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Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: Plant and human lymphocytes

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ABSTRACT

The environmental fate and behaviour of titanium dioxide (TiO_2) nanoparticles is a rapidly expanding area of research. There is a paucity of information regarding toxic effect of TiO_2 nanoparticles in plants and to certain extent in humans. The present study focuses on the effect of exposure of TiO_2 nanoparticles in two trophic levels, plant and human lymphocytes. The genotoxicity of TiO_2 nanoparticles was evaluated using classical genotoxic endpoints, comet assay and DNA laddering technique. DNA damaging potential of TiO_2 nanoparticles in *Allium cepa* and *Nicotiana tabacum* as representative of plant system could be confirmed in the comet assay and DNA laddering experiments. In *Allium* micronuclei and chromosomal aberrations correlated with the reduction in root growth. We detected increased level of malondialdehyde (MDA) concentration at 4 mM (0.9 μM) treatment dose of TiO_2 nanoparticles in *Allium cepa*. This indicated that lipid peroxidation could be involved as one of the mechanism leading to DNA damage. A comparative study of the cytotoxic and genotoxic potential of TiO_2 nanoparticles and bulk TiO_2 particles in human lymphocytes also reveal interesting results. While TiO_2 nanoparticles were found to be genotoxic at a low dose of 0.25 mM followed by a decrease in extent of DNA damage at higher concentrations; bulk TiO_2 particles reveal a more or less dose dependent effect, genotoxic only at dose 1.25 mM and above. The study thus confirms the genotoxic potential of TiO_2 nanoparticles in both plant and human lymphocytes.

1. Introduction

With the advent of newer nanomaterials and their use in the industry ranging from health care products, to cosmetics to dietary supplements human exposure to nanomaterials is a driving concern. The increasing concentrations of nanomaterials in groundwater and soil may present the most significant exposure avenues for assessing environmental risk (Colvin, 2003). The novel properties such as smaller size, large surface area, higher reactivity that imparts nanomaterials such interesting property are a major point of concern. Nanomaterials when released into the environment through industrial or domestic waste might tend to disrupt the microflora of soil and water. This in turn might lead to alteration of the food chain and disrupt plant productivity by disrupting nitrogen assimilation and metabolism and can damage aquatic organisms too (Oberdorster et al., 2007).

Amongst the manufactured nanoparticles, TiO_2 nanoparticles are used in a broad range of products as food colorant, in sunscreen and in cosmetics. In the recent past, it has also been used in certain sectors of agriculture and is one of the most widely used nanomaterials. Recent evidences have shown TiO_2 nanoparticles to induce inflammatory and genotoxic response in different animal and human cell lines (Wang et al., 2006; Reeves et al., 2007; Xu et al., 2009). In the present study, the DNA damaging potential of TiO_2 nanoparticles in two different trophic levels, in plants (*Allium cepa* and *Nicotiana tabacum*) and in human lymphocytes was evaluated. The genotoxicity of TiO_2 nanoparticles was evaluated using classical genotoxic endpoints like *Allium* test, comet assay and DNA laddering technique in *A. cepa* roots; and using comet assay and DNA laddering technique in *N. tabacum*. Amongst different test systems used for genotoxicity testing, *A. cepa* and *N. tabacum* were selected, as they are known to be efficient assays for chemical screening and *in situ* monitoring for genotoxicity of environmental contaminants. Apart from *Allium* test, *A. cepa* has been used successfully in our laboratory as a biomarker of genotoxicity using comet assay (Chakraborty et al., 2009). The DNA damaging potential of TiO_2 nanoparticles was evaluated and compared to that of bulk TiO_2 particles in human lymphocytes as representative of human test system. Comet assay is a simple, sensitive and effective technique used to quantitatively express the extent of genotoxicity of many

Abbreviations: TiO_2, titanium dioxide; MDA, malondialdehyde; TCA, trichloroacetic acid; TBAR, thiobarbituric acid reaction; CA, chromosomal aberration; MI, mitotic index; MN, micronuclei; RPMI-1640, Roswell Park Memorial Institute.

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hazardous agents. It has been used widely in genotoxicity monitoring of the environment and has been found to be as sensitive as sister chromatid exchange (SCE) or micronucleus (MN) (Yuquan et al., 1997; Gichner et al., 2009). To further ascertain the genotoxic potential of TiO_2 nanoparticles DNA laddering was used. Report from a previous investigation had shown that TiO_2 nanoparticles induce genotoxicity in animal cells mediated by the production of hydroxyl radicals (Reeves et al., 2007). In this study, the possible role of lipid peroxidation in inducing DNA damage in *A. cepa* was also evaluated.

2. Materials and methods

2.1. Chemicals

Titanium dioxide nanoparticles (CAS No. 13463-67-7), ethyl methanesulphonate (EMS, CAS No. 62-50-0), normal melting point agarose (NMPA), low melting point agarose (LMPA), di-sodium salt of ethylene diamine tetra acetic acid (EDTA), Tris buffer, ethidium bromide (EtBr), histopaque, thiobarbituric acid (TBAR), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), Trizma base, Triton X-100, DMSO were purchased from Sigma–Aldrich Co. (USA). Phosphate buffered saline (Ca^{2+}, Mg^{2+} free; PBS), 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. Cell proliferation reagent WST-1 was purchased from Roche, Lewes, UK. All other chemicals like sodium hydroxide, sodium chloride, acetic acid, orcein, chloroform, iso amyl alcohol were obtained locally and were of analytical grade.

2.2. Preparation and size characterization of TiO_2 nanoparticle solution

2.2.1. Preparation of solution

TiO_2 nanoparticles were obtained from Sigma–Aldrich Co. (USA). The physical characteristics as obtained from the supplier were: particle size (100 nm), surface area ($14.0\ m^2\ g^{-1}$), mp (350 °C), and density ($4.26\ g\ mL^{-1}$ at 25 °C). Appropriate amount of TiO_2 nanoparticles were suspended in filter sterilized double distilled water and were sonicated to prepare stock solutions. Working solutions were made by serial dilution, followed by sonication and vigorous vortexing as and when required.

The criterion for dose selection for each assay was based on earlier study reports, EC_{50} value and cytotoxicity test performed for plant system (Kumari et al., 2009) and human lymphocytes (Wang et al., 2006; Reeves et al., 2007).

2.2.2. Size and characterization of TiO_2 nanoparticles using atomic force microscopy (AFM)

Size and surface topography of the drop coated film of TiO_2 nanoparticles were investigated using AFM (NANOSCOPE (R) 111a Veeco multimode, USA) and high resolution surface images were produced. In AFM characterization, the contact mode (NP10) with a silicon probe over scan sizes of 10 μm was used.

