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Investigating the impact of Multiwalled Carbon Nanotubes exposure on enzymatic activities and histopathological variations in *Swiss albino mice*

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Present study was conducted to evaluate the detrimental impacts of exposure of Multi-walled Carbon Nanotubes (MWCNT-NP) on enzymatic activities and tissue structures in Swiss albino mice. The experimental groups of mice received MWCNT-NP for specific time period (seven or fourteen days). Two distinct doses of the MWCNT-NP solution were given orally: 0.45 µg and 0.90 µg, and the distilled water was given to the control group. Serum samples were extracted at 7 and 14 days after the experiment by centrifuging whole blood for 15 min at 3,000 rpm. An enzyme-linked immunosorbent test (ELISA) was used to measure many enzyme assays, such as Angiotensin Converting Enzymes (ACE), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase enzyme. Hematoxylin and Eosin (H&E) staining of tissue samples was done along with a histopathological examination. During a 14-day exposure, ACE, NADPH Oxidase, ALT, and AST enzyme levels were significantly higher in the exposed groups (0.45 µg and 0.90 µg) than in the control group ($p < 0.05$). Male mice exposed to MWCNT-NP showed substantial histological damage in the relevant organs as well as elevated enzyme activity levels. Present study showed a comprehensive and practical assessment of the toxicity associated with MWCNT-NP of different geometries and functionalization.

Keywords Nanoparticles, Multi-walled Carbon nanotubes, Toxicity, 3Rs Principle

Nanoparticles are described as tiny particles that could dissolve and had one or more dimensions larger than their exterior ones. In addition, because of their unique attributes, nanoparticles have drawn a lot of interest and are being used in a variety of industries and medical domains. Additionally, a wide range of sectors have employed nanoparticles such as electronics industry, rubber sector, food additives, biosensors, paints, dyes, inks, as well as the cosmetics industry and sunscreens¹. Although nanoparticles have various applications, it is essential to take into account their size, shape, and chemical composition. Nanoparticles have numerous advantages in the field of electronics. Additionally, cellular network connections, the endocytic route, and the absorption process have been cited as potential sources of cytotoxicity and disruptions to cellular homeostasis². The danger posed by nanoparticles is strongly correlated with their size, which plays a crucial role in their interaction with biological systems. Smaller nanoparticles may be capable of absorbing a higher concentration of chemical compounds due to their larger surface area per mass unit. MWCNTs have diameters of 5 to 100 nm, whereas polyhedral diameters can exceed 100 nm, primarily due to the number of nanotube wall layers and functional groups attached to them. Due to increased cellular reactivity, there are consequently more toxicological effects³. Toxicity is also affected by the surface area of the nanoparticles because surface area and absorption efficiency are related⁴. Hence, the objective of nanotoxicity research is to decide how much these attributes could imperil the climate or the existence of living things. Nanomedicines are being developed to reduce the toxic effects

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of medications while simultaneously increasing their bioavailability and biocompatibility. The production of MWCNT raises safety concerns because of the potential threats these industrial waste-derived nanoparticles may pose risk to the environment and workplace. While research on potential risks associated CNTs have been centered around skin contact and inhalation, it's fundamental to remember that workers might swallow CNTs orally in situations where CNTs are utilized in various applications. The nature of the work environment can also lead to ingestion exposure. Industrial workers may unintentionally come into contact with CNT-containing products on their hands since they handle them frequently⁵. When eating or drinking, there is a real possibility that contaminated hands could transfer CNTs to the mouth if proper hygiene measures and personal protective equipment are not taken⁶. Even though research on the health effects of carbon nanotube exposure is still ongoing, oral consumption is a possibility that should not be ignored. To protect workers' health in this rapidly changing technological world, adequate training programs, stringent safety regulations, and industry-wide risk management strategies are required⁷. Numerous researches on nanotoxicity are being carried out in order to guarantee safe industrial production and lessen the influence of these nanomaterials on the environment. The purpose of these investigation is to address worries about the possible toxicity of multiwalled carbon nanotubes (MWCNT), which can provide serious exposure risks in environmental and occupational contexts. As highlighted in several publications, scientists have discovered that nanomaterials mostly enter the human body by ingestion, inhalation, or skin contact⁸.

The toxicity of MWCNTs is determined by the degree of surface functionalization and the toxicity of the functional groups. MWCNTs' toxicity is significantly influenced by their bioavailability. Studies and attention must be paid to metabolism, degradation, dissolution, clearance, and bioaccumulation in order to fully comprehend the limitations of nanoparticles as medicines. Utilizing carbon nanotubes has been found to be problematic in terms of biocompatibility. CNTs interact with organs and body fluids once they enter the body. Carbon nanotubes typically enter the human body through the mouth, nose, or skin, as stated by Staron⁹. The respiratory and digestive systems are then attacked, and skin erosion is the result. The organ-specific toxicity of CNTs has been demonstrated by research. CNTs' biodistribution within the body may result in a variety of toxicities based on their concentration, composition, size, shape, and functionalization¹⁰. Importantly, the interference of CNTs with luminescence-based tests, which are frequently used to determine a variety of cytotoxic effects¹¹. MWCNTs might get into the hands of workers during production, use, and disposal. Severe control conventions and individual personal protective equipment are required while taking care of nano objects and agglomerates greater than 100 nm (NOAA). There is still a lack of comprehensive research on the health effects of several nanomaterials. The majority of studies have focused on demonstrating pulmonary damage, such as inflammation and fibrosis, following oral, intratracheal, or inhalation exposures^{12–14}, genotoxicity^{15–18}, cytotoxicity^{19–21}, hepatotoxicity^{22–24}.

Present research was conducted to comprehend the adverse impacts of multi-walled carbon nanotubes (MWCNT-NP) exposure on visceral organs in Swiss albino mice. The study accomplishes a comprehensive analysis of the expected enzymatic interruptions inside the testicles, lungs, and liver by utilizing a scope of MWCNT-NP dosage, exposure durations, and particle sizes. To better comprehend the long-term effects of these nanomaterials on enzyme function and tissue structure, present study used both low and high dosages. This dual approach will significantly contribute to the evaluation of MWCNT-NP safety and provide valuable insights into the potential effects of prolonged exposure.

Materials and methods

Characterization of Multiwalled Carbon Nanotube nanoparticle (MWCNT-NP)

Multiwalled Carbon Nanotubes purchased from Sigma Aldrich under the CAS number 308068-56-6, are as a dry powder, involving more than 98% carbon content. These Multiwalled Carbon Nanotube Nanoparticles (MWCNT-NPs) have an average diameter of between 50 and 90 nanometers, as stated in the specifications provided by the manufacturer. Various methods, such as Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM-EDS), Energy Dispersive X-ray (EDX) analysis with elemental mapping, X-ray Diffraction (XRD), and Fourier Transmission Infrared Spectroscopy (FTIR), were used to determine size and morphology of MWCNT-NPs.

Animal specifications

In the present study, a total of twenty adult male Swiss albino mice, aged 7–8 weeks, were employed. The mice were housed in cages, with four mice per cage and two mice per cage in a separate control cage, within a standard laboratory environment maintained at a temperature range of 20 to 24 degrees Celsius. The average body weight of the chosen mice was 33.7 ± 5 g. In keeping with the 3R principle (Replacement, Reduction, Refinement) which aims to reduce the number of animals needed for research and protect animal welfare, only male mice were used in this experiment. The goal was to reduce the number of animals subjected to experimentation while ensuring the reliability of the results through the use of appropriate statistical methods with a minimal sample size. The experimental procedures and activities were designed to minimize any potential discomfort or distress to the animals. Additionally, selecting mice of a single-sex helped further reduce the number of animals used in the study. The chosen group size was carefully determined to strike a balance between obtaining scientifically robust data and adhering to ethical guidelines for the humane treatment of research animals. The mice were allowed a day to adjust to their new surroundings and were allowed unlimited access to food and water before the start of the experiment.

Experimental design

Sixteen mice were receiving treatment in the trial, divided into four different groups of four mice each. Two vehicle groups with two mice each were among these groups. In addition, the two exposure periods in the study design—7 and 14 days—were included. Furthermore, applying two distinct exposure doses (based on the calculated LD₅₀ of 1.8 µg) before the final experimentation, the LD₅₀ equivalent to 25% of the lethal dose was assigned as MWCNT-NPs low dose (MWC-L). MWCNT-NPs high dose (MWC-H) was defined as the dose that corresponded to 50% of the LD₅₀. MWCNT-NPs low dose (MWC-L) and MWCNT-NPs high dose (MWC-H) were the two different exposure dosages used. The first group: was treated with a low dose of MWCNT-NPs (0.45 µg) for 7 days; the second group: was treated with the same treatment for 14 days; the third group: served as vehicle control, receiving no treatment. For seven and fourteen days, respectively, the fourth and fifth groups were treated with a high dose of MWCNT-NPs (0.90 µg), whereas the sixth group received no treatment and served as the vehicle control. The oral gavage was used to administer each treatment. During the oral gavage procedure, the mice were gently secured with one hand, and a 3 ml suspension was carefully administered using a glass syringe fitted with a disposable polyurethane feeding needle. After the gavage process, the mice were then weighed and returned to their respective cages. The vehicle groups functioned as control, helping to contextualize the study's conclusions. This thorough experimental design allowed for the evaluation of the effects of MWCNT-NPs at various dosages and exposure times through oral exposure.

