Int. J. Pharm. Investigation, 2021;11(1):82-87 *Original Article*

Advanced Development of Carboxylic Acid Functionalized Multiwall Carbon Nanotubes as Safe Inhalation Drug Carrier

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ABSTRACT

Background: Multiwall Carbon Nanotubes (MWCNTs) have innovative characteristic features strongly associated with nanotechnology and drug delivery applications. **Objectives:** The current work was performed with an objective to assess the inhalation toxicity of multi-walled carbon nanotubes. **Methods:** Functionalization of multi-walled carbon nanotubes was carried out by the initial basic treatment with hydrochloric acid and nearly assessed by Infrared (IR) spectroscopy, mass spectroscopy, and Transmission electron microscopy (TEM) in comparison with pure multi-walled carbon nanotubes. The acute inhalation toxicity of these functionalized and pure multi-walled carbon nanotubes was assessed in Wistar rats. The parameters: hematology, liver function test, kidney function test, and histopathology observed, evaluated and interpreted for the toxicity study. **Results:** The functionalized multi-walled carbon nanotubes and pure multi-walled carbon nanotubes were nearly assessed by IR spectroscopy, mass spectroscopy, and TEM. The assessments affirmed and anticipated that the pure multi-walled carbon nanotube undergoes successful functionalization. Results obtained showed a remarkable difference in the ranges of hemoglobin and platelet. The acute inhalation toxicity study did not show any toxicity of functionalized multi-walled carbon nanotubes when compared to pure multi-walled carbon nanotubes. The results of acute inhalation toxicity studies did not show any significant changes also not demonstrated hazard potential in rats following acute exposures to the functionalized multi-walled carbon nanotubes and there is no significant toxicity. Because of basic treatment during functionalization all impurity gets removed. During functionalization addition of oxygen containing group and removal of carbon dioxide group occurs. **Conclusion:** Functionalized multi-walled carbon nanotubes can prove to be a novel, safe, and non-toxic carrier for the delivery of drugs by inhalation route.

Key words: Carbon nanotubes, Functionalization, Acute inhalation toxicity, Dry powder inhaler, Liver function test, Kidney function test.

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DOI: 10.5530/ijpi.2021.1.15

INTRODUCTION

Carbon Nanotubes (CNTs) are carbon allotropes with a nanostructure having a length-to-diameter ratio of more than 1,000,000. These cylindrical carbon molecules have innovative characteristic features, making them possibly auxiliary in numerous nanotechnology applications. Produced from the graphene sheet they exhibit distinctive mechanical features like high durability and high multifarious moduli.[1] Multi-functional nanoparticles have been of great importance in various biomedical applications, such as delivery systems, biosensors, bio imaging and tissue engineering.[2] Among the numerous materials for nanoparticles, materials based on carbon have expanded biomedical building applications, including nano diamonds (NDs), carbon nanotubes, graphene, carbon nano fibers, fullerenes and nano horns[3] Carbon nanotubes (CN) have numerous structures, length differences, kind of helicity; number of layers and thickness.[4] There are two main types of CN that can have high structural perfection, these are Single-walled carbon nanotubes (SWCNT) consisting of a single graphite sheet seamlessly wrapped into a cylindrical tube and Multiwall carbon nanotubes (MWCNT) comprising of several graphenes wrapped together into a tube of cylindrical shape.[5]

