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RESEARCH ARTICLE

HEMATOLOGICAL INVESTIGATIONS ON THE EFFECTS OF FUNCTIONALIZED MULTI-WALLED CARBON NANOTUBES IN WISTAR RAT

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ABSTRACT

Multi-walled carbon nanotubes (MWCNTs) have been proposed for various biomedical applications which require their systemic administration. Therefore, the present study was undertaken to investigate the effects of carboxyl group functionalized multi-walled carbon nanotubes (COOH-MWCNT) on hematological parameters and morphology of erythrocytes of Wistar rat. Forty eight adult rats of both the sexes were used for the study. Rats of experimental groups were intraperitoneally injected with 0.5 ml of COOH-MWCNT suspension in double distilled water containing 1% tween-80 at a dose of 2.5, 5 and 10 mg/kg body weight on alternate days. Rats of control group received 0.5 ml of vehicle. Half of the animals of each group were sacrificed after 30 days and remaining half after 60 days of treatment. The results of present study indicate a significant reduction in the number of total erythrocytes, hemoglobin levels and hematocrit values while significant increment in the number of leukocytes in MWCNT administered rats at both the durations of the study as compared to control rats. Mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) remain significantly unaltered while Mean corpuscular volume (MCV) decreased in high dose group only at 60 days of treatment duration. Differential leukocyte count revealed a decrease in the percentage of neutrophils while increase in the percentage of monocytes and lymphocytes. Scanning electron micrograph of blood of treated rats showed presence of echinocytes and acanthocytes. Thus, results of our study suggest that intraperitoneal administration of COOH-MWCNTs have adverse effect on the hematological parameters of rats.

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INTRODUCTION

Nanotechnology is a rapidly emerging field which involves the manipulation of matter at nanometer (one billionth of a meter) scale. It has led to the discovery and development of novel materials in the field of optical, electronical, material and biological science. Among these nanomaterials, carbon nanotubes (CNTs) are one of the most promising and uniquely engineered nanoparticles. A carbon nanotube (CNT) can be visualized as a graphene sheet (single layer of sp^2 bonded carbon atoms tightly packed together in hexagonal honeycomb lattice) folded into cylindrical shape (Madani *et al*., 2013). In general, CNTs are classified as single-walled CNTs (SWCNTs) when the cylindrical structure is single layered and multi-walled CNTs (MWCNTs) when composed of many concentric cylinders.

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Since their discovery by Iijima in 1991 (Iijima, 1991), CNTs have been exhaustively explored due to their bizarre electrical, thermal and physio-chemical properties. Because of their light weight, high tensile strength, high surface to volume ratio, high aspect ratio, good thermal and electrical conductivities, CNTs offer a wide range of applications ranging from manufacturing of paints, coatings, sporting goods, rechargeable batteries and solar cells to super capacitors, flexible TFTs (Thin film transistors), microwave generators, nanosized electronic devices, biosensors and scanning probe tips (Baughman *et al*., 2002 and De Volder *et al*., 2013). The potential applications of CNTs in biological and biomedical field include therapeutics, diagnostics, photodynamic therapy (Murakami *et al*., 2016 and Wang *et al*., 2013), molecular imaging (Keren *et al*., 2008 and Zerda, 2011), optical imaging (Robinson *et al*., 2012 and Hong *et al*., 2012), drug delivery (Bianco *et al*., 2005) protein delivery (Kam and Dai, 2005) vaccine delivery (In het Panhuis, 2003) and gene delivery (Liu *et al*., 2005; Cai *et al*., 2005) Due to widespread commercialization and their application in biomedical tools & diagnosis, exposure to CNTs

is likely to increase in the near future which may be hazardous to human health.

The pristine CNTs are generally not soluble in aqueous solutions because of their highly hydrophobic surfaces, therefore, for biomedical applications, surface modification or functionalization is required to solubilize CNTs and to render biocompatibility & low toxicity (Liu *et al*., 2006) Accidental exposure of CNTs by inhalation or ingestion via skin or mucosa, can potentially lead to the translocation of CNTs in the bloodstream. On the other hand, many of the proposed biomedical applications will require direct injection into the body via different routes of administration which will probably lead to direct contact with blood components (Bussy *et al*., 2013).

