CHAPTER 6: DISCUSSION

The results of the toxicity of the following nanoparticles have been reported and discussed with reference to the available literature:

- **Characterization of the Nanomaterials**
  - Silver nanoparticles (Ag-np)
  - Multiwalled carbon nanotubes (MWCNT)
  - Titanium dioxide nanoparticles (TiO$_2$-np)
  - Zinc oxide nanoparticles (ZnO-np)

- **Toxicity Evaluation in Plant Systems**
  - Silver nanoparticles (Ag-np)
  - Multiwalled carbon nanotubes (MWCNT)
  - Titanium dioxide nanoparticles (TiO$_2$-np)
  - Zinc oxide nanoparticles (ZnO-np)

- **Toxicity (Cyto and Genotoxicity) of the Nanoparticles in Human Lymphocyte Cells in Vitro**
  - Silver nanoparticles (Ag-np)
  - Multiwalled carbon nanotubes (MWCNT)
  - Titanium dioxide nanoparticles (TiO$_2$-np)
  - Zinc oxide nanoparticles (ZnO-np)

- **Evaluation of In Vivo Cytotoxicity and Genotoxicity in Swiss Albino Male Mice**
  - Silver nanoparticles (Ag-np)
  - Multiwalled carbon nanotubes (MWCNT)
  - Titanium dioxide nanoparticles (TiO$_2$-np)
  - Zinc oxide nanoparticles (ZnO-np)

- **Nanoparticle Uptake**
  - Silver nanoparticles (Ag-np)
  - Multiwalled carbon nanotubes (MWCNT)
  - Titanium dioxide nanoparticles (TiO$_2$-np)
  - Zinc oxide nanoparticles (ZnO-np)
The primary focus of the present study was to evaluate the fate and behaviour of certain commonly used nanoparticles (Silver, Multiwalled Carbon nanotubes, TiO₂, and ZnO) at different trophic levels (plants and animals). To achieve that the following study approach was used:

- Characterization of the nanomaterials
- Toxicity evaluation in plant systems
- Toxicity (cyto and genotoxicity) of the nanoparticles in human lymphocyte cells in vitro
- Evaluation of in vivo cytotoxicity and genotoxicity in Swiss albino male mice
- Nanoparticle uptake.

CHARACTERIZATION OF NANOMATERIALS

An initial characterization of the engineered nanoparticle is important before screening for toxicology (Dhawan and Sharma, 2010). However most of the ecotoxicological studies have lacked adequate characterization. Hence the nanoparticles were characterized by TEM, SEM-EDX, XRD, DLS, in addition to the BET and Raman spectroscopy data provided by the manufacturer.

Silver nanoparticles: Ag-np (≤ 100 nm) was purchased and characterized using transmission electron microscopy (TEM), scanning electron microscope (SEM) and X-ray diffraction (XRD), diffraction light scattering (DLS) and UV visible spectrophotometry for size and dispersion. In TEM and SEM analysis of Ag-np powder, an average particle size of 120 nm was noted. EDX and XRD analysis revealed that the Ag-np sample was devoid of any impurity. DLS and UV visible spectrophotometric study revealed of an unstable suspension with a tendency to form aggregates at higher concentrations.
MWCNT: SEM and TEM photograph of MWCNT revealed an average diameter to be \( \sim 21.55 \) nm. Dynamic light scattering measurements 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 \( \sim 40 \% \) MWCNT in the size range of 246 nm.

**TiO\textsubscript{2}-np:** Characterization of TiO\textsubscript{2} nanoparticle powder sample was done by SEM and XRD and the average size was found to be \( \sim 50 \) nm. TEM of TiO\textsubscript{2} nanoparticles revealed an average size of \( \sim 51 \) nm. The size in suspension was analyzed using AFM and was found to be in the range of 90–110 nm. Nanoparticles have a tendency to aggregate in solvent and hence an increase in size was observed when the particles were characterized using AFM. DLS and UV visible spectrophotometric study further revealed of an unstable suspension with a tendency to form agglomerates at higher concentrations. Formation of larger aggregates in suspension could be studied using DLS. A PDI of 1.53 indicated of unstable suspension with a tendency to form aggregates.

**ZnO-np:** Characterization of the ZnO-np powder sample revealed a size range of 75-85 nm. The EDX profile of the nanoparticle characterized by absence of major peaks of any other elements confirmed the purity of the sample. DLS of the ZnO-np in suspension indicated of a tendency to agglomerate.

This difference in sizes could be attributed to the different principles involved in these measurement techniques. The nanoparticles have a tendency to agglomerate because of van der Waal’s forces which dominate at the nanoscale due to the increased surface area to volume ratio. During the DLS measurement, the agglomeration becomes more evident in the aqueous medium, thereby giving the size of clustered particles rather than the individual particles. It (DLS)
measures Brownian motion and subsequent size distribution of an ensemble collection of particles in solution and gives mean hydrodynamic diameter which is usually larger than TEM diameter as it includes a few solvent layers. It reports an intensity weighted average hydrodynamic diameter of a collection of particles so any poly-dispersity of the sample will skew the average diameter towards larger particle sizes.

