GENOTOXICITY AND HEPATOTOXICITY OF MULTI-WALLED CARBON NANOTUBES IN *Mus musculus*: ASSESSMENT AND NATURAL DETOXIFICATION

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**GENOTOXICITY AND HEPATOTOXICITY OF MULTI-WALLED CARBON NANOTUBES IN *Mus musculus*: ASSESSMENT AND NATURAL DETOXIFICATION**" is based on the results of investigations carried out by me in the **Zoology discipline** at the **School of Biological Sciences and Biotechnology**, **Goa University**, under the Supervision of **Dr. Avelyno D'Costa**, and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "**GENOTOXICITY AND HEPATOTOXICITY OF MULTI-WALLED CARBON NANOTUBES IN *Mus musculus*: ASSESSMENT AND NATURAL DETOXIFICATION**" is a bonafide work carried out by **Ms. Riddhi Ramdas Savoiverekar** under my supervision in partial fulfilment of the requirements for the award of the degree of **Masters of Science** in the **Zoology Discipline** at the **School of Biological Sciences and Biotechnology**, **Goa University**.

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CONTENTS

PREFACE

Carbon nanotubes are promising materials with various applications in biomedicine. These nanotubes have unique physicochemical properties that make them attractive for drug delivery, tissue repair, semiconductors, aerospace, sewage disposal, diagnostics etc. However, concerns have been raised about their potential toxicity, particularly in the liver and lungs. It is crucial to investigate the genotoxicity and hepatotoxicity of multi-walled carbon nanotubes in order to understand their potential adverse effects and develop appropriate safety measures. The toxicity of Multi-walled carbon nanotubes in organisms, specifically in Swiss albino mice, is an important field of study to assess the potential risks associated with their use. Detoxification via natural methods has not been precisely studied, with respect to the toxicity caused by MWCNTs.

The following dissertation report discusses the potential effects of MWCNTs on liver and DNA damage, and possible mechanisms for natural detoxification in Swiss albino strain of *Mus musculus*. The report is divided into 5 chapters. The first chapter is an introduction that discusses the properties, sources and applications of carbon nanotubes, specifically about MWCNTs. It also mentions the background of toxicity problems caused by MWCNTs, along with the lacunae, objectives and significance of the study.

The second chapter is a review of literature presented on the general toxicity of MWCNTs, along with mentions about specific studies carried out on the hepatotoxicity and genotoxicity of MWCNTs and other methods of detoxification studied.

The third chapter focuses on the materials and methods that mentions the experimental setup and analysis of morphological and behavioral changes induced by sub-acute exposure to two doses of

MWCNTs. It also provides the methods used to estimate various biochemical molecules, enzymes and antioxidants, along with genotoxicity test procedures.

The fourth chapter is results, where the effect of exposure of different doses of MWCNTs is seen on different parameters, and analyzed using statistical significance.

The fifth chapter is a discussion that provides an insight on the possible reasons for hepatotoxicity and genotoxicity with reference to other literature.

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LIST OF GRAPHS

-

-

LIST OF TABLES

-

-

LIST OF FIGURES

-

LIST OF PLATES

-

LIST OF ABBREVIATIONS

TERM	ABBREVIATION
Nanoparticles	NPs
Carbon nanotubes	CN
Multi walled carbon nanotubes	MWCNTs
Single walled carbon nanotubes	SWCNTs
Lithium	Li
Reactive oxygen species	ROS
Deoxyribonucleic acid	DNA
National Institute for Occupational Safety and Health	NIOSH
Carbon nanotubes and nanofibers	CNT/F
Vascular endothelial growth factor	VEGF
Oxidized multiwalled carbon nanotubes	O-MWCNT
Tween-80 dispersed multiwalled carbon nanotubes	T-MWCNT
Protein kinase B	AKT
Micronucleus	MN
Carbon nanoblack	CB

Alanine transaminase	ALT
Alkaline phosphatase	ALP
Aspartate aminotransferase	AST
Non-alcoholic fatty liver disease	NAFLD
Lead acetate	PbAc
Activated protein kinase	AMPK
Simvastatin	SD
1,2-dioleoyl-sn-glycero-3-phosphocholine	DOPC
Lactate dehydrogenase	LDH
Caffeic acid	CA
Malondialdehyde	MDA
Committee for control and supervision on experiment on animals	CPCSEA
Sodium chloride	NaCl
Ethylenediaminetetraacetic acid	EDTA
Sodium hyrdoxide	NaOH
Magnesium chloride	$MgCl_2$
Phosphate buffer	PB
Phosphate buffer saline	PBS
Hydrochloric acid	HCl
2,4-dinitrophenyl hydrazine	DNPH
Hydrogen peroxide	H_2O_2

Thiobarbituric acid reactive substances	TBARS
Thiobarbituric acid	TBA
Trichloroacetic acid	TCA
Superoxide dismutase	SOD
Nitroblue tetrazolium	NBT
Double distilled water	DDW
Reduced glutathione	GSH
5,5’-dithiobis-(2-nitrobenzoic acid)	DTNB
Potassium chloride	KCl
Monosodium phosphate	NaH_2PO_4
Monopotassium phosphate	KH_2PO_4
Dimethyl sulfoxide	DMSO

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ABSTRACT

This study aimed to evaluate the genotoxic and hepatotoxic effects of MWCNTs in Swiss Albino mice. 12 Male Swiss Albino mice were orally gavaged with low (50 mg/kg of body weight) and high (100 mg/kg of body weight) doses of MWCNTs, for an exposure period of 14 days .Morphological and behavioral analysis was done using general standard observations, wherein dose-dependent decrease in body weight, increase of liver organ index and behaviors such as drowsiness, lethargy, isolation and abnormal feeding frequencies were observed. Hepatotoxicity was evaluated by measuring dose-dependent decrease in concentrations of total proteins, increasing levels of carbohydrates, decrease in levels of free sugars and decrease in albumin levels. Different liver function markers were also estimated, and there were noted dose-dependent decrease in levels of catalase and increase in levels of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD). Genotoxicity did not occur. Natural detoxification was studied, by studying the same parameters, after an additional period of 7 days without exposure to the toxicant. Significant detoxification of liver biomolecules was observed.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

1.1 CARBON NANOTUBES

CNTs, short for carbon nanotubes, have garnered significant attention in the scientific community due to their remarkable physio-chemical, electrical, mechanical and thermal properties. Carbon nanotubes represent one of the fastest developing nanoparticle materials with production set to increase rapidly as a consequence of the useful properties of this material (Donaldson et al. 2006). These unique characteristics have sparked extensive research aimed at exploring their potential applications in various fields, such as semiconductors, solar cells, and optical instruments, that has an immense potential to improve consumer and industrial products (Karousis et al. 2010).

Carbon nanotubes are highly promising materials, especially in the field of electronics, energy storage, aerospace, sewage disposal, water purification, disinfection and biomedical industries. Their large surface area to volume ratio and the ability to functionalize their surfaces make carbon nanotubes versatile for various functions such as drug delivery, sensing applications, and reinforcement in composites materials. The controllable synthesis and mass production of carbon nanotubes with desired properties is crucial for their widespread application in these fields (Hanim et al. 2022).

By employing combinatorial methodologies, the discovery and optimization of carbon nanomaterials and catalyst formulations can be accelerated, leading to advancements in nanoscale science and technology. In recent years, there has been increasing interest in the use of carbon nanotubes in various biomedical applications such as drug delivery systems, imaging techniques, and tissue engineering (Huang et al. 2011).

Single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are two types of carbon allotrope nanomaterials that consist of one or multiple layers of graphene sheets, in the form of tubes.

1.2 MULTIWALLED CARBON NANOTUBES (MWCNTs)

Multi-walled nanotubes (MWNTs) consist of multiple rolled layers (concentric tubes) of graphene. The diameters of MWNT are typically in the range of 5-50 nm. The interlayer distance in MWNT is close to the distance between graphene layers in graphite. MWNT is easier to produce in high volume quantities than SWNT. However, the structure of WNT is less well understood because of its greater complexity and variety. Regions of structural imperfection may diminish its desirable material properties (Ganji et al. 2015).

Even relatively small amounts of MWCNTs are mixed into normally insulating materials, the materials can gain significant conductivity. Applications include things like conductive latex gloves for touchscreen use or antistatic conveyor rollers, which can dissipate the static charge. While traditional conductive additives such as carbon black can also impart conductivity, the length-to-diameter ratio of multi wall carbon nanotubes makes it possible to achieve that at much smaller amounts, because the long tubes can contact each other over longer distances and form a conductive network through the material (Flahaut et al. 2003).

MWCNTs also have high thermal conductivity and can be used in normally insulative materials to increase their ability to transmit heat. This can be useful in applications where heat needs to be dissipated, such as in electronics. In cases where the use of metals is prohibited due to unwanted stresses or chemical instability, thermally conductive ceramics can be advantageous. Again,

SWCNTs can achieve a similar effect to traditional conductive additives but at lower dosages, minimizing the possible negative effect on other properties of materials.

Added into materials, carbon nanotubes can improve various mechanical properties. In tension, MWCNTs are stronger than steel, with a tensile strength of 10 to 50 GPa compared to 0.5 GPa for mild steel. When MWCNTs are used as an additive, even a small fraction inside a material can significantly strengthen it. SWCNTs are even more impressive, with a tensile strength of 50 to 100 GPa.

1.3 SOURCES AND APPLICATIONS OF MWCNTs

There exists several practical applications of CNTs, that includes its usage in enhancing properties of other materials. MWCNTs increase electrical conductivity of materials such as plastic. This property allows it to be used as additives in conducting coatings, conductive ink for printing and creating transparent, conductive films for touchscreens (Piao et al. 2011).

MWCNTs are also used in vehicle manufacturing, to produce lower weight materials with higher strength, for eg. Carbon-fiber bicycle parts, sporting goods such as tennis racquets and golf clubs. Combination of properties of MWCNTs and SWCNTs results in production of silicon anodes for Li-ion batteries, which makes them resilient. For these purposes, SWCNTs are better suited.

1.4 BACKGROUND OF THE PROBLEM

Due to its numerous applications in material science, there are various forms in which these nanoparticles can enter the environment. From industrial waste disposal, hospital disposal and other waste sources, these nanotubes enter into the aquatic bodies and soil, thus migrating into

surface and groundwater. These environmental toxicants further reach into the human body, thus inducing toxicity (Petersen et al. 2011). The mobility and absorptive capacity of these nanoparticles, allow them to act as vectors of other pollutants into the environment. The biological interactions of nanotubes are not well understood, and the field is open to continued toxicological studies.

Although it is widely believed that various properties influence the toxicity of CNTs, the precise mechanisms responsible for their potential toxicity have yet to be fully elucidated. It is important to evaluate their toxic effects as they may directly or indirectly affect many organs.

Carbon nanotubes have adverse effects on human health and the environment that needs to be focused on before their widespread use, and thus should also undergo testing, to ensure safety and reduce risks from exposure.

1.5 TOXICITY OF MWCNTs

Toxicological profile of MWCNTs is important to assess its potential health and environmental impacts. MWCNTs persist in biological tissues (Kasai et al. 2015). MWCNTs agglomerate due to weak Van der Waals force that affects its dispersion and stability, crucial for understanding the potential risks associated with MWCNT exposure (Zulkifli et al. 2022). Carboxylated MWCNTs accumulate in macrophages that help in assessing its interaction with biological systems (Huynh et al. 2021). Modified MWCNTs have potential biocompatibility, due to its functionalization (Dhekale et al. 2021).

SWCNTs easily penetrate the blood-brain barrier via endocytosis, which results in signs of inflammation, apoptosis and oxidative stress (Bardi et al. 2013). On exposure to MWCNTs, a

similar effect is seen, that includes increase in neuroinflammatory cytokines and activation of glial cells (Kafa et al. 2015). Van et al., (2013) stated that kidneys are at higher risk of toxicity. A study on human embryo kidney cells, exposed to SWCNTs, showed increased levels of cellular apoptosis and hindrance of the cell cycle (Cui et al. 2005). On exposure to MWCNTs, there was an increase in levels of interleukin 6 and 8, DNA damage and mitochondrial damage (Tang et al. 2012).

Garibaldi et al., (2006) in their study informed that exposure to SWCNTs resulted in cardiac cell proliferation, muscle damage, low blood flow, vascular atherosclerosis, decrease in blood pressure and heart rate, due to oxidative stress and inflammation. Similar studies were seen in MWCNTs exposure (Ibrahim et al. 2006).

1.6 LACUNAE OF THE STUDY

There exists a lack of long-term toxicity studies (sub-acute and sub-chronic) of multi-walled carbon nanotubes (MWCNTs). Precise in-vivo studies and bioaccumulation of MWCNTs in mice, is yet to be fully elucidated. Natural detoxification of MWCNTs over 14 days has not been extensively studied.

1.7 HYPOTHESES

Multi-walled carbon nanotubes induce genotoxic and hepatotoxic effects in mice.

Multi-walled carbon nanotubes undergo natural detoxification in the bodies of mice.

1.8 OBJECTIVES OF THE STUDY

1. To analyze the morphological and behavioral changes induced by multi-walled carbon nanotubes (MWCNTs) in *Mus musculus*.

2. To evaluate genotoxicity and hepatotoxicity of MWCNTs in *Mus musculus*.

3. To understand the natural detoxification of MWCNTs.

1.9 SCOPE OF THE STUDY

A better understanding of the toxicity profile of CNTs in model organisms is of significant importance to improve their biological safety in humans and in the environment. It also facilitates their wide biological application and for the successful commercial application. Due to the lack of long-term toxicity studies of MWCNTs in mice , the ability to comprehend later effects are hindered. The study of genotoxic and hepatotoxic effects of MWCNTs can be used to understand the lethality of these nanoparticles, especially by corresponding it with the expected effects in humans. The detoxification of MW-CNTs has not been touched upon precisely and thus requires further research.

Since most of the nanotoxicology studies were focused on in vitro models and only few research groups have dealt with in vivo systems. Toxicology studies with in vivo systems carry greater significance pertaining to their diversity in physiology and anatomy (Balakrishna et al. 2011).

CHAPTER 2: LITERATURE REVIEW

2. LITERATURE REVIEW

Rapid development of nanotechnology has led multi-walled carbon nano-tubes (MWCNTs) to emerge as a highly promising material due to their distinct physical, chemical, and mechanical properties. However, despite their impressive characteristics, the potential toxicity of MWCNTs has become a major focus of interest. The toxicity of MWCNTs is a topic of extensive research due to their growing utilization in various consumer goods and industrial processes. While MWCNTs have diverse applications like drug delivery, tissue engineering, and electronics, the possible negative impacts on human health and the environment need to be understood and addressed. Research on the toxicity of MWCNTs covers a wide range of studies, including in vitro cellular experiments, animal models, epidemiological studies, and environmental assessments. These studies seek to elucidate the effects of MWCNTs exposure on cellular responses, organ functions, the immune system, genotoxicity, and the potential to cause cancer. A thorough review of the existing literature on MWCNT toxicity is essential for identifying knowledge gaps, significant concerns, and future research goals in this area. The purpose of the review is to improve understanding of the dangers associated with MWCNT exposure through a thorough literature review, as well as to offer techniques for reducing these risks of this nanoparticle.

2.1 GENERAL TOXICITY STUDIES

The pulmonary toxicity of single-walled and multi-walled carbon nanotubes delivered at high doses and dose rates to the lower respiratory tract of rats and mice induced a high acute inflammatory response with granuloma formation and fibrosis as late effects (Warheit et al. 2004).

Monteiro-Riviere et al. (2005) found multi-walled carbon nanotubes (MWCNT) in the cytoplasmic vacuoles of human epidermal keratocytes in vitro (up to 3.6 μm long), a decrease in cell viability and a significant increase in an inflammation marker (interleukin8).

Deng et al. (2008) investigated the toxicity of water-soluble multi-walled carbon nanotubes (S-MWCNTs) on mouse spleens. The researchers investigated carbon clearance, oxidative stress, histology, and electron microscopy of the spleen. Over the course of two months, phagocytic activity and levels of glutathione, superoxide dismutase, and malondialdehyde were stable. However, S-MWCNTs collected in the spleen and moved from the red to the white pulp, potentially triggering an adaptive immune response.

Numerous studies have shown that long time inhalation of CNTs result in persistent inflammation, fibrosis, development of lung cancer, respiratory tumors and persistent inflammation in their lungs (Han et al. 2010).

Exposure to NPs induce DNA breaks, micronuclei formation and lung mutations, in acute and chronic exposures. Some studies also suggest a link between ROS production and DNA damage. Increased levels of lung carcinomas, especially bronchoalveolar carcinoma and combined carcinoma and adenoma, is seen in long term exposures. Some studies also suggested that MWCNTs act as promoters of lung cancer (Wang et al. 2011).

There are no clinical signs of dermal toxicity or formation of lesions in Wistar rats and New Zealand rabbits, upon application of MWCNT1 and 2. Yet, they exhibited conjunctival redness, chemosis and discharge, which recovered after 5 days (Balakrishna et al. 2011).

SWCNTs exhibit oxidative stress and cell inflammation of dermal tissue (Seaton et al., 2010). 3 weeks exposure to MWCNT resulted in granuloma formation in the subcutaneous tissues of mice (Koyama et al., 2006). Adhesion and migration of cells is also affected due to MWCNTs exposure (Murray et al., 2009). Wound healing properties of dermal fibroblasts is reduced (Stadelmann et al., 1998).

The length of CNT fibers affect the inflammation, that is, longer fibers result in more permanent inflammation. Bronchiolar and alveolar epithelial cells proliferate following exposure to CNTs (Porter et al. 2013).