The arc-discharge technique produces high-quality MWCNTs and SWCNTs. MWCNTs do not need a catalyst for growth, while SWCNTs can only be grown in the presence of a catalyst making MWCNT less toxic than that of SWCNT. Thus, MWCNT is generally the carrier of choice for delivery of drugs for most of the researchers. The MWCNT is composed of numerous rolled coatings of sheets of graphene with internal diameters as little as those of the SWCNTs that may be up to 10 nm. MWCNTs have an interlayer separation of roughly 0.34 nm, which is rather more noteworthy than graphite (0.335 nm) 4. However, MWCNT exhibit advantages over SWCNT, such as ease of mass production and low product cost per unit.[6] Nano carriers can release the active drug directly into the cells and surmount the biological hurdles to offer categorical tissue targeting hence drug distribution at the target is possible.[7] The solubility and compatibility of CNTs can be amended to a great extent by functionalization. Nanotubes provide the ideal and unique surrounding for a drug molecule until it reaches the desired site as well as avoids degradation and reaction with healthy cells. Nanotubes also provide a means of delivery without the need to include solvents.[8] Use of SWCNTs and MWCNT's for the delivery of anti-cancer drugs is a major development in the nanotechnology. Management of cancer by conventional chemotherapeutic agents generally harms the healthy tissues. In such case, delivery of drug through SWCNTs and MWCNTs frameworks are a promising way for conveying anti-cancer drugs.[9] Crossing the barrier of blood-brain is also an important application of these carriers that is utilized in different drug deliveries, as many drugs suffer from an inability to reach tumors (the blood-brain barrier prevents drugs from destroying brain tumors.[10,11] MWCNT based systems help in improving the controlled release of BCS class 2 drugs and enhance the solubility of class 4 drugs. The effect of CNTs on the entrapment efficiency is very pronounced. The entrapment efficiency increases significantly as an increase in MWCNTs concentration which may be

due to the interaction among the drug and MWCNTs. These carriers reduce the general drug leakage from the formulation with entrapping more amount of drug.[12] Better drug loading is possible due to network like structure or high acceptance ratio as well as needle like structure shows better drug deposition. In general, electrical and mechanical features of MWCNT can alter when they are functionalized, because of the structural defects that arise by bond breakage of organic groups; throughout chemical processes.[13] The functionalization of carbon nanotubes is based on confirmation of organic moiety linkages to their tubular structure. Through the functionalization of carbon nanotubes, it is possible to modulate their physico-chemical properties, increasing their ease of dispersion, manipulation and process ability among others.[14] For the solubilization of carbon nanotubes, the attachment of relatively large functional groups to the nanotubes is required.[15] In this work the CNTs were functionalized using organic groups such as carbonyl (C=O) and hydroxyl (O-H). The characterization of the transformed structures was done by FT-IR spectroscopy, mass spectroscopy and TEM technique. A dry-powder inhaler (DPI) is a device that conveys drug to the lungs in the form of dry powder. DPIs are generally utilized to treat respiratory problems, for example, asthma, bronchitis and respiratory viruses.[16] The system is capable of continuously generating consistent concentrations (3–12mg/m^3) of an MWCNT like the DPI for extended periods (5h/day, 5 days/week, for up to 4 weeks). MWCNT was employed as an inhalation carrier involved in targeted delivery and selective controlled release to optimize the efficacy of the proposed drug and minimize the systemic as well as local toxicity. The concentration-dependent increase in the retention of pure MWCNT in the lung is associated with the severity of toxicity and after functionalization this toxicity may be reduced or might be non-toxic. Importantly, MWCNT fibers are also observed in the sub pleural area and diaphragm which is the reason behind toxicity. This indicates that inhaled MWCNT get deposited deep inside the respiratory tract and may be translocate into the pleural cavity, and that exposure to MWCNT induces pathological transmutations in the lung and chest cavity.[17,18] In this research work, acute inhalation toxicity of pure and functionalized multi wall carbon nano tube was evaluated by performing different parameters like hemogram, liver function test, kidney function test, and histopathology study on Wistar albino rat. In past multi-walled carbon nanotubes have never been utilized as aerosol for inhalation purpose, and none research showed its toxicity or safety profile,[19] also carbon nanotubes have not been analyzed as a carrier in DPI. Thus, there was a need to do such research and to determine its safety which is focused here.

MATERIALS AND METHODS

Materials

MWCNTs were purchased from Applied Science Innovations Pvt. Ltd., Pune, India. All the other chemicals used for synthesis were procured from Sigma Aldrich Chemicals Inc., Bangalore, India, and distilled water was prepared from de ionized water in the lab.