TOXICITY EVALUATION IN PLANT SYSTEM

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). The effect of nanoparticle uptake on plant system is poorly understood. DNA damage induced by nanomaterials is not just relevant to human species but also to other components of the environment (Singh et al., 2009). Currently very little data are available regarding the toxicity of nanomaterials in systems other than human (Singh et al., 2009). In the present thesis we make an effort to study the genotoxicity of commonly used nanoparticles in plant system.

Silver nanoparticle: Previous studies in A. cepa have shown that Ag-np could enter plant system, affect cell division exhibiting cytotoxic response and cause chromosomal aberrations (Babu et al., 2008; Kumari et al., 2009; Panda et al., 2011). In the present study, in A. cepa and N. tabacum a higher extent of DNA damage was observed in roots than in the leaves. This could be due to the direct exposure of roots to the treatment chemical. Furthermore lesser amount of nanomaterials would have been translocated to leaf than in roots within the same period of time. An increase in DNA damaging effect of Ag-np was observed at certain concentrations in A. cepa.
(50 µg/ml) as well as in N. tabacum (75 µg/ml) roots. There was a gradual decrease in % Tail DNA with increase in Ag-np concentration. 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 Ag-np from interacting with the test systems. A number of studies have also shown that Ag-np treatment induced DNA damaging effects on aquatic organism (Wise et al., 2010) and plant cells (Kumari et al., 2009) with impairment of cell-division. In the present study a significant accumulation of cells in Sub G0 phase of cell cycle was observed in A. cepa root cells.

MWCNT: Genotoxicity studies of multiwalled carbon nanotubes (MWCNT) in plant are scarce and often contradictory (Canas et al., 2008; Tan et al., 2007; Serag et al., 2010; Lin and Xing, 2007; Khodakovskaya et al., 2009). A majority of these studies have reported of enhanced seed germination and growth in crop plants (Lin and Xing, 2007; Khodakovskaya et al., 2009). From the present study 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 that are manifestation of spindle aberration. Aberrations such as binucleate cells arise as a result of inhibition of cell plate formation or even mitotic irregularities (Grant, 1978). Presence of micronuclei (at interphase) is 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 (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 chromosome aberration is equally important revealing both the genotoxicity and mechanism of action of the genotoxicant (Rank and Nielsen, 1993). 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 MWCNT was investigated. *Allium 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 diffusion assay was performed to study the induction of apoptosis in *A. cepa*. DNA diffusion assay is a simple, sensitive and rapid method for estimating apoptosis in single cell (Singh, 2005). Apoptotic cells when tested using DNA diffusion 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. In the present study, results of DNA diffusion 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 (Oberhammer et al., 1993). The presence of internucleosomal
fragments in the size range of 100-400 bp clearly indicated the induction of apoptosis. Flow cytometric estimation of different cell cycle phases revealed an increase in cell population in sub G0 fraction confirming apoptotic cell death. A G0/G1 arrest was also evident from the result. These results could be correlated with the presence of a large number of “black dots” distributed throughout the cytoplasm A. cepa. The study in A. cepa root cells revealed a significant increase in percentage of sub G0 cell population, suggesting induction of cell death which was confirmed from DNA laddering assay.

TiO$_2$-np: The study reveals the genotoxic potential of TiO$_2$-np in the plant systems. Allium cepa and Nicotiana 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 Allium 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 % tail DNA in Nicotiana 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$_2$-np from interacting with the plant system. The genotoxicity of TiO$_2$-np in Allium cepa roots were further confirmed qualitatively using DNA laddering. A greater amount shearing of DNA was noted at 4 mM treatment concentration in Allium cepa roots whereas in Nicotiana 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. Though some studies have claimed TiO$_2$-np 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$_2$-np. The clastogenicity of TiO$_2$-np 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 *Allium 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). A limited number of studies in plant have highlighted similar toxic effect of TiO$_2$-np (Castiglione et al., 2011; Ghosh et al., 2010; Mandeh et al., 2012).

