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Multi-walled carbon nanotubes (MWCNT): Induction of DNA damage in plant and mammalian cells

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ABSTRACT

Increasing use of multiwalled carbon nanotubes (MWCNT) necessitates an improved understanding of their potential impact on environment health. In the present study we evaluated the genotoxicity of MWCNT on plant and mammalian test systems. Genotoxic responses such as chromosomal aberrations and DNA strand breakages were studied in *Allium cepa*, human lymphocytes, mouse bone marrow cells and pBR322 plasmid DNA. Results showed that MWCNT could cause chromosomal aberrations, DNA fragmentation and apoptosis in *Allium* root cells that could be correlated with the internalization of MWCNT in the plant cells. In human lymphocytes significant genotoxic response was observed at the concentration 2 μg/ml. Higher concentrations led to a decrease in values of the tail DNA percent that may be due to the formation of crosslinks. Annexin V-FITC–PI staining indicated only a small percentage of cells were undergoing apoptosis. Genotoxic effects were shown by micronuclei (MN) frequencies in experiments on mouse bone marrow cells. In the cell free DNA system (plasmid pBR322), a strong correlation between DNA strand break and concentration was observed. Based on the findings of the present study MWCNT may have significant impact on genomic activities.

1. Introduction

Carbon nanotubes have won enormous popularity in nanotechnology and the effect of their release into the environment is of prime concern [1,2]. The reports on their toxicity in animal system either *in vivo* [3–5] or *in vitro* [6–14] are often divergent. A number of studies have shown carbon nanotubes to be cytotoxic and genotoxic, capable of producing ROS and inducing apoptosis [2,9,14–18]. While a fair number of studies has shown negligible cytotoxic effect of these nanomaterials [13,18,19]. Genotoxicity studies of multiwalled carbon nanotubes (MWCNT) in plant are scarce and often contradictory [20–24]. A majority of these studies have reported of enhanced seed germination and growth in crop plants [23,24].

Increasing production and expanding application of MWCNT in multiple aspects of life necessitate reliable safety assessment. One important component of the safety assessment process, irrespective of its application, is the testing of genotoxicity using a battery of tests. Therefore the *in vitro* and *in vivo* genotoxicity of MWCNT was examined at different trophic levels. DNA strand breaks in pBR322 plasmid DNA were studied using agarose gel electrophoresis. Classical genotoxic endpoints like *Allium* test, comet assay, DNA diffusion assay and DNA laddering technique were evaluated in *Allium cepa* roots. Cytotoxicity was measured by the trypan blue dye exclusion assay in human lymphocytes. Genotoxicity was studied using comet assay *in vitro* (human lymphocytes) and *in vivo* (bone marrow cells of Swiss albino male mice). To study induction of apoptosis DNA diffusion assay and flow cytometric analysis (Annexin V-FITC–PI) was performed. In addition *in vivo* micronucleus assay was performed in bone marrow cells of Swiss albino male mice.

The study provides evidence of genotoxicity of MWCNT. Induction of DNA damage in cell free DNA system (plasmid pBR322) in absence of any cellular enzymes helps understand whether DNA damage mediated by MWCNT is a result of direct interaction or is mediated by metabolic activation. The genotoxicity, DNA-crosslink formation and induction of apoptosis by MWCNT in the plant system could be supported by the internalization of the nanoparticles in the plant cell. Data indicates of cytotoxicity and genotoxicity in human lymphocytes. The results of micronucleus assay and comet assay in Swiss albino male mice suggest of genotoxicity of MWCNT *in vivo*.

Abbreviations: MWCNT, multiwalled carbon nanotubes; CA, chromosome aberration; MI, mitotic index; MN, micronuclei; SEM, scanning electron microscope; DLS, dynamic light scattering; (RPMI)-1640, Roswell Park Memorial Institute.

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2. Materials and methods

2.1. Chemicals

Multi-walled carbon nanotubes (MWCNT; CAS Number: 308068-56-6), Ethyl methanesulphonate (EMS, CAS No. 62-50-0), Normal melting point agarose (NMPA), low melting point agarose (LMPA), di-sodium salt of EDTA, Tris buffer, ethidium bromide (EtBr), Histopaque, trypan blue were purchased from Sigma–Aldrich Co. (USA). Cetyl trimethyl ammonium bromide (CTAB, CAS No. 57-09-0), polyvinylpyrrolidone (PVP, CAS No. 9003-39-8) were purchased from Hi-Media Ltd., Mumbai, India. All other chemicals like sodium hydroxide, sodium chloride, acetic acid, orcein, chloroform, isoamyl alcohol were obtained locally and were of analytical grade.

2.2. Preparation of MWCNT solution

MWCNTs were obtained from Sigma–Aldrich, USA (Product code: 694185-1G). The physical characteristics of MWCNT according to the manufacturers' data are: average wall thickness 3–19 graphene layers, bundle diameter ~44–800 nm (TEM); produced by CVD method; Outer diameter × inner diameter × length: 7–15 nm × 3–6 nm × 0.5–200 μm; melting point ~3652–3697 °C; density ~2.1 g/ml at 25 °C. To prepare stock solutions appropriate amount of MWCNT were suspended in filter sterilized double distilled water and were sonicated on ice at 100 W, 30 kHZ (UP100H Ultrasonic processor, Hielscher Ultrasound Technology, Germany), for 20 min. Working solutions was made by serial dilution in filter sterilized double distilled water, followed by sonication and vigorous vortexing (5 min) as and when required.

2.3. Characterization of MWCNT

2.3.1. Characterization of MWCNT powder

MWCNT as obtained from Sigma–Aldrich, USA (Product code: 694185-1G), was characterized using scanning electron microscopy (Hitachi S-415A electron microscope at 25 kV).

2.3.2. Characterization of MWCNT in dispersion

The nanoparticle suspension was characterized for size and dispersity. This was accomplished using dynamic light scattering (DLS). Measurements were made using Malvern NanoZS (Worcestershire, United Kingdom) where particle size distributions were determined on the basis of number, volume and scattering intensity.

2.4. Genotoxicity of MWCNT in pBR322 plasmid DNA

pBR322 plasmid DNA (400 ng) obtained from Chromous Biotech (India) was incubated with different concentration of MWCNT (0, 5, 10, 15, 20 and 25 μg/ml) for a period of 30 min at 37 °C, and resolved on 1% agarose gel in 1xTAE buffer at 100 V for 30 min.