Knudsen et al. (2019) conducted a study on the pulmonary toxicity of Multi-walled carbon nanotubes (MWCNT) by evaluating histological changes in lung tissue of female C57BL/6N BomTac mice exposed to 11 types of MWCNT. The study used a dose equivalent to three times the NIOSH exposure limit and found that thin and entangled MWCNT caused pulmonary inflammation, while thicker ones did not. Analysis showed that larger diameter and higher iron content predicted less histopathological changes, while higher cobalt content predicted more. No fibrosis or tumors were found, but genotoxicity in the liver was observed in one type of MWCNT. The study suggests that the physicochemical properties of MWCNT play a crucial role in inducing long-term pulmonary changes, with diameter size and cobalt content identified as key factors in toxicity.

Fraser et al. (2021) highlighted the limited number of comparative studies on MWCNTs and nanofibers, particularly those with larger diameters, leading to uncertainty regarding the toxicity of various CNT/F types and their associated histopathological effects. Exposure of 40 µg of nine CNT/F variants to Male C57BL/6 mice with diameters ranging from 6 to 150 nm resulted in the

induction of histopathological changes, with varying degrees of severity observed, linked to physical dimensions and agglomeration patterns of the materials.

In a comprehensive analysis conducted by Chetyrikina et al. (2022) on the potential toxicity of carbon nanotubes in in-vitro studies, focusing on their interactions with biological systems, stated that there is a need for standardizing testing materials and methodologies in routine laboratory procedures to assess the safety and potential toxic effects of materials. Parameters like the diameter, length, purification process, and synthesis of carbon nanotubes can significantly impact their toxicity levels.

MWCNTs disrupted endothelial cell activity in a concentration-dependent manner, affecting functions like tube formation and cell migration. Angiogenesis was significantly reduced in MWCNT-treated mice, and VEGF expression was found to be decreased in the MWCNT group. Protein chip analysis indicated a decrease in AKT and eNOS expression, which was alleviated by VEGF treatment. The study concluded that MWCNTs influence angiogenesis through the VEGF-Akt-eNOS axis, and VEGF treatment can rescue endothelial cell function (Dai et al. 2022).

Solorio Rodriguez et al. (2023) conducted a study comparing the effects of SWCNTs and MWCNTs on pulmonary toxicity in mice. They found that MWCNTs were more genotoxic than SWCNTs and identified a potentially fibrogenic SWCNT that requires further testing.

2.2 HEPATOTOXICITY STUDIES

Ji et al. (2009) conducted a study on the hepatotoxicity of two types of multi-walled carbon nanotubes (MWCNTs) in Kunming mice. The findings showed that mice exposed to MWCNTs

exhibited reduced body-weight gain, elevated levels of certain markers of liver damage, and significant liver damage, with the Tween-80-dispersed MWCNTs causing more severe damage compared to the acid-oxidized MWCNTs. The study also revealed alterations in gene expressions related to liver function, metabolism, and immune response in the MWCNT-exposed groups, indicating that high doses of Tween-80-dispersed MWCNTs can be hepatotoxic in mice, while acid-oxidized MWCNTs seem to be less harmful.

In another study by Zhang et al. (2018), exposure of adult mice to MWCNTs significantly reduced the weight of offspring mice and caused liver tissue damage. The expression of genes related to liver fat synthesis was significantly increased, and inflammatory reactions were observed. This exposure led to disruption of liver function and accumulation of lipid droplets in hepatocytes in the offspring mice, possibly due to an imbalance in liver macrophage phenotypes.

Shah et al. (2021) conducted a study on the toxicity of carbon nanotubes (CNTs), graphene-family nanomaterials, and fullerenes, revealing their harmful effects on liver and spleen tissues by breaching blood barriers. Factors such as concentration, functionalization, and dimensions influence their toxicity, impacting various human body systems. The inhalation of multi-walled carbon nanotubes (MWCNT) in mice, particularly after exposure to DNA-damaging agents, increased the risk of lung tumors, highlighting their carcinogenic potential.

2.3 GENOTOXICITY STUDIES

In a study conducted by Szendi et al. (2008), it was observed that there exists an insufficiency of data on the genotoxicity of CNTs. It was observed that there was no mutagenicity of statistical significance in the Ames test, micronucleus test (MN) and cell kinetics between control and exposed groups indicating the nonexistence of genotoxic effects.

According to study conducted by Georgia et al. (2011), both CNTs and CB are confirmed to be cytotoxic and genotoxic to RAW 264.7 mouse macrophages, causing ROS release, necrosis, chromosomal aberrations, ultra structural damage, and apoptosis. The harmful effects of CNTs are influenced by factors such as purity, fiber length, and degree of aggregation-dispersion, making it challenging to design a comprehensive model to explain CNT toxicity.

In this study, the effects of carboxylated functionalized multi-walled carbon nanotubes on hepatotoxicity and oxidative stress biomarkers in mice were examined via intraperitoneal injection of different doses for five days. Results demonstrated reduced body-weight gain, increased ROS, elevated serum ALT/AST and ALP levels, higher lipid hydroperoxide levels, and significant liver tissue morphological changes in exposed mice, indicating potential hepatotoxicity induction through oxidative stress mechanisms, necessitating further animal exposure studies (Patlolla et al. 2011).

Awasthi et al. (2015) conducted a research study exploring the harmful effects of smaller-sized Ag NPs and MWCNTs on liver cells of mice through oral administration for 28 days, resulting in increased DNA damage, indicating cytotoxicity and genotoxicity, thus harming cellular components.

Fang et al. (2018) investigated the toxic effects of nanoparticles (NP), pristine MWCNTs, and a combination of MWCNTs and NP in male mice. The study assessed oxidative stress levels, genotoxicity, and mitochondrial damage in the liver and sperm of the mice after exposure. The results indicated that high doses of MWCNTs, particularly when combined with NP, induced oxidative damage in the liver and DNA damage in sperm, highlighting the importance of assessing the toxicological effects of MWCNTs.

Liu et al. (2020) examined the hepatotoxic effects of simultaneous exposure to MWCNTs and lead ions in individuals with NAFLD. The combined exposure resulted in liver dysfunction, exacerbation of NAFLD-related conditions, apoptosis in liver cells, lipid peroxidation, compromised antioxidant defense, and increased inflammation in NAFLD mice, potentially through AMPK/PPARγ pathway inhibition.

According to Horibata et al. (2022), MWCNT-7 has been categorized as Group 2B by The International Agency for Research on Cancer due to its inflammation-producing properties. Genotoxicity was investigated through erythrocyte micronucleus assay following inhalation and intratracheal exposure. The findings revealed a significant rise in micronucleated reticulocytes, showing a potential genotoxicity of MWCNT-7. Lung cells were isolated, treated with MWNT-7, and an increase in micronucleated cells was observed, indicating DNA damage.

2.4 DETOXIFICATION STUDIES

Due to the body's natural defense mechanism to eliminate toxic substances, there is a potential for natural detoxification of these NPs. These include metabolism, excretion and detoxifying enzymes (Lam et al. 2003). Understanding this natural detoxification allows us to assess safety guidelines. Evaluating the detoxification techniques allows understanding ways to reduce harmful effects of MWCNTs (Mamidi, 2019). Further research needs to be analyzed and effective strategies need to be developed for safe handling and use of these NPs (Martin et al. 2011).

Liu et al. (2008) discussed the methods to eliminate bioavailable iron and nickel from commercial carbon nanotubes, aiming to reduce their toxicity. The study emphasizes the importance of comprehending and regulating the presence of bioavailable metals in nanotube

samples to ensure their safe utilization. Various purification techniques, including acid treatment and oxidation, are examined to decrease the bioavailability of metals in carbon nanotubes.

In murine studies, the MWCNTs are observed to penetrate the subpleural tissue upon inhalation, and persist in the tissues (Ryman-Rasmussen et al. 2009). This distribution highlights the need to understand clearance mechanisms through natural detoxification in the body, which could be further correlated with that of the human body systems. Serum-borne bioactivity caused due to pulmonary exposure, induces neuroinflammation via blood-brain barrier (Aragon et al. 2017).

Jafar et al. (2016) proposed various methods to reduce carbon nanotube (CNT) toxicity, such as understanding CNT properties, enzymatic degradation using horseradish peroxidase, and a novel detoxification approach involving coating CNTs with recombinant globular heads. These strategies aim to enhance the safety of CNTs for drug delivery applications, but further research is needed to evaluate their effectiveness and explore additional techniques like chemical functionalization and employing a "shielding strategy" with PEGylated CNTs for controlled degradation and reduced inflammatory responses.

Simvastatin (TD) offers therapeutic benefits for oMWCNT-induced damages. Factors like nanoparticle size, structure, and functional groups play a role in determining the toxicity risks associated with carbon nanotubes. By investigating the effects of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and TD on oMWCNT toxicity, the research aims to provide insights into mitigating the harmful impacts of these nanomaterials. The study indicates that while DOPC and TD at low doses do not affect oMWCNT distribution, they can

influence each other's metabolism and impact on erythrocytes, with TD showing promise in treating tissue damage caused by oMWCNTs (Qi et al. 2017).

Requardt et al. (2019) investigated the impact of surface defects on Multi-walled Carbon Nanotubes (MWCNTs) on their toxicity, suggesting that extracting iron from the MWCNT structure could mitigate harmful effects. The study evaluated the cytotoxicity of MWCNTs with and without iron catalyst in lung epithelial and hepatocyte cells, revealing decreased cell viability, compromised membrane integrity, and increased oxidative stress over time, particularly with heat-treated MWCNTs showing heightened adverse effects despite reduced surface defects.

Single-walled carbon nanotubes (SWCNTs) induced oxidative stress in pancreatic islets, but the effects of multi-walled carbon nanotubes (MWCNTs) remained unexplored. This study aimed to evaluate how MWCNTs affect islet oxidative stress and how caffeic acid (CA) as an antioxidant may offer protection, as demonstrated through various assays measuring ROS, MDA, antioxidant enzyme activities, and insulin secretion. The findings suggest that MWCNTs can induce oxidative stress in pancreatic islets, potentially contributing to diabetes, while CA treatment can counteract these effects by enhancing the antioxidant defense system in islets, underscoring the need for further research in this area (Ahangarpour et al. 2021).

These findings have highlighted the importance of investigating the natural detoxification pathways of MWCNTs in the body, to eliminate the adverse effects, and to get a brief idea about the complex processes involved in distribution, persistence and systemic effects. Further research is needed to elucidate the mechanisms underlying the clearance of MWCNTs (Kobayashi et al. 2017).

CHAPTER 3: MATERIALS AND METHODS

3. Materials and Methods

3.1 GLASSWARES

Various types of laboratory glassware such as test tubes, measuring cylinders, beakers, reagent bottles, centrifuge tubes etc. were issued and utilized. The glasswares were immersed in chromic acid overnight, followed by rinsing with distilled water and sterilization in a hot air oven prior to usage. All the prepared reagents were stored according to ambient conditions, and covered in aluminum foil during use.

3.2 INSTRUMENTATION

Different instruments such as Weighing balance (Wensar PGB 200), Water bath, pH meter (TOSHCON TMP3), Vortex (CM 101), Refrigerator, Deep freezer (-20°C), Cold Centrifuge (REMI R-24), Hot air oven (MIC-165), Light microscope (Olympus BX53), Colorimetric spectrophotometer, UV-visible spectrophotometer, Fluorescence microscope (ProgRes CF), Homogenizer, Hot plate, Magnetic stirrer, Sonicator, Incinerator, were available in the Zoology discipline, Goa University.

3.3 CHEMICALS

All chemicals that were acquired and utilized were of Analytical grade from Thermo-Fisher, Sigma-Aldrich, Hi-media, Merck etc. that were available in the Zoology discipline, Goa University.

3.4 PROCUREMENT OF NANOPARTICLES

5 grams of Multi-walled carbon nanotubes (MWCNTs) were acquired from AdNano Technologies (CAS No: 308068-56-6) (Fig 8.18) with the following specifications:

AD-MWCNT	DESCRIPTION
PURITY	≈ 99%
LENGTH	≈ 100 µm
AVERAGE DIAMETER	≈10-20 nm
BULK DENSITY	0.03g/cm³
PHYSICAL FORM	Fluffy powder
COLOUR	Black
SURFACE AREA	≈ 230 m2/g
CAS NO:	308068-56-6

3.5 EVALUATION OF MWCNT TOXICITY

3.5.1 Animal Model: Swiss Albino mouse (*Mus musculus*)

Phylum: Animalia

Class: Mammalia

Order: Rodentia

Family: Muridae

Subfamily: Murinae

Genus: Mus

Species: *Mus musculus* (Linnaeus, 1758)

Mice demonstrate considerable homology with humans and can be easily maintained under controlled laboratory conditions, thus making them a widely accepted experimental animal model. Laboratory mice, which are typically members of standardized inbred strains, are chosen for their stability of specific deleterious mutations. They serve as valuable model organisms in both genetic and medicinal investigations, due to their lifespan, low cost and easy availability. (Miller and Miller, 1975)

3.5.2 Procurement and maintenance of animals

18 healthy male Swiss albino mice (*Mus musculus*), aged 5 weeks with an average body weight ranging from 37-45 g, were procured from The National Institute of Biosciences in Pune, India. Prior to the commencement of experimentation, the essential ethical clearance was obtained from the Animal Ethics Committee of Goa University (Reference No: GUZ/IAEC/23-24/N4, dated 12/09/2023) for the utilization of these animals.

All 18 Swiss albino mice were housed at the animal house facility of the Department of Zoology, Goa University (Reg. No: 2104/G0/Re/S/20/CPCSEA dt. 10/08/2020). Following the CCSEA guidelines, the mice were acclimatized for 1 week, and separated in different polypropylene cages with stainless steel lid, according to weight and behavior, with adequate provision of

standard mice pellet food and tap water. Standard bedding of paddy husk was used and replaced twice to thrice a week. Ambient temperature of 21°C and 12h dark-light cycle was maintained. (CPCSEA guidelines).

3.5.3 Experimental set-up

The mice were assigned in 3 different groups, comprising 6 individuals in each group. The groups included 1 control and 2 experimental groups of different dosages (Fig 8.21). The doses were selected as per Awasthi et al. (2013). Experimental-1 dose of MWCNTs was prepared by sonicating 232.2mg of MWCNTs in 60ml of 0.9% NaCl saline. Experimental-2 dose of MWCNTs was prepared by sonicating 435.6 mg of MWCNTs in 60ml of 0.9% NaCl saline (Fig 8.20). Prior to dosage, the mice were fasted for 3-4 hours and weighed. Suspension of 2 concentrations of MWCNTs (50 mg/kg of body weight for experimental group 1 and 100 mg/kg of body weight for experimental group 2) was orally gavaged to the two experimental groups, using a suitable feeding tube, once a day, around the same time, for a period of 14 days. The control group was provided with distilled water.

3.5.4 General examination

Over a duration of 14 days, the mice underwent regular observations to assess their survival rate, behavior, motor functions, and any morphological alterations in terms of fur quality and color, skin texture and color, tail spots etc. The body weight was measured once in 7 days to analyze the effect of the nanoparticle.

3.5.6 Behavior

Any possible behavioral modifications such as lethargy, drowsiness, isolation, and abnormal feeding patterns were observed and noted over a 14-day monitoring period (Subakanmani 2015).

3.6 COLLECTION OF TISSUES

After a 14-day exposure period, the mice were euthanized using cervical dislocation, wherein pressure was exerted on the neck to dislocate the spinal column from the skull. Subsequently, blood samples were obtained via cardiac puncture via 1 ml EDTA washed syringe and transferred to microfuge tubes washed with EDTA, for serum collection (Diehl et al. 2001) Following this, the general viscera were exposed, and the liver tissue was excised and rinsed with 0.9% NaCl saline solution to eliminate any remaining blood residues (Baratta et al. 2009). The liver tissue was preserved at -20°C for subsequent biochemical analysis. Additionally, bone marrow from both the femurs was flushed out with cold PBS and stored in microfuge tubes for the genotoxicity test (D'Costa et al. 2019).

3.7 ESTIMATION OF BIOMOLECULES

Liver tissues of the different groups that were stored at -20 degrees were weighed and used to prepare various tissue homogenates for different biochemical tests. Blood collected through cardiac puncture was centrifuged at 2400 rpm at 27°C for 30 minutes, to separate the serum, stored at -4°C, which was further utilized for analysis. (Diehl et al. 2001)

3.7.1 Total Carbohydrates (Ludwig and Goldberg, 1956)

Anthrone test is used to detect carbohydrates. Addition of anthrone dehydrates the carbohydrates along with concentrated sulphuric acid, to form furfural. Furfural reacts with

anthrone to form a dark bluish-green complex that is quantified spectrophotometrically at 620 nm.

Extraction:

10% liver tissue homogenate was prepared in ice-cold water that was further deproteinized using equal amounts of 0.3N Barium hydroxide and 5% Zinc sulphate. This was centrifuged at 800 xg for 15 minutes. The supernatant collected was used for estimation.

Reagents:

1. Anthrone reagent – 0.2 gm Anthrone was dissolved in 100 ml of concentrated sulphuric acid.

Estimation:

0.1ml of deproteinized supernatant was taken in different test tubes, of different experimental and control groups. The homogenate was diluted using 0.9 ml distilled water. 4 ml of Anthrone reagent was added and the solution was incubated for 15 minutes in a boiling water bath. The intensity of color was measured at 620 nm using UV-Visible spectrophotometer against a suitable blank. Quantification of carbohydrate content was carried out using a standard curve of 100 µg/ml D-glucose.

3.7.2 Free sugars (CMFRI,1981)

Free sugars on reacting with alkaline copper reagent forms cuprous oxide. Cuprous oxide provides a blue coloured complex with arsenomolybdate reagent, that is measured at 540 nm.

Extraction:

The deproteinized homogenate prepared for carbohydrates was also used for estimation of free sugars.

