CHAPTER 5: RESULTS

Four nanoparticles were studied for their toxicity in plant and animal systems, \textit{in vitro} \& \textit{in vivo}.

Section I: Silver nanoparticles (Ag-np)

Section II: Multiwalled carbon nanotubes (MWCNT)

Section III: Titanium dioxide nanoparticles (TiO$_2$-np)

Section IV: Zinc oxide nanoparticles (ZnO-np)
Section I: Silver Nanoparticles (Ag-np)

Ag-np (≤ 100 nm) was purchased and characterized. For in vitro study, human lymphocytes were used for cytotoxicity (trypan blue dye exclusion, MTT and WST assays) and genotoxicity (comet assay) studies. In addition, flow cytometry was performed to measure the mode of cell death and uptake of Ag-np in human lymphocytes. The in vitro results were further substantiated by in vivo studies. Bone marrow cells of mice and root or leaf tissues of Allium and Nicotiana plants were used in the comet assay. TEM was performed to study structural modifications and uptake of Ag-np in cells.

A. Characterization of Ag-np

B. Toxicity in Plant System

C. In Vitro Toxicity of Ag-np in Human Lymphocyte Cells

D. In Vivo Toxicity of Ag-np in Mice

E. TEM Study: Uptake and Structural Alterations

F. Inference
Figure 11a: Transmission electron microscope image of Ag-np

Figure 11b: Scanning electron microscope image of Ag-np
Figure 11c: EDX analysis of Ag-np

Figure 11d: XRD image of Ag-np
Figure 11e: Zeta potential of Ag-np in suspension

Figure 11f: UV visible spectra of Ag-np suspensions
B. Toxicity in plant system

DNA damage in Allium cepa and Nicotiana tabacum: In nuclei isolated from A. cepa shoot, the DNA strand breaks increased and reached plateau at concentrations of 50µg/ml and above. Whereas in roots it increased steadily till 50µg/ml followed by a gradual decrease (Fig. 12). The effects of treatment in shoots were statistically significant (P<0.05) at concentrations 25 and 50 µg/ml. Genotoxicity study in N. tabacum plantlets indicated of a greater extent of DNA damage in roots as compared to that in leaves. Responses were statistically significant at most treatment concentrations (Fig. 12).

Effect on cell cycle in plant system: Effect of Ag-np on cell cycle progression was studied in A. cepa root cells. The flow cytometry histogram for control plants (Figure 14) displayed peaks, corresponding to nuclei at G0/G1 phase (58%), G2 phase (22%), S phase (5%) and sub G0 (4%). In roots exposed to Ag-np, a significant (P ≤ 0.05) decrease in G0/G1 peak values (Figure 14) was observed at 100 µg/ml treatment concentration (1.11-fold). A significant (P ≤ 0.05) accumulation of cells in the Sub G0 phase was observed for the treated samples.
Figure 12: Comet data (% Tail DNA) of nanosilver treated *Allium cepa* and *Nicotiana tabacum* root and shoot (24 h treatment); * P ≤ 0.05; in inset images of cells at different concentrations, showing varying extent of DNA damage as analyzed by comet assay.

Figure 13: Representative image of Ag-np treated *A. cepa* root nuclei
**Figure 14:** Effect of Ag-np treatment in *Allium cepa* root cells

<table>
<thead>
<tr>
<th>Ag-np (µg/ml)</th>
<th>Sub G0</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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C. In vitro toxicity of Ag-np in human lymphocyte cells

Cytotoxicity: The result of cytotoxicity tests revealed dose dependent cytotoxicity at all the concentrations tested. The Trypan blue dye exclusion method revealed a dose dependent decrease in viability of cells, significant at 150μg/ml (72.54 %) and above. The measurement of the activity of the mitochondrial dehydrogenases by the MTT and WST assays implicated concentration dependent decrease of cell viability, significant above 25 and 50 µg/ml respectively (Fig. 15).

DNA damage: Ag-np induces DNA breakage in human lymphocyte cells (Fig. 16). The tail intensity (% tail DNA) was higher than the control at all concentrations. There was a sharp increase at the lowest concentration (25µg/ml) with a gradual decrease with increase in concentration of Ag-np. Responses were statistically significant (P<0.05) at concentrations 25, 50 and 200 pg/ml. The results of MMS (100 µM) - induced DNA break was ~ 18 fold higher than the background value.

Effect on mitochondrial membrane potential: Decrease in mitochondrial membrane potential (Fig. 17) was observed at all the treatment concentrations. However the decrease was statistically significant (p < 0.05) at 25 and 100 µg/ml.

ROS generation in human lymphocytes: A significant increase in ROS generation was observed at all the concentrations tested. A ~ 3-5 fold increase (Fig. 18) in fluorescence intensity (DCFDA) was observed.
Figure 15: MTT and WST-1 cell viability assay, cytotoxicity induced in Ag-np treated human lymphocyte cells. * $P < 0.05$

Figure 16: Comet data (% Tail DNA) of human lymphocytes treated with different concentrations of nanosilver; * $P < 0.05$
Figure 17: Reduction of mitochondrial membrane potential in Ag-np treated human lymphocyte cells; * $P \leq 0.05$

Figure 18: Production of ROS in Ag-np treated human lymphocyte cells; * $P \leq 0.05$
**Mode of cell death:** To assess the extent and mode of cell death, annexin-V FITC – PI staining was used. Based on the percentages of unstained cells (live cells), and those with red fluorescence (necrotic cells), green fluorescence (early apoptotic cells), and dual stained cells (late apoptotic cells) were analyzed. Annexin-V staining experiment indicated that a small percentage of cells were undergoing apoptosis at the concentrations (~ 1.5 fold) as compared to control (Fig. 19). A significant increase in the number of necrotic cells was observed, at the concentration 25 µg/ml and above (Fig. 19).