2.5. Genotoxicity of MWCNT in common onion (Allium cepa) bulbs

2.5.1. Treatment of A. cepa bulbs

Equal-sized onion bulbs, from a population of a local market variety of *A. cepa* were used. The bulbs were allowed to germinate on sand in earthen pots [25] at room temperature (28 ± 1 °C) under a 12 h light/dark cycle. The *A. cepa* bulbs were then exposed to different concentrations (0, 10, 20 and 50 μg/ml) of MWCNT according to the schedule given below:

(i) 3 h exposure to MWCNT followed by 24 h in absence of MWCNT for a period of one cell cycle (*A. cepa* ~18 h).
(ii) 24 h exposure to MWCNT
(iii) 24 h exposure to MWCNT followed by 1 h exposure to EMS

In set (i) roots were processed 24 h post-treatment of a 3 h exposure to MWCNT and in the set (ii) roots were processed after 24 h exposure to MWCNT. Two different treatment schedules were adopted to understand the effect of MWCNT – exposure over a period of one cell cycle [26]. In the set (iii) a combined treatment of EMS and MWCNT was carried out to detect DNA-crosslinks induced by MWCNT [27]. Following MWCNT treatment, *A. cepa* bulbs were subjected to 1 h treatment with EMS (4 mM). For all set of experiments 5 bulbs per concentration were used. The genotoxic potential of MWCNT was evaluated using the classical *Allium* test, comet assay, DNA diffusion assay and DNA laddering as endpoints.

2.5.2. Allium test

Following treatment, 10–15 roots from set (i) and (ii) were chosen at random and excised. The excised root tips were fixed immediately in acetic acid/ethanol (1:3). After a period of 3 h the root tips were hydrolyzed and stained in 9:1, 2% aceto-orcein – (N) HCl mixture. Slides were prepared from each of the roots following the squash technique of Sharma and Sharma [28], and coded to prevent observer bias. The mean values for mitotic index, binucleated cell, micronucleus, chromosomal aberrations (breaks and fragments, early separation, laggard and anaphase – telophase bridges) at each point were scored and the standard deviation was calculated accordingly [29]. Mean values for each aberration were calculated per concentration of exposure and were statistically correlated.

2.5.3. Comet assay and DNA diffusion assay in A. cepa

Comet assay was carried out according to the method previously published [29–31]. Roots from set (i), (ii) and (iii) were placed for 2 min on ice to keep them turgid. For isolation of nuclei, roots, treated or untreated as appropriate, were placed in a 60 mm Petri plate containing 400 μl of cold 400 mM Tris-buffer, at pH 7.5. Using a fresh razor blade, the roots were finely and gently sliced allowing isolation of nuclei into the buffer. Taking the nuclear suspension, slides were prepared in triplicates per concentration. The slides were subjected to 20 min unwinding, followed by electrophoresis at 4 °C for 30 min at 26 V adjusted to 300 mA, in alkaline electrphoresis buffer (300 mM NaOH and 1 mM EDTA; pH > 13). Slides were neutralized thrice in 0.4 M Tris pH 7.5 for 5 min and finally rinsed in water.

Nuclei were isolated from set (ii) and slides were prepared as previously described. Diffusion assay was performed according to method of Singh [32] with slight modification [33]. The slides with the isolated nuclei embedded in agarose were placed for 5 min in a Coplin jar containing a cold lysing and denaturation solution (2.5 M NaCl, 1% sodium sarcocinate, 100 mM Na_2EDTA, 10 mM Tris, pH 10 and 0.2% DMSO, 0.3N NaOH freshly added) before rinsing in cold water. The slides were placed for 1 h in a solution of 50% ethanol and 50% Tris buffer (400 mM, pH 7.4), with a final concentration of 1 mg spermine/ml to remove the precipitated salts and detergents while retaining DNA in the agarose. The slides were air-dried at room temperature and stored till scoring.

2.5.4. DNA extraction and laddering

DNA was isolated from *A. cepa* roots (set-(iii)) post 24 h treatment of MWCNT (0, 10, 20 and 50 μg/ml), using a modified CTAB method [34,35]. The roots were weighed and frozen at −20 °C till further use. The roots were ground in extraction buffer containing 100 mM Tris buffer pH 8, 25 mM EDTA, 2 M NaCl, 3% CTAB, 3% PVP. The suspension was gently mixed and incubated at 65 °C for 20 min and then cooled to room temperature. An equal volume of chloroform:isoamyl alcohol (24:1) was added. The mixture was

centrifuged at 12,000 rpm for 5 min. The clear upper aqueous phase was then transferred to a new tube, to which 2/3 volume of ice-cold isopropanol was added, and incubated at −20 °C for 30 min. The nucleic acid was collected by centrifugation at 10,000 rpm for 10 min. The resulting pellet was washed twice with 75% ethanol. The pellet was air-dried and the nucleic acid was dissolved in TE (10 mM Tris buffer pH 8, 1 mM EDTA) at room temperature and stored at 4 °C until use. RNA was eliminated by treating the sample with RNase A (10 mg/ml) for 30 min at 37 °C. DNA purity was determined by measuring the absorbance of the diluted DNA solution at 260 and 280 nm.

The isolated DNA samples were resolved on 2.5% agarose gel in 1xTAE (Tris–acetate–EDTA) buffer at 100 V, for 90 min at 4 °C. A lane was loaded with 100 bp ladder for reference. DNA was stained with aqueous solution of EtBr, visualized and photographed under a UV transilluminator.

2.6. Cytotoxicity and genotoxicity of MWCNT in human lymphocytes

2.6.1. Isolation of lymphocytes from human peripheral blood

Human peripheral blood was obtained by venipuncture from healthy volunteers (20–25 year old male donors, non-smokers, non-alcohol consuming and not undergoing any medication) into heparinised vacutainers. Lymphocytes were isolated from fresh blood according to the method of Boyum [36], using Histopaque. The cells were washed with PBS and resuspended in RPMI-1640 media at a concentration of 10^6 cell/ml and were immediately processed for further experiment. All experiments were conducted in accordance with the institutional guidelines.

2.6.2. Dose selection

The dose selection for genotoxicity study was based on initial screening using trypan blue dye exclusion method. MWCNT was screened for cytotoxicity over a wide range of concentration (0–500 μg/ml). The cut-off point as suggested by Henderson et al., [37] was 70%. Following the initial screening final treatment concentrations (0, 1, 2, 5 and 10 μg/ml) were selected for further experiments.

2.6.3. Treatment of lymphocytes

Freshly isolated lymphocytes were incubated for 3 h at 37 °C in RPMI-1640 media with different concentration of MWCNT (0, 1, 2, 5 and 10 μg/ml). Following treatment, the lymphocytes were processed for cytotoxicity study and detection of DNA damage as assessed by the alkaline comet assay.

