16
100	80.52	82.93	73.69	78.71	75.12	77.77	60.05	74.75	60.22	62.82	49.51	55.77
200	76.14	77.75	69.58	75.38	72.88	71.25	57.25	72.52	56.30	56.54	46.15	54.24
400	72.64	73.00	66.84	72.06	70.89	64.73	53.43	57.67	47.61	52.61	40.86	43.57

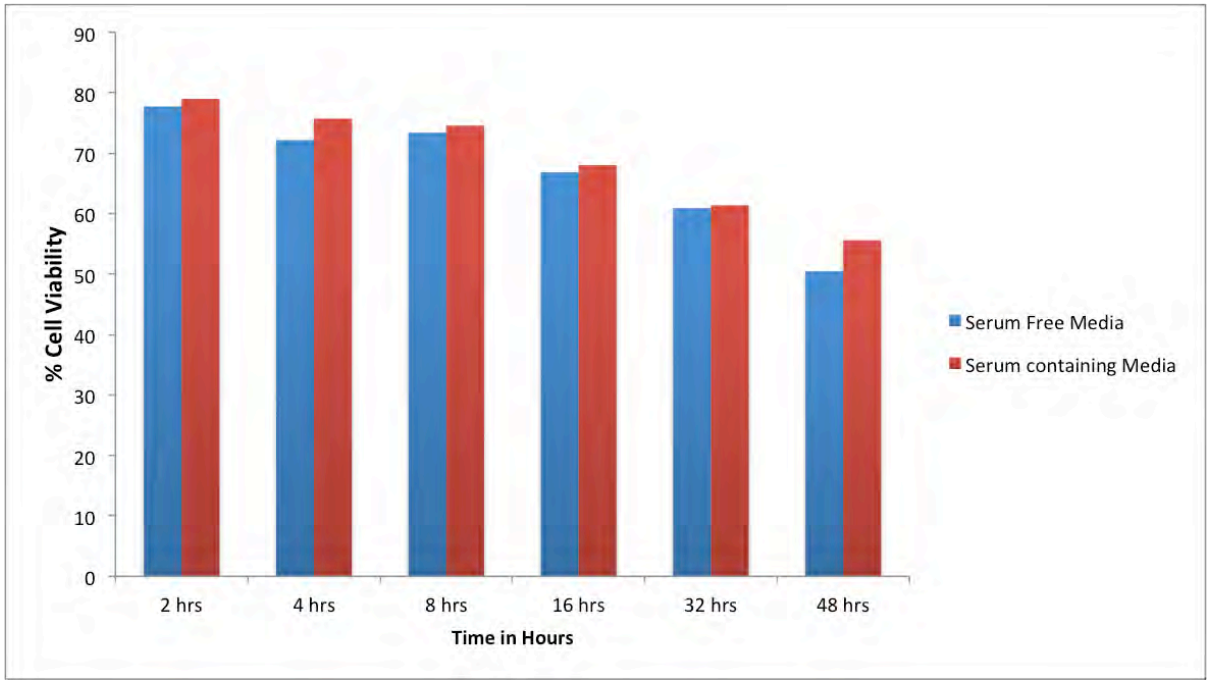

Figure 17: MTT Assay Results for MWCNT Exposure Concentration of 400 µg/ml in Phosphate Buffer

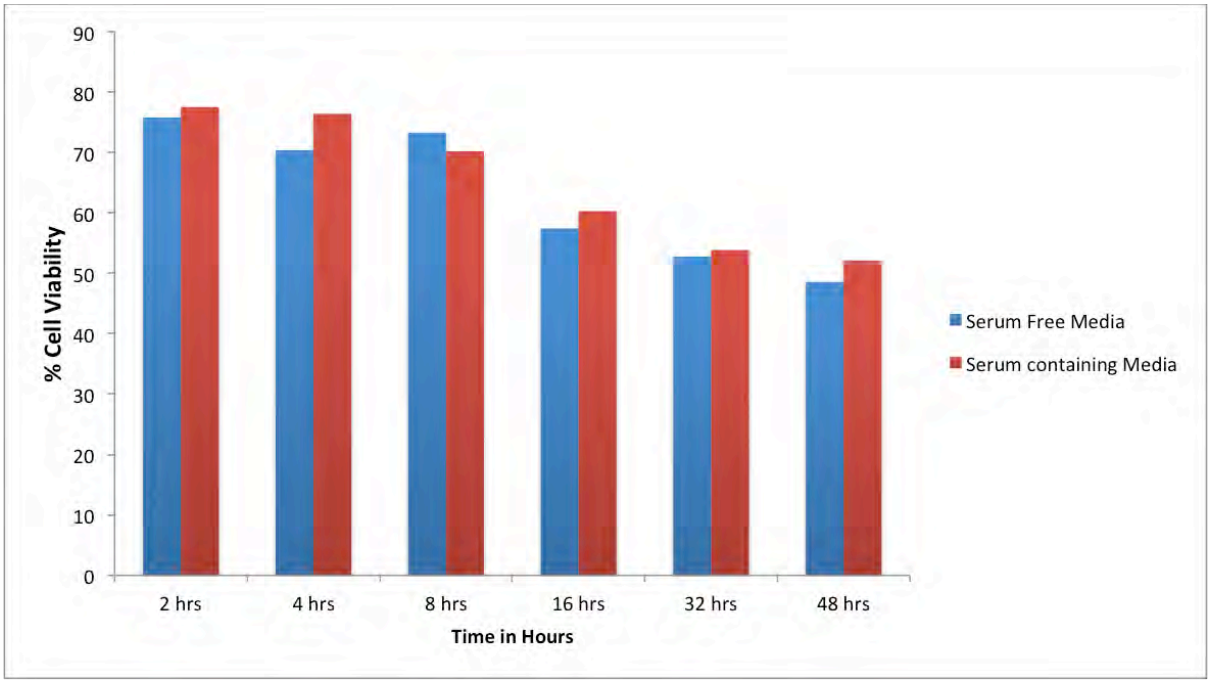

Figure 18: MTT Assay Results for MWCNT Exposure Concentration of 400 µg/ml in Ethanol

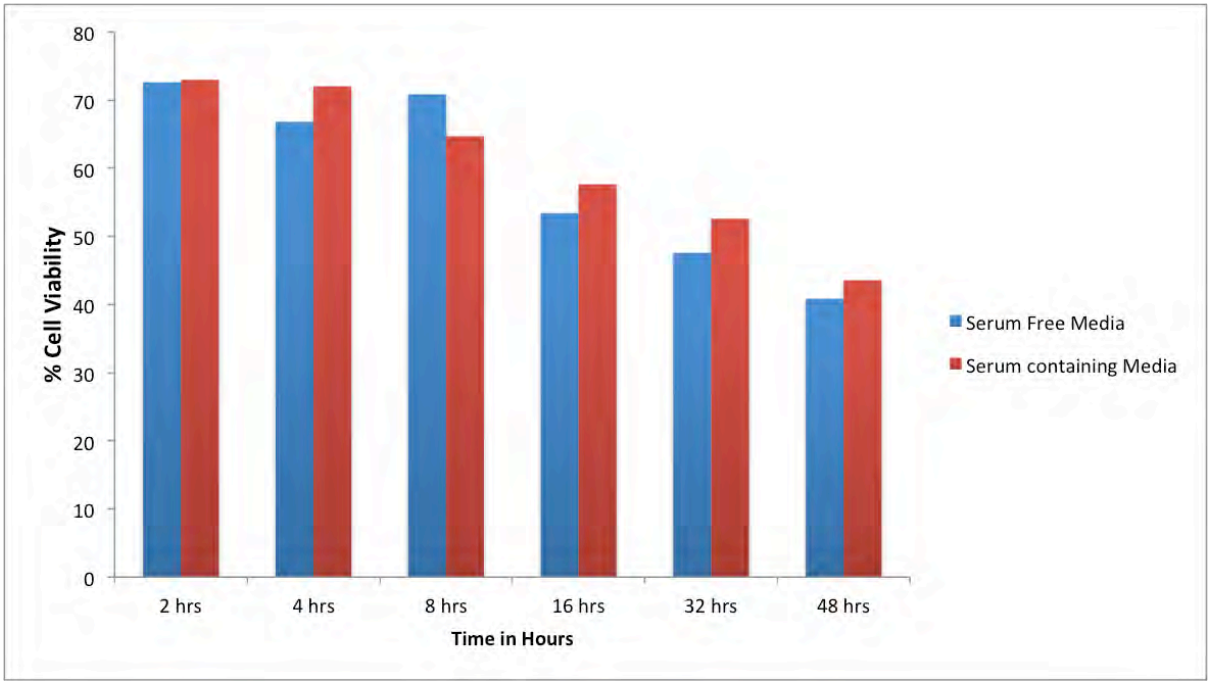

Figure 19: MTT Assay Results for MWCNT Exposure Concentration of 400 µg/ml in Sterile Water

Scientists (Cui et al. 2005; Magrez et al. 2006; Davoren et al. 2007; Zhang et al 2007; Muller et al. 2008; Simon-Deckers et al. 2008; Tabet et al. 2009; Patlolla et al 2010; Thurnherr et al. 2011) have performed MTT assay in order to measure the cytotoxicity of CNTs with varying exposure concentrations. The studies indicated decrease in cell viability with increasing concentrations of CNTs.

HEK 293 cell viability decreased in time and dose dependent manner after exposing to 0.78125-200 µg/mL concentration of SWCNTs (Cui et al 2005). The cell viability was inversely proportional to the dosage of MWCNTs in H 596 human lung tumor cells (Magrez et al. 2006). Maximum cytotoxicity of SWCNTs on A549 cells was observed at a concentration of 800 µg/mL after 24 hr exposure to 800µg/mL of SWCNTs. However the percentage inhibition was more in the absence of serum (45%) when compared to its presence (32%) at 800µg/mL (Davoren et al. 2007). Reduction in cell viability was 60 % at a dose of

100 µg/ml of MWCNTs after one day in Rat Lung Epithelial cells (Muller et al. 2008). Cell death is 50 % at a concentration of 0.5 µg/mL of MWCNTs after two days in A549 cells (Simon-Deckers et al. 2008). Decrease in cell viability is 60 % at a concentration of 100 µg/mL of MWCNTs in both A549 & MeT5A cells (Tabet et al. 2009). Dose dependent decrease in mitochondrial activity of A549 cells is due to acute exposure to 3.2, 6.25, 12.5, 25 and 30 µg/mL MWCNT concentration for an exposure period of one day. (Thurnherr et al. 2011). The decrease in cell viability in all the studies might be due to the mitochondrial damage and interruption of NAD(P)H flux.

5.4 XTT Assay Results

For the XTT assay, MWCNTs were dispersed in PBS, ethanol and sterile water. MWCNT effects on A549 lung epithelial cell viability was different for all the three dispersion media. The XTT results shown in table 14, 15 & 16 indicate that dispersion in PBS media is more toxic as compared to other dispersion media. The cell viability has decreased significantly from 100 µg/ml to 400 µg/ml. This decrease was upto 50% for 200 µg/ml and 400 µg/ml at 48 hr post exposure. No change in XTT was observed when cells were incubated upto 50 µg/ml concentration. The same experiments were conducted in serum containing and serum free media. After the incubation of MWCNT's in an aqueous solution of fetal bovine serum, and in the absence of fetal bovine serum the results indicated that the cells in serum free media were effected by MWCNTs rather than serum containing media. The proteins present in fetal bovine serum will probably mask the surface the MWCNTs making them less toxic. The obtained XTT assay results for the three different dispersion media at 400µg/ml concentration was depicted in figure 20, 21 and 22.

Table 14: % of cell viability of XTT assay of A549 lung cells in serum containing & serum free media after exposure to various concentrations of MWCNTs dispersed in Phosphate Buffer Saline for different durations.

Exposure	Duration in hours											
	2		4		8		16		32		48	
Concentration in µg/ml	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media
6.25	98.65	99.11	96.29	97.74	94.05	94.71	73.41	79.75	80.97	82.60	69.76	76.17
12.5	97.08	96.00	94.90	95.71	87.87	90.80	67.59	76.54	74.55	79.53	66.66	71.69
25	95.29	94.66	90.74	93.67	80.44	82.06	64.05	73.33	73.52	76.72	62.38	69.33
50	92.37	93.55	86.11	90.51	75.49	79.08	62.78	70.61	71.97	74.93	59.76	66.74
100	91.03	92.66	83.10	88.71	73.26	76.55	58.98	63.70	68.89	73.14	57.85	60.61
200	88.11	86.88	79.62	83.29	71.03	73.56	56.70	60.00	66.58	69.05	53.80	57.31
400	82.06	83.33	76.62	80.58	67.32	71.72	53.67	57.77	60.92	61.38	50.71	54.71

Table 15: % of cell viability of XTT assay of A549 lung cells in serum containing & serum free media after exposure to various concentrations of MWCNTs dispersed in Ethanol for different durations.

Exposure	Duration in hours											
	2		4		8		16		32		48	
Concentration in µg/ml	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media
6.25	96.48	97.69	92.43	95.90	85.10	87.85	73.23	76.51	73.05	77.43	64.69	70.35
12.5	94.84	96.30	91.59	94.09	81.79	84.04	72.22	73.23	70.00	74.35	60.37	63.76
25	92.27	93.76	87.39	92.04	80.14	81.19	62.62	71.21	68.33	73.33	57.14	60.23
50	89.22	91.22	84.03	89.31	76.59	79.04	58.83	69.19	65.27	67.69	53.63	58.35
100	84.07	89.14	80.11	86.81	75.41	77.14	56.56	62.87	61.94	63.58	46.90	55.76
200	80.56	82.90	72.54	80.45	70.92	71.66	55.05	60.10	56.66	57.94	45.28	52.23
400	77.75	79.44	68.62	76.13	61.46	64.76	51.51	57.07	52.77	53.84	40.97	50.58

Table 16: % of cell viability of XTT assay of A549 lung cells in serum containing & serum free media after exposure to various concentrations of MWCNTs dispersed in sterile water for different durations.

Exposure	Duration											
	2		4		8		16		32		48	
Concentration in µg/ml	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media	Serum Free Media	Serum containing Media
6.25	93.37	96.53	88.13	90.00	79.95	83.58	71.61	75.06	71.98	75.39	56.82	68.95
12.5	90.94	95.88	86.44	88.22	76.73	80.59	68.54	72.56	68.06	67.27	53.65	64.45
25	88.52	94.15	84.74	84.88	72.52	77.36	62.40	69.57	64.70	65.70	51.70	62.08
50	79.47	90.04	75.98	79.11	69.55	73.63	59.59	66.33	61.62	64.92	49.02	59.95
100	77.92	87.01	70.33	76.66	63.61	65.42	56.26	60.33	60.22	62.82	46.34	53.08
200	75.49	79.22	66.10	74.44	59.15	62.93	53.96	58.10	56.30	56.54	40.97	49.76
400	72.84	77.83	62.71	69.55	54.45	60.94	51.15	55.36	47.61	52.61	37.07	42.65

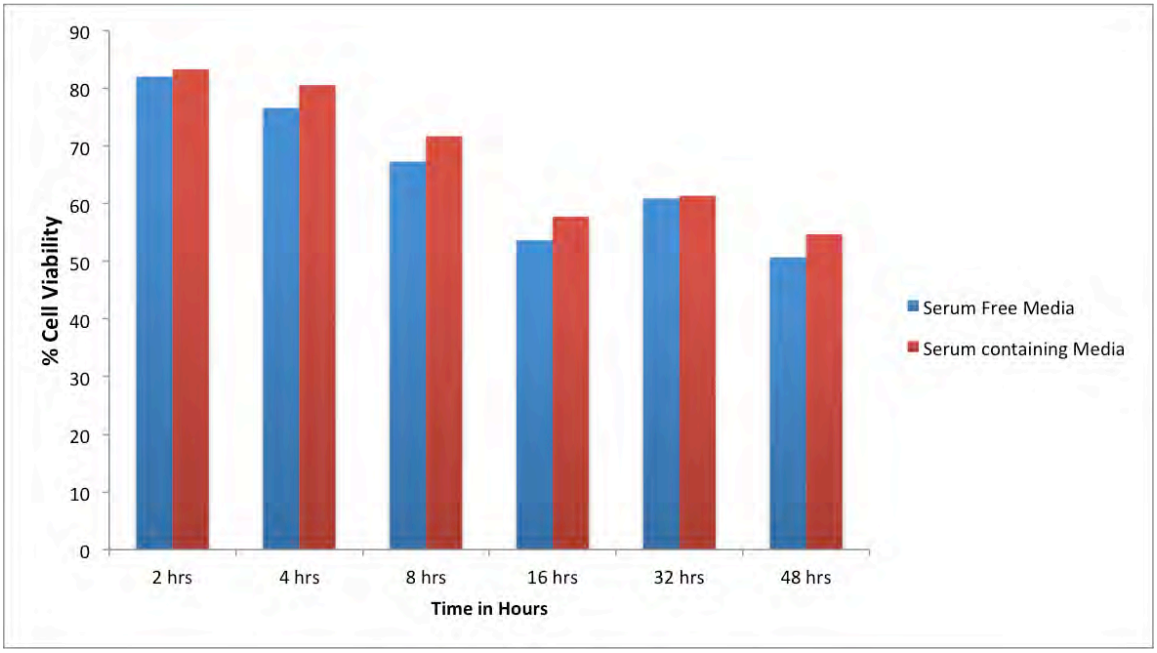

Figure 20: XTT Assay Results for MWCNT Exposure Concentration of 400 µg/ml in Phosphate Buffer