**Figure 19:** Flow cytometric analysis of annexin V, FITC- PI stained lymphocyte cells showing induction of apoptosis and necrosis at different nanosilver treatment concentrations; representative scatter plots of annexin V, FITC- PI stained lymphocyte cells at (a) Control, (b) 25 µg/ml Ag-np, (c) 100 µg/ml Ag-np and (d) 200 µg/ml Ag-np treatment concentrations.
D. *In vivo* toxicity of Ag-np

**Chromosome aberration and DNA damage in mouse bone marrow cells:** The comparative data on the percentage of aberrant cells and number of aberration per cell are provided in table 6. The aberrations scored were mainly found to be of chromatid breaks, while in animals treated with the positive compound (mitomycin C) both chromatid and chromosome type of aberrations were recorded. ANOVA test revealed the frequency of aberrant cells and the number of breaks per cell to be significantly higher (*P* ≤ 0.05) than the control.

**Table 6:** Chromosomal aberrations of mice bone marrow cells following treatment with different doses of Ag-np.

<table>
<thead>
<tr>
<th>Test chemical</th>
<th>Dose (mg/kg)</th>
<th>Total chromosome aberrations*</th>
<th>% aberrant cells*</th>
<th>Number of aberration/ cell (mean ± SD)</th>
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</thead>
<tbody>
<tr>
<td>Negative control (distilled water)</td>
<td>-</td>
<td>6</td>
<td>5</td>
<td>2.00±0.81</td>
</tr>
<tr>
<td>Ag-np</td>
<td>1C</td>
<td>7</td>
<td>1</td>
<td>41</td>
</tr>
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<td>Mitomycin C</td>
<td>2.5</td>
<td>26</td>
<td>12</td>
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</table>

*G*, *G*': Chromatid and isochromatid gaps; *B*, *B*': chromatid and isochromosome breaks; RR: Chromatid rearrangements.

* 50 metaphase cells/animal (5 animals/dose)

* Percentage of cells with damaged metaphase (excluding gaps)

* Number of chromosome aberration/ cell (excluding gaps)

* *P* ≤ 0.05
The data of comet assay in mouse bone marrow cells are presented in Table 7. An increase in DNA damage in Ag-np treatment groups were observed. The comet parameter (% tail DNA) showed no further increase in DNA damage beyond the concentration of 20 mg/kg body weight. In the positive control the values of % tail DNA was ~ 16 fold higher than the background value.

Table 7: Comet parameter (% Tail DNA) of mice bone marrow cells (5 animals/dose) following treatment with different doses of Ag-np. * P ≤ 0.05

<table>
<thead>
<tr>
<th>Test chemical</th>
<th>Dose (mg/kg)</th>
<th>% Tail DNA (mean ± SD)</th>
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<tr>
<td>Control (water)</td>
<td>-</td>
<td>1.99±0.43</td>
</tr>
<tr>
<td>Ag-np</td>
<td>10</td>
<td>6.79±1.53*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10.17±1.06*</td>
</tr>
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<td></td>
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<td></td>
<td>80</td>
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<tr>
<td>Cyclophosphamide</td>
<td>20</td>
<td>32.53±8.11*</td>
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Effect on mitochondrial membrane potential: Ag-np did not induce significant alteration in mitochondrial membrane potential in bone marrow cells.

ROS generation in mouse bone marrow cells: ROS generation by Ag-np in bone marrow cells were quantified using flow cytometry. The results indicated of significant ROS generation at concentrations 10 and 20 mg/kg body weight. A ~1.5 and 1.4 fold increase in fluorescence intensity was observed at 10 and 20 mg/kg body weights respectively. ROS generation in the subsequent concentrations were negligible and comparable to that of control set.

E. TEM study: Uptake and structural alterations

Human blood cells (in vitro), incubated with Ag-np was compared to control for structural alterations and Ag-np accumulation. Ag-np treated cells (Fig. 21 B-C) show marked difference from control cells (Fig. 21 A). Cells incubated with Ag-np (Fig. 21 B-C) were characterized by different degrees of deformity (Fig. 21 B), damaged cell membrane (Fig. 21 B) and vacuolation (Fig. 21 C). In A. cepa root, cells from control set show normal cellular organization, with proper nuclear and organellar structures (Fig. 21 D). Features like extensive vacuolation, loss of nuclear organization, ruptured plasma membrane and shrinkage of the protoplast (Figure 21 E) were observed in sets that were exposed to Ag-np. Localization of nanoparticles was observed in the vacuoles (Figure 21 E) of the cells.
Figure 21: TEM images of ultrathin sections of blood cells (A-C) and plant cells (D-E) showing effect of Ag-np treatment; A- normal blood cells from control set, B- Ag-np treatment set showing deformed cells and cells with damaged membrane, C- Ag-np treated blood cells showing large vacuolation; D A. cepa root cells in absence of treatment with normal cellular organization, well defined nuclear and organellar structures, E- Ag-np treated A. cepa root cells with extensive vacuolation, loss of nuclear organization, ruptured plasma membrane and shrinkage of the protoplast.
Increasing use of multiwalled carbon nanotubes (MWCNT) necessitates an improved understanding of their potential impact on environmental 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 and mouse bone marrow cells.