2.6.4. Viability of lymphocytes using trypan blue dye exclusion method

Prior to alkaline comet assay and DNA diffusion assay, cell viabilities were checked by trypan blue dye exclusion to avoid artefacts due to cytoxicity [38]. Viability was measured both before and after treatment.

2.6.5. DNA damage analysis using Comet assay and DNA difussion assay in lymphocytes

The DNA damage studies were carried out following the comet assay according to the method of Singh et al., [30]. Slides were prepared in triplicates per concentration. A total of 100 μl of cell suspension was mixed with 100 μl of 1% low melting agarose onto microscope slides. The suspension was pipetted onto the precoated slides. Slides were immersed in cold lysis solution at pH 10 (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% Triton X-100, and 10% DMSO) and kept at 4 °C for 60 min. To allow denaturation of DNA, the slides were placed in alkaline electrophoresis buffer at pH > 13 (1 mM Na_2EDTA/300 mM NaOH) and left for 20 min and subsequently were transferred to an electrophoresis tank with fresh alkaline electrophoresis buffer, and electrophoresis was performed at a field strength of 0.7 V/cm for 30 min at 4 °C (20 V–300 mA). Slides were neutralized in 0.4 M Tris pH 7.5 for 5 min,thrice and finally rinsed in water.

For DNA diffusion assay the lymphocytes were processed immediately according to the method of Singh [32] with modifications as has been described previously.

2.7. Detection of apoptosis/necrosis using Annexin V-FITC–PI staining

Annexin V-FITC–PI staining was performed to differentiate apoptosis from necrotic cell death. Annexin V has a high affinity for phosphatidyl serine, translocated from the inner to the outer leaflet of the plasma membrane at an early stage of apoptosis. Its conjugation with the fluorescent probes FITC and facilitates measurement by flow cytometric analysis. Using propidium iodide (PI) helps distinguish between apoptosis and necrosis due to difference in permeability of PI through the cell membranes of live and damaged cells. Treated cells were harvested and washed twice in PBS. The staining was carried out as per manufacturer's instruction (FITC Annexin V Apoptosis Detection Kit, BD Pharmingen, USA). Data analyses were done using BD FACS Calibur™ (BD Bioscience, USA).

2.8. Genotoxicity of MWCNT in Swiss albino male mice, in vivo

The study was conducted on Swiss albino male mice, *Mus musculus* (8–10 weeks old, weighing 20–25 g). The animals were obtained from departmental animal house, housed in polycarbonate cages, bedded with rice husk and acclimatized under laboratory conditions (20–22 °C, humidity 50–60%, 12 h light/dark photoperiod) for at least a week prior to experiment and fed with standard rodent pellet (M/S Hindustan Lever foods, India) and water was provided ad libitum. All the experiments were done in accordance to the University Ethical committee guidelines (University of Calcutta, Kolkata, India).

2.8.1. MWCNT treatment doses

After acclimatization, the animals were divided into 5 groups of 3 male mice each; each group implying to a particular treatment dose.

In vivo micronucleus assay and comet assay in bone marrow cells of Swiss albino male mice was performed according to the protocol of Bowen et al., [39]. For *in vivo* micronucleus assay animals were administered (ip. injection) with different concentrations (2, 5 and 10 mg/kg body weight) of test chemicals and vehicle and positive controls (MMC) at 0 and 24 h. Animals were sacrificed 24 h after the final dose administration. For comet assay in bone marrow cells *in vivo,* animals were administered (ip. injection) with different concentrations (2, 5 and 10 mg/kg body weight) of test chemicals and vehicle and positive controls at 0 and 24 h and the animals were sacrificed 3 h after the final dose administration. The dose selection was based on *in vitro* genotoxicity data obtained.

2.8.2. Micronucleus test in bone marrow cells

The animals were sacrificed by cervical dislocation. Bone marrow cells from femurs were flushed into individual centrifuge tubes containing 1% sodium citrate. The cells were pelleted by centrifugation at 1000 rpm for 5 min and slides were prepared by making smears. The slides were coded and stained in May-Gruenwald/Giemsa stain and processed for scoring.

Scoring was carried out under blinded conditions. Initially the relative proportions of polychromatic erythrocytes (PCE) and NCE were determined until a total of 1000 cells had been scored. Then

2000 PCE/animal were examined for the presence of MN. Percentage PCE, Frequency of MN-PCE (MN/2000 PCE) and percentage of MN-PCE for each animal were calculated and compared with the vehicle control and studied for evidence of bone-marrow toxicity.

2.8.3. Comet assay in bone marrow cells

Bone marrow cells from the femur were flushed into individual centrifuge tubes with phosphate-buffered saline (PBS, pH 7.4). The bone marrow cells were dispersed by gentle pipetting and collected by centrifugation at 1500 rpm for 10 min at 4 °C. Cell pellet was resuspended in PBS and used for further analysis using comet assay. The DNA damage studies were carried out following the comet assay according to the method previously described in Section 2.6.4. Slides were prepared in triplicates per concentration and images of 150 cells per concentration were analysed.

2.9. Scoring of slides of comet assay

Slides stained with ethidium bromide (EtBr; 20 μg/ml) were scored using image analysis system (Kinetic imaging; Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters (N2.1). The microscope was connected to a computer through a charge-coupled device (CCD) camera to transport images to software (Komet 5.5) for analysis. The final magnification was 100×. Among the comet parameters we report the % of DNA in the tail [% tail DNA] which gave us a clear indication of the extent of DNA damage induced by the test compound. Images of 75 (25 × 3) nuclei for plant and 150 (50 × 3) nuclei for human lymphocyte cells and bone marrow cells per concentration were analysed for comet assay. The median values of each concentration with respect to the comet parameter (% tail DNA) was calculated. For DNA diffusion assay average nuclear area were scored per concentration. From each replicate experiment, the percentage of diffused nuclei (in human lymphocytes) and the average nuclear area (plant and human lymphocytes) were used to express the nuclear DNA diffusion.

2.10. Statistical analysis

For mitotic index, micronuclei, % chromosomal aberration, students '*t*' test was performed. For comet assay, one way analysis of variance (ANOVA) test was performed. For all statistical tests Sigma Stats.3 software (SPSS Inc., Chicago, Illinois, USA) was used. The level of significance was established at $P \leq 0.05$.

3. Results

3.1. Characterization of MWCNT

3.1.1. Characterization of MWCNT powder

SEM photograph of MWCNT (Fig. 1a) revealed of an average diameter to be ~21.55 nm.