Reagents:

1. Alkaline copper reagent – a) 12g anhydrous sodium carbonate and 6 g sodium potassium tartarate were dissolved in 125 ml distilled water.

b) 2 g copper sulphate was dissolved in 25 ml distilled water.

Solutions a) and b) were mixed, along with addition of 8 g sodium bicarbonate to form Solution A

90 g anhydrous sodium sulphate was dissolved in 250 ml distilled water, boiled and cooled to room temperature to form Solution B.

Solution A and Solution B were mixed and volume was made up to 500 ml with distilled water.

2) Arsenomolybdate color reagent – 25 g ammonium molybdate was dissolved in 450 ml distilled water along with slow addition of 21 ml concentrated sulphuric acid. To this, 3 g disodium hydrogen arsenate that was dissolved in 25 ml distilled water was added. This was stored in an amber bottle at room temperature.

Estimation:

0.1 ml of deproteinized samples of different groups were taken in different test tubes. To this 1 ml of alkaline copper reagent was added and incubated in a boiling water bath for 20 minutes. After cooling to room temperature, 1 ml of arsenomolybdate color reagent was added to the test tubes, that was diluted with 7 ml distilled water. The intensity of color was measured at 540 nm

against a suitable blank. Quantification of free sugars was calculated using a standard curve of 200 µg/ml D-glucose.

3.7.3 Total proteins (Lowry et al., 1951)

Protein concentration is estimated by reactivity of nitrogen with copper 2 ions in alkaline condition. Amino acids reacts with tungstic acid present in Folin Cio-Calteau reagent, to provide a blue color that is proportional to the amount of phenolic group of tyrosine and tryptophan, measured at 690 nm.

Extraction:

The liver tissue was weighed and homogenized with PBS to produce 10% tissue homogenate. The homogenate was cold centrifuged at 4 degrees at 3000 rpm for 15 minutes. The supernatant was stored at -4°C for further analysis.

Reagents:

1. Lowry's reagent- 98 ml of 4% sodium carbonate was mixed with 1ml of 2% copper sulphate and 1 ml of 4% sodium potassium tartarate, to form 100 ml solution.

2. Follin's reagent- equal amounts of follin's reagent and distilled water was used, according to required volume.

Estimation:

0.1 ml tissue homogenate was diluted with 0.5 ml distilled water. 5 ml of Lowry's reagent was added and incubated at room temperature for 10 minutes. 0.5 ml of Folin Cio-Calteau reagent was added and incubated again at room temperature for 10 minutes. Blue coloured complex was

measured at 690 nm against a suitable blank. Quantification of protein content was carried out using a standard curve of 100 µg/ml bovine serum albumin in 1N NaOH.

3.7.4 Albumins (Doumas et al., 1971)

Albumin reacts with bromocresol green at low pH, to form a green coloured complex, measured at 640 nm.

Extraction:

Tissue homogenate prepared for protein estimation, was also used for estimation of albumins.

Reagents:

Albumin reagent- 8.85 g succinic acid, 0.1 g sodium azide, 0.108 g bromocresol green and 4 ml of Triton X-100 were dissolved in 900 ml of distilled water. Using 1 N NaOH, the pH was maintained at 4.1. Final volume was made up to 1000 ml using appropriate amount of distilled water.

Estimation:

5 ml of albumin reagent was added to 0.05 ml of homogenate, and incubated at 37 degrees for 10-15 minutes. The color intensity was measured at 640 nm against a suitable blank. Quantification of albumin was measured using a standard curve of 4g/dl of albumin in 0.1g/dL of sodium azide.

3.8 ENZYME AND ANTIOXIDANT ASSAY

3.8.3 Catalase activity (Aebi,1984)

The catalase reaction involves the breakdown of hydrogen peroxide into water and molecular oxygen. Any residual hydrogen peroxide that remains after catalase activity will interact with dichromate, resulting in the formation of a blue precipitate of perchromic acid. This compound is known to be unstable and upon heating, it decomposes into a green-colored stable compound, which can be quantified by measuring its absorbance at 620 nm.

Extraction:

Blood serum was used.

Reagents:

1. Dichromate acetic acid reagent- 5% potassium dichromate and glacial acetic acid was used in 1:3 ratio.

Estimation:

1 ml of 0.01 M PB (pH 7) was added to 0.1 ml of serum. To this, 0.5 ml of 0.2 M H_2O_2 was added and reaction was stopped using 2 ml of Dichromate acetic acid reagent. The solution was incubated in a boiling water bath for 10 minutes, and color intensity was measured at 620 nm using a suitable blank. Enzyme activity was quantified using a standard curve of 0.05g/ml catalase, and expressed in μg/mg of total protein.

3.8.4 Thiobarbituric acid reactive substances (TBARS) (Beuge and Aust, 1978)

Water soluble malondialdehyde reacts with thiobarbituric acid in 1:2 ratio to form coloured complex measured at 535 nm.

Extraction:

2% liver tissue homogenate was prepared in Tris HCl buffer (pH 7) and centrifuged at 4000 rpm for 15 minutes. The supernatant was used for analysis of TBARS.

Reagents:

1. 0.67% TBA – 0.134 g of TBA was added in 20 ml of glacial acetic acid

2. 5% TCA- 5 ml TCA was added in 95 ml of distilled water

Estimation:

To 0.1 ml of homogenate, 1 ml of 5% TCA and 1 ml of 0.67% TBA was added, and boiled for 10 minutes to produce the coloured complex, measured at 535 nm against a suitable blank. Quantification of TBARS was done using a standard curve of 0.1g/ml of malondialdehyde.

3.9 GENOTOXICITY TEST

3.9.1 Comet assay test (Ferraro et al., 2004)

In this method, a suspension of eukaryotic blood cells in low-melting-point agarose undergoes cell lysis through detergents and high salt treatment to remove cell content except DNA. The liberated DNA is then electrophoresed under alkaline conditions to unwind from breakage sites. Cells with higher levels of DNA damage exhibit increased DNA migration from the nucleus towards the anode under an electric current, creating a "comet tail" appearance when viewed under fluorescent dye (ethidium bromide).

Extraction:

Bone marrow was flushed from the femur using cold PBS, that was used for analysis.

Reagents:

1. 0.5% Low melting Agarose: 0.25 g Low melting Agarose was dissolved in 50 ml of 1x PBS

2. 1% Normal melting Agarose: 0.5 g Normal melting Agarose was dissolved in 50 ml of 1x PBS

3. 1x PBS (pH 7.4) : 8g NaCl, 0.2 g KCl, 1.4 g Na_2HPO_4 and 0.27g KH_2PO_4 were dissolved in 100 ml distilled water, to prepare 10x stock solution that was diluted to 1x.

4. Lysis buffer (pH 10): 14.6g NaCl (2.5M), 3.74g disodium salt of EDTA (100 mM) 0.12g of Tris HCl was mixed in 100 ml distilled water. 89 ml of this stock was mixed in 0.1ml of 1% Triton-X and 10 ml of 10% DMSO was added after adjusting the pH.

5. 1% Triton-X : 1 ml Triton-X (10 mM) was diluted to 100 ml using distilled water

6. 10% DMSO: 10 ml DMSO was diluted to 100 ml using distilled water.

7. Neutralisation buffer (pH 7.5): 4.84g Tris Base (400mM) was added in 100 ml distilled water after adjusting pH to 7.5. It was stored at room temperature and chilled before use.

8. Electrophoresis buffer: 20 g of NaOH was mixed in 50 ml of distilled water, 1.49g disodium salt of EDTA was mixed in 20 ml distilled water after adjusting the

pH to 10. Working solution of 27 ml NaOH, 4.5ml of disodium EDTA salt and 1.8 ml of DMSO was prepared that was diluted to 1L of distilled water.

9. Ethidium bromide dye: 0.01g Ethidium bromide was added in 50 ml distilled water and stored in an amber bottle. From this stock, 0.1 ml was diluted to 1 ml distilled water to prepare 1x working solution, following utmost care.

Procedure:

A layer of 500 μL of 1% Normal Melting Agarose was spread on clean frosted slides and covered with a coverslip. Following solidification of the gel, a layer of 200 μL of bone marrow sample was blended with 600 μL of 0.5% Low Melting Agarose and spread over the existing layer. After the gel suspension solidified, a third layer of 500 μL of 0.5% Low Melting Agarose was added and allowed to solidify. The coverslip was removed, and the frosted slides were immersed in Lysis buffer (pH 10) at 4°C overnight. Subsequent to lysis, the slides were placed in an unwinding buffer (electrophoresis buffer, pH 10) for 30 minutes to unwind the DNA and Electrophoresis was conducted for 20 minutes at 280mA and 25V. The slides were then transferred to a pre-cooled Neutralisation buffer (pH 7.5) for 5 minutes, excess buffer cleaned off. The slides were stained with 100 ml 1X Ethidium Bromide solution, covered with a coverslip, and examined under a fluorescence microscope (Olympus BX53) at 20x magnification using a red filter. The slide was meticulously examined for the presence of comets and was analyzed using CASP software to calculate the % tail DNA.

3.10 STATISTICAL ANALYSIS

All the samples were analyzed as triplicates and data were presented as the mean ± S.D. Statistical analysis was performed with Graphpad Prism 9 software for biochemical and morphological tests. CASP software was used to analyze comets and measure the tail DNA %. Normal distribution of data was checked using the Shapiro-Wilk test. Significant differences between each group were analyzed using two-way ANOVA, followed by post-hoc Tukey's Honest Significant Difference test, when F values of ANOVA were significant. Pearson's correlation was carried out to check positive (+ value) and negative (- value) correlations between different parameters. A p value ≤ 0.05 was considered statistically significant.

CHAPTER 4: RESULTS

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4.1 EFFECT OF MWCNTs ON MORPHOLOGY OF *Mus musculus*

Following exposure to two different doses of multi-walled carbon nanotubes (MWCNTs) in two separate experimental groups of mice, a continuous monitoring of both morphological and behavioral alterations was conducted over a 14-day exposure period. Observations were extended for an additional 7-day period to allow for a natural detoxification phase in all three groups: the control group, Experimental Group 1, and Experimental Group 2.

4.1.1 BODY WEIGHT

The average weight of six mice in each group was assessed, with measurements taken once every seven days. The initial average weight of the control group showed an increase over the span of 21 days. The average body weight of three mice in both Experimental Groups 1 and 2 decreased over a 14-day period. Similarly, the remaining three mice designated for natural detoxification experienced a comparable decline in weight over the subsequent seven days. (Fig 8.1)

4.1.2 WEIGHT OF LIVER

Upon dissection, the liver weight was measured for all three groups. An average of 3 liver tissue weights was calculated for each group: control, experimental 1, and experimental 2.The liver weight was found to be slightly higher in experimental groups 1 and 2 compared to the control group, although this difference was not statistically significant ($p=0.5405$ for experimental 1, $p=0.9691$ for experimental 2). However, a non-significant decrease in liver weight was observed

in experimental group 2 compared to experimental group 1 (p=0.8576). After a 7-day detoxification period, the liver weight in both experimental groups was observed to have increased slightly, although this increase was not statistically significant when compared to both the control group and the average liver weight of the three experimental groups post-exposure period (p= 0.9953 for experimental 1, p=0.9423 for experimental 2). (Fig 8.2)

4.1.3 FUR AND TAIL MORPHOLOGY

Fur morphology in terms of texture, color, density was intact and normal for all the groups, over a course of 14 days exposure period and 7 days of natural detoxification period. There were no abnormal tail spots observed as well in any of the groups during the same period. (Fig 8.15)

4.2 EFFECT OF MWCNTS ON BEHAVIOUR OF *Mus musculus*

Behavioral analysis was conducted to identify signs of drowsiness, lethargy, isolation, and feeding frequency. In the control group, no abnormal behavioral changes were noted. Experimental Group 1 mice exhibited heightened drowsiness and lethargy after 14 days, while no isolating behavior was observed. A decrease in feeding frequency was also noted. Experimental Group 2 mice displayed similar signs of increased drowsiness, lethargy, and isolation, accompanied by reduced feeding frequency. After a 7-day detoxification period, Experimental Group 1 mice showed no significant behavioral differences except for an increase in feeding frequency. Similarly, Experimental Group 2 showed no significant changes for the subsequent 2 days, except for increased feeding frequency. (Fig 8.16)

4.3 EFFECT OF MWCNTS ON LIVER AND BLOOD SERUM BIOMOLECULES

The changes in the concentration of different biomolecules and enzymes of mice exposed to 2 different concentrations of MWCNTs, for an exposure period of 14 days, and after a 7 days detoxification period, are revealed.

Mice administered MWCNTs nanoparticles via gavage exhibited a notable dose-dependent reduction in protein concentration in both Experimental Groups 1 and 2 compared to the control group ($p \leq 0.05$ and $p \leq 0.01$, respectively). Furthermore, the detoxification batch of Experimental Groups 1 and 2 also displayed a significant decrease in protein concentration compared to the control group ($p \leq 0.01$). The protein concentration in Experimental Group 1 was markedly higher than that in Experimental Group 2 ($p \leq 0.01$). Upon transitioning the experimental groups to the detoxification phase, a significant decrease in protein concentration was observed compared to the period of toxicant exposure ($p \leq 0.01$). (Fig 8.3)

The total carbohydrates concentration in experimental 1 and 2 groups showed a significant increase compared to the control group, even after natural detoxification ($p \leq 0.05$). The carbohydrate concentration however decreased in experimental 2, compared to experimental 1 in a non-significant dose-dependent manner ($p=0.3041$). The levels of carbohydrates were seen to have remained elevated post detoxification, compared to control as well as during the toxicant exposure period ($p \leq 0.01$). (Fig 8.5)

Significant dose-dependent reductions were observed in the concentrations of free sugars in both experimental groups following nanoparticle exposure, in comparison to the control group ($p \leq 0.01$), as well as during the post-detoxification period ($p \leq 0.01$). These lower levels persisted even after the detoxification phase, when compared to the initial concentration of free sugars post-exposure ($p \leq 0.01$). However, there was a notable elevation in the concentration of free

sugars in Experimental Group 2 compared to Experimental Group 1, showing significant difference ($p \leq 0.01$). (Fig 8.6)

Significant decreases in albumin concentrations were observed in both experimental groups following exposure and after the detoxification period, in comparison to the control group ($p \leq 0.05$). However, there was a noteworthy difference in albumin concentration between Experimental Group 2 and Group 1, with Experimental Group 2 exhibiting significantly higher levels ($p \leq 0.05$). Additionally, the albumin levels post-detoxification were significantly lower compared to the levels observed post-exposure in Experimental Group 1. A non-significant increase in albumin levels was seen in Experimental Group 2 following the detoxification phase ($p = 0.999$). (Fig 8.4)

The catalase levels exhibited a significant decrease in both Experimental Groups 1 and 2 compared to the control ($p \leq 0.01$). However, Experimental Group 2 showed a notably higher catalase level than Experimental Group 1 ($p \leq 0.01$). Moreover, there was a significant increase in catalase levels observed post-detoxification compared to both the control group and the post-exposure groups ($p \leq 0.01$). (Fig 8.7)

TBARS levels exhibited a non-significant increase in both experimental groups when compared to the control ($p = 0.2817$ for experimental group 1, $p = 0.1315$ for experimental group 2). However, post-detoxification, these levels decreased non-significantly compared to the control ($p = 0.9794$ for experimental group 1, $p = 0.6640$ for experimental group 2) and the post-exposure period group ($p = 0.1073$ for experimental group 1, $p = 0.7816$ for experimental group 2). Additionally, the TBARS level was non-significantly higher in experimental group 2 compared to experimental group 1 ($p = 0.9908$). (Fig 8.9)

4.4 EFFECT OF MWCNTs ON GENETIC MATERIAL

DNA damage was assessed using the comet assay on bone marrow cells from mice exposed to two different doses of MWCNTs nanoparticles. According to the test results, the percentage of tail DNA observed in the cells of both experimental groups showed a non-significant increase compared to the control group during the post-exposure period ($p = 0.9970$ for experimental group 1, $p = 0.8346$ for experimental group 2) and during the post-detoxification period in experimental group 1 ($p = 0.9762$), while a significant increase was observed in experimental group 2 ($p \leq 0.01$). A non-significant increase was noted in experimental group 2 compared to experimental group 1 ($p = 0.9508$). In experimental group 1, the tail DNA percentage exhibited a non-significant increase following a 7-day natural detoxification period ($p = 0.999$). However, a significant increase in tail DNA percentage was observed in experimental group 2 following the same detoxification duration ($p \leq 0.05$). (Fig 8.13)

4.5 CORRELATION AMONG TESTS

The biochemical tests were correlated to each other, to understand any significance or whether there is a consistent pattern of association between the concentrations of different biomolecules across multiple samples or experimental conditions. The r coefficient value, generated for all the tests, is provided in Fig. 8.17 and the graphs are shown in Fig 8.14.

CHAPTER 6: DISCUSSIONS

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5.1 EFFECT OF MWCNTs ON MORPHOLOGY OF *Mus musculus*

Morphological changes in experimental 1 group (50 mg/kg per body weight dose) and experimental 2 group (100 mg/kg per body weight dose) of Swiss albino strain of *Mus musculus* were observed for a period of 14 days, that was compared to the that of the control group, fed upon standard feed and water. There were no visible signs of damage observed on the fur and tail morphology of the mice, that is in terms of presence of change in color, texture and density of fur, and possible appearance of tail spots. No loss of hair and change in color and texture are indicative that these nanoparticles are incapable of damaging the hair follicles that result in excessive hair shedding and impaired hair growth. The appearance and progression of tail spotting can also be influenced by factors such as genetics, housing conditions, and overall health. DNA damage can disrupt cellular processes involved in hair growth and maintenance, leading to hair abnormalities (Ungvari et al. 2024).