2.3. Treatment of plant test systems (A. cepa and N. tabacum)

Equal-sized bulbs, from a population of a local market variety of *A. cepa* L. were used. The onions were allowed to germinate on sand taken in earthen pots (Fiskesjö, 1985; Panda et al., 1990) at room temperature (28 ± 1 °C) under a 12 h light/dark cycle. The *A. cepa* bulbs were then exposed to different concentrations (0, 2, 4, 6, 8 and 10 mM) of TiO_2 nanoparticles diluted in filter sterilized double distilled water, with five bulbs for each concentration. The roots after specific treatment periods (3, 6 and 24 h) were used for comet assay and other tests as applicable. *N. tabacum* seeds were allowed to germinate in well watered earthen pots (30 cm diameter) containing garden soil. The plantlets were used when they reached the 4th leaf stage. They were then exposed to different concentrations (0, 2, 4, 6, 8 and 10 mM) of TiO_2 nanoparticles diluted in filter sterilized double distilled water. The 4th leaf from the treated plant (24 h treatment) was used for comet assay and DNA isolation.

2.4. DNA damage analysis using comet assay in A. cepa and N. tabacum

The DNA damage studies were carried out using comet assay according to Singh et al. (1988) with slight modifications (Tice et al., 2000; Chakraborty et al., 2009). The roots or leaves of the plants were placed for 2 min on ice to keep them turgid. For isolation of nuclei, root and leaf tissues, 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 and leaves were finely and gently sliced allowing isolation of nuclei. The isolated nuclei were collected in the buffer. Taking the nuclear suspension, slides were prepared in triplicates per concentration. The slides were placed in alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA; pH > 13) for 15 min to allow unwinding of the DNA in a horizontal gel tank (Life Technologies, California, USA) followed by electrophoresis at 4 °C for 20 min at 26 V adjusted to 300 mA by adjusting the buffer level in the tank. Slides were neutralized in 0.4 M Tris pH 7.5 for 5 min and finally rinsed in water. Each experiment was repeated twice.

2.5. Isolation of mononuclear cells from 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 (1976), using Histopaque. The cells were washed with PBS and resuspended in RPMI-1640 media at a concentration of 10^6 cell mL^{-1} for further use. All experiments were conducted in accordance with the institutional guidelines.

2.6. Treatment of the lymphocytes

Freshly isolated human lymphocytes were incubated for 3 h at 37 °C in RPMI-1640 media with different concentrations of TiO_2 nanoparticles and bulk TiO_2 particles (0, 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2 mM). Following treatment, the lymphocytes were processed for cytotoxicity study and detection of DNA damage as assessed by the alkaline comet assay.

2.7. Viability of lymphocytes

2.7.1. Trypan blue dye exclusion method

The viability tests were performed using Trypan blue dye exclusion test to avoid artefacts due to toxicity (Tennant, 1964). The cut-off point suggested by Henderson et al. (1997) was 70%. Viability was measured both before and after treatment and range of viable cells was within 75–90% in all experiments.

2.7.2. MTT assay

Cells with treatment concentrations of TiO_2 nanoparticles (0, 0.25, 0.50, 0.75, 1, 1.25 mM) were seeded onto 96-well culture plates at 1×10^5 cells per well, incubated at 37 °C for 3 h. After incubation, cells were treated with $0.5\ mg\ mL^{-1}$ solution of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 100 μL/well) at 37 °C for 3 h. The number of viable cells was determined by uptake of MTT. Optical density (OD) was read on iMark™ Microplate Absorbance Reader (BIO-RAD, USA) at 570 nm, with 630 nm as a reference wavelength. All experiments were performed at least in triplicate on three separate occasions. Data are presented as mean ± SD.

2.7.3. WST-1 assay

For cell viability assays using WST-1, cells with treatment concentrations of TiO_2 nanoparticles (0, 0.25, 0.50, 0.75, 1, 1.25 mM) were seeded onto 96-well culture plates at 1×10^5 cells per well and incubated at 37 °C for 3 h. Cells were then washed twice in PBS and incubated in normal growth medium for 6 h before cell viability assays were carried out using Cell Proliferation Reagent WST-1 according to the manufacturer's instructions. OD was read on iMark™ Microplate Absorbance Reader (BIO-RAD, USA).

2.8. DNA damage analysis using comet assay in lymphocytes

The DNA damage studies were carried out following the comet assay according to the method of Singh et al. (1988) with modifications (Tice et al., 2000; Bandyopadhyay et al., 2008). Slides were prepared in triplicates per concentration. Slides were immersed in cold lysis solution at pH 10. The lysis solution consisted of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X-100, 10% DMSO and kept at 4 °C for 60 min. After lysis the DNA was allowed to unwind in the electrophoresis buffer (300 mM NaOH:1 mM Na_2EDTA at pH 13.5) for 20 min. This was followed by electrophoresis conducted at a constant voltage of 25 V and 300 mA at 4 °C. Slides were neutralized in 0.4 M Tris (pH 7.5) for 5 min and finally rinsed in water. Each experiment was repeated twice.

2.9. Scoring of slides of comet assay

The slides were stained with EtBr (20 $\mu g\ mL^{-1}$) and rinsed in water to wash off excess stain. Slides 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 percent of DNA in the tail [tail DNA (%)]. This will give us a clear indication of the extent of DNA damage induced by the test chemical. Images of 75 (25 × 3) cells per concentration were analysed for plant system and 150 (50 × 3) cells per concentration were analysed for human lymphocytes. The median values of each concentration with respect to the comet parameter were calculated.

2.10. Root growth inhibition test and determination of EC_{50} using *A. cepa*

The root growth inhibition test was performed according to Fiskesjö (1985) and Rank (2003) with slight modifications. The *A. cepa* bulbs were allowed to germinate on sand for 48 h. The root lengths (longest and smallest five roots per bulb) were recorded prior to treatment with the test reagents. The bulbs were then exposed to different concentrations (0, 2, 4, 6, 8 and 10 mM) of test chemicals (pH 7.4) diluted in sterile double distilled water. The test solutions were replaced by fresh solutions every 24 h with final root measurement taken at the end of 48 h of exposure. The EC_{50} (50% inhibition of root length) was obtained by interpolation from the graph with concentration against root length expressed as percent of control.

2.11. Allium test

The germinated bulbs were exposed to different concentrations (0, 2, 4, 6, 8 and 10 mM) of TiO_2 nanoparticles (pH 7.4) diluted in sterile double distilled water. After 48 h of exposure, root meristems (10–15 in number), chosen at random from five bulbs per exposure, were excised and fixed immediately for 3 h in acetic acid/ethanol (1:3). The remaining roots were used for the comet assay. The excised root tips were hydrolyzed and stained in 9:1, 2% aceto-orcein – (N) HCl mixture. Slides were prepared from each of the root meristem following the squash technique of Sharma and Sharma (1980), and coded to prevent observer bias. At least 4000 cells from root meristems per exposure were scored. The mean values for mitotic index (MI, percent of mitosis), micronucleus and chromosomal aberrations (breaks and fragments, early separation, laggard and anaphase – telophase bridges) at each point were scored and the standard deviation was calculated accordingly (Mukherjee and Sharma, 1988). Mean values for each criterion were calculated per concentration of exposure and were statistically correlated. The experiments were repeated at least once in order to establish the reproducibility of the results.