3.1.2. Characterization of MWCNT in dispersion

Dynamic light scattering measurements using Malvern NanoZS (Worcestershire, United Kingdom) performed on the stock suspension showed a majority of MWCNT forming agglomerates, with an average hydrodynamic diameter of 1895 nm. DLS of the stock suspension revealed ~40% MWCNT in the size range of 246 nm (Fig. 1b).

3.2. Genotoxicity of MWCNT in pBR322

Agarose gel electrophoresis of treated pBR322 (Fig. 2) showed a dose dependent induction of DNA strand break, characterized by

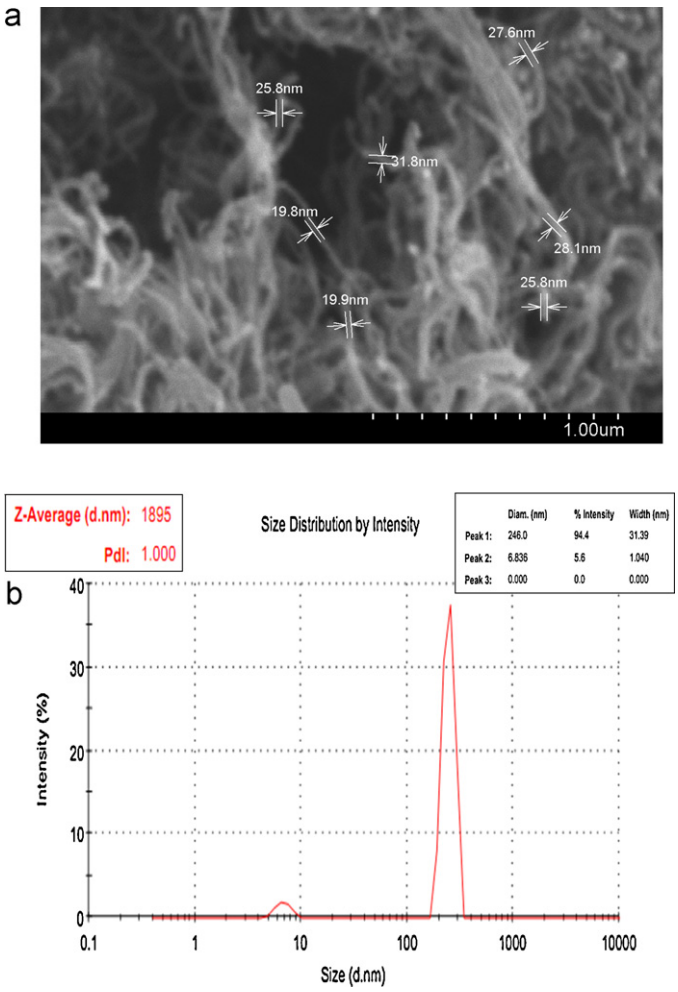

Fig. 1. a: SEM image of the MWCNT. b: DLS measurement of MWCNT suspension.

absence of super coiled form in the highest dose. DNA strand scission induced by MWCNT lead to a gradual decrease in the amount of super-coiled DNA. Though an initial increase in relaxed form of DNA was observed upto treatment concentration of 15 μg/ml, the subsequent higher doses were marked by extensive shearing of DNA.

3.3. Genotoxicity of MWCNT in plant

3.3.1. Allium test

The genotoxic effect of MWCNT was evaluated on the basis of *Allium* test results (mitotic index, micronucleus, and chromosomal aberrations). Formations of micronuclei were detected in interphase cells at all concentrations (Table 1). Treatment with MWCNT in set (i) revealed an increase in chromosomal aberration as well as in the number of micronuclei at the highest dose. The values were significantly high ($P \leq 0.05$.) at 50 μg/ml. Mitotic index that was determined in control and treatments (range: 3.38–7.36%) was statistically significant .In the experimental set (ii) MWCNT treatment induced chromosomal aberrations (Fig. 3a), micronuclei and binucleate cells. ANOVA test showed an absence of statistically significant variation amongst the concentrations tested (Table 1). Following a 24 h exposure to MWCNT suspension the divisional frequency decreased from a value of 8.83% in control to a value of 5.22% in 50 μg/ml. Localization of MWCNT within the cells could be confirmed by the presence of a large number of "black dots" distributed throughout the cytoplasm (Fig. 3b). Moreover, treated cells

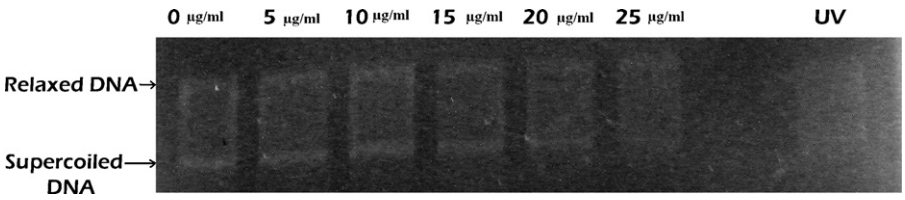

Fig. 2. Agarose gel electrophoresis of plasmid pBR322 treated with different concentrations of MWCNT.

revealed presence of large number of vacuoles (Fig. 3c), retracted cytoplasm, smaller and more condensed nuclei (pyknotic cells) showing no nucleolus (Fig. 3c).

3.3.2. DNA damage analysis using comet assay in A. cepa

After treatment of *A. cepa* for 3 h or 24 h with MWCNT (0,10,20,50 μg/ml) nuclei from roots were isolated either immediately after the treatment,or after a recovery period of 24 h (sets (i) and (ii)). Comet parameter (% tail DNA) clearly indicated a significant ($P \leq 0.05$) dose depedent increase in DNA migration (set (i)). On the other hand, 24 h treatment with MWCNT revealed a remarkably distinct dose response.Tail DNA percent decrease was dependent on the concentrations (Fig. 4a). The results of set (iii) treatment revealed an increase in DNA migration, significant ($P \leq 0.05$) at the highest dose (50 μg/ml) (Fig. 4a).

3.3.3. DNA diffusion assay

DNA diffusion assay revealed a dose dependent decrease in average nuclear area (Fig. 4b) in post 24 h MWCNT treatment (set-(ii)). The values in 20 μg/ml MWCNT were significantly ($P \leq 0.05$) lower than the negative control. Presence of apoptotic and necrotic cells along with normal cells were detected in certain treatment concentrations (Fig. 4c).

3.3.4. DNA laddering

DNA laddering clearly indicated DNA fragmentation and presence of DNA fragments in the size range of 100–400 bp, in MWCNT (10, 20 and 50 μg/ml) – treated *A. cepa* roots. In the control sample, DNA was characterized by the presence of a single prominent band corresponding to its genomic DNA without DNA fragmentation (Fig. 4d).