A. CHARACTERIZATION OF MWCNT

B. TOXICITY IN PLANT SYSTEM

C. IN VITRO TOXICITY OF MWCNT IN HUMAN LYMPHOCYTE CELLS

D. IN VIVO TOXICITY OF MWCNT IN MICE

E. TEM STUDY: UPTAKE AND STRUCTURAL ALTERATIONS

F. INFECTION
A. Characterization of MWCNT

SEM (Fig. 22 a) and TEM (Fig. 22 b) photograph of MWCNT revealed an average diameter to be ~21.55 nm. 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. 22 c).

Figure 22: Characterization of MWCNT, (a) SEM image of MWCNT, (b) TEM image of MWCNT, (c) DLS of MWCNT in suspension

B. Toxicity in plant system

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 8). 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. 23a), micronuclei and binucleate cells. ANOVA test showed an absence of statistically significant
variation amongst the concentrations tested (Table 8). 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 50µg/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. 24). Moreover, treated cells revealed presence of large number of vacuoles (Fig. 25), retracted cytoplasm, smaller and more condensed nuclei (pyknotic cells) showing no nucleolus (Fig. 25).

Table 8: Mitotic index (MI), number of micronuclei / 1000 cells and % chromosomal aberrations revealing the genotoxic potential of MWCNT in Allium cepa roots as analyzed by Allium test and Allium anaphase - telophase chromosome aberration assay: * significant at \( P < 0.05 \).
Figure 23: Nuclear aberrations induced by MWCNT treatment in *Allium cepa* root cells; a-c: anaphase bridge and vagrants, d: telophasic fragments, e-f: nuclear buds, g-h: micronuclei, i: binucleate cells.
Figure 24: Cells of *Allium cepa* showing 1- “black dots” representing deposition of MWCNT inside the cell, 2- untreated cells

Figure 25: 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
DNA DAMAGE ANALYSIS USING COMET ASSAY IN A. CEPA: After treatment of A. cepa for 3h or 24h with MWCNT (0, 10, 20, 50 µg/ml) nuclei from roots were isolated either immediately after the treatment, or after a recovery period of 24h. Comet parameter (% Tail DNA) clearly indicated a significant ($P \leq 0.05$) dose dependent increase in DNA migration. 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. 26). The results of MWCNT-EMS treatment revealed an increase in DNA migration, significant ($P \leq 0.05$) at the highest dose (50 µg/ml) (Fig. 26).

![Figure 26: Comet data (% Tail DNA) of MWCNT treated (0, 10, 20 and 50 µg/ml) Allium cepa root over different periods of treatment time (3, 24 and 24 + 1 h 4 mM EMS); * $P \leq 0.05$]
DNA DIFFUSION ASSAY: DNA diffusion assay revealed a dose dependent decrease in average nuclear area (Fig. 27) in post 24 h MWCNT treatment. 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. 28).

![Figure 27: DNA diffusion assay in MWCNT treated *Allium cepa* root nuclei; * $P \leq 0.05$](image1)

**Figure 27:** DNA diffusion assay in MWCNT treated *Allium cepa* root nuclei; * $P \leq 0.05$

![Figure 28: DNA diffusion assay in MWCNT treated *Allium cepa* root nuclei; 1- control nuclei, 2- apoptotic nuclei, 3- necrotic nuclei](image2)

**Figure 28:** DNA diffusion assay in MWCNT treated *Allium cepa* root nuclei; 1- control nuclei, 2- apoptotic nuclei, 3- necrotic nuclei
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. 29).

*Figure 29:* Agarose gel electrophoresis of control and MWCNT treated *Allium cepa* DNA; bands corresponding to ladder from 100 – 400 bp indicate induction of apoptosis
Effect on cell cycle in plant system: Effect of MWCNT on cell cycle progression was studied in *A. cepa* root cells. The flow cytometry histogram for control plants (Fig. 30) displayed peaks, corresponding to nuclei at G0/G1 phase (58%), G2 phase (22%), S phase (5%) and sub G0 (4%). In roots exposed to MWCNT, a significant ($P \leq 0.05$) decrease in G0/G1 peak values (Fig. 30) was observed at 20 µg/ml treatment concentration (1.23-fold). A significant ($P \leq 0.05$) accumulation of cells in the Sub G0 phase was observed for the treated samples. A~2.4 fold increase in sub G0 population was observed at 20 µg/ml treatment set over control.

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<tr>
<th>MWCNT (µg/ml)</th>
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<td>0</td>
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<td>9.7</td>
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*Figure 30: Effect of MWCNT on cell cycle progression in *A. cepa* root cells*
C. Toxicity of MWCNT in Human Lymphocyte Cells In Vitro

Cytotoxicity: 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. 31).

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. 32). The values of % tail DNA were higher in 2, 5 and 10 µg/ml than the negative control (4.8±0.85 %). But was statistically significantly (P < 0.05) in 2 µg/ml (12.5±1.92 %) only.