2.12. DNA extraction and laddering

DNA was isolated from roots of *A. cepa* bulb and *N. tabacum* leaves post treatment schedule using a modified CTAB method (Khan et al., 2007; Al-Qurainy, 2009). The roots/leaves were weighed and frozen at −20 °C till further use. The roots/leaves were ground in extraction buffer (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 with occasional mixing. The suspension was then cooled to room temperature and 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 under a sterile laminar hood 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 used. RNA was eliminated by treating the sample with RNase A (10 mg mL^{-1}) for 30 min at 37 °C. DNA purity was determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm.

The isolated DNA from all the treated samples were resolved on 2.5% agarose gel in 1 × TAE (Tris–acetate–EDTA) buffer at 100 V, 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.

2.13. AFM image of DNA extracted from *N. tabacum* leaf

Size and surface topography of the drop coated film of isolated DNA solution from *N. tabacum* leaf treated with TiO_2 nanoparticles was investigated using AFM (NANOSCOPE (R) 111a Veeco multimode, USA) and high resolution surface images were produced. In AFM characterization, the contact mode (NP10) with a silicon probe over scan sizes of 10 μm was used.

2.14. Lipid peroxidation (LPO) assay in *A. cepa*

LPO was determined by measuring the amount of malondialdehyde (MDA) according to Ohkawa et al. (1979) with modifications (Çelik et al., 2008). Each experiment was performed with three replicates. A total of 0.2 g of root tissues from control and treated plants were cut into small pieces and homogenized by the addition of 1 mL of 5% trichloro-acetic acid (TCA) solution. The homogenates were then transferred into fresh tubes and centrifuged at 12,000 rpm for 15 min at room temperature. Equal volumes of each supernatant and 0.5% TBAR in 20% TCA solution (freshly prepared) were added into a new tube and incubated at 96 °C for 25 min. The tubes were transferred into ice bath and then centrifuged at 10,000*g* for 5 min. The absorbance of the supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm; 0.5% TBAR in 20% TCA solution was used as the blank. Malondialdehyde (MDA) content was determined using the extinction coefficient of 155 mM^{-1} cm^{-1}.

2.15. Statistical analyses

For mitotic index, micronuclei, chromosomal aberrations, 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 $P \leqslant 0.05$.

3. Results

3.1. Size and characterization of TiO_2 nanoparticles using AFM

The AFM images (Fig. 1) obtained of TiO_2 nanoparticles revealed the particles to be symmetric, spherical in shape, well distributed without much aggregation and in the size range of 90–110 nm as specified by the supplier (TiO_2 nanoparticles, size ~100 nm; Sigma–Aldrich, USA).

3.2. DNA damage evaluation using comet assay in *A. cepa* and *N. tabacum*

The DNA damage induced in *A. cepa* root nuclei by TiO_2 nanoparticles showed a similar pattern in dose response over different treatment times (3, 6 and 24 h). Comet parameters for all the treatment sets (3, 6 and 24 h) showed an initial increase in DNA damage up to a treatment concentration of 4 mM followed by a gradual decrease up to the highest dose (Fig. 2a). Though a uniform pattern of dose response was observed in *A. cepa* at all treatment schedules, no particular time dependent effect was noticed. Similarly TiO_2 nanoparticles treated (24 h) *N. tabacum* leaf nuclei showed an initial increase in extent of DNA damage followed by a gradual decrease up to the highest dose (Fig. 2b). The value was statistically significant ($p \leqslant 0.05$) at 2 mM.

3.3. Viability of lymphocytes

3.3.1. Trypan blue dye exclusion method

Trypan blue dye exclusion method did not indicate of any significant amount of cytoxicity in human lymphocytes treated with different concentrations (0, 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2 mM) of TiO_2 nanoparticles and bulk TiO_2. The percent viability (Fig. 3a) varied between ~93% in control set to ~82–85% at the highest treatment doses for both the test compounds.

3.3.2. MTT and WST-1 assays

MTT and *WST-1* assay (Fig. 3b) revealed a significant increase in cytotoxicity with increase in treatment doses as compared to control. Both the assays revealed a very similar trend, with LC_{50} ranging between 1.0 and 1.25 mM.

3.4. DNA damage analysis using comet assay in lymphocytes

The percentage of tail DNA (% tail DNA) in lymphocytes treated with different concentrations of (0, 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2 mM) of TiO_2 nanoparticles and bulk TiO_2 revealed a distinct pattern of genotoxicity (Fig. 3c). While TiO_2 nanoparticles showed signs of significant DNA damage only at lower concentration (0.25 mM) followed by gradual decrease, bulk TiO_2 particles induced a dose dependent increase in extent of DNA damage with significant ($p \leqslant 0.05$) damage observed at concentrations above 1 mM.

3.5. Root growth inhibition test and determination of EC_{50} using *A. cepa*

The EC_{50} value of TiO_2 nanoparticles as obtained from root growth inhibition test was found to be ~4 mM. The test also re-

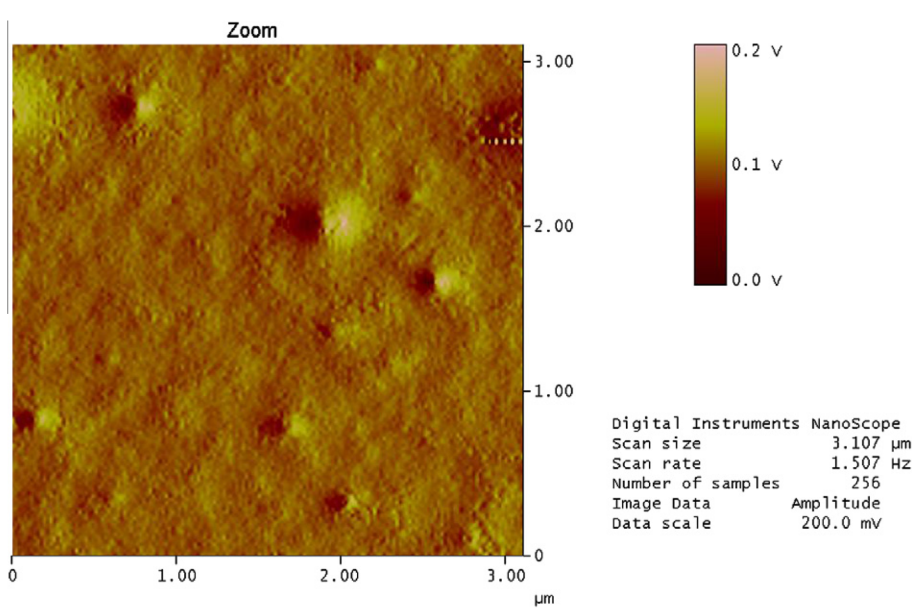

Fig. 1. Characterization of TiO_2 nanoparticles; AFM image showing TiO_2 nanoparticles in size range of ~90–110 nm.

