Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 10 [5] April 2021 : 266-272
©2021 Academy for Environment and Life Sciences, India
Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Hepatotoxicity of hydroxyl-functionalized multiwalled carbon nanotubes in Wistar rats

Naresh K. Nirmal[1], Kumud K. Awasthi[1,2], Placheril J. John[1*]
1Department of Zoology, University of Rajasthan, Jaipur, 302004 India
2Department of Life Sciences, Vivekananda Global University, Jaipur, 303012 India
Email:nir.poet@gmail.com; kkantawasthi@gmail.com; *placheriljohn@gmail.com

ABSTRACT
The current study analyzed effects of hydroxyl-functionalized MWCNTs on liver of adult Wistar rats. Four equal sets of rats were intraperitoneally administered with increasing doses of the nanomaterial for a period of 30 days. General health parameters, liver function and oxidative stress assays were performed along with histological study. Inflammation, vacuolations, agglomerates of MWCNTs around the veins and hepatocytic injury were major anomalies observed in histological sections of treated liver. The exposure was associated with significant changes in liver function enzymes and oxidative stress level. These findings indicate that hydroxyl-functionalized MWCNTs are harmful to rodent liver.
Keywords*: Multiwalled carbon nanotubes, Hydroxyl-functionalized MWCNTs, Toxicity, Histopathology, Hepatotoxicity.*

Received 18.02.2021 Revised 23.03.2021 Accepted 15.04.2021

INTRODUCTION

Carbon in its nano-form is highly utile material. One such material, carbon nanotubes (CNTs)is made up of tiny, hollow cylindrical tubes with one or more walls having diameter less than 100 nm. Carbon nanotubes have become a topic of intense research activity in recent years owing to their unique physico-chemical attributes. CNTs possess high tensile strength and elasticity, excellent thermal stability with ample electric conductance, easy functionalization potential, much surface area with extremely small diameter which make CNTs wonderous materials for wide ranging industrial and biomedical applications [1]. Global market of CNTs is projected to increase with compound annual growth rate of 16.70% from USD 3.95 billion in 2017 to USD 9.84 billion by 2023 according to a report by Markets and Markets (2018).

Studies have suggested that MWCNTs are capable of entering into cells through diverse mechanisms and producing several biochemical changes which are deleterious in nature such as overproduction of reactive oxygen species (ROS), DNA damage, mitochondrial damage and cell cycle impairment [2, 3]. *In vivo* exposure to MWCNTs inflict organ specific damage and has potential to disturb homeostatic mechanisms of the body. Awasthi *et al.* (2013)studied impacts of MWCNTs in Swiss albino mice and found that after 28 days, hepatic tissue exhibited inflammation, necrosis and blood coagulation [4]. MWCNTs are engulfed by macrophages in lungs and cause frustrated phagocytosis. Frustrated phagocytosis leads to inflammation in the tissue which in turn is a factor for increased oxidative stress [5]. Our previous reports have also shown that without affecting overall health, MWCNTs generate toxic effects on reproductive organs of male Wistar rats [6].

Despite of the above reports, toxicity assessments of MWCNTs are challenging and far from conclusive because their toxicity is greatly affected by various factors like route of administration, dose level, length and size of MWCNTs, presence of variety of surface functional groups among others. Therefore, in the current study, hydroxyl functionalized MWCNTs(OH-*f* MWCNTs) having average diameter of10-20 nm were used and their toxicity was tested over a sub-acute duration by intraperitoneal route of exposure.

MATERIAL AND METHODS

OH-f MWCNTs

OH-*f* MWCNTs were procured from Sisco Research Laboratories (SRL) Pvt. Ltd. (Andheri (E), India).The nanotubes had average diameter in the range of 10 to 20 nm while the length was 10-30 µm. Detailed characterization data has already been published elsewhere[6].

Animal maintenance and exposure

A total of 24 healthy adult male Wistar rats (age 10-12 weeks; body weight 175±10 g) were grouped in four equal sets and were exposed to increasing dose of OH-*f* MWCNTs. Dose levels were 0.0, 0.4 (low), 2.0 (mid) and 10.0 (high) mg/Kg body weight. Experimental sets were named according to the dose level, namely control group (vehicle only sterilized normal saline containing 0.1% Tween-80), low-dose (LD) group, mid-dose (MD) group, high-dose (HD). Intraperitoneal injections with chosen doses were given on alternate days for a duration of 30 days. MWCNTs were suspended in sterilized normal saline containing 0.1% Tween-80 and sonicated well before each dose (Labman-probe sonicator, PRO-250).Experimental animals were euthanized after the treatment period of 30 days. Serum was separated from the blood and used for the assay of liver function enzymes. A part of the liver tissue was preserved in Bouin's fixative for histopathology. Rest of the organ was kept at -80^{0}C until used for biochemical parameters. The work had the approval of the Institutional Animal Ethical Committee (1678/GO/a/12CPCSEA).

Liver function assays

Liver function assays viz. aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) were carried out using commercial kits (AccuZyme GOT, AccuZyme GPT and AccuZyme ALP respectively) procured from Accurex Biomedical Pvt. Ltd. (Mumbai).

Preparation of liver homogenate and mitochondrial fractions

Liver homogenate was prepared in a mixture of cold PBS (50mM, pH 7.4) and 0.25M sucrose (20% w/v homogenate). Homogenate filtered through a four-layered cheesecloth was treated as crude homogenate. Crude homogenate was further centrifuged at 600 g for 10 minutes at 4^{0}C. Supernatant thus obtained was then subjected to centrifugation at 10,000 g for 5 minutes. Pellet was washed three times in chilled PBS and suspended again for further experimentation as mitochondrial fraction (MF).

Catalase (CAT) assay

CAT activity was measured according to protocol of Aebi [7].Reaction mixture was prepared by mixing 100µl of MF (containing 0.4-0.5 mg protein) and 2.9 ml of 50 mM PBS (pH 7.0) having 12 mM H_2O_2. Absorbance was taken immediately by UV-Vis spectrophotometer at 240 nm every 15 seconds for 2 minutes. Results were written as µmoles of H_2O_2 decomposed/min/mg protein.

Malondialdehyde (MDA) assay

Total MDA concentration was estimated by the method given by Ohkava *et al*. (1979).[8] 1.0 ml of 0.15 M Tris-HCl buffer (pH 7.4), 0.3 ml of 10 mM KH_2PO_4 were mixed with 0.2 ml sample (crude homogenate containing 1-1.5 mg of protein) and 0.5 ml of DW. 10% TCA (1.0 ml) was added after a 20-minute incubation at 37^{0}C with constant shaking. 1.5 ml 1% TBA was then added and the mixture was kept in a boiling water bath for 1 hour. Test tubes were incubated at 4^{0}C for 1hour followed by n-butanol and pyridine (15:1 v/v) extraction. Absorbance was taken spectrophotometrically at 532 nm. Extinction coefficient of $1.56X10^6$ M^{-1} $cm^{-1}$was used for calculation. Unit of concentration was reported as nmol MDA/mg protein.

Histopathology

Organ samples were fixed in Bouin's solution for 24 hours followed by thorough washing in running water. Dehydration was done in graded alcohol series. Paraffin embedded tissues were cut into sections of 4-5 µm by a microtome and stained with hematoxylin and eosin. Microscopic observations and microphotography were performed and recorded (DM 1000, Leica Microsystems, Germany).

Statistical analysis

Values were documented as mean ± standard deviation (SD). Data was statistically analyzedby GraphPad Prism (version 8.0.2). One-way ANOVA was used to test the statistical significance and Tukey's honest significant difference test was applied for multiple comparisons between control and treated groups. $p \leq 0.05$ was considered significant.

RESULTS

General health

Exposure to OH-*f*MWCNTs did not produce significant change in behavior. Animals displayed aggression, lethargic body movements and piled up in cage corners after injections in exposed as well as control group. Aggression and pile-up behavior were most pronounced in HD group. Other clinical symptoms such as excessive salivation, heightened thirst, food avoidance and shivering were not observed (Table 1). Rats consumed water and food in normal quantities throughout the study period (Figure 1a, b). Liver index did not vary significantly in treated animals as compared tocontrol (Figure1c).External surface of liver showed darkening with entangled MWCNTs in visceral membranes of HD treated rats (Figure 2d).

Biochemical parameters

Enzyme activities of ALT, AST and ALP in blood sera were elevated significantly in mid- and high-dose treated animals in comparison to control (Figure 3a, b, c). Liver tissue homogenates from MD and HD

treated rats had significantly altered CAT activity as shown in figure 3d.Liver extracts from mid- and high-dose treated groups were found to contain higher levels of malondialdehyde in comparison to the control as well as LD group (Figure 3e).