Mice typically stabilize their body weight after an initial adjustment period in the animal facility. Significant fluctuations or consistent weight loss may indicate stress or health issues (Jeong et al. 2013). In the control group, an increase in body weight was observed over a span of two weeks and in the following 1 week period kept for natural detoxification. However, upon exposure to MWCNTs, mice from experimental groups 1 and 2 exhibited a decrease in body weight after a 14-day exposure period. This decrease persisted slightly even after the mice underwent a natural detoxification period of another 7 days. The reduction in body weight could be connected to the repeated oral gavaging of MWCNTs, which led to a decrease in their feeding frequency on the standard pellet provided. A significant amount of feed remained untouched during the exposure

period. Although feeding frequency slightly increased during the natural detoxification period, it was not significant enough to increase body weight. Increased doses frequently result in more significant effects on body weight and other physiological parameters. These mechanisms can influence body weight directly or indirectly by impacting metabolic processes, organ functions, or appetite regulation. Nanoparticles can engage with various biological systems, causing systemic effects extending in areas away from the site of exposure (Noga et al. 2023). These interactions might interfere with hormonal regulation, immune responses, or nutrient absorption, all of which may affect body weight.

Upon dissection, the weight of the liver was measured of the control and experimental groups, after the 14 days exposure period and after 7 days natural detoxification period, and an average was considered of 3 liver tissues of each group. A non-significant increase in the liver weight was observed in the experimental groups, compared to that of the control. The liver weight persisted to increase even after the period of no exposure to the toxicant, indicating a probability of retention or accumulation of these nanoparticles in the hepatocytes (Witkowska et al. 2022). Excessive exposure to CNTs can cause liver damage and inflammation, altering liver size and weight. Prolonged exposure worsens toxicity, potentially changing the liver organ index. Chronic exposure may lead to liver fibrosis, hepatocellular hypertrophy, or other histopathological changes, impacting liver size and weight (Dong et al. 2019). However, the average weight of liver tissue of experimental group 1 was non-significantly lower compared to the experimental 1 group. This could either occur due to procedural error, or due to probability of selecting random liver tissue samples.

5.2 EFFECT OF MWCNTS ON BEHAVIOUR OF *Mus musculus*

Behavioral studies have not been frequently carried out to analyze the possible effects of these nanotubes on the Swiss albino strain of mice. The behavior analyzed was based on standard procedures to report the absence, presence or normal behaviors of drowsiness, lethargy, isolation and feeding frequency of the mice in both the experimental groups (Subakanmani 2015).

Upon exposure to different doses of MWCNTs, a notable presence of drowsiness and lethargy was observed in the mice, in both the experimental groups, when observed at the end of the exposure period. These behaviors persisted even in the remaining 3 mice from each experimental group during the natural detoxification period. CNT exposure can induce systemic inflammation and immune responses (Boyles et al. 2014). Inflammatory cytokines released in can trigger sickness behavior, such as lethargy and drowsiness, as part of the body's defense mechanisms. Additionally, CNTs have been found to generate reactive oxygen species (ROS), leading to oxidative stress in various tissues (Manke et al.2013). This oxidative stress can impair cellular function and contribute to fatigue and lethargy. However, individual responses to CNT exposure may vary due to differences in genetics, age, sex, and health status.

Mice from experimental 1 group did not exhibit any initial signs of isolating behavior in the first two weeks of exposure, and maintained this behavior even during the detoxification period. Experimental 2 group however, showed isolating behavior from week 1 of the toxicant exposure period, itself, that persisted up to the end of the exposure and detoxification period. Sick or injured mice may isolate themselves from their cage mates as a survival strategy or to minimize the spread of disease within the group. Isolation can also be a sign of pain or discomfort in mice.

The nanotubes, suspended in saline, were administered orally via gavage repeatedly for a 14-day period, consistently at the same time each day. This repetitive administration may have contributed to the observed decrease in feeding frequency among the mice in the experimental groups. The standard feed provided per cage remained largely untouched for most of the days. Although an increase in water intake was noted, the reduced feeding frequency persisted. However, upon completion of the exposure period, the feeding frequency of the experimental mice returned to normal. Depending on the duration and nature of nanoparticle exposure, mice may experience residual effects even after dosing is stopped. It's possible that these residual effects could influence feeding behavior, either positively or negatively, until the effects reduce completely. The control groups showed normal behavioral signs for the entire duration of study.

5.3 EFFECT OF MWCNTS ON LIVER AND BLOOD SERUM BIOMOLECULES

The liver plays a crucial role in metabolizing foreign substances, including nanoparticles, entering the body (Zhang et al. 2016). Through enzymatic reactions, it processes and detoxifies these substances, affecting their toxicity. Nanoparticles, especially those administered systemically, tend to accumulate in the liver due to its high blood flow and filtration capabilities, making it a key target organ for toxicity assessment (Ngo et al. 2022). Liver enzymes (e.g., ALT, AST), pro-inflammatory cytokines, and oxidative stress markers are important biomarkers of liver toxicity. Monitoring changes in these biomarkers can help in assessing nanoparticle-induced liver injury and toxicity. Nanoparticles, administered via different routes (e.g., intravenous, oral, inhalation), spread throughout the body via bloodstream. Blood serum acts as a reservoir for these nanoparticles and their byproducts, facilitating the assessment of systemic exposure and distribution. It contains biomarkers that show physiological and pathological changes from nanoparticle exposure, including liver enzymes (e.g., ALT, AST), kidney function

markers (e.g., creatinine), inflammatory cytokines, oxidative stress markers, and lipid profiles (Uges et al. 1988). These changes provide insights into nanoparticle-induced toxicity and its mechanisms, highlighting dysfunction in specific organs like the liver, kidneys, heart, and lungs, helping in target organ identification and assessment of toxicity.

The total protein concentration showed a significant dose-dependent decrease in the experimental groups 1 and 2 when compared to the control groups as well as liver homogenate samples taken after 14 days exposure period, of the respective experimental groups. The concentration in experimental 1 group was higher compared to that of experimental 2 mice, due to a lower dose administration. Certain nanoparticles have the potential to disrupt cellular machinery responsible for protein synthesis, such as ribosomes or transcription factors (Kulasza et al. 2021). This disruption can interfere with normal protein production in liver cells, resulting in alterations in protein concentration. Additionally, nanoparticles may trigger specific intracellular signaling pathways in liver cells, influencing gene expression and protein synthesis. These alterations can ultimately modify the protein composition of liver tissue.

However, a similar result was seen in terms of albumin levels, with the exception that the concentration of albumin was higher in the second experimental group compared to the first one, while the experimental 2 mice post natural detoxification showed a non-significant increase in the albumin concentration when compared to those levels in the liver homogenate after exposure period. Nanoparticles can damage the liver, reducing albumin production by hepatocytes. Factors like oxidative stress, inflammation, and ER stress hinder albumin synthesis (Almanza et al. 2019). This leads to decreased albumin levels in liver tissue.

When the concentrations of carbohydrates were measured in the groups, a significant increase was noted in the liver tissue in experimental 1, and non-significant increase in experimental 2. The levels remained at the higher end, up to the end of the study duration of exposure and detoxification period. Carbohydrates were found to be higher in the first experimental group than that of the second group. Nanoparticle exposure can stimulate glycogen breakdown (glycogenolysis) in the liver, increasing glucose levels and raising total carbohydrate content in liver tissue along with activation of gluconeogenesis, synthesizing glucose from non-carbohydrate sources like amino acids and glycerol (Martin et al. 2023).

However, the levels of free sugars in the liver tissue showed a significant decrease upon exposure in experimental group 1 and 2, after a period of 14 days of repeated dosage and 7 days of natural detoxification (ref). While, the levels of free sugars was seen to be significantly higher in experimental 2 group, when compared to that of experimental 1. This could be due to dysfunction in liver cells, potentially disrupting metabolic processes involved in sugar metabolism. If key enzymes or transporters involved in sugar metabolism are affected by nanoparticle toxicity, this could lead to decreased levels of free sugar in the liver tissue.

Catalase levels significantly started increasing in the experimental groups, after the end of the detoxification period, with higher concentrations in the experimental 2 mice. Elevated catalase levels in the blood serum reflects response to increased reactive oxygen species (ROS) production, as catalase is an important antioxidant enzyme that helps neutralize hydrogen peroxide (H2O2) and protect cells from oxidative damage (Florek et al. 2023).

Meanwhile, an increase in the levels of TBARS was seen in the experimental groups 1 and 2, after 14 days, that non-significantly decreased during the detoxification period of experimental 1

mice while remained elevated in the second group when compared to that of their respective experimental groups upon exposure.The higher dosed mice showed higher levels of TBARS. Thiobarbituric acid reactive substances (TBARS) are a measure of lipid peroxidation, which is a common consequence of oxidative stress (Fraga et al. 1988). Increased TBARS levels indicate that liver cells are experiencing oxidative stress. It also suggests that lipid molecules in liver cell membranes have undergone peroxidation, a process initiated by ROS (Weinberger et al. 2002).

5.4 GENOTOXICITY OF MWCNTs

The tail DNA % represents the proportion of total DNA content that has migrated into the tail of the comet, indicating the extent of DNA damage. DNA damage was observed in both the experimental groups, upon sub-acute exposure to MWCNTs, that further increased even after a natural detoxification period. CNTs have a high surface area and unique physicochemical properties that enable them to interact directly with DNA molecules and can adsorb onto DNA strands, leading to structural distortions, strand breaks, and other types of DNA damage (Ema et al. 2013). Inflammatory mediators, ROS, as well as DNA-damaging enzymes can contribute to DNA damage. Prolonged exposure to CNTs may overwhelm cellular DNA repair mechanisms, leading to accumulation of DNA damage (Pacurari et al. 2008). Impaired DNA repair processes, such as base excision repair, nucleotide excision repair, and double-strand break repair, can lead to CNT-induced genotoxicity (Darne et al. 2014).

Some toxicants can be metabolized by cellular enzymes into reactive metabolites that can induce DNA damage (Martin et al. 2008). While detoxification processes aim to eliminate these toxicants, they may also produce reactive intermediates or metabolites that can cause DNA

damage themselves (Wan et al.2012).Therefore, even after the end of exposure and natural detoxification, residual reactive metabolites may continue to induce DNA damage.

5.5 NATURAL DETOXIFICATION OF MWCNTS

The liver plays a crucial role in the natural detoxification of nanoparticles by liver enzymes, particularly those belonging to the cytochrome P450 (CYP) family (Grant et al. 1991). These enzymes catalyze various reactions, including oxidation, reduction, and conjugation, which can modify the chemical structure of nanoparticles to facilitate their elimination from the body (Ismail et al. 2010). Hepatocytes contain a variety of detoxification enzymes, including glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs), which catalyze the conjugation of nanoparticles with molecules such as glutathione or glucuronic acid (Li et al. 2015). Conjugation reactions increase the water solubility of nanoparticles and facilitate their excretion via urine or bile. Hepatocytes contain high levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, which help neutralize reactive oxygen species (ROS) generated by nanoparticle exposure and prevent oxidative damage to cellular components (Ighodaro et al. 2018). The liver is a major immunological organ involved in immune surveillance and clearance of foreign particles, including nanoparticles. Kupffer cells and other immune cells within the liver can recognize and phagocytose nanoparticles, leading to their removal from circulation and subsequent processing or excretion (Sadauskas et al. 2007).

CHAPTER 6 : CONCLUSION

6. CONCLUSION

In conclusion, exposure to multiwalled carbon nanotubes via ingestion in two different concentrations resulted in genotoxic and hepatotoxic effects in Swiss albino mice during a sub-acute exposure period of 14 days. Following a week of natural detoxification, a decrease in toxicity biomarkers was observed, indicating a level of detoxification carried out naturally by the liver. However, DNA damage appeared to persist and even increase. This suggests that while some detoxification mechanisms may be activated, they may not fully counteract the genotoxic effects of the nanoparticles. There is a need for further research to understand the underlying mechanisms of hepatotoxic effects, the accumulation or retention of nanoparticles in the body, the reasons for persistent DNA damage, and explore alternative methods for detoxifying these nanoparticles. This research will provide valuable insights into mitigating the adverse effects of nanoparticle exposure and enhancing the safety of nanomaterials in various applications.

CHAPTER 7: REFERENCES

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1. Aebi, H. (1984). [13] Catalase in vitro. In *Methods in Enzymology* (Vol. 105, pp. 121–126). Elsevier. https://doi.org/10.1016/S0076-6879(84)05016-3

2. Baršienė, J., Andreikėnaitė, L., & Bjornstad, A. (2010). Induction of micronuclei and other nuclear abnormalities in blue mussels *Mytilus edulis* after 1-, 2-, 4- and 8-day treatment with crude oil from the North Sea. *Ekologija*, *56*(3), 124–131. https://doi.org/10.2478/v10055-010-0018-4

3. Chetyrkina, M. R., Fedorov, F. S., & Nasibulin, A. G. (2022). *in vitro* toxicity of carbon nanotubes: A systematic review. *RSC Advances*, *12*(25), 16235–16256. https://doi.org/10.1039/D2RA02519A

3. CMFRI, Kochi (1981) *Manual of research methods for crustacean biochemistry and physiology*. CMFRI Special Publication, 7. pp. 1-172.

5. Deng, X., Wu, F., Liu, Z., Luo, M., Li, L., Ni, Q., Jiao, Z., Wu, M., & Liu, Y. (2009). The splenic toxicity of water soluble multi-walled carbon nanotubes in mice. *Carbon*, *47*(6), 1421–1428. https://doi.org/10.1016/j.carbon.2008.12.032

6. Di Giorgio, M. L., Bucchianico, S. D., Ragnelli, A. M., Aimola, P., Santucci, S., & Poma, A. (2011). Effects of single and multi walled carbon nanotubes on macrophages: Cyto and

genotoxicity and electron microscopy. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *722*(1), 20–31. https://doi.org/10.1016/j.mrgentox.2011.02.008

7. Doumas, B. T., Ard Watson, W., & Biggs, H. G. (1971). Albumin standards and the measurement of serum albumin with bromcresol green. *Clinica Chimica Acta*, *31*(1), 87–96. https://doi.org/10.1016/0009-8981(71)90365-2

8. Ferraro, D., Anselmi-Tamburini, U., Tredici, I. G., Ricci, V., & Sommi, P. (2016). Overestimation of nanoparticles-induced DNA damage determined by the comet assay. *Nanotoxicology*, *10*(7), 861–870. https://doi.org/10.3109/17435390.2015.1130274

9. Horibata, K., Takasawa, H., Hojo, M., Taquahashi, Y., Shigano, M., Yokota, S., Kobayashi, N., Sugiyama, K., Honma, M., & Hamada, S. (2022). In vivo genotoxicity assessment of a multiwalled carbon nanotube in a mouse ex vivo culture. *Genes and Environment*, *44*(1), 24. https://doi.org/10.1186/s41021-022-00253-2

10. Horie, M., Sugino, S., & Ohno, T. (2023). Follow the time course of inflammation caused by intraperitoneal administration of multi-wall carbon nanotubes in mice. International Journal of Immunopathology and Pharmacology, 37, 03946320231176402. https://doi.org/10.1177/03946320231176402

11. Jafar, A., Alshatti, Y., & Ahmad, A. (2016). Carbon nanotube toxicity: The smallest biggest debate in medical care. *Cogent Medicine*, *3*(1), 1217970. https://doi.org/10.1080/2331205X.2016.1217970

12. Karmakar, R., Banik, S., Bandyopadhyay, S., & Chatterjee, M. (1998). Cadmium-induced alterations of hepatic lipid peroxidation, glutathione S-transferase activity and reduced glutathione level and their possible correlation with chromosomal aberration in mice: A time course study. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *397*(2), 183–190. https://doi.org/10.1016/S0027-5107(97)00209-1

13. Karousis, N., Tagmatarchis, N., & Tasis, D. (2010). Current progress on the chemical modification of carbon nanotubes. *Chemical Reviews*, *110*(9), 5366–5397. https://doi.org/10.1021/cr100018g

14. King, E. J., Abul-Fadl, M. A. M., & Walker, P. G. (1951). King-armstrong phosphatase estimation by the determination of liberated phosphate. *Journal of Clinical Pathology*, *4*(1), 85–91. https://doi.org/10.1136/jcp.4.1.85

15. Liu, X., Guo, L., Morris, D., Kane, A. B., & Hurt, R. H. (2008). Targeted removal of bioavailable metal as a detoxification strategy for carbon nanotubes. *Carbon*, *46*(3), 489–500. https://doi.org/10.1016/j.carbon.2007.12.018

16. Lowry, OliverH., Rosebrough, NiraJ., Farr, A. L., & Randall, RoseJ. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, *193*(1), 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

17. Ludwig, T. G., & Goldberg, H. J. V. (1956). The anthrone method for the determination of carbohydrates in foods and in oral rinsing. *Journal of Dental Research*, *35*(1), 90–94. https://doi.org/10.1177/00220345560350012301

18. Mohanta, D., Patnaik, S., Sood, S., & Das, N. (2019). Carbon nanotubes: Evaluation of toxicity at biointerfaces. *Journal of Pharmaceutical Analysis*, *9*(5), 293–300. https://doi.org/10.1016/j.jpha.2019.04.003

19. Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95*(2), 351–358. https://doi.org/10.1016/0003-2697(79)90738-3

20. Qi, W., Tian, L., An, W., Wu, Q., Liu, J., Jiang, C., Yang, J., Tang, B., Zhang, Y., Xie, K., Wang, X., Li, Z., & Wu, W. (2017). Curing the toxicity of multi-walled carbon nanotubes through native small-molecule drugs. *Scientific Reports*, *7*(1), 2815. https://doi.org/10.1038/s41598-017-02770-5

21. Requardt, H., Braun, A., Steinberg, P., Hampel, S., & Hansen, T. (2019). Surface defects reduce Carbon Nanotube toxicity in vitro. *Toxicology in Vitro*, *60*, 12–18. https://doi.org/10.1016/j.tiv.2019.03.028

22. Shah, P., Lalan, M., & Jani, D. (2021). Toxicological aspects of carbon nanotubes, fullerenes and graphenes. *Current Pharmaceutical Design*, *27*(4), 556–564. https://doi.org/10.2174/1381612826666200916143741

23. Solorio-Rodriguez, S. A., Williams, A., Poulsen, S. S., Knudsen, K. B., Jensen, K. A., Clausen, P. A., Danielsen, P. H., Wallin, H., Vogel, U., & Halappanavar, S. (2023). Single-walled vs. Multi-walled carbon nanotubes: Influence of physico-chemical properties on toxicogenomics responses in mouse lungs. *Nanomaterials*, *13*(6), 1059. https://doi.org/10.3390/nano13061059

24. Szendi, K., & Varga, C. (2008). Lack of genotoxicity of carbon nanotubes in a pilot study. *Anticancer Research*, *28*(1A), 349–352. https://ar.iiarjournals.org/content/28/1A/349

25. Test No. 407. (2008) Repeated Dose 28-Day Oral Toxicity Study in Rodents.*OECD Guidelines for the Testing of Chemicals*. Section 4,OECD Publishing. https://doi.org/10.1787/20745788

26. Test No. 425. (2016) Acute Oral Toxicity: Up-and-Down Procedure. *OECD Guidelines for the Testing of Chemicals* .Section 4, OECD Publishing. https://doi.org/10.1787/20745788

27. Test No.473. (2016) In Vitro Mammalian Chromosomal Aberration Test. *OECD Guidelines for the Testing of Chemicals.* Section 4, OECD Publishing. https://doi.org/10.1787/20745788

28. Wilkinson, J. H., Baron, D. N., Moss, D. W., & Walker, P. G. (1972). Standardization of clinical enzyme assays: A reference method for aspartate and alanine transaminases. *Journal of Clinical Pathology*, *25*(11), 940–944. https://doi.org/10.1136/jcp.25.11.940

29. Beuge JA and Aust SD (1978): Microsomal lipid peroxidation. In methods in enzymology. Academic Press NY and London, 52:302-310.