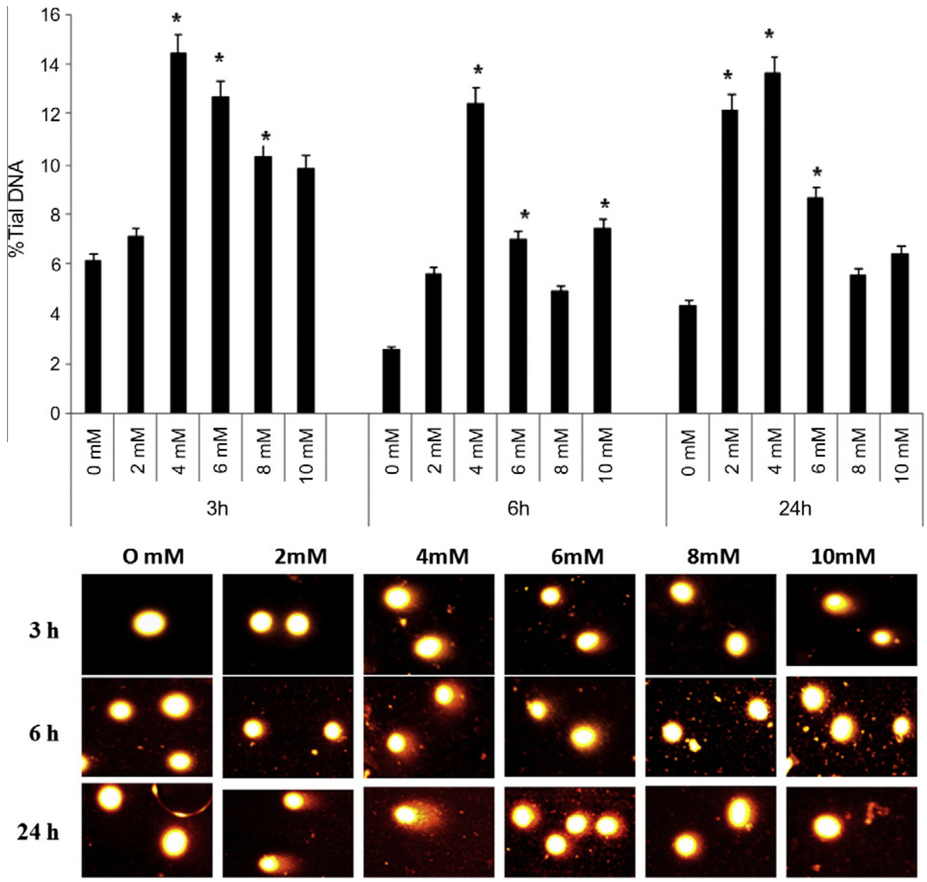

Fig. 2a. Comet data (% tail DNA) of TiO_2 nanoparticles treated (0, 2, 4, 6, 8 and 10 mM) *Allium cepa* root over different treatment times (3, 6 and 24 h); $^{*}p \leqslant 0.05$; in inset images of cells at different concentrations, showing varying extent of DNA damage as analyzed by comet assay.

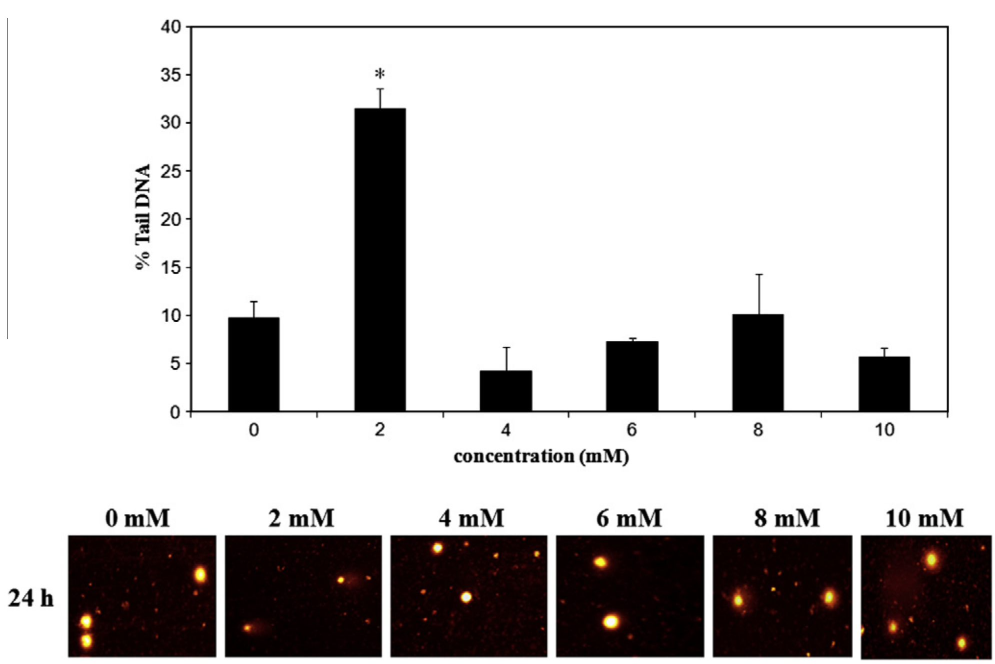

Fig. 2b. Comet data (% tail DNA) of TiO_2 nanoparticles treated (0, 2, 4, 6, 8 and 10 mM) *Nicotiana tabacum* leaf (24 h treatment); $^{*}p \leqslant 0.05$; in inset images of cells at different concentrations, showing varying extent of DNA damage as analyzed by comet assay.

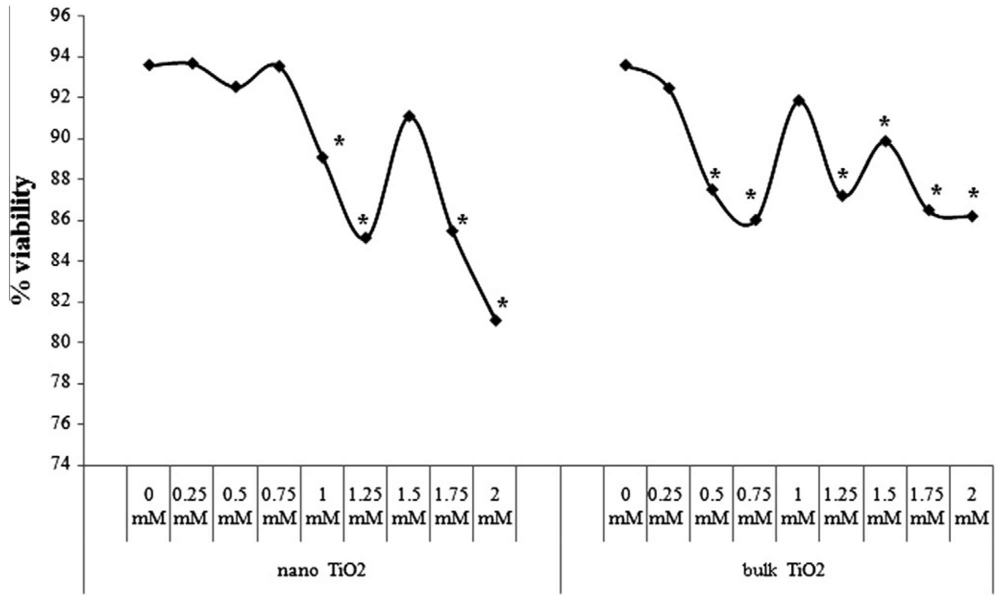

Fig. 3a. Comparative representation of cell viability of bulk TiO_2 and TiO_2 nanoparticles in human lymphocytes treated with TiO_2 nanoparticles using trypan blue dye exclusion method.