Histopathological observations

Histological analysis from control and low-dose group revealed well-arranged hepatic lobules with intact cords of hepatocytes. Tissue had healthy hepatocytes, Kupffer cells and normal sinusoidal spaces (Figure 4a, b).Mid- and high-dose tissues exhibited various pathological signs apart from normal structural aspects. In many instances,edema filled spaces were observed. Mild inflammatory areas around the portal and central veins were observed in high-dose treated rats (Figure4c,d). Agglomerated mass of MWCNTs could be seen at various locations in mid- and high-dose groups.The agglomerates were lodged into the extracellular space. Agglomerates in most cases were surrounded by Kupffer cells. Hepatocyte shape change, loss of nuclei and cytoplasm showed clear evidence of injury to cells (Figure4e, f and h). Pathology score showed adose-dependent increase in structural anomalies as compared to control. However, statistically significant change was observed in case of mid-and high-dose groups (Figure 4i).

DISCUSSION

The current study was undertaken to study *in vivo*toxicity of hydroxyl-functionalized MWCNTs over a sub-acute exposure schedule. Despite of high dose levels, administered nanotubes did not affect general health of rats. Intraperitoneally administered nanoparticles reach in liver through hepatic portal vein and accumulate there depending upon the nature of the particle [9]. OH-*f* MWCNTs accumulated in liver of treated rats in the present study. Signs of accumulation can be inferred by darkening of liver in the photographs of liver from HD group rats and by the presence of MWCNTs agglomerates or aggregates observed in histopathology. Bai et al. (2010)analyzed toxicity of carboxylated (COOH)- and amino (NH_2)-functionalized MWCNTs (diameter20-30 nm; length0.5-2.0 μm, 5 repeated doses at 5 mg/kg dose level) via intravenous route in male mice and reported that treated micehad similar body weight gains and their relative organ weights did not change significantly except lung and spleen organ weight [9]. Contrasting results with dose 10 and 60 mg/kg of MWCNTs was reported by Ji *et al.* (2009) where body-weight gain was decreased significantly as compared to control group after a 60-day intravenous exposure in Kunming mice [10]. Such a contrast is possibly be a result of differences in route of dose administration, dose level and nature of carbon nanotube used.

Liver inflammation and damage is strongly correlated with increased levels of ALT, AST and ALP. Mice were intraperitoneally injected with carboxyl-functionalized MWCNTs (with an outer diameter of 15-30 nm and lengths of 15-20 μm) at 0.25, 0.5, and 0.75 mg/kg/day for 5 days. The study recorded that 0.75 mg/kg/day dose resulted in significant deviation in the activities of ALT and ALP. However, level of AST was found to be unchanged in comparison to control.[11]In the present study, all three enzymes exhibited increased activities as compared to control at 2.0 and 10.0 mg/Kg dose levels.This is an evidence of liver inflammation and injury.Our report also indicates that the hydroxyl functionalization is not sufficient to reduce the toxicity of MWCNTs. This *in vivo* observation does not corroborate with an *in vitro* study done by Liu et al. (2014). The group reported that conjugating pristine MCNTswith hydroxyl group reduces their cytotoxicity. It not only prevented disturbance of the mitochondrial membrane potential but also avoided the release of cytochrome c and caspases activation [12].

Most studies with nanomaterials agree that ROSoverproduction and resulting cellular damage is one of the primary mechanisms of their toxicity. Therefore, CAT activity and lipid peroxidation was assessed in the present study. Results demonstrated decrease in CAT activity and elevation in MDA concentration in liver extracts as compared to control which is a sign of significant ROS production and oxidative stress. These results are in line with the findings of Ji *et al.* (2009). Tween-80 dispersed MWCNTs were shown to result in mild reversible oxidative stress in liver of treated mice after 15 days of intravenous administration [10]. Adedara *et al.* (2018) investigated hepatotoxicity of COOH-MWCNTs. Adult rats were treated at 0, 0.25, 0.50, 0.75 and 1.0 mg/kg for 5 consecutive days. Inflammatory markers and oxidative stress parameters were found to be increased including serum activities of AST, ALT, ALP and gamma glutamyl transferase when compared with control [13].

Patlolla *et al.* (2011) found a dose related impact on histological structure of liver following exposure to 0.25, 0.5 and 0.75 mg/kg/day of functionalized MWCNT. Structure anomalies included hepatocellular vacuolations, hepatocyte injury, disruption of central vein etc. At higher doses liver atrophy was reported which is a sign of severe toxicity [11]. In an interesting work by Xu *et al.* (2016), MWCNTs (average diameter, 30 nm; length, ≥1 μm) were administered intravenously in a fatty liver rat model. The exposure enhanced the steatohepatitis and oxidative stress in liver with chronic hepatitis [14]. The present study observed mild inflammatory areas and vacuolations around the central vein and portal veins. Agglomerates lodged into the extracellular spaces clearly showed that multiple injections of MWCNTs at

2.0 and 10.0 mg/Kg dose results in accumulation and agglomerate or aggregate formation inside the tissue. Although, possibility of agglomerates in the dispersion prior to treatment, despite of ultrasonication, cannot be completely denied. It was interesting to note that hepatocytes near these agglomerates exhibited altered morphology and Kupffer cells were found adjacent to these areas. These observations indicate that OH-*f* MWCNTs generatetoxicity to hepatic tissue in a localized manner. Toxicity outcomes are greatly affected by diameter, length, functionalization and surface defects of MWCNTs. For instance, toxicity to rat lung tissue increases considerably as the length of nanotubes decreases from 2.77 μm to 0.40 μm [15]. Diverging results due to above mentioned factors is a serious challenge in toxicity analysis of OH-*f* MWCNTs. Meticulously designed studies considering physico-chemical characteristics are needed to fully address biocompatibility issue of these highly beneficial nanomaterials.

Table 1. Behavioral signs as recorded at 1, 6, 12 and 24 hours after the exposure of OH-*f* MWCNTs.

Behavioral responses	Time Interval															
	1 hour				**6 hours**				**12 hours**				**24 hours**			
	C	**LD**	**MD**	**HD**	**C**	**LD**	**MD**	**HD**	**C**	**LD**	**MD**	**HD**	**C**	**LD**	**MD**	**HD**
Thirst	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salivation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aggressiveness	+	+	+	+++	+	+	++	++	+	+	+	+	-	-	-	+
Change in gait	-	+	+	++	-	-	-	+	-	-	-	-	-	-	-	-
Shivering	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Itching	+	+	+	++	-	-	-	+	-	-	-	-	-	-	-	-
Food avoidance	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lethargy	-	-	+	++	-	-	+	++	-	-	+	+	-	-	-	+
Piling	+	+	++	+++	-	+	+	++	-	-	+	+	-	-	-	+

++++ Very severe; +++ Severe; ++ Moderate; + Mild; - Absent
C=Control; LD=Low-dose group; MD=Mid-dose group; HD=High-dose group

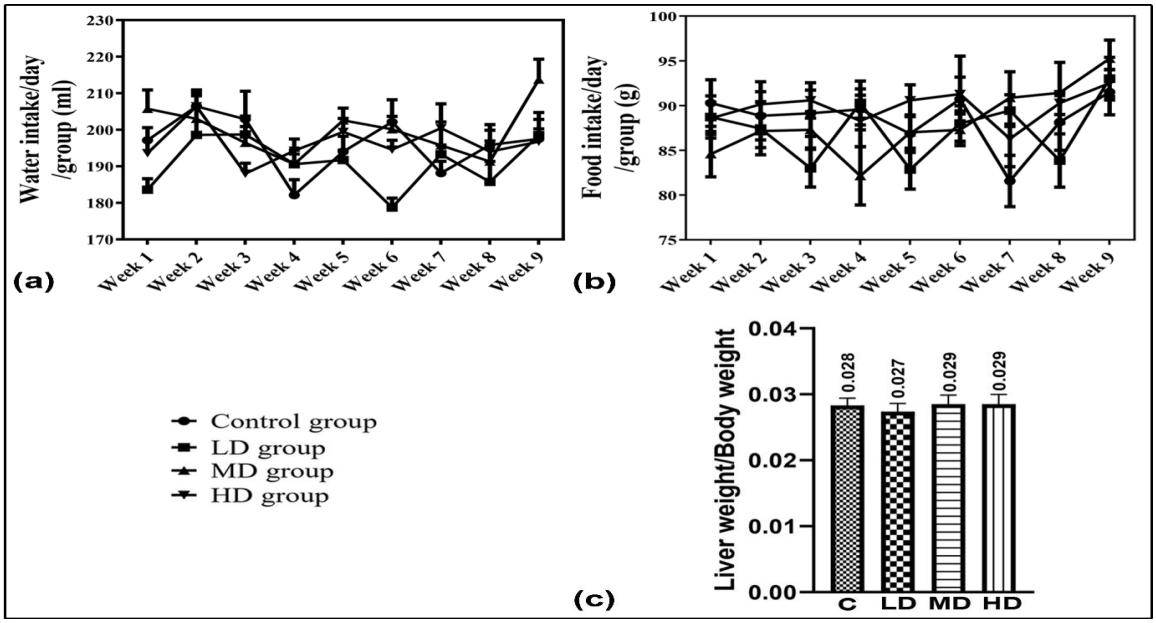

Figure 1. Water and food intake observed in control and treated animals from a simultaneous experiment in which animals were studied for a duration of 60 days.**(a)**Water intake (in ml/day/group) by control and OH-*f*MWCNT-exposed Wistar rats. Mean values represent the average of water consumption by a group of 6 rats in a period of 7 days. Values are expressed as Mean±SEM.**(b)** Food consumption (in g/day/group) pattern. Mean values represent the average of food consumption by a group of 6 rats in a period of 7 days. Values are expressed as Mean±SEM.**(c)** Liver index (liver weight/body weight) calculated in experimental groups. Values are mean ± SD.

