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Acute Toxicological Effects of Multi-Walled Carbon Nanotubes (MWCNT)

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1. Introduction

Health effects of nanoparticles are attracting considerable and increasing concern of the public and government worldwide. Carbon nanotubes represent one of the fastest developing nanoparticle materials with production set to increase rapidly as a consequence of the useful properties of this material (Donaldson et al., 2006). The pulmonary toxicity of single-walled and multi-walled carbon nanotubes delivered at high doses and dose rates to the lower respiratory tract of rats and mice induced a high acute inflammatory response with granuloma formation and fibrosis as late effects (Warheit et al. 2004; Muller et al. 2005). However, the reports about toxicological research of multi-walled carbon nanotubes by the oral, dermal and ocular routes are not yet published. The oral, dermal and ocular routes represents an important portal of entries for MWCNT since several consumer which are on the market, already contain multi-walled carbon nanotubes.

Few studies provided only scanty insights into the interaction of MWCNT with the human body after entering via different portals. Hydroxylated single-walled carbon nanotubes (SWCNT) administered by gavage in mice (100 μ L of a 15 μ g/mL solution) are distributed to most of the organs and tissues, except the brain (Wang et al., 2004). The study by Wang et al., (2004) shows in mice that the radiomarked hydroxylated singled-walled carbon nanotubes administered intraperitoneally (100 μ L of a 15 μ g/mL solution) are distributed throughout the body, except the brain, pass through several compartments and are retained in the bones. Monteiro-Riviere et al. (2005) found multi-walled carbon nanotubes (MWCNT) in the cytoplasmic vacuoles of human epidermal keratocytes in vitro (up to 3.6 μ m long), a decrease in cell viability and a significant increase in an inflammation marker (interleukin-8). This demonstrates the capability of MWCNT to penetrate the cell membrane. Huczko and Lange (2001) studied the effects on the skin and eyes of exposure to carbon nanotubes. The application of a saturated filter of a solution containing nanotubes did not cause irritation or allergy in volunteers. Ocular instillation of an aqueous suspension of nanotubes in rabbits did not cause irritation.

In the light of the increased production and proposed use of MWCNT in consumer products, there is a need for screening the potential toxicity of these nanoparticles. In the present study, we have made an attempt to investigate the acute oral, dermal, acute dermal irritation, eye irritation and skin sensitization potential of two different sizes (140 \pm 30, 10-15nm) of MWCNT.

2. Materials and methods

2.1 Nanomaterials

Two different sizes of multi-walled carbon nanotubes (MWCNT 1: 5–8 microns in length with 3–8nm inside diameter and outside diameter of 140 ± 30 nm; Product No. 659258) and (MWCNT 2: 1–10 microns in length with 2–6nm inside diameter and outside diameter of 10–15 nm; Product No. 677248) purchased from Sigma–Aldrich, USA, were used to compare size dependent toxicity.

2.2 Particle-types and physicochemical characterization

The multi-walled carbon nanotubes used in the study was produced by catalytic chemical vapor deposition (CCVD). The MWCNT were composed of 99.9+% carbon with small amount (<0.1% Fe) of iron. The manufacturer specification for nanomaterial characterization was confirmed by the following techniques. MWCNT characteristics were assessed in the as-synthesized form prior to use for experiments or after dispersion in the vehicle (distilled water) for dosing. Solution characteristics were measured with dynamic light scattering (DLS).

2.2.1 Size, morphology

Multi-walled carbon nanotubes size was determined with scanning electron microscopy (SEM). In this study, Hitachi S-520 SEM was used at an accelerating voltage of 10,000V after depositing the samples onto aluminum stubs with double-sided carbon adhesive tape. Over 100 particles were counted and measured to determine average sizes and size distributions.

2.2.2 Dynamic light scattering (DLS)

Particle size measurements in distilled water were determined with dynamic light scattering as described by Murdock et al. (2008) on a Malvern Instruments Zetasizer. Photon correlation spectroscopy or DLS is an analytical technique capable of measuring the size of very small particles, at low sample concentrations.

2.3 Animals and housing conditions

Experimental animals were obtained from in house animal facility. All procedures using animals were reviewed and approved by the institutional animal ethics committee.