30. Kono Y (1978) Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. Arch. Biochem. Biophy., 186: 189 -195.

31. Miller, O.J. and D.A. Miller (1975) Cytogenetics of the mouse, Ann. Rev. of Genetics; 9, 285 – 303

32. Baratta, J. L., Ngo, A., Lopez, B., Kasabwalla, N., Longmuir, K. J., & Robertson, R. T. (2009). Cellular organization of normal mouse liver: A histological, quantitative immunocytochemical, and fine structural analysis. *Histochemistry and Cell Biology*, *131*(6), 713–726. https://doi.org/10.1007/s00418-009-0577-1

33. D'Costa, A., Praveen Kumar, M. K., & Shyama, S. K. (2019). Genotoxicity assays. In *Advances in Biological Science Research* (pp. 291–301). Elsevier. https://doi.org/10.1016/B978-0-12-817497-5.00018-5

34. Diehl, K., Hull, R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J., & Vorstenbosch, C. V. D. (2001). A good practice guide to the administration of substances and removal of blood, including routes and volumes. *Journal of Applied Toxicology*, *21*(1), 15–23. https://doi.org/10.1002/jat.727

35. Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, *82*(1), 70–77. https://doi.org/10.1016/0003-9861(59)90090-6

36. Molina, L. M., Krutsenko, Y., Jenkins, N. E. C., Smith, M. C., Tao, J., Wheeler, T. B., Watkins, S. C., Watson, A. M., & Monga, S. P. (2022). LiverClear: A versatile protocol for

mouse liver tissue clearing. *STAR Protocols*, *3*(1), 101178. https://doi.org/10.1016/j.xpro.2022.101178

37. Subakanmani, S. (2015). Evaluation of neuropsychopharmacological effects of hypericum hookerianum extracts on swiss albino mice. *University*. https://shodhganga.inflibnet.ac.in:8443/jspui/handle/10603/115190

38. Singh, K. (2003). Studies on the effect of monosodium glutamate MSG on the oxidative stress in certain tissues of adult mice. *University*. https://shodhganga.inflibnet.ac.in:8443/jspui/handle/10603/87920

39. Ahangarpour, A., Alboghobeish, S., Oroojan, A. A., & Dehghani, M. (2021). Caffeic acid protects mice pancreatic islets from oxidative stress induced by multi-walled carbon nanotubes (Mwcnts). *Veterinary Research Forum*, *12*(1). https://doi.org/10.30466/vrf.2019.94666.2279

40. Awasthi, K. K., Awasthi, A., Verma, R., Soni, I., Awasthi, K., & John, P. J. (2015). Silver nanoparticles and carbon nanotubes induced dna damage in mice evaluated by single cell gel electrophoresis. *Macromolecular Symposia*, *357*(1), 210–217. https://doi.org/10.1002/masy.201500018

41. Balakrishna, P., Sairam, A., & Surekh, P. (2011). Acute toxicological effects of multi-walled carbon nanotubes(Mwcnt). In M. Naraghi (Ed.), *Carbon Nanotubes—Growth and Applications*. InTech. https://doi.org/10.5772/18984

42. Dai, X., Ren, L., Yan, L., Zhang, J., Dong, Y., Qing, T., Shi, W., Li, J., Gao, F., Zhang, X., Tian, Y., Zhu, Y., Zhu, J., & Chen, J. (2022). Vascular toxicity of multi-walled carbon nanotubes targeting vascular endothelial growth factor. *Nanotoxicology*, *16*(5), 597–609. https://doi.org/10.1080/17435390.2022.2125849

43. Fang, H., Cui, Y., Wang, Z., & Wang, S. (2018). Toxicological assessment of multi-walled carbon nanotubes combined with nonylphenol in male mice. *PLOS ONE*, *13*(7), e0200238. https://doi.org/10.1371/journal.pone.0200238

44. Fraser, K., Hubbs, A., Yanamala, N., Mercer, R. R., Stueckle, T. A., Jensen, J., Eye, T., Battelli, L., Clingerman, S., Fluharty, K., Dodd, T., Casuccio, G., Bunker, K., Lersch, T. L., Kashon, M. L., Orandle, M., Dahm, M., Schubauer-Berigan, M. K., Kodali, V., & Erdely, A. (2021). Histopathology of the broad class of carbon nanotubes and nanofibers used or produced in U.S. facilities in a murine model. *Particle and Fibre Toxicology*, *18*(1), 47. https://doi.org/10.1186/s12989-021-00440-z

45. Ji, Z., Zhang, D., Li, L., Shen, X., Deng, X., Dong, L., Wu, M., & Liu, Y. (2009). The hepatotoxicity of multi-walled carbon nanotubes in mice. *Nanotechnology*, *20*(44), 445101. https://doi.org/10.1088/0957-4484/20/44/445101

46. Knudsen, K. B., Berthing, T., Jackson, P., Poulsen, S. S., Mortensen, A., Jacobsen, N. R., Skaug, V., Szarek, J., Hougaard, K. S., Wolff, H., Wallin, H., & Vogel, U. (2019). Physicochemical predictors of Multi-Walled Carbon Nanotube–induced pulmonary histopathology and toxicity one year after pulmonary deposition of 11 different Multi-Walled Carbon Nanotubes in mice. *Basic & Clinical Pharmacology & Toxicology*, *124*(2), 211–227. https://doi.org/10.1111/bcpt.13119

47. Liu, E., Wang, X., Li, X., Tian, P., Xu, H., Li, Z., & Wang, L. (2020). Co-exposure to multi-walled carbon nanotube and lead ions aggravates hepatotoxicity of nonalcoholic fatty liver via inhibiting AMPK/PPARγ pathway. *Aging*, *12*(14), 14189–14204. https://doi.org/10.18632/aging.103430

48. Patlolla, A. K., Berry, A., & Tchounwou, P. B. (2011). Study of hepatotoxicity and oxidative stress in male Swiss-Webster mice exposed to functionalized multi-walled carbon nanotubes. *Molecular and Cellular Biochemistry*, *358*(1–2), 189–199. https://doi.org/10.1007/s11010-011-0934-y

49. Zhang, H., Chen, R., Shao, Y., Wang, H., & Liu, Z. (2018). Effects of exposure of adult mice to multi-walled carbon nanotubes on the liver lipid metabolism of their offspring. *Toxicology Research*, *7*(5), 809–816. https://doi.org/10.1039/C8TX00032H

50. Aragon, M. J., Topper, L., Tyler, C. R., Sanchez, B., Zychowski, K., Young, T., Herbert, G., Hall, P., Erdely, A., Eye, T., Bishop, L., Saunders, S. A., Muldoon, P. P., Ottens, A. K., & Campen, M. J. (2017). Serum-borne bioactivity caused by pulmonary multiwalled carbon nanotubes induces neuroinflammation via blood–brain barrier impairment. *Proceedings of the National Academy of Sciences*, *114*(10). https://doi.org/10.1073/pnas.1616070114

51. Dhekale, K., Patil, S., & Kamble, R. (2021). Advanced development of carboxylic acid functionalized multiwall carbon nanotubes as safe inhalation drug carrier. *International Journal of Pharmaceutical Investigation*, *11*(1), 82–87. https://doi.org/10.5530/ijpi.2021.1.15

52. Ganji, D. D., & Kachapi, S. H. H. (2015). Semi nonlinear analysis in carbon nanotube. In *Application of Nonlinear Systems in Nanomechanics and Nanofluids* (pp. 13–70). Elsevier. https://doi.org/10.1016/B978-0-323-35237-6.00002-9

53. Huang, J., Zhang, Q., Zhao, M., & Wei, F. (2012). A review of the large-scale production of carbon nanotubes: The practice of nanoscale process engineering. *Chinese Science Bulletin*, *57*(2–3), 157–166. https://doi.org/10.1007/s11434-011-4879-z

54. Huynh, M. T., Mikoryak, C., Pantano, P., & Draper, R. (2021). Scavenger receptor a1 mediates the uptake of carboxylated and pristine multi-walled carbon nanotubes coated with bovine serum albumin. *Nanomaterials*, *11*(2), 539. https://doi.org/10.3390/nano11020539

55. Kasai, T., Umeda, Y., Ohnishi, M., Mine, T., Kondo, H., Takeuchi, T., Matsumoto, M., & Fukushima, S. (2015). Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. *Particle and Fibre Toxicology*, *13*(1), 53. https://doi.org/10.1186/s12989-016-0164-2

56. Kobayashi, N., Izumi, H., & Morimoto, Y. (2017). Review of toxicity studies of carbon nanotubes. *Journal of Occupational Health*, *59*(5), 394–407. https://doi.org/10.1539/joh.17-0089-RA

57. Lam, C., James, J. T., McCluskey, R., Holian, A., & Hunter, R. L. (2003). Toxicity of carbon nanotubes and its implications for occupational and environmental health. In C. S. S. R. Kumar (Ed.), *Nanotechnologies for the Life Sciences* (1st ed.). Wiley. https://doi.org/10.1002/9783527610419.ntls0053

58. Mamidi, N. (2019). Cytotoxicity evaluation of carbon nanotubes for biomedical and tissue engineering applications. In H. El-Din Saleh & S. Moawad Mohamed El-Sheikh

(Eds.), *Perspective of Carbon Nanotubes*. IntechOpen. https://doi.org/10.5772/intechopen.85899

59. Martin, D., Minchin, R. F., Belkina, M., Milev, A., & Kamali Kannangara, G. S. (2011). Toxicity and regulatory perspectives of carbon nanotubes. In *Polymer–Carbon Nanotube Composites* (pp. 621–653). Elsevier. https://doi.org/10.1533/9780857091390.2.621

60. Ryman-Rasmussen, J. P., Cesta, M. F., Brody, A. R., Shipley-Phillips, J. K., Everitt, J. I., Tewksbury, E. W., Moss, O. R., Wong, B. A., Dodd, D. E., Andersen, M. E., & Bonner, J. C. (2009). Inhaled carbon nanotubes reach the subpleural tissue in mice. *Nature Nanotechnology*, *4*(11), 747–751. https://doi.org/10.1038/nnano.2009.305

61. Zulkifli, N. A., Ismail, N. H., Jaafar, M., & Othman, A. R. (2022). Effect of carbon nanotubes on the flexural properties and flammability of carbon fibre/epoxy multi-scale composites. *Materialwissenschaft Und Werkstofftechnik*, *53*(12), 1544–1550. https://doi.org/10.1002/mawe.202100284

62. Donaldson, K., Aitken, R., Tran, L., Stone, V., Duffin, R., Forest, G., & Alexander, A. (2006).Carbon nanotubes: A review of their properties in relation to pulmonary toxicology and workplace safety, Toxicological Sci 92, 1, pp. 5-22.

63. Warheit, D.B., Laurence, B.R., Reed, K.L., Roach, D.H., Reynolds, G.A.M., & Webb, T.R.(2004). Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicological Sci, 77,pp.117-125.

64. Monteiro-Riviere, N.A., Namanich, R., Inman, A., Wang, Y., & Riviere, J. (2005). Multi-walled carbon nanotube interactions with human epidermal keratinocytes. ToxicolLett, 155, pp. 377-384.

65. Azmah Hanim, M. A., Hadirah, S. N., Dele-Afolabi, T. T., Jung, D. W., Sapuan, S. M., & Aiza Jaafar, C. N. (2022). Microstructure and hardness properties of aluminum matrix composites reinforced with iron (Iii) oxide nanoparticles and carbon nanotubes. *Materialwissenschaft Und Werkstofftechnik*, *53*(9), 1121–1127. https://doi.org/10.1002/mawe.202100246

66. Balakrishna, P., Sairam, A., & Surekh, P. (2011). Acute toxicological effects of multi-walled carbon nanotubes(Mwcnt). In M. Naraghi (Ed.), *Carbon Nanotubes—Growth and Applications*. InTech. https://doi.org/10.5772/18984

67. Bardi, G., Nunes, A., Gherardini, L., Bates, K., Al-Jamal, K. T., Gaillard, C., Prato, M., Bianco, A., Pizzorusso, T., & Kostarelos, K. (2013). Functionalized carbon nanotubes in the

brain: Cellular internalization and neuroinflammatory responses. *PLoS ONE*, *8*(11), e80964. https://doi.org/10.1371/journal.pone.0080964

68. Cui, D., Tian, F., Ozkan, C. S., Wang, M., & Gao, H. (2005). Effect of single wall carbon nanotubes on human HEK293 cells. *Toxicology Letters*, *155*(1), 73–85. https://doi.org/10.1016/j.toxlet.2004.08.015

69. Flahaut, E., Bacsa, R., Peigney, A., & Laurent, C. (2003). Gram-scale CCVD synthesis of double-walled carbon nanotubes. *Chemical Communications*, *12*, 1442. https://doi.org/10.1039/b301514a

70. Garibaldi, S., Brunelli, C., Bavastrello, V., Ghigliotti, G., & Nicolini, C. (2006). Carbon nanotube biocompatibility with cardiac muscle cells. *Nanotechnology*, *17*(2), 391–397. https://doi.org/10.1088/0957-4484/17/2/008

71. Han, S. G., Andrews, R., & Gairola, C. G. (2010). Acute pulmonary response of mice to multi-wall carbon nanotubes. *Inhalation Toxicology*, *22*(4), 340–347. https://doi.org/10.3109/08958370903359984

72. Ibrahim, J., Berk, B. C., & Hughes, A. D. (2006). Comparison of simultaneous measurements of blood pressure by tail-cuff and carotid arterial methods in conscious

spontaneously hypertensive and wistar-kyoto rats. *Clinical and Experimental Hypertension*, *28*(1), 57–72. https://doi.org/10.1080/10641960500386817

73. Kafa, H., Wang, J. T.-W., Rubio, N., Venner, K., Anderson, G., Pach, E., Ballesteros, B., Preston, J. E., Abbott, N. J., & Al-Jamal, K. T. (2015). The interaction of carbon nanotubes with an in vitro blood-brain barrier model and mouse brain in vivo. *Biomaterials*, *53*, 437–452. https://doi.org/10.1016/j.biomaterials.2015.02.083

74. Koyama, S., Endo, M., Kim, Y.-A., Hayashi, T., Yanagisawa, T., Osaka, K., Koyama, H., Haniu, H., & Kuroiwa, N. (2006). Role of systemic T-cells and histopathological aspects after subcutaneous implantation of various carbon nanotubes in mice. *Carbon*, *44*(6), 1079–1092. https://doi.org/10.1016/j.carbon.2005.08.006

75. Murray, A. R., Kisin, E., Leonard, S. S., Young, S. H., Kommineni, C., Kagan, V. E., Castranova, V., & Shvedova, A. A. (2009). Oxidative stress and inflammatory response in dermal toxicity of single-walled carbon nanotubes. *Toxicology*, *257*(3), 161–171. https://doi.org/10.1016/j.tox.2008.12.023

76. Petersen, E. J., Zhang, L., Mattison, N. T., O'Carroll, D. M., Whelton, A. J., Uddin, N., Nguyen, T., Huang, Q., Henry, T. B., Holbrook, R. D., & Chen, K. L. (2011). Potential release pathways, environmental fate, and ecological risks of carbon nanotubes.