Functionalization of multi-walled carbon nanotubes

Functionalization of MWCNT was carried out by the initial basic treatment followed by treatment with hydrochloric acid. This generated functionalized MWCNTs covalently. By this method, the primary essential treatment with hydrogen peroxide and ammonium hydroxide created oxidized MWCNTs and later the treatment with HCl delivered carboxylated MWCNTs. 500 mg of MWCNT was dispersed in 25 ml of the mixture of 25 % ammonium hydroxide and 30% hydrogen peroxide (V: V = 1:1) in a 100 ml round bottom flask, furnished with a condenser and dispersion was heated to 80°C and kept for 5 h. The residue thus obtained was then washed thoroughly with water until neutral pH was attained and dried overnight in vacuum at 40°C.[20] The treatment with hydrochloric acid produced carboxylated MWCNTs. In this method 500mg of MWNCTs was placed in round bottom flask and 200 ml of HCl was added. The resultant reaction mixture was stirred using magnetic stirrer for 2 h, then diluted, filtered and washed with ultrapure water dried in vacuum overnight.[11]

Characterization of pure and functionalized multi-walled carbon nanotubes

Infrared (IR) spectrum of pure and functionalized MWCNT was obtained on a JASCO, V-530 FTIR in anhydrous IR grade potassium bromide. The morphology was examined by Transmission electron microscopy (TEM). The mass spectrum was recorded employing electrospray ionization technique on G6460A Triple Quad LC/MS/MS System (Agilent Technologies) at Poona College of Pharmacy, Food Testing Laboratory, Pune, India.

Acute inhalation toxicity study of multi-walled carbon nanotubes and functionalized multi-walled carbon nanotubes

For the determination of MWCNT and FMWCNT toxicity study, the research experiment was performed on healthy Wistar albino rats weighing between 180 g and 250 g (National Institute of Biosciences, Pune, India). They were trained in cages with a 12:12 hr dark/light cycle and humidity (44 – 55 %) controlled environment and were provided free access to standard food and tap water. The studies were performed in accordance to Organization for Economic Cooperation and Development (OECD) and Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines. All studies were approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy (1703/PO/C/13/CPCSEA) Pune, India bearing protocol number CPCSEA, PCP/PCT 08/2017-2018. The experiments were conducted under the provisions of approved protocol only. The animals were fasted but provided free access to water overnight before first day of dosing. Animals were divided into three groups for each dose i.e. lower and higher (n = 6) each consisting of 3 males and 3 females. First group (control group) received distilled water orally. Other groups should have been given lower to higher dose i.e. 0.5 mg/kg, however according to OECD guideline there is no need to perform the study for lower dose, hence it was performed for higher dose 5 mg/kg nose-only exposure by fabricated apparatus. After the first dosing daily parameters were noted i.e. body weight, food intake, and water intake. After 28 days, blood sample withdrawn by retro-orbital puncture from the rats, was collected in EDTA tubes from each animal under anaesthesia and used to determine cellular count.[21] Each sample was centrifuged at 500 rpm for 10 min at 4°C; the cells in the pellet were washed in 0.5 mL saline and total cells were counted using automated cell counter (KX -21, Sysmex, India). In order to perform differential analysis, aliquots of the cells were placed onto slides and stained with Field's stain to identify eosinophil's, lymphocytes, macrophages or neutrophils using standard morphologic determinants. At the end of the procedure and as per protocol, the animals were anaesthetized using CO_2 and euthanasia was performed on randomly selected one animal of each group to analyse the macroscopic external features of the lung, kidney, and heart tissue. These tissues were carefully removed and fixed in 10 % buffered formalin and embedded in paraffin. Histology sections (5 µm thick) were stained with haematoxylin and examined under a light microscope.

Hematological and biochemical parameter analysis

The parameters observed, evaluated and interpreted for the toxicity study were as hematological parameters (including Red blood cells [RBC.],

white blood cells [W.B.C], hemoglobin [Hb], Platelet, packed cell volume [PCV], mean corpuscular volume [MCV], corpuscular hemoglobin [MCH] and mean corpuscular hemoglobin concentration [MCHC]), biochemical parameters (Gamma-glutamyltransferase [GGT], Total protein, albumin, globulin, serum glutamic-oxaloacetic transaminase [SGOT], serum glutamic-pyruvic transaminase [SGPT], alkaline phosphatase [ALP], Total Bilirubin, conjugated bilirubin, unconjugated bilirubin, creatinine, Blood urea nitrogen [BUN], sodium, potassium, urea, chloride and calcium.