Hematological profile is a measure of general health and plays a critical role in diagnosis and assessing the health condition of an organism. In the past decade, various independent studies have been conducted on MWCNTs which have reported toxicity of CNTs both *in vitro* and *in vivo,* but majority of them have focused on pulmonary toxicity (Muller *et al*., 2005; Roda *et al*., 2011 and Kayat *et al*., 2011), cytotoxicity (Monteiro-Riviere *et al*., 2005 and Patlolla *et al*., 2010), genotoxicity (Naya *et al*., 2011 and Kato *et al*., 2013), and common response (Nygaard *et al*., 2009 and Delogu *et al*., 2012). However, adverse effects of CNTs on hematological parameters have been rarely studied. Thus, there is a need for better understanding of the toxic effects and safety concerns related to CNTs before introduction into the body.

The present investigation was undertaken to assess the effects of COOH-functionalized MWCNTs after intraperitoneal injection on hematological parameters of Wistar rat.

MATERIALS AND METHODS

Dose preparation

Carboxyl group functionalized multi-walled carbon nanotubes (COOH-MWCNT) were purchased from Nanobeach (Delhi, India). According to the Data sheet provided by Nanobeach along with COOH-MWCNT, these were synthesized by chemical vapour deposition (CVD) method [Average outer diameter - 20 nm; average length of COOH-MWCNT - 20 µm as determined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). COOH-MWCNTs were suspended in double distilled water using Tween-80 (1%) as surfactant with the help of physical mixing and ultrasonication using water bath ultrasonicator (Labman LMUC-2A) for 40 minutes at 40 kHz.

Animals

Adult, colony bred rats of both sexes of the Wistar strain, weighing 160-180 g each, were used for the study. The rats were left under normal healthy conditions in the animal house of the department. Animals were housed in polypropylene cages under standard laboratory conditions of 12 h-12 h light-dark cycle and a temperature of 22 ± 3°C. They were provided standard rat diet (Aashirwad Food Industries, Chandigarh, India) and water *ad libitum*. The animals were maintained as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations. The study was approved by the Institutional Ethical Committee, Department of Zoology, University of Rajasthan, Jaipur, India.

Experimental design

Animals were randomly divided into four groups (12 in each group) including control and experimental groups. The animals of the experimental group were intraperitoneally (i.p.) exposed with COOH-MWCNT (2.5, 5 and 10 mg/kg body weight) suspension in a value of vehicle 0.5 mL/rat on alternate days. The animals of the control group were treated with vehicle in the same way as that of the animals of the experimental groups. Half of the animals of each group were sacrificed after 30 days and remaining half after 60 days of treatment.

Autopsy

At the end of the respective experimental period, animals were fasted overnight and sacrificed under mild ether anesthesia. The blood samples were collected through cardiac puncture in EDTA vials from rats of all the groups.

Hematological study

Total erythrocyte (RBC) and total leukocyte (WBC) counts (Lynch *et al*., 1969), differential leukocyte count (Ghai, 2013) hemoglobin concentration (Crosby *et al*., 1954), and hematocrit value (Strumia *et al*., 1954) were determined by standard laboratory methods. Red blood cell indices *viz.* mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated using standard formulae.

$$\text{MCV (in femtolitres)} = \frac{\text{Hematocrit (\%)} \times 10}{\text{No. of RBC (millions/mm}^3\text{)}}$$

$$\text{MCH (in picograms)} = \frac{\text{Hemoglobin (g/dL)} \times 10}{\text{No. of RBC (millions/mm}^3\text{)}}$$

$$\text{MCHC (in g/dL)} = \frac{\text{Hemoglobin (g/dL)} \times 100}{\text{Hematocrit (\%)}}$$

Morphological study

For morphological study of erythrocytes, a drop of fresh blood was fixed in 2% glutaraldehyde (in 0.1 M phosphate buffer) for 30 minutes. It was then centrifuged, washed in 0.1 M phosphate buffer (pH 7.4), washed and centrifuged in double distilled water 5 times and resuspended in double distilled water. A thin film was then applied onto cut glass slide pieces (1 cm X 1 cm), dried, sputter coated with gold and finally observed under scanning electron microscope (30 kV, EVO-18 Carl Zeiss).

Statistical analysis

All values were reported as mean ± standard error mean (SEM) for all experiments. Statistical analysis of the data was performed by one way analysis of variance (ANOVA)

followed by Tukey's post-hoc test using version 20 of IBM SPSS statistics software for windows. The significance level was set at $P \leq 0.05$.