Environmental Science & Technology, *45*(23), 9837–9856. https://doi.org/10.1021/es201579y

77. Piao, Y., Chen, C.-F., Green, A. A., Kwon, H., Hersam, M. C., Lee, C. S., Schatz, G. C., & Wang, Y. (2011). Optical and electrical properties of inner tubes in outer wall-selectively functionalized double-wall carbon nanotubes. *The Journal of Physical Chemistry Letters*, *2*(13), 1577–1582. https://doi.org/10.1021/jz200687u

78. Porter, D. W., Hubbs, A. F., Chen, B. T., McKinney, W., Mercer, R. R., Wolfarth, M. G., Battelli, L., Wu, N., Sriram, K., Leonard, S., Andrew, M., Willard, P., Tsuruoka, S., Endo, M., Tsukada, T., Munekane, F., Frazer, D. G., & Castranova, V. (2013). Acute pulmonary dose–responses to inhaled multi-walled carbon nanotubes. *Nanotoxicology*, *7*(7), 1179–1194. https://doi.org/10.3109/17435390.2012.719649

79. Seaton, A., Tran, L., Aitken, R., & Donaldson, K. (2010). Nanoparticles, human health hazard and regulation. *Journal of The Royal Society Interface*, *7*(suppl_1). https://doi.org/10.1098/rsif.2009.0252.focus

80. Stadelmann, W. K., Digenis, A. G., & Tobin, G. R. (1998). Physiology and healing dynamics of chronic cutaneous wounds. *The American Journal of Surgery*, *176*(2), 26S-38S. https://doi.org/10.1016/S0002-9610(98)00183-4

81. Tang, S., Tang, Y., Zhong, L., Murat, K., Asan, G., Yu, J., Jian, R., Wang, C., & Zhou, P. (2012). Short- and long-term toxicities of multi-walled carbon nanotubes *in vivo* and *in vitro*. *Journal of Applied Toxicology*, *32*(11), 900–912. https://doi.org/10.1002/jat.2748

82. Van Angelen, A. A., Glaudemans, B., Van Der Kemp, A. W. C. M., Hoenderop, J. G. J., & Bindels, R. J. M. (2013). Cisplatin-induced injury of the renal distal convoluted tubule is associated with hypomagnesaemia in mice. *Nephrology Dialysis Transplantation*, *28*(4), 879–889. https://doi.org/10.1093/ndt/gfs499

83. Wang, X., Katwa, P., Podila, R., Chen, P., Ke, P. C., Rao, A. M., Walters, D. M., Wingard, C. J., & Brown, J. M. (2011). Multi-walled carbon nanotube instillation impairs pulmonary function in c57bl/6 mice. *Particle and Fibre Toxicology*, *8*(1), 24. https://doi.org/10.1186/1743-8977-8-24

84. Awasthi, K. K., John, P. J., Awasthi, A., & Awasthi, K. (2013). Multi walled carbon nano tubes induced hepatotoxicity in Swiss albino mice. *Micron*, *44*, 359–364. https://doi.org/10.1016/j.micron.2012.08.008

85. Almanza, A., Carlesso, A., Chintha, C., Creedican, S., Doultsinos, D., Leuzzi, B., Luís, A., McCarthy, N., Montibeller, L., More, S., Papaioannou, A., Püschel, F., Sassano, M. L., Skoko, J., Agostinis, P., De Belleroche, J., Eriksson, L. A., Fulda, S., Gorman, A. M., …

Samali, A. (2019). Endoplasmic reticulum stress signalling – from basic mechanisms to clinical applications. *The FEBS Journal*, *286*(2), 241–278. https://doi.org/10.1111/febs.14608

86. Boyles, M., Stoehr, L., Schlinkert, P., Himly, M., & Duschl, A. (2014). The significance and insignificance of carbon nanotube-induced inflammation. *Fibers*, *2*(1), 45–74. https://doi.org/10.3390/fib2010045

87. Darne, C., Terzetti, F., Coulais, C., Fontana, C., Binet, S., Gaté, L., & Guichard, Y. (2014). Cytotoxicity and genotoxicity of panel of single- and multiwalled carbon nanotubes: *In vitro* effects on normal syrian hamster embryo and immortalized v79 hamster lung cells. *Journal of Toxicology*, *2014*, 1–15. https://doi.org/10.1155/2014/872195

88. Dong, J., & Ma, Q. (2019). Integration of inflammation, fibrosis, and cancer induced by carbon nanotubes. *Nanotoxicology*, *13*(9), 1244–1274. https://doi.org/10.1080/17435390.2019.1651920

89. Ema, M., Masumori, S., Kobayashi, N., Naya, M., Endoh, S., Maru, J., Hosoi, M., Uno, F., Nakajima, M., Hayashi, M., & Nakanishi, J. (2013). *In vivo* comet assay of multi-walled carbon nanotubes using lung cells of rats intratracheally instilled. *Journal of Applied Toxicology*, *33*(10), 1053–1060. https://doi.org/10.1002/jat.2810

90. Florek, E., Witkowska, M., Szukalska, M., Richter, M., Trzeciak, T., Miechowicz, I., Marszałek, A., Piekoszewski, W., Wyrwa, Z., & Giersig, M. (2023). Oxidative stress in long-term exposure to multi-walled carbon nanotubes in male rats. *Antioxidants*, *12*(2), 464. https://doi.org/10.3390/antiox12020464

91. Fraga, C. G., Leibovitz, B. E., & Tappel, A. L. (1988). Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: Characterization and comparison with homogenates and microsomes. *Free Radical Biology and Medicine*, *4*(3), 155–161. https://doi.org/10.1016/0891-5849(88)90023-8

92. Grant, D. M. (1991). Detoxification pathways in the liver. *Journal of Inherited Metabolic Disease*, *14*(4), 421–430. https://doi.org/10.1007/BF01797915

93. Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (Sod), catalase (Cat) and glutathione peroxidase (Gpx): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, *54*(4), 287–293. https://doi.org/10.1016/j.ajme.2017.09.001

94. Ismail, N., Okasha, S., Dhawan, A., Abdel-Rahman, A., Shaker, O., & Sadik, N. (2010). Antioxidant enzyme activities in hepatic tissue from children with chronic cholestatic liver disease. *Saudi Journal of Gastroenterology*, *16*(2), 90. https://doi.org/10.4103/1319-3767.61234

95. Jeong, J. Y., Lee, D. H., & Kang, S. S. (2013). Effects of chronic restraint stress on body weight, food intake, and hypothalamic gene expressions in mice. *Endocrinology and Metabolism*, *28*(4), 288. https://doi.org/10.3803/EnM.2013.28.4.288

96. Ji, Z., Zhang, D., Li, L., Shen, X., Deng, X., Dong, L., Wu, M., & Liu, Y. (2009). The hepatotoxicity of multi-walled carbon nanotubes in mice. *Nanotechnology*, *20*(44), 445101. https://doi.org/10.1088/0957-4484/20/44/445101

97. Kaplowitz, N. (1981). The importance and regulation of hepatic glutathione. *The Yale Journal of Biology and Medicine*, *54*(6), 497–502.

98. Kong, Y., Ding, Z., Zhang, Y., Zhou, P., Wu, C., Zhu, M., & Ye, J. (2019). Types of carbohydrate in feed affect the growth performance, antioxidant capacity, immunity, and activity of digestive and carbohydrate metabolism enzymes in juvenile Macrobrachium nipponense. *Aquaculture*, *512*, 734282. https://doi.org/10.1016/j.aquaculture.2019.734282

99. Kulasza, M., & Skuza, L. (2021). Changes of gene expression patterns from aquatic organisms exposed to metal nanoparticles. *International Journal of Environmental Research and Public Health*, *18*(16), 8361. https://doi.org/10.3390/ijerph18168361

100. Li, S., Tan, H.-Y., Wang, N., Zhang, Z.-J., Lao, L., Wong, C.-W., & Feng, Y. (2015). The role of oxidative stress and antioxidants in liver diseases. *International Journal of Molecular Sciences*, *16*(11), 26087–26124. https://doi.org/10.3390/ijms161125942

101. Manke, A., Wang, L., & Rojanasakul, Y. (2013). Mechanisms of nanoparticle-induced oxidative stress and toxicity. *BioMed Research International*, *2013*, 1–15. https://doi.org/10.1155/2013/942916

102. Martin, L. J. (2008). Dna damage and repair: Relevance to mechanisms of neurodegeneration. *Journal of Neuropathology & Experimental Neurology*, *67*(5), 377–387. https://doi.org/10.1097/NEN.0b013e31816ff780

103. Martín-Pardillos, A., & Martin-Duque, P. (2023). Cellular alterations in carbohydrate and lipid metabolism due to interactions with nanomaterials. *Journal of Functional Biomaterials*, *14*(5), 274. https://doi.org/10.3390/jfb14050274

104. Mohamadi-Nejad, A., Moosavi-Movahedi, A. A., Hakimelahi, G. H., & Sheibani, N. (2002). Thermodynamic analysis of human serum albumin interactions with glucose: Insights into the diabetic range of glucose concentration. *The International Journal of Biochemistry & Cell Biology*, *34*(9), 1115–1124. https://doi.org/10.1016/S1357-2725(02)00031-6

105. Moradian, R., Najdegerami, E. H., Nikoo, M., & Nejati, V. (2022). Ameliorating effects of bioactive peptides extracted from litopenaeus vannamei wastes on oxidative stress,

glucose regulation, and autophagy gene expression in nonalcoholic fatty liver-induced rats. *Evidence-Based Complementary and Alternative Medicine*, *2022*, 1–12. https://doi.org/10.1155/2022/2679634

106. Ngo, W., Ahmed, S., Blackadar, C., Bussin, B., Ji, Q., Mladjenovic, S. M., Sepahi, Z., & Chan, W. C. W. (2022). Why nanoparticles prefer liver macrophage cell uptake in vivo. *Advanced Drug Delivery Reviews*, *185*, 114238. https://doi.org/10.1016/j.addr.2022.114238

107. Noga, M., Milan, J., Frydrych, A., & Jurowski, K. (2023). Toxicological aspects, safety assessment, and green toxicology of silver nanoparticles (Agnps)—critical review: State of the art. *International Journal of Molecular Sciences*, *24*(6), 5133. https://doi.org/10.3390/ijms24065133

108. Pacurari, M., Yin, X. J., Ding, M., Leonard, S. S., Schwegler-berry, D., Ducatman, B. S., Chirila, M., Endo, M., Castranova, V., & Vallyathan, V. (2008). Oxidative and molecular interactions of multi-wall carbon nanotubes (Mwcnt) in normal and malignant human mesothelial cells. *Nanotoxicology*, *2*(3), 155–170. https://doi.org/10.1080/17435390802318356

109. Pan, L., Sui, J., Xu, Y., Zhao, Q., Cai, Y., Sun, G., & Xia, H. (2023). Effect of fine particulate matter exposure on liver enzymes: A systematic review and meta-analysis.

International Journal of Environmental Research and Public Health, *20*(4), 2803. https://doi.org/10.3390/ijerph20042803

110. Sadauskas, E., Wallin, H., Stoltenberg, M., Vogel, U., Doering, P., Larsen, A., & Danscher, G. (2007). Kupffer cells are central in the removal of nanoparticles from the organism. *Particle and Fibre Toxicology*, *4*(1), 10. https://doi.org/10.1186/1743-8977-4-10

111. Sharma, C. S., Sarkar, S., Periyakaruppan, A., Barr, J., Wise, K., Thomas, R., Wilson, B. L., & Ramesh, G. T. (2007). Single-walled carbon nanotubes induces oxidative stress in rat lung epithelial cells. *Journal of Nanoscience and Nanotechnology*, *7*(7), 2466–2472. https://doi.org/10.1166/jnn.2007.431

112. Uges, D. R. A. (1988). Plasma or serum in therapeutic drug monitoring and clinical toxicology. *Pharmaceutisch Weekblad Scientific Edition*, *10*(5), 185–188. https://doi.org/10.1007/BF01956868

113. Ungvari, A., Kiss, T., Gulej, R., Tarantini, S., Csik, B., Yabluchanskiy, A., Mukli, P., Csiszar, A., Harris, M. L., & Ungvari, Z. (2024). Irradiation-induced hair graying in mice: An experimental model to evaluate the effectiveness of interventions targeting oxidative stress, DNA damage prevention, and cellular senescence. *GeroScience*, *46*(3), 3105–3122. https://doi.org/10.1007/s11357-023-01042-7

114. Urushihara, Y., Kawasumi, K., Endo, S., Tanaka, K., Hirakawa, Y., Hayashi, G., Sekine, T., Kino, Y., Kuwahara, Y., Suzuki, M., Fukumoto, M., Yamashiro, H., Abe, Y., Fukuda, T., Shinoda, H., Isogai, E., Arai, T., & Fukumoto, M. (2016). Analysis of plasma protein concentrations and enzyme activities in cattle within the ex-evacuation zone of the fukushima daiichi nuclear plant accident. *PLOS ONE*, *11*(5), e0155069. https://doi.org/10.1371/journal.pone.0155069

115. Wan, R., Mo, Y., Feng, L., Chien, S., Tollerud, D. J., & Zhang, Q. (2012). Dna damage caused by metal nanoparticles: Involvement of oxidative stress and activation of atm. *Chemical Research in Toxicology*, *25*(7), 1402–1411. https://doi.org/10.1021/tx200513t

116. Weinberger, B., Watorek, K., Strauss, R., Witz, G., Hiatt, M., & Hegyi, T. (2002). [No title found]. *Critical Care*, *6*(6), 521. https://doi.org/10.1186/cc1547

117. Witkowska, M., Florek, E., & Mrówczyński, R. (2022). Assessment of pristine carbon nanotubes toxicity in rodent models. *International Journal of Molecular Sciences*, *23*(23), 15343. https://doi.org/10.3390/ijms232315343

118. Yang, X., Zhang, X., Shu, X., Zhang, W., Kai, J., Tang, M., Gong, J., Yang, J., Lin, J., Chai, Y., & Liu, J. (2022). Effects of multi-walled carbon nanotubes in soil on earthworm

growth and reproduction, enzymatic activities, and metabolomics. *Ecotoxicology and Environmental Safety*, *246*, 114158. https://doi.org/10.1016/j.ecoenv.2022.114158

119. Zhang, X., Jiao, L., Li, M., Ren, F., Tao, X., Jin, M., Zhang, L., Liu, W., & Zhou, Q. (2023). Effects of different dietary carbohydrate-to-protein ratios on the growth performance, antioxidant capacity and energy metabolism in Marsupenaeus japonicus. *Aquaculture Reports*, *30*, 101626. https://doi.org/10.1016/j.aqrep.2023.101626

120. Zhang, Y.-N., Poon, W., Tavares, A. J., McGilvray, I. D., & Chan, W. C. W. (2016). Nanoparticle–liver interactions: Cellular uptake and hepatobiliary elimination. *Journal of Controlled Release*, *240*, 332–348. https://doi.org/10.1016/j.jconrel.2016.01.020

CHAPTER 8: APPENDIX

APPENDIX I

8.1 GRAPHS

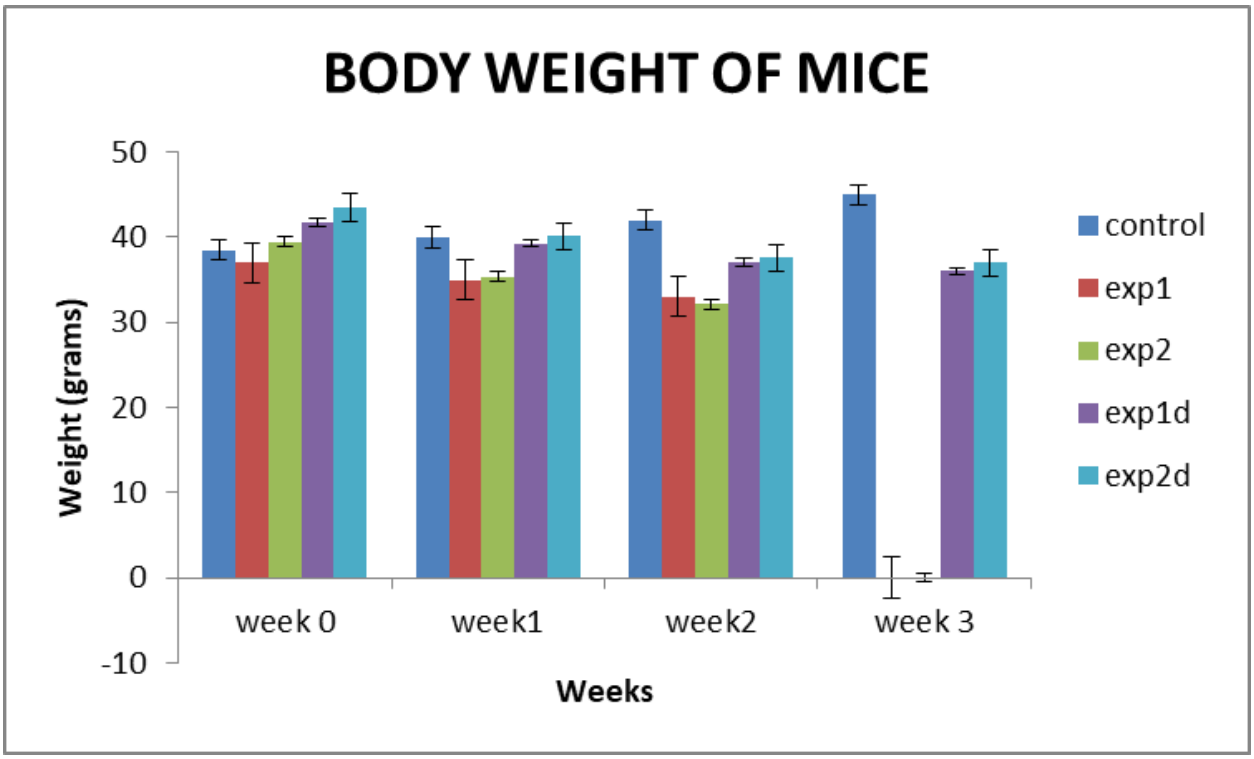

Fig.8.1 Body weight of mice after MWCNTs exposure

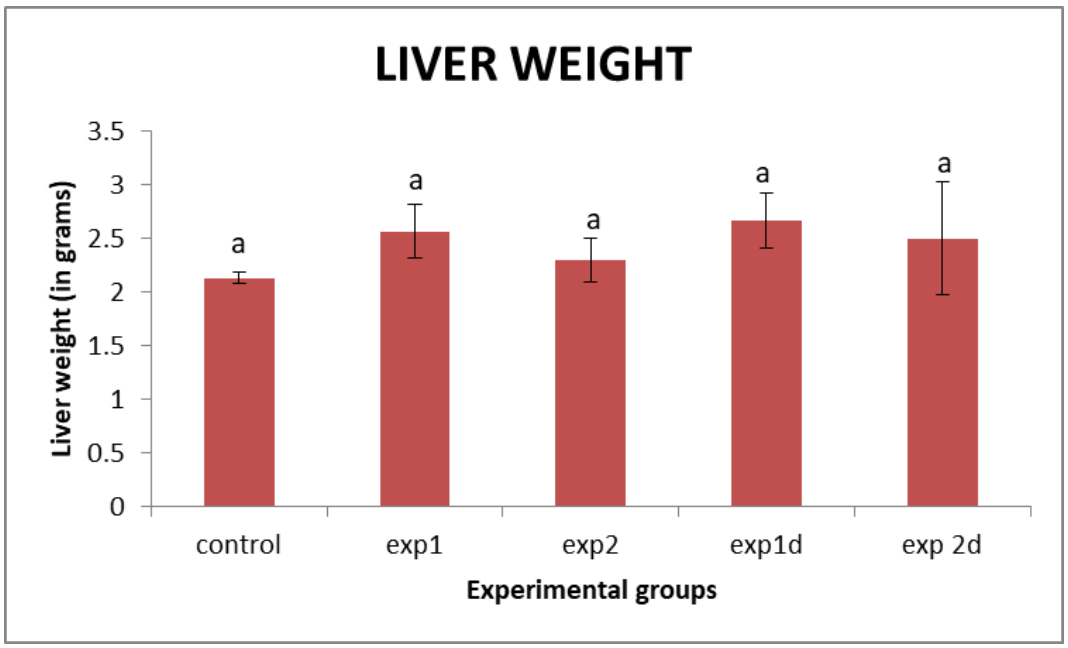

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Fig.8.2 Liver organ index (same letter indicates non-significant differences as per Tukey's test)

