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RESEARCH ARTICLE

Effects of Hydroxyl Functionalized Multiwalled Carbon Nanotubes on Histology of Various Organs in Wistar Rats

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Abstract

Multiwalled carbon nanotubes (MWCNTs) are important nanomaterials having great industrial and commercial value. Their industrial production has grown up tremendously in recent years. Several studies have documented adverse effects of MWCNTs on living systems. The present study examined the toxic effects of MWCNTs on hematology and histopathology of various organs in Wistar rats. The results have demonstrated that these nanotubes alter some parameters of blood like total number of RBC and mean corpuscular volume at higher doses. Histopathological analysis has shown that 2.0 and 10.0 mg/kg bw doses of MWCNTs led to histological changes both in kidney and spleen. However, the exposure did not affect histological features of lungs.

Keywords : Hematology, Histology, Multiwalled carbon nanotubes, Toxicity

Introduction

Multiwalled carbon nanotubes are two-dimensional cylindrical tubes having a diameter less than 100 nm. Owing to unique physical and chemical properties, their commercial applications have expanded fueling bulk level production in many industries. According to a report, the carbon nanotubes market is expected to grow from USD 4.55 billion in 2018 to USD 9.84 billion by 2023, with a CAGR 16.7% (Markets and Markets report, 2018). Most important commercial applications of MWCNTs are for the development of integrated circuits, field emission displays, hydrogen storage, lithium batteries, fuel cells and drug delivery. New research is being done to find applications in food, agriculture and cosmetics industries. Therefore, it will not be an exaggeration to argue that in near future we are going to be exposed to these materials in a big way (Pertersen *et al.*, 2011).

Ecotoxicological studies have shown that MWCNTs bioaccumulate and reach up to higher trophic levels (Cheng *et al.*, 2009; Jackson *et al.*, 2013). Many studies demonstrated potential toxic effects on mammalian models. Awasthi *et al.* (2013) reported that MWCNTs lead to hepatic tissue inflammation, necrosis and blood coagulation. Our previous reports demonstrated testicular toxicity of MWCNTs in adult Wistar rats (Nirmal *et al.*, 2017). A recent *in vitro* report suggests that small diameter MWCNTs generated cellular stress and autophagy dysfunction in endothelial cells (Zhao *et al.*, 2019).

The current study was done with the objective to demonstrate sub-acute toxicity of hydroxyl functionalized MWCNTs (OH-*f*MWCNTs) to various mammalian tissues following intraperitoneal (IP) administration. Three incremental doses were selected to test the influence of various dose levels on toxicity outcomes. The average diameter of the tubes was 10-20 nm.

Materials and Methods

OH-*f* MWCNTs

OH-*f*MWCNTs were commercially purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Andheri (E), India). The carbon nanotubes were 10 to 20 nm thick and average length was 10-30 μm. Physical characterization details have already been published elsewhere (Nirmal *et al.*, 2017).

Animal Maintenance and Exposure

Adult male Wistar rats (body weight 175±10 g) were assorted in four groups (6 animals each) according to the dose level. There were three dose levels and a control group. Doses were kept 0.4 (low dose), 2.0 (mid dose) and 10.0 (high dose) mg/Kg body weight. The control group received vehicle (sterilized normal saline containing 0.1% Tween-80) only. A total of 15 IP injections were given to each test animal. The work was approved by the Institutional Animal Ethical Committee (1678/GO/a/12CPCSEA).

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Hematology

Fresh blood samples were collected and stored in fresh EDTA coated vials at 4^0C. Hematological measurements were taken by automated blood analyzer (Accurex; CBC 360 Plus). Blood parameters studied were: total red blood cells (RBC), white blood cells (WBC), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), hemoglobin (Hb), platelets and hematocrit (HCT).

Histopathology

Histological sections were prepared using standard double staining procedures (Nirmal *et al.*, 2017). Microscopic observations and microphotography were carried out (DM 1000, Leica Microsystems, Germany).

Statistical Analysis

Values were calculated and presented as mean ± standard deviation (SD). Data was subjected to statistical analysis using GraphPad Prism (version 8.0.2). One-way ANOVA was used to test the statistical significance and multiple comparisons were performed by Dunnetts multiple comparison test ($p \leq 0.05$).

Results and Discussion

General Health, Organ Indices and Abdominal Cavity

Exposure to OH-*f* MWCNTs did not produce significant change in behavior such as aggression, body movements, salivation, lacrimation, piling etc. Water and food consumption patterns were found similar in both control and treated groups (data not shown). Relative organ weights of lung, kidney and heart did not show significant changes. Relative spleen weight in high dose treated rats declined significantly as shown in table 1.