3.4. Cytotoxicity and genotoxicity of MWCNT in human lymphocytes

3.4.1. Viability of lymphocytes

The initial cell viability screening was performed over wide dose range (0–500 μg/ml). A significant decrease in cell viability was observed at treatment concentrations of 50–500 μg/ml (range: 74.92–55.08%) as compared to control (96.56%).

Cell viability determined in treatments (1, 2, 5 and 10 μg/ml) revealed a decrease in % viability (range: 90.47–87.28%). Percentage cell viability was lowest at 5 μg/ml treatment dose (Fig. 5a).

3.4.2. DNA damage analysis using comet assay in lymphocytes

DNA damage analysis in human lymphocytes using comet assay did not reveal any dose dependent effect (Fig. 5b). The values of % tail DNA were higher in 2, 5 and 10 μg/ml than the negative control ($4.8 \pm 0.85\%$). But was statistically significantly ($P \leq 0.05$) in 2 μg/ml ($12.5 \pm 1.92\%$) only.

3.4.3. DNA diffusion assay

DNA diffusion assay in human lymphocytes revealed a dose dependent decrease in nuclear area (Fig. 5c) as well as in the percent of diffused nuclei (Fig. 5d).

3.5. Detection of apoptosis/necrosis using Annexin V-FITC–PI staining

To assess the extent and mode of cell death, Annexin V-FITC–PI staining was used. Based on the percentages of unstained cells (viable cells), and those with red fluorescence (necrotic cells), green fluorescence (apoptotic cells), and dual stained cells (late apoptosis) were analysed. Annexin V staining experiment indicated that only a small percentage of cells were undergoing apoptosis at treatment concentrations (~1.5 times) as compared to control (Fig. 6). There was an increase (~22 fold with respect to control) in the number of necrotic cells up to the highest treatment concentration (Fig. 6).

3.6. Genotoxicity of MWCNT in Swiss albino male mice, in vivo

3.6.1. Micronucleus test in bone marrow cells

A decrease in percentage of polychromatic erythrocytes (% PCE) was observed, however the decrease was not statistically significant. Statistically significant ($P \leq 0.05$) increase in micronucleated polychromatic erythrocytes was observed at all treatment doses (2, 5 and 10 mg/kg body weight) tested (Table 2). Micronuclei formation was highest at 2 mg/kg body weight followed by 5 and 10 mg/kg body weight.

Table 1
Mitotic index (MI), number of micronuclei/1000 cells and % chromosomal aberrations revealing the genotoxic potential of MWCNT in *Allium cepa* roots as analysed by *Allium* test and *Allium* anaphase – telophase chromosome aberration assay.

Treatment time	MWCNT (ppm)	No. of cells examined	MI	Micronuclei/1000 cells	% Chromosomal aberration
3 h treatment + 24 h recovery	0	1745	7.36	0.43	0.05
	10	1301	5.45	2.30*	0.23*
	20	1056	3.21*	1.89	0.18
	50	1950	3.38*	2.92*	0.26*
24 h treatment	0	4745	8.826	0.67	0.07
	10	4145	8.04	1.44	0.43*
	20	4082	7.23	1.29	0.13
	50	4132	5.22	0.73	0.17