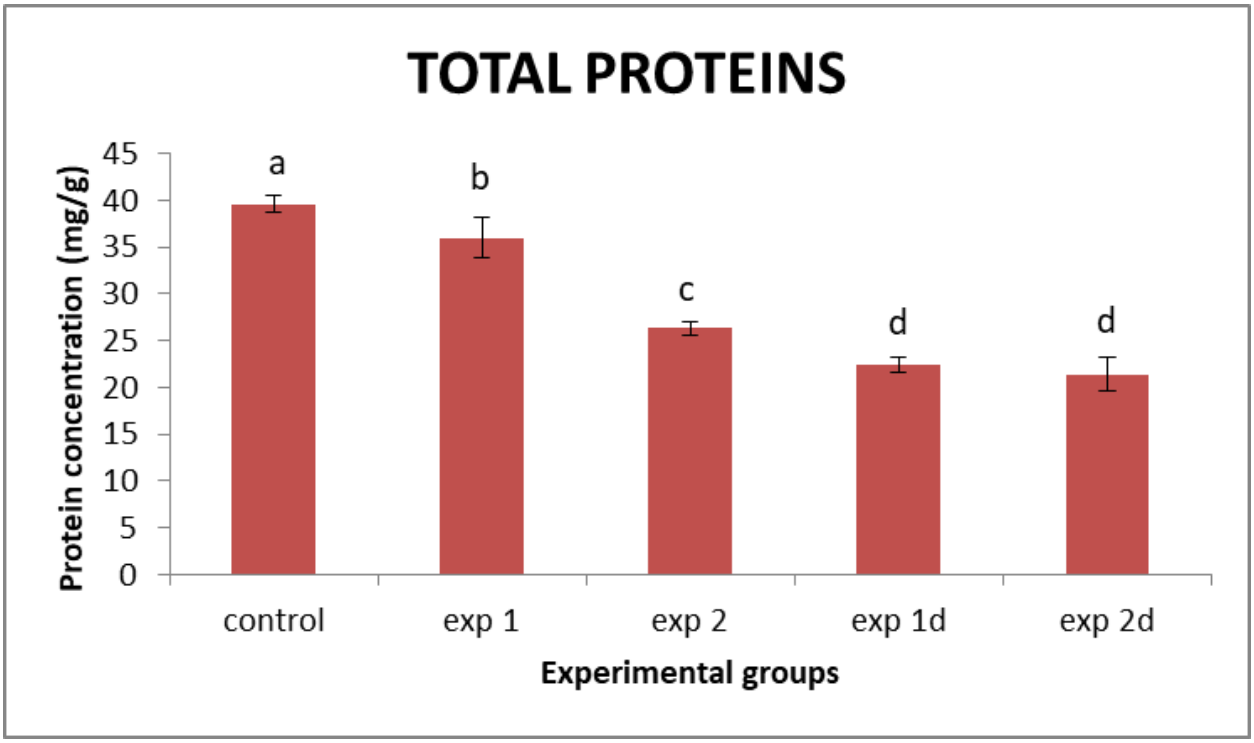


Fig. 8.3 Effect of MWCNTs on Total proteins content in the liver of mice. (Different letters indicate significant difference between groups as per Tukey's test at $p \leq 0.05$.Same letters indicate non-significant difference as per Tukey's test.)

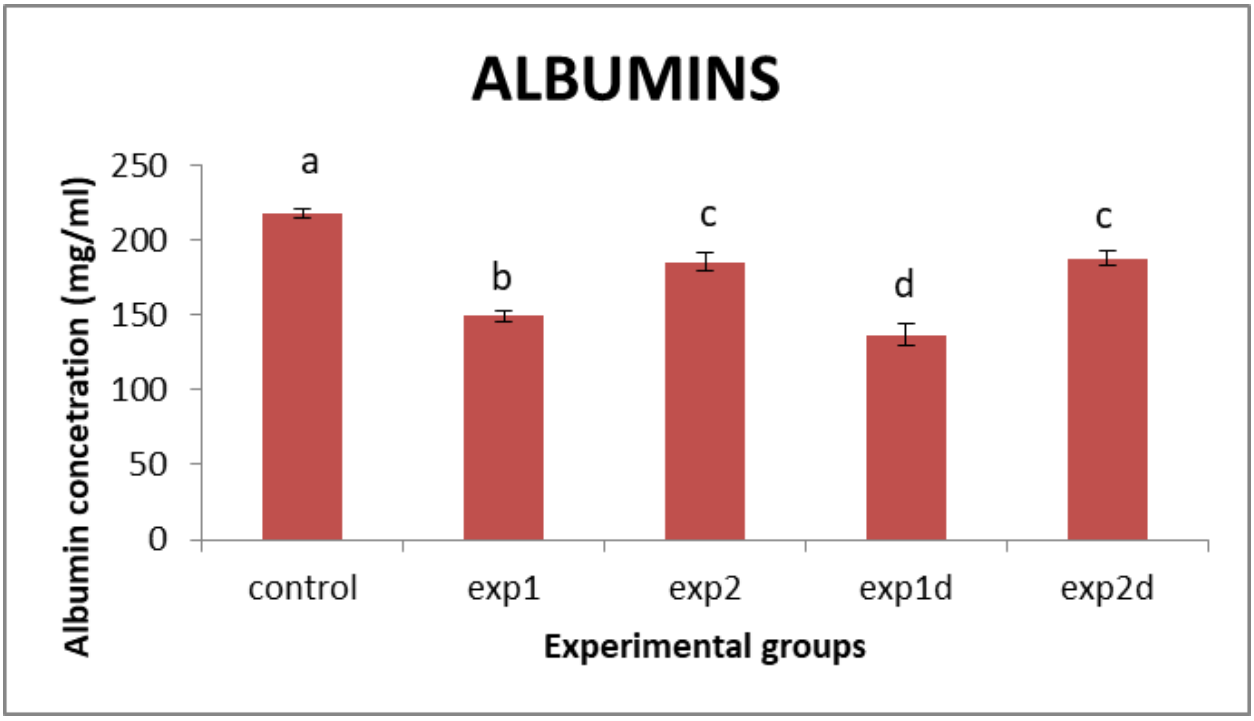


Fig. 8.4 Effect of MWCNTs on Albumin content in the liver of mice. (Different letters indicate significant difference between groups as per Tukey's test at $p \leq 0.05$.Same letters indicate non-significant difference as per Tukey's test.)

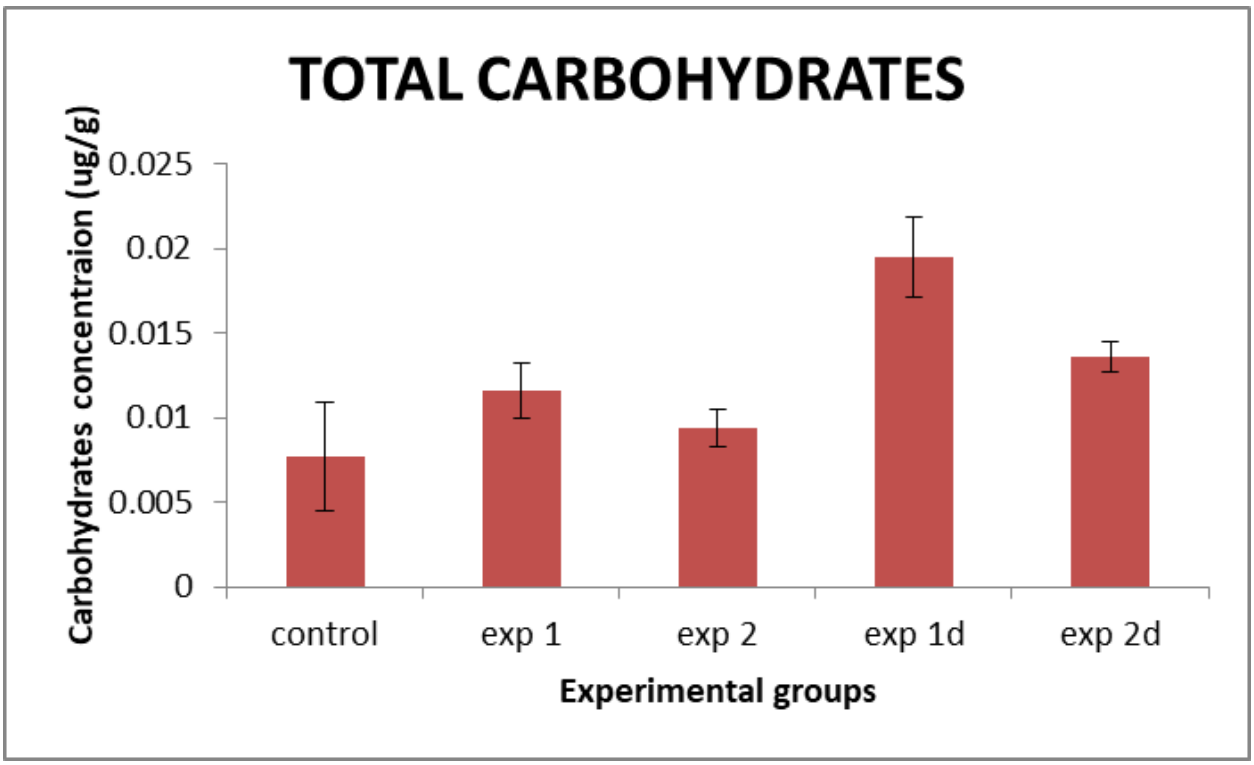


Fig. 8.5 Effect of MWCNTs on Total Carbohydrates content in the liver of mice. (Different letters indicate significant difference between groups as per Tukey's test at $p \leq 0.05$.Same letters indicate non-significant difference as per Tukey's test.)

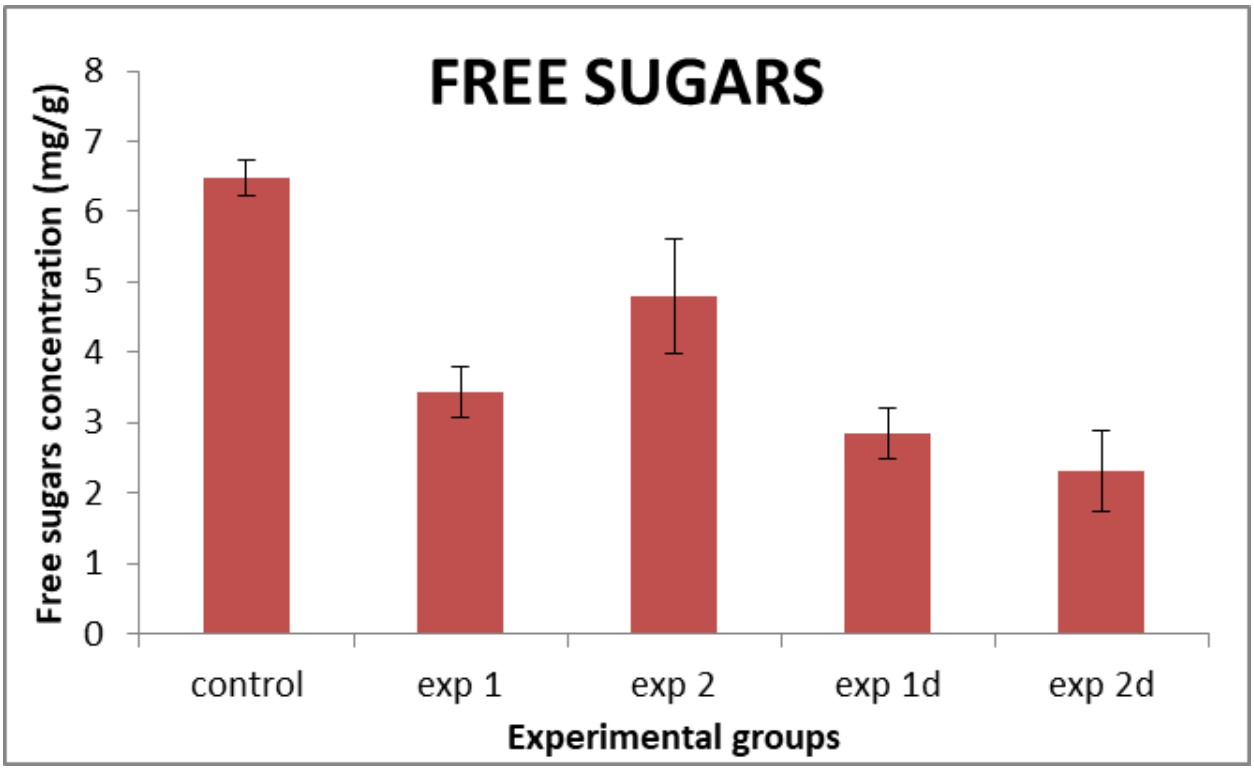


Fig. 8.6 Effect of MWCNTs on Free sugars content in the liver of mice. (Different letters indicate significant difference between groups as per Tukey's test at $p \leq 0.05$.Same letters indicate non-significant difference as per Tukey's test.)

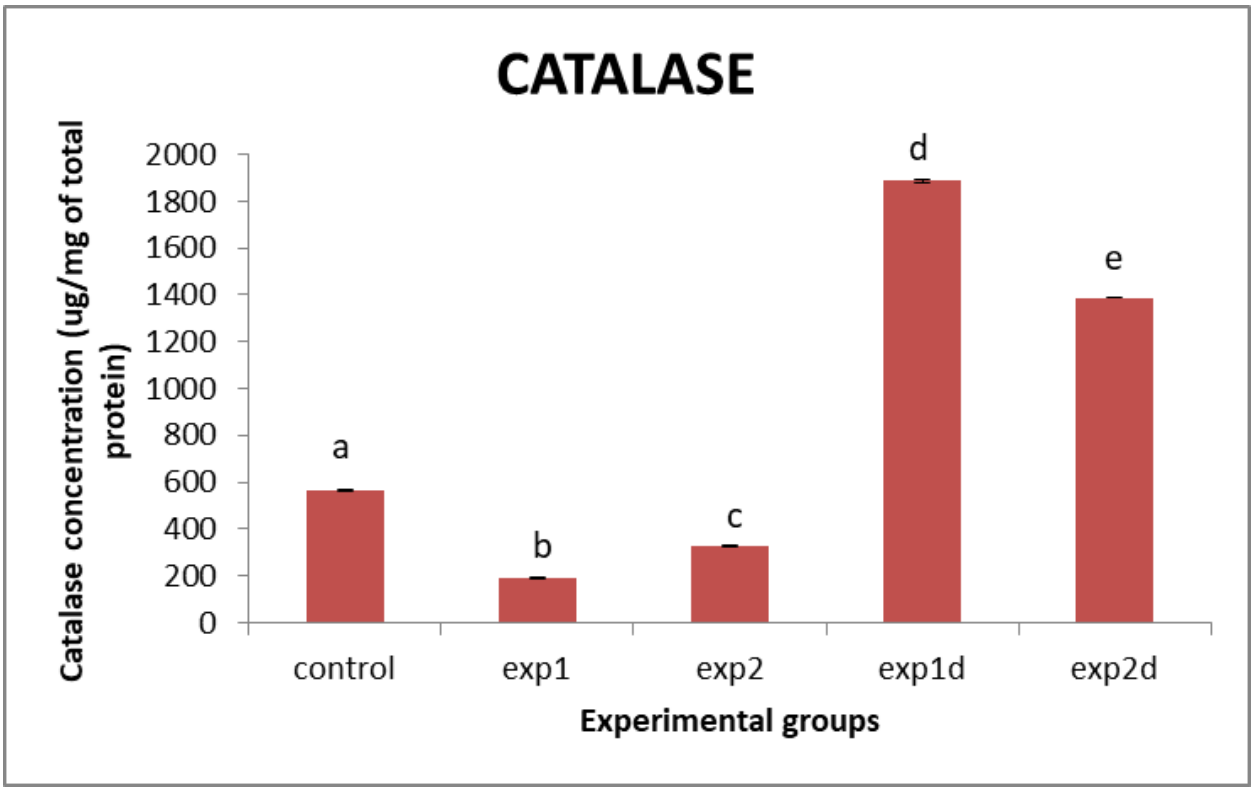


Fig. 8.7 Effect of MWCNTs on Catalase content in the blood serum of mice. (Different letters indicate significant difference between groups as per Tukey's test at $p \leq 0.05$.)