DNA Diffusion Assay: DNA diffusion assay in human lymphocytes revealed a dose dependent decrease in nuclear area as well as in the percent of diffused nuclei (Fig. 33).
Figure 31: Cell viability study using trypan blue dye exclusion method in MWCNT treated human lymphocyte cells.

Figure 32: Comet parameter % Tail DNA indicating of extent of DNA fragmentation induced by MWCNT treatment in human lymphocyte cells; * $P < 0.05$
Figure 33: DNA diffusion assay indicating of a decrease in % diffused nuclei with increase in treatment concentration of MWCNT in human lymphocyte cells: * $P \leq 0.05$

**Effect on Mitochondrial Membrane Potential:** MWCNT treatment in lymphocyte cells induced reduction of mitochondrial membrane potential, significant ($P < 0.05$) at all treatment concentrations (Fig. 34).

**ROS Generation in Human Lymphocytes:** MWCNT treatment in human lymphocyte induced ROS production, ~1.2-1.6 fold higher compared to control (Fig. 35). The increase was significant on at 50 μg/ml.
Figure 34: Reduction of mitochondrial membrane potential in MWCNT treated human lymphocyte cells; * $P \leq 0.05$

Figure 35: Production of ROS in MWCNT treated human lymphocyte cells; * $P \leq 0.05$
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 analyzed. 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. 36). There was an increase (~ 22 fold with respect to control) in the number of necrotic cells up to the highest treatment concentration (Fig. 36).

**Figure 36:** Flow cytometric analysis of annexin V, FITC- PI stained lymphocyte cells showing induction of apoptosis and necrosis at different MWCNT treatment concentrations.
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 9). Increase in % tail DNA was significant (*P* ≤ 0.05) at doses 2 mg/kg body weight (32.46±5.16 %) and 5 mg/kg body weight (10.49±0.48 %).

EFFECT ON MITOCHONDRIAL MEMBRANE POTENTIAL: Rhodamine 123 uptake in the mitochondria was not affected in the bone marrow cells under the effect of MWCNT (Fig. 38).

![Figure 38](image)

**Figure 38:** Reduction of mitochondrial membrane potential in bone marrow cells of MWCNT treated Swiss albino male mice *in vivo*

ROS GENERATION IN BONE MARROW CELLS: MWCNT induced a dose dependent increase in ROS generation in bone marrow cells (Fig. 39). A statistically significant (*P* < 0.05) increase in ROS production was observed at the highest concentration (10 mg/kg).
Figure 39: ROS generation in bone marrow cells of MWCNT treated Swiss albino male mice *in vivo*, \( ^* P < 0.05 \)

E. TEM STUDY: UPTAKE AND STRUCTURAL ALTERATIONS

Ultrathin sections of root tissue of *Allium cepa* root cells provided evidences of MWCNT uptake. Extensive vacuolation, cytoplasmic shrinkage and absence of well organized nuclei were observed in root cells treated with MWCNT (Figure 40).

Figure 40: TEM images of ultra thin sections of *Allium cepa* root cells, showing (A) Control cells (B-C) effect of MWCNT treatment.
F. INFECTION

- SEM and TEM images of MWCNT revealed an average diameter of 21.55 nm. DLS revealed an average hydrodynamic diameter of 1895 nm.

- MWCNT could cause chromosomal aberrations, DNA fragmentation and apoptosis in *A. cepa* root cells. Localization of MWCNT in root cells was characterized by the presence of “Black dots”. Cell cycle analysis revealed a significant decrease in G2/M cells. Significant increase in sub G0 cells suggested apoptotic cell death.

- In human lymphocytes cytotoxic response was not significant. Genotoxic response was observed at the concentration 2 μg/ml. ROS generation was significantly increased and mitochondrial membrane potential was compromised by MWCNT treatment. Annexin V-FITC-PI staining indicated only a small percentage of cells were undergoing apoptosis.

- In mouse bone marrow cells genotoxic effects were revealed by micronuclei (MN) frequencies, and comet assay. ROS generation was observed only at the highest treatment dose. Mitochondrial membrane potential was not affected significantly.
SECTION III: TITANIUM DIOXIDE NANOPARTICLES (TiO$_2$-np)

Amongst the manufactured nanoparticles, TiO$_2$-np is used in a broad range of products as food colorant, in sunscreen and in cosmetics. In the present study the DNA damaging potential of TiO$_2$-np in two different trophic levels, in plants (*Allium cepa* and *Nicotiana tabacum*) and in human lymphocytes was evaluated. The genotoxicity of TiO$_2$-np was evaluated using classical genotoxic endpoints like *Allium* test, comet assay and DNA laddering technique in *Allium cepa* roots; and using comet assay and DNA laddering technique in *Nicotiana tabacum*.