Table 1. Relative organ weights or indices of lung, kidney, spleen and heart in control and OH-f MWCNTs-treated Wistar rats.

Relative organ weight (ROW)	Control	LD group (0.4 mg/kg bw)	MD group (2.0 mg/kg bw)	HD group (10 mg/kg bw)
Lung	0.915±0.05	0.920±0.05	0.938±0.11	0.987±0.06
Kidney	0.517±0.04	0.543±0.04	0.527±0.04	0.565±0.05
Spleen	0.628±0.05	0.583±0.06	0.555±0.03	0.530±0.05**
Heart	0.540±0.02	0.555±0.03	0.542±0.02	0.537±0.03

Values are presented as the mean ± SD (n = 06; adult male rats); Relative organ weight was calculated as (organ weight/body weight) × 100; $p \leq 0.05$ using one-way ANOVA followed by Dunnetts multiple comparison test; ** significant.

Visible observation of abdominal cavities in mid and high dose treated rats clearly revealed presence of entangled mass of MWCNTs in visceral membranes. External appearance of most of the organs was found normal except that of liver which showed darkening of external surface (Fig. 1)

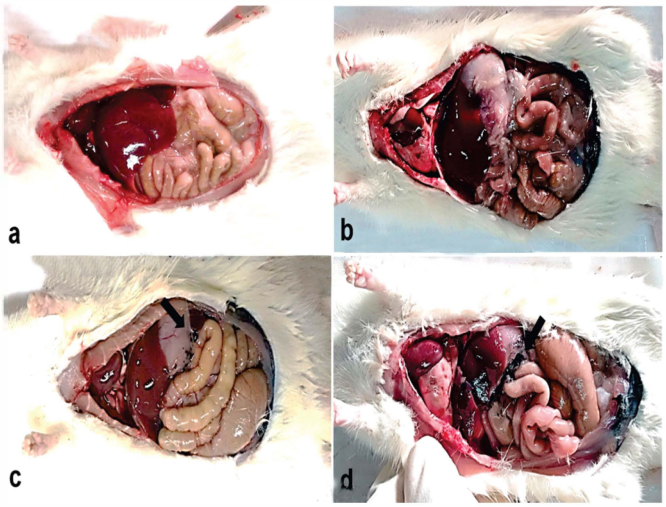

Fig. 1. Abdominal cavity exposed on the day of autopsy. a Control, showing clean organs and visceral membranes. b LD group abdominal cavity. c MD group shows black mass of agglomerated MWCNTs (arrow). d HD group also showing black mass of agglomerated MWCNTs (arrow) near liver.

Hematological Parameters

OH-*f* MWCNTs exposure was associated with changes in few hematological indicators. High dose resulted in significant reduction of RBC and MCV. Remaining other indicators including WBC, hematocrit, platelets, MCH, MCHC and hemoglobin content did not show any significant change compared to control. Low dose and mid dose did not affect the parameters in any of the test groups (Fig. 2).

Histopathological Observations

Lung histology exhibited normal features in all the treated group as in the control group (Fig. 3).

Lung tissue in control group contained normal histoarchitecture with thin interalveolar septa, normal fibrous tissue and clearly seen alveolar sacs. There were no major lesions, inflammation or evidence of fibrosis in lung tissue of treated rats. Spleen and kidney histology revealed mild damage in mid and high dose treatment groups (Fig. 4 and 5).

In the histology of spleen, control group exhibited normal architecture with normal periarterial lymphoid sheath, red pulp, white pulp and lymphoid follicles. In case of treated rats, mid- and high-dose group showed mild increase in white pulp area with the increase of compartments in white pulp (Fig. 4). Any major lesions were not observed in splenic sections. In case of kidney,

mild damage was observed in mid and high dose treated rats. Glomerular damage was clearly seen (Fig. 5c & d, arrowhead). Few instances of mild inflammatory cell aggregation were also found (Fig. 5d, arrow).