Histopathology

Sacrificed the animals at end of study, organs were removed, weighed and finally processed for histopathology of kidney, heart, and lung.[22,23]

RESULTS

Functionalization of Carbon Nanotubes

Initial MWCNTs treated with ammonium hydroxide and hydrogen peroxide for oxidization of MWCNTs and again carboxylated by treatment with hydrochloric acid. HCl was used to produce covalently functionalized MWCNTs after successful functionalized characterize analytically.

IR Spectroscopy

The FTIR spectra of MWCNT and FMCNT revealed few common peaks at 2397.08 cm^{-1} and 2394.19 cm^{-1} respectively, however the FTIR spectrum of MWCNT showed peaks at 1691.27 (C=O) and 3287.07 cm^{-1} (-OH) confirming the attachment of COOH group after functionalization. The pure MWCNT had no obvious characteristic absorption peaks. IR spectra of pure and functionalized MWCNT obtained are as shown in Figure 1.

Transmission electron microscopy

The TEM image showed more bents with closed tips in functionalized MWCNT group as compared with the pure MWCNT group. TEM micrograph of pure MWCNT is Figure 2.

Mass spectroscopy

The results obtained from mass spectroscopy indicated the maintenance of the graphitic structure of MWCNTs after functionalization. Results for the pure MWCNT group showed the mass to charge ratio of 526.39 *m/z* as presented in Figure 3 A, whereas for functionalized MWCNT group it was 942.49 *m/z* as Figure 3 B.

Acute inhalation toxicity study of MWCNT and FMWCNT

Daily parameters: Body weight (grams), water intake (mL), and food intake (grams) were the daily parameters that were recorded, the results obtained showed that there was variation in body weight (grams), water intake (mL), and food intake (grams) of both male and female Wistar rats after 28 days in pure MWCNT whereas functionalized MWCNT group and control group exhibited negligible difference.

Post sacrifices parameters: The results obtained for various parameters after 28 days study was as follows:

Hemogram

The values estimated for all hematological parameters of FMWCNT treated groups were comparable to that of control group as shown in Table 1. FMWCNT notably increased the Hb, WBC, platelets, PCV and MCH counts and lowered the MCV and MCHC counts compared to MWCNT indicating there were no alterations in any of the hematological parameters for control group and functionalized MWCNT group.

Liver function test

All the important liver function markers were quantified for all the three groups and the results obtained are Table 2. The results demonstrated that there was distinguished variation in all the parameters except the

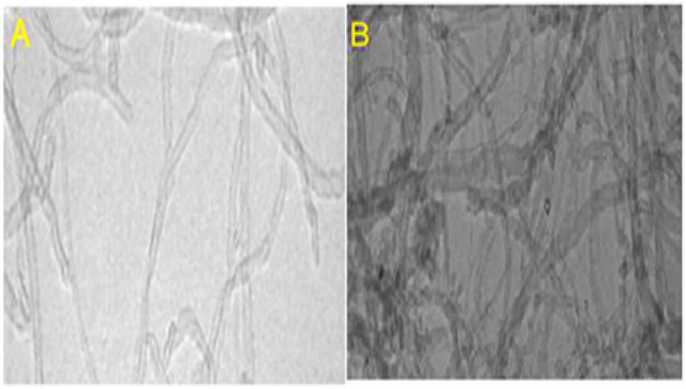

Figure 2: Morphology of pure MWCNT (TEM) (A) and functionalized MWCNT (TEM) (B).