**ZnO-np:** The effect of nanoparticle uptake on plant system is poorly understood. Over the last decade, researches have reported ZnO-np to be toxic to microbes, algae and plants (Huang et al., 2008; Kasemets et al., 2009; Simon-Deckers et al., 2009; Manzo et al., 2011; Jiang et al., 2008; Kumar et al., 2011; Franklin et al., 2007; Navarro et al., 2008; Heinlaan et al., 2008; Miao et al., 2010; Brayner et al., 2010; Lin and Xing, 2008). A few more recent studies on plant system have addressed the
effects of ZnO-np with respect to phenotypic symptoms such as seed germination and root elongation (Ghodake et al., 2011; Ma et al., 2013), indicative of their toxicity. Our results demonstrate that exposure to ZnO-np cause cytotoxicity, genotoxicity and oxidative stress to *Allium cepa*, *Nicotiana tabacum* and *Vicia faba* plants. Decrease of mitotic frequency and increase of Evans blue uptake in *A. cepa* and *V. faba* root cells indicated cytotoxic effect of ZnO-np. The genotoxicity of ZnO-np in meristem root cells of *A. cepa* and *V. faba* was evaluated by the cell cycle dependent chromosomal aberrations (CA) and micronucleus (MN) assays. The parameters helped to understand the mode (clastogenic and/or aneugenic) of DNA damage induced by ZnO-np. Increase in percentage CA was observed in both *A. cepa* and *V. faba*. Similar genotoxic effects of ZnO-np was reported in *A. sativum* (Shaymurat et al., 2012) and *V. faba* (Manzo et al., 2011) by other workers. In our experiment, DNA fragmentation (Comet assay) as biomarker for genotoxicity has been detected in both *A. cepa* and *N. tabacum* plants. Since oxidative damage was confirmed by enhanced MDA level in *A. cepa* we assume that oxidative attack to DNA has generated structural alterations that were resolved as strand breaks due to alkali condition of the Comet assay. Effect of ZnO-np on cell cycle progression was studied in *A. cepa* root cells. The flow cytometry histogram revealed a decrease in G0/G1 peak values, significant accumulation of cells in the Sub G0, S, and G2/M phase. The results demonstrate a change in cell cycle dynamics of roots exposed to different ZnO-np concentrations. An arrest of cell cycle at the G2/M checkpoint is part of the cell strategy to cope with DNA damage, giving the cell extra time to either repair the damage or activate an apoptosis-like program.

**TOXICITY (CYTO- AND GENOTOXICITY) OF THE NANOPARTICLES IN HUMAN LYMPHOCYTE CELLS IN VITRO**
Silver nanoparticle: The effect of nanoparticle size and shape on cyto- and genotoxicity has been reported by several authors (Jiang et al., 2008; Bar-Ilan et al., 2009; Yen et al., 2009). There are a significant number of studies on the genotoxicity and cytotoxicity of silver nanoparticles on mammalian and human cell lines (Braydich-Stolle et al., 2005; Hussain et al., 2005; Park et al., 2007; Arora et al., 2008; Hsin et al., 2008; Asharani et al., 2008; Asharani et al., 2009; Kawata et al., 2009; Rahman et al., 2009; Farkas et al., 2010; Sharma et al., 2010; Scrown et al., 2010). In the present study cytotoxicity was evident in MTT, WST and trypan blue dye exclusion tests. Jiang et al., (2008) reported that gold and silver nanoparticles within the 2-100 nm size were found to alter signalling processes essential for basic cell functions including cell death, and 40 and 50-nm nanoparticles demonstrated the greatest effect. We conducted comet assay as conventional testing method in vitro on human lymphocytes. The tail intensity (% tail DNA) was higher at all concentrations. There was a 4-fold increase at the lowest concentration (25 µg/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 µg/ml. Flow cytometric evaluation revealed a significant decrease in mitochondrial membrane potential. Rhodamine 123 uptake by mitochondria was reduced by ~40% at all the concentrations tested. Fluorescence intensity also revealed a significant increase in ROS production at all concentrations tested. Cytotoxicity and genotoxicity of starch-coated silver nanoparticles was studied using normal human lung fibroblast (IMR-90) and human glioblastoma (U251) cells by Asharani et al, (2008; 2009). They observed an increase in DNA damage with increase in Ag-np concentration in cancer cells, whereas in the fibroblast cells they found increase in DNA damage only beyond a concentration of 100µg/ml. Their choice of capping agent was done based on the stability of Ag-np in cell culture medium. For risk assessment it should be ecologically relevant to use natural aggregated
nanoparticles (Handy et al., 2008). Most of the manufactured nanoparticles have not been
designed to disperse readily in water, further more they will aggregate in many types of natural
water. To assess the extent and mode of cell death, annexin-V FITC – PI staining was used.
Compared to control a small percentage of cells were undergoing apoptosis at the treatment
concentration (~1.5 fold). A significant increase in the number of necrotic cells was observed, at
the concentration 25 µg/ml and above. Thus from the study we could conclude the major reason
for Ag-np induced cell death is necrotic. Previously published data (Asharani et al., 2008) on
normal human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) indicated
only a small percentage of cells undergoing apoptosis and necrosis at higher concentrations of
Ag-np.