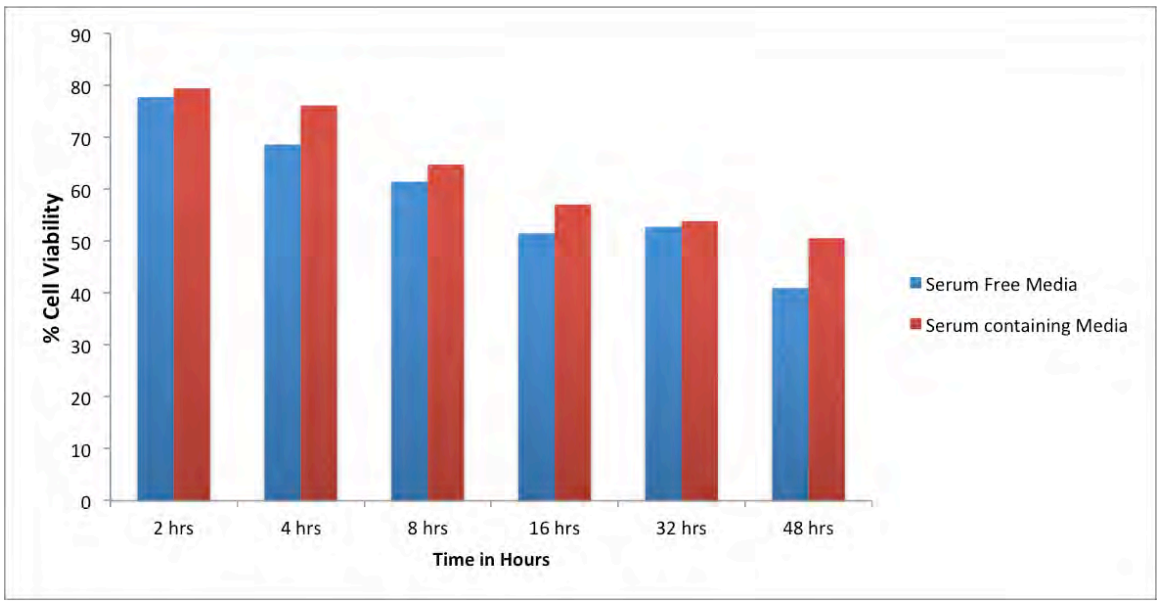

Figure 21: XTT Assay Results for MWCNT Exposure Concentration of 400 µg/ml in Ethanol

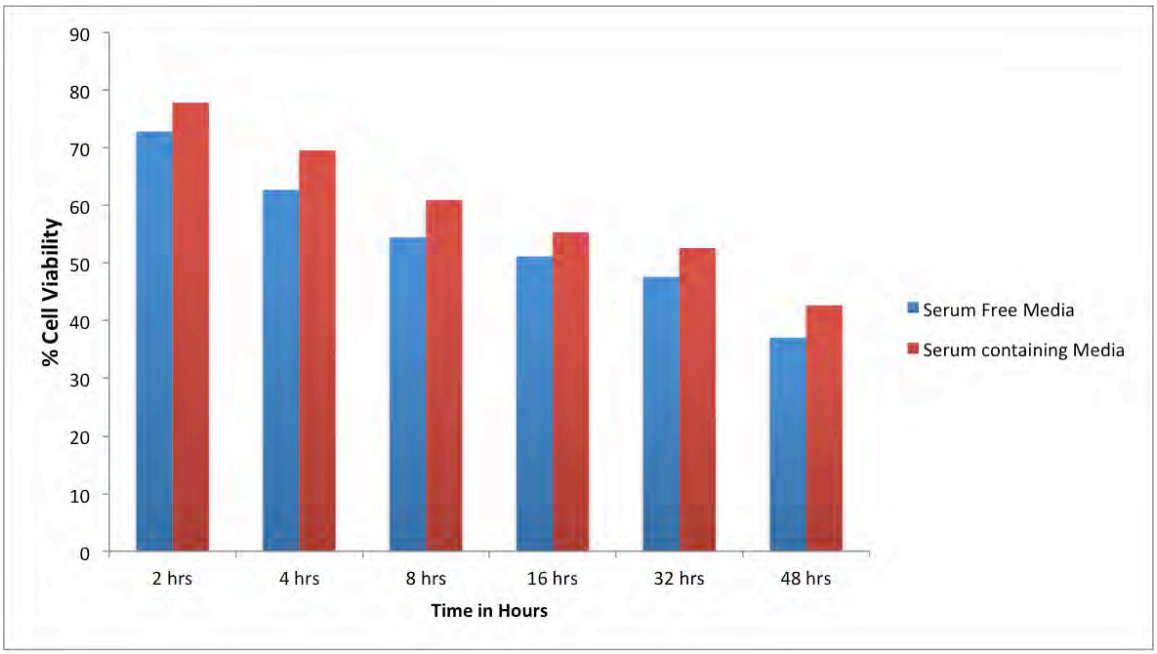

Figure 22: XTT Assay Results for MWCNT Exposure Concentration of 400 µg/ml in Sterile Water

Cell death was 10 % after two days exposure to MWCNTs in A549 cells at a concentration of 50 µg/mL, which was quantified by XTT assay (Simon-Deckers et al. 2008).

5.5 Apoptosis Assay Results

The MTT and XTT assays revealed the impaired mitochondrial metabolism. This impaired mitochondrial metabolism might result in decrease in cell number after incubation with MWCNTs. This was evaluated using DAPI staining. The percentage of apoptotic cells in MWCNT exposed A549 epithelial cells was higher after 48 hours of exposure period. This decrease was more significant in PBS dispersion media as shown in figures 23 and 24. The number of apoptotic cells was less in serum containing media than in serum free media. This apoptosis may be due to the increased levels of reactive oxygen species in concentration dependent manner. Reactive Oxygen Species induction and decreased mitochondrial activity might have affected the cell morphology of MWCNT treated cells.

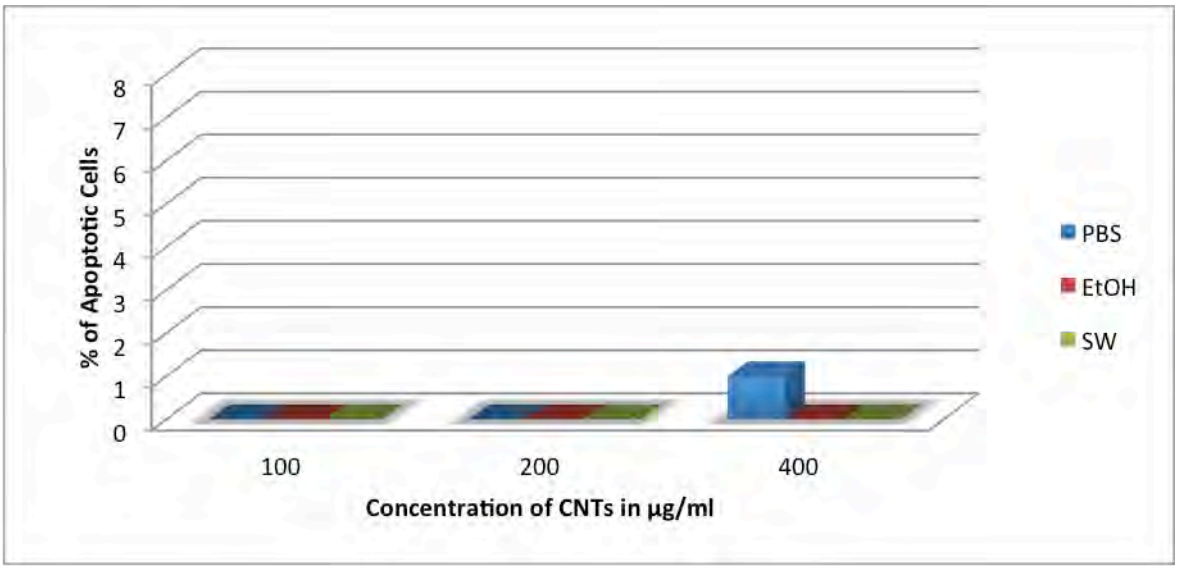

Figure 23: % of Apoptotic cells after treating with DAPI in Serum Containing Media – 48 hrs Stimulation in PBS, EtoH, SW.

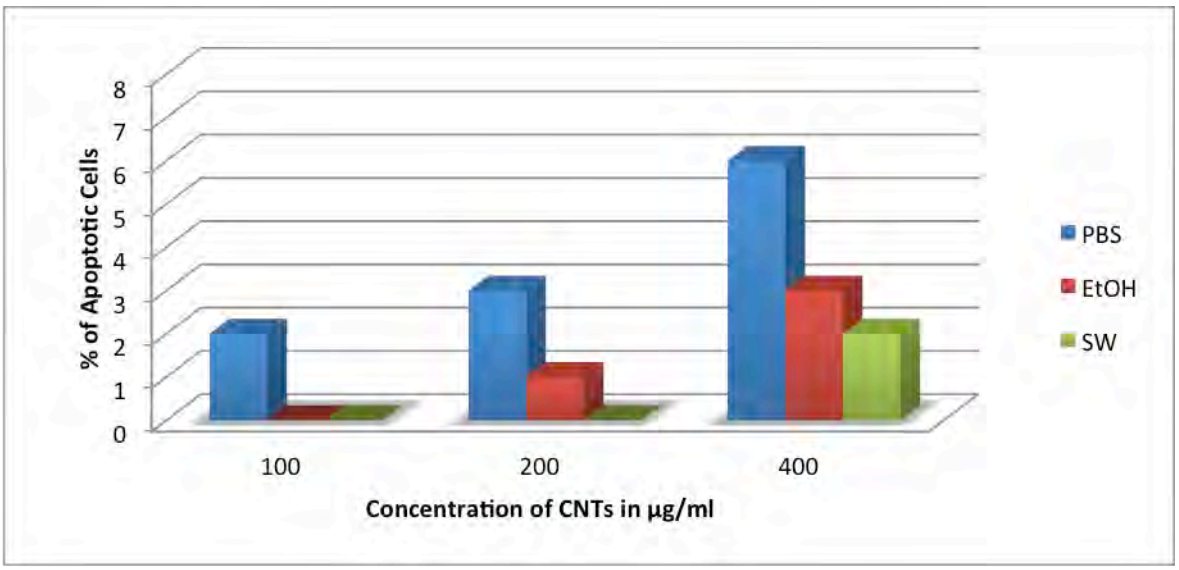

Figure 24: % of Apoptotic Cells After Treating with DAPI in Serum Free Media – 48hrs Stimulation in PBS, EtoH, SW.

Six hours exposure to MWCNTs induced a significant apoptotic response at 50μg/mL concentration in RLE cells (Muller et al. 2008). Exposure of A549 cells to 30 μg/mL MWCNTs for six days has increased the number of apoptotic cells (Thurnherr et al. 2011). Incubation of A549 with 100 μg/mL MWCNTs for one, two and three days has resulted in 15-20 % reduction in cell number (Tabet et al. 2009). Induction of apoptosis was started from a dosage of 40 μg/mL MWCNTs. A significant dose dependent increase in the percentage of apoptotic cells was observed after exposure to MWCNTs (40, 200, 400 μg/mL) for two days (Patlolla et al. 2010).

5.6 Optical Microscopy

Optical Microscopic observation reveals that MWCNTs were agglomerated and these agglomerates were of different sizes. These agglomerates were localized to cytoplasm. This indicates that MWCNTs have strong tendency to bundle together in ropes due to Vanderwaals forces. These bundles may contain many tens of nanotubes and can be considerably longer and wider than the nanotubes from which they are formed. This is very important factor in modifying the toxicity. Aggregates might have a much larger aerodynamic diameter than singlet MWCNT and so could be less respirable. The number of aggregates are proportionately increasing with increase in the concentration and exposure period of MWCNTs as depicted in figures 25, 26, 27 and 28 (100 μg/ml and 400 μg/ml for an exposure period of 2 and 4 hr).

The microscopic observation of A549 cell exposed to 100μg/ml concentration of MWCNTs for 8 hr and 400 μg/ml for 48 hr is shown in figures 29 & 30. The agglomerates are more in number with increasing concentration of MWCNTs. This is evident from the figures 29 &

30. It was observed that agglomerates of MWCNT had showed negative effects on cell viability and cell proliferation. The study reveals that the degree of dispersion and agglomeration was able to modify the MWCNT toxicity. The optical microscopic studies further revealed that MWCNTs interaction with A549 cell line caused morphological changes.

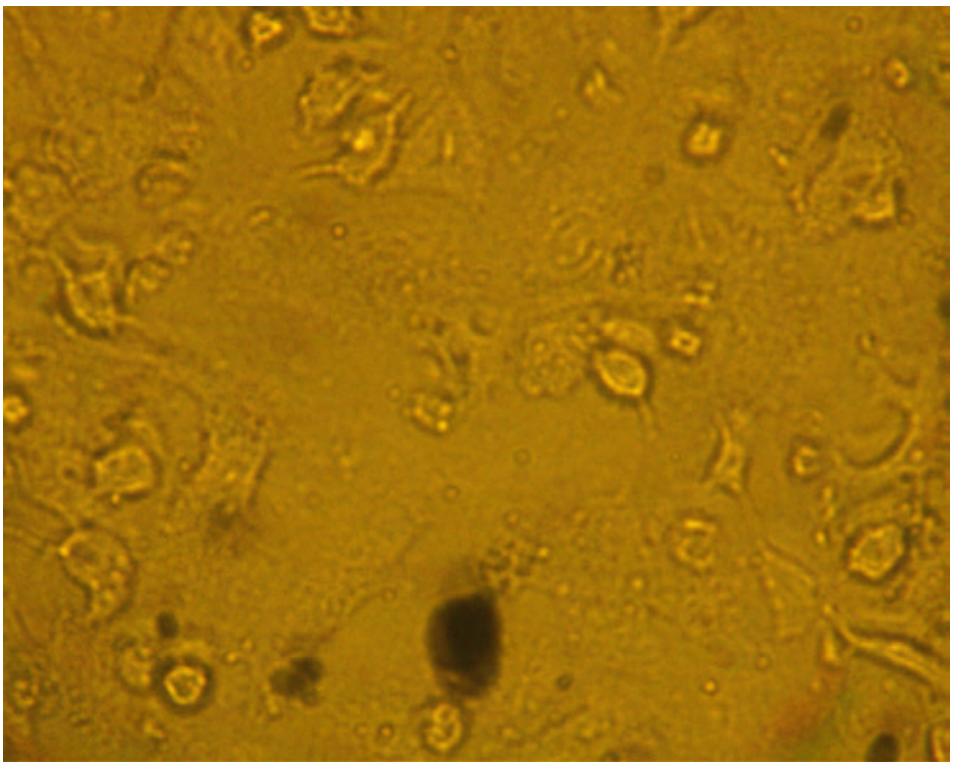

Figure 25: Microscopic Observation of A549 Lung Cells Exposed 100 µg/ml Concentration of MWCNT for an exposed period of 2 hr.