RESULTS

Table 1 depicts the mean values of the various hematological parameters of control and COOH-MWCNT administered rats at different doses and durations. The erythrocyte counts, hemoglobin level and hematocrit value remain significantly unchanged in the low dose group at both the treatment durations compared to the control group. However, there was significant decline in the erythrocyte counts in medium group only at 60 days and in high dose group at both 30 and 60 days durations of the treatment compared to the control group. A significant reduction in the hemoglobin levels and hematocrit values were also observed in both medium and high groups of COOH-MWCNT treated rats at 30 as well as 60 days duration when compared to control group. There were no statistically significant alterations observed in the values of MCH and MCHC in all the three dose groups at both the durations of the study. MCV values also remained significantly unchanged in the low and medium dose groups at both 30 and 60 days treatment duration but reduced significantly in the high dose group compared to control at 60 days duration of the treatment. In medium dose group the number of circulating leukocytes increased significantly only at 60 days duration of the treatment while in highest dose group their numbers increased significantly at both 30 and 60 days of durations as compared to control.

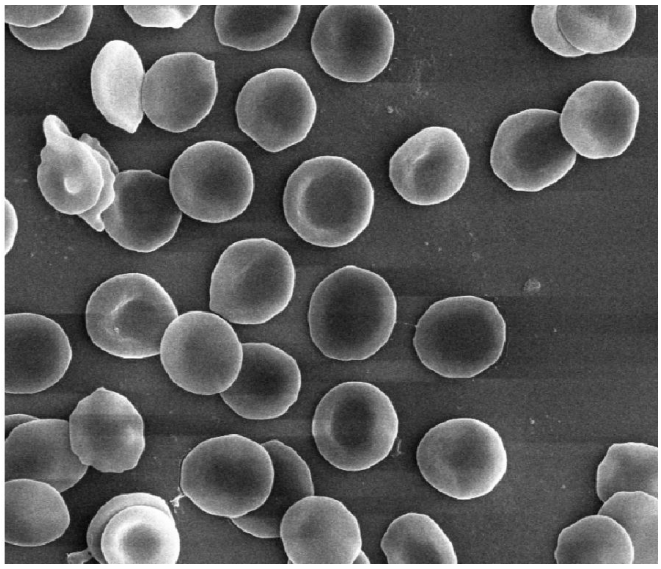

Figure 1. Scanning electron micrograph of blood of control (17.43 RX)

Table 2 represents differential leukocyte counts of control and COOH-MWCNT administered rats at different doses and durations. Statistically non-significant changes were observed in the percentage of subpopulations of leukocytes in all the experimental groups at 30 days treatment duration except that of neutrophils which were reduced significantly in high dose group compared to control. In 60 days treatment study, the percentage of lymphocytes increased significantly only in high dose group, monocytes increased significantly in medium dose group while neutrophils decreased significantly in both medium and high dose groups compared to control. Eosinophils and basophils percentage showed non-significant change in all the experimental groups at both treatment durations. Figure 1 and 2 depicts scanning electron micrographs of blood of control and highest dose COOH-MWCNT administered (10 mg/kg b.wt.) rats of 60 days experimental duration. Micrographs of control rat showed normal morphology of erythrocytes having smooth surface and rounded shape. On the other hand, COOH-MWCNT administered rats showed presence of echinocytes and acanthocytes (spiculated erythrocytes) with wavy appearance of their membrane and spicule like projections on cell surface.

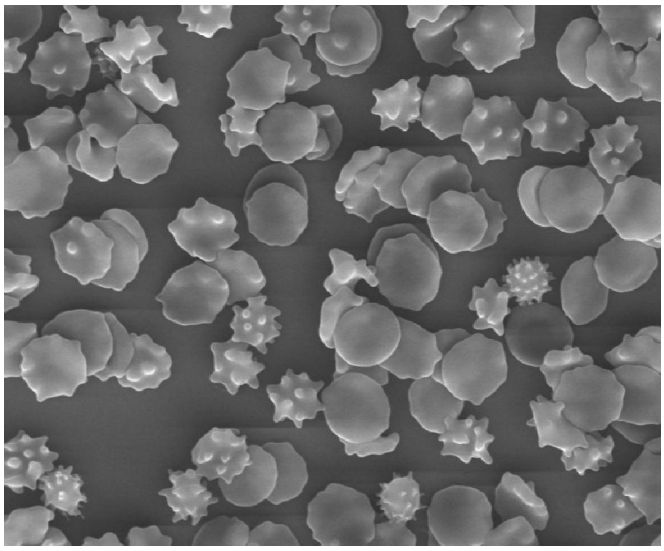

Figure 2. Scanning electron micrograph of blood of rat administer with 10mg/kg b wt. of COOH-MWCNT for 60days experimental duration (15.58 RX)

DISCUSSION

Carbon nanotubes have attracted researchers worldwide because of their unique properties which can be utilized in diverse fields including diagnosis and drug delivery. In recent years, the use of CNTs has increased tremendously and is expected to increase more rapidly in coming time. Biomedical applications require systemic administration of CNTs, and the toxicity of the CNTs reaching the blood stream, together with their impact on different tissues is of vital importance (Clichici *et al*., 2012).