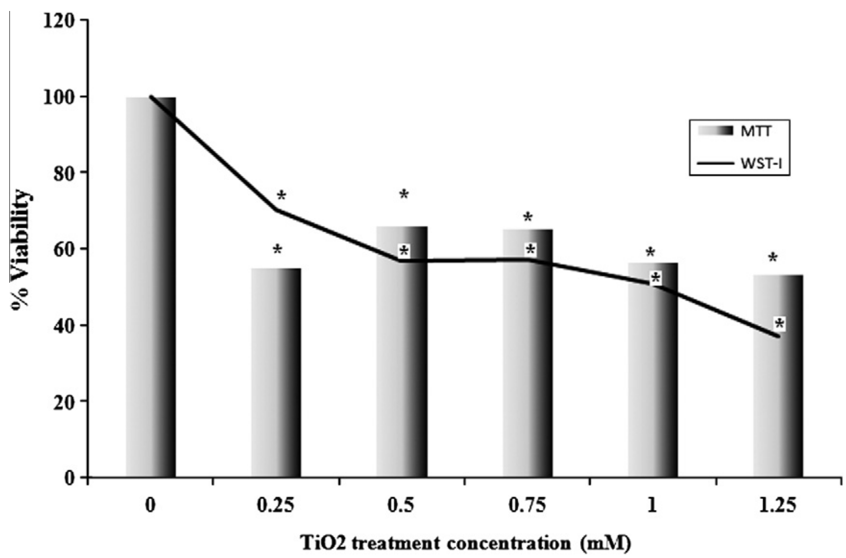

Fig. 3b. MTT and WST-I cell viability assay, cytotoxicity induced in human lymphocytes treated with TiO_2 nanoparticles.

vealed significant reduction in root elongation at treatment doses 6 mM (−4.81%) and 8 mM (0%) as compared to the control set.

3.6. Allium test

The clastogenic effect of TiO_2 nanoparticles was evaluated on the basis of *Allium* test results (mitotic index, micronucleus, chromosomal aberrations). MI that was determined in control and treatments (range: 5.72–9.16%) did not show statistical significance. This indicated normal mitotic progress. TiO_2 nanoparticles induced micronuclei at all doses compared to control, with the highest being observed at 8 mM treatment dose (Table 1). TiO_2 nanoparticles revealed a dose dependent increase in chromosomal aberrations, significant at treatment dose of 6 mM and above. Of the chromosomal aberrations anaphase–telophase bridges were predominant. Almost a 6-fold increase in.% of chromosomal aberrations was observed at 10 mM (0.29 ± 0.02%) treatment dose as compared to control set (0.05 ± 0.01%).

3.7. DNA laddering

The DNA damaging effect of TiO_2 nanoparticles in *A. cepa* was further evaluated qualitatively using DNA laddering. The result obtained can very well be correlated with that obtained from comet assay. While the negative control set showed presence of undamaged genomic DNA represented by a thick band on the agarose gel, the highest extent of DNA damage was observed at treatment concentration of 4 mM. The gel (Fig. 4a) also clearly indicated of an initial increase in DNA damage up to 4 mM followed by subsequent reduction in extent of DNA damage with increasing treatment concentrations. At 4 mM treatment dose, large number of fragments of sizes less than 1 kb was observed, unlike in the other concentrations which were characterized by the presence of larger DNA frag-

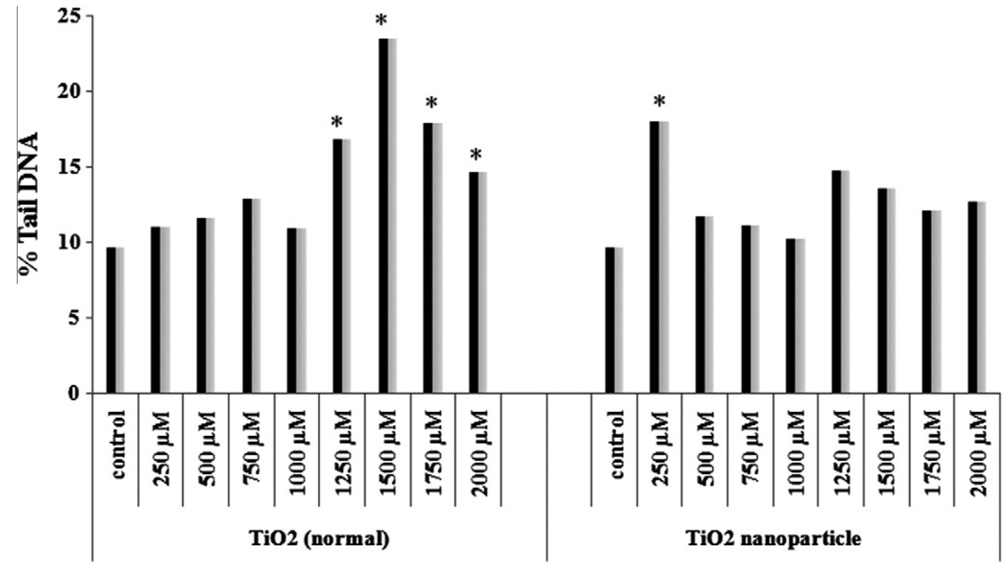

Fig. 3c. Comparative genotoxicity of bulk TiO_2 and TiO_2 nanoparticles; Comet data (% tail DNA) of human lymphocytes treated with different concentrations of bulk TiO_2 and TiO_2 nanoparticles; $^*p \leqslant 0.05$.

Table 1
Mitotic index (MI), number of micronuclei/1000 cells and % chromosomal aberrations revealing the genotoxic potential of TiO_2 nanoparticles in *Allium cepa* roots as analyzed by *Allium* test and *Allium* anaphase–telophase chromosome aberration assay.

TiO_2 nanoparticles (mM)	No. of cells examined	Cells in mitosis	MI (±SD)	Cells with micronuclei	Micronuclei/1000 cells	% Chromosomal aberrations
0	4191	263	6.28 ± 0.78	0	0	0.05 ± 0.01
2	4130	271	6.57 ± 1.23	1	0.24	0.05 ± 0.01
4	4010	270	6.73 ± 1.51	3	0.67	0.06 ± 0.02
6	4241	388	9.16 ± 0.73	2	0.45	0.14 ± 0.01*
8	4101	234	5.72 ± 0.68	13	3.15	0.20 ± 0.03*
10	4109	236	5.76 ± 1.15	4	0.99	0.29 ± 0.02*

* Significant at $p \leqslant 0.05$.