The healthy Wistar (CrI:WI) rats of both sex, aged between 8 to 10 weeks and body weights of 200 – 250 g were used for oral and dermal studies respectively. Females were nulliparous and non pregnant. The animal's parent stock was procured from Charles River, USA. Animals were housed in poly propylene cages with stainless steel grills, sieved and sterilized paddy husk used as bedding. Bedding material, cages, grills and water bottles were changed on alternate days. Animals were housed individually sex wise and group wise. Animals were acclimated for minimum period of five days in the controlled environment (temperature: 22 ± 3 °C; relative humidity: $50\pm 20\%$ and light: 12 h light/dark cycle) and *ad libitum* supply of reverse osmosis water and a standard rodent pellet food (supplier: M/s. Tetragon Chemie Pvt. Ltd., Bangalore, India). In case of oral study, prior to the dosing over-night and following dosing, for a period of 3 hours food was withheld.

The following acute toxicity studies were conducted with two different sizes of MWCNT. A brief description of each of the test was provided in the methodology section below.

2.4 Acute oral toxicity test in Wistar rats

Acute oral toxicity –up and down procedure was conducted as per OECD 425 guidelines (OECD, 2006) with slight modifications in terms of usage of sexes and animal number. The limit test dose is selected (2000mg/kg b.w.) as per the OECD 425. A single dose of each test material (two different sizes of MWCNT) suspended in distilled water was administered by oral gavage to group of rats at a dose of 2000 mg/ kg b.w. with minimum of 48 h time interval. The test solution was prepared shortly prior to the administration. The dose volume maintained for all the groups was maximum 10 ml/kg b.w. Similarly, control group of animals (5 males and 5 females) were dosed in a sequential fashion with distilled water alone. Animals were observed for mortality/morbidity, clinical signs of toxicity and weekly body weight during the experimental period. Gross pathology was performed at the end of experimental period (day 14).

2.5 Acute dermal toxicity study in Wistar rats

The acute dermal toxicity test was conducted according to OECD 402 guidelines (OECD, 1987). A limit dose of 2000 mg/ kg b.w. of each test material (two different sizes of MWCNT) mixed with minimum volume (0.25ml) of distilled water was applied uniformly to a clipped 10% body surface area of different groups of rats comprising of 5 males and 5 females/ group. The test substance was held in contact with skin with a porous gauze dressing and bandaged with non-irritating adhesive tape up to 24 h. Following this, the residual test substance was wiped off gently from the skin using cotton soaked in water. Restrainer was used to prevent the ingestion of the test substance from the application site. Control group of animals (5 males and 5 females) were handled similarly without any treatment. Animals were observed for mortality/morbidity, clinical signs of toxicity and weekly body weight during the experimental period. Gross pathology was performed at the end of experimental period (day 14).

2.6 Acute dermal and eye irritation studies in New Zealand white rabbits

2.6.1 Animals and housing conditions

The female New Zealand white rabbits, 2.0 to 3.0 kilograms body weight ranges were used for dermal and eye irritation experiments. Females were nulliparous and non pregnant. Animals were housed individually in standard stainless steel cages and sterilized paddy husk as bedding. Bedding material was changed daily, whereas water bottles were changed on alternate days. Animals were acclimated for minimum period of five days in the controlled environment (temperature: 20±3 °C; relative humidity: 50±20% and light: 12 h light/dark cycle) and *adlibitum* supply of UV treated water and standard rabbit pellet food (supplier: M/s. Amrut Laboratory Animal Feed, Pune).

2.7 Acute dermal irritation study in New Zealand white rabbits

The acute dermal irritation test was conducted according to OECD 404 guidelines (OECD, 2002). A single dermal dose of 0.5 gram of each test material (two different sizes of MWCNT) moistened with minimum volume (0.25 ml) of distilled water was applied to a 6 cm² clipped area of skin. The application area was covered with 2-ply gauze square which

was held in place with non-irritating tape and covered with porous tape for a semi-occlusive dressing. The rabbits were exposed to the test substance for 4 h after which the test substance was removed. Test sites were evaluated by Draize score for signs of dermal irritation approximately 60 min, 24, 48, and 72 h after test substance removal. Initially test was carried out using one animal. The negative response was confirmed using two additional animals. After 72h of observation the animals were euthanized using carbon dioxide.