Hemotoxic profiling of a chemical can be considered as the identification of the possible adverse effects resulting from its interaction with blood components including cells and proteins (Bussy *et al*., 2013), Deviation from the normal values of hematological parameters reflects alteration in the physiological state. In the present study, the hematological alterations induced by COOH functionalized multi-walled carbon nanotubes in adult Wistar rats have been investigated.

The results of our study revealed a dose and duration dependent decline in both the total erythrocyte count and hemoglobin level in COOH-MWCNT treated groups as compared to control groups. This decline in the erythrocyte

Table 1. Hematological parameters of control and COOH-MWCNT administered rats at different doses and durations

Parameters	30 days				60 days			
	Control	COOH-MWCNT			Control	COOH-MWCNT		
		2.5 mg/kg b.wt.	5 mg/kg b.wt.	10 mg/kg b.wt.		2.5 mg/kg b.wt.	5 mg/kg b.wt.	10 mg/kg b.wt.
RBC (million/mm^3)	5.61 ± 0.15^a	5.33 ± 0.24^a	4.76 ± 0.27^{ab}	4.38 ± 0.24^b	5.50 ± 0.17^a	5.21 ± 0.27^{ab}	4.40 ± 0.23^{bc}	4.15 ± 0.27^c
WBC (thousand/mm^3)	6.48 ± 0.16^a	6.68 ± 0.28^a	7.08 ± 0.40^{ab}	7.71 ± 0.32^b	6.59 ± 0.10^a	6.79 ± 0.27^{ab}	7.38 ± 0.19^{bc}	7.93 ± 0.20^c
Hemoglobin (g/dL)	12.88 ± 0.17^a	12.07 ± 0.34^{ab}	11.07 ± 0.56^b	10.43 ± 0.55^b	13.02 ± 0.24^a	11.92 ± 0.37^{ab}	10.40 ± 0.54^{bc}	9.45 ± 0.49^c
Hematocrit (%)	43.05 ± 1.14^a	41.10 ± 3.66^{ab}	35.64 ± 2.65^{bc}	31.32 ± 1.33^c	42.74 ± 1.05^a	40.14 ± 1.12^a	32.96 ± 1.24^b	28.60 ± 1.67^b
MCV (fL)	76.70 ± 0.57^a	77.75 ± 3.59^a	74.50 ± 2.51^a	72.06 ± 3.27^a	78.01 ± 2.44^a	77.67 ± 2.65^{ab}	75.25 ± 2.25^{ab}	69.11 ± 1.42^b
MCH (pg)	23.02 ± 0.63^a	22.81 ± 0.88^a	23.28 ± 0.34^a	24.01 ± 1.24^a	23.73 ± 0.40^a	23.03 ± 0.64^a	23.65 ± 0.56^a	22.90 ± 0.68^a
MCHC (%)	30.02 ± 0.82^a	29.54 ± 1.30^a	31.43 ± 1.15^a	33.51 ± 1.85^a	30.52 ± 0.68^a	29.70 ± 0.58^a	31.58 ± 1.32^a	33.20 ± 1.13^a

Values represent mean ± standard error mean (n = 6). Values in a row with different letter in superscript indicates significant difference according to Tukey's post-hoc multiple comparison test.

Table 2. Differential leukocyte count (DLC) of control and COOH-MWCNT administered rats at different doses and durations

Parameters	30 days				60 days			
	Control	COOH-MWCNT			Control	COOH-MWCNT		
		2.5 mg/kg b.wt.	5 mg/kg b.wt.	10 mg/kg b.wt.		2.5 mg/kg b.wt.	5 mg/kg b.wt.	10 mg/kg b.wt.
Lymphocytes (%)	73.67 ± 0.80^a	74.17 ± 2.24^a	76.83 ± 1.28^a	79.33 ± 1.65^a	73.83 ± 0.70^a	76.50 ± 0.85^{ab}	77.00 ± 1.10^{ab}	78.67 ± 1.12^b
Monocytes (%)	2.17 ± 0.31^a	1.83 ± 0.40^a	2.83 ± 0.48^a	2.17 ± 0.48^a	2.00 ± 0.36^a	1.83 ± 0.31^a	3.33 ± 0.33^b	2.33 ± 0.33^{ab}
Neutrophils (%)	20.50 ± 0.85^a	20.83 ± 1.64^a	16.33 ± 0.88^{ab}	15.83 ± 1.17^b	20.83 ± 0.70^a	18.17 ± 1.08^{ab}	15.33 ± 0.49^b	15.67 ± 0.88^b
Eosinophils (%)	3.17 ± 0.31^{ab}	2.50 ± 0.50^{ab}	3.83 ± 0.31^a	2.33 ± 0.33^b	3.17 ± 0.48^a	3.17 ± 0.54^a	4.33 ± 0.49^a	3.17 ± 0.40^a
Basophils (%)	0.50 ± 0.22^a	0.67 ± 0.33^a	0.17 ± 0.17^a	0.33 ± 0.21^a	0.17 ± 0.17^a	0.33 ± 0.21^a	0.00 ± 0.00^a	0.17 ± 0.08^a