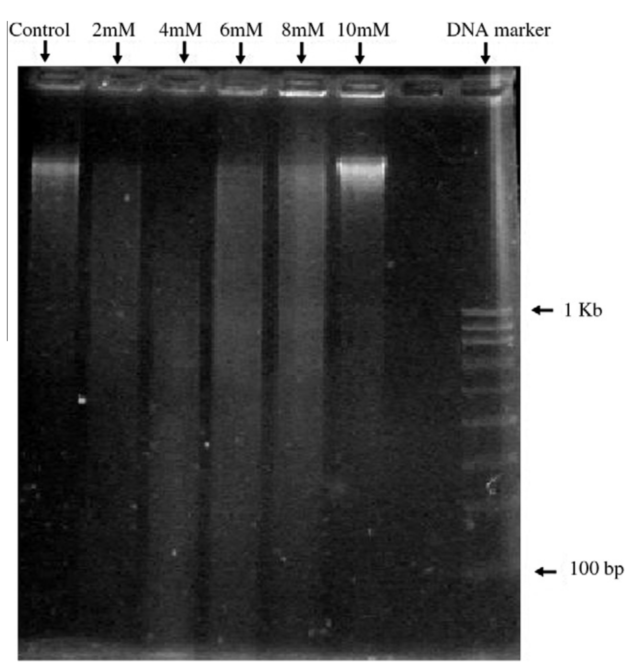

Fig. 4a. DNA laddering of *Allium cepa* root DNA treated with different concentrations of TiO_2 nanoparticles; 2.5% agarose gel showing different degrees of DNA shearing at different treatment concentrations.

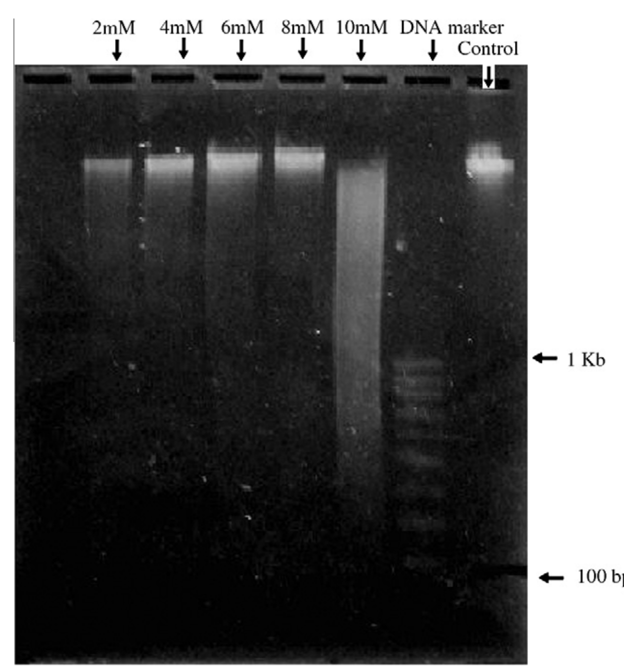

Fig. 4b. DNA laddering of *Nicotiana tabacum* leaf DNA treated with different concentrations of TiO_2 nanoparticles; 2.5% agarose gel showing different degrees of DNA shearing at different treatment concentrations.

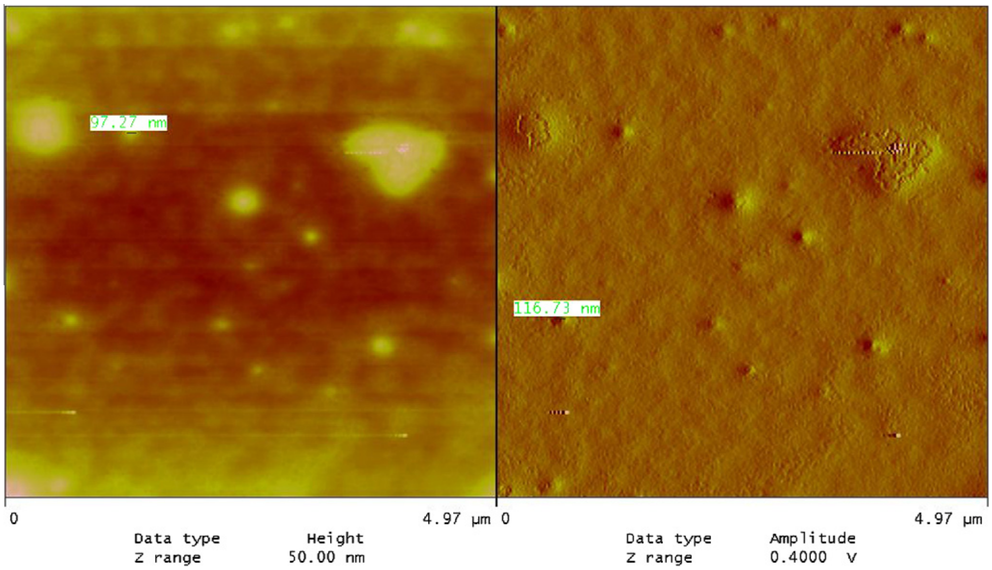

Fig. 5. AFM image of interaction of DNA with TiO_2 nanoparticles, in *Nicotiana tabacum* leaf.

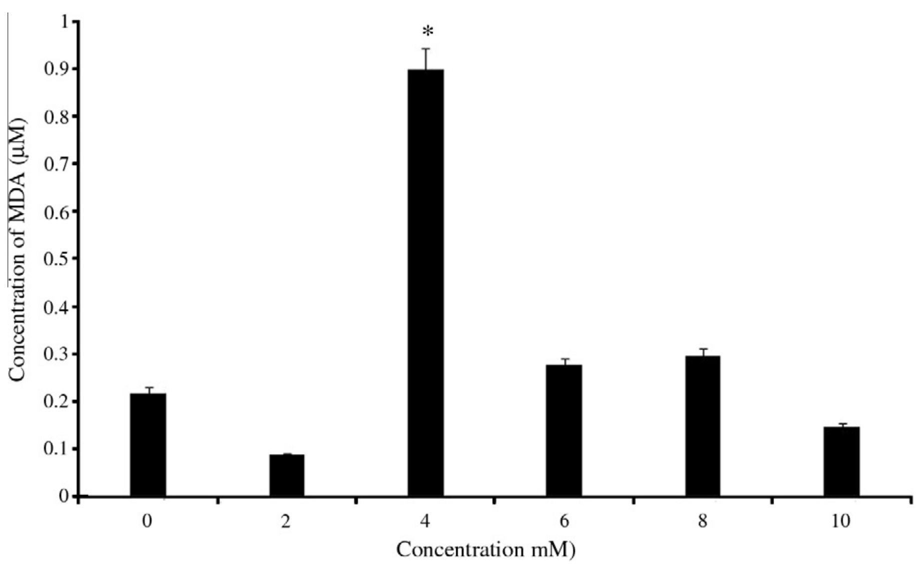

Fig. 6. Lipid peroxidation assay in *Allium cepa*; MDA concentration as plotted against TiO_2 nanoparticles (0, 2, 4, 6, 8 and 10 mM) post 24 h treatment time; $^{*}p \leqslant 0.05$.

ments comparable to negative control. Similarly agarose gel electrophoresis of *N. tabacum* leaf DNA (Fig. 4b) show varying extent of DNA fragmentation, the highest being observed at the highest concentration (10 mM).