2.8 Acute eye irritation study in New Zealand white rabbits

The acute eye irritation/corrosion test was conducted according to OECD 405 guidelines (OECD, 2002). A single dose of 0.1 gram of each test material (two different sizes of MWCNT) was applied into the conjunctival sac of left eye of each animal after gently pulling the lower lid away from the eyeball. The lids were then gently held together for about one second in order to prevent loss of the material. The right eye, which remains untreated, served as control. Initially test was carried out using one animal. The eyes of the test animals were washed with distilled water at 24 hours following the application of test materials and the responses obtained in the initial test were confirmed with two additional animals in the confirmatory test. The conjunctiva, iris, and cornea of both treated and control eyes were evaluated and scored according to Draize method at the end of 1, 24, 48, and 72 h following application of the test materials.

2.9 Skin sensitization potential test in Guinea pigs

The Skin sensitization potential test in Guinea pigs was conducted according to OECD 406 guidelines (OECD, 1992). The Dunkin Hartley guinea pigs, 300 to 400 grams body weight ranges were used in the experiments. The treatment group consists of 20 males whereas control group consist 10 males. The test was conducted in two different sizes of MWCNT. Animals were housed individually in standard stainless steel cages and sterilized paddy husk as bedding. Bedding material was changed daily, whereas water bottles were changed on alternate days. Animals were acclimated for minimum period of five days in the controlled environment (temperature: 20 ± 3 °C; relative humidity: $50\pm 20\%$ and light: 12 h light/dark cycle) and *ad libitum* supply of UV treated water and standard rabbit pellet food (supplier: M/s. Amrut Laboratory Animal Feed, Pune). The diet was supplemented with ascorbic acid. The food and water was routinely analyzed and are considered not to contain any contaminants that could reasonably be expected to affect the purpose or integrity of the study. One flank of each animal was closely clipped free of hair, without any abrasion, 24 hours before the induction exposure. A cotton pad about 4 - 6 cm² in size was loaded with the test substance at a dose of 400 mg to all the animals of treatment group. The cotton pad was applied to the clipped area of animals of treatment group and held in contact with the skin by an occlusive patch and bandage dressing for a period of 6 h. The same treatment was repeated on day 7 and 14. Similarly, the animals in control group were treated with distilled water. On day 28, to the closely clipped posterior flank of both treatment and control groups, the nanomaterials were applied in cotton pad at a dose of 200 mg. As in induction exposure, the cotton pads were held in contact with the skin for 6 h by an occlusive patch and bandage dressing. The skin reactions were observed and scored at 30 h after application of the challenge Patch and 54 h after application of the challenge patch. The skin reactions were graded as per the Buehler sensitization scoring scale. On termination, the experiment animals were euthanized using carbon dioxide.

3. Results

3.1 Physicochemical characterization

3.1.1 Size

The average size of the MWCNT 1 is 166nm and MWCNT 2 is 100nm in SEM analysis. The morphology of the MWCNT (1 and 2) is open or closed tube. The MWCNT formed compact aggregates, making it difficult to determine individual particle size by SEM; however, a rough estimate for average size of the MWCNT 1 is 166nm and MWCNT 2 is 100nm (Figures 1(a)-(d))