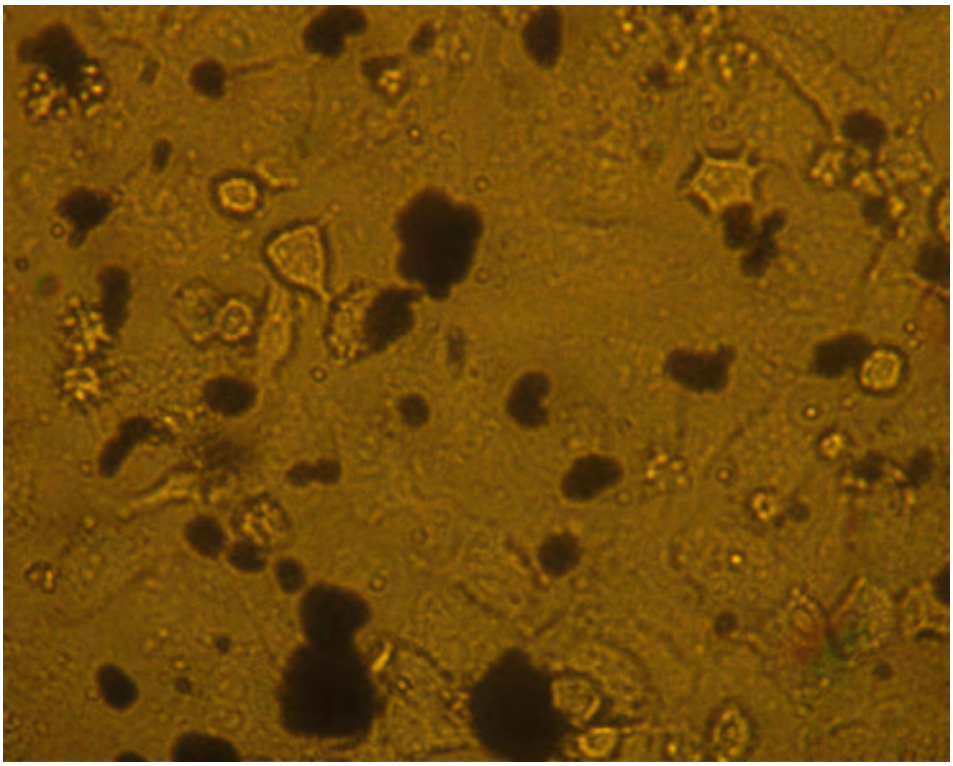

Figure 26: Microscopic Observation of A549 Lung Cells Exposed 400 µg/ml Concentration of MWCNT for an exposed period of 2 hr.

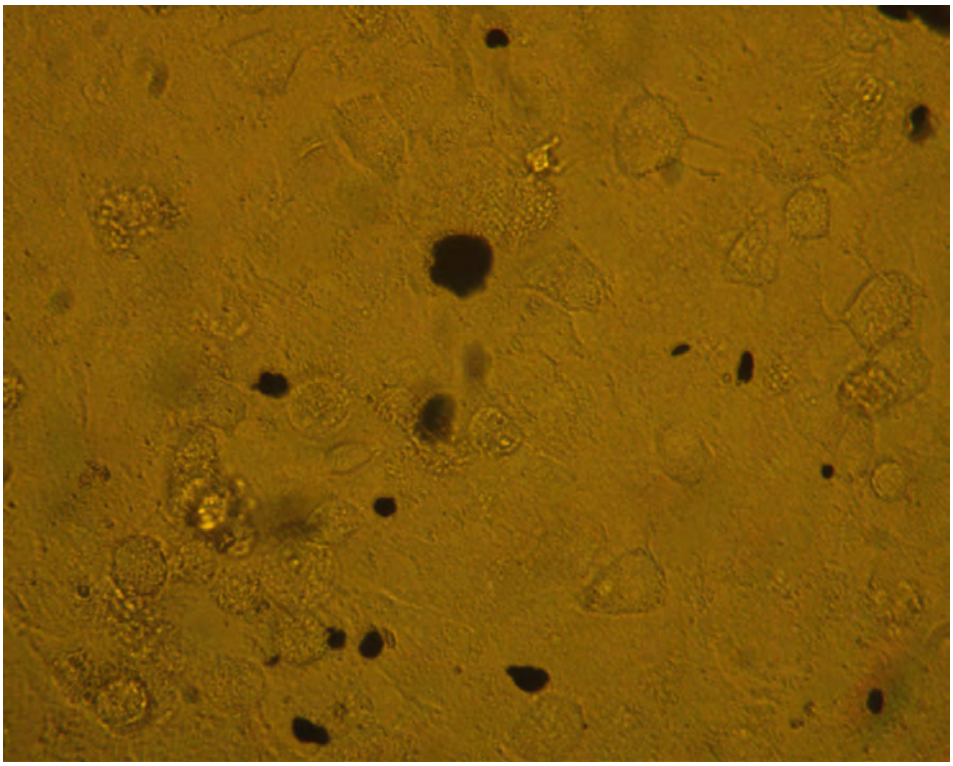

Figure 27: Microscopic Observation of A549 Lung Cells Exposed 100 µg/ml Concentration of MWCNT for an exposed period of 4 hr

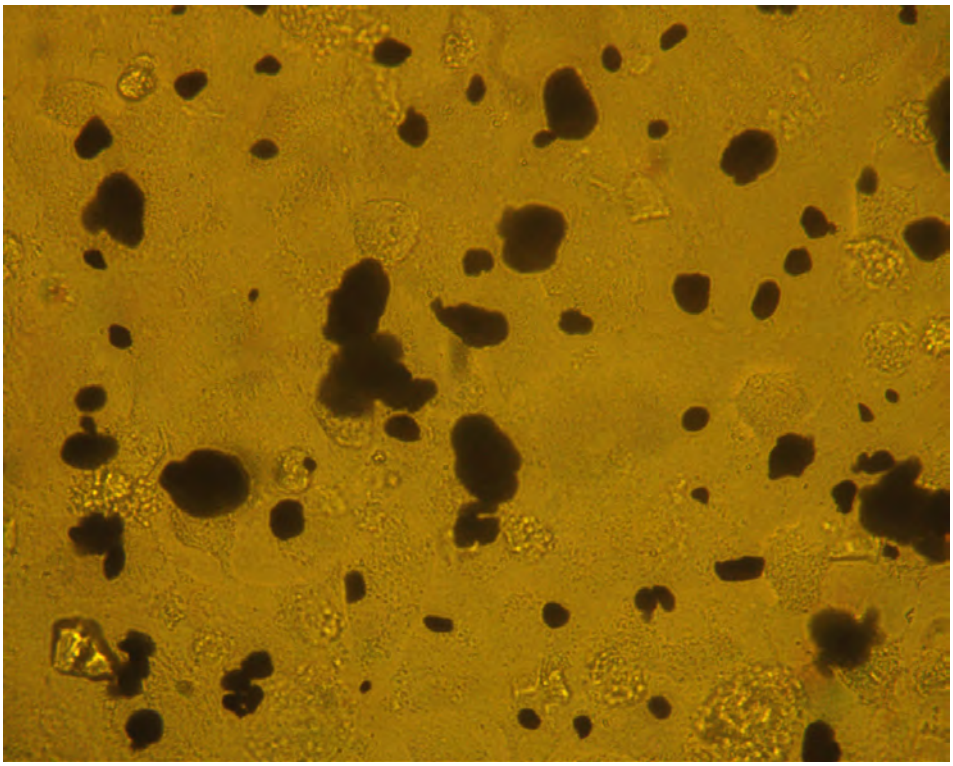

Figure 28: Microscopic Observation of A549 Lung Cells Exposed 400 µg/ml Concentration of MWCNT for an exposed period of 4 hr

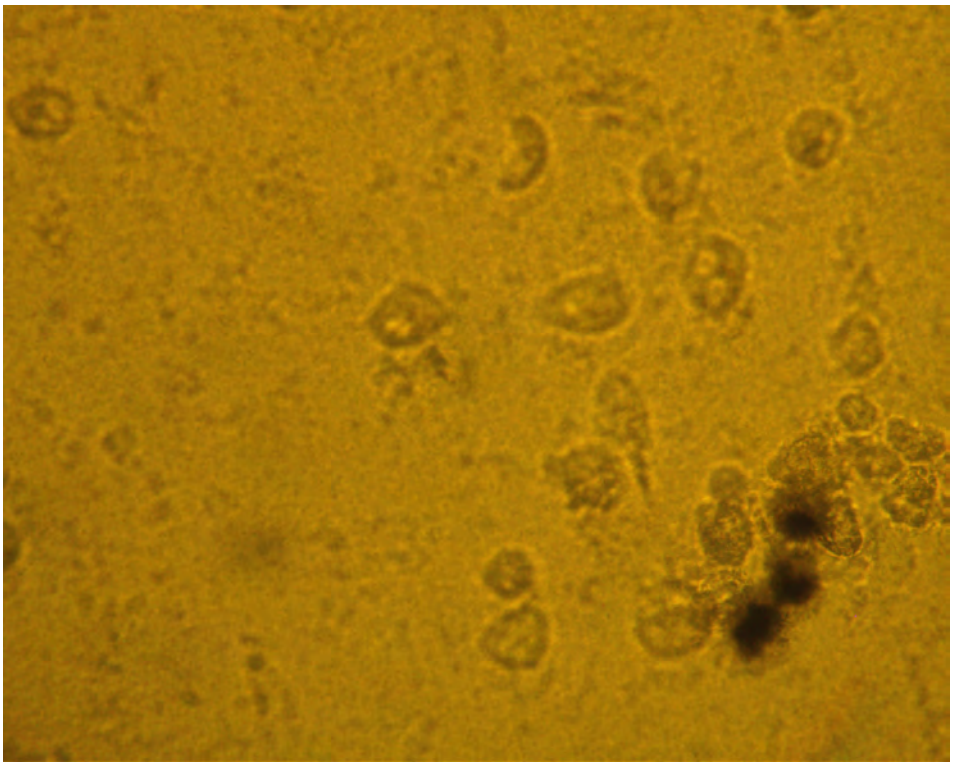

Figure 29: Microscopic Observation of A549 Lung Cells Exposed 100 µg/ml Concentration of MWCNT for an exposed period of 8 hr

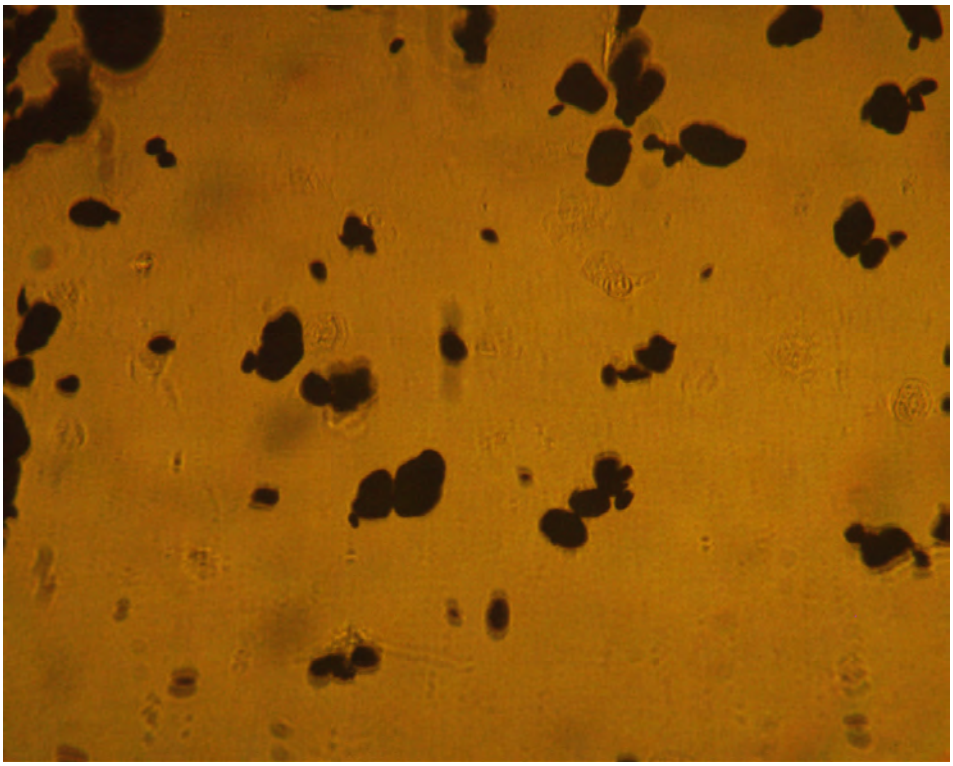

Figure 30: Microscopic Observation of A549 Lung Cells Exposed 400 µg/ml Concentration of MWCNT for an exposed period of 48 hr.

---End of Chapter 5---

Chapter-6

6. Results & Discussion - B

In real world, workers are exposed to MWCNTs through environmental media. The properties of MWCNTs may be altered by physical and chemical processed occurring in the environment. Keeping this in view, occupational exposure of MWCNTs was measured in an industrial air environment and occupational risk was estimated in the present chapter.

6.1 Field Study Results

The MWCNT concentrations were estimated at five different activities in the industry. The results obtained are presented in the table 17. The major emission source in the production facility was production unit i.e. near the reactor. Only a small difference was observed between samples collected during May, July and September months. But the concentrations of samples collected at various sampling points in the industry were varying. This might be due to significant variation in spatial distribution of particles. Improper mixing of air in the production area might be the reason for this spatial variation in particle distribution. The particle concentration was relatively low in the air at the site of packaging.

Table 17: Field Study Results of MWCNTs Concentrations

S.No	Sampling Location	MWCNTs Concentration during the months of			Background corrected concentration of MWCNTs		
		May	**July**	**September**	**May**	**July**	**September**
1	Near the reactor	2.1 $\mu g/m^3$	1.9 $\mu g/m^3$	2.0 $\mu g/m^3$	2.0 $\mu g/m^3$	1.8 $\mu g/m^3$	1.9 $\mu g/m^3$
2	During reactor cleaning	2.2 $\mu g/m^3$	2.0 $\mu g/m^3$	2.1 $\mu g/m^3$	2.1 $\mu g/m^3$	1.9 $\mu g/m^3$	2.0 $\mu g/m^3$
3	At the site of powder handling	1.5 $\mu g/m^3$	1.4 $\mu g/m^3$	1.4 $\mu g/m^3$	1.4 $\mu g/m^3$	1.3 $\mu g/m^3$	1.3 $\mu g/m^3$
4	At the site of packaging	1.2 $\mu g/m^3$	1.4 $\mu g/m^3$	1.3 $\mu g/m^3$	1.1 $\mu g/m^3$	1.3 $\mu g/m^3$	1.2 $\mu g/m^3$
5	During workplace cleaning	1.8 $\mu g/m^3$	1.7 $\mu g/m^3$	1.8 $\mu g/m^3$	1.7 $\mu g/m^3$	1.6 $\mu g/m^3$	1.7 $\mu g/m^3$
Background Concentration (Under non- Production conditions)		0.1 $\mu g/m^3$					

Note: The sampling location was same for all the three months and the values given in the table are the mean of at least two measurements.

The measured concentrations near the MWCNT emission sources were utilized for the estimation of exposure risk. MWCNT inhalation exposure of workers who directly handles MWCNT powder can be estimated using the assumed exposure scenario. The table 18 shows the classification of exposure potential according to material forms, exposure control, working scales and exposure frequencies. The estimated ambient air concentrations and assumed exposure scenario was used to estimate the amount of MWCNT powder that a worker will be exposed to by inhalation without exposure control. Assuming the exposure frequency to be high (8 hr per day × 5 days per week) the amount of exposure concentration was roughly estimated. In the working environment particle emission and exposure occur simultaneously. The environmental fate has to be considered in alleviating the exposure.

The results of the onsite investigation were close to exposure concentrations and the decrease in concentrations due to environmental fate was ignored and the exposure control absence was assumed. Keeping these things in mind the same concentrations were used for exposure assessment studies.

Table 18: Classification of Exposure of Nanomaterials Based on Material Forms, Exposure Control and Working Scale

Class	Material Form	Exposure control	Working scale[4] (or exposure Frequency)	Exposure potential (low-high, 1-5)
A	Fixed state (e.g., mixed in rasins)	-	-	1
B	Nanomaterials in liquids[a]	-	-	2
C	Dry nanomaterial powder	Closed system/unattended operation/automatization[b]	-	1
D1		Local ventilation equipment[c]	Small (low)	2
D2			Large (high)	3
E1		Only personal protective equipment[c]	Small (low)	3
E2			Large (high)	4
F1		No exposure control[c]	Small (low)	4
F2			Large (high)	5

(Source: NEDO project on Research and development of nanoparticle characterisation methods)

a: Exposure can occur when the liquid itself is splashed (e.g., during agitation, ultrasonication, processes involving foaming and spraying).

b: If an operation involves the opening of a closed system (sample collection, maintenance, cleaning, etc.), it will be regarded as Class D-F

c: Class D-F operations in which workers directly handle the nanomaterial powder include the following: unpacking, weighing, subdividing, scooping, blending, charging into manufacturing/processing equipment's, collection from manufacturing/processing equipment's, transferring to other containers, packing/bagging, cleaning/maintenance, treatment of wastes, etc.

d: Examples of the working scale: laboratories (small); industrial production (large).

6.2 Exposure Assessment

MWCNT inhalation exposure of workers who directly handles CNT powder was estimated using the assumed exposure scenario. The concentrations near the MWCNT emission source was experimentally estimated. The estimated industrial air concentrations and assumed exposure scenario was used to estimate the amount of MWCNT powder that a worker will be exposed to by inhalation without exposure control. In estimating the exposure, the amount of MWCNTs deposited on the pulmonary alveoli has to be calculated. MWCNTs with an aerodynamic diameter of 20 nm will have high deposition rate, approximately 40%, while those with an aerodynamic diameter of more than 10 μm had a deposition rate of 0%. In estimating the exposure potential, the alveolar deposition fraction was assumed to be 10%. The workers would be exposed to MWCNT particles at a high exposure frequency i.e. 8 h/day and six days per week. Breathing rate of 20 m^3/hr and a body weight of 70 kg was assumed. Then, the amount of MWCNTs deposited on the pulmonary alveoli per day per body weight is expressed as follows

Amount of exposure = Exposure concentration ×Alveolar deposition fraction × Exposure frequency × Breathing rate.