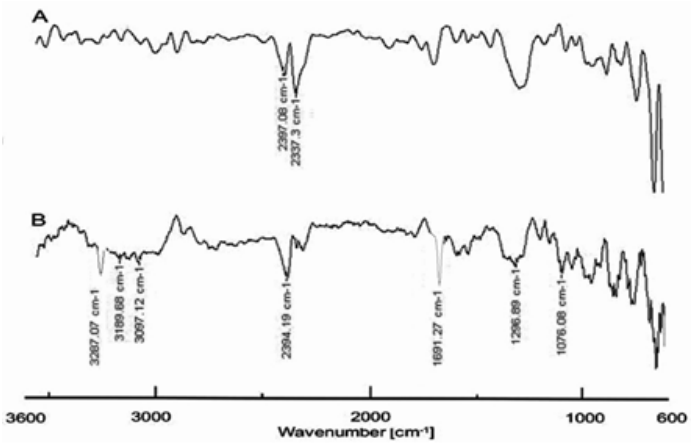

Figure 1: IR spectroscopy of pure MWCN (A) and functionalized MWCNT (B).

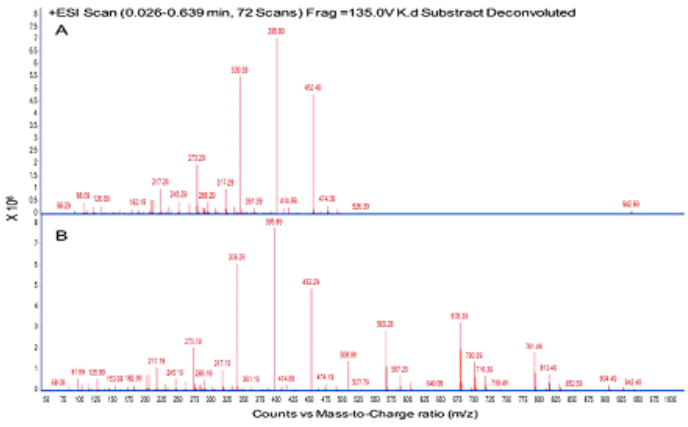

Figure 3: Mass spectroscopy of pure MWCNT (A) and functionalized MWCNT (B).

GGT in pure MWCNT group compared to control and functionalized group.

Kidney function test

Kidney function test parameters were studied and results are depicted in Table 3. The values of creatinine, sodium, chloride, urea and BUN indicated alterations in MWCNT group compared to control and FMWCNT treated group, whereas levels of potassium were similar in all three groups.

Table 1: **Haemogram study.**

Parameter	Group 1 Control group	Group 2 MWCNTs	Group 3 FMWCNTs
Hb (g/dl)	13.10±0.29	9.10±0.92#	13.11±0.47ns
WBCs (103/mm3)	9.50 ± 0.15	8.03± 0.10#	9.51 ± **
RBCs (103/mm3)	6.4 ± 0.2	6.4 ± 0.47ns	6.4 ± 0.15ns
Platelets (103/ mm3)	480±2.31	350±2.11#	482±1.31 ns
PCV (mg/dl)	39±1.3	37±2.6#	39 ± 1.4 ns
MCV (µm3)	61 ± 1.70	63±0.79#	60±0.94 ns
MCH (mmg)	20±0.34	17±0.31#	20±0.26 ns
MCHC (mg/dl)	34±0.50	37±0.23#	35±1.10*

Data are expressed as mean (n = 6) and analyzed by one-way ANOVA followed by Dunnetts test for each parameter of FMWCNTs group 3 separately. P < 0.05, **P < 0.01, ***P < 0.001 and FMWCNTs group 2 separately #P < 0.05, ##P < 0.01, ###P < 0.001, as compared to control group and. ns non significant as compared to Normal group*