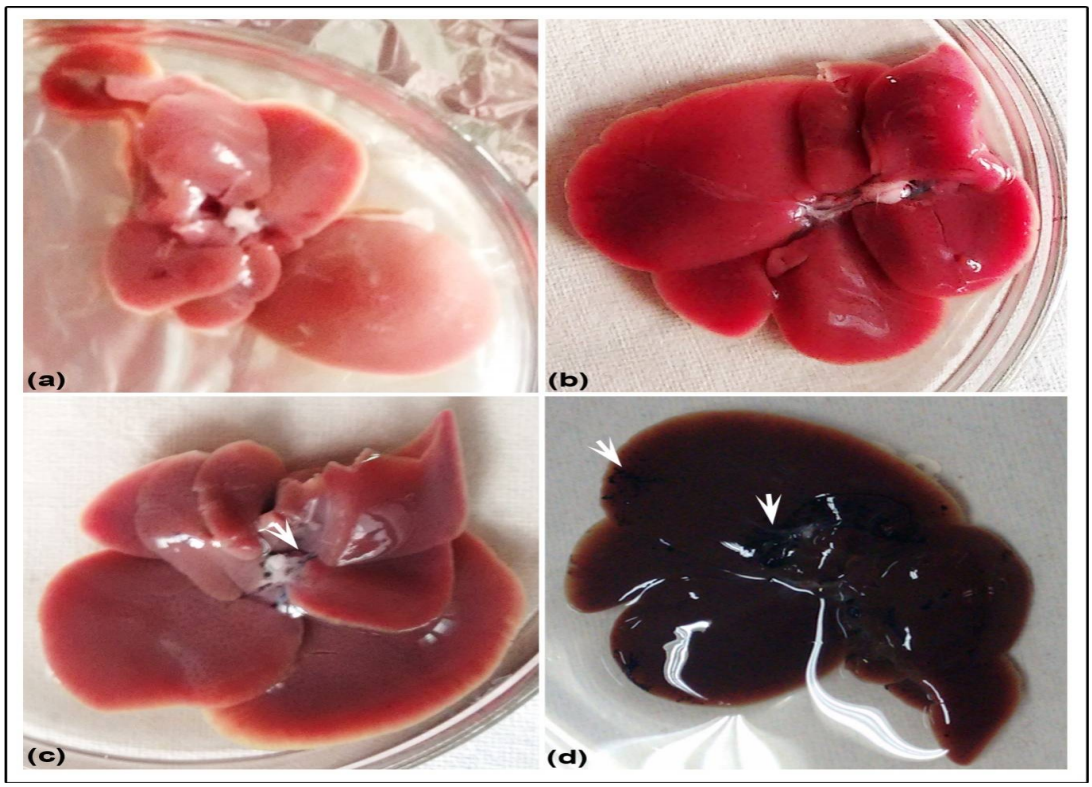

Figure 2. Representativephotographs of liver from control and treated rats showing external morphology. Images from; **(a)** control group,**(b)** LD group,**(c)** MD group,**(d)** HD group showing blackish color and entangles mass of OH-*f* MWCNTs (white arrow).

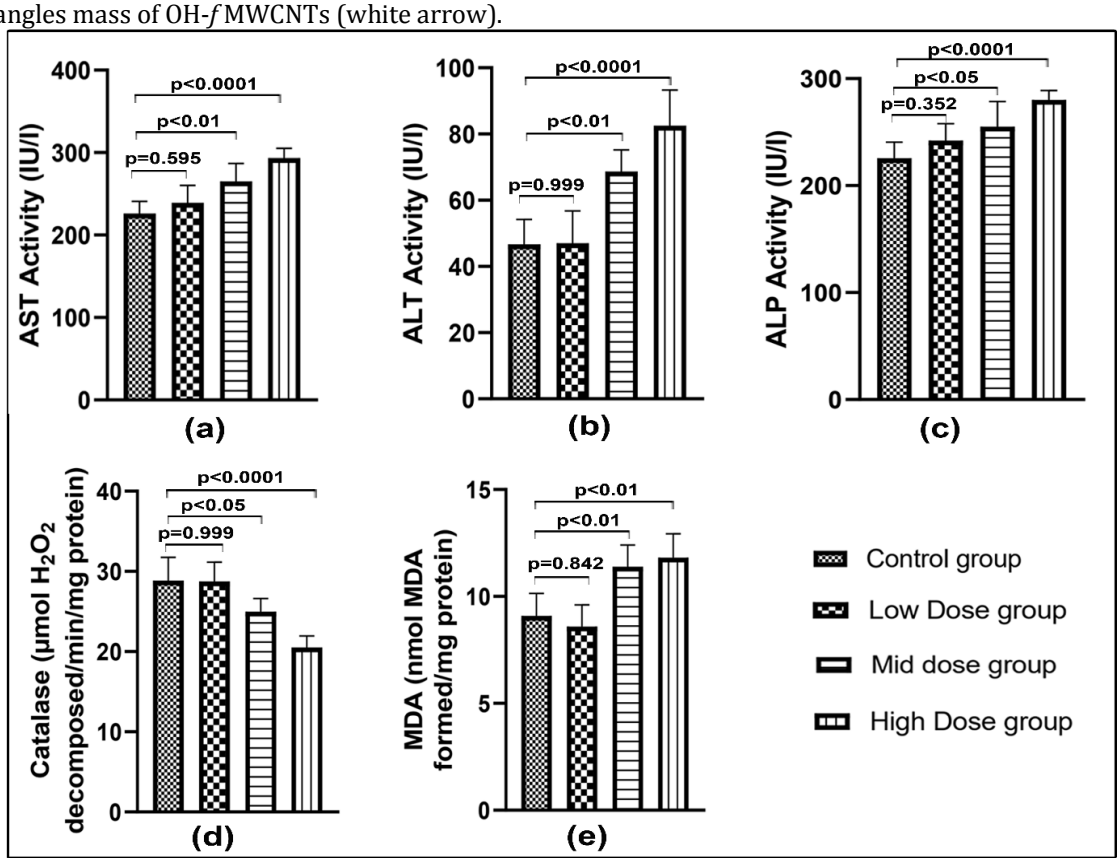

Figure 3. Biochemical assays.**(a)** AST enzyme activity in sera of control and treated groups. **(b)** Activity of ALT. **(c)** ALP activity as observed in control and exposed groups. **(d)**Specific activity of CAT in mitochondrial fraction of control and treated rats. **(e)** Concentrations of MDA in tissues of control and treated rats. Data presented as mean ± SD. P<0.05 was considered significant difference from the control.