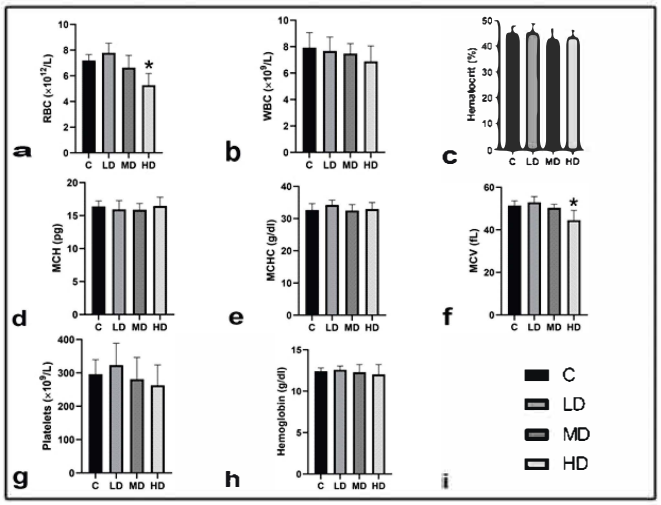

Fig. 2. Hematological indicators of rats after the intraperitoneal exposure of OH-f MWCNTs.
Each value represents mean ± standard deviation for each treatment group (n=6). Bars with asterisk (*) show statistically significant difference as compared to control, $p \leq 0.05$. a RBC, red blood cells. b WBC, white blood cells. c hematocrit. d MCH, mean corpuscular hemoglobin. e MCHC, mean corpuscular hemoglobin concentration. f MCV, mean corpuscular volume. g platelets. h Hb, hemoglobin. i legends .

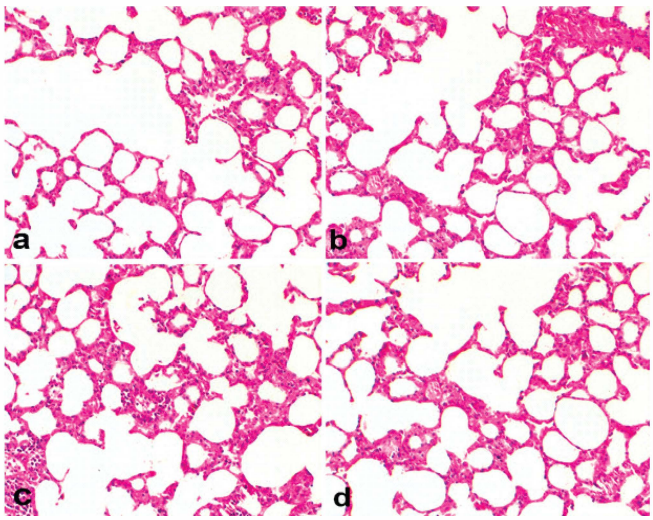

Fig. 3. Representative photographs showing histopathology of lung. a Control. b LD group. c MD group. d HD group. Magnification is 200×.

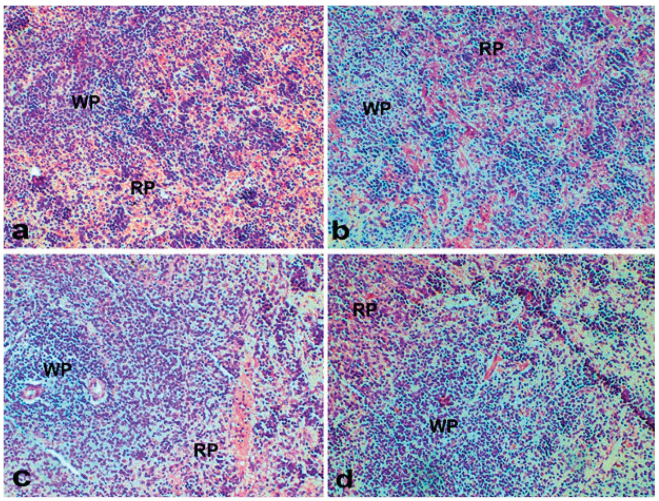

Fig. 4. Representative photographs showing histopathology of spleen. a Control. b LD group. c MD group. d HD group. Magnification is 200×. RP=Red pulp. WP=White pulp.

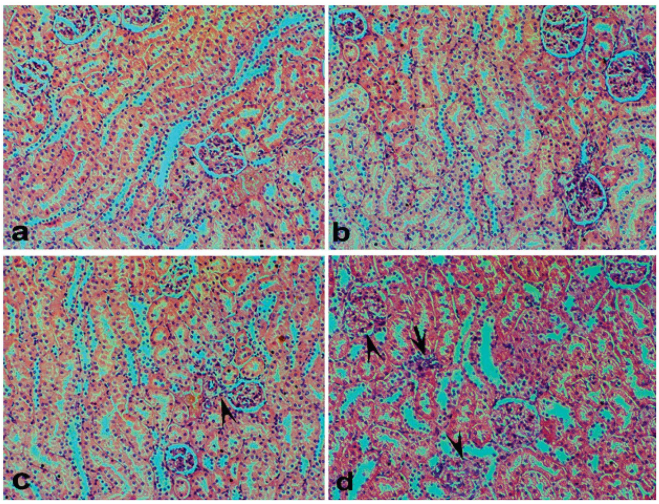

Fig. 5. Representative photographs showing histopathology of kidney. a Control. b LD group. c MD group. d HD group. Magnification is 200×. Arrow and arrowheads point towards the abnormal aspects of histology.