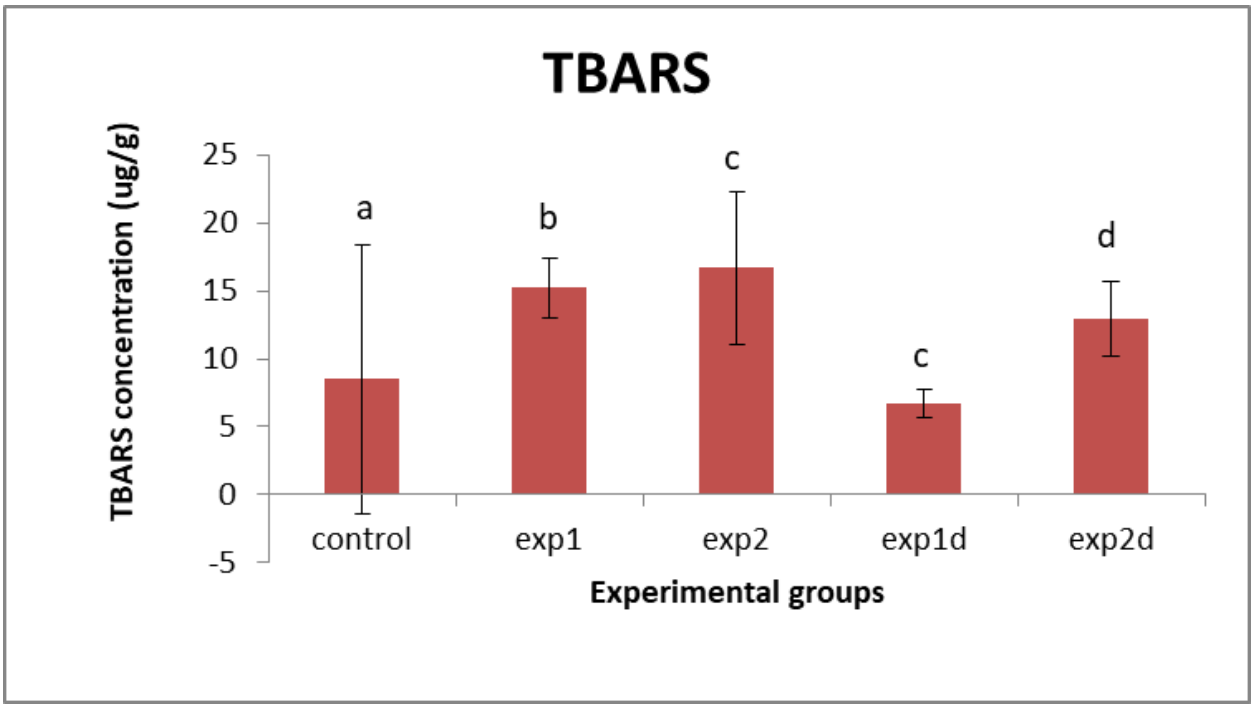


Fig. 8.9 Effect of MWCNTs on TBARS content in the liver tissue of mice. (Different letters indicate significant difference between groups as per Tukey's test at $p \leq 0.05$. Same letters indicate non-significant difference as per Tukey's test.)

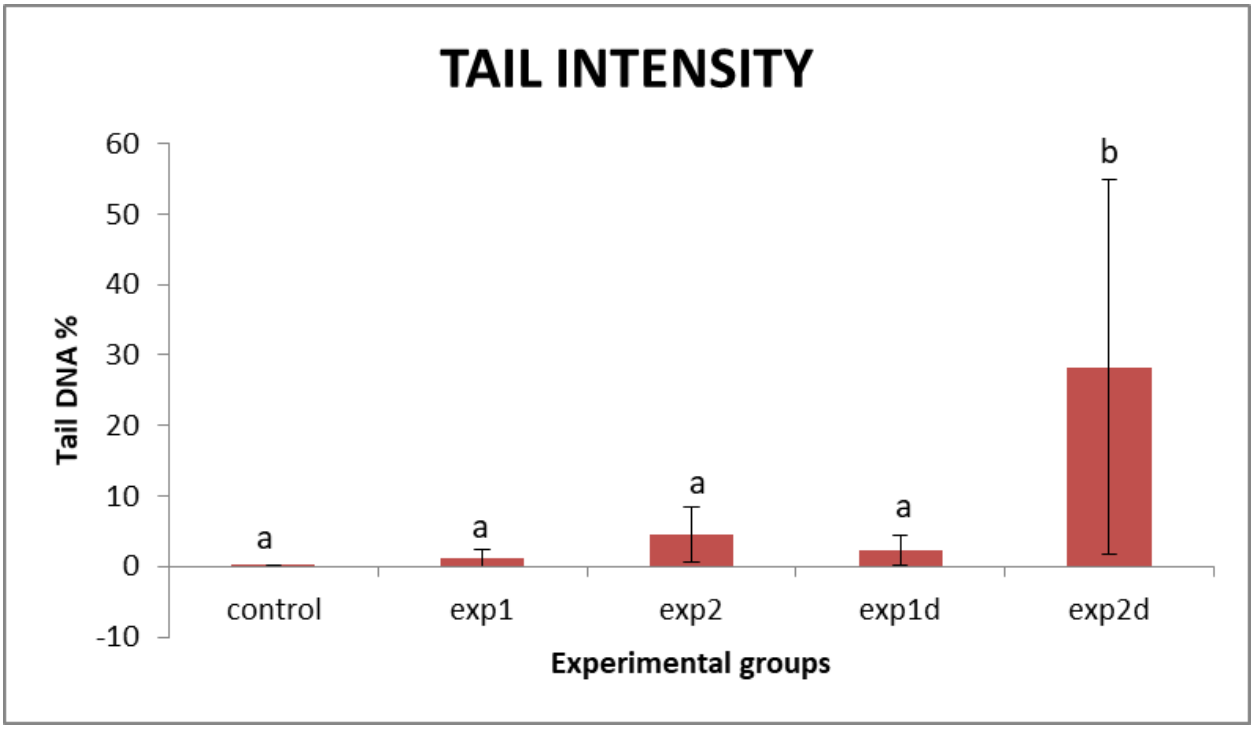


Fig. 8.13 Tail intensity in Comet assay (Different letters indicate significant difference between groups as per Tukey's test at $p \leq 0.05$. Same letters indicate non-significant difference as per Tukey's test.)

APPENDIX II

8.2 TABLES

Morphology	exp1	exp2	exp1d	exp2d	control
	week1	week2	week1	week2	
Fur density	normal	normal	normal	normal	normal
Fur color	white	white	white	white	white
Fur texture	smooth	smooth	smooth	smooth	smooth
Tail spots	nil	nil	nil	nil	nil

Fig. 8.15 Morphological changes observed in mice after exposure and natural detoxification of MWCNTs

Behavior	Experiment 1		Experiment 2		Exp 1 d	Exp 2 d	Control
	Week 1	Week 2	Week 1	Week 2	Week 3	Week 4	
Drowsiness	normal	increased	normal	increased	increased	increased	normal
Lethargy	normal	increased	normal	increased	increased	increased	normal
Isolation	normal	normal	normal	increased	normal	increased	normal
Feeding frequency	normal	decreased	normal	decreased	increased	increased	normal

Fig. 8.16 Behavioral changes observed in mice after exposure and natural detoxification of MWCNTs

APPENDIX III

8.3 FIGURES

Fig. 8.18 MWCNTs acquired from AdNano technologies

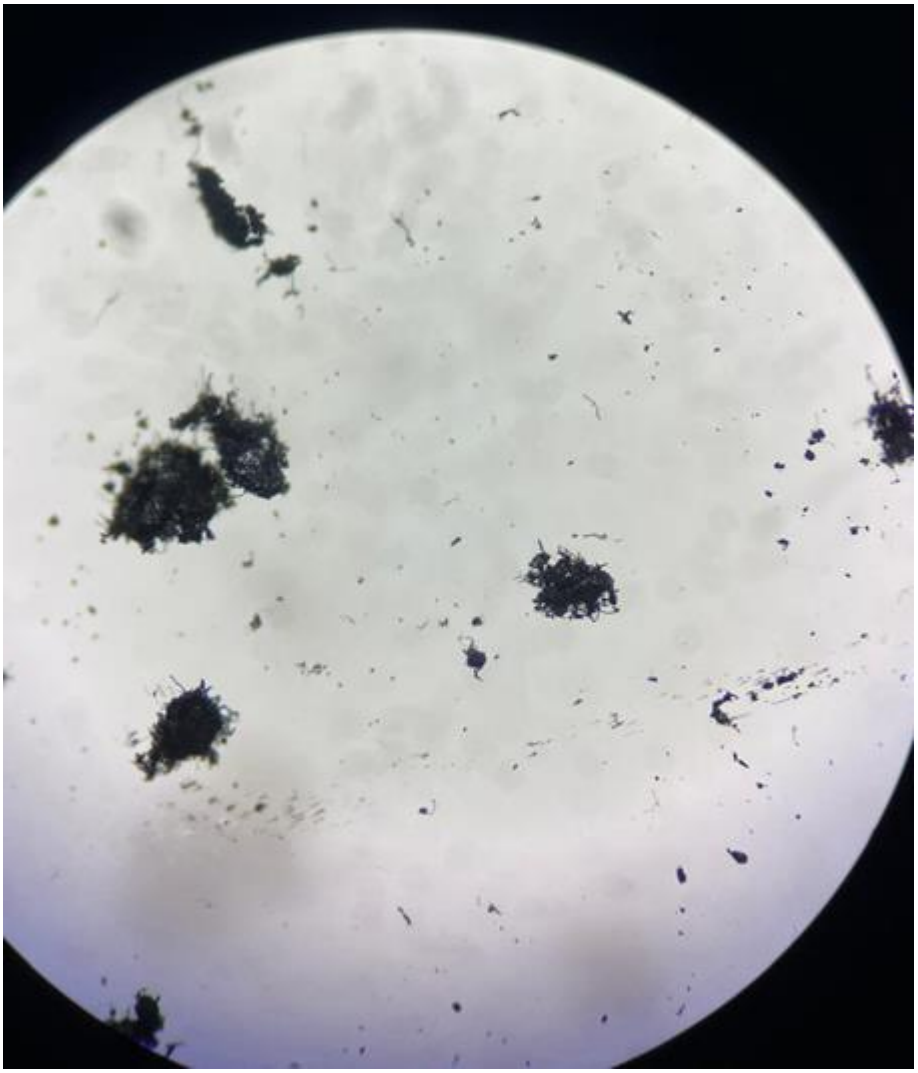


Fig. 8.19 MWCNTs observed under stereomicroscope

Fig. 8.20 MWCNTs doses prepared (a:50 mg/kg of body weight b:100 mg/kg of body weight)

Fig. 8.21 Experimental set-up (a: Experimental groups 1 and 2 b:Control group)

APPENDIX IV

8.4 PLATES

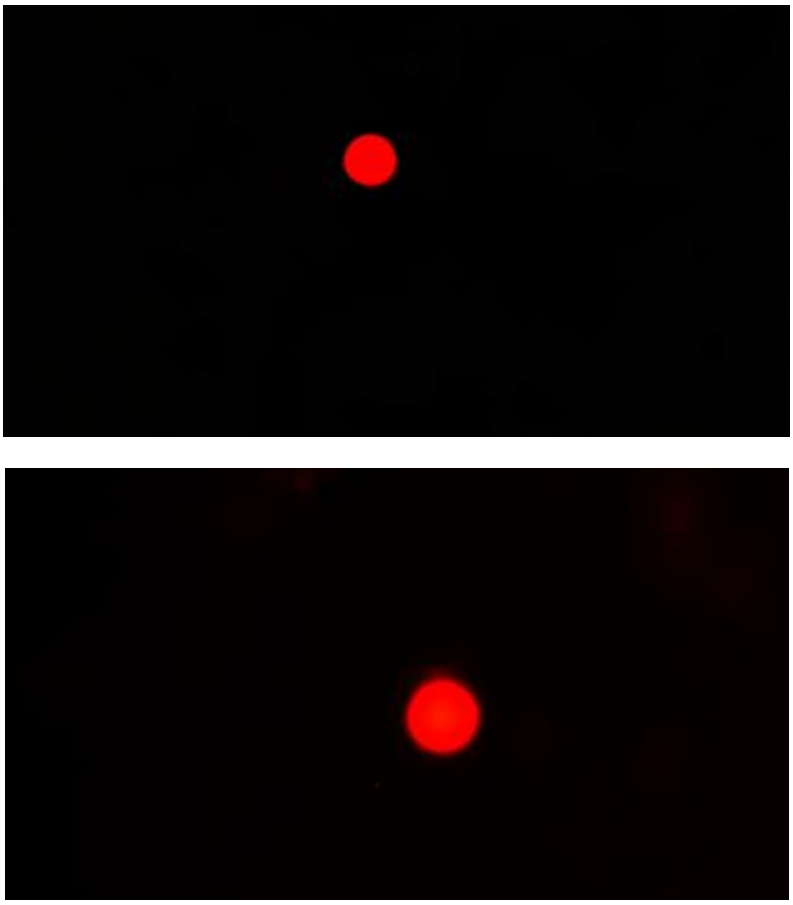
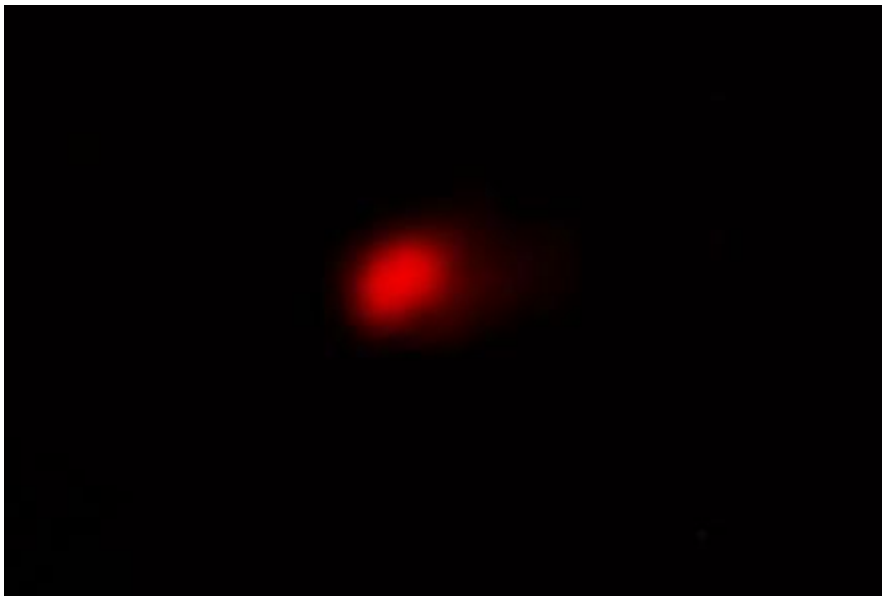


Plate 1: Bone marrow cells of control group under 20x magnification

Plate 2: Bone marrow cells of experimental groups after sub-acute exposure to MWCNTs under 20x magnification (a: Experimental 1 b:Experimental 2)



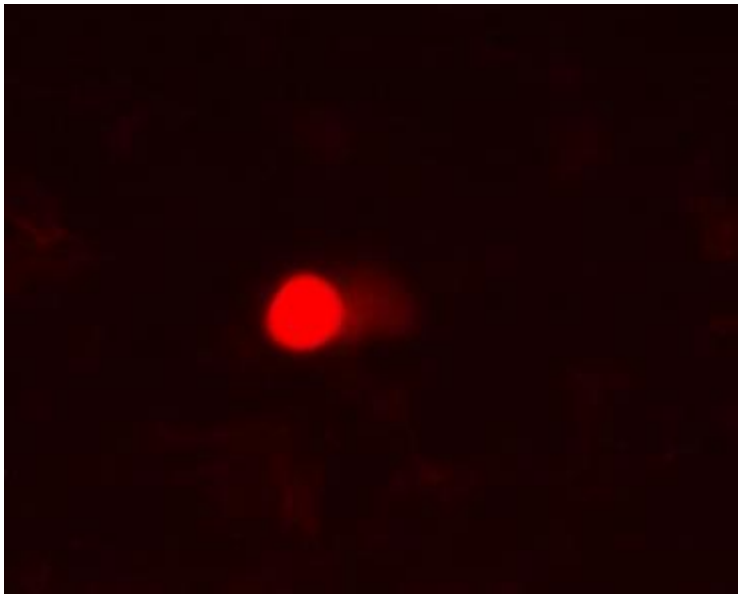


Plate 3: Bone marrow cells of experimental groups after natural detoxification under 20x magnification (a: Experimental 1 b:Experimental 2)

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