Dose Preparation

An ultrasonic liquid processor operating at 4 °C and 30% amplitude was used to disperse MWCNT-NPs over 30 min, with pulse measurements of one second on and one second off. Before being dispersed, MWCNT-NPs were suspended and sonicated in a sterile saline solution containing 1% tween 20. Before being given by oral gavage, the MWCNT-NP suspensions were subjected to a 15-minute sonication procedure to prevent them from aggregating. The treated mice were hence given these MWCNT-NP suspensions consistently by gavage for seven or fourteen days straight. Previous research guided the dosage and method of administration of MWCNT-NP^{25,26}.

Samples collection

Swiss albino mice were euthanized using halothane in a controlled environment. Halothane was inhaled in a closed container and ensuring the minimal animal stress. Exsanguination was performed after confirming the mice were fully unconscious and had ceased all signs of life. The mice were exsanguinated on days 7 and 14, and the blood was taken from the heart and placed in sterile, closed blood collection tubes (4.5 mL) that needed to be kept up-right for 30 min. The serum was then carefully transferred to a sterile, clean tube, securely capped, and stored at 4 °C until analysis. Before analysis, the serum was centrifuged for ten minutes at 3000 rpm to get rid of any clots. The lungs, liver, and testes were removed immediately following dissection, cleaned with PBS, and preserved in 10% formalin for histological analysis.

Enzymatic assays

The serum level of liver, lungs and testes enzymes; Alanine aminotransferase (ALT), aspartate aminotransferase (AST), Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase enzyme and Angiotensin Converting Enzyme (ACE) were per-formed in the ELISA using commercially available kits (BT Lab, China).

Histopathology

Tissues were immersed in 10% buffered formalin for a duration of 24 h. Tissue samples were then positioned on histopathology cassettes and underwent processing using an automated tissue processor. The tissue underwent progressive dehydration with increasing concentration of ethyl alcohol solutions (ranging from 80 to 99.8%), followed by cleaning in xylene, and finally, embedding in paraffin. In order to identify histological alterations, the paraffin-embedded tissue was sliced into 5 µm sections using a rotary microtome (LEICA RM 2125). These sections were subsequently affixed to slides, subjected to hematoxylin and eosin staining, and observed under a light micro-scope to identify and capture images of any histopathological changes.

Statistical analysis

The data from the enzymatic tests were statistically analyzed using analysis of variance (ANOVA), with a significance level of $p < 0.05$. The Least Significant Difference (LSD = 0.05) was utilized for post-hoc comparisons. The graphical representations' bars were labeled with alphabetically (a, b, c, d) to indicate significant variations in doses and exposure durations.

Institutional review board statement

The study was carried out in compliance with guidelines of EU Directive 2010/63/EU for animal experiments and in accordance with the ARRIVE guidelines or the National Research Council's Guide for the Care and Use of Laboratory Animals and received approval from the Ethical Committee of the Department of Environmental Sciences at Lahore College for Women University under the approval number ENV.SCI. /LCWU21.

Results

Characterization of Multiwalled Carbon Nanotube Nanoparticle

Advanced techniques such as X-ray Diffraction (XRD), Fourier Transmission Infrared Spectroscopy (FTIR), and particle size analyzer with the help of zeta potential were employed to characterize the MWCNT NPs, ensure the validation of the commercialized synthesis method and confirm the material's authenticity. Figure 1 (a-c)

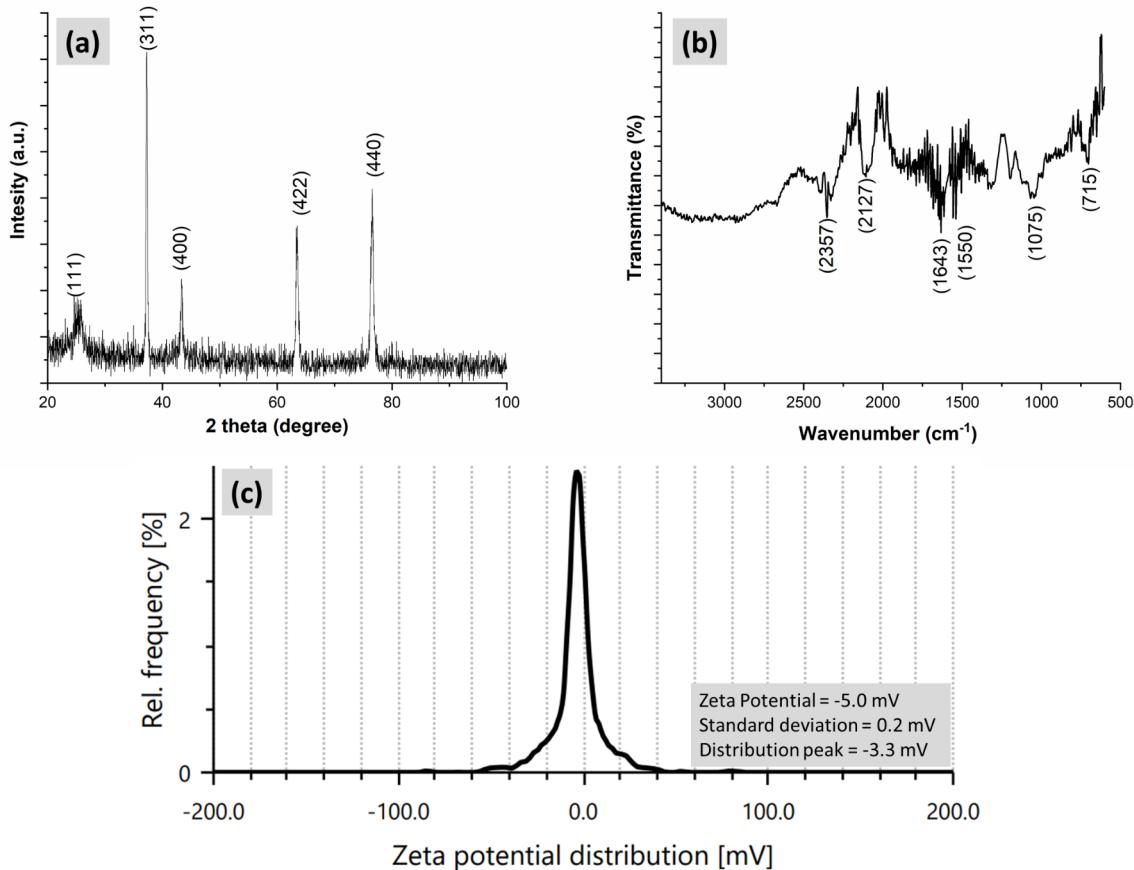


Fig. 1. X-ray diffraction analyses (a), (b) FTIR analysis, and particle size (c) of commercialized MWCNTs NPs.

presents a de-tailed depiction of various physiochemical properties of the commercialized MWCNT-NP. The X-ray diffraction analysis showed specific peaks at 2θ values of 26° , 38° , 43° , 63° , and 78° . These correspond to the (111), (311), (400), (422), and (440) planes, respectively (Fig. 1a). This affirms the presence of a specific structure in the MWCNT-NP. The narrow peak width in the high-intensity diffraction pattern of MWCNT-NP confirms that it's well-crystallized and has very few impurities. These results are consistent with earlier findings of MWCNT-NP^{27,28}. The average size of the nanoparticles, measured using the particle size analyzer and zeta potential, ranged from 50 to 90 nm, with most of the MWCNT-NP being under 100 nm. A high zeta potential value is indicative of a stable dispersion, ensuring an even distribution of dispersed MWCNT-NP. Conversely, a low zeta potential results in an attractive force surpassing the repulsive force, leading to the flocculation of MWCNT-NP. Studies have shown that under conditions of low potential, the attractive force may dominate, causing coagulation and flocculation of the dispersion. In contrast, a high zeta potential, whether negative or positive, contributes to electrical stabilization^{29–31}.

During the FTIR analysis, the structure and identified changes in functional groups within the MWCNT-NP were examined. This analysis revealed the presence of phenolic compounds, alkynes, terpenoids, and flavonoids, indicating their involvement in the formation process of MWCNT-NP. Figure 1c shows the FTIR spectra of the commercialized MWCNT-NP. In the range of 2000 to 2500 cm^{-1} , we observed two distinct peaks at (2357) and (2127) cm^{-1} . This indicates the presence of alcohol, phenolic, or water molecules with specific stretches and groups in the extract. Additionally, strong absorption peaks at 1647 cm^{-1} in the 1500 to 1700 cm^{-1} region suggest specific vibrations of surface groups. The signal at 1075 cm^{-1} corresponds to the stretching vibration of nitrate ions (NO_3^-). The most pronounced stretching vibration of MWCNT-NP is observed at 715 cm^{-1} . The coordination of MWCNT-NP with certain groups likely helps stabilize and cap the nanoparticles^{32–35}.