* Significant at $p \leq 0.05$

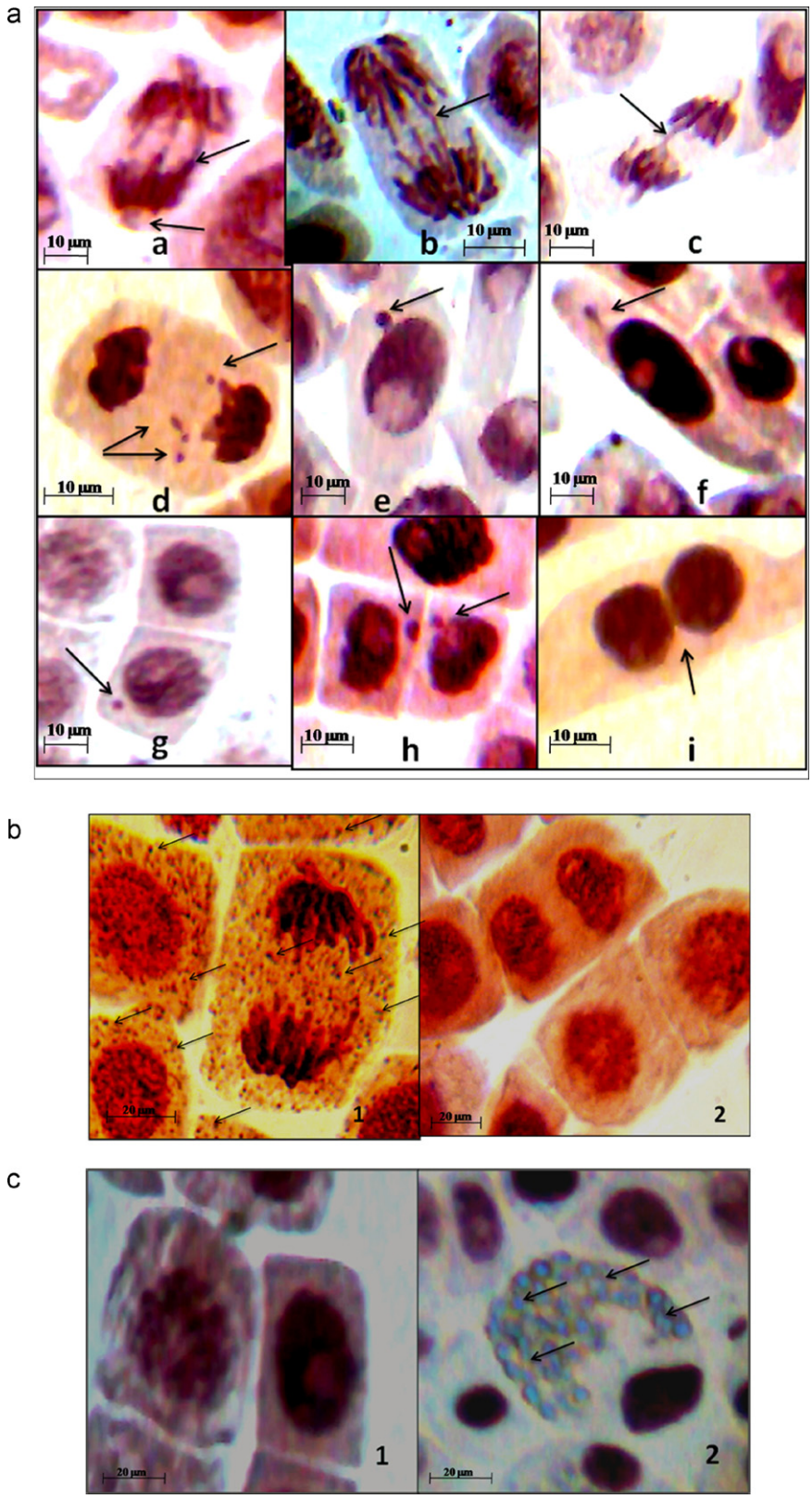

Fig. 3. a: Nuclear aberrations induced by MWCNT treatment in *Allium cepa* root cells; a–c: anaphase bridge and vagrants, d: telophasic fragments, e and f: nuclear buds, g and h: micronuclei, i: binucleate cells. b: Cells of *Allium cepa* showing 1 – "black dots" representing deposition of MWCNT inside the cell, 2 – untreated cells. c: Cells of *Allium cepa* showing 1 – normal nuclei with proper nucleolus, 2 – MWCNT treated cells showing presence of vacuolation and constricted, dark stained nuclei, marked by absence of nucleolus.

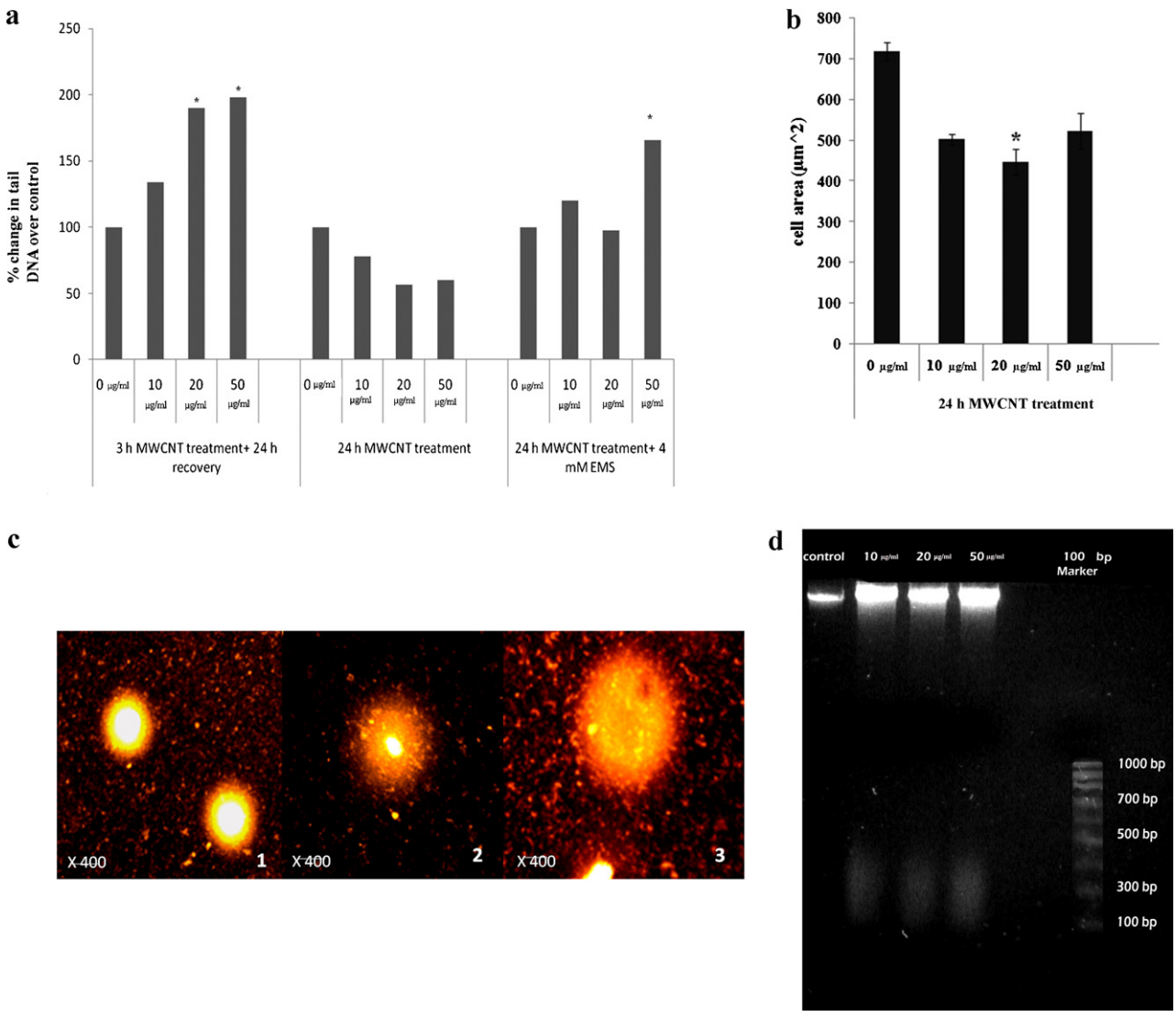

Fig. 4. a: Comet data (% tail DNA) of MWCNT treated (0, 10, 20 and 50 μg/ml) *Allium cepa* root over different periods of treatment time (3 h, 24 h and 24 h + 1 h 4 mM EMS); $^{*}p < 0.05$. b: DNA difussion assay in MWCNT treated *Allium cepa* root nuclei; $^{*}p < 0.05$. c: DNA difussion assay in MWCNT treated *Allium cepa* root nuclei; 1 – cotnrol nuclei, 2 – apoptotic nuclei, 3 – necrotic nuclei. d: Agarose gel electrophoresis of control and MWCNT treated *Allium cepa* DNA; bands corresponding to ladder from 100 to 400 bp indicate of induction of apoptosis.

3.6.2. Comet assay in bone marrow cells

In vivo comet assay results in bone marrow cells indicated of similar dose response as observed *in vitro*. The values of % tail DNA were higher at all treatment doses (2, 5 and 10 mg/kg body weight) as compared to the negative control (2.75 ± 0.86%) (Table 2). Increase in % tail DNA was significant ($P \leq 0.05$) at doses 2 mg/kg body weight (32.46 ± 5.16%) and 5 mg/kg body weight (10.49 ± 0.48%).

4. Discussion

DNA damage induced by nanomaterials is not just relevant to human species but also to other components of the environment [40]. Currently very little data are available regarding the toxicity of nanomaterials in systems other than human [40]. In this study we make an effort to study the genotoxicity on naked DNA (plasmid pBR322), in plant (*A. cepa*),

Table 2
DNA damage determined by the bone marrow micronucleus assay and Comet assay in Swiss albino male mice, exposed to different concentrations of multiwalled carbon nanotubes (MWCNT).