Table 2: **Liver function test.**

Parameter	Group 1 Control group	Group 2 MWCNTs	Group 3 FMWCNTs
Total Protein (g/l)	8.55±1.06	7.85±0.49###	8.35±0.91***
Albumin (g/dl)	4±0.02	6±0.01#	4.85±0.02***
Globulin (g/dl)	6.85±0.07	6.4±0.28#	6.85±0.35***
Alkaline phosphotase (u/l)	168±6.79	158±21.21###	162±14.14**
SGPT (u/l)	95±8.48	93.5±9.19###	92.5±10.60**
SGOT (u/l)	252.5±24.74	249.5±33.23###	247±22.62 **
Total Bilirubin (mg/dl)	0.2±0.02	0.05±0.01#	0.185±0.02 ***
Conjugated Bilirubin (mg/dl)	0.0375±0.01	0.0255±0.00#	0.039±0.00*
Unconjugated bilirubin (mg/dl)	0.165±0.03	0.095±0.03#	0.155±0.00*
GGTP (u/l)	8±1.4	8±2.8 ns	8.65±1.2 ***

Data are expressed as mean (n = 6) and analyzed by one-way ANOVA followed by Dunnetts test for each parameter of FMWCNTs group 3 separately. P < 0.05, **P < 0.01, ***P < 0.001 and FMWCNTs group 2 separately #P < 0.05, ##P < 0.01, ###P < 0.001, as compared to control group and. ns non significant as compared to Normal group*

Histopathology

Histopathological investigation of sections of rat lung, kidney, and heart taken from the MWCNT-administered group displayed histological deterioration in the form of inflammation, blood clots, necrosis etc. Whereas there were no variations in tissue sections of FMWCNT when compared with control group as shown in Figures 4-6.

DISCUSSION

Functionalization of MWCNT to FMWCNT was undertaken with the purpose of reducing the toxicity.[24] It was performed via initial basic treatment with ammonium hydroxide and hydrogen peroxide produced oxidized MWCNTs then simple acidic treatment procedure

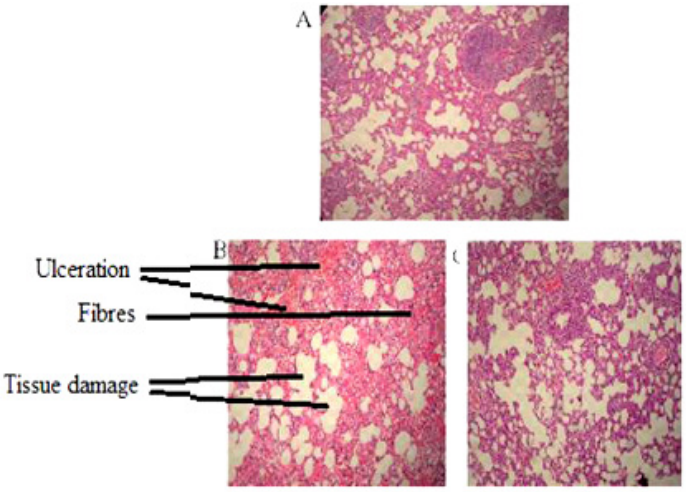

Figure 4: Histopathology of lung (A: control group, B: pure MWCNT, C: functionalized MWCNT, TD: tissue damage, U: ulceration).

Table 3: **Kidney function test.**

Parameter	Group 1 Control group	Group 2 MWCNTs	Group 3 FMWCNTs
Creatinine (mg/dl)	0.83±0.08	1.29±0.04#	0.82±0.05***
Sodium (m mol/lit)	124±31.11	118.5±23.33###	125.5±30.40 ns
Potassium (m mol/lit)	4.35±0.35	4.3±0.28 ns	4.5±0.28ns
Chloride (m mol/lit)	99±0.00	96.5±2.12#	100±0.00***
Urea (mg/dl)	46.5±4.94	50.5±6.36 ##	45±5.65 ns
BUN (blood urea nitrogen) (mg/dl)	16±4.24	22.5±0.70#	16.5±3.53 ns
BicarbonatemEq/l	22.5±2.12	22±1.41###	22.5±2.12 ns
Calcium (mmol/L)	9.65±0.49	9.45±0.49#	9.65±1.13 ns
Phosphorus (mg/dL)	6.8±0.98	6.75±1.27 ns	6.78±1.20 ns
Uric acid (mg/dL)	0.98±0.01	2.45±0.07#	1±0.00***