Surface morphology and spot analysis using SEM-EDX analysis

An important analytical tool for examining the surface morphology, structure, and elemental makeup of industrial materials is the Energy Dispersive X-ray Spectroscopy (EDS) equipped with scanning electron microscope (SEM-EDS). This study employed Spot analysis, EDS elemental mapping, and SEM-EDS techniques to precisely determine the elemental composition and co-occurring elements of commercially available MWCNT-NP. As shown in Figure 2, the SEM-EDS results showed that the MWCNT-NP tends to form larger clusters and has a spherical shape. In Fig. 2, the MWCNT-NP elemental mapping and EDS spot analysis provide

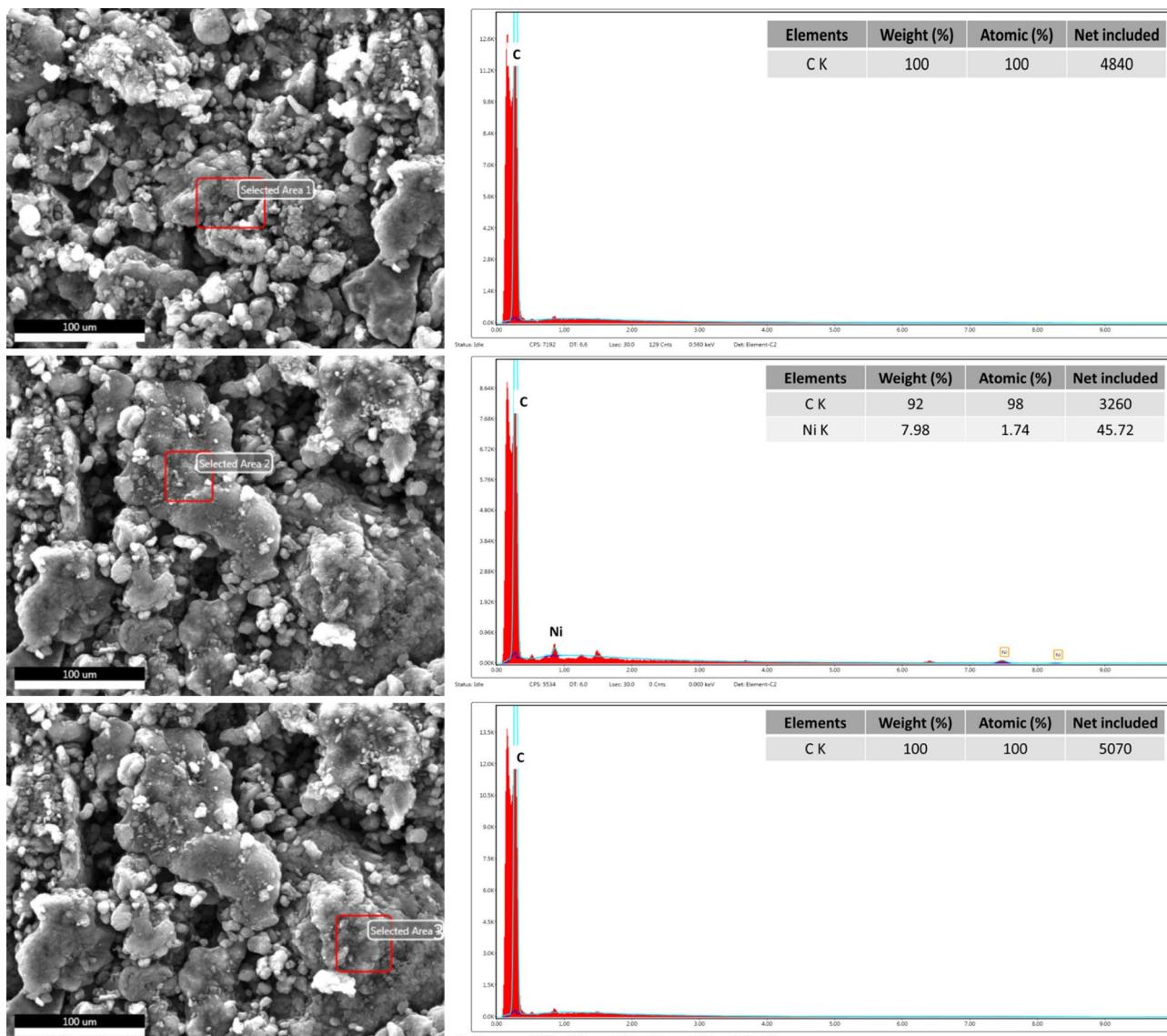


Fig. 2. Scanning electron microscopy attached with energy dispersive X-ray spectroscopy (SEM-EDS) spot analysis of MWCNT NPs nanoparticles.

a comprehensive breakdown of the elemental composition at S1, S2, and S3. The peaks observed in the X-ray spectra correspond to the energy levels of each element, enabling the identification of the elemental composition of the commercialized NPs through the EDS spot analysis technique. In Fig. 2A's EDS spot analysis spectrum, it is evident that the MWCNT-NP exhibit a maximum of 100% carbon content at S1 and S3. However, at S2, there is approximately 98% carbon and 1.74% nickel present in atomic percentages. These findings suggest that the carbon element maintains a high level of purity throughout the material. The elemental composition and the proportions of co- occurring elements in the pure carbon MWCNT-NP were precisely characterized using spot analysis, EDS elemental mapping, and SEM-EDX methods.

Relative body weight

According to results, animals didn't exhibit any significant clinical changes during the 7 and 14 days of repeated doses or the intervals before sample collection. No animal deaths during the period of the treatment were seen as a result of exposures, but mice at high doses had impaired mobility. For both doses of MWCNT-NP administered for 7 and 14 days, it was discovered that animals displayed a considerable reduction in body weight as shown in Fig. 3. Among all the groups, only the high-dose MWCNT-NP group (0.9 μ g) exhibited a significant reduction in relative body weight gain within 7 days when compared to the baseline.

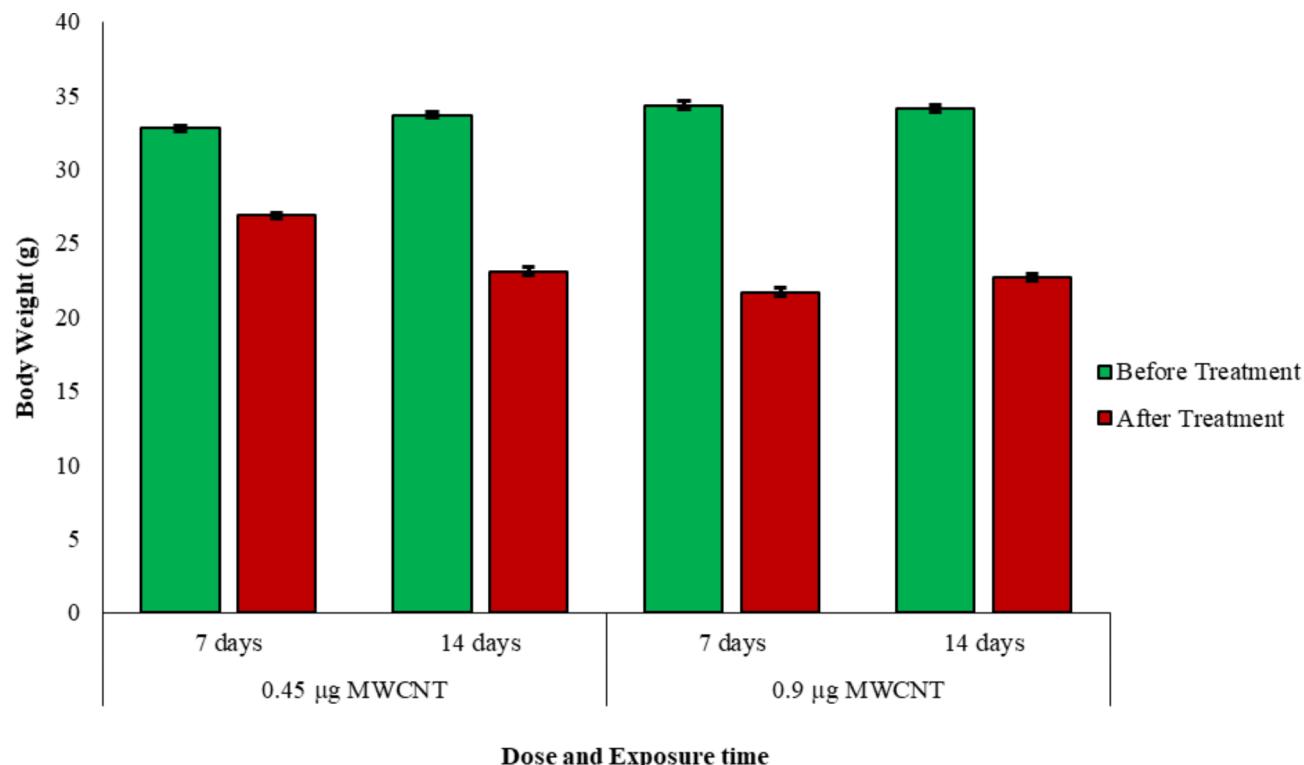


Fig. 3. Relative Body Weight (g) of male mice with before and after treatment of MWCNT during the experimental period of 7- and 14-days exposure.