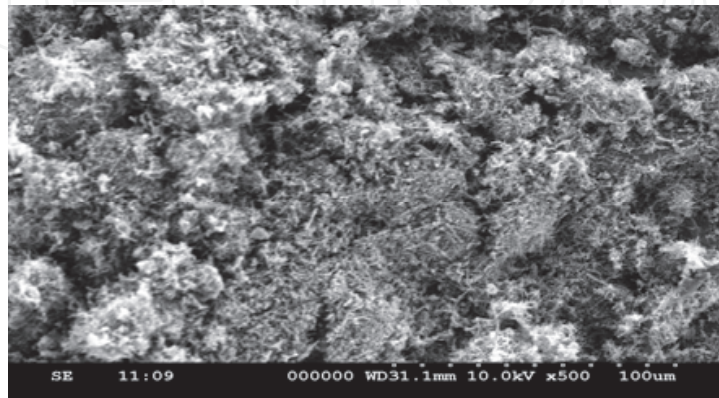


Fig. 1. a) Scanning Electron Micrograph (SEM) of MWCNT 1

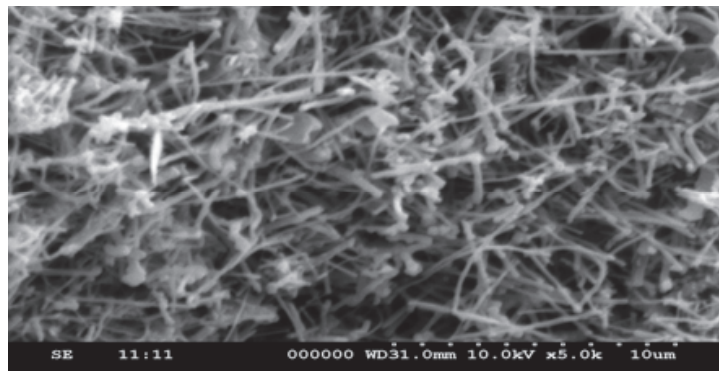


Fig. 1. b) Scanning Electron Micrograph (SEM) of MWCNT 1

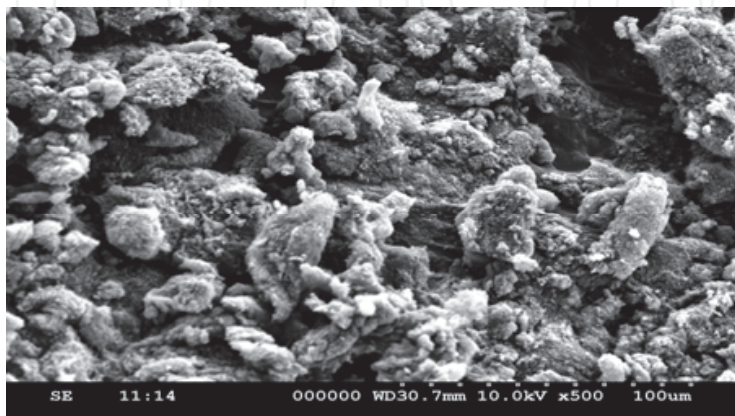


Fig. 1. c) Scanning electron micrograph (SEM) of MWCNT 2

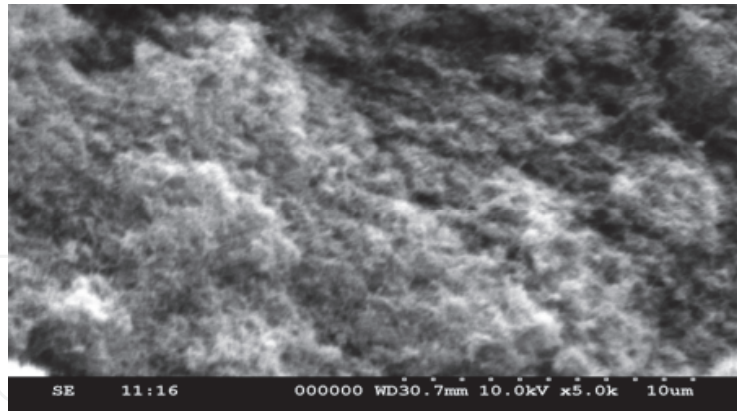


Fig. 1. d) Scanning electron micrograph (SEM) of MWCNT 2

3.1.2 Dynamic light scattering

The solution properties of nanomaterials (two different sizes of MWCNT) in distilled water were examined for changes in size due to agglomeration with dynamic light scattering (Murdock et al., 2008). Average size was calculated by the software from the intensity, volume and number distributions measured (Table 1). The polydispersity index (PDI) given is a measure of the size ranges present in the solution with a scale ranging from 0 to 1, with 0 being monodisperse and 1 being polydisperse (Wagner et al., 2007). The DLS results illustrate that depending on the material, once the nanomaterials are in solution they do not necessarily retain their “nano-size”(Murdock et al., 2008). The average size values (in solution) of 901nm for MWCNT 1, 554nm for MWCNT 2 were found along with high PDI readings. The formation of very strong nanomaterials aggregates due to vander waals forces is expected to occur for unmodified carbon nanomaterials in solution, which is shown for the nano-sized carbon black nanoparticles, which have much larger sizes than their primary size (Murdock et al., 2008).