Concentrations (mg/kg body weight)	Micronucleus test			Comet assay
MWCNT	% PCE	Mn-PCE/2000 PCE	% Mn-PCE	% Tail DNA ± SD
0	62.84	5.80	0.29	2.75 ± 0.86
2	68.48	57.28*	2.86*	32.46 ± 5.16*
5	59.76	46.31*	2.32*	10.49 ± 0.48*
10	51.06	42.22*	2.11*	6.23 ± 2.54
MMC (5)	24.75	83.92	4.19	–
Cyclophosphamide (20)	–	–	–	32.53 ± 8.11

* Significant at $p \leq 0.05$

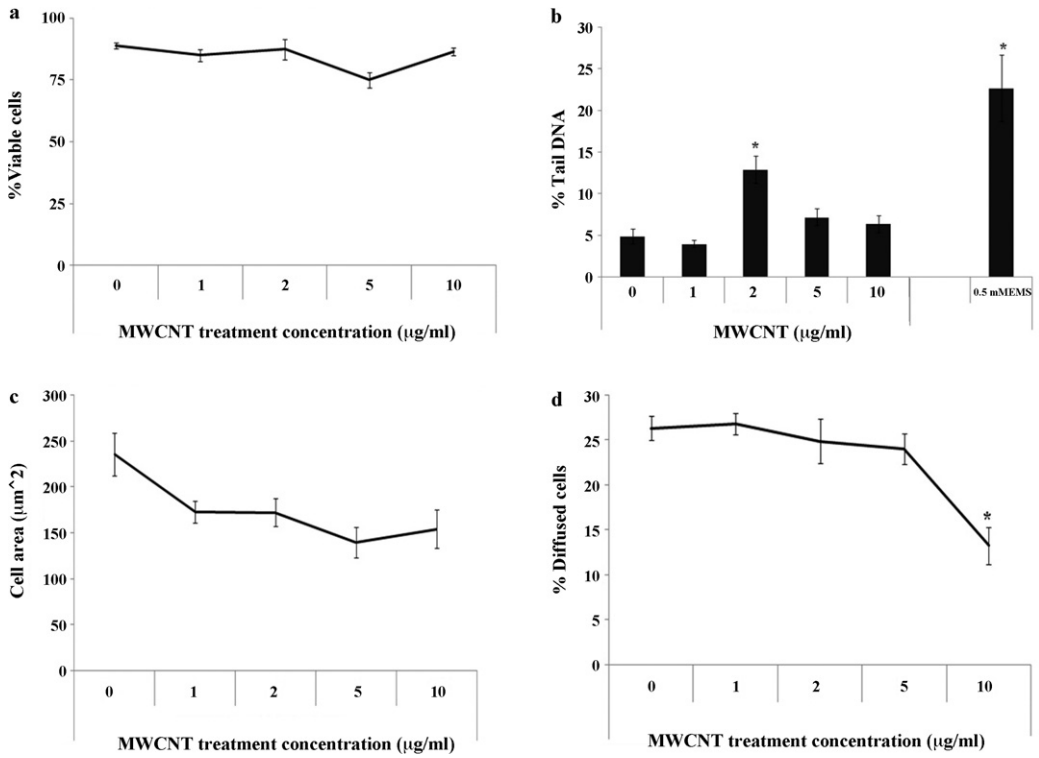

Fig. 5. a: Cell viability study using trypan blue dye exclusion method in MWCNT treated human lymphocyte cells. b: Comet parameter % tail DNA indicating of extent of DNA fragmentation induced by MWCNT treatement in human lymphocyte cells; $^{*}p < 0.05$. c: DNA difussion assay indicating of dose dependent decrease in cell area with increase in treatment concentration of MWCNT in human lymphocyte cells; $^{*}p < 0.05$. d: DNA difussion assay indicating of a decrease in % difussed nuclei with increase in treatment concentration of MWCNT in human lymphocyte cells; $^{*}p < 0.05$.

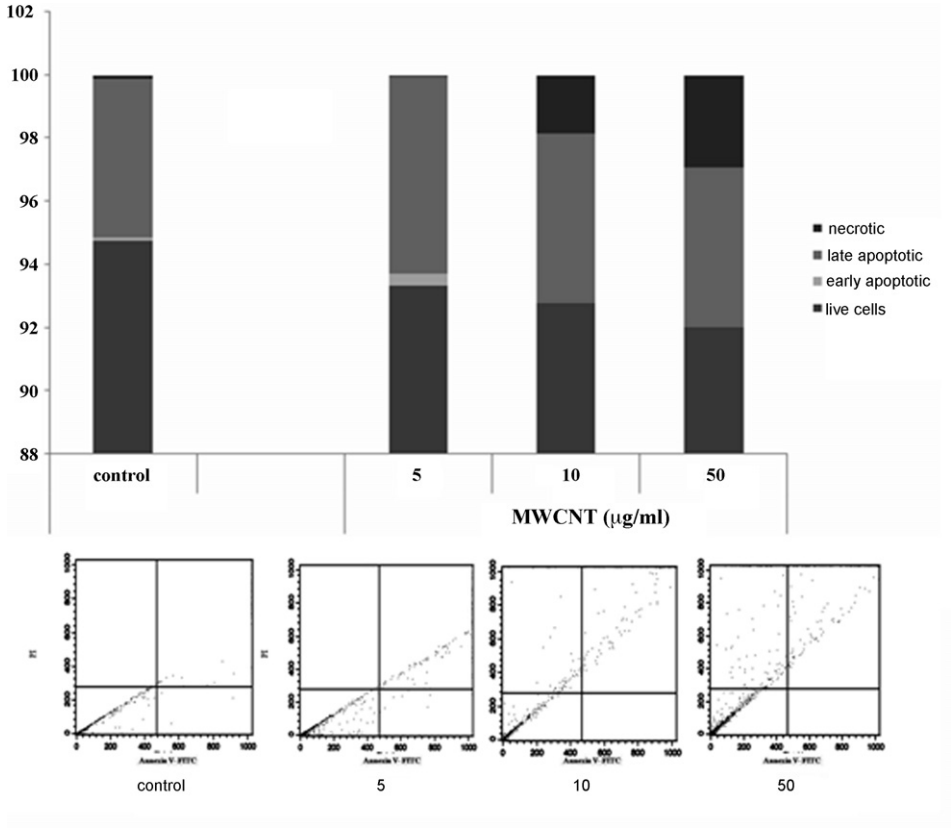

Fig. 6. Flow cytometric analysis of Annexin V-FITC–PI stained lymphocyte cells showing induction of apoptosis and necrosis at different MWCNT treatment concentrations.

human lymphocytes *in vitro* and in Swiss albino male mice *in vivo*.