Data are expressed as mean (n = 6) and analyzed by one-way ANOVA followed by Dunnetts test for each parameter of FMWCNTs group 3 separately. P < 0.05, **P < 0.01, ***P < 0.001 and FMWCNTs group 2 separately #P < 0.05, ##P < 0.01, ###P < 0.001, as compared to control group and. **Ns** non significant as compared to Normal group.*

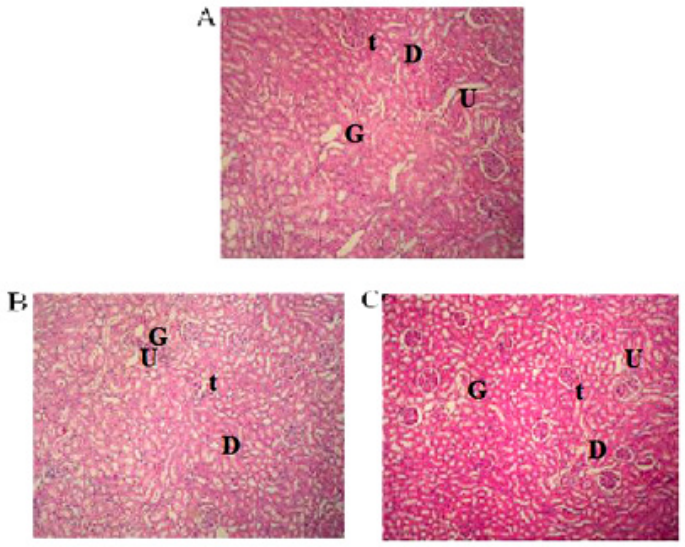

Figure 5: Histopathology of kidney (A: control group, B: pure MWCNT, C: functionalized MWCNT) G – glomerulus, U – urinary space, t – proximal convoluted tubule, D – distal convoluted tubule

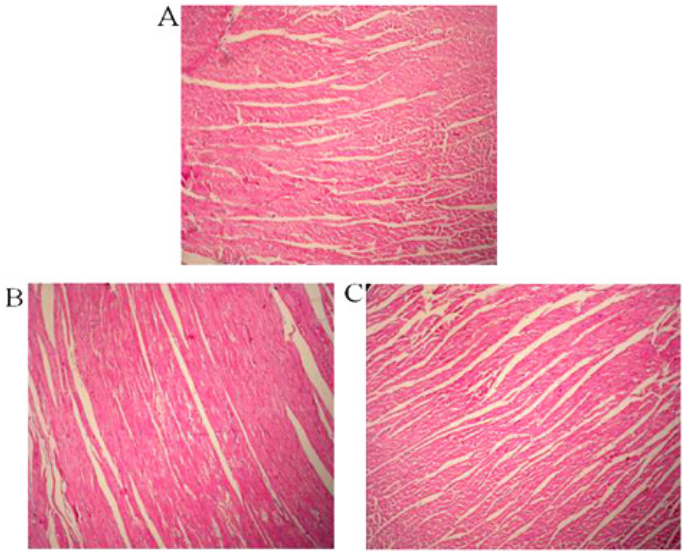

Figure 6: Histopathology of heart (A: control group, B: pure MWCNT, C: functionalized.