A. CHARACTERIZATION OF TiO$_2$-np

B. TOXICITY IN PLANT SYSTEM

C. IN VITRO TOXICITY OF TiO$_2$-np IN HUMAN LYMPHOCYTE CELLS

D. IN VIVO TOXICITY OF TiO$_2$-np IN MICE

E. TEM STUDY: UPTAKE AND STRUCTURAL ALTERATIONS

F. INFERENCES
Figure 41: Typical TEM image of TiO₂ nanoparticle captured using Jeol JEM-2100 LaB6, 200 kV Transmission electron microscope; particles in the size range of 35 – 56 nm, with an average size of ~51 nm

Figure 42: SEM image of TiO₂ nanoparticle captured using Hitachi S-415A, 25 kV scanning electron microscope; the nanoparticles revealed an average size of ~ 50 nm
Figure 45: Size and surface topography of the drop coated film of TiO$_2$-np were investigated using AFM (NANOSCOPE (R) 111a Veeco multimode, USA). AFM images revealed the nanoparticle to be symmetric, spherical in shape, well distributed without much aggregation and in the size range of 90–110 nm.
B. Toxicity in plant system

Root growth inhibition test and determination of EC\textsubscript{50} using \textit{Allium cepa}: The EC\textsubscript{50} value of TiO\textsubscript{2}-np as obtained from root growth inhibition test was found to be \textasciitilde 4 mM. The test also revealed significant reduction in root elongation at treatment doses 6 mM (-4.81\%) and 8 mM (0\%) as compared to the control set.

Comet assay: The DNA damage induced in \textit{Allium cepa} root nuclei by TiO\textsubscript{2}-np 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 at a treatment concentration of 4 mM followed by a gradual decrease at 6 mM and above (Fig. 46). Though a uniform pattern of dose response was observed in \textit{Allium cepa} at all treatment schedules, no particular time dependent effect was noticed. Similarly \textit{Nicotiana tabacum} exposed to TiO\textsubscript{2}-np for 24 h showed an initial increase in extent of DNA damage followed by a gradual decrease up to the highest dose (Fig. 47). The value was statistically significant ($P < 0.05$) at 2 mM.
Figure 46: Comet data (% Tail DNA) of TiO$_2$-np treated (0, 2, 4, 6, 8 and 10 mM) *Allium cepa* root over different treatment times (3, 6 and 24 h); * $P \leq 0.05$; in inset images of cells at different concentrations, showing varying extent of DNA damage as analyzed by comet assay.
Figure 47: Comet data (% Tail DNA) of TiO$_2$-np treated (0, 2, 4, 6, 8 and 10 mM) *Nicotiana tabacum* leaf (24 h treatment); *P < 0.05; in inset images of cells at different concentrations, showing varying extent of DNA damage as analyzed by comet assay.

**ALLIUM TEST- CHROMOSOME ABERRATION AND MICRONUCLEUS ASSAY:** The clastogenic effect of TiO$_2$-np 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 to 9.16 %) did not show statistical significance. This indicated normal mitotic progress. TiO$_2$-np induced micronuclei at all doses compared to control, with the highest being observed at 8 mM treatment dose (Table 10). TiO$_2$-np 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 %).

Table 10: Mitotic index (MI), number of micronuclei / 1000 cells and % chromosomal aberrations revealing the genotoxic potential of TiO_2-np in *Allium cepa* roots as analyzed by *Allium* test and *Allium* anaphase - telophase chromosome aberration assay

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

* significant at \( P < 0.05 \)

**DNA LADDERING:** The DNA damaging effect of TiO_2-np in *Allium 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 single thick band on the agarose gel, the highest extent of DNA damage was observed at treatment concentration of 4 mM. The gel (Fig. 48) 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 fragments comparable to negative control. Similarly agarose gel electrophoresis of *Nicotiana tabacum* leaf DNA (Fig. 48) show varying extent of DNA fragmentation, the highest being observed at the highest concentration (10 mM).

Fig. 48. DNA laddering of *Allium cepa* root DNA and *Nicotiana tabacum* leaf DNA treated with different concentrations of TiO$_2$-np; 2.5% agarose gel showing different degrees of DNA shearing at different treatment concentrations.

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C. IN VITRO TOXICITY OF TiO$_2$-np IN HUMAN LYMPHOCYTE CELLS

Cytotoxicity: Trypan blue dye exclusion method did not indicate any significant amount of cytotoxicity 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$_2$-np. The cell viability (Fig. 49) varied between 93% in control to 82-85% at the highest treatment dose.

MTT and WST-1 assay (Fig. 50) 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 -1.25 mM.

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$_2$-np revealed genotoxicity (Fig. 51). TiO$_2$-np showed signs of significant DNA damage only at lower concentration (0.25 mM) followed by gradual decrease in extent of DNA damage.
**Figure 49:** Cell viability of TiO$_2$-np in human lymphocytes treated with TiO$_2$-np using trypan blue dye exclusion method. * significant at $P \leq 0.05$

**Figure 50:** MTT and WST-1 cell viability assay, cytotoxicity induced in human lymphocytes treated with TiO$_2$-np. * significant at $P \leq 0.05$
Figure 51: Genotoxicity of TiO₂-np: Comet data (% Tail DNA) of human lymphocytes treated with different concentrations of TiO₂-np; * significant at $P \leq 0.05$

**Effect on Mitochondrial Membrane Potential:** The effect of TiO₂ nanoparticle on MMP was evaluated in lymphocyte. Cells exposed to TiO₂ nanoparticle for 3 h were immediately assayed for Rhodamine 123 uptake by flow cytometry. The results indicated that there was a significant decrease of MMP at all the concentrations tested (Fig. 52).