Nanomaterial	Average size in water ^a	Polydispersity index (PDI)	PH	Specific surface area (SSA)
MWCNT 1 (O.D.X I.D X L: 110-170 nm × 3-8nm X 5-9 μm)	901 nm	1.000	7.2	10-15 m ² /g
MWCNT 2 (O.D. × I.D. × L 10-15 nm × 2-6 nm × 0.1-10 μm)	554 nm	1.000	7.1	30-45 m ² /g

^a Measured before dosing in distilled water

Table 1. Multi-walled carbon nanotubes (MWCNT) Size in solution

3.2 Acute oral toxicity test in Wistar rats

There was no mortality evident in the study. Animals of treated groups showed normal and consistent gain in body weight when compared to the control group of animals (Figure 2a and 2b). No gross lesions were observed. Based on the above findings, the oral LD₅₀ for MWCNT of two different sizes was greater than 2000 mg/kg b.w for Wistar rats.

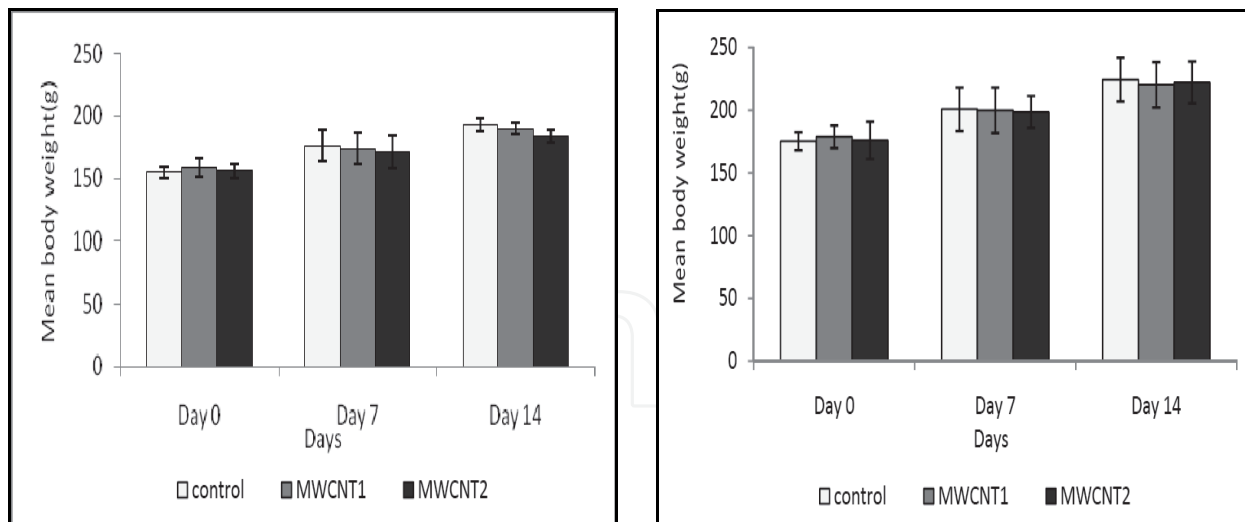


Fig. 2. a) & b) Acute oral toxicity test in rats - Mean body weight data (Males&Females)

3.3 Acute dermal toxicity study in Wistar rats

No mortality was evident in the study. Animals of treated groups showed normal and consistent gain in body weight when compared to the control group of animals (**Figure 3a and 3b**). No gross lesions were observed. Based on the above findings, the dermal LD₅₀ for MWCNT of two different sizes was greater than 2000 mg/kg b.w for Wistar rats.