and attachment of carboxylic functional group on the pure MWCNT was confirmed from the presence of characteristic peaks of C=O and O-H seen in IR spectrum of FMWCNT and absence of these peaks in the spectra of pure MWCNT. On account of functionalization several functional groups were attached which were observed in the form of bents in the TEM image.[25] Notably pure MWCNTs were seen to have closed tips; it was found that fewer bents were present inside the structure, even after bent washing with water, thus confirming functionalization. From the increase in mass to charge ratio noted in mass spectrum of FMWCNT compared to pure MWCNT it was inferred that attachment of carboxylic functional group increased the molecular weight. The toxicity studies included quantification of various parameters like hematological, liver function markers, kidney function markers and histopathology. Significant diminution in levels of Hb, WBC, platelets, PCV and MCH counts and elevated levels of MCV and MCHC are consequences of underlying toxicity and were prominent in MWCNT treated group.[26-28] In the present study, treatment with FMWCNT could successfully restore the hematological parameters comparable to the control group, signifying its ameliorating effect. Estimation of liver function markers demonstrated that there was distinguished variation in albumin and total bilirubin count of the pure MWCNT group compared with control and functionalized group.[29] This can be attributed to the fact that when liver is in inflamed condition, it fails to process bilirubin and hence its levels rise in blood. Likewise elevated levels of albumin are associated with exposure to toxicants or may be due to dehydration and were observed in MWCNT treated group.[30-32] Albumin levels in FMWCNT treated group were however similar to control group suggesting that FMWCNT does not cause any toxic alterations. The kidney function can be measured by estimating concentration of electrolytes sodium, potassium, chloride etc. and concentration of metabolites like urea and creatinine. The normal ranges of these parameters are confirmation of healthy renal functioning. In our study these levels were found to be disturbed in case of MWCNT- administered group. However, FMWCNT-administered group did not show variations in levels of creatinine, chloride, urea and BUN (similar to control group) which further ascertained the safety of FMWCNT. In histological examination of MWCNT treated rat lungs, signs of inflammation like ulceration, blood clots, tissue damage, inflammatory infiltrate and dense eosinophils were evident Figure 4. Images of control and FMWCNT treated rat lungs indicated intact cyto-architecture without any abnormality. (Figure 5) represents histopathology of the kidney, where exposures of MWCNT in rats produced significant tubular necrosis which was characterized by death of tubular epithelial cells of the kidney and interstitial nephritis.[33] Which is swelling in between the kidney tubules at a dose of 5 mg/kg at 28 days in pure MWCNT whereas no variation was observed in functionalized MWCNT in comparison with control group. From results of histopathology of heart shown in Figure 6. It can be stated that pure and functionalized MWCNT showed no significant changes with 5 mg/kg dose in comparison with control group. Thus, no signs of toxicity were observed in FMWCNT-treated rat kidney and heart images, confirming its safety *in vivo* MWCNT in comparison with control group.[24,34] The current research described the results of *in vivo* toxicity studies conducted on functionalized (FMWCNT) and pure multi-walled carbon nanotubes (MWCNT). The justification for these particular tests rests on the criteria: (i.e., Daily parameters, Histopathology, Hb, WBC, RBC, platelets, PCV, MCV, MCH, ad MCHC), liver function test and kidney function test in Table 1-3. And the results of these acute studies that did not show any significant changes also not demonstrated hazard potential in rats following acute exposures to the functionalized multi-walled carbon nanotubes (MWCNT) and no significant toxicity was observed. This may be accredited to the fact that acidic treatment during functionalization removes all impurities. During functionalization addition of oxygen containing group and removal of carbon dioxide group occurs. This may be the reason that FMWCNT is non-toxic.

CONCLUSION

Functionalization of MWCNT was performed successfully to assess material inhalation toxicity taking into consideration various parameters known to quantify toxicity. It was observed that the functionalization of MWCNT reduced material toxicity. Additionally, it can be concluded that MWCNT after functionalization is safe advanced material carrier and can act as potential drug carrier in inhalation drug delivery systems such as dry powder inhalers. Briefly, all these consequences open a new door for advanced carbon material in pulmonary drug delivery.

ACKNOWLEDGEMENT

Authors would like to thank to Dhande Pathlab Diagnostic Pvt. Ltd. Pune for their kind support during hematological and all other lab studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

FMWCNT: Functionalization of mult-walled carbon numbers; **CNTs:** Carbon Nanotubes; **SWCNT:** Single walled carbon nanotubes; **IAEC:** Institutional Animal Ethics Committee; **ARRIVE:** Animal Research: Reporting *in vivo* Experiments.

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Article History: Submission Date : 16-12-2020; Revised Date : 17-01-2021; Acceptance Date : 27-02-2021
Cite this article: Dhekale K, Patil S, Kamble R. Advanced Development of Carboxylic Acid Functionalized Multiwall Carbon Nanotubes as safe Inhalation Drug Carrier. Int. J. Pharm. Investigation. 2021;11(1):82-7.