**ROS Generation in Human Lymphocytes:** Experimental results indicated significant increase in ROS production at all treatment concentrations tested (Fig. 53). There was a $3-5$ fold increase in ROS generation.
Figure 52: Reduction of mitochondrial membrane potential in TiO₂-np treated human lymphocyte cells; * significant at $P \leq 0.05$

Figure 53: Production of ROS in TiO₂-np treated human lymphocyte cells; * significant at $P \leq 0.05$
MODE OF CELL DEATH: To assess the extent and mode of cell death, annexinV-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 analyzed. Experimental results indicate of induction of apoptosis at all treatment concentrations compared to control (Fig. 54). Significant increases in percentage of apoptotic cells were observed. Only a small percentage of cells were found to undergo necrosis at concentrations of 50 mM and above.

Figure 54: Flow cytometric analysis of annexin V, FITC- PI stained lymphocyte cells showing induction of apoptosis and necrosis at different TiO2-np treatment concentrations
Figure 56: Effect of TiO₂-np treatment on mitochondrial membrane potential in bone marrow cells, *P < 0.05

Figure 57: Effect of TiO₂-np treatment on ROS generation in bone marrow cells, *P < 0.05
E. TEM STUDY: UPTAKE AND STRUCTURAL ALTERATIONS

Gross ultrastructural changes were observed in the TiO$_2$-np treated *A. cepa* root cells (Fig. 58) compared to control cells (Fig. 58). Extensive vacuolation were observed in root cells treated with TiO$_2$-np (Fig. 58). Depositions of TiO$_2$-np were observed along the vacuolar membrane (Fig. 58).

![Figure 58](image)

**Figure 58:** TEM images of ultra thin sections of *Allium cepa* root cells showing (a) normal cells and (b-d) TiO$_2$-np treated cells showing extensive vacuolation and particle deposition.
Section IV: Zinc Oxide Nanoparticles (ZnO-np)

In spite of the wide spread use, relatively very few studies have reported the environmental impact of ZnO-np. We therefore focused our recent investigations on this aspect.

A. CHARACTERIZATION OF ZnO-np

B. TOXICITY IN PLANT SYSTEM

C. IN VITRO TOXICITY OF ZnO-np IN HUMAN LYMPHOCYTE CELLS

D. IN VIVO TOXICITY OF ZnO-np IN MICE

E. TEM STUDY: UPTAKE AND STRUCTURAL ALTERATIONS

F. INFERENCE
and above. Statistically significant ($P < 0.05$) increase in number of binucleate cells was observed at all the concentrations tested across all treatment schedules.

**Table 11:** Mitotic index (MI), % chromosomal aberrations, number of micronuclei/1000 cells (MN/1000 cells) and binucleate cells/1000 cells (BN/1000 cells) revealing the genotoxic potential of ZnO-np in *Allium cepa* and *Vicia faba* roots; * significant at $P \leq 0.05$

<table>
<thead>
<tr>
<th>Test system</th>
<th>Time schedule</th>
<th>Concentration (mM)</th>
<th>Total no of cells</th>
<th>Mitotic index (MI) (mean ± SD)</th>
<th>% Chromosomal aberration</th>
<th>MN/1000 cells</th>
<th>BN/1000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Allium cepa</em></td>
<td>24 h</td>
<td>0</td>
<td>3257</td>
<td>6.78 ± 1.67</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>3202</td>
<td>1.59 ± 0.78*</td>
<td>-</td>
<td>- 7.57*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>3164</td>
<td>4.62 ± 0.56</td>
<td>0.189*</td>
<td>3.16*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0</td>
<td>3005</td>
<td>6.45 ± 0.64</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>3077</td>
<td>0.42 ± 0.08*</td>
<td>0.032</td>
<td>- 5.19*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>3155</td>
<td>0.852 ± 0.03*</td>
<td>0.09</td>
<td>0.32*</td>
<td>1.58*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>3200</td>
<td>0.625 ± 0.043*</td>
<td>-</td>
<td>0.49*</td>
<td>7.93*</td>
</tr>
<tr>
<td><em>Vicia faba</em></td>
<td>24 h</td>
<td>0</td>
<td>3101</td>
<td>4.91 ± 1.30</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>3082</td>
<td>2.62 ± 0.86</td>
<td>0.19*</td>
<td>1.62*</td>
<td>2.89*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>3013</td>
<td>0.38 ± 0.05*</td>
<td>-</td>
<td>1.93*</td>
<td>0.96*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>3191</td>
<td>1.22 ± 0.25*</td>
<td>-</td>
<td>0.62*</td>
<td>11.28*</td>
</tr>
</tbody>
</table>

**Figure 60:** Figures showing (A) binucleate cell, (B) anaphase bridge, (C-D) micronucleus in *Allium cepa* root cells treated with ZnO-np

**COMET assay:** To study the induction of DNA strand breaks comet assay was performed in *A. cepa* and *N. tabacum*. The effect of ZnO-np exposure (3, 24 and 48 h) on DNA fragmentation was studied in *A. cepa* root cells. Exposure for 3 h and 48 h did not induce significant DNA
Effect on Cell Cycle in Plant System: Effect of ZnO-np on cell cycle progression was studied in *A. cepa* root cells. The flow cytometry histogram for control plants (Fig. 62) displayed peaks, corresponding to nuclei at G0/G1 phase (58.3%), G2 phase (22.2%), S phase (2.5%) and sub G0 (4%). In roots exposed to ZnO-np, a significant ($P < 0.05$) decrease in G0/G1 peak values (Fig. 62) was observed for 2.5 mM (1.98-fold), 5 mM (2.62-fold) and 10 mM (1.30-fold). A significant ($P < 0.05$) accumulation of cells in the Sub G0 and S phases were observed for the treated samples. An increased accumulation of cells in G2/M phase was observed in cells exposed to 10 mM ZnO-np only.