Present study examined the sub-acute toxicity of MWCNTs in Wistar rats post 15 repeated IP injections. Overall health was not significantly altered due to the exposure except a decrease in spleen relative weight at higher dose level. A few studies have produced similar findings. Bai *et al.* (2010) studied carboxylated (COOH)- and amino (NH_2)-functionalized MWCNTs with diameter 20-30 nm (5 repeated doses at 5 mg/kg bw dose level) in male mice. The group found that lung and spleen relative weights were declined significantly in treated

mice. In another study, 10 and 60 mg/kg bw of MWCNTs were associated with decrease in body weight gain of treated animals compared to control group after a 60-day intravenous exposure in Kunming mice (Ji *et al.*, 2009).

Hemocompatibility is an important aspect in toxicology studies. Blood is the tissue which becomes a vehicle for the distribution of test material to rest of the body. In the process, it itself is exposed to the material. In the current study, RBC and MCV were affected due to the exposure of MWCNTs as shown in the results section. Rest other parameters showed insignificant changes in their mean values as compared to the control groups. Kavosi *et al.* (2018) have reported toxicities of single walled CNTs (SWCNTs) and MWCNTs in BALB/c female mice. Both the CNTs were injected in mice once at different doses (0.5 and 1.0 mg/kg bw). After 7 days, the hemoglobin, RBC, WBC and hematocrit counts remained in normal range. Animals exposed with 1mg/kg SWCNT showed a significant elevation in MCH and MCHC levels ($P<0.0001$), but MWCNTs did not affect these parameters at similar dose level. All the exposed mice with SWCNT and MWCNT demonstrated significant decrease in MCV count ($P=0.0025$) and increase in platelet count ($P<0.0001$) compared to the control group. SWCNTs owing to their sharp and needle shaped morphology may adversely affect blood cells while MWCNTs which have multiple layers inflict lesser damage.

MWCNTs owing to nano size is capable of entering into various organs and tissues of the body. Distribution to various organs not only depends upon route of administration but the nature of the nanomaterial to be administered. For instance, water-soluble functionalized MWNTs were found to distribute in various body tissues and compartments of mice after IP injections (Guo *et al.*, 2007). Therefore, MWCNTs have the potential to induce adverse effects on tissues. In the current study, 2.0 and 10.0 mg/kg IP doses of hydroxyl functionalized MWCNTs induced mild tissue damage in spleen and kidney as revealed by histopathological analysis. The present findings are consistent with the results obtained by Qi *et al.* (2017). They injected 12.4 mg/kg MWCNTs intravenously in model mice. Examination of various biochemical parameters and histology revealed a significant dose-dependent toxicity. In lung tissue, edema and alveoli rupture were observed. Spleen showed fibrosis and damage. Similarly, kidney tissue also showed damages such as disordered kidney tubules, damage to podocytes and parietal cells. Liver and heart tissues were not affected in the study. In contrast to the above report, Kovasi *et al.* (2018) show that 1.0 mg/kg dose of MWCNTs did not affect most of the organs (lungs, kidney, spleen, brain, heart) except evidence of cell shrinkage in liver. The difference in findings clearly depends upon the dose levels. Direct instillation of MWCNTs in lungs is associated with lung fibrosis, inflammation and DNA damage (Gate *et al.* 2019).

Conclusion

The present work examined the toxicity of OH-*f*MWCNTs in adult Wistar rats in a sub-acute time frame and repeated dosing schedule. Intraperitoneal injections led to exposure related toxic changes in the parameters of study. Major altered parameters included RBC number, MCV, spleen histology and kidney histology. One point that is apparent from results is that toxic changes were dose dependent. Mid (2.0 mg/kg bw) or high (10.0 mg/kg bw) or both resulted in toxicity while, low dose (0.4 mg/kg bw) did not affect any parameter. The current report suggests that MWCNTs have the potential to produce toxicity to various tissues and care should be exercised before these materials enter our daily life in substantial manner.

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Conflict(s) of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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