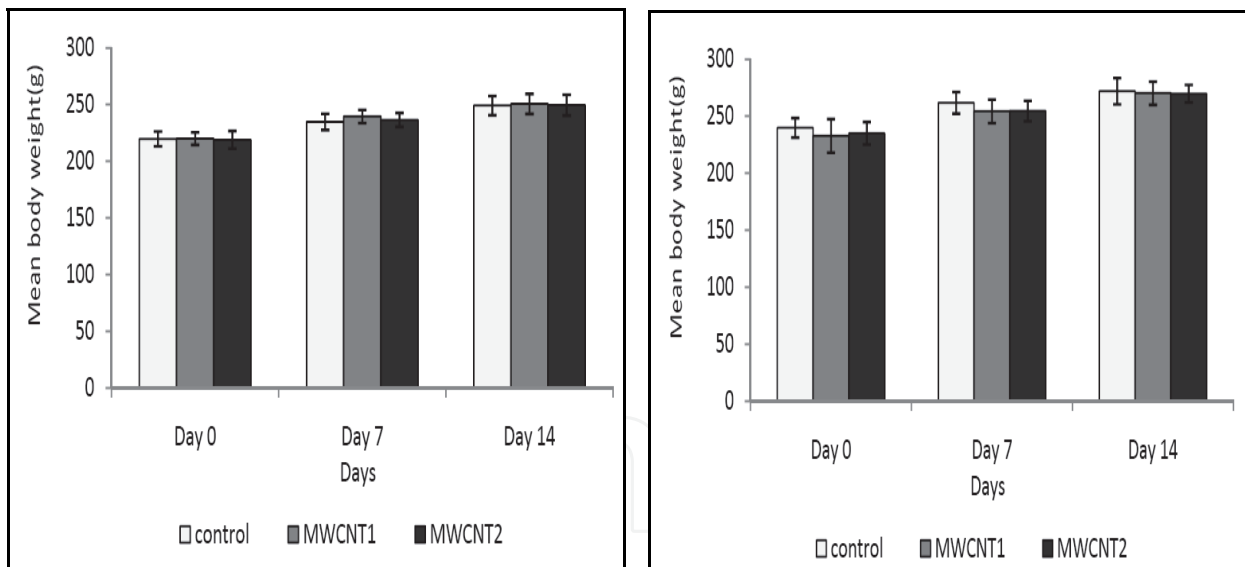


Fig. 3. a) & b) Acute dermal toxicity test in rats - Mean body weight data (Males & Females)

3.4 Acute dermal irritation study in New Zealand white rabbits

None of the rabbits treated with test material (two different sizes of MWCNT) showed any dermal reactions at 60 min, 24, 48 and 72 h after patch removal. None of the animals showed clinical signs of toxicity. Body weight gain of the individual animal was normal during the study period. Thus the Primary Irritation Index (PII) of two different sizes of MWCNT's is zero. Hence based on the above findings MWCNT 1 and 2 were considered as non irritant to the skin of the New Zealand white rabbits.

3.5 Acute eye irritation study in New Zealand white rabbits

The eyes of the rabbits were examined at 1, 24, 48, 72, 96 hours and 5th day after treating with test material. The maximum mean score for ocular lesions observed was 8.0 and 10.0 for MWCNT 1 and MWCNT 2 respectively (Table 2 and 3). Animals exhibited conjunctival redness, chemosis and discharge (score 1 or 2) from 1hour onwards. All the animals recovered from above ocular lesions on 5th day. Hence the MWCNT 1 and 2 were considered as minimally irritating to the eye of the New Zealand white rabbits.

Time of Observation	MWCNT 1 - Ocular Lesions			
	Total Score		Mean \pm S.D.	
	Treated (left eye)	Control (right eye)	Treated (left eye)	Control (right eye)
1 hour	12	0	4.0 \pm 0.00	0
24 hour	24	0	8.0 \pm 0.00	0
48 hour	14	0	4.7 \pm 1.15	0
72 hour	8	0	2.7 \pm 1.15	0
96 hour	4	0	1.3 \pm 1.15	0
5 th Day	0	0	0	0

Table 2. Acute eye irritation test in New Zealand White rabbits - Mean ocular lesions - MWCNT 1

Time of Observation	MWCNT 2 - Ocular Lesions			
	Total Score		Mean \pm S.D.	
	Treated (left eye)	Control (right eye)	Treated (left eye)	Control (right eye)
1 hour	16	0	4.7 \pm 1.15	0
24 hour	30	0	10.0 \pm 0.00	0
48 hour	18	0	6.0 \pm 2.00	0
72 hour	10	0	3.3 \pm 1.15	0
96 hour	4	0	1.3 \pm 1.15	0
5 th Day	0	0	0	0

Table 3. Acute eye irritation test in New Zealand White rabbits - Mean ocular lesions - MWCNT 2

3.7 Skin sensitization potential test in Guinea pigs

None of the animals treated with both the sizes of MWCNT did not exhibited any dermal reactions. Hence both the sizes of MWCNT could be considered as non sensitizer.