<table>
<thead>
<tr>
<th></th>
<th>Sub G0</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>4.0</td>
<td>58.3</td>
<td>2.5</td>
<td>22.2</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>18.7</td>
<td>29.4</td>
<td>6.2</td>
<td>14.5</td>
</tr>
<tr>
<td>5 mM</td>
<td>16.1</td>
<td>22.2</td>
<td>5.3</td>
<td>21.6</td>
</tr>
<tr>
<td>10 mM</td>
<td>16.4</td>
<td>44.6</td>
<td>2.8</td>
<td>28.3</td>
</tr>
</tbody>
</table>

Figure 62: Effect of ZnO-np on cell cycle progression in *Allium cepa* root cells
C. **IN VITRO TOXICITY OF ZNO-np IN HUMAN LYMPHOCYTE CELLS**

**Cytotoxicity**: Trypan blue dye exclusion method was used to study the effect of ZnO-np on membrane integrity of PBMCS. Compared to control no statistically significant change was observed in the treated sets (Fig. 63). The results of WST -1 (Fig.63) assay did not reveal any significant alteration in mitochondrial dehydrogenase activity. The results of neutral red uptake assay (Fig. 63) did not indicate of lysosomal fragility. ZnO-np did not induce significant alteration in metabolic activity as evident from Resazurin assay results (Fig. 63). ZnO-np was not cytotoxic at the concentrations tested.

**Comet assay**: Genotoxicity of ZnO-np to PBMC were not evident from the comet assay results (Fig. 64). Though an increase in comet parameter (% Tail DNA) were observed at certain concentrations, the results were not significant compared to control.

**Effect on mitochondrial membrane potential**: The effect of ZnO-np on mitochondrial membrane potential (MMP) was evaluated in PBMC. Cells exposed to 0, 0.25, 0.5 and 1 mM ZnO-np for 3 h were immediately assayed for Rhodamine 123 uptake by flow cytometry. The results indicated that there was a significant decrease of MMP at all the concentrations tested (Fig. 65).

**ROS generation**: Flow cytometric studies revealed significant increase in ROS production at all concentrations tested (Fig. 66). The treatment concentration revealed a 1.2-1.8 fold increase in ROS generation compared to control.
Figure 63: Cytotoxicity induced by ZnO-np in PBMCs studied using trypan blue dye exclusion, WST-1, neutral red uptake and Resazurin assays

Figure 64: Comparative representation of cytotoxicity [trypan blue dye exclusion assay] and genotoxicity [comet assay] induced by ZnO-np in PBMCs
Figure 65: ROS induction by ZnO-np measured by flow cytometry using DCFDA assay. Representative histograms obtained from flow cytometry, showing plots of changing fluorescence intensity across ZnO-np treatment concentrations, * $P < 0.05$

Figure 66: Effect of ZnO-np on mitochondrial membrane potential. Representative histograms obtained from flow cytometry, showing plots of changing fluorescence intensity across ZnO-np treatment concentrations, * $P \leq 0.05$
**MODE OF CELL DEATH:** 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 analyzed. Experimental results indicate of induction of apoptosis at all treatment concentrations compared to control (Fig. 67). Induction of apoptosis was evident at all concentrations tested, significant at the highest treatment concentration (2 mM). A small percentage of cells were also found to undergo necrosis at the highest treatment concentrations (2 mM).

**Figure 67:** Graphical representation of Annexin V-FITC/ PI stained PBMCs showing induction of apoptosis/necrosis, representative scatter plots of annexin V, FITC- PI stained PBMCs at different concentrations of ZnO-np.
CHROMOSOME ABERRATION IN BMC: The comparative data on the percentage of aberrant cells and number of aberration per cell have been provided in Table 12. The aberrations scored were mainly found to be of chromatid breaks, while in animals treated with the positive compound (mitomycin C) both chromatid and chromosome type of aberrations were recorded. ANOVA test revealed the frequency of aberrant cells and the number of breaks per cell to be significantly higher (P<0.05) than the control. A ~10 fold increase in aberrations was observed at the highest treatment concentration (25 mg/kg body weight) compared to control.

Table 12: Chromosomal aberrations in mice bone marrow cells following treatment with different doses of ZnO-np

<table>
<thead>
<tr>
<th>Test chemical</th>
<th>Dose (mg/kg body weight)</th>
<th>Total chromosome aberrations*</th>
<th>% aberrant cells*</th>
<th>Number of aberration/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G'</td>
<td>G''</td>
<td>B'</td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ZnO-np</td>
<td>25</td>
<td>6</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>26</td>
<td>12</td>
<td>130</td>
</tr>
</tbody>
</table>

G', G'': Chromatid and isochromatid gaps; B', B'': chromatid and isochromosome breaks; RR: Chromatid rearrangements.

* 50 metaphase cells/animal (5 animals/dose)

b Percentage of cells with damaged metaphase (excluding gaps)

c Number of chromosome aberration/ cell (excluding gaps)

* P< 0.05
Micronucleus formation in BMC: 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 (0, 25, 50 and 100 mg/kg body weight) tested (Table 13). Micronuclei formation was highest at 100 mg/kg body weight revealing a ~5 fold increase compared to control.