Exposure concentration = 2.2 μg/m^3

Alveolar deposition fraction = 0.1

Exposure frequency = (8h/24h) × (6days/7days) = 0.286

Breathing rate = 20 m^3/day/70 Kg

Amount of Exposure = 2.2 ×0.1× 0.286 × 20 /7= 0.179 μg/kg/day

The Amount of CNTs that will be deposited on the pulmonary alveoli / day / body weight is 0.179 μg/kg/day.

6.3 Correlation of Field and Laboratory Results

The expected revolution of carbon nanotubes in industry is raising questions about their impact on human health. In the present study MWCNT treated and untreated human lung cells were incubated for definite time period in order to observe acute toxicity on cell viability. The laboratory results concluded that MWCNTs are effecting the mitochondrial activity of lung epithelial cells there by causing oxidative stress. Intracellular reactive oxygen species were increasing in dose and time dependent manner. The oxidative stress triggers inflammation. The CNTs that have entered into the lung cells might have the same influence on the human lung. The optical microscopic studies revealed that MWCNT interaction with lung cell line caused morphological changes in cells. It was also observed that agglomeration of CNTs showed effect on cell viability and cell proliferation. The MWCNTs have three main properties that are clearly associated with pathogenicity of CNTs, they are fiber shaped and so might behave like asbestos which have toxicity associated with their needle like shape. There are essentially graphitic and so are expected to be biologically persistent.

In CNT manufacturing industries, handling of CNTs is releasing aerosols into the atmosphere. But how these MWCNTs react inside human beings is not answered completely till today. MWCNT exposure scenarios were useful in establishing exposure standards for the workers working in MWCNT manufacturing industries.

---End of Chapter 6---

Chapter-7

7. Summary, Conclusions & Recommendations

The chapter concludes current research work and noticeable contributions are mentioned in this section. Recommendations of the research and the benefits in implementing research findings to all companies of MWCNT manufacturing are given in this section.

7.1 Summary

As the CNTs possess unique physico-chemical properties and high economic potential they are being used in many products and their production is also being increased to meet the market demand. Due to their small size, the nanotubes can get entry easily into the human body through lungs, skin and gut. Compared to other organs as lung is the most sensitive organ, most of the studies were conducted on lung cell model. It is evident from the literature that CNTs are toxic to humans and there exists inconsistency among the reports on cytotoxicity of CNTs. It may be due to variation in the synthesis methods, purification method, mode of CNTs exposure i.e., as suspension in the media (or) immobilization (or) aerosol etc., route of administration, dimensions, metallic content, dispersion media, membrane permeability of a particular cell line and dosage of CNTs used and surface chemistry of the nanotubes and experimental materials used in the study. CNTs are able to

cause oxidative stress, inflammation, cell damage, granulomas etc., and these effects are dose and time dependent. Even though many studies have been conducted there is no clear evidence for the cytotoxicity of CNTs. Owing to their similarity to asbestos and other pathogenic fibers which have toxicity associated with their needle-like shape, further research is needed in this area in order to release the nanobased products into the market safely. Workers who are exposed to airborne CNTs need to take proper measures in order to protect themselves from the effects of CNTs on their health. The end users of CNT based products must also keep the effects of CNTs in mind before using them and their usage must be limited.

The present study investigated the exposure assessment of human health hazards that were caused by exposure to MWCNT's. The occupational workers and consumers are exposed to MWCNT's during MWCNT manufacturing and consumption of products that contain MWCNTs respectively. Potential health hazards caused by the inhalation of MWCNTs is oxidative stress of lung epithelial cells. The short term cell viability studies conducted in the laboratory for exposure assessment of MWCNTs indicate that mitochondrial activity was effected in the cells which were stimulated with various concentrations of MWCNTs. The health effect of workers working in CNT manufacturing industry was surveyed with a questionnaire proforma. The prepared questionnaire was subjected to Cronbach alpha analysis for reliability of the responses. The obtained value 0.792 was highly promising. The direct application of health effects collected by the questionnaire response is difficult as there is no data of emissions. The short-term cell viability studies conducted in the laboratory for exposure assessment of MWCNTs indicate that mitochondrial activity of A549 lung cells was effected due to stimulation caused by various concentrations of MWCNTs. Potential health hazard caused by the inhalation of MWCNTs is oxidative stress of lung epithelial cells. Even

at higher concentration, MWCNTs have not induced major acute toxicity in A549 lung epithelial cells.

The emission concentrations were estimated for an industry and exposure concentrations were evaluated by assuming lung deposition potential of 10%. The concentrations of MWCNTs were measured at an industrial site and the measured concentrations were utilized for calculating the exposure to workers, who are working in that industry. The highest concentration was observed at the reactor site as 2.2 $\mu g/m^3$. Hence, this concentration was used for calculating the exposure potential of the MWCNTs. The amount of CNTs that will be deposited on the pulmonary alveoli / day / body weight is 0.179 μg/kg/day. This might lead to potential health hazards if there are no safety precautions. In the industry in which we have measured the concentrations, they are following highest safety standards.

7.2 Conclusion & Recommendations

MWCNTs are fibre like forms of carbon and have structural resemblance with asbestos. Asbestos is known cause of many health effects on humans. The shape, biopersistance and insolubility of MWCNTs have raised concerns about the possible health effects for humans. The present investigation was carried out to understand the possible health effects of MWCNTs on human lung cells. The present study has investigated acute (two days) effects of MWCNTs on A549 human pulmonary epithelial cells, which was taken as in-vitro model for studying MWCNT inhalation toxicity. In addition, exposure assessment was carried out for workers working in an MWCNT manufacturing industry. The laboratory results were correlated to field studies.

The researchers have established contacts with CNT manufacturing units. In total, the employees belonging to 22 different industries have participated in the survey. The survey responses were analyzed using Minitab software for Chronbach Alpha value and are found to be 0.793 indicating that the responses are relatively consistent.

The MTT and XTT cytotoxicity assays were performed on MWCNT treated A549 human epithelial lung cell line. In both the assays the cell viability is decreasing with increasing concentration of MWCNT. The cell viability is also decreasing with increase in exposure period. The study reveals that the cytotoxicity of MWCNTs is time and dose dependent. The IC_{50} values are found to be 200 μg/ml and 400 μg/ml for serum free and serum containing media respectively. The MWCNTs dispersed in phosphate buffer solution have showed greater toxicity than MWCNTs dispersed either in ethanol or in sterile water, indicating that MWCNT dispersion plays an important role in cytotoxicity of MWCNTs. The impairment of mitochondrial activity is leading to apoptosis, was higher in cells treated with 400 μg/ml MWCNTs for 48 hr. The optical microscopic observations of treated cells reveals that the MWCNTs will form into clumps and these clumps might affect the cell viability of MWCNT thereby affecting cell proliferation.

Though the MWCNTs are fibre shaped, they were agglomerated to form particle like structures. The stable aggregates formed might mask the availability of nutrients to cells, which in turn will deprive the cells for nutrients. This might cause indirect toxicity to the cells. The study concludes that the toxicity of MWCNTs is dependent on presence or absence of serum.

Workers are more prone to exposure to manufactured MWCNTs than general public. The duration of exposure to MWCNTs and their release, dispersion and transformation would dictate the occupational risk. The field measurements of MWCNT exposure indicated significant aerosolization of MWCNTs. MWCNTs measurements during the study showed MWCNTs mass concentration in the range of 1.2 to 2.1 $\mu g/m^3$. The released particles have the tendency to agglomerate in the work place environment for a long period and most of these particles were respirable.

Appropriate measures have to be taken to limit the exposure of MWCNTs by workers. Local exhaust ventilation, fume hoods and wet cutting of composites can reduce the exposure to CNTs. National Institution for Occupational Safety and Health has proposed an exposure limit of 1 $\mu g/m^3$ for CNT exposure. Some studies have suggested an occupational exposure limit of 1 – 50 $\mu g/m^3$. This limit is based on quantitative risk assessment and short-term animal studies. The present study recommends the application of prevention strategies and control measures to minimize exposure of workers to CNTs. Workers should be trained to identify potentially hazardous nanomaterials in the workplace. Workers being exposed to CNTs should be evaluated periodically for potential health risks. Precise measurement methods are needed to obtain workplace exposure data and establishment of exposure standards. The long-term inhalation studies are required to determine time course and dose response for possible development of adverse health effects.

---End of Chapter 7---

Chapter-8

8. Scope For Future Work

Although current research itself is an exhaustive work, there is definite possibility to extend current research to the below mentioned topics for complete understanding of MWCNT exposure assessment.

8.1 Scope For Future Work

- The extent of entry of CNTs into the blood stream through alveolar passage still remains unexplained.
- The potential health hazards are caused by inhalation, oral & dermal exposure to MWCNTs, where as the present proposal has concentrated only on the hazards caused by the inhalation of MWCNTs.
- In-depth characterisation of MWCNTs is crucial for analysis and interpretation of toxicological assessment.
- Toxicological effects of different types of CNTs can be compared by a set of toxic equivalency factors. Hence, it would be useful to develop a set of toxic equivalency factors for CNTs by considering nanotube specific properties such as particle size, aspect ratio and agglomeration.
- Investigation of toxicity mechanisms of MWCNTs at the cellular level is required.

- Elimination mechanisms like biodegradation of CNTs have to be investigated, as they are highly hydrophobic in nature.

---End of Chapter 8---

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---End of References Section---

Appendix

Filled in Questionnaire Proforma

Attached are few samples of filled in pro-forma sent by the employees of MWCNT manufacturing industries.

Department of HSE University of Petroleum and Energy Studies, Dehradun		Department of Biotechnology GITAM Institute of Technology, GITAM University, Visakhapatnam
Proforma		
1	**Name of the company:**	Nanoshel (Intelligent Materials Pvt Ltd)
2.	**Address:**	# 117, Industrial Area Ph-2 Panchkula (Hry)
3.	**Contact number:**	+91-9779880077
4.	**E-Mail:**	nanoshel2@gmail.com
5.	**Age:**	5years
6.	**Sex (Male/Female):**	Company
7.	**Date of birth:**	27th January 2007
8.	**Name the of organization:**	Nanoshel
9.	**Department:**	Sales
10.	**Occupation (Supervisor /Technician/ Engineer/ worker /Administration):**	Administration
11.	**Work Nature (Physical / Mental):**	Mental
12.	**Severity of the work (Light/ Moderate/ heavy):**	Heavy
13.	**Duration of the work (hours/day):**	8-10 hours
14.	**Date of joining (Date/month/year):**	Since Beginning
15.	**Work experience (in years):**	8 years
16.	**Nature of Nano particles manufactured or used in the company (carbon/metal/oxide):**	Carbon Nano Tubes / Oxide Nano
17.	**Method of carbon nanotube production:**	NA (SWCNT- arc-discharge, MWCNTs-Thermal Chemical vapour deposition)
18.	**In which form carbon nanotubes are produced (raw/pure/extra pure):**	Pure / Extra Pure
19.	**Quantity of Carbon nanotubes produced or utilised:**	NA
20.	**Type of carbon nanotubes manufactured or used (Single walled/ multiwalled/ double walled/ side-wall functionalized):**	SWCNT/ MWCNT/Surface Modified SWCNT and MWCNT

21.	**Use of Carbon nanotubes in the industry (pure form/ along with other metals):**	Polymer, Coating, Defence, R&D etc
22.	**Approximate size of nano particle (nm):**	20-100nm
23.	**Health problems if any before. Please mention (No, Dermal, Allergic Ocular, Pulmonary, Cardiac):**	No
24.	**Does exposure to nanoparticles is causing any skin irritation/allergy? (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No (Never)
25.	**Breathing complaints if any (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
26.	**Heart related complaints. Please mention (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
27.	**Any incidences of cancer (No, Stage I, Stage II, Stage III):**	No
28.	**Are you practicing any safety practices. Please mention (High, Moderate, Light, Negligible, Not Applicable):**	Yes (To highest safety levels as prescribed by SHE)
29.	**Health check-ups if any: monthly/yearly? Please mention (Very high (monthly), High (3 months), low (6 months), very low (yearly), No, Not Applicable):**	After 3 months
30.	**Any other information which you would like to share:**	No

Thank you for your co-operation. This information is very valuable to us. We will email you the findings of our research.

Kindly email this filled in form to the e-mail id khasimb@gitam.edu. or akbarziauddin@gmail.com

Department of HSE University of Petroleum and Energy Studies, Dehradun		Department of Biotechnology GITAM Institute of Technology, GITAM University, Visakhapatnam
Proforma		
1	**Name of the company:**	David Carnahan
2.	**Address:**	179 Bear Hill Road, Waltham, MA 02451 USA
3.	**Contact number:**	781 609 2722
4.	**E-Mail:**	dcarnahan@nano-lab.com
5.	**Age:**	44
6.	**Sex (Male/Female):**	Male
7.	**Date of birth:**	Jan 7,1969
8.	**Name the of organization:**	Nano Lab, Inc.
9.	**Department:**	Multiple
10.	**Occupation (Supervisor /Technician/ Engineer/ worker /Administration):**	Engineering + Admin
11.	**Work Nature (Physical / Mental):**	50/50
12.	**Severity of the work (Light/ Moderate/ heavy):**	Light
13.	**Duration of the work (hours/day):**	8-9
14.	**Date of joining (Date/month/year):**	Jan 21, 2000
15.	**Work experience (in years):**	23
16.	**Nature of Nano particles manufactured or used in the company (carbon/metal/oxide):**	Carbon nanotubes
17.	**Method of carbon nanotube production:**	CVD
18.	**In which form carbon nanotubes are produced (raw/pure/extra pure):**	All
19.	**Quantity of Carbon nanotubes produced or utilised:**	10 kg/yr
20.	**Type of carbon nanotubes manufactured or used (Single walled/ multiwalled/ double**	All

	walled/ side-wall functionalized):	
21.	**Use of Carbon nanotubes in the industry (pure form/ along with other metals):**	Pure form
22.	**Approximate size of nano particle (nm):**	15nm x 20 um
23.	**Health problems if any before. Please mention (No, Dermal, Allergic Ocular, Pulmonary, Cardiac):**	No
24.	**Does exposure to nanoparticles is causing any skin irritation/allergy? (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
25.	**Breathing complaints if any (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
26.	**Heart related complaints. Please mention (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
27.	**Any incidences of cancer (No, Stage I, Stage II, Stage III):**	No
28.	**Are you practicing any safety practices. Please mention (High, Moderate, Light, Negligible, Not Applicable):**	Yes, P99 cartridge respirators
29.	**Health check-ups if any: monthly/yearly? Please mention (Very high (monthly), High (3 months), low (6 months), very low (yearly), No, Not Applicable):**	Nothing beyond standard yearly checkup
30.	**Any other information which you would like to share:**	Skin exposure to nanotubes does not seem to be a particular hazardous route of entry, as we handled them extensively without special protection in the early years of the company, from 2000 on. Our CNT are curly, and often found in aggregates 100 microns in diameter, so they are difficult to aerosolize.

Thank you for your co-operation. This information is very valuable to us. We will email you the findings of our research.

Kindly email this filled in form to the e-mail id khasimb@gitam.edu. or akbarziauddin@gmail.com

Department of HSE University of Petroleum and Energy Studies, Dehradun		Department of Biotechnology GITAM Institute of Technology, GITAM University, Visakhapatnam
Proforma		
1	**Name of the company:**	NANOSHEL LLC
2.	**Address:**	3422 Old Capitol Suit 1305 Willmington DE
3.	**Contact number:**	+1 302 652 3464
4.	**E-Mail:**	avena@nanoshel.com
5.	**Age:**	12 years
6.	**Sex (Male/Female):**	Female
7.	**Date of birth:**	1st May 2001
8.	**Name the of organization:**	NANOSHEL LLC
9.	**Department:**	Customer Care
10.	**Occupation (Supervisor /Technician/ Engineer/ worker /Administration):**	Administration
11.	**Work Nature (Physical / Mental):**	Official
12.	**Severity of the work (Light/ Moderate/ heavy):**	Moderate
13.	**Duration of the work (hours/day):**	6 Hours
14.	**Date of joining (Date/month/year):**	1st April 2007
15.	**Work experience (in years):**	5
16.	**Nature of Nano particles manufactured or used in the company (carbon/metal/oxide):**	Metal Nano Particles / Carbon Nano
17.	**Method of carbon nanotube production:**	CVD / Arc Discharge
18.	**In which form carbon nanotubes are produced (raw/pure/extra pure):**	Pure
19.	**Quantity of Carbon nanotubes produced or utilised:**	NA
20.	**Type of carbon nanotubes manufactured or used (Single walled/ multiwalled/ double walled/ side-wall functionalized):**	SWCNT/ MWCNT/Surface Modified SWCNT and MWCNT

21.	**Use of Carbon nanotubes in the industry (pure form/ along with other metals):**	Industrial, Defence & R&D etc
22.	**Approximate size of nano particle (nm):**	20-100nm
23.	**Health problems if any before. Please mention (No, Dermal, Allergic Ocular, Pulmonary, Cardiac):**	Nil
24.	**Does exposure to nanoparticles is causing any skin irritation/allergy? (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No (Never)
25.	**Breathing complaints if any (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
26.	**Heart related complaints. Please mention (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
27.	**Any incidences of cancer (No, Stage I, Stage II, Stage III):**	No
28.	**Are you practicing any safety practices. Please mention (High, Moderate, Light, Negligible, Not Applicable):**	Yes (To highest safety levels as prescribed by SHE)
29.	**Health check-ups if any: monthly/yearly? Please mention (Very high (monthly), High (3 months), low (6 months), very low (yearly), No, Not Applicable):**	After 3 months
30.	**Any other information which you would like to share:**	No

Thank you for your co-operation. This information is very valuable to us. We will email you the findings of our research.