Assessment of enzymatic assays and its histopathological evaluation

Underlying mechanism of testicular toxicity

Multiwalled carbon nanotubes can travel through the bloodstream to the testes and taken up by Sertoli cells, Leydig cells, and germ cells after being exposed to them. Internalization causes the production of reactive oxygen species (ROS), which in turn causes oxidative stress, cellular damage, and an inflammatory response marked by the production of pro-inflammatory cytokines. Significant histological changes, such as an expanded interstitial space, the loss of microorganism cells, disturbances in the seminiferous tubules, and degeneration of Sertoli and Leydig cells, can be triggered by oxidative pressure and persistent aggravation in the testicles³⁶. Changes in enzymes action go with these shifts, for example, an expansion in the movement of apoptotic and steroidogenic catalysts and a lessening in the action of cell reinforcement proteins. Be-sides, changed angiotensin changing over protein activity can provoke reduced testosterone levels and appalling sperm creation, and cause infertility as displayed in Fig. 4.

Testicular enzyme (angiotensin converting enzymes: ACE)

Serum biochemical parameters were analyzed to determine the toxicity of MWCNT-NP. One of the enzymes examined in the testes was angiotensin converting enzyme (ACE), which has been linked to male fertility. According to the findings of the study, the mice that were given MWCNT-NP at a concentration of 0.90 g for 14 days had higher levels of ACE than the mice that were given MWCNT-NP at a concentration of 0.45 g for 7 days. As can be seen in Fig. 5, the ACE levels of the MWCNT-exposed groups and the control group were significantly higher. These results show that exposure to MWCNT-NP, especially within the electronics industry, could raise concerns regarding occupational safety. The elevated ACE levels observed in the exposed groups raise concerns regarding the potential impact on male fertility.

Testicular histological analysis

Histopathological changes in the testes are shown in Fig. 6. Severe degeneration of seminiferous tubules and Leydig cell hyperplasia significantly increased in 14 days exposure of both dose groups MWCNT- NP treated mice as compared to vehicle control. There were mild epididymitis changes were observed in 7 days exposure of 0.90 µg dose group as compared to low dose 0.45 µg. Similar results were observed ACE enzyme (Fig. 4) that low dose of 7 days exposure has low values as compared to other dose and exposure time. So, the high dose with long term occupational exposure cause in-fertility in males' workers of industries.

Angiotensin-converting enzyme (ACE) plays a pivotal role in regulating cardiovascular homeostasis and fertility. There are two isoforms of ACE1: somatic ACE1 (sACE1) and testicular ACE1 (tACE1), both transcribed from the same gene but regulated by different promoters. Research has highlighted that ACE1, as a seminal fluid protein, helps preserve sperm before and after translocation to females. In contrast, tACE1 plays a critical role

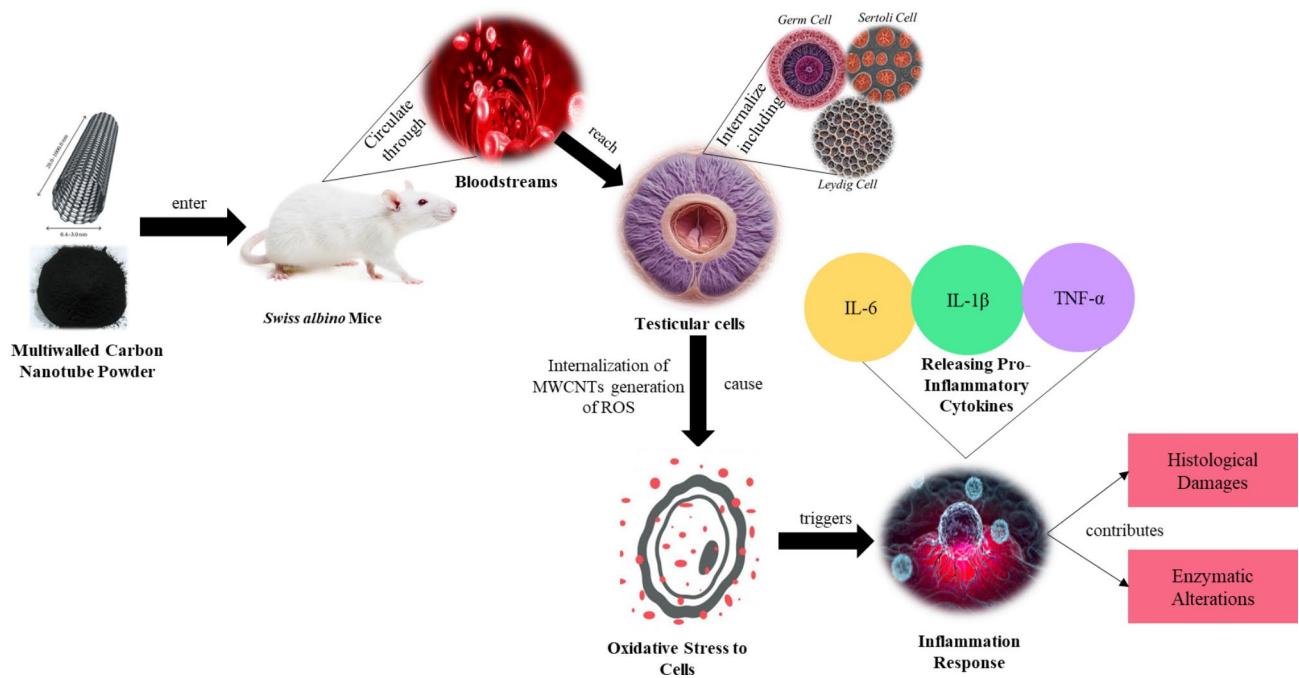


Fig. 4. The mechanisms that underlie the histological and enzymatic changes in testicular toxicity that occur when Multiwalled Carbon Nanotubes are exposed.

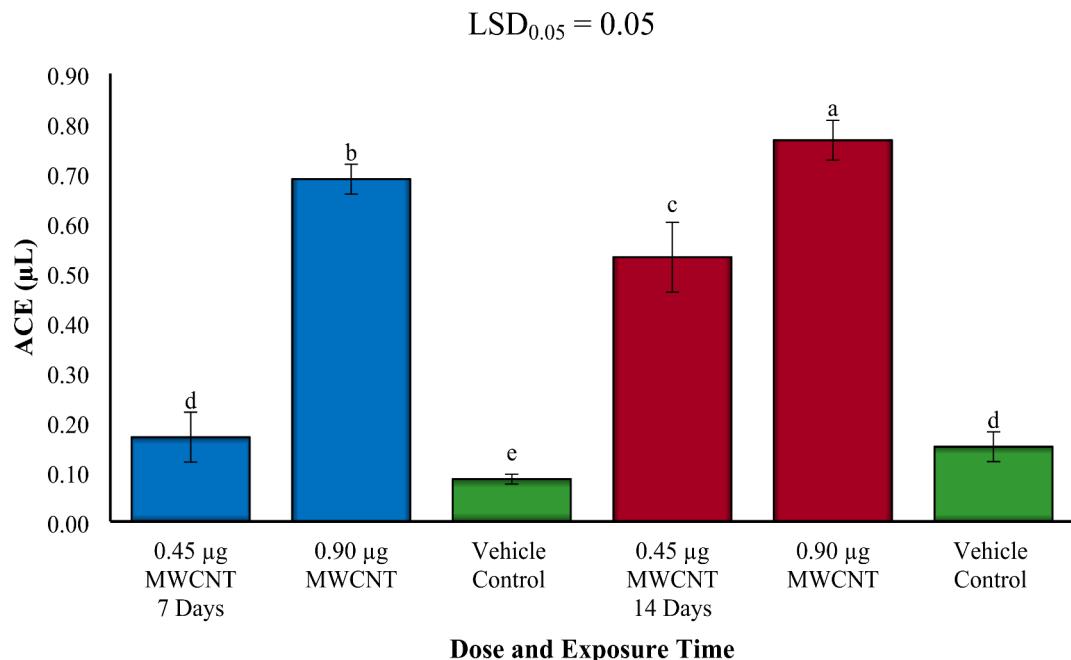


Fig. 5. Serum angiotensin converting enzyme (ACE) action in male mice presented to MWCNTs over the trial time of 7 and 14 days. Each test group was compared with the control group. Letters (e.g., a, b, c, d and e) show that values are significantly different.

in sperm migration and adhesion to the zona pellucida (ZP), a crucial step in fertilization. Interestingly, studies show that male mice lacking sACE1 are fertile, while those without tACE1 are sterile, underscoring the distinct functions of these isoforms in reproduction. Recently cloned ACE2 is predominantly expressed in Leydig cells within the testes, as well as in the kidney and heart's epithelial and endothelial cells³⁷. Its presence in the testes suggests that it regulates steroidogenesis, which impacts germ cells and overall reproductive health³⁸. ACE2 deficiency has been shown to impair both basal and luteinizing hormone-stimulated testosterone production,