In *A. cepa*, the clastogenicity of MWCNT is evident from chromosome breaks and formations of micronuclei at interphase. Chromosomal aberrations were characterized by presence of anaphase/telophase bridges and early/late separations (Fig. 3a) that are manifestation of spindle aberration. Aberrations such as binucleate cells (Fig. 3a) arise as a result of inhibition of cell plate formation or even mitotic irregularities [41]. Presence of micronuclei (at interphase) (Fig. 3a) are manifestation of chromosome fragmentation that occurred in the previous cell cycle and was evident from both the treatment schedules. Previous studies have established the fact that chromosome aberrations, such as fragments and chromosome losses, can result in micronucleated cells [42]. According to Ma et al., [43] micronuclei are the most effective parameter of studying cytological damages resulting from environmental contamination. Analysis of chromosome aberration is equally important revealing both the genotoxicity and mechanism of action of the genotoxicant [44]. Results of comet assay in *A. cepa* revealed an initial dose dependent increase in DNA damage over a short period of time (3 h) followed by decrease with increasing time of exposure (24 h). The increase in dose and time of exposure might have enhanced the interaction of MWCNT with DNA, that could be responsible for the crosslinking at 24 h as compared to 3 h. To understand this distinct dose response a probable DNA crosslinking potency of MWCNTwas investigated. *A. cepa* bulbs were post-treated for an hour with a positive DNA damaging agent EMS (4 mM) following a 24 h exposure to MWCNT. EMS treatment revealed an increase in DNA migration, indicating formation of crosslink (MWCNT–DNA and/or DNA–DNA crosslink).

DNA difussion assay was performed to study the induction of apoptosis in *A. cepa*. DNA difussion assay is a simple, sensitive and rapid method for estimating apoptosis in single cell [45]. Apoptotic cells when tested using DNA difussion assay are characterized by presence of a dense central zone and a lighter hazy outer halo-like zone of granular nucleosome sized DNA fragments. Difussed nuclei formed due to necrosis show presence of a distinct outer boundary [33]. In the present study, results of DNA difussion assay did not indicate induction of apoptosis or necrosis. The decrease in nuclear area may be due to the formation of DNA crosslinks (MWCNT-DNA and/or DNA–DNA). Certain treatment concentrations demonstrated presence of both apoptotic and necrotic cells along with normal cells.

In addition, DNA laddering was performed in *A. cepa*. It is known that apoptosis is characterized by the biochemical production of 180–200 bp internucleosomal DNA fragments, resulting from endonucleolytic cleavage [46]. The presence of internucleosomal fragments in the size range of 100–400 bp clearly indicated the induction of apoptosis.

These results could be correlated with the presence of a large number of "black dots" distributed throughout the cytoplasm *A. cepa*. Previous studies with mammalian cell lines also demonstrated that the nanoparticles penetrated subcellular structures such as mitochondria and nucleus [47,48]. MWCNT treated cells also showed the presence of a large number of vacuoles. This could be associated with apoptosis as has been seen in a number of studies [49–51]. Presence of large number of cells with retracted cytoplasm, having smaller and more condensed nuclei (pyknotic cells) showing no nucleolus, has been previously reported in animal cells [16].

The genotoxicity of MWCNT was further evaluated with respect to human lymphocytes. The trypan blue dye exclusion method revealed decrease in cell viability (membrane integrity) at the treatment doses, compared to control. Comet parameter (% tail DNA) did not reveal any dose dependent effect. Lymphocytes demonstrated significant genotoxic response at 2 μg/ml treatment dose, followed by a gradual decrease in extent of DNA migration in the rest of the concentrations. The dose response could be either owing to crosslinking or agglomeration of MWCNT in aqueous media and the effective concentration that the cell is exposed to in case of agglomeration. The DNA diffusion assay in human lymphocyte revealed a dose dependent decrease in nuclear area as well as in the percent of diffused nuclei. As has been already studied in *A. cepa*. MWCNT induces formation of crosslinks which hinders DNA migration and could be attributed to the dose response. Induction of apoptosis in human lymphocytes was confirmed using Annexin V-FITC–PI staining.

In vivo genotoxicity of MWCNT in Swiss albino male mice was studied in bone marrow cells using micronucleus assay and comet assay as endpoints. Comet parameter (% tail DNA) revealed of induction of DNA fragmentation induced by MWCNT, significant at treatment doses 2 and 5 mg/kg body weight. Significant increase in micronucleated polychromatic erythrocytes was observed at all treatment doses tested, highest being observed at treatment dose 2 mg/kg body weight. The *in vivo* results were supportive of the *in vitro* genotoxicity analysis performed in human lymphocyte cells.

From our study thus far in plant system, human lymphocyte and Swiss albino male mice *in vivo*, it has been observed that MWCNT is capable of inducing DNA damage. While, formation of large number of vacuoles, internucleosomal fragments and pyknotic cells indicated of apoptosis in plants, flow cytometric analysis in human lymphocyte revealed induction of apoptosis/necrosis. Induction of micronuleus formation in both plant and Swiss albino male mice *in vivo* also indicated of persistant DNA amage induced by MWCNT treatment.

With regard to mechanism of genotxicity, the distinct dose response could be attributed to crosslinking and agglomeration of MWCNT. With increase in concentration and depending upon the hydrophobicity of MWCNT, greater agglomeration might reduce cellular uptake or reduce potential of MWCNT to cross biological barrier. Nanomaterials have a tendency to form agglomerates under physiological conditions. In our study DLS measurements revealed a similar tendency of MWCNT, with an average hydrodynamic diameter of ∼1.5 μm. Uses of surfactant to avoid agglomeration were avoided, as carbon nanotubes when stabilized with surfactant are known to induce higher cytotoxic response [40]. Previous studies have also clearly elaborated the affinity of MWCNT to DNA in water solute environment [52] and this could be the possible reason behind the genotoxic potential of MWCNT in cells' environment.In the present study formation of crosslink could be supported by a recent report. The study revealed dose dependent DNA aggregations resulting in formation of DNA network, on carbon nanoparticle exposure, both *in vivo* and *in vitro* [53].

In conclusion, our results demonstrate that MWCNT can interact with DNA and careful monitoring of toxicity studies is essential for risk assessment. The findings also suggest that plants as an important component of the ecosystem need to be included when evaluating toxicity of engineered nanomaterials.

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