Kindly email this filled in form to the e-mail id khasimb@gitam.edu. or akbarziauddin@gmail.com

Department of HSE University of Petroleum and Energy Studies, Dehradun	Department of Biotechnology GITAM Institute of Technology, GITAM University, Visakhapatnam

Proforma

1	**Name of the company:**	Julia
2.	**Address:**	Nanothinx S.A, Stadious street,Platani, P.O. Box 1414, Riopatras 26504, Greece
3.	**Contact number:**	+30-2610-965208
4.	**E-Mail:**	info@nanothinx.com
5.	**Age:**	45years
6.	**Sex (Male/Female):**	Female
7.	**Date of birth:**	29th September1968
8.	**Name the of organization:**	Nanothinx
9.	**Department:**	Customer care
10.	**Occupation (Supervisor /Technician/ Engineer/ worker /Administration):**	Technician
11.	**Work Nature (Physical / Mental):**	Mental
12.	**Severity of the work (Light/ Moderate/ heavy):**	Moderate
13.	**Duration of the work (hours/day):**	8-10 hours
14.	**Date of joining (Date/month/year):**	5/7/2004
15.	**Work experience (in years):**	10 years
16.	**Nature of Nano particles manufactured or used in the company (carbon/metal/oxide):**	Carbon
17.	**Method of carbon nanotube production:**	NA
18.	**In which form carbon nanotubes are produced (raw/pure/extra pure):**	Pure
19.	**Quantity of Carbon nanotubes produced or utilised:**	NA
20.	**Type of carbon nanotubes manufactured or used (Single walled/ multiwalled/ double**	NA

	walled/ side-wall functionalized):	
21.	**Use of Carbon nanotubes in the industry (pure form/ along with other metals):**	Coating, R&D
22.	**Approximate size of nano particle (nm):**	20-100nm
23.	**Health problems if any before. Please mention (No, Dermal, Allergic Ocular, Pulmonary, Cardiac):**	No
24.	**Does exposure to nanoparticles is causing any skin irritation/allergy? (Not Applicable, No, Negligible, Light, Moderate, Severe):**	Not Applicable
25.	**Breathing complaints if any (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
26.	**Heart related complaints. Please mention (Not Applicable, No, Negligible, Light, Moderate, Severe):**	Not Applicable
27.	**Any incidences of cancer (No, Stage I, Stage II, Stage III):**	No
28.	**Are you practicing any safety practices. Please mention (High, Moderate, Light, Negligible, Not Applicable):**	Moderate
29.	**Health check-ups if any: monthly/yearly? Please mention (Very high (monthly), High (3 months), low (6 months), very low (yearly), No, Not Applicable):**	High
30.	**Any other information which you would like to share:**	No

Thank you for your co-operation. This information is very valuable to us. We will email you the findings of our research.

Kindly email this filled in form to the e-mail id khasimb@gitam.edu. or akbarziauddin@gmail.com

Department of HSE University of Petroleum and Energy Studies, Dehradun		Department of Biotechnology GITAM Institute of Technology, GITAM University, Visakhapatnam
Proforma		
1	**Name of the company:**	Mathew
2.	**Address:**	Rosseter holding ltd, 4 Nikiforou lytra, P.O.Box 57220, 3310 Limassol, Cyprus
3.	**Contact number:**	+35799460171
4.	**E-Mail:**	contact@e-nanoscience.com
5.	**Age:**	31 years
6.	**Sex (Male/Female):**	Male
7.	**Date of birth:**	22 January 1983
8.	**Name the of organization:**	Rosseter holdings ltd
9.	**Department:**	Production
10.	**Occupation (Supervisor /Technician/ Engineer/ worker /Administration):**	Worker
11.	**Work Nature (Physical / Mental):**	Physical
12.	**Severity of the work (Light/ Moderate/ heavy):**	Heavy
13.	**Duration of the work (hours/day):**	9 hours
14.	**Date of joining (Date/month/year):**	16/10/2010
15.	**Work experience (in years):**	3 years
16.	**Nature of Nano particles manufactured or used in the company (carbon/metal/oxide):**	Carbon Nano Tubes
17.	**Method of carbon nanotube production:**	Chemical vapour deposition
18.	**In which form carbon nanotubes are produced (raw/pure/extra pure):**	Pure
19.	**Quantity of Carbon nanotubes produced or utilised:**	1.5 tonnes per annum
20.	**Type of carbon nanotubes manufactured or used (Single walled/ multiwalled/ double**	SWCNT, MWCNT

	walled/ side-wall functionalized):	
21.	**Use of Carbon nanotubes in the industry (pure form/ along with other metals):**	Pure
22.	**Approximate size of nano particle (nm):**	10-100nm
23.	**Health problems if any before. Please mention (No, Dermal, Allergic Ocular, Pulmonary, Cardiac):**	No
24.	**Does exposure to nanoparticles is causing any skin irritation/allergy? (Not Applicable, No, Negligible, Light, Moderate, Severe):**	Light
25.	**Breathing complaints if any (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
26.	**Heart related complaints. Please mention (Not Applicable, No, Negligible, Light, Moderate, Severe):**	No
27.	**Any incidences of cancer (No, Stage I, Stage II, Stage III):**	No
28.	**Are you practicing any safety practices. Please mention (High, Moderate, Light, Negligible, Not Applicable):**	High
29.	**Health check-ups if any: monthly/yearly? Please mention (Very high (monthly), High (3 months), low (6 months), very low (yearly), No, Not Applicable):**	After 6 months
30.	**Any other information which you would like to share:**	No

Thank you for your co-operation. This information is very valuable to us. We will email you the findings of our research.

Kindly email this filled in form to the e-mail id khasimb@gitam.edu. or akbarziauddin@gmail.com

---End of Appendix---

Publications

Publications Based on Research Work

Based on the research work, the scholar has published three research papers in reputed journals.

Published In International Journals

1. Akbar Ziauddin, Nihal Anwar Siddiqui, S.K. Beebi. (2014), "Carbon Nanotubes – Production, Properties and Health Effects" *International Journal of Advanced Enginering Technology* Vol. V, No. 3: 42-49.

2. Akbar Ziauddin, Nihal Anwar Siddiqui, S.K. Beebi. (2014), "Occupational Exposure Assessment of Multiwalled Carbon Nanotubes" *Asian Journal of Engineering Research* Vol. II, No. 4: 01-02.

3. Akbar Ziauddin, S.K. Beebi, Nihal Anwar Siddiqui. (2014), "Acute Toxicity Effects of MWCNTs on Human Lung Cells" *Asian Journal of Microbiology, Biotechnology & Environmental Science. Vol. 16, No. (4): 1207-1212.*

Research Paper

CARBON NANOTUBES- PRODUCTION, PROPERTIES AND HEALTH EFFECTS

[a]A. Ziauddin, [b]*Dr. S.K Beebi, [a]Dr. N.A Siddiqui , [a]Dr. Kanchan

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ABSTRACT

Nanotechnology is the new emerging technology which stands forefront in the science research and technology development. Carbon nanotubes (CNTs) are one of the most promising materials in the field of nanotechnology and are being produced by three methods: electric arc discharge, laser ablation and chemical vapour deposition. CNTs possess unique electrical, mechanical, chemical, thermal and optical properties, due to which these materials are suitable for various applications. The CNTs production is increasing in proportion to their usage. Since materials at the nanoscale behave differently than they do in their massive form, hence these CNTs are subjected to intense toxicological scrutiny. Research has proved that exposure to CNTs have negative effects on human health. The present paper attempts to review the production, properties and impact of CNTs on human health. This knowledge will be useful in formulating exposure standards for CNTs.

KEY WORDS: carbon nanotubes; production methods; properties; health effects; toxicity.

INTRODUCTION

Nanotechnology is the emerging field of science, which deals with nanoparticles ($1nm=10^{-9}$ m) and their production. CNTs are nanoparticles with a size range of 1-100 nm (ISO/TS 27687, 2008), with unique electrical, thermal, mechanical and vibrational properties, having a wide range of applications in the fields of electronics, computers, aerospace and other industries. Humans get exposed to high concentrations of these particles during the manufacturing process and usage of nano based products. CNTs are a form of carbon with a cylindrical shape and are first observed by Endo (1975), and later by Lijima (1991) in the soot produced by the arc-discharge synthesis of fullerenes. These tubes are made up of thick sheets of carbon called graphene which were rolled up to form a seam less cylinder. On the basis of number of tubes present, the CNTs are classified as Single walled (SWCNTs), Double walled (DWCNTs) and Multiwalled (MWCNTs) carbon nanotubes.

PRODUCTION

CNTs are finding wide range of applications, there by their production is also being increased by the companies. More than 100 companies in the world today are manufacturing CNTs and this number may increase to more than 200 in the next five years. Currently, CNTs account for 28 % market share of overall nanomaterials demand (Table 1). By 2016, the market demand amounts to 333,043 metric tons with a revenue of $2.4 billions, a five year compound annual growth rate of 19.2 % in unit terms and 20.9 % in value terms. In the coming five years, the CNT production capacities may increase enormously in some manufacturing units (2011 Nanotechnology research review, 2012).

Table: 1 Annual production capacities of CNTs in manufacturing companies (2010)
(Global carbon nanotubes market-industry beckons, 2011).

S. No	Type of CNTs	Manufacturing company	Annual production capacity (metric tonnes)	Production method
1.	SWCNTs	Mitsubishi Rayon Co. Ltd	1.2	Chemical vapour deposition (CVD)
2.		Kleancarbon Inc.	1.0	CVD
3.		Unidym, Inc	1.5	High-pressure carbon monoxide (HiPCO)
4.		Toray Industries, Inc	1.5	Catalytic chemical vapour deposition (CCVD)
5.		SouthWest Nano Technologies Inc	1.0	Cobalt molybdenum catalyst (CoMoCat)
6.	MWCNTs	Showa Denko K.K	500	CCVD
7.		CNano Technology Limited	500	CCVD
8.		Nanocyl S.A	400	CCVD
9.		Bayer Material science AG	260	CCVD
10.		Arkema Inc	50	CCVD
11.		Hyperion Catalysis International, Inc	50	CVD

Synthesis

CNTs are synthesized by various methods like arc-discharge, laser ablation, chemical vapour deposition (CVD), high pressure carbon monoxide (HiPCO), CoMoCat etc. An energy source (electricity, heat from a furnace or high light intensity) is added to carbon source for the synthesis of CNTs, which may vary depending on the synthesis method (Donaldson et al. 2006). Though the CNTs are synthesized by different methods (Table 2), CVD, HiPCO and CoMoCat are the most widely used methods for production of CNTs due to their high yield, purity and low cost of production and the reason for fewer yields of CNTs in arc discharge and laser ablation is due to the evaporation of carbon source at high temperatures.

1. Electrical arc discharge

It is the oldest method used for the production of CNTs. Pure and metal doped graphite electrodes are used for the synthesis of MWCNTs and SWCNTs

respectively (Popov 2004). The electrodes are temporarily brought into contact and an arc is struck. Low pressure (between 50 and 700 mbar) and controlled atmosphere composed of inert (like helium or argon) gases are maintained for the production. In the inter electrode zone, high temperature is maintained, so that the carbon sublimes from positive electrode (anode) and is consumed. A constant gap (1mm) is maintained between the two electrodes which can be done by adjusting the position of anode. Plasma formed between the electrodes, can be maintained constant for prolonged periods by controlling the distance between the two electrodes and the voltage (25-40 V). After de-pressurisation and cooling the reaction chamber, nanotubes together with by-products can be collected (Shifrina 2011; Szabo et al. 2010).

2. Laser ablation

In the year 1995, Richard E Smalley and his group used laser ablation for the first time to produce high quality CNTs. In this process, a graphite target gets vaporized by a pulsed laser in a tube furnace which is heated to 1200°C. Inert gases like helium or argon were sent into the chamber to carry the grown nanotubes to the copper collector. Nanotubes develop on the cooler surfaces of the reactor as the vaporized carbon condenses (Shifrina 2011). MWCNTs can be produced by using pure electrodes and SWCNTs can be produced by using metal-doped electrodes (Dai 2002).

3. HiPCO

In the year 1999, Richard E Smalley and his co-workers developed a high pressure carbon monoxide method (HiPCO) for the synthesis of CNTs. In this process a continuous gas phase carbon monoxide acts as feedstock and iron carbon monoxide ($Fe(CO)_5$) acts as a catalyst. Thinner SWCNTs with high purity, less structural defects and high intrinsic selectivity were obtained (Shifrina 2011).

4. CoMoCat

In 2000, Kitiyanan suggested another method in which a mixture of cobalt and molybdenum was used as a catalyst and hence, this process was named after the unique catalyst. At high temperatures (between 700°C-950°C) carbon monoxide decomposes into simple carbon and carbon dioxide (Shifrina 2011). The advantage of this method is that it reduces the formation of by-products as compared to arc discharge and laser ablation methods.

5. Chemical vapour deposition

The catalyst material (most commonly nickel, cobalt, iron or a combination) is heated to high temperature in a tube furnace and a hydrocarbon gas is passed through the reactor in controlled manner for a definite period of time. The hydrocarbon gas dissociates into individual components in the furnace and supplies the necessary carbon atoms for the CNT growth. Low temperatures (500-800°C) yield MWCNTs and high temperatures (600-1200°C) yield SWCNTs. Commercially available CNTs are often synthesized by this process.

Table: 2 % Yield of CNTs obtained by different production methods
(Shifrina (2011) Synthesis of carbon nanotubes)

S.No	Production method	Energy source	Substrate used	Catalyst used	Conditions maintained	% Yield	Cost of production of SWCNTs (per gram) in 2009
1.	Electric arc discharge	Electric arc	Pure or metal (Fe, Co, Ni, Mo, Y) doped graphite electrode	-	Low pressure (between 50-700 mbar)	60	$ 1,906
2.	Laser ablation	Pulsed laser	Graphite	Co, Cu, Nb, Ni, Pt	High temperature (1200°C)	70	-
3.	HiPCO	Heat	Carbon monoxide	$Fe(CO)_5$	High temperature (1000-1100°C), high pressure	90	$ 485
4.	CoMoCat	High temperature	Carbon monoxide	Co-Mo	700-950°C, 1-10 atm pressure	80-90	-
5.	Chemical vapour deposition	Heat from furnace	Carbon monoxide	Fe, Co, Ni, Mo	Temperature (500-1100°C), atmospheric pressure	90	$ 1,706

Properties

CNTs, due to their tiny size exhibit many interesting and unique properties.

1. Mechanical

CNTs are made up of sheets of graphene and the C-C bond in a graphene layer is probably the strongest chemical bond known in nature. CNTs are the strongest and stiffest materials yet discovered in terms of tensile strength and elastic modulus.

a. Tensile Strength

The tensile strength of CNTs is due to the covalent sp^2 bonds formed between the individual carbon atoms. The CNTs can sustain extremely high tension force of about 130 GPa (gigapascals) where as the steel can withstand <5 GPa. Yu et al. (2000) tested the tensile strength of SWCNTs and MWCNTs and is found to be 13-52 GPa and 63 GPa respectively. The tensile strength of a single layer of MWCNTs is 100 times stronger than that of steel.

b. Elasticity

The CNTs are elastic and they can withstand stress. The elasticity can be measured experimentally by calculating the Young's modulus. Lourie and Wagner (1998) reported Young's modulus of 2.8-3.6 TPa (terra pascal) for SWCNTs and 1.7-2.4 TPa for MWCNTs.

2. Electronic

CNTs possess unusual electronic properties and act as conductors of energy. The diameter and helicity (n, m) of carbon atoms in the nanotube shell are believed to determine their conductivity (metallic or semiconductor). Theoretical calculations for

electronic properties by Hamada et al. 1992; Mintmire et al. 1992; Saito et al. 1992 showed that CNTs are very sensitive due to their geometrical structure. Theoretically it was determined that metallic nanotubes (where the energy gap between the valence and conducting states is zero) can carry an electric current density of 4×10^9 A/cm^2 (ampere per square centimetre) which is 1000 times higher than copper (Hong et al. 2007).