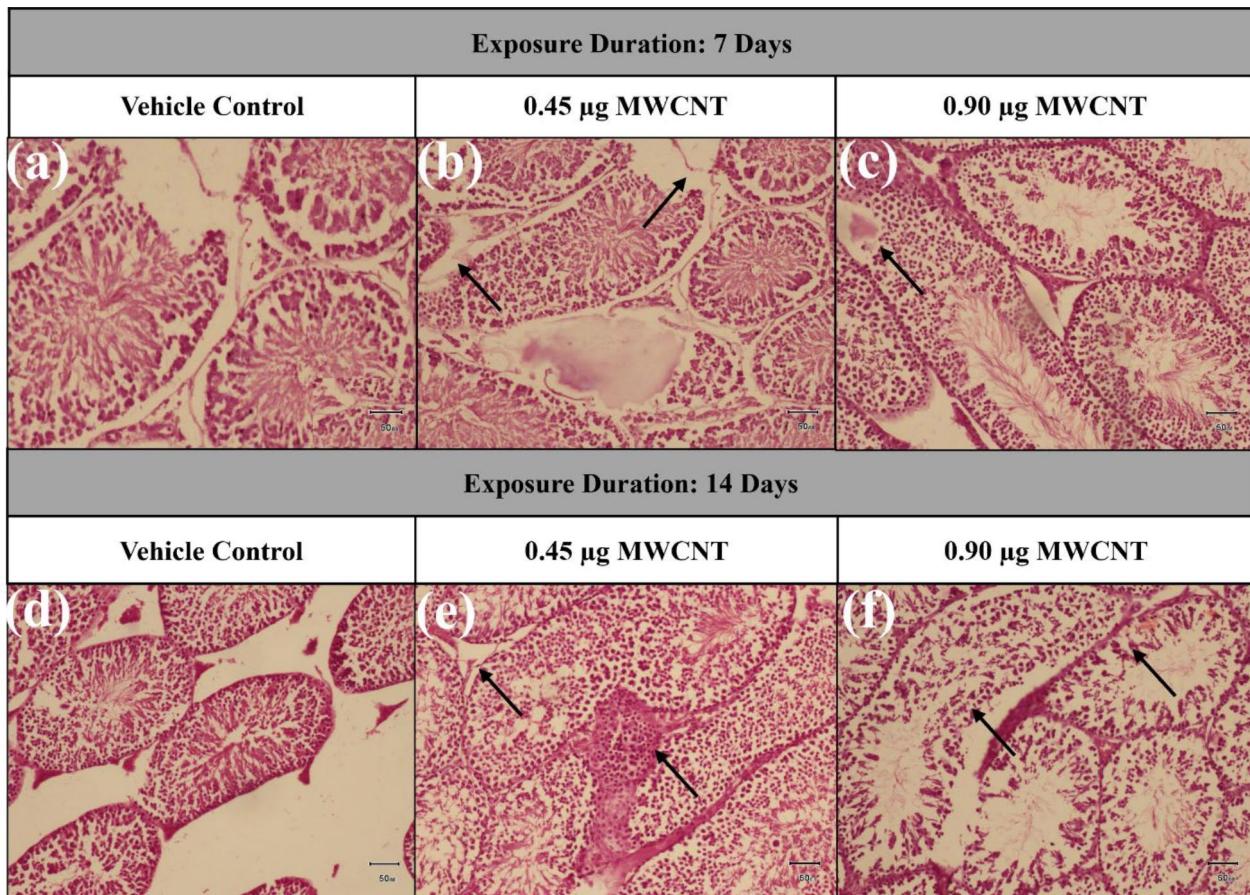


Fig. 6. Mice testes exhibit histological alterations by oral administration of MWCNTs-NPs. (a and d) Normal tissue from mice exposed to a vehicle control for seven and fourteen days. After 7-day exposure period, (b) the treated group (0.45 µg) with MWCNT low dose exhibits mild seminiferous tubule degeneration. (c) The group that received a high dose of MWCNT (0.90 µg) exhibits mild epididymitis and significant degeneration of the seminiferous tubules. After 14 days of exposure: (e) MWCNT low dose (0.45 µg) treated group exhibits minor Leydig cell hyperplasia and rupture cell lining. (f) The group that received a high dose of MWCNT (0.90 µg) exhibits hyperplasia of Leydig cells.

significantly affecting male fertility³⁹. Additionally, ACE2 has been implicated in maintaining testicular microvasculature and influencing fluid movement and permeability, although testosterone may mitigate some of these effects⁴⁰. The correlation between elevated ACE levels and reduced reproductive function suggests that exposure to MWCNT-NP (Multi-Walled Carbon Nanotubes-Nanoparticles) could lead to infertility, particularly with prolonged exposure and higher doses. Previous studies have also documented significant histological and stereological changes in the testes following exposure to CNTs (Carbon Nanotubes). Oxidative stress and mitochondrial dysfunction, as reported by⁴¹, are believed to play a key role in the reproductive toxicity caused by CNTs, affecting both sperm quality and testicular health. Additionally⁴², explored the impact of functionalization and nanotube layers on the acute and chronic toxicological effects of CNTs on various organs, particularly the male reproductive system. Histopathological findings from these studies include vacuolization, degeneration, edema, and reduced germinal epithelium thickness in the seminiferous tubules. A reduction in spermatogenic cells was also observed, likely due to tubular degeneration. These effects were more pronounced in chronic exposure phases compared to acute phases, indicating that prolonged exposure to CNTs exacerbates testicular damage.

Underlying mechanism of pulmonary toxicity

Oral ingestion exposure to multiwalled carbon nanotubes (MWCNTs), which causes their deposition in lung tissue. Reactive oxygen species (ROS) and oxidative stress are produced when lung cells, such as alveolar macrophages, epithelial cells, and fibroblasts, internalize the MWCNTs. This oxidative pressure brings about cell harm and an inflammatory reaction, described by the release of cytokines and enlistment of inflammatory cells. Fibrosis and tissue remodeling, characterized by excessive collagen deposition by activated fibroblasts, are the outcomes of chronic inflammation. Alveolar wall thickening, inflammatory cell infiltration, granuloma formation, and fibrosis are histological changes in the lungs⁴³. These processes cause structural damage and

impaired lung function, both of which contribute to respiratory problems and reduce lung capacity as a whole as shown the mechanism in Fig. 7.

Pulmonary enzyme (Nicotinamide Adenine Dinucleotide Phosphate Oxidase: NADPH oxidase)

The findings demonstrated that in mice exposed to MWCNT-NP, notable effects were observed in the lungs. The group exposed to MWCNT-NP at a concentration of 0.90 µg for 14 days exhibited higher levels of the NADPH oxidase enzyme in comparison to the group exposed to MWCNT-NP at a concentration of 0.45 µg for 7 days. Additionally, both exposed groups showed significantly higher NADPH oxidase enzyme levels compared to the control group (Fig. 8). These findings suggest that MWCNT-NP exposure in the electronics industry requires occupational safety measures due to the potential for elevated NADPH oxidase levels, which may lead to increased ROS production can disrupt lung functioning by promoting inflammation, oxidative damage, fibrosis, and antioxidant depletion. These effects can impair respiratory function and increase the risk of developing lung disorders.

Pulmonary histological analysis

Figure 9 shows representative overviews of hematoxylin and eosin (H&E) stained lung sections, embedded in paraffin, from mice exposed to MWCNT-NP for both 7 and 14 days, across two dose groups (0.45 µg and 0.90 µg). In Fig. 9(f), MWCNT-NP were predominantly observed as black aggregates within macrophages or granulomas. Histopathological evaluation revealed significant changes induced by the two doses and exposure times of MWCNT-NP compared to the control group. These changes included varying degrees of histopathological alterations, such as pulmonary edema in alveoli and severe congestion, which can affect breathing and cause airway blockage. This condition is known as acute lung injury (ALI), a frequent acute respiratory disorder that is primarily characterized by increased permeability, pulmonary edema, and respiratory function impairment.

Elevated NADPH oxidase (NOX) enzyme levels in the lungs can impair lung function by promoting the generation of reactive oxygen species (ROS). ROS plays a critical role in cellular metabolism, signaling, and immune responses. Superoxide, a major source of cellular ROS, is primarily produced by NOX enzymes and the mitochondria through oxidative phosphorylation⁴⁴. The NADPH oxidase (NOX) family consists of seven distinct isoforms, which are responsible for the majority of ROS production. Under diseased conditions in the lungs, several NOX isoforms play crucial roles. For example, NOX-2 has been strongly linked to ovalbumin (Ova)-induced allergic asthma, while NOX-4 is elevated in patients with asthma. External factors, such as air pollution, ionizing radiation, certain medications (e.g., antibiotics and anticancer drugs), specific trace elements, and alcohol, can also increase ROS levels, leading to chronic oxidative stress. This oxidative stress, triggered by