4. Discussion

Among 580 consumer nanotechnology-based products, one of the most common materials mentioned in the product descriptions is MWCNT based nanoparticles (Woodrow Wilson

International Center for Scholars, 2007). However, MWCNT remain one of the most controversial research areas regarding their toxicity to biological systems.

However, the effect of MWCNT *in vivo* has not been studied extensively. Therefore, we have made an attempt to scrutinize the acute toxicological potential of MWCNT. The adequate characterization of nanomaterials represents one of the key aspects of toxicity screening strategies. Adequate NM characterization is needed prior to the initiation of toxicological experimentation to ascertain the possible cause for the toxicity of NM and in absence of this would have limited significance (Warheit, 2008). Hence, we performed prior to toxicology experiments the physicochemical characterization of MWCNT like, size in dry state and distilled water, surface area to ascertain the possible cause for the toxicity. The two different sizes of MWCNT in distilled water showed increase in the size. Murdock et al., (2008) reported that once the nanomaterials are in water they do not necessarily retain their nano-size. Both the sizes (140±30, 10-15 nm) of MWCNT exhibited similar kind of results in acute oral, dermal and ocular irritation tests. Hence, there is no size dependant toxicity occurred in current acute toxicity experiments.

In our present acute oral and dermal toxicity studies in Wistar rats of two different sizes with MWCNT did not show any toxicity. Based on these results the acute oral and dermal LD₅₀ is greater than 2000 mg/kg b.w. for both the sizes of MWCNT. The acute dermal irritation study in New Zealand white rabbits with two different sizes of MWCNT, exhibited no dermal reactions. Under the conditions of this test, both the sizes of MWCNT were considered to be non-irritating to the skin. However, acute eye irritation study with both the sizes of MWCNT produced conjunctival redness and discharge in the treated eye of the rabbits and the lesions were reversible. No skin reactions were observed in Guinea Pigs treated with both the sizes of MWCNT. Based on these findings, both the sizes of MWCNT could be classified as minimally-irritating to the eye of the rabbits.

In conclusion, the results of these acute toxicity studies demonstrated low hazard potential in mammals following acute exposures to the MWCNT in oral and dermal routes. The MWCNT screened in these studies may serve as a base-line data for risk assessment. Further studies are required to investigate the impact of multi-walled carbon nanotubes to cellular components, including genetic material.

5. Conclusion and future remarks

Despite of the wide usage of Multi-walled carbon nanotubes the toxicological information and potential *in vivo* health hazards are still fragmentary. Hence we have made an attempt to understand the toxicological potential of these nanomaterials in four different *in vivo* models since most of the nanotoxicology studies were focused on *in vitro* models and only few research groups have dealt with *in vivo* systems. Toxicology studies with *in vivo* systems carry greater significance pertaining to their diversity in physiology and anatomy.

The current research work describes the results of four different *in vivo* toxicity studies conducted on multi-walled carbon nanotubes (MWCNT). The studies included are acute oral and dermal toxicity in Wistar rats, acute dermal and eye irritation study in New Zealand White rabbits and skin sensitization in Guinea pigs. Justification for these particular tests rests on the following criteria: potential routes of exposures (i.e., oral, dermal, and/or ocular) and the results of these acute studies demonstrated low hazard potential in mammals following acute exposures to the multi-walled carbon nanotubes (MWCNT) and there is no significant size dependent toxicity.

6. Acknowledgements

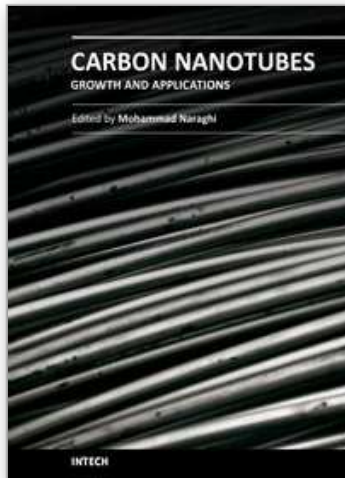
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7. Conflict of interest

The authors declare no conflict of interest

8. References

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Carbon Nanotubes are among the strongest, toughest, and most stiff materials found on earth. Moreover, they have remarkable electrical and thermal properties, which make them suitable for many applications including nanocomposites, electronics, and chemical detection devices. This book is the effort of many scientists and researchers all over the world to bring an anthology of recent developments in the field of nanotechnology and more specifically CNTs. In this book you will find:

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