3. Thermal

CNTs are very good thermal conductors due to their geometrical structure. The thermal conductivity of CNTs was evaluated both theoretically and experimentally at room temperature. Theoretically it was predicted that CNTs exhibit a thermal conductivity of 6600 W/m K which is larger than graphite (> 2000 W/m K) or diamond (3320 W/m K) (Berber et al. 2000). The measured value of thermal conductivity for bulk samples of SWCNTs is over 200 W/m K (Watts per meter Kelvin) and for individual MWCNTs is over 3000 W/m K (Hone 2004).

4. Optical

CNTs possess unique optical properties and can be studied using a variety of theoretical tools. The calculated optic and nonlinear properties are important for various applications. Light absorption, photoluminescence and Raman spectroscopy measurements are needed to observe the optical properties. The optical properties can be detected by spectroscopic studies. The optical properties of CNTs can be derived from electronic transitions within one-dimensional density of states. Optical responses of semiconducting species are greater than the metallic nanotubes. CNTs have light emitting capacity and vary between metallic and semiconducting CNTs.

Health effects of CNTs

Though the CNTs have unique properties and are useful for many industrial applications, effects on human health were investigated because materials at the nanoscale behave differently from their original form. CNTs can enter into the human body through various routes like skin, lungs and digestive tract. After gaining entry, they can accumulate in different body parts and can bring out changes. Many CNT toxicity studies have been conducted both *in vivo* and *in vitro* to determine the fate and effect of CNTs in the body. However among them, most of the studies were conducted in lung cell model as it is the most sensitive organ.

Toxic effects of CNTs:

To investigate the effects of CNTs, researchers exposed various cell lines and animal subjects to CNTs, produced by different methods possessing diverse lengths, diameters and aspect ratios. The parameters, specific surface area and size of CNTs were measured using Brunauer Emmet Teller method and Transmission electron microscopy respectively. The toxicity effects of SWCNTs & MWCTs on living cells and systems were analysed.

1. Cytotoxicity

Cytotoxicity of CNTs (SWCNTs & MWCNTs) can be assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH), sodium 3'-(phenyl amino-carbonyl-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulphonic acid hydrate (XTT), Alamar Blue (AB), neutral red, Alkaline Phosphatase (ALP) assays and staining techniques such as trypan blue exclusion, Hoechst, propidium iodide, YO-PRO1, Diamino-2-Phenyl Indole dihydrochloride (DAPI), annexin V , and Bromodeoxy uridine (BrdU) antibody stains and methods like DNA fragmentation, caspase-3 and 3/7 activity measurement etc.

a) Cell Viability

Scientists (Cui et al. 2005; Magrez et al. 2006; Davoren et al. 2007; Zhang et al 2007; Muller et al. 2008; Simon-Deckers et al. 2008; Tabet et al. 2009; Patlolla et al 2010; Thurnherr et al. 2011) have performed MTT assay in order to measure the cytotoxicity of CNTs with varying exposure concentrations. The studies indicated decrease in cell viability with increasing concentrations of CNTs.

MTT is a calorimetric assay, which is used to assess the cell viability. Live cells were able to convert yellow soluble tetrazolium dye to purple insoluble formazan with the help of NAD(P)H- dependent oxidoreductases. All this process occurs in mitochondrial cytosol.

HEK 293 cell viability decreased in time and dose dependent manner after exposing to 0.78125-200 µg/mL of SWCNTs (Cui et al 2005). The cell viability was inversely proportional to the dosage of MWCNTs in H596 human lung tumor cells (Magrez et al. 2006). Maximum cytotoxicity of A549 cells was observed at 800 µg/mL after 24 h exposure to1.56-800µg/mL SWCNTs, however the percentage inhibition was more in the absence of serum (45%) when compared to its presence (32%) at 800µg/mL (Davoren et al. 2007). Reduction in cell viability is 60 % at a dose of 100 micrograms per millilitre (µg/mL) of MWCNTs after one day in RLE cells (Muller et al. 2008). Cell death is 50 % at a concentration of 0.5 µg/mL MWCNTs after two days in A549 cells (Simon-Deckers et al. 2008). Decrease in cell viability is 60 % at a concentration of 100 µg/mL of MWCNTs in both A549 & MeT5A cells (Tabet et al. 2009). Patlolla et al 2010 observed time and dose dependent decrease of human dermal fibroblasts viability after 24, 48, 72 and 96h exposure to 40, 200 and 400 µg/mL MWCNTs and the reduction in cell viability is 50 % at 400 µg/mL. Dose dependent decrease in mitochondrial activity of A549 cells is due to acute exposures (one day) (3.2, 6.25, 12.5, 25 and 30 µg/mL) of MWCNTs (Thurnherr et al. 2011).

The decrease in cell viability in all the studies might be due to the mitochondrial damage and interruption of NAD(P)H flux.

Cell death was 10 % after two days exposure to MWCNTs in A549 cells at a concentration of 50 µg/mL which was quantified by XTT assay (XTT is also based on reduction of tetrazolium to formazan but requires an electron coupling reagent for efficient reduction) (Simon-Deckers et al. 2008). Tabet et al. (2009) did not observe any significant alteration in cell viability through neutral red assay (Neutral red is a vital stain, which can be incorporated into lysosomes of only live cells and not dead cells). Shvedova et al. (2003) used Alamar blue bio assay (The metabolic condition of mitochondria can be detected by using Alamar blue assay. Alamar blue cell viability reagent is used to measure the proliferation of various cells quantitatively and thereby deciding the cell health. Live cells maintain a

reducing environment in the cytosol of the cell. Alamar blue contains an active ingredient resazurin, which is non-toxic, cell permeable, blue in color and non-fluorescent. Resazurin is effectively reduced in mitochondria. When cells are treated with Alamar blue the resazurin is reduced to resorufin, a compound which is red in color and highly fluorescent in the presence of NADPH or NADH dehydrogenase and NADPH or NADH as reductant. Live cells constantly convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding the cells) to determine cell viability of human epidermal keratinocytes by exposing them to 60, 120 and 240 µg/mL SWCNTs for 18 h and observed a decrease in cell viability of 11.3 %, 24.5 % and 37.2 % respectively indicating the concentration dependent decrease in cell viability. Bottini et al. 2006 exposed T lymphocytes to MWCNTs (oxidized and pristine) at a concentration of 0.001 and 0.01 µg up to five days and assessed the cell viability by trypan blue staining. Dead cells take up the dye and appear in blue color whereas the viable cells don't take up the dye as they possess intact cell membrane. Oxidized MWCNTs were found to be more toxic than pristine MWCNTs. Zhang et al. (2007) used ALP assay to investigate the potential effects of CNTs on osteoblasts differentiation and the study indicated the inhibition of ALP activity at all concentrations of CNTs in a time dependent manner without any dose dependence. Alkaline phosphatase (ALP) dissociates p-Nitrophernyl Phosphate, an early marker of osteoblasts differentiation. Zein et al 2008 exposed Human peripheral blood lymphocytes to 5, 10, 25, 50 µg/mL concentrations of SWCNTs for one, two and three days and tested the cell viability with trypan blue staining. Dose-dependent decrease in cell viability was observed, which was significant at 25 and 50 µg/mL concentrations. The effect of soluble MWCNTs on neural cells was studied by Bang et al. (2011) at two concentrations i.e., 190 and 295 µg/mL. Reduction in cell viability was indicated by resazurin reduction assay and *in situ* Hoechst and propidium iodide double staining assay of plasma membrane integrity. The condensed chromatin of apoptotic cells was stained more brightly than normal cells by Hoechst dye, Whereas PI is taken up only by dead cells. Normal, apoptotic and dead cells can be differentiated by flow cytometry and fluorescent microscope. 40 % decrease of resazurin conversion capacity was observed after one day exposure to CNTs, and was more pronounced after two days. Less than half of the treated cells had intact plasma membrane by second day.

Sayes et al. (2006) evaluated the dependence of cytotoxicity of functional SWCNTs on human dermal fibroblasts. Higher degree of functionalization has reduced the effect of CNTs and their interactions with biomembranes have increased their biocompatibility. The non toxicity of functional CNTs is due to the specific groups which reduce the availability of reactive sites on the surface of CNTs.

The effect of CNTs on cell viability varies due to the use of different cell lines, varied concentrations & type of CNTs and presence of catalyst impurities (Simon Deckers et al. 2008; Tabet et al. 2009).

b) Cell damage: The extent of damage to the cell can be determined by LDH release assay (cell damage marker) (Muller et al. 2008, Simon-Deckers et al. 2008; Zein et al 2008; Patlolla et al 2010; Cesta et al. 2010; Fenoglio et al. 2012;). The cell damage was dose-dependent in RLE cells and was significant at 100 and 150 µg/mL of MWCNTs (Muller et al. 2008). Exposure to 100 µg/mL MWCNTs for two days has resulted in 35-40 % cell damage in A549 cells (Simon-Deckers et al. 2008). Zein et al 2008 observed a slight increase in LDH in human peripheral blood lymphocytes after two days exposure to 5, 10, 25 and 50 µg/mL SWCNTs. Patlolla et al. (2010) exposed human dermal fibroblasts to MWCNTs and quantified the cell damage by performing LDH release assay. The release of LDH increased in time and in dose-dependent manner. The extent of cell damage is also dependent on the thickness of CNTs as evident from the study performed by Fenoglio et al. (2012) in which thin $MWCNTs_{9.4}$ were found to be more toxic than thicker ones ($MWCNT_{70}$).

c) Apoptosis

Apoptotic cells can be identified using annexin V staining. Six hours exposure to MWCNTs induced a significant apoptotic response at 50 µg/mL concentration in RLE cells (Muller et al. 2008). Exposure of A549 cells to 30 µg/mL MWCNTs for six days has increased the number of apoptotic cells (Thurnherr et al. 2011). In addition to annexin V, Thurnherr et al. (2011) used propidium iodide stain to detect late apoptotic/ necrotic cells. Exposure to 30 µg/mL MWCNTs for three or six days did not induce apoptosis. Incubation of A549 with 100 µg/mL MWCNTs for one, two and three days have resulted in 15-20 % reduction in cell number (Tabet et al. 2009).

Apoptosis and necrosis were induced in MWCNT treated skin fibroblast cells in a dose dependent manner. This response is due to a type I INF response which will lead to apoptosis and cell death (Ding et al. 2005). Induction of apoptosis was started from a dosage of 40 µg/mL. A significant dose dependent increase in the percentage of apoptotic cells was observed after exposure to MWCNTs (40, 200, 400 µg/mL) for two days (Patlolla et al. 2010).

Translocation of phosphotidyl serine phospholipids from the cytoplasmic face to the extracellular face of the plasma membrane is one of the earliest events in apoptosis. MWCNTs stimulated apoptosis/necrosis was tested using a double-stain flow cytometric assay. In neural cells CNTs induced cell death was apoptotic and not necrotic and apoptosis occurs more slowly. Apoptosis can be induced by two major pathways; the receptor mediated extrinsic pathway and the mitochondrial activated intrinsic pathway. Both the pathways involve the activation of the executioner protein Caspase 3, which shares substrate specificity with Caspase 7. Measurement of active Caspase 3/7 levels in CNT treated cells will indicate the extent of cell damage and apoptosis. The observation of Bang et al. (2011) suggests that the Caspase 3/7 activity is 20-30 % higher in CNT treated cells than controls and is maximum after three days. SWCNTs treated HEK 293 cells displayed morphological changes and became apoptotic at 25 µg/mL concentration after one day. The apoptotic cells have formed nodular structures encapsulating SWCNTs and exhibited apoptotic features such as membrane vesicles, nucleus condensation,

fragmentation and apoptotic bodies. Chromatin condensation and membrane bleeding are the phenomena of the cells undergoing apoptosis. Binding of annexin V to phosphotidyl serine present on the extracellular surface of cells reflects apoptosis. Methods like DNA fragmentation and annexinV-FITC (fluorescein isothiocyanate) staining, Caspase-3 and 3/7 activity were used by Bang et al. (2011); Bottini et al. (2006); Cui et al. (2005) and Zeni et al. (2008) to identify apoptotic cells. Cui et al. (2005) after exposing HEK 293 cells to various concentrations of MWCNTs for 6 days observed apoptosis after one day at 25 µg/mL concentration which was increased gradually with increase in time. Dose dependent stimulation of apoptotic cells were monitored by Bottini et al. (2006) after five days exposure to MWCNTs exposure. Zeni et al. (2008) did not observe any increase in Caspase-3 activity after treating with SWCNTs at 25 and 50 µg/mL concentrations for seven h and one day. Bang et al. (2011) monitored CNT exposed neural cells after three days and found high activity levels of Caspase-3/7. He further observed increase in percentage of hypodiploid cells with increase in time after three days exposure to CNTs.

d) Cell proliferation

Proliferation of skin fibroblasts can be measured by incorporating BrdU and the cell proliferation in CNT treated cells was delayed because of G2/M block and S phase delay during cell cycle. Cell proliferation was reduced by ~50 % in MWCNT treated fibroblasts after two days at 0.06 µg /mL (Ding et al. 2005). Davoren et al. 2007 used Alamar blue assay to test the cell proliferation inhibition of SWCNTs exposed A549 lung cells both in the presence and absence of serum. The effect of low concentrations of SWCNTs on A549 lung cells was considerably less in the presence of serum which might be due to the availability of nutrients, whereas at higher concentrations of SWCNTs the effect is independent of serum.

d) Oxidative stress

Oxidative stress was induced when the human epidermal keratinocytes were exposed to SWCNTs for 15 min. This was confirmed by the formation of free radical species, decrease in vitamin E, total antioxidant reserves, accumulation of peroxidative products and reduction of protein thiols in the CNT treated cells (Shvedova et al. 2003).

2. Genotoxic effects

Exposure to CNTs may effect the genetic material of the cells, which are called genotoxic effects and can be evaluated by MN assay, comet assay and DNA ladder analysis. Scientists (Ding et al. (2005), Muller et al. (2008), Thurnherr et al. (2011)) performed studies to determine the genotoxic effects of CNTs in various cell lines and animal models.

Muller et al. (2008) used female Wistar rats for *in vivo* studies and rat lung epithelial cells for *in vitro* studies to test the toxicity of MWCNTs (10, 25, 50, 100, 150 µg/mL; 0.5,2 mg/rat) by micronucleus (MN) assay. The formation of micronucleus is an indication of genotoxic effects. A dose dependent increase in multinucleated pneumocytes and micronucleus were observed *in vivo* and *in vitro* studies respectively. The two major mechanisms accountable for MN formation are breaks in DNA and loss of chromosomes thereby causing clastogenic and aneugenic effects. Thurnherr et al. (2011) studied the effects of MWCNTs in two different cell types A549 lung epithelial cells and Jurkat T lymphocytes by exposing to various concentrations of MWCNTs (0.5, 3.2, 6.25, 12.5, 25, 30 µg/mL) for different time intervals (one & 180 days) and genotoxic effects were assessed by MN assay. Neither DNA damage nor chromosomal damage was observed, which might be due to the variation in cells selected and concentrations used for the study.

MWCNT treatment causes changes in gene expression which are involved in down regulation and up regulation. CNT treatment will alter the promoters of genes. Nanomaterial treated cells trigger responses from the activated P^{38} and ERK MAPK cascades. Cells treated with high dose of CNTs caused more changes in gene expression than lower dosage. The cellular response to CNTs treatment mimics to the response of viral infection as the dimensions of the CNTs are similar to that of the virus. At high doses CNTs induces innate immune response and the carbon atoms released may participate in cells metabolic pathways (Ding et al. 2005). The genomic DNA damage was increased with the increase of CNT dosage which can be measured by Comet assay and DNA ladder analysis. Patlolla et al. (2010) monitored CNT treated human dermal fibroblasts and the cells have shown significant increase in percent tail DNA (2.96 %-16.39 %) and DNA damage with increase in dosage. Patlolla et al. (2010) used high dose of MWCNTs when compared to Ding et al. (2005), this might be the reason for high DNA damage in human dermal fibroblasts, whereas in the latter, it was only alteration in gene expression.

3. Inflammation and Fibrosis of lung cells

Exposure to CNTs developed inflammation and fibrosis in experimental animal used for the study (Warheit et al. 2004; Shvedova et al. 2005; Magnum et al. 2006 ; Mercer et al. 2008; Ma-Hock et al. 2009; Cesta et al. 2010; Fenoglio et al. 2012). Inflammation was due to the accumulation of total protein, LDH and inflammatory cells like neutrophils & macrophages.