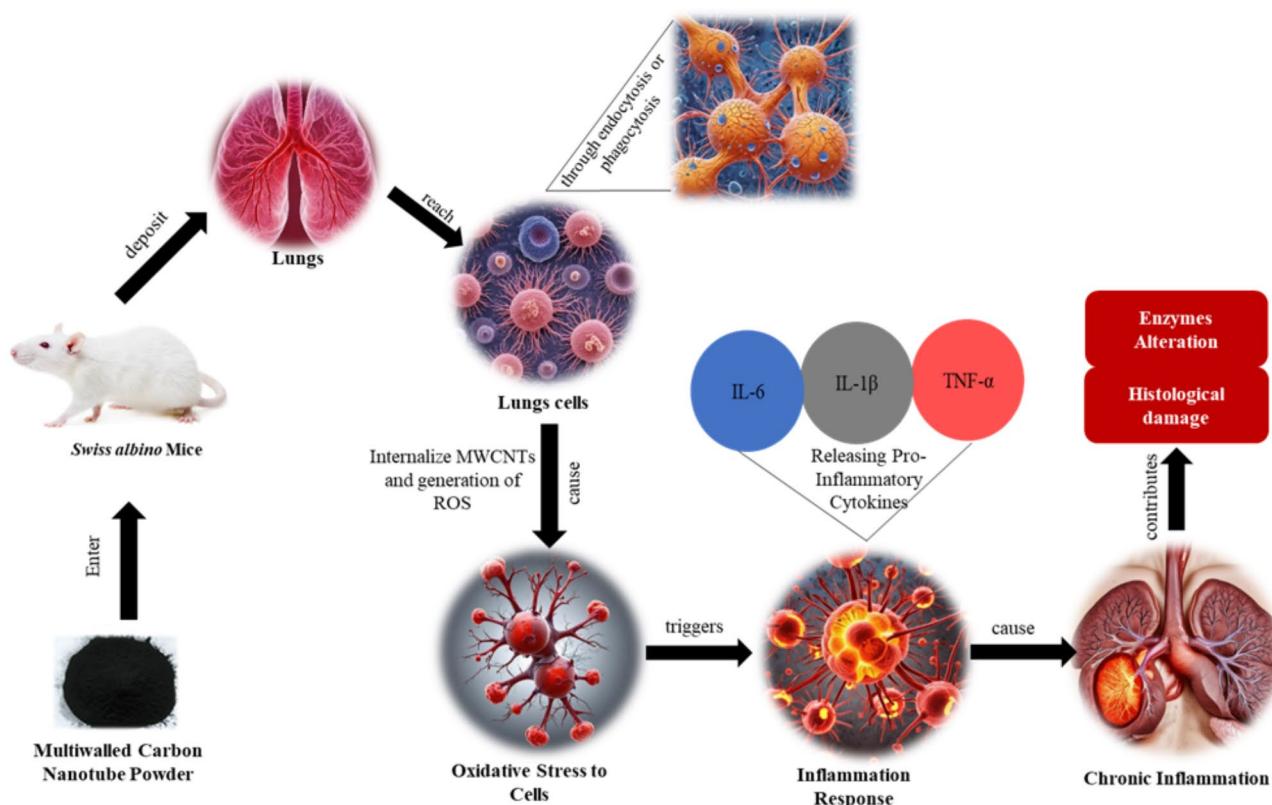


Fig. 7. The mechanisms behind histological and enzymatic alterations of pulmonary toxicity with exposure to Multiwalled Carbon Nanotubes.

$$\text{LSD}0.05 = 0.07$$

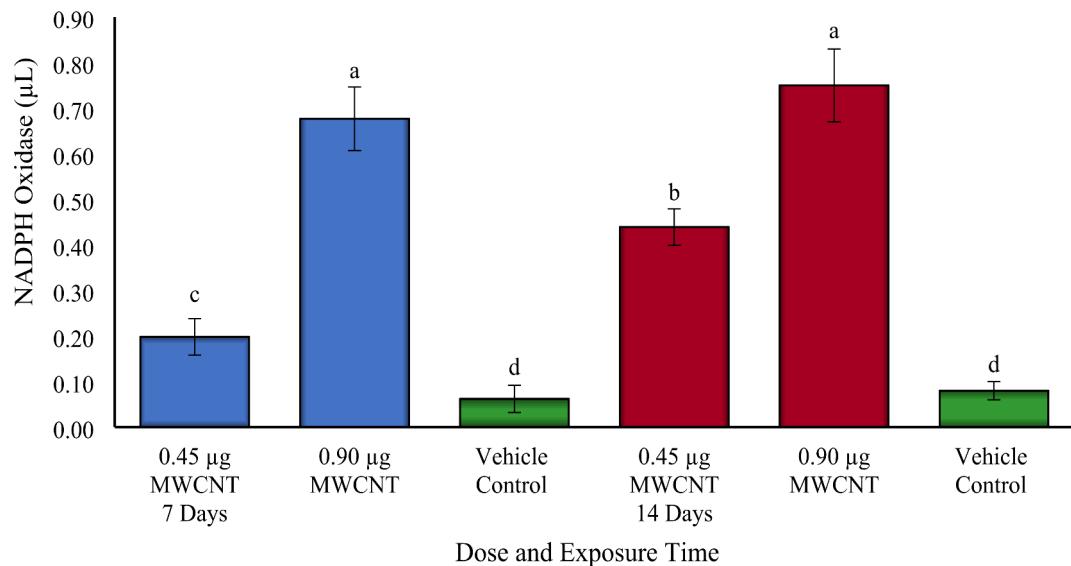


Fig. 8. Serum NADPH Oxidase activity in male mice exposed to MWCNTs and the control group over the experimental period of 7 and 14 days. Each test group was compared with the control group. Letters (e.g., a, b, c and d) show that values are significantly different.

environmental oxidants, can worsen allergic inflammation and disrupt normal cellular functions, contributing to lung damage, impaired lung function, and exacerbated asthmatic airway inflammation⁴⁵.

Nanoparticles (NPs) are known to cause significant toxicities in the lungs, as evidenced by numerous studies. A study by⁴⁶ revealed that C_{60} fullerene-treated lung tissue exhibited increased inflammatory cell counts, alveolar collapse, septal thickening, congestion, and pulmonary edema. Similarly⁴⁷, investigated the pulmonary toxicity of graphene nanomaterials and observed significant pulmonary edema. The cytotoxicity of nanoparticles is influenced by various factors, including dose, size, shape, and chemical composition. Several studies have shown that nanoparticle toxicity can result from multiple mechanisms, such as ROS generation, DNA damage, inflammation, pulmonary edema, cell death, and extreme congestion⁴⁸.

Underlying mechanism of hepatic toxicity

Multiwalled carbon nanotubes (MWCNTs) are distributed into the bloodstream and transported to the liver, where they are absorbed by hepatocytes, Kupffer cells, and hepatic stellate cells. This causes oxidative stress, which damages cells and triggers an inflammatory response marked by the release of pro-inflammatory cytokines through the production of reactive oxygen species (ROS). Activated Kupffer cells exacerbate inflammation and ROS production, further harming liver tissue. During chronic inflammation, activated hepatic stellate cells deposit too much collagen, causing fibrosis, which causes tissue remodeling and scarring. Histological changes in the liver include hepatocyte necrosis, inflammatory cell infiltration, fibrosis, and lipid accumulation. As shown in Fig. 10, these processes result in the release of the liver enzymes ALT and AST, which serve as biomarkers for liver damage^{49,50}.

Hepatic enzyme (ALT: Alanine aminotransferase and AST: aspartate aminotransferase)

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) are compounds dominantly present in hepatocytes, which are liver cells. These enzymes are released into the bloodstream when the liver is damaged or injured, causing their levels to rise. As a result, elevated ALT and AST levels are important signs of liver damage. Similarly, Fig. 11 (a–b) shows that ALT and AST levels of 0.45 $\mu\text{g}/\text{mouse}$ group and 0.90 $\mu\text{g}/\text{mouse}$ group are higher than those of the control group and it demonstrates that MWCNT-NP induced hepatic injury at 7- and 14-days exposure. So, it indicates that liver cells have been damaged or are undergoing cell death. However, it's important to note that the ratio of ALT to AST elevation can provide additional insights into the specific cause and severity of liver injury.

Hepatic histological analysis

As illustrated in Fig. 12, the liver showed several histological alterations. In comparison to controls, histological alterations of the inflammatory, degenerative, and necrotic forms were seen in mice exposed to MWCNT-NP. For mice exposed to MWCNT-NP, there was a noticeable variation in the type of coagulative necrosis observed, and the occurrences were equivalent across dose groups. The mild cellular swelling, atrophy of hepatocytes changes and hydropic degeneration in the two dose groups of 7 days exposure were observed. The significant and severe cellular swelling and coagulative necrosis of hepatocytes were observed in 14 days exposure of two dose