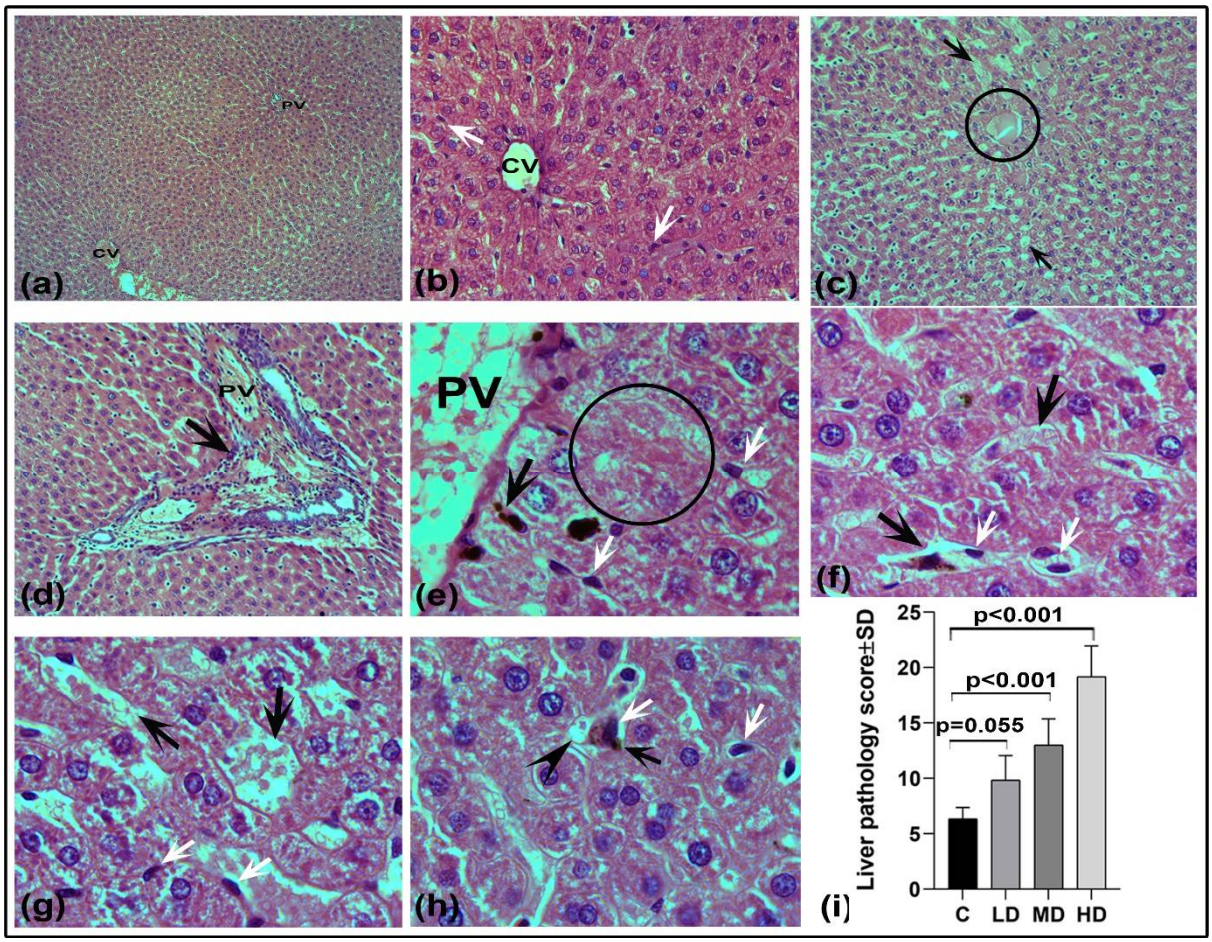

Figure 4. Representative photomicrographs showing normal and pathological aspects of liver histology. **(a)** and **(b)** Normal histology of liver at different magnifications (100X in 'a' and 400X in 'b'). Sections show good lobular arrangement. The cords of hepatocytes are well organized with normal sinusoidal spaces. White arrows show Kupffer cells. CV,Central Vein; PV, Portal Vein. **(c)** Liver histology at 200X magnification showing exudate (circle) and presence of vacuoles (black arrows) a feature frequently encountered in treated groups. **(d)**Image showing mild inflammation around the portal vein (black arrow). Magnification is 200X. **(e)** Agglomerates of MWCNTs as seen at 1000X near the portal vein boundary (black arrow). White arrows show presence of Kupffer cells. The agglomerates are lodged into the extracellular space. Hepatocytes in the image are showing loss of nuclei (area inside the circle). **(f)** and **(g)**Show change in morphology of hepatocytes along with vacuolated area around cells (black arrows). Agglomerates of MWCNTs are also seen. Kupffer cells (white arrow) are visible near the agglomerates or around the vacuoles (1000X). **(h)**Shows a hepatocyte with damaged structure and MWCNTs agglomerate (black arrow). Kupffer cell(white arrow) is also observed adjacent to the agglomerate (arrowhead)(1000X). **(i)** Histopathology score from all the groups. The histological sections (10 per animal) were scored for the presence of various anomaliesincluding inflammatory areas, vacuolated sinusoids, altered cell shape, agglomerates of MWCNTs, presence of edema and exudate etc. Presence of pathologicalsign was allotted a score of 1 while the absence was noted as 0. The resultant scores for each group (mean ± SD) are displayed in the form of a bar graph. $P<0.05$ was considered as statistically significant variation as compared to the control.

SUMMARY

The current study analyzed toxicity of OH-*f* MWCNTs in adult Wistar rats in a sub-acute and repeated exposure scheme. Results of the study clearly demonstrated a dose-related toxicity outcome. Liver function enzymes were significantly elevated indicating inflammatory damage to liver. Histopathological observations correlated with biochemical parameters. Observed hepatic toxicity may be a result of increased oxidative stress as suggested by altered MDA and CAT levels. More studies are required to decipher the mechanistic aspect of the toxicity. Findings in our study are expressive of the fact that OH-*f*

MWCNTs have significant toxicity to mammalian liver and efforts are needed to alleviate the toxicity of these nanotubes.

ACKNOWLEDGEMENT
We acknowledge Indian Council of Medical Research, New Delhi for partial financial assistance as Junior Research Fellowship (3/1/3/JRF-2011/HRD 107/81242) to Naresh Kumar Nirmal.

CONFLICT(S) OF INTEREST
The author(s) declared no potential conflicts of interest with respect tothe research, authorship, and/or publication of this article.

REFERENCES
1. Sharma, P., Mehra, N. K., Jain, K., et al., (2016). Biomedical Applications of Carbon Nanotubes: A Critical Review. *Curr. Drug.Deliv.*, **13**(6), 796 – 817.
2. Fanizza, C., Casciardi, S., Incoronato, F., et al., (2015). Human epithelial cells exposed to functionalized multiwalled carbon nanotubes: interactions and cell surface modifications. *J.Microsc.*, **259**, 173-84.
3. Hiraku, Y., Guo, F., Ma, N., Yamada, T., et al., (2016). Multi-walled carbon nanotube induces nitrative DNA damage in human lung epithelial cells via HMGB1-RAGE interaction and Toll-like receptor 9 activation. *Part.Fibr.Toxicol.*, , **13**, 16.
4. Awasthi, K. K., John, P. J., Awasthi A., et al., (2013). Multi walled carbon nanotubes induced hepatotoxicity in Swiss albino mice. *Micron.*, **44**, 359-364.
5. Boyles, M. S. P., Young, L., Brown, D. M., et al., (2015). Multi-walled carbon nanotube induced frustrated phagocytosis, cytotoxicity and pro-inflammatory conditions in macrophages are length dependent and greater than that of asbestos. *Toxicol. In Vitro*., **29**, 1513-1528.
6. Nirmal, N.K., Awasthi, K.K. and John, P.J., (2017). Effects of hydroxyl-functionalized multi-walled carbon nanotubes on sperm health and testes of Wistar rats. *Toxicol. Ind. Health*, **33**(6),519-529.
7. Aebi, H., Catalase in Vitro. *Methods Enzymol.*, 1984, **105**, 121-26.
8. Ohkawa, H., Ohishi, N. and Yagi, K., (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal.Biochem.*, **95**, 351-358.
9. Bai, Y., Zhang, Y., Zhang, J., et al., (2010). Repeated carbon nanotube administrations in male mice cause reversible testis damage without affecting fertility. *Nat Nanotechnol*., **5**, 683-89.
10. Ji, Z., Zhang, D., Li, L., et al., (2009). The hepatotoxicity of multi-walled carbon nanotubes in mice. *Nanotechnology*., **20**(44), 445101.
11. Patlolla, A. K., Berry, A. and Tchounwou, P. B., (2011). Study of hepatotoxicity and oxidative stress in male Swiss-Webster mice exposed to functionalized multi-walled carbon nanotubes. *Mol. Cell Biochem.*, **358**(1-2),189-99.
12. Liu, Z., Liu, Y. and Peng, D., (2014). Hydroxylation of multi-walled carbon nanotubes reduces their cytotoxicity by limiting the activation of mitochondrial mediated apoptotic pathway. *J. Mater. Sci. Mater. Med.*, **25**, 1033-1044.
13. Adedara, I. A., Anao, O. O., Forcados, G. E., et al., (2018). Low doses of multi-walled carbon nanotubes elicit hepatotoxicity in rats with markers of oxidative stress and induction of pro-inflammatory cytokines. *Biochem.Biophys. Res. Commun*., **503**(4), 3167-3173.
14. Xu, Y. Y., Juan, G., Zhang, M. H., et al., (2016). Intravenous administration of multi-walled carbon nanotubes aggravates high-fat diet-induced non-alcoholic steatohepatitis in Sprague-Dawley rats.*Int. J.Toxicol.*, **35**(6), 634-643.
15. Ema, M., Takehara, H., Naya, M., et al., Length effects of single-walled carbon nanotubes on pulmonary toxicity after intratracheal instillation in rats. *J.Toxicol. Sci.*, 2017, **42**(3), 367-378.