Warheit et al. (2004) instilled rats with SWCNTs ranging from 1000-5000 µg/kg and observed the effects upto 90 days. Post instillation of SWCNTs has increased the LDH levels and short-term pulmonary inflammatory response due to increase in neutrophils after 1 day. Dose independent multifocal granulomas were also observed. Shvedova et al. (2005) observed the pulmonary responses of mice by exposing them to SWCNTs at a concentrations of 10, 20, 40 µg/mouse for 60 days. BAL cell counting and Sirius red staining indicated increased accumulation of neutrophils followed by sequential appearance of lymphocytes and macrophages. Magnum et al. (2006) counted cells, analyzed total protein and LDH levels in BALF (broncho alveolar lavage fluid) and observed the formation of fibrotic lesions in rat lungs after 21 days of exposure to SWCNTs (CVD, ~300-600 m^2/g surface area, 896 % carbon, 2.6 % cobalt and 1.7 % molybdenum) (200 µg/kg). Increased PDGF levels suggest their role in the formation of SWCNT induced fibrotic lesions. The dispersion of SWCNTs (HiPCO, diameter 0.69-3.7 µm with <2 wt % of contaminants) will increase the thickness of the alveolar wall and number of alveolar macrophages

(Mercer et al. 2008). Muller et al. (2008) observed inflammatory response in rats exposed to MWCNTs (500, 2000 µg/rat) due to dose-dependent increase in levels of LDH, protein, macrophages and neutrophils. Inhalation of 500 and 2500 µg/m^3 (microgram per cubic meter) concentration of MWCNTs resulted in increase in lung weight, inflammation (multifocal granulomatous, histiocytic), neutrophilic and intra-alveolar lipoproteinosis in the lung and lung associated lymph nodes and slight blood neutrophilia at 25 µg/m^3 (Ma-Hock et al. 2009). Cesta et al. (2010) exposed rats to MWCNTs (4000 µg/kg) (microgram per kilogram) for a period of 21 days and observed significant fibrosis & lesions in the lungs. Prior exposure to 2500 µg/kg lipo polysaccharide (LPS) of E.*coli* enhanced the effect of MWCNTs by inducing fibrosis, increasing total protein and platelet derived growth factor (PDGF-AA) by two-fold and three to four folds respectively after one day. Fenoglio et al. (2012) tested the inflammatory effect of MWCNTs (2000 µg/rat) of two different diameters (9.4 and 70 nm) by measuring LDH activity and protein content in BAL (broncho alveolar lavage) and inflammatory cell count (neutrophils, eosinophils and lymphocytes) in alveoli. The effect of MWCNTs decreased with increase in diameter.

In all studies the exposure to CNTs (SWCNTs or MWCNTs) caused inflammation, which is an immune reaction exhibited by host to the target particle and the increase in inflammatory levels is different in each study and is due to variation in dosage & exposure routes.

4. Cellular internalization of CNTs

Exposure of lung cells to CNTs has resulted in the internalization of nanotubes. Transmission electron microscopy (TEM) observations of MWCNT exposed A549 cells have revealed the presence of two to three micrometer or smaller MWCNTs in the cytoplasm, in an isolated manner, and altered the morphology of cells (Simon- Deckers et al. 2008). In contrast, agglomerates of MWCNTs with different sizes were found in the cytoplasm of murine alveolar macrophage (MH-S) cells (Fenoglio et al. 2012).

Although many toxicity studies were conducted for assessing the health effects of CNTs, only few studies have indicated the presence of CNTs within the lung cells which may be due to the membrane permeability of a particular cell line and high dosage of CNTs used in that study. The formation of agglomerates within the cell depends on surface chemistry of CNTs.

Table 3: Summary table of carbon nanotubes- production method, properties, impurities and exposure concentrations
In vitro studies

S. No	CNTs Type	Production Method	Diameter (nm)	Length (µm)	SSA (m^2/g)	Metallic impurities (%)	Cell line	Exposure dose/concentration	Reference
1.	SWCNTs	HiPCO	-	-	-	Fe-30	HaCaT cells (Human Keratinocyte)	60, 120, 240 µg/mL	Shvedova et al. (2003)
2.	SWCNTs	-	-	-	-	-	HEK293 (human embryonic kidney)	0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 150, 200 µg/mL	Cui et al. (2005)
3.	MWCNTs	CVD	-	-	-	-	Human skin fibroblasts	0.06, 0.6 µg/mL	Ding et al. (2005)
4.	MWCNTs	-	20-40	1-5	-	< 5	Human T lymphocytes	0.001, 0.01 µg/cell	Bottini et al. (2006)
5.	MWCNTs	CVD	20	-	-	-	H596, (Lung-tumor cell line)	0.002-0.2 µg/mL	Magrez et al. (2006)
6.	SWCNTs	HiPCO	1-4	-	1,040	Fe-0.23	RAW 264.7 macrophages	100 µg/mL	Shvedova et al. (2005)
7.	SWCNTs	HiPCO	1	0.4	-	Residual metals - < 1	Human Dermal Fibroblasts	3-30,000 µg/mL	Sayes et al. (2006)
8.	SWCNTs	HiPCO	0.8-1.2	-	-	Fe-10	Human A549 lung cells	1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400, 800 µg/mL	Davoren et al. (2007)
9.	SWCNTs MWCNTs	CVD	< 2 < 10	5-15	-	-	Primary osteoblasts	0.1, 1, 10, 50, 100 µg/mL	Zhang et al. (2007)
10.	MWCNTs	-	11.3	0.7	-	Co-1, Fe--0.5 catalysts	Rat lung epithelial cell (RLE)	10, 25, 50, 100, 150 µg/mL	Muller et al. (2008)
11.	MWCNTs	CVD	10-160	0.1-12 0.1-3.5	42±2	Fe (raw) -4.24 Fe (annealed)-0.08	A549 (Human pulmonary cells)	0.25-100 µg/mL	Simon-Deckers et. al (2008)
12.	SWCNTs	-	1.1	50	-	Al-0.08, Cl-0.41, Co-2.91 and S-0.29	HPBL (Human peripheral blood lymphocytes)	1-50 µg/mL	Zein et al. (2008)
13.	MWCNTs	CVD	12	0.1-13	219.2	Al-2.4, Fe-2	A549 (Pulmonary), MeT5A (Mesothelial)	0.1-100 µg/mL	Tabet et al. (2009)
14.	MWCNTs	CVD	10-30	0.3-50	109	Ni-0.34, La-0.03	Rat lung fibroblasts	10 µg/cm^2	Cesta et al. (2010)
15.	MWCNTs	CVD	15-30	15-20	41	COOH-2-7	Human Dermal Fibroblasts	40, 200, 400 µg/mL	Patlolla et al. (2010)
16.	MWCNTs	CVD	10-20 85-115	10-30 -	-	Fe-< 0.1	NS-1 (Neuroscreen)	190 and 295 µg/mL	Bang et al. (2011)
17.	MWCNTs	-	6-24	2-5	-	metallic residues- < 0.4-	A549 (Human pulmonary cells, Jurkat T lymphocytes	3.2, 6.25, 12.5, 25, 30 µg/mL) 0.5 µg/mL	Thurnherra et al. (2011)
18.	MWCNTs	CVD	9.4±0.3 70±2	0.1-1 1-3	240 60	Al-0.06, Fe-0.08, Co-0.07 Al-0.06, Fe -0.05	MH-S (Murine alveolar macrophages)	260 µg/mL	Fenoglio et al. (2012)

In vivo studies

S. No	Type of CNTs	Production Method	Diameter (nm)	Length (μm)	SSA (m^2/g)	Metallic impurities (%)	Animal subject used	Exposure dose/concentration	Reference
1.	SWCNTs	Laser ablation	1.4	>1	-	Ni and Co-5	Rat (Intratracheal instillation)	1000, 5000 μg/kg	Warheit et al. (2004)
2.	SWCNTs	HiPCO	1-4	-	1,040	Fe-0.23	Mice (Pharyngeal aspiration)	10, 20, 40 μg/mouse	Shvedova et al. (2005)
3.	SWCNTs	CVD	~ 2	0.5-40	~300-600	Co-2.6, Mo-1.7	Rats (Oropharyngeal aspiration)	2000 μg/kg	Magnum et al. (2006)
4.	SWCNTs	HiPCO	690-3700	-	-	< 2	Mice (Aspiration)	10 μg/mouse	Mercer et al. (2008)
5.	MWCNTs	-	11.3	0.7	-	Co-1, Fe--0.5 catalysts	Rat (Intratracheal instillation)	500, 2000, 5000 μg/rat	Muller et al. (2008)
6.	MWCNTs	CVD	5-15	0.1-10	250-300	Al_2O_3 9.6, traces of Fe and Co-0.4	Rats (Inhalation)	100, 500, 2500 $\mu g/m^3$	Ma-Hock et al. (2009)
7.	MWCNTs	CVD	10-30	0.3-50	109	Ni-0.34, La-.03	Rats (Intratracheal instillation)	4000 μg/kg	Cesta et al. (2010)
8	MWCNTs	CVD	9.4±0.3 70±2	0.1-1 1-3	240 60	Al-0.06, Fe-0.08,Co-0.07 Al-0.06, Fe-.05	Rats (Trans oral aspiration)	2000 μg/rat	Fenoglio et al. (2012)

CONCLUSION

As the CNTs possess unique physico-chemical properties and high economic potential they are being used in many products and their production is also being increased to meet the market demand. Due to their small size, the nanotubes can get entry easily into the human body through lungs, skin and gut. Compared to other organs as lung is the most sensitive organ, most of the studies were conducted on lung cell model. It is evident from the literature that CNTs are toxic to humans and there exists inconsistency among the reports on cytotoxicity of CNTs. It may be due to variation in the synthesis methods, purification method, mode of CNTs exposure i.e., as suspension in the media (or) immobilisation (or) aerosol etc., route of administration, dimensions, metallic content, dispersion media, membrane permeability of a particular cell line and dosage of CNTs used in that particular study and surface chemistry of the nanotubes and experimental materials used in the study. CNTs are able to cause oxidative stress, inflammation, cell damage, granulomas etc., and these effects have been also observed as dose and time dependent. Even though many studies have been conducted there is no clear evidence for the cytotoxicity of CNTs. Owing to their similarity to asbestos and other pathogenic fibres which have toxicity associated with their needle-like shape, further research is needed in this area in order to release the nanobased products into the market safely. Workers who are exposed to airborne CNTs need to take proper measures in order to protect themselves from the effects of CNTs in the body. The end users of CNT based products must also keep the effects of CNTs in mind before using them and their usage must be in controlled level.

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Research Paper

OCCUPATIONAL EXPOSURE ASSESSMENT OF MULTIWALLED CARBON NANOTUBES

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ABSTRACT

Pulmonary toxicity of CNTs is well established by many laboratory investigations. The toxicity studies were challenging due to lack of occupational exposure assessments. In the present study exposure assessment of MWCNTs was carried out by measuring the mass concentrations in personal breathing zone of a manufacturing facility using filter sampling procedure. The measured concentrations were used to calculate the amount of MWCNTs that can be deposited on the alveoli of human lung. The obtained results indicate that the amount of CNTs that will be deposited on the pulmonary alveoli / day / body weight is 0.179µg/kg/day.

KEY WORDS: carbon nanotubes; production methods; properties; health effects; toxicity.

INTRODUCTION

CNT's have diameters of 100 nm or less with high structural integrity. Though these CNTs comprises of only carbon atoms, they have excellent mechanical properties and semiconducting characteristics. The quantum of production will increase to greater magnitude in near future. Potential human health hazards are caused by inhalation, dermal and oral exposure to CNTs. Among all the exposures, pulmonary toxicity of carbon nanotubes is well established. Many laboratory studies were conducted for understanding the cytotoxicity of Multiwalled carbon nanotubes. All these studies lack correlation to occupational exposure in workers mainly due to the lack of human exposure assessment data. At present the scale of the industries manufacturing and handling is small, but in future it is expected to develop extensively. This scenario is due to the fact that small size of workforce is prevailing in carbon nanotube manufacturing industry. Exposure assessment methods and exposure metrics such as particle number, surface area and mass which are correlating with adverse health effects are not in consensus with each other. This study was conducted to assess exposure concentrations of MWCNTs at different locations in a MWCNT manufacturing facility, located at Noida.

MATERIALS AND METHODS

Filter based sampling was employed to assess exposure concentrations in the industrial air environments. An aerosol monitor was used to monitor particle mass concentration. An impact or with a cutoff of 1µm was used to allow sampling from 0.1 to 1 µm as the mass measurement is dominated by larger sized particles. A filter testing chamber was designed to determine mask filter particle retention in the workplace during realtime operating conditions and exposure situations. Particle laden air during regular production was drawn in with a vacuum pump and controlled by a rotameter. Pressure drop between two compartments was measured with a manometer. The data set included measurements under background and production conditions. These are the two diverse environments with respect to concentration levels. Five sampling locations were chosen to capture the spatial and temporal variation in particle levels. The sampling inlets were positioned at 1.2m above the ground. This level will quantify the breathing concentration between standing and sitting working position. Worker's activity includes reactor maintenance and cleaning, mechanical adjustments to the reactor system and its operation, powder handling and packaging and work place cleaning. Background levels were determined each day prior to the production start. Temporal measurements were also made with the same sampling system by collecting samples during the months of May and June. The filters were analysed gravimetrically for the mass concentration of MWCNTs using Pyris 1 TGA.

RESULTS AND DISCUSSION

The MWCNTs concentrations were estimated at five different activities in the industry. The results obtained are presented in the table 1. The major emission source in the production facility is production unit i.e., near the reactor. Only a small difference was observed between samples collected during May and July months. But the concentrations of samples collected at various sampling points in the industry are varying. There is significant variation in particle distribution spatially. This distribution could be because of improper mixing of air in the production area. Low concentration in inhalable dust fraction is observed at the site of packaging.

Table 1: Exposure concentrations in the selected facility

S. No	Sampling location	MWCNTs Concentration		Background corrected concentration of MWCNTs	
		During the month of May	During the month of July	During the month of May	During the month of July
1	Near the reactor	2.1µg/m³	1.9 µg/m³	2.0µg/m³	1.8µg/m³
2	During reactor cleaning	2.2 µg/m³	2.0 µg/m³	2.1 µg/m³	1.9 µg/m³
3	At the site of powder handling	1.5µg/m³	1.4 µg/m³	1.4µg/m³	1.3µg/m³
4	At the site of packaging	1.2 µg/m³	1.4 µg/m³	1.1µg/m³	1.3µg/m³
5	During workplace cleaning	1.8 µg/m³	1.7 µg/m³	1.7µg/m³	1.6µg/m³
Background concentration		0.1 µg/m³			

The measured concentrations near the MWCNT emission sources are utilized for the estimation of exposure. MWCNT inhalation exposure of workers who directly handles MWCNT powder can be estimated using the exposure scenario. The table 2 shows the classification of exposure potential according to material forms, exposure control, working scales and exposure frequencies. The estimated ambient air concentrations and assumed exposure scenario was used to estimate the amount of MWCNT powder that a worker will be exposed to by inhalation without exposure control. Assuming the exposure frequency to be high (8 hrs per day × 5 days per week) the amount of exposure concentration was roughly estimated. In the working environment particle emission and exposure occur simultaneously. The environmental fate has to be considered in alleviating the exposure. The results of the onsite investigation are close to exposure concentrations. And the decrease in concentrations due to environmental fate was ignored and the exposure control absence was assumed. Keeping these things in mind the same concentrations were used for exposure assessment studies.

Table 2: Classification of exposure potential of nanomaterials based on material forms, exposure control and working scale

Class	Material Form	Exposure control	Working scale[d] (or exposure Frequency)	Exposure potential (low-high, 1-5)
A	Fixed state (e.g., mixed in resins)	-	-	1
B	Nanomaterials in liquids[a]	-	-	2
C	Dry nanomaterial powder	Closed system/unattended operation/automatization[b]	-	1
D1		Local ventilation equipment[c]	Small (low)	2
D2			Large (high)	3
E1		Only personal protective equipment[c]	Small (low)	3
E2			Large (high)	4
F1		No exposure control[c]	Small (low)	4
F2			Large (high)	5

(Source: NEDO project on Research and development of nanoparticle characterization methods)

a: Exposure can occur when the liquid itself is splashed (e.g., during agitation, ultrasonication, processes involving foaming and spraying).

b: If an operation involves the opening of a closed system (sample collection, maintenance, cleaning, etc.), it will be regarded as Class D-F

c: Class D-F operations in which workers directly handle the nanomaterial powder include the following: unpacking, weighing, subdividing, scooping, blending, charging into manufacturing/processing equipment's, collection from manufacturing/processing equipment's, transferring to other containers, packing/bagging, cleaning/maintenance, treatment of wastes, etc.

d: Examples of the working scale: laboratories (small); industrial production (large).