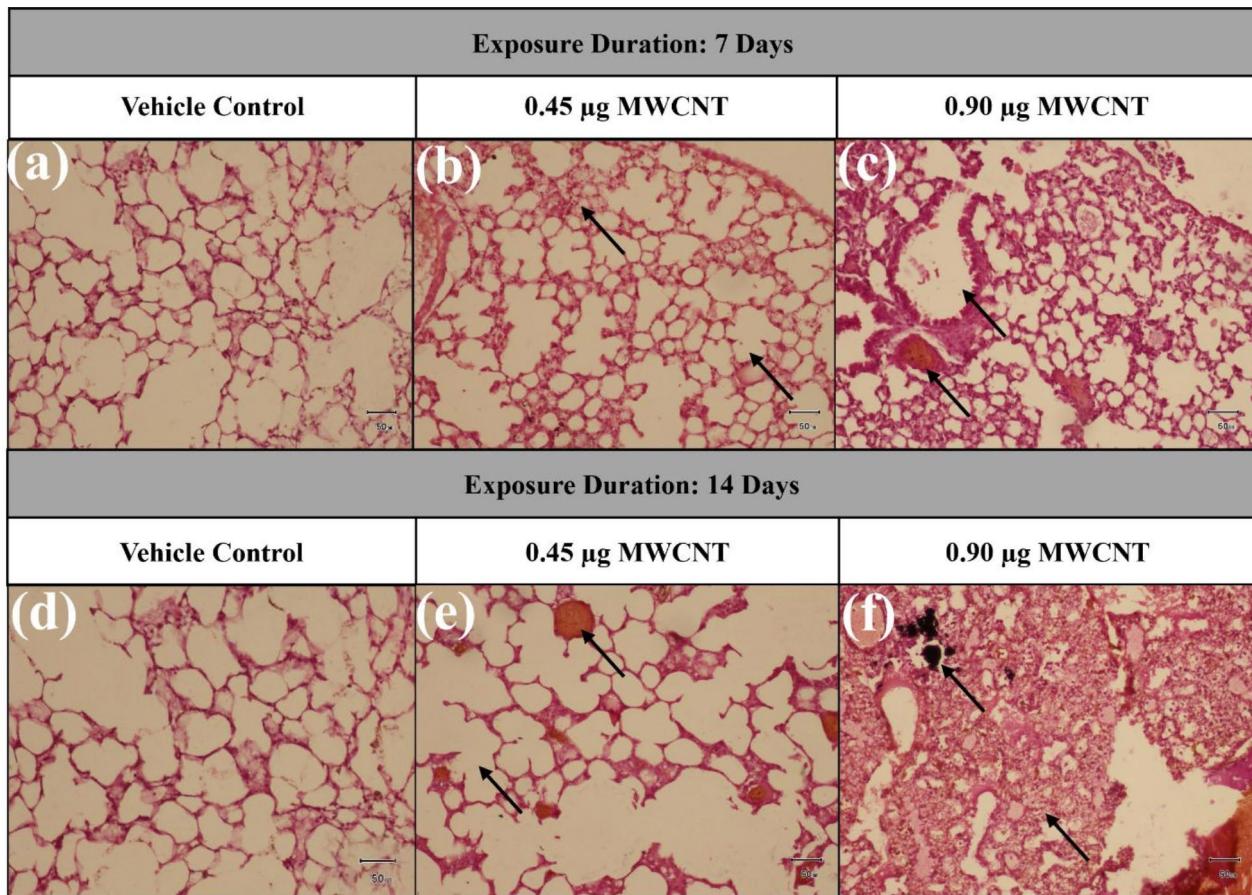


Fig. 9. Mice lungs exhibit histological alterations by oral administration of MWCNTs-NPs. (a and d) Normal tissue from mice exposed to a vehicle control for seven and fourteen days. After 7-day exposure period, (b) Treated group with MWCNT low dose (0.45 µg) shows mild pulmonary edema in many alveoli. (c) Treated group with MWCNT high dose (0.90 µg) shows absence of intact alveoli structures. After 14 days exposure period: (e) Treated group with MWCNT low dose (0.45 µg) shows moderate congestion and pulmonary edema. (f) Treated group with MWCNT high dose (0.90 µg) shows bulk of black aggregates within macrophages and absence of alveoli structure and ruptured of cell linings.

groups. Nanoparticles deposited in liver tissue have the potential to impact liver cells and crucial physiological processes, as well as the biochemical functioning.

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ -glutamyltransferase (GGT) are liver enzymes commonly used as reliable indicators of liver health. Serum ALT and AST, predominantly found in the liver, are particularly considered specific markers of hepatic injury. Elevations in ALT and AST levels typically indicate liver damage or disease⁵¹, as these enzymes are released when liver cells (hepatocytes) are injured. Hepatocytes play vital roles in detoxification, metabolism, and protein synthesis⁴², making their integrity crucial to liver function. A study on adult rats exposed to nanoparticles revealed dose-dependent hepatotoxicity, marked by significant increases in ALT and AST activity. Histopathological changes included congestion in the central vein and hepatic sinusoids, as well as hepatocyte degeneration, indicating damage to liver tissue⁵². Such damage disrupts normal liver functions, including detoxification and protein synthesis. Liver inflammation, often associated with conditions like viral hepatitis (e.g., hepatitis A, B, or C), is another common cause of elevated ALT and AST levels⁵³. This inflammation compromises liver function and can lead to tissue damage, further impairing essential processes like protein synthesis, fluid balance (due to reduced albumin production), and enzyme activity⁵⁴. Prolonged liver damage can eventually result in cirrhosis, a condition characterized by scar tissue formation in the liver, which severely affects liver function^{55–57}. Further studies on nanoparticle toxicity support these findings. In a study involving rats exposed to CuO/ZnO nanoparticles, histopathological observations included slight cellular enlargement, granular cytoplasmic degradation of hepatocytes, and necrosis in the peripheral zones of hepatic lobules⁵⁸. Interestingly, a lower dose administered over a longer period caused more severe liver abnormalities than a higher dose, suggesting that smaller, prolonged exposures may be more harmful. This highlights the potential risks for occupational workers exposed to nanoparticles. Overall, the evidence clearly demonstrates that MWCNT-NP exposure has adverse effects on vital organs, including the liver, lungs, and testes, primarily through disruption of enzymatic function

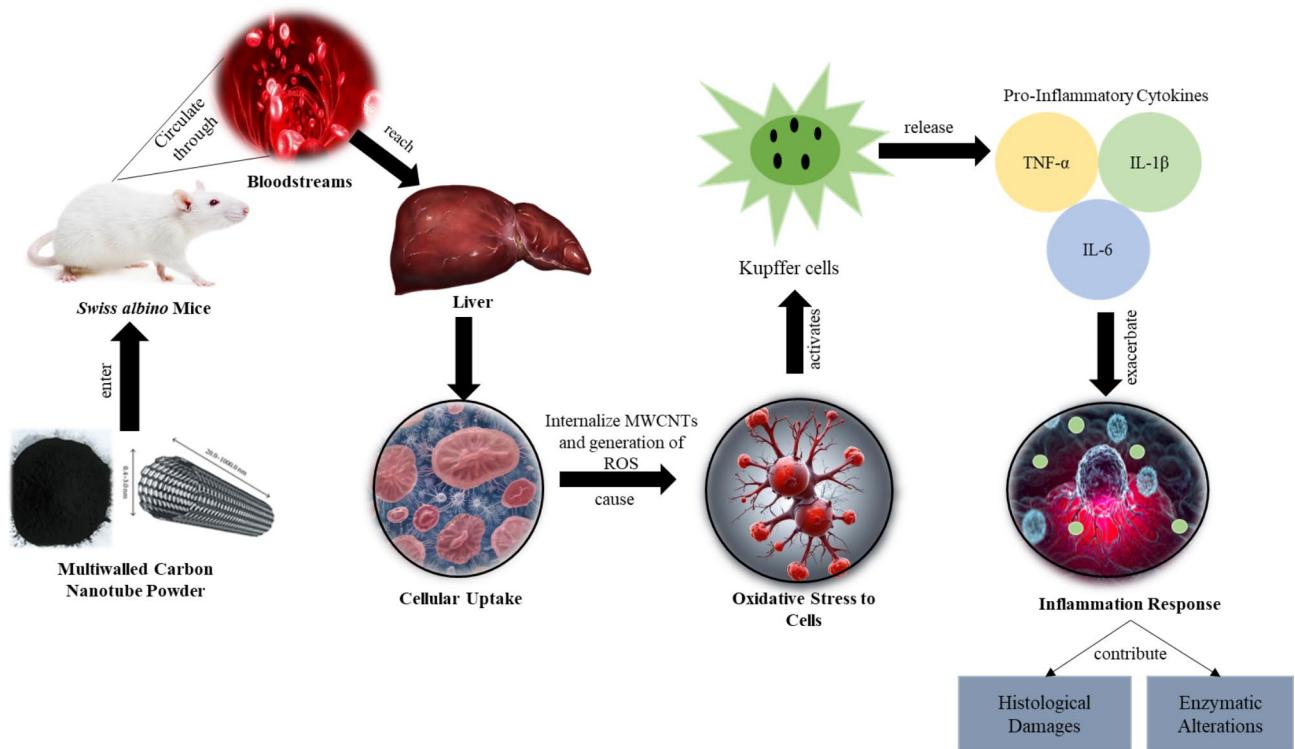


Fig. 10. The underlying mechanism behind histological and enzymatic adjustments of hepatic toxicity with exposure to Multiwalled Carbon Nanotubes.

and tissue damage. These findings are particularly relevant for industrial workers frequently exposed to such nanoparticles.

Conclusions

Multi-walled Carbon Nanotubes (MWCNT-NP) possess unique physicochemical properties, including electrical, mechanical, and thermal attributes, making them valuable for various industrial applications, particularly in electronics. This study investigated the toxicological effects of MWCNT-NP on enzymatic activity in key organs, with a focus on their potential impact on male reproductive health. The elevated levels of angiotensin-converting enzyme, along with histopathological changes such as Leydig cell hyperplasia and seminiferous tubule degeneration, indicated that prolonged exposure could impair male fertility. Furthermore, increased NADPH oxidase levels in the lungs indicated oxidative stress, which could lead to lung damage, including pulmonary edema and congestion. In the liver, higher ALT and AST enzyme levels, coupled with histological signs of hepatocyte degeneration and necrosis, showed significant liver injury. These findings highlighted the significance to monitor MWCNT-NP exposure, especially among workers in industries that utilize such materials. Reducing exposure is essential to minimize health risks and promote safer working environments.