Values represent mean ± standard error mean (n = 6). Values in a row with different letter in superscript indicates significant difference according to Tukey's post-hoc multiple comparison test.

count might be due to suppression of the activity of hematopoietic tissues or accelerated destruction of erythrocytes by COOH-MWCNT induced oxidative stress. It has been reported that administration of functionalized SWCNTs causes oxidative stress in blood (Clichici *et al*., 2011).

Scanning electron micrographs of the blood in the current study also support adverse impact of COOH-MWCNTs on morphology of erythrocytes as evidenced by presence of echinocytes and acanthocytes (spiculated erythrocytes) with wavy appearance of their membrane and spicule like projections on the surface in contrast with the normal rounded morphology of the erythrocytes in control group. Crenation of erythrocytes due to MWCNT might be responsible for morphological changes of the erythrocytes and formation of echinoctes and acanthocytes. These results are in accordance with the findings of Meng *et al.* (2012), who also reported disturbances in the morphology, shrinkage and membrane disruption of human erythrocytes after interaction with aminated and carboxylated MWCNTs (Meng *et al*., 2012).

Low levels of hemoglobin after COOH-MWCNT administration observed in the present study might be either due to increased rate of RBC destruction or decreased hemoglobin synthesis. Parallel to our finding, Sachar and Saxena (2011), also observed transient anemia in mice characterized by significant decline in the blood erythrocyte count and hemoglobin levels after intravenous administration of single dose (100 µg) of acid functionalized SWCNTs. They also reported dose and time dependent lysis of erythrocytes in *in vitro* condition when incubated with acid functionalized SWCNTs (Sachar and Saxema, 2011).

The dose dependent reduction in hematocrit values in rats after administration of COOH-MWCNTs observed in our study might be attributed to the low erythrocyte count and/or reduction in the size of erythrocyte induced by MWCNT stress.

It is well known that red blood cell indices *viz.* MCV, MCH and MCHC depend on the erythrocyte count, hemoglobin level and hematocrit value, any change in these parameters may have an effect on blood cell indices. In our study, we didn't find any remarkable change in the values of red blood cell indices except significant reduction in the MCV value of rats treated with 10 mg/kg b.wt. COOH-MWCNT for 60 days as compared to control rats. Reduction in MCV value is indicative of microcytic anemia (Sarma, 1990).

The results of our study indicated that administration of COOH-MWCNTs to rats caused a systemic immune or inflammatory response as evident by significant increase in the number of circulating total leukocytes, as well as in the percentage of monocytes and lymphocytes in blood. These results are supported by the findings of Yamaguchi *et al.* (2012), who also reported sustained systemic inflammation caused by single intraperitoneal administration of MWCNTs along with the increase in the number of leukocytes, granulocytes, lymphocytes and monocytes from 1 to 20 weeks after MWCNT administration (Yamaguchi *et al*., 2012). In the present study, a significant reduction in the percentage of neutophils was observed in experimental rats as compared to control rat. These results are corroborated with the findings of Magaye *et al.* (2014), who also reported significant reduction in the number of neutrophils and an increase in the number of leukocytes and monocytes as a result of nickel nanoparticle induced inflammatory condition (Magaye *et al*., 2014). According to Tian *et al.* (2013), neutrophils and macrophages present the front-line of early inflammatory responses against foreign materials and as neutrophils are short lived; accordingly their number decreases rapidly while the number of monocytes and lymphocytes increases in the later phase of inflammation (Tian *et al*., 2013).

In conclusion, the results of our study suggest that COOH-MWCNT administration could adversely alter the hematological parameters of rats in dose and duration dependent manner. Further, *in vivo* studies are needed to investigate the interaction of COOH-MWCNT with blood cells and blood components.

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Declaration of Conflicting Interests

The author(s) declare no conflicts of interest.

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