3.8. AFM image of DNA extracted from TiO_2 nanoparticles treated N. tabacum leaf

The uptake of TiO_2 nanoparticles by *N. tabacum* leaf was visualized by AFM. The AFM image (Fig. 5) clearly indicates presence of nano-sized (90–110 nm), spherical particles similar to the ones observed from AFM image of TiO_2 nanoparticles. The image (Fig. 5) further shows the presence of a few larger aggregations appearing to be that of TiO_2 nanoparticles and *N. tabacum* leaf DNA.

3.9. Lipid peroxidation (LPO) assay in A. cepa

The roots were also analyzed for LPO post 24 h treatment to TiO_2 nanoparticles. The concentration of MDA thus obtained from the TiO_2 nanoparticles treated *A. cepa* roots showed an initial increase in concentration of MDA up to a treatment concentration of 4 mM (0.9 µM of MDA) followed by a decrease up to the highest treatment concentration of 10 mM (0.148 µM of MDA). The result thus obtained from LPO assay (Fig. 6) in *A. cepa* could be correlated with the results obtained from comet assay as well as that obtained from DNA laddering.

4. Discussion

The study reveals the genotoxic potential of TiO_2 nanoparticles in the plant systems, *A. cepa* and *N. tabacum*. The pattern of dose response shows an initial increase in DNA damaging effect followed by a decrease up to the highest treatment concentration.

In *A. cepa* comet parameter indicated DNA damage. The pattern of dose response showed a ~3.5-fold increase in extent of DNA damage at 4 mM concentration. This was followed by a gradual decrease in amount of DNA damage with respect to increasing treatment dose. The highest dose (10 mM) strikingly showed a similar result comparable to that obtained in control set. The percent tail DNA in *N. tabacum,* shows an initial increase in genotoxicity (2 mM), followed by a decrease up to the highest treatment dose (10 mM). This could be attributed to a property of nanomaterials to form agglomerates by virtue of which, with increase in treatment concentration the nanoparticles had a tendency to precipitate. The greater interaction of nanoparticles amongst themselves that could have increased owing to increase in treatment concentration might have limited the free TiO_2 nanoparticles from interacting with the plant system.

The genotoxicity of TiO_2 nanoparticles in *A. cepa* roots were further confirmed qualitatively using DNA laddering. A greater amount shearing of DNA was noted at 4 mM treatment concentration in *A. cepa* roots whereas in *N. tabacum* leaves the highest amount of DNA fragmentation was observed at the highest dose (10 mM). This discrepancy cannot be explained at the moment. A possible explanation could be in the difference of the tissues being processed.

AFM image of DNA isolated from *N. tabacum* leaf, confirms the uptake of TiO_2 nanoparticles. This indicates interaction of TiO_2 nanoparticles with DNA that might be responsible for the DNA damage.

Though some studies have claimed TiO_2 nanoparticles to be genotoxic and cytotoxic this to the best of our knowledge is the first work of the kind in plant system.

The cell cycle dependent classical *Allium* test affirms the genotoxicity of TiO_2 nanoparticles. The clastogenicity of TiO_2 nanoparticles is evident from chromosomal aberrations and interphase micronuclei. Presence of micronuclei (at interphase) is manifestation of chromosome fragmentation that occurred in the previous cell cycle. Earlier studies have established the fact that chromosome aberrations, such as fragments and chromosome losses, can result in micronucleated cells (Leme and Marin-Morales, 2009). According to Ma et al. (1995) micronuclei are the most effective parameter of studying cytological damages resulting from environmental contamination. Analysis of chromosomal aberrations is equally important revealing both the genotoxicity and mechanism of action of the genotoxicant (Rank and Nielsen, 1993). Results of the chromosomal aberrations and micronuclei do not show similar trend/or a good correlation with that of the comet assay, in *A. cepa*. The difference could be because induction of chromosomal aberrations and micronuclei are strictly cell cycle dependent whereas the comet assay is not (Gichner et al., 2009).

The study further reveals the *in vitro* cytotoxicity and genotoxicity of TiO_2 in human lymphocytes. The Trypan blue exclusion test showed cytotoxic response for both TiO_2 nanoparticles and bulk TiO_2 particles. This was further confirmed using MTT and WST-1 assays, indicating possible induction of mitochondrial damage by TiO_2 nanoparticles. Comet assay revealed two distinct patterns of DNA damage. While bulk TiO_2 particles revealed dose dependent genotoxicity, significant at 1.25 mM and above; TiO_2 nanoparticles revealed a dose response pattern similar to that observed in plant system. Lymphocytes demonstrated significant genotoxic response at 0.25 mM that was followed by a gradual decrease in extent of DNA damage. This could be explained for, owing to agglomeration property of TiO_2 nanoparticles. Recent studies in animal models both *in vitro* and *in vivo* have shown TiO_2 nanoparticles to induce DNA damage and cause genetic instability (Kang et al., 2008; Vevers and Jha 2008; Trouiller et al., 2009; Bernardeschi et al., 2010).

From previous study reports in animal cell lines it is evident that TiO_2 nanoparticles generate large amount of hydroxyl free radical thereby leading to DNA damage (Reeves et al., 2007; Zhu et al., 2008). It has been proposed that the genotoxicity of metals and metal oxides results from non-direct formation of reactive oxygen species (ROS). Abiotic stress (including heavy metals) may result in DNA damage to plant cells either directly or indirectly (Zhang et al., 2005; Kumari et al., 2009). Protonation of radical of O_2 can produce the hydroperoxyl radical ($^{\cdot}OH$, H_2O_2), which can convert fatty acids to toxic lipid peroxides, destroying biological membranes (Zhang et al., 2005). MDA formation is used as a general indicator of the extent of lipid peroxidation resulting from oxidative stress. In the present study, MDA contents in *A. cepa* roots increased $\sim$4.5 times after exposure to TiO_2 nanoparticles at 4 mM concentration. This suggests that TiO_2 nanoparticles indirectly lead to excessive generation of superoxide radicals resulting in increased lipid peroxidation and oxidative stress. Subsequent decrease of MDA concentration up to 10 mM was similar to that observed in the genotoxicity studies. Considering the positive findings of different tests in the two trophic levels, possible consequence of the release of nanoparticles like TiO_2 warrants further investigation.