CITATION OF THIS ARTICLE
Naresh K. Nirmal, Kumud K. Awasthi, Placheril J. John. Hepatotoxicity of hydroxyl-functionalized multiwalled carbon nanotubes in Wistar rats. Bull. Env. Pharmacol. Life Sci., Vol 10[5] April 2021: 266-272

Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 10 [5] April 2021 : 266-272
©2021 Academy for Environment and Life Sciences, India
Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Hepatotoxicity of hydroxyl-functionalized multiwalled carbon nanotubes in Wistar rats

Naresh K. Nirmal[1], Kumud K. Awasthi[1,2], Placheril J. John[1*]
1Department of Zoology, University of Rajasthan, Jaipur, 302004 India
2Department of Life Sciences, Vivekananda Global University, Jaipur, 303012 India
Email:nir.poet@gmail.com; kkantawasthi@gmail.com; *placheriljohn@gmail.com

ABSTRACT
The current study analyzed effects of hydroxyl-functionalized MWCNTs on liver of adult Wistar rats. Four equal sets of rats were intraperitoneally administered with increasing doses of the nanomaterial for a period of 30 days. General health parameters, liver function and oxidative stress assays were performed along with histological study. Inflammation, vacuolations, agglomerates of MWCNTs around the veins and hepatocytic injury were major anomalies observed in histological sections of treated liver. The exposure was associated with significant changes in liver function enzymes and oxidative stress level. These findings indicate that hydroxyl-functionalized MWCNTs are harmful to rodent liver.
Keywords*: Multiwalled carbon nanotubes, Hydroxyl-functionalized MWCNTs, Toxicity, Histopathology, Hepatotoxicity.*

Received 18.02.2021 Revised 23.03.2021 Accepted 15.04.2021

INTRODUCTION

Carbon in its nano-form is highly utile material. One such material, carbon nanotubes (CNTs)is made up of tiny, hollow cylindrical tubes with one or more walls having diameter less than 100 nm. Carbon nanotubes have become a topic of intense research activity in recent years owing to their unique physico-chemical attributes. CNTs possess high tensile strength and elasticity, excellent thermal stability with ample electric conductance, easy functionalization potential, much surface area with extremely small diameter which make CNTs wonderous materials for wide ranging industrial and biomedical applications [1]. Global market of CNTs is projected to increase with compound annual growth rate of 16.70% from USD 3.95 billion in 2017 to USD 9.84 billion by 2023 according to a report by Markets and Markets (2018).

Studies have suggested that MWCNTs are capable of entering into cells through diverse mechanisms and producing several biochemical changes which are deleterious in nature such as overproduction of reactive oxygen species (ROS), DNA damage, mitochondrial damage and cell cycle impairment [2, 3]. *In vivo* exposure to MWCNTs inflict organ specific damage and has potential to disturb homeostatic mechanisms of the body. Awasthi *et al.* (2013)studied impacts of MWCNTs in Swiss albino mice and found that after 28 days, hepatic tissue exhibited inflammation, necrosis and blood coagulation [4]. MWCNTs are engulfed by macrophages in lungs and cause frustrated phagocytosis. Frustrated phagocytosis leads to inflammation in the tissue which in turn is a factor for increased oxidative stress [5]. Our previous reports have also shown that without affecting overall health, MWCNTs generate toxic effects on reproductive organs of male Wistar rats [6].

Despite of the above reports, toxicity assessments of MWCNTs are challenging and far from conclusive because their toxicity is greatly affected by various factors like route of administration, dose level, length and size of MWCNTs, presence of variety of surface functional groups among others. Therefore, in the current study, hydroxyl functionalized MWCNTs(OH-*f* MWCNTs) having average diameter of10-20 nm were used and their toxicity was tested over a sub-acute duration by intraperitoneal route of exposure.

MATERIAL AND METHODS

OH-f MWCNTs

OH-*f* MWCNTs were procured from Sisco Research Laboratories (SRL) Pvt. Ltd. (Andheri (E), India).The nanotubes had average diameter in the range of 10 to 20 nm while the length was 10-30 µm. Detailed characterization data has already been published elsewhere[6].

Animal maintenance and exposure

A total of 24 healthy adult male Wistar rats (age 10-12 weeks; body weight 175±10 g) were grouped in four equal sets and were exposed to increasing dose of OH-*f* MWCNTs. Dose levels were 0.0, 0.4 (low), 2.0 (mid) and 10.0 (high) mg/Kg body weight. Experimental sets were named according to the dose level, namely control group (vehicle only sterilized normal saline containing 0.1% Tween-80), low-dose (LD) group, mid-dose (MD) group, high-dose (HD). Intraperitoneal injections with chosen doses were given on alternate days for a duration of 30 days. MWCNTs were suspended in sterilized normal saline containing 0.1% Tween-80 and sonicated well before each dose (Labman-probe sonicator, PRO-250).Experimental animals were euthanized after the treatment period of 30 days. Serum was separated from the blood and used for the assay of liver function enzymes. A part of the liver tissue was preserved in Bouin's fixative for histopathology. Rest of the organ was kept at -80^{0}C until used for biochemical parameters. The work had the approval of the Institutional Animal Ethical Committee (1678/GO/a/12CPCSEA).

Liver function assays

Liver function assays viz. aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) were carried out using commercial kits (AccuZyme GOT, AccuZyme GPT and AccuZyme ALP respectively) procured from Accurex Biomedical Pvt. Ltd. (Mumbai).

Preparation of liver homogenate and mitochondrial fractions

Liver homogenate was prepared in a mixture of cold PBS (50mM, pH 7.4) and 0.25M sucrose (20% w/v homogenate). Homogenate filtered through a four-layered cheesecloth was treated as crude homogenate. Crude homogenate was further centrifuged at 600 g for 10 minutes at 4^{0}C. Supernatant thus obtained was then subjected to centrifugation at 10,000 g for 5 minutes. Pellet was washed three times in chilled PBS and suspended again for further experimentation as mitochondrial fraction (MF).

Catalase (CAT) assay

CAT activity was measured according to protocol of Aebi [7].Reaction mixture was prepared by mixing 100µl of MF (containing 0.4-0.5 mg protein) and 2.9 ml of 50 mM PBS (pH 7.0) having 12 mM H_2O_2. Absorbance was taken immediately by UV-Vis spectrophotometer at 240 nm every 15 seconds for 2 minutes. Results were written as µmoles of H_2O_2 decomposed/min/mg protein.

Malondialdehyde (MDA) assay

Total MDA concentration was estimated by the method given by Ohkava *et al*. (1979).[8] 1.0 ml of 0.15 M Tris-HCl buffer (pH 7.4), 0.3 ml of 10 mM KH_2PO_4 were mixed with 0.2 ml sample (crude homogenate containing 1-1.5 mg of protein) and 0.5 ml of DW. 10% TCA (1.0 ml) was added after a 20-minute incubation at 37^{0}C with constant shaking. 1.5 ml 1% TBA was then added and the mixture was kept in a boiling water bath for 1 hour. Test tubes were incubated at 4^{0}C for 1hour followed by n-butanol and pyridine (15:1 v/v) extraction. Absorbance was taken spectrophotometrically at 532 nm. Extinction coefficient of $1.56X10^6$ M^{-1} $cm^{-1}$was used for calculation. Unit of concentration was reported as nmol MDA/mg protein.

Histopathology

Organ samples were fixed in Bouin's solution for 24 hours followed by thorough washing in running water. Dehydration was done in graded alcohol series. Paraffin embedded tissues were cut into sections of 4-5 µm by a microtome and stained with hematoxylin and eosin. Microscopic observations and microphotography were performed and recorded (DM 1000, Leica Microsystems, Germany).

Statistical analysis

Values were documented as mean ± standard deviation (SD). Data was statistically analyzedby GraphPad Prism (version 8.0.2). One-way ANOVA was used to test the statistical significance and Tukey's honest significant difference test was applied for multiple comparisons between control and treated groups. $p \leq 0.05$ was considered significant.

RESULTS

General health

Exposure to OH-*f*MWCNTs did not produce significant change in behavior. Animals displayed aggression, lethargic body movements and piled up in cage corners after injections in exposed as well as control group. Aggression and pile-up behavior were most pronounced in HD group. Other clinical symptoms such as excessive salivation, heightened thirst, food avoidance and shivering were not observed (Table 1). Rats consumed water and food in normal quantities throughout the study period (Figure 1a, b). Liver index did not vary significantly in treated animals as compared tocontrol (Figure1c).External surface of liver showed darkening with entangled MWCNTs in visceral membranes of HD treated rats (Figure 2d).

Biochemical parameters

Enzyme activities of ALT, AST and ALP in blood sera were elevated significantly in mid- and high-dose treated animals in comparison to control (Figure 3a, b, c). Liver tissue homogenates from MD and HD

treated rats had significantly altered CAT activity as shown in figure 3d.Liver extracts from mid- and high-dose treated groups were found to contain higher levels of malondialdehyde in comparison to the control as well as LD group (Figure 3e).