EXPOSURE ASSESSMENT

In estimating the exposure, the amount of MWCNTs deposited on the pulmonary alveoli has to be calculated. MWCNTs with an aerodynamic diameter of 20 nm will have high deposition rate, approximately 40%, while those with an aerodynamic diameter of more than 10 µm had a deposition rate of 0%. In estimating the exposure potential, the alveolar deposition fraction was assumed to be 10%. The workers would be exposed to MWCNT particles at a high exposure frequency i.e., 8 h/day and six days per week. Breathing rate of 20 m^3/hr and a body weight of 70 kg was assumed. Then, the amount of MWCNTs deposited on the pulmonary alveoli per day per body weight is expressed as follows

Amount of exposure =

Exposure concentration ×Alveolar deposition fraction × Exposure frequency × Breathing rate.

Exposure concentration	= 2.2 µg/m^3
Alveolar deposition fraction	= 0.1
Exposure frequency	= (8h/24h) × (6days/7days) = 0.286
Breathing rate	= 20 m^3/day/70 Kg
Amount of Exposure	= 2.2 ×0.1×0.286 × 20 /7 = 0.179 µg/kg/day

The Amount of CNTs that will be deposited on the pulmonary alveoli/day/body weight is 0.179µg/kg/day.

CONCLUSION

The concentrations of MWCNTs were measured at an industrial site and the measured concentrations were utilized for calculating the exposure amounts by workers, who are working in that industry. The highest concentration was observed at the reactor site as 2.2 µg/m^3. Hence, this concentration was used for calculating the exposure potential of the MWCNTs. The amount of CNTs that will be deposited on the pulmonary alveoli / day / body weight is 0.179µg/kg/day. This might lead to potential hazards if there are no safety precautions. In the industry in which we have measured the concentrations, they are following highest safety standards. Safety is given priority in that premises.

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ACUTE TOXICITY EFFECTS OF MWCNTs ON HUMAN LUNG CELLS

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Key words : *MTT, XTT, Apoptosis, MWCNTs, Cytotoxic Assay.*

Abstract - CNTs are bio-persistent and have the potential to induce severe inflammatory and fibrotic reactions. The published in-vitro studies have given a diverging data. Several studies demonstrated that MWCNTs are able to induce cytotoxic effects and Apoptosis in different cell types. Other studies indicated that there is a very low or no toxic effect. The reason for these discrepancies is not evident probably this may be due to different experimental protocols and interferences with test systems. Hence the present study has standardized the experimental protocol and used the same for all the experiments. Cell viability was analyzed with the MTT, XTT, Apoptosis assays. These tests were conducted after the incubation of MWCNT's in an aqueous solution of fetal bovine serum and in the absence of fetal bovine serum. The results indicated that the cells in serum free media were affected greatly by MWCNTs rather than serum containing media.

INTRODUCTION

Nanotechnology is the branch of science that will deal with making and use of materials, devices and systems in Nano scale, which possess 0.1 to 100 nm dimensions (1nm=10^{-9} m). This branch is now-a-days gaining lot of importance as Nanomaterials are being used widely in many industrial sectors for manufacturing different types of Nano based products. Nano materials can be synthesized by two different approaches i.e. "Bottom-up" and "Top-down". CNTs are finding wide range of applications; thereby the manufacturing companies are also increasing their production. More than 100 companies in the world today are manufacturing CNTs and this number may increase to more than 200 in the next five years. Currently, CNTs account for 28% market share of overall nanomaterial demand. Many scientists are concerned about the toxicity of the CNTs because of their structural resemblance to asbestos. Inhalation of asbestos fibers is known to induce asbestosis, lung cancer and malignant mesothelioma. The size, aspect ratio and surface charges are influencing the toxicity of asbestos and these are similar to CNTs. Particularly biological applications like drug delivery are getting much attention, as the concern for its potential hazards related to CNT exposure is still being debated. Careful evaluation of health effects is required before the revolution of nanotechnology. The primary route for the entry of CNTs is inhalation and lung is the target organ for the deposition and accumulation of CNTs. To explore the effect of CNTs on lung cells, we exposed different concentrations of MWCNTs on A549 lung cells. Pulmonary toxicity is of major concern as nano materials are very small and when airborne, a large fraction of these particles will be deposited within the alveolar region of the lungs. Hence the present study mainly focused on MWCNTs toxicity on lung cells to analyze the effects of MWCNTs on human lung cells by exposing them to various concentrations of MWCNTs.

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MATERIALS AND METHODS

For cytotoxicity assays cell culture density of 1×10^5 cells/ml was used. 100 µL of A549 cells were seeded in 96-well microplates. The microplates were incubated for 24 hours for the attachment of cells. Then, these wells were treated with 100 µL of MWCNTs dispersed solution at varied concentrations. For reference wells, only medium was added instead of MWCNTs solution. Cells, which are in log phase, were exposed to varied concentrations of MWCNTs, i.e. 6.25, 12.5, 25, 50, 100, 200, 400 µg/mL for 2, 4, 8, 16, 32 and 48 hr. After incubating for a specified exposure period the MTT, XTT and apoptotic assays were performed to know the viability of the cells. All these tests were also conducted in serum free media in order to understand the effect of serum on MWCNTs toxicity.

MTT assay

The cells were diluted to the required concentration (5×10^5 cell/mL) in complete medium and 100 µl of cell suspension was seeded into 95 wells of a 96-well tissue culture plates. In the remaining well only 100 µL of culture medium was added (blank). Cells were diluted in medium and were plated out and incubated fora period of 24 hr. The culture medium was replaced with complete medium containing the MWCNTs and incubated for 2, 4, 8, 16, 32 and 48 hrs.For each exposure period 6.25, 12.5, 25, 50, 100, 200, 400 µg/mL MWCNTs concentrations were added into 7 wells and a reference sample in the 8th well. This platewas incubated at 37°C for defined exposure period. 10 µL of MTT solution and 100 µL of DMSO solution was added to each well and agitated for 5 min in a plate shaker. The plates were read at 550 nm and 620 nm using scanning multiwell spectrophotometer. The optical density of each well sample was recorded and corrected with reference to the blank. Percentage of cell survival was calculated as: (absorbance of treated cells/ absorbance of control cells) × 100. The results recorded were the mean of three experimental determinations.Dose response relation was calculated for MWCNTs over the tested range of concentrations. This enables us to obtain IC_{50} values.

XIT Assay

A seeding density of 1×104 cells were seeded in 200 µL of medium per well of the microplate and the cells were allowed to adhere to the wells for an incubation period of 24 hr. 10 µL solution of MWCNTs with various concentrations, i.e. 6.25, 12.5, 25, 50, 100, 200, 400 µg/mL were added and incubated for 2, 4, 8, 16, 32 and 48 hrs. After the required time of incubation, 100 µL of XTT solution was added per well and incubated for 12hr. Optical density was measured at 500 nm, with a reference wavelength of 650 nmand optical density was plotted against the concentration of MWCNTs.

Apoptosis Assay

Apoptosis was examined by 4, 6-diamidino-2-phenylindole di-hydro-chloride (DAPI) staining coupled to fluorescence microscope was used to count the cell number. Cells were seeded into microplate reader and were exposed for 2, 4, 8, 16, 32 and 48hr to 100, 200, 400 µg/mL of MWCNTs or suspension medium alone. At the end of stimulation, the cells were fixed with 4 % paraformaldehyde in PBS for 25 min at room temperature. DAPI solution (1 µg/ml) was added for 5 min at 37°C. The cells were observed under fluorescence microscope.

Optical Microscopy

Optical microscopy was performed on cells exposed for 24 and 48 hours to 400 µg/mL MWCNT's. At the end of the stimulation cells were fixed in acetone and strained with Harris haematoxylin. The stained cells were observed under microscope.

Statistical Analysis

The MTT, XTT and apoptosis results were analysed using statistica software and the results were given as the means ± S.E.M. The data was analysed by simple regression. The significance for all measurements was accepted at $p<0.05$.

RESULTS AND DISCUSSIONS

MTT Assay Results

Cytotoxicity was evaluated by MTT assay performed on human lung epithelial cells after exposure to MWCNTs. MWCNTs were dispersed in PBS, ethanol and sterile water. For the MTT assay, MWCNT effects on A549 lung epithelial cell viability were different for 3 dispersion media.

In this assay, dark blue formazan crystals will be accumulated inside living cells after their

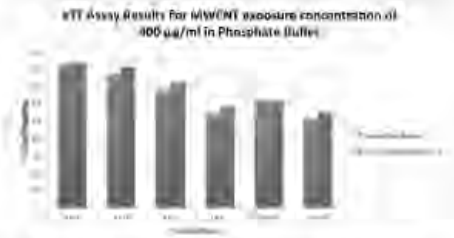

Fig. 1 XTT Assay Results for MWCNT exposure concentration of 400 µg/mL in Phosphate Buffer

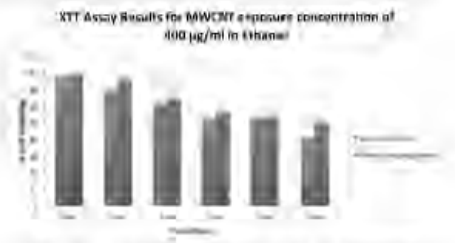

Fig. 2 XTT Assay Results for MWCNT exposure concentration of 400 µg/mL in Ethanol

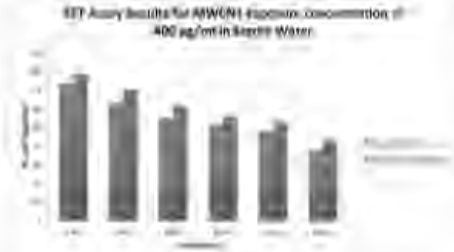

Fig. 3 XTT Assay Results for MWCNT exposure concentration of 400 µg/ml in Sterile Water

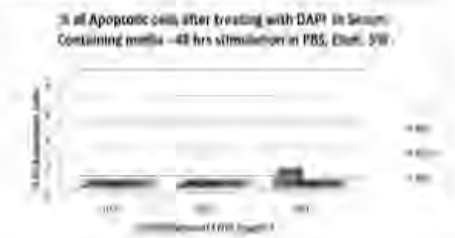

Fig. 4 % of Apoptotic cells after treating with DAPI in Serum containing media- 48 hrs stimulation in PBS, EtoH, SW.

Fig. 5 % of Apoptotic cells after treating with DAPI in Serum free media - 48 hrs stimulation in PBS, EtoH, SW.

treatment with MTT. Addition of DMSO will destruct the cell membrane, which will result in the liberation and solubilization of the crystals. The viable cell number is directly proportional to the quantity of formazan product proportional to the quantity of formazan product formed. The formazan is quantified by measuring absorbance at 570 nm. A linear relationship was established between cell no's and optical density of exposed A549 cells, and control cells. This allows for an accurate quantification of changes in the rate of cell proliferation.

The results presented in Table 1,2,3 reveals that exposure to MWCNTs, the no. of viable cells decreases as a function of MWCNT dosage for all tested concentrations. No change in MTT was observed when cells were incubated upto 50 µg/mL concentration. MTT values decreased significantly from 100 µg/mL to 400 µg/mL. This decrease is upto 50% for 200 µg/mL and 400 µg/mL at 48 hr post exposure. The proteins present in Fetal bovine serum will probably mask the surface of the MWCNTs making them less toxic. The MTT values indicate that dispersion in PBS media is more toxic as shown in tables. CNTs are known to interfere with the MTT assay at high concentrations but the interference was minimal for the highest studied concentrations.

XTT Assay Results

For the XTT assay, MWCNTs were dispersed in PBS, ethanol and sterile water. MWCNT effects on A549 lung epithelial cell viability was different for all three dispersion media. The XTT values indicate

Table 1. % of cell viability of A549 lung cells in serum containing and serum free media after exposure to various concentrations of MWCNTs dispersed in Phosphate Buffer Saline for different durations.

Exposure	Duration in hours											
	2		4		8		16		32		48	
Concentration in µg/mL	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media
6.25	99.33	99.55	98.41	99.10	91.62	98.42	85.42	89.92	80.97	82.60	75.35	78.97
12.5	98.21	98.45	96.38	98.20	87.93	97.29	83.16	86.39	74.55	79.53	72.27	78.27
25	95.76	96.46	95.24	95.28	85.22	95.49	79.64	82.36	73.52	76.72	68.72	75.70
50	93.09	93.58	89.59	90.56	81.77	92.79	75.87	78.33	71.97	74.93	61.84	69.15
100	89.53	92.47	84.38	86.74	80.78	91.21	72.86	76.07	68.89	73.14	60.42	66.12
200	83.51	82.30	74.20	84.04	77.09	89.18	67.83	74.30	66.58	69.05	54.26	62.61
400	77.72	78.98	72.17	75.73	73.39	83.78	66.83	68.01	60.92	61.38	50.47	55.60

Table 2. % of cell viability of A549 lung cells in serum containing and serum free media after exposure to various concentrations of MWCNTs dispersed in Ethanol for different durations.

Exposure	Duration in hours											
	2		4		8		16		32		48	
Concentration in µg/mL	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media
6.25	98.40	99.10	94.02	97.05	87.47	95.03	80.60	87.93	73.05	77.43	72.98	77.23
12.5	96.57	98.42	90.76	91.15	82.74	93.38	78.33	85.92	70.00	74.35	69.90	74.41
25	93.60	95.05	87.50	90.24	80.85	90.54	74.81	81.40	68.33	73.33	59.24	70.89
50	89.04	90.78	86.41	87.30	79.66	83.21	65.49	78.39	65.27	67.69	57.58	68.07
100	86.98	88.98	83.96	83.44	78.72	79.19	64.23	75.37	61.92	63.58	56.39	62.67
200	82.42	82.02	74.18	80.72	76.59	73.52	62.21	73.36	56.66	57.94	52.60	59.38
400	75.79	77.52	70.38	76.41	73.28	70.21	57.43	60.30	52.77	53.84	48.57	52.11

Table 3. % of cell viability of A549 lung cells in serum containing and serum free media after exposure to various concentrations of MWCNTs dispersed in sterile water for different durations.

Exposure	Duration in hours											
	2		4		8		16		32		48	
Concentration in µg/mL	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media	Serum Free Media	Serum Containing Media
6.25	98.24	98.70	92.60	93.34	85.32	94.20	78.88	86.38	71.98	75.39	70.67	70.58
12.5	92.77	92.87	87.94	88.24	83.83	85.99	75.06	79.95	68.06	67.27	56.97	66.88
25	85.99	86.82	85.75	86.47	80.59	84.78	67.93	76.98	64.70	65.70	53.60	64.48
50	83.80	86.17	79.17	83.14	77.36	81.15	64.37	75.95	61.62	64.92	51.68	58.16
100	80.52	82.93	73.69	78.71	75.12	77.77	60.05	74.75	60.22	62.82	49.51	55.77
200	76.14	77.75	69.58	75.38	72.88	71.25	57.25	72.52	56.30	56.54	46.15	54.24
400	72.64	73.00	66.84	72.06	70.89	64.73	53.43	57.67	47.61	52.61	40.86	43.57

that dispersion in PBS media is more toxic as compared to other dispersion media. XTT values shown in Figures 1, 2 & 3 decreased significantly with 100 µg/mL to 400 µg/mL. This decrease is upto 50% for 200 µg/mL and 400 µg/mL at 48 hrs post exposure. No change in XTT was observed when cells were incubated upto 50 µg/ml concentration. The same experiments were conducted in serum containing and serum free media. After the incubation of MWCNT's in an aqueous solution of fetal bovine serum, and in the absence of fetal bovine serum the results indicated that the cells in serum free media were effected by MWCNTs rather than serum containing media. The proteins present in Fetal bovine serum will probably mask the surface the MWCNTs making them less toxic.

Apoptosis Assay Results

The MTT and XTT assays reveal the impaired mitochondrial metabolism. This impaired mitochondrial metabolism might result in decrease in cell number after incubation with MWCNTs. This was evaluated using DAPI staining. The percentage of apoptotic cells in MWCNT exposed A549 epithelial cells was higher after 48 hours of exposure period. This decrease is more significant in PBS dispersion media. This apoptosis may be due to the increased levels of reactive oxygen species in concentration dependent manner. Reactive Oxygen Species induction and decreased mitochondrial activity might have affected the cell morphology of MWCNT treated cells.

CONCLUSION

The short term cell viability studies conducted in the laboratory for exposure assessment of MWCNTs indicate that mitochondrial activity was effected in the cells which were stimulated with various concentrations of MWCNTs. Potential health hazard caused by the inhalation of MWCNTs is oxidative stress of lung epithelial cells.Even at higher concentration, MWCNTs have not induced major acute toxicity in A549 lung epithelial cells.

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---End of published papers---

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- "Trends of Noise Levels of A Developing City", Jr. of Pollution Research, (ISSN: 0257-8050), Vol. 31, No.1; PP 51 - 56 (2012).
- "Spatial Variation of Air Quality in Visakhapatnam City", Environmental Pollution Control Jr. (ISSN 0972- 1541) Vol.11 No.; Jul - Aug 2008; pp 51 - 53.
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---End of Scholar Profile---