5. Conclusion

Genotoxicity of TiO_2 nanoparticles was assessed *in vivo* in plants and *in vitro* in human lymphocytes. TiO_2 used in this study were mainly nano-sized but also showed a strong tendency to aggregate in spite of sonication of the suspension. DNA fragmentation as a marker for genotoxicity was determined by comet assay and DNA laddering. Trypan blue dye exclusion test, MTT and WST-1 assays were used as a measure of cytotoxicity. In addition MDA was measured for oxidative stress. Results from this study demonstrate that TiO_2 nanoparticles were genotoxic to both plant and human lymphocytes. Formation of MDA as a consequence of lipid peroxidation could be a possible reason for the genotoxic potential of TiO_2 nanoparticles.

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References

Al-Qurainy, F., 2009. Toxicity of heavy metals and their molecular detection on *Phaseolus vulgaris* (L.). Aust. J. Basic Appl. Sci. 3, 3025–3035.
Bandyopadhyay, A., Ghoshal, S., Mukherjee, A., 2008. Genotoxicity testing of low-calorie sweetners: aspartame, acesulfame-K, and saccharin. Drug. Chem. Toxicol. 31, 447–457.
Bernardeschi, M., Guidi, P., Scarcelli, V., Frenzilli, G., Nigro, M., 2010. Genotoxic potential of TiO_2 on bottlenose dolphin leukocytes. Anal. Bioanal. Chem. 396, 619–623.
Boyum, A., 1976. Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol. 5, 9–15.
Çelik, A., Ünyayar, S., Çekiç, F.O., Güzel, A., 2008. Micronucleus frequency and lipid peroxidation in *Allium sativum* root tip cells treated with gibberellic acid and cadmium. Cell. Biol. Toxicol. 24, 159–164.
Chakraborty, R., Mukherjee, A.K., Mukherjee, A., 2009. Evaluation of genotoxicity of coal fly ash in *Allium cepa* root cells by combining comet assay with the *Allium* test. Environ. Monit. Assess. 153, 351–357.
Colvin, V.L., 2003. The potential environmental impact of engineered nanomaterials. Nat. Biotechnol. 21, 1166–1170.
Fiskesjö, G., 1985. The *Allium* test as a standard in environmental monitoring. Hereditas 102, 99–112.
Gichner, T., Znidar, I., Wagner, E.D., Plewa, M.J., 2009. The use of higher plants in the Comet assay. In: Dhawan, A., Anderson, D. (Eds.), The Comet Assay in Toxicology. Royal Society of Chemistry, UK, pp. 98–119.
Henderson, L., Jones, E., Brooks, T., Chetelat, A., Ciliutti, P., Freemantle, M., Howard, C.A., Mackay, J., Phillips, B., Riley, S., Roberts, C., Wotton, A.K., van de Waart, E.J., 1997. Industrial genotoxicology group collaborative trial to investigate cell cycle parameters in human lymphocyte cytogenetic studies. Mutagenesis 12, 163–167.
Kang, S.J., Kim, B.M., Lee, Y.J., Chung, H.W., 2008. Titanium dioxide nanoparticle trigger p53-mediated damage response in peripheral blood lymphocytes. Environ. Mol. Mutagen. 49, 399–405.

Khan, S., Qureshi, M.I., Kamaluddin, Alam, T., Abdin, M.Z., 2007. Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. Afr. J. Biotechnol. 6, 175–178.
Kumari, M., Mukherjee, A., Chandrasekaran, N., 2009. Genotoxicity of silver nanoparticles in *Allium cepa*. Sci. Total Environ. 407, 5243–5246.
Leme, D.M., Marin-Morales, M.A., 2009. *Allium cepa* test in environmental monitoring: a review on its application. Mutat. Res. 682, 71–81.
Ma, T.H., Xu, Z., Xu, C., McConnell, H., Rabago, E.V., Arreola, G.A., Zhang, H., 1995. The improved *Allium*/*Vicia* root tip micronucleus assay for clastogenicity of environmental pollutants. Mutat. Res. 334, 185–195.
Mukherjee, A., Sharma, A., 1988. Effects of cadmium and selenium on cell division and chromosomal aberrations in *Allium sativum*. Water Air Soil Pollut. 37, 433–438.
Oberdorster, G., Stone, V., Donaldson, K., 2007. Toxicology of nanoparticles: a historical perspective. Nanotoxicology 1, 2–25.
Ohkawa, H., Ohishi, N., Yagi, Y., 1979. Assay of lipid peroxides in animal tissue by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Panda, K.K., Lenka, M., Panda, B.B., 1990. Monitoring and assessment of mercury pollution in the vicinity of a chloralkali plant. 1. Distribution, availability and genotoxicity of sediment mercury in the Rushikulya estuary, India. Sci. Total Environ. 96, 281–296.
Rank, J., 2003. The method of *Allium* anaphase–telophase chromosome aberration assay. Ekologija 38, 42.
Rank, J., Nielsen, M.H., 1993. A modified *Allium* test as a tool in the screening of the genotoxicity of complex mixtures. Hereditas 18, 49–53.
Reeves, J.F., Davies, S.J., Dodd, N.J.F., Jha, A.N., 2007. Hydroxyl radicals ($^{\cdot}OH$) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytoxicity and oxidative DNA damage in fish cells. Mutat. Res. 640, 113–122.
Sharma, A.K., Sharma, A., 1980. Chromosome Techniques – Theory and Practice, third ed. Butterworths, London. p. 711.
Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantification of low levels of DNA damage in individual cells. Exp. Cell. Res. 175, 184–191.
Tennant, J.R., 1964. Evaluation of the trypan blue technique for determination of cell viability. Transplantation 2, 685–694.
Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.
Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., Schiestl, R.H., 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability *in vivo* in mice. Cancer Res. 69, 8784–8789.
Vevers, W.F., Jha, A.N., 2008. Genotoxic and cytotoxic potential of titanium dioxide (TiO_2) nanoparticles on fish cells *in vitro*. Ecotoxicology 17, 410–420.
Wang, J.J., Sanderson, B.J.S., Wang, H., 2006. Cyto- and geno-toxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. Mutat. Res. 628, 99–106.
Xu, A., Chai, Y., Nohmi, T., Hei, T.K., 2009. Genotoxic response to titanium dioxide nanoparticles and fullerene in gpt transgenic MEF cells. Part. Fiber Toxicol. 6, 3.
Yuquan, L.U., Tatsuya, T., Kanehisa, M., 1997. Single-cell gel electrophoresis (SCG) – a review and discussion. Environ. Health Prev. Med. 2, 53–58.
Zhang, H., Jiang, Y., He, Z., Ma, M., 2005. Cadmium accumulation and oxidative burst in garlic (*Allium sativum*). J. Plant Physiol. 162, 977–984.
Zhu, R., Wang, S., Chao, J., Sun, X., Yao, S., 2008. Oxidative and binding effect of nano-TiO_2 on plasmid DNA and pepsin. In: The 2nd International Conference on Bioinformatics and Biomedical Engineering, pp. 1013–1016.