Histopathological observations

Histological analysis from control and low-dose group revealed well-arranged hepatic lobules with intact cords of hepatocytes. Tissue had healthy hepatocytes, Kupffer cells and normal sinusoidal spaces (Figure 4a, b).Mid- and high-dose tissues exhibited various pathological signs apart from normal structural aspects. In many instances,edema filled spaces were observed. Mild inflammatory areas around the portal and central veins were observed in high-dose treated rats (Figure4c,d). Agglomerated mass of MWCNTs could be seen at various locations in mid- and high-dose groups.The agglomerates were lodged into the extracellular space. Agglomerates in most cases were surrounded by Kupffer cells. Hepatocyte shape change, loss of nuclei and cytoplasm showed clear evidence of injury to cells (Figure4e, f and h). Pathology score showed adose-dependent increase in structural anomalies as compared to control. However, statistically significant change was observed in case of mid-and high-dose groups (Figure 4i).

DISCUSSION

The current study was undertaken to study *in vivo*toxicity of hydroxyl-functionalized MWCNTs over a sub-acute exposure schedule. Despite of high dose levels, administered nanotubes did not affect general health of rats. Intraperitoneally administered nanoparticles reach in liver through hepatic portal vein and accumulate there depending upon the nature of the particle [9]. OH-*f* MWCNTs accumulated in liver of treated rats in the present study. Signs of accumulation can be inferred by darkening of liver in the photographs of liver from HD group rats and by the presence of MWCNTs agglomerates or aggregates observed in histopathology. Bai et al. (2010)analyzed toxicity of carboxylated (COOH)- and amino (NH_2)-functionalized MWCNTs (diameter20-30 nm; length0.5-2.0 μm, 5 repeated doses at 5 mg/kg dose level) via intravenous route in male mice and reported that treated micehad similar body weight gains and their relative organ weights did not change significantly except lung and spleen organ weight [9]. Contrasting results with dose 10 and 60 mg/kg of MWCNTs was reported by Ji *et al.* (2009) where body-weight gain was decreased significantly as compared to control group after a 60-day intravenous exposure in Kunming mice [10]. Such a contrast is possibly be a result of differences in route of dose administration, dose level and nature of carbon nanotube used.

Liver inflammation and damage is strongly correlated with increased levels of ALT, AST and ALP. Mice were intraperitoneally injected with carboxyl-functionalized MWCNTs (with an outer diameter of 15-30 nm and lengths of 15-20 μm) at 0.25, 0.5, and 0.75 mg/kg/day for 5 days. The study recorded that 0.75 mg/kg/day dose resulted in significant deviation in the activities of ALT and ALP. However, level of AST was found to be unchanged in comparison to control.[11]In the present study, all three enzymes exhibited increased activities as compared to control at 2.0 and 10.0 mg/Kg dose levels.This is an evidence of liver inflammation and injury.Our report also indicates that the hydroxyl functionalization is not sufficient to reduce the toxicity of MWCNTs. This *in vivo* observation does not corroborate with an *in vitro* study done by Liu et al. (2014). The group reported that conjugating pristine MCNTswith hydroxyl group reduces their cytotoxicity. It not only prevented disturbance of the mitochondrial membrane potential but also avoided the release of cytochrome c and caspases activation [12].

Most studies with nanomaterials agree that ROSoverproduction and resulting cellular damage is one of the primary mechanisms of their toxicity. Therefore, CAT activity and lipid peroxidation was assessed in the present study. Results demonstrated decrease in CAT activity and elevation in MDA concentration in liver extracts as compared to control which is a sign of significant ROS production and oxidative stress. These results are in line with the findings of Ji *et al.* (2009). Tween-80 dispersed MWCNTs were shown to result in mild reversible oxidative stress in liver of treated mice after 15 days of intravenous administration [10]. Adedara *et al.* (2018) investigated hepatotoxicity of COOH-MWCNTs. Adult rats were treated at 0, 0.25, 0.50, 0.75 and 1.0 mg/kg for 5 consecutive days. Inflammatory markers and oxidative stress parameters were found to be increased including serum activities of AST, ALT, ALP and gamma glutamyl transferase when compared with control [13].

Patlolla *et al.* (2011) found a dose related impact on histological structure of liver following exposure to 0.25, 0.5 and 0.75 mg/kg/day of functionalized MWCNT. Structure anomalies included hepatocellular vacuolations, hepatocyte injury, disruption of central vein etc. At higher doses liver atrophy was reported which is a sign of severe toxicity [11]. In an interesting work by Xu *et al.* (2016), MWCNTs (average diameter, 30 nm; length, ≥1 μm) were administered intravenously in a fatty liver rat model. The exposure enhanced the steatohepatitis and oxidative stress in liver with chronic hepatitis [14]. The present study observed mild inflammatory areas and vacuolations around the central vein and portal veins. Agglomerates lodged into the extracellular spaces clearly showed that multiple injections of MWCNTs at

2.0 and 10.0 mg/Kg dose results in accumulation and agglomerate or aggregate formation inside the tissue. Although, possibility of agglomerates in the dispersion prior to treatment, despite of ultrasonication, cannot be completely denied. It was interesting to note that hepatocytes near these agglomerates exhibited altered morphology and Kupffer cells were found adjacent to these areas. These observations indicate that OH-*f* MWCNTs generatetoxicity to hepatic tissue in a localized manner. Toxicity outcomes are greatly affected by diameter, length, functionalization and surface defects of MWCNTs. For instance, toxicity to rat lung tissue increases considerably as the length of nanotubes decreases from 2.77 μm to 0.40 μm [15]. Diverging results due to above mentioned factors is a serious challenge in toxicity analysis of OH-*f* MWCNTs. Meticulously designed studies considering physico-chemical characteristics are needed to fully address biocompatibility issue of these highly beneficial nanomaterials.

Table 1. Behavioral signs as recorded at 1, 6, 12 and 24 hours after the exposure of OH-*f* MWCNTs.

Behavioral responses	Time Interval															
	1 hour				**6 hours**				**12 hours**				**24 hours**			
	C	**LD**	**MD**	**HD**	**C**	**LD**	**MD**	**HD**	**C**	**LD**	**MD**	**HD**	**C**	**LD**	**MD**	**HD**
Thirst	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salivation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aggressiveness	+	+	+	+++	+	+	++	++	+	+	+	+	-	-	-	+
Change in gait	-	+	+	++	-	-	-	+	-	-	-	-	-	-	-	-
Shivering	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Itching	+	+	+	++	-	-	-	+	-	-	-	-	-	-	-	-
Food avoidance	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lethargy	-	-	+	++	-	-	+	++	-	-	+	+	-	-	-	+
Piling	+	+	++	+++	-	+	+	++	-	-	+	+	-	-	-	+

++++ Very severe; +++ Severe; ++ Moderate; + Mild; - Absent
C=Control; LD=Low-dose group; MD=Mid-dose group; HD=High-dose group

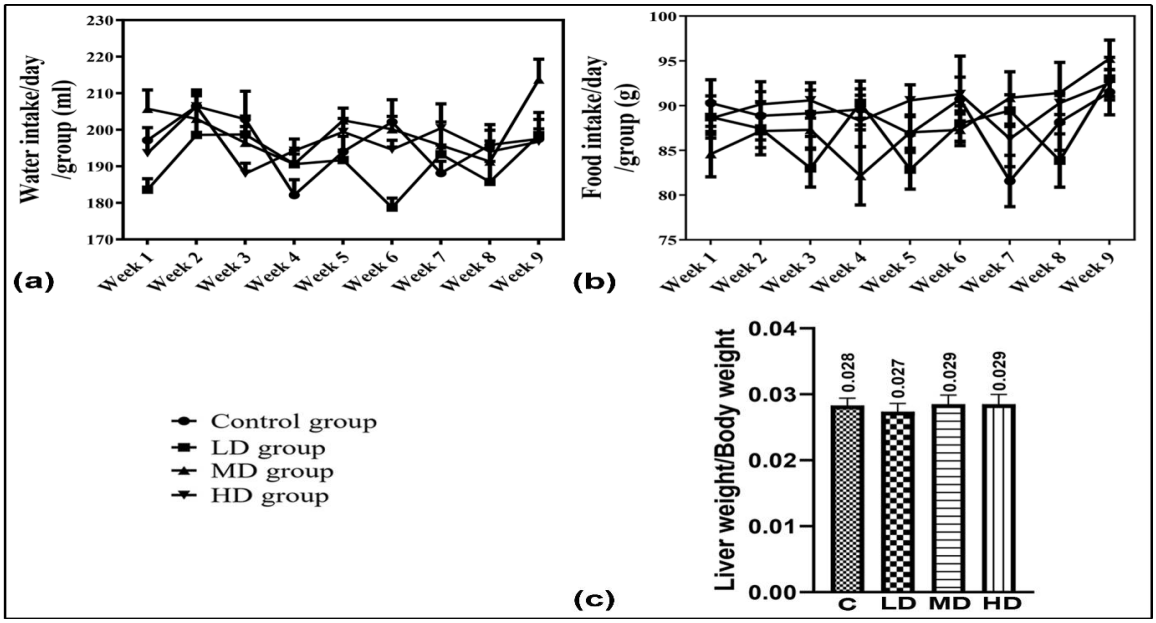

Figure 1. Water and food intake observed in control and treated animals from a simultaneous experiment in which animals were studied for a duration of 60 days.**(a)**Water intake (in ml/day/group) by control and OH-*f*MWCNT-exposed Wistar rats. Mean values represent the average of water consumption by a group of 6 rats in a period of 7 days. Values are expressed as Mean±SEM.**(b)** Food consumption (in g/day/group) pattern. Mean values represent the average of food consumption by a group of 6 rats in a period of 7 days. Values are expressed as Mean±SEM.**(c)** Liver index (liver weight/body weight) calculated in experimental groups. Values are mean ± SD.