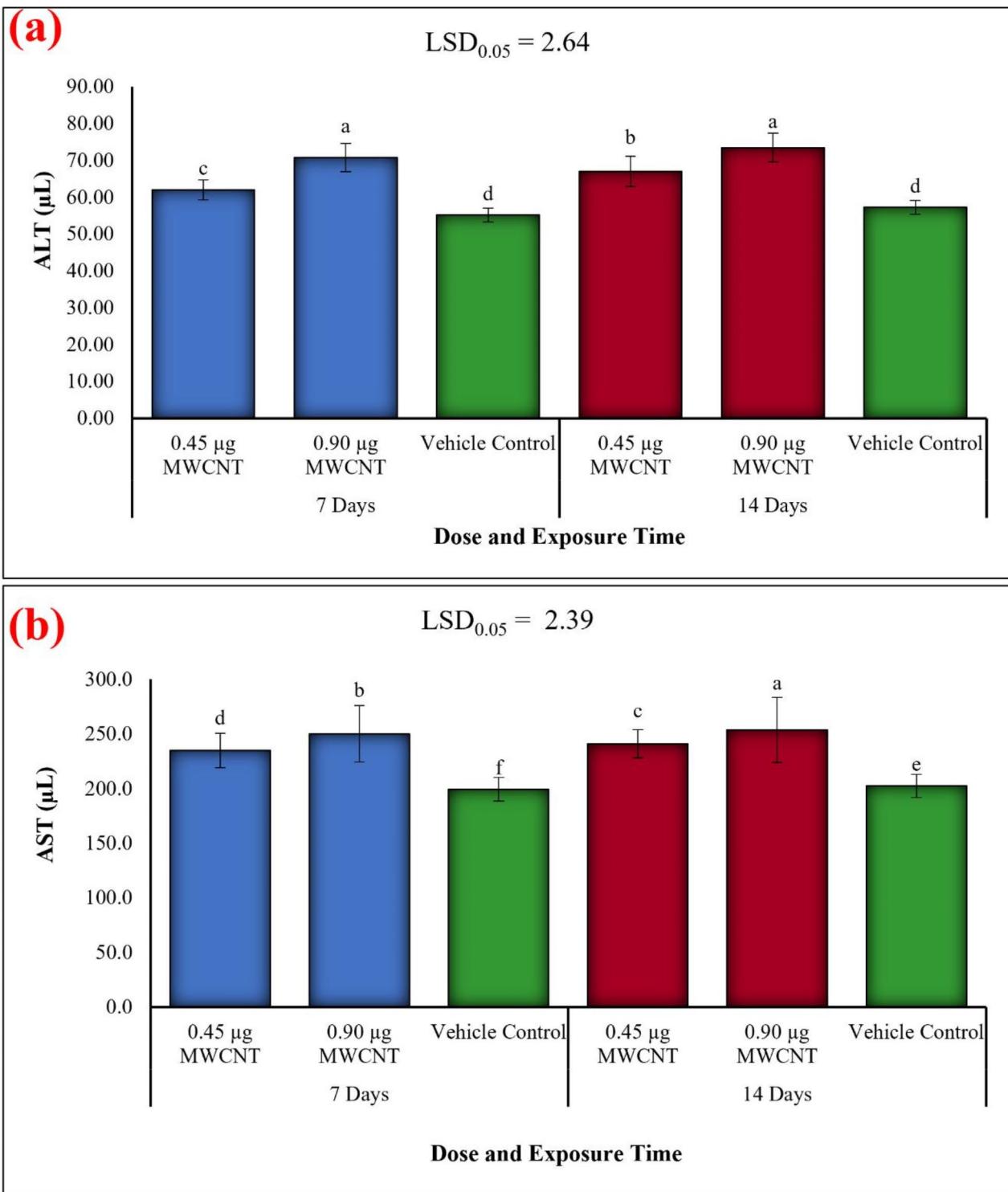


Fig. 11. (a) Serum alanine aminotransferase (ALT) (b) Serum aspartate aminotransferase (AST) activity in male mice exposed to MWCNTs and the control group over the experimental period of 7 and 14 days. Each test group was compared with the control group. Letters (e.g., a, b, c, d, e and f) show that values are significantly different.

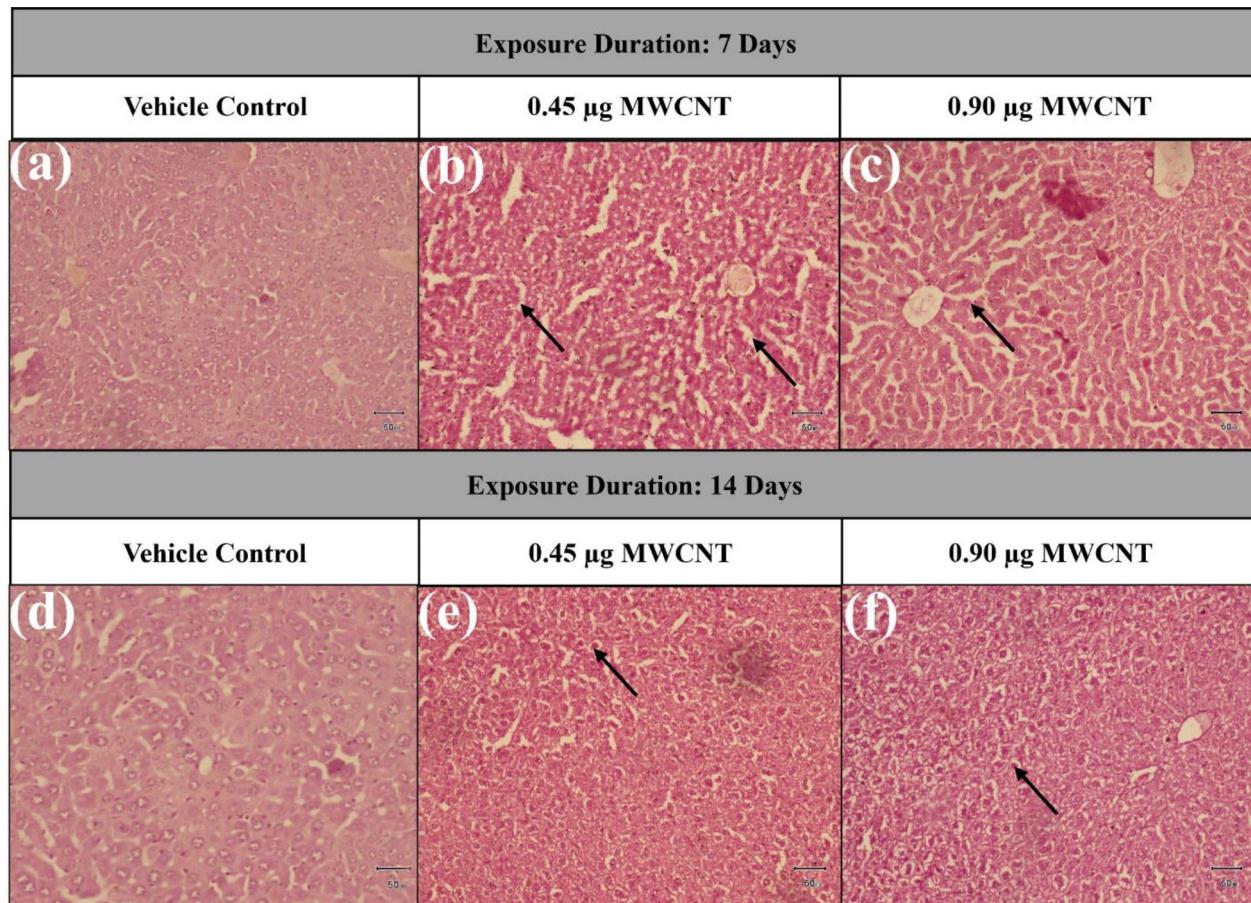


Fig. 12. Mice liver exhibit histological alterations by oral administration of MWCNTs-NPs. (a and d) Normal tissue from mice exposed to a vehicle control for seven and fourteen days. After 7-day exposure period: (b) Treated group with MWCNT low dose (0.45 µg) has mild cellular swelling and atrophy of hepatocytes seen (c) Treated group with MWCNT high dose (0.90 µg) shows few hepatocytes are normal and some show mild hydropic degeneration. After 14 days exposure period: (e) Treated group with MWCNT low dose (0.45 µg) shows moderate cellular swelling and coagulative necrosis of hepatocytes (f) Treated group with MWCNT high dose (0.90 µg) severe cellular swelling and coagulative necrosis seen in hepatocyte.

Data availability

All the data is contained in the manuscript. This paper is part of Ph.D. dissertation of first author. Academic Thesis is submitted to Higher Education Commission Repository(https://www.turnitin.com/download_for_mat_select.asp?oid=2384662046&fn=Plagiarsim_File_Iqra_Nasim.docx&p=1&ft=docx&svr=6&lang=en_us&r=79.8792976933825).

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Author contributions

I.N; methodology, formal analysis, investigation, original writing draft and data curation. N.G; Conceptualization, writing—review and editing, resources, supervision and project administration. R.N; editing, resources, and supervision; E.M. Writing - review & editing, Data Curation, Visualization, Validation; Y.A. B. J: Funding acquisition, Investigation, Project administration, Formal analysis, Writing - review & editing. All authors contributed to the article and approved the submitted version.

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Declarations

Competing interests

The authors declare no competing interests.

Institutional review board statement

The study was carried out in compliance with guidelines of EU Directive 2010/63/EU for animal experiments and in accordance with ARRIVE guidelines or the National Research Council's Guide for the Care and Use of Laboratory Animals and received approval from the Ethical Committee of the Department of Environmental Sciences at Lahore College for Women University, Lahore (Approval Number ENV.SCI. /LCWU21).

Additional information

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