Table 13: DNA damage determined by the bone marrow micronucleus assay in Swiss albino male mice, exposed to different concentrations of ZnO-np

<table>
<thead>
<tr>
<th>Dose (mg/kg body weight)</th>
<th>% PCE</th>
<th>MN-PCE 1000 PCE</th>
<th>% MN-PCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnO-np</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>67.06</td>
<td>2.95</td>
<td>0.29</td>
</tr>
<tr>
<td>25</td>
<td>65.20</td>
<td>5.79*</td>
<td>0.58*</td>
</tr>
<tr>
<td>50</td>
<td>66.01</td>
<td>5.51*</td>
<td>0.75*</td>
</tr>
<tr>
<td>100</td>
<td>57.51</td>
<td>15.02*</td>
<td>1.50*</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>5</td>
<td>21.75*</td>
<td>4.19*</td>
</tr>
</tbody>
</table>

* Significant at $P \leq 0.05$

Reduction of mitochondrial membrane potential and ROS generation in BMC: The effect of ZnO-np on MMP was evaluated in PBMC. The results indicated that there was a significant decrease of MMP at all the concentrations tested (Fig. 69). The MMP at 100 mg/kg body weight treatment concentration was 45 % as compared to control.

Flow cytometric studies revealed significant dose dependent increase in ROS production at all treatment concentrations (Fig. 70). The highest treatment concentration (100 mg/kg body weight) revealed a 2.3 fold increase in ROS generation compared to control.
Figure 69: Flow cytometric estimation of alteration of mitochondrial membrane potential in bone marrow cells, at different concentrations of ZnO-np; * $P \leq 0.05$. Representative histograms obtained from flow cytometry, showing plots of changing fluorescence intensity across ZnO-np treatment concentrations.

Figure 70: Flow cytometric estimation of ROS generation in bone marrow cells, at different concentrations of ZnO-np; * $P \leq 0.05$. 
**CELL CYCLE PROGRESSION IN BMC:** The flow cytometry histogram for control cells (Fig. 71) displayed peaks, corresponding to nuclei at G0/G1 phase (50.45%), G2 phase (6.60%), S phase (12.5%) and sub G0 (13.45%). In BMC of mice exposed to ZnO-np, an increase in G0/G1 peak values (Fig. 71) was observed for 25 mg/kg (1.15-fold), 50 mg/kg (1.13-fold) and 100 mg/kg (1.10-fold) body weight. Accumulation of cells in the Sub G0 phases was observed for the treated samples. A statistically significant decrease in number of cells in G2/M and S phase was observed in cells exposed to ZnO-np (50 and 100 mg/kg body weight) as compared to control.

<table>
<thead>
<tr>
<th></th>
<th>Sub G0</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>13.45</td>
<td>50.45</td>
<td>12.55</td>
<td>6.00</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>11.40</td>
<td>58.30</td>
<td>11.55</td>
<td>5.15</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>14.85</td>
<td>57.20</td>
<td>8.90</td>
<td>3.95</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>14.85</td>
<td>55.85</td>
<td>9.30</td>
<td>4.30</td>
</tr>
</tbody>
</table>

Figure 71: Effect of *in vivo* ZnO-np treatment on cell cycle in mice bone marrow cells, * P ≤ 0.05
E. TEM Study: Uptake and Structural Alterations

Nanoparticle uptake in Allium root cells was studied using TEM. Untreated cells revealed normal cellular organization, with normal nuclear and organellar structures (Fig. 72). Features like extensive vacuolation, loss of nuclear organisation, ruptured plasma membrane and shrinkage of the protoplast (Fig. 72) were observed in cell exposed to ZnO-np. Localization of nanoparticle was observed all along the plasma membrane (Fig. 72).

Figure 72: TEM images of ultrathin sections of Allium cepa root cells (A-C) A. cepa root cells in absence of treatment with normal cellular organization, well defined nuclear and organellar structures, (D-F) ZnO-np treated A. cepa root cells with extensive vacuolation, loss of nuclear organization, ruptured plasma membrane and shrinkage of the protoplast, arrow showing nanoparticle deposition.
F. INFERENCES

- TEM image and SEM image revealed an average size of 80 nm. BET revealed a surface area of 15-25 m²/g. The purity of the substance was confirmed by EDX analysis. DLS revealed an average hydrodynamic diameter of 5 µm with a polydispersity index of 1.38.

- ZnO-np treatment led to a decrease in MI, increase in chromosome aberration and micronucleus formation in *Vicia faba* and *Allium cepa* root cells. DNA damage was induced in root cells of *A. cepa* and root and leaf cells of *N. tabacum*. An increased accumulation of cells in G2/M phase was observed in *A. cepa* root cells along with an increase in sub G0 population. TEM studies revealed significant alteration of cellular organization in *A. cepa* root cells.

- In human lymphocyte cells *in vitro*, ZnO-np was not cytotoxic. There was an increase in % Tail DNA. However, the increase was not significant. ZnO-np induced significant increase in ROS generation, caused decrease in mitochondrial membrane potential and manifested apoptotic cell death.

- In bone marrow cells and liver cells, *in vivo*, ZnO-np induced significant genotoxic response. Significant increase in ROS generation and decrease in mitochondrial membrane potential was observed. ZnO-np induced G0/G1 arrest in bone marrow cells and an accumulation of cells in subG0 fraction.