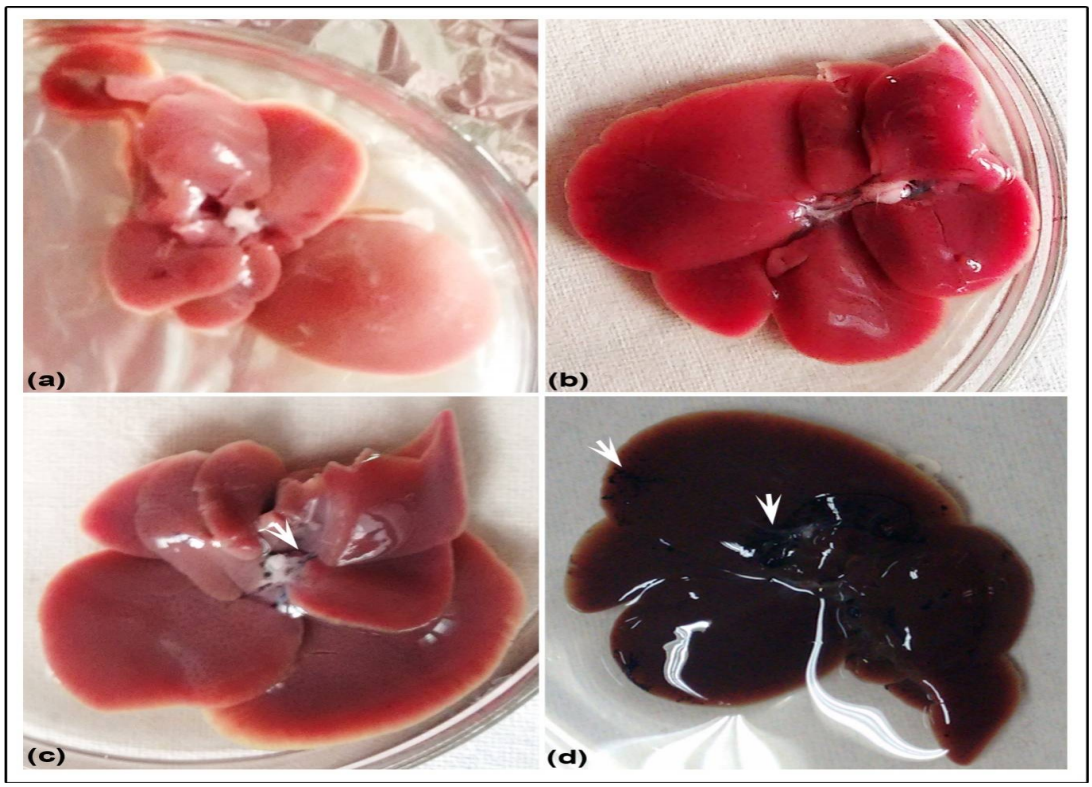

Figure 2. Representativephotographs of liver from control and treated rats showing external morphology. Images from; **(a)** control group,**(b)** LD group,**(c)** MD group,**(d)** HD group showing blackish color and entangles mass of OH-*f* MWCNTs (white arrow).

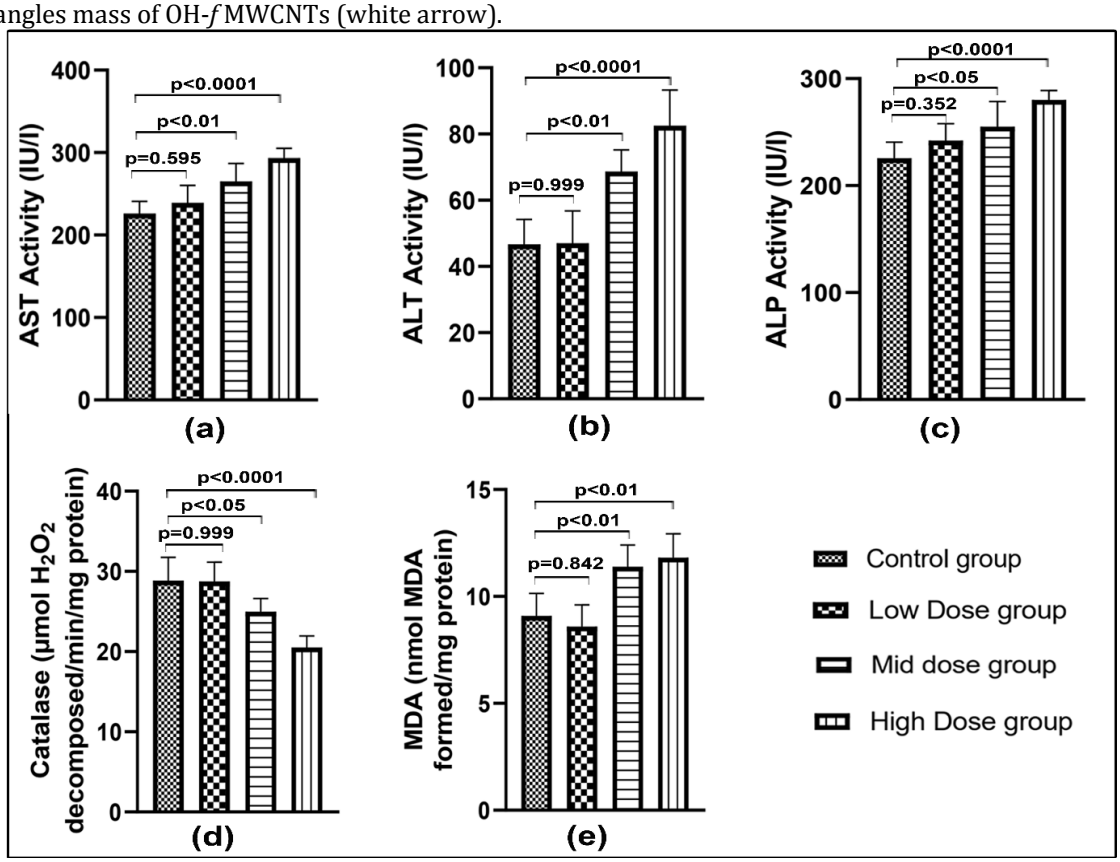

Figure 3. Biochemical assays.**(a)** AST enzyme activity in sera of control and treated groups. **(b)** Activity of ALT. **(c)** ALP activity as observed in control and exposed groups. **(d)**Specific activity of CAT in mitochondrial fraction of control and treated rats. **(e)** Concentrations of MDA in tissues of control and treated rats. Data presented as mean ± SD. P<0.05 was considered significant difference from the control.

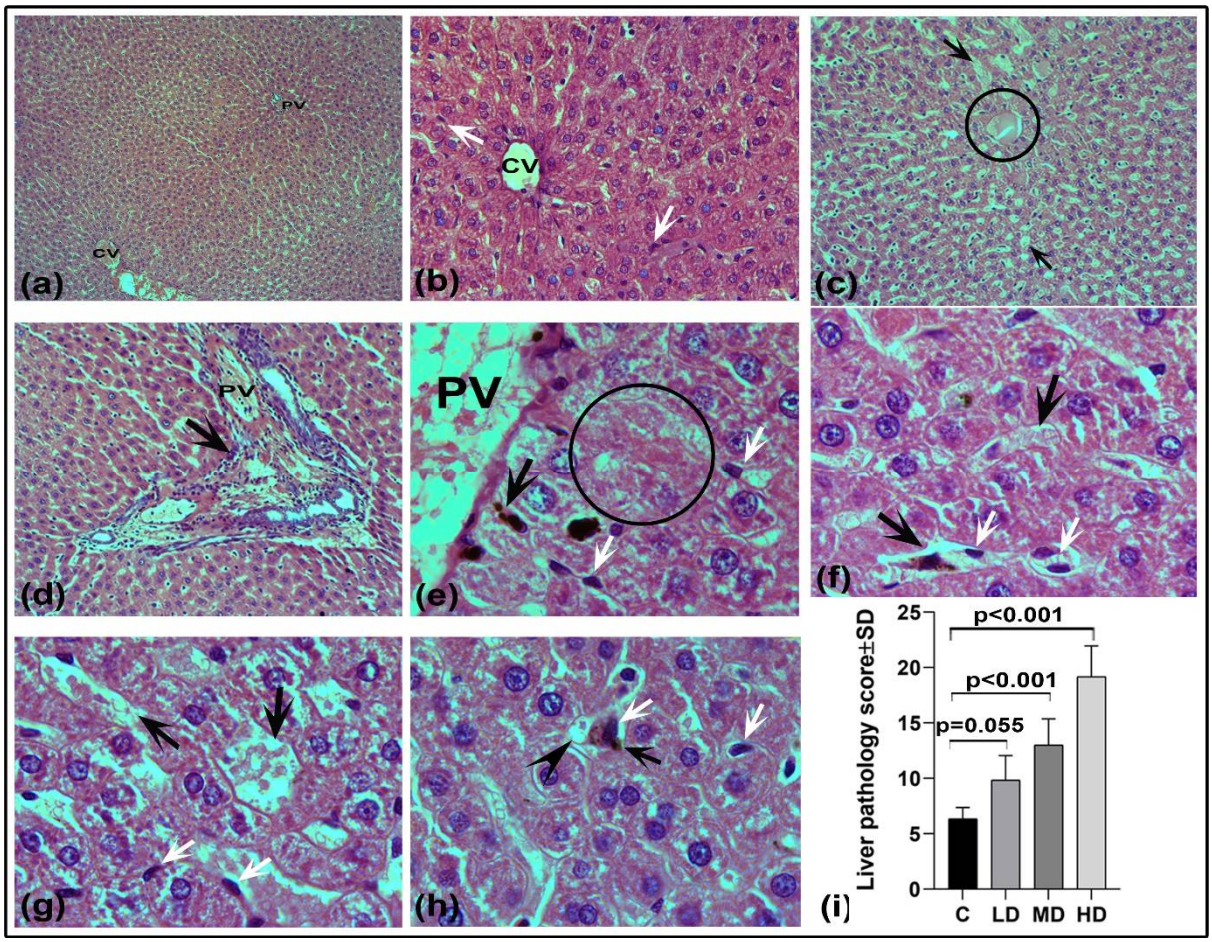

Figure 4. Representative photomicrographs showing normal and pathological aspects of liver histology. **(a)** and **(b)** Normal histology of liver at different magnifications (100X in 'a' and 400X in 'b'). Sections show good lobular arrangement. The cords of hepatocytes are well organized with normal sinusoidal spaces. White arrows show Kupffer cells. CV,Central Vein; PV, Portal Vein. **(c)** Liver histology at 200X magnification showing exudate (circle) and presence of vacuoles (black arrows) a feature frequently encountered in treated groups. **(d)**Image showing mild inflammation around the portal vein (black arrow). Magnification is 200X. **(e)** Agglomerates of MWCNTs as seen at 1000X near the portal vein boundary (black arrow). White arrows show presence of Kupffer cells. The agglomerates are lodged into the extracellular space. Hepatocytes in the image are showing loss of nuclei (area inside the circle). **(f)** and **(g)**Show change in morphology of hepatocytes along with vacuolated area around cells (black arrows). Agglomerates of MWCNTs are also seen. Kupffer cells (white arrow) are visible near the agglomerates or around the vacuoles (1000X). **(h)**Shows a hepatocyte with damaged structure and MWCNTs agglomerate (black arrow). Kupffer cell(white arrow) is also observed adjacent to the agglomerate (arrowhead)(1000X). **(i)** Histopathology score from all the groups. The histological sections (10 per animal) were scored for the presence of various anomaliesincluding inflammatory areas, vacuolated sinusoids, altered cell shape, agglomerates of MWCNTs, presence of edema and exudate etc. Presence of pathologicalsign was allotted a score of 1 while the absence was noted as 0. The resultant scores for each group (mean ± SD) are displayed in the form of a bar graph. $P<0.05$ was considered as statistically significant variation as compared to the control.

SUMMARY

The current study analyzed toxicity of OH-*f* MWCNTs in adult Wistar rats in a sub-acute and repeated exposure scheme. Results of the study clearly demonstrated a dose-related toxicity outcome. Liver function enzymes were significantly elevated indicating inflammatory damage to liver. Histopathological observations correlated with biochemical parameters. Observed hepatic toxicity may be a result of increased oxidative stress as suggested by altered MDA and CAT levels. More studies are required to decipher the mechanistic aspect of the toxicity. Findings in our study are expressive of the fact that OH-*f*

MWCNTs have significant toxicity to mammalian liver and efforts are needed to alleviate the toxicity of these nanotubes.

ACKNOWLEDGEMENT
We acknowledge Indian Council of Medical Research, New Delhi for partial financial assistance as Junior Research Fellowship (3/1/3/JRF-2011/HRD 107/81242) to Naresh Kumar Nirmal.

CONFLICT(S) OF INTEREST
The author(s) declared no potential conflicts of interest with respect tothe research, authorship, and/or publication of this article.

REFERENCES
1. Sharma, P., Mehra, N. K., Jain, K., et al., (2016). Biomedical Applications of Carbon Nanotubes: A Critical Review. *Curr. Drug.Deliv.*, **13**(6), 796 – 817.
2. Fanizza, C., Casciardi, S., Incoronato, F., et al., (2015). Human epithelial cells exposed to functionalized multiwalled carbon nanotubes: interactions and cell surface modifications. *J.Microsc.*, **259**, 173-84.
3. Hiraku, Y., Guo, F., Ma, N., Yamada, T., et al., (2016). Multi-walled carbon nanotube induces nitrative DNA damage in human lung epithelial cells via HMGB1-RAGE interaction and Toll-like receptor 9 activation. *Part.Fibr.Toxicol.*, , **13**, 16.
4. Awasthi, K. K., John, P. J., Awasthi A., et al., (2013). Multi walled carbon nanotubes induced hepatotoxicity in Swiss albino mice. *Micron.*, **44**, 359-364.
5. Boyles, M. S. P., Young, L., Brown, D. M., et al., (2015). Multi-walled carbon nanotube induced frustrated phagocytosis, cytotoxicity and pro-inflammatory conditions in macrophages are length dependent and greater than that of asbestos. *Toxicol. In Vitro*., **29**, 1513-1528.
6. Nirmal, N.K., Awasthi, K.K. and John, P.J., (2017). Effects of hydroxyl-functionalized multi-walled carbon nanotubes on sperm health and testes of Wistar rats. *Toxicol. Ind. Health*, **33**(6),519-529.
7. Aebi, H., Catalase in Vitro. *Methods Enzymol.*, 1984, **105**, 121-26.
8. Ohkawa, H., Ohishi, N. and Yagi, K., (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal.Biochem.*, **95**, 351-358.
9. Bai, Y., Zhang, Y., Zhang, J., et al., (2010). Repeated carbon nanotube administrations in male mice cause reversible testis damage without affecting fertility. *Nat Nanotechnol*., **5**, 683-89.
10. Ji, Z., Zhang, D., Li, L., et al., (2009). The hepatotoxicity of multi-walled carbon nanotubes in mice. *Nanotechnology*., **20**(44), 445101.
11. Patlolla, A. K., Berry, A. and Tchounwou, P. B., (2011). Study of hepatotoxicity and oxidative stress in male Swiss-Webster mice exposed to functionalized multi-walled carbon nanotubes. *Mol. Cell Biochem.*, **358**(1-2),189-99.
12. Liu, Z., Liu, Y. and Peng, D., (2014). Hydroxylation of multi-walled carbon nanotubes reduces their cytotoxicity by limiting the activation of mitochondrial mediated apoptotic pathway. *J. Mater. Sci. Mater. Med.*, **25**, 1033-1044.
13. Adedara, I. A., Anao, O. O., Forcados, G. E., et al., (2018). Low doses of multi-walled carbon nanotubes elicit hepatotoxicity in rats with markers of oxidative stress and induction of pro-inflammatory cytokines. *Biochem.Biophys. Res. Commun*., **503**(4), 3167-3173.
14. Xu, Y. Y., Juan, G., Zhang, M. H., et al., (2016). Intravenous administration of multi-walled carbon nanotubes aggravates high-fat diet-induced non-alcoholic steatohepatitis in Sprague-Dawley rats.*Int. J.Toxicol.*, **35**(6), 634-643.
15. Ema, M., Takehara, H., Naya, M., et al., Length effects of single-walled carbon nanotubes on pulmonary toxicity after intratracheal instillation in rats. *J.Toxicol. Sci.*, 2017, **42**(3), 367-378.

CITATION OF THIS ARTICLE
Naresh K. Nirmal, Kumud K. Awasthi, Placheril J. John. Hepatotoxicity of hydroxyl-functionalized multiwalled carbon nanotubes in Wistar rats. Bull. Env. Pharmacol. Life Sci., Vol 10[5] April 2021: 266-272