MWCNT: Growing popularity and release of carbon nanotubes into the environment is of prime
concern (Patlolla et al., 2010; Oberdorster et al., 2007). The reports on their toxicity in animal
system in vitro (Shvedova et al., 2005; Cui et al., 2005; Jia et al., 2005; Manna et al., 2005;
Sayes et al., 2006; Tian et al., 2006; Brown et al., 2007; Pulskamp et al., 2007; Muller et al.,
2008) are often divergent. A number of studies have shown carbon nanotubes to be cytotoxic and
genotoxic, capable of producing ROS and inducing apoptosis (Manna et al., 2005; Botinni et al.,
2006; Magrez et al., 2006; Zhu et al., 2007; Muller et al., 2008; Wirnitzer et al., 2009; Patlolla et
al., 2010). While a fair number of studies has shown negligible cytotoxic effect of carbon
nanotubes (Davoren et al., 2007; Pulskamp et al., 2007; Wirnitzer et al. 2009). The toxicity of
MWCNT was 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. MWCNT treatment in lymphocytes lead to a significant decrease in mitochondrial membrane potential. Flow cytometric estimation also revealed a significant increase in ROS generation. Induction of apoptosis in human lymphocytes was confirmed using annexinV-FITC – PI staining. With regard to mechanism of genotoxicity, 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 ~1.5μm. Uses of surfactant to avoid agglomeration were avoided, as carbon nanotubes when stabilized with surfactant are known to induce higher cytotoxic response (Singh et al., 2009). Previous studies have also clearly elaborated the affinity of MWCNT to DNA in water solute environment (Gao et al., 2003) 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.

**TiO$_2$-np:** The cytotoxic potential of TiO$_2$ nanoparticle in human lymphocyte was evaluated using more than one endpoint, to get more reliability. In a number of studies it has been reported that
classical dye based assays could produce false positive/ negative results and hence in the present study several cytotoxicity assays were used to eliminate variability arising from one of the assays, as suggested by Dhawan and Sharma, 2010. Trypan blue dye exclusion method was used to study the effect on membrane integrity. Trypan blue dye exclusion method did not reveal any significant alteration in cell viability, indicating that TiO$_2$ nanoparticle did not alter membrane integrity. MTT and WST-1 assays provided evidence of alteration of mitochondrial activity in cells exposed to TiO$_2$ nanoparticles. A significant dose dependent decrease in mitochondrial dehydrogenase activity could be attributed to decrease in reduction of tetrazolium salts (MTT and WST-1) to formazan dyes (Mosmann, 1983). Alterations of the cell surface or the sensitive lysosomal membrane leads to lysosomal fragility and other changes that gradually becomes irreversible. Changes produced by toxic substances result in decreased uptake and binding of neutral red, making it possible to distinguish between viable, damaged, or dead cells via spectrophotometric measurements. However neutral red release assay did not reveal any significant change from that of the control set. Resazurin or alamar blue is a water-soluble dye that has been previously used for quantifying in vitro viability of various cells (Fields and Lancaster, 1993; Ahmed et al., 1994). The number of viable cells correlates with the magnitude of dye reduction (Ahmed et al., 1994; Goegan et al., 1995; Nociari et al., 1998) giving an overview of cell’s metabolic condition. TiO$_2$ nanoparticle treatment did not induce significant alteration in metabolic activity of the cell as studied by resazurin assay. Genotoxicity of TiO$_2$ nanoparticle was evident from comet assay results. A significant increase in the DNA damage at 25 µg/ml was followed by a gradual decrease in extent of DNA damage. This could be explained for, owing to agglomeration property of TiO$_2$ nanoparticles (Ghosh et al. 2010). 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; Falck et al., 2009; Huang et al., 2009; Trouiller et al., 2009; Bernardeschi et al., 2010). It has been proposed that the genotoxicity of metals and metal oxides results from formation of reactive oxygen species (ROS). Shi et al., (2010) reported that TiO$_2$ nanoparticle enhances photocatalysis, oxidative stress, DNA adducts, DNA strand breaks, and chromosome damage. Earlier reports in animal cells have also suggested that TiO$_2$ nanoparticles generate large amount of hydroxyl free radical thereby leading to DNA damage (Reeves et al., 2007; Zhu et al., 2008; Ghosh et al., 2010). In our study DCFHDA assay in human lymphocyte cells also suggested of significant increase in ROS generation with increase in concentrations. Turkez et al., (2007) demonstrated that TiO$_2$ nanoparticles were capable of causing genotoxicity by inducing sister chromatid exchange and micronucleus formation in human white blood cells. However, Bhattacharya et al., (2009) reported that TiO$_2$ nanoparticle treatment did not induce DNA breakage measured by the comet-assay in human lung cell lines. Study reports by Driscoll et al., 1997 and Linnainmaa et al., 1997 also suggested TiO$_2$-np/ultrafine particles were not genotoxic to certain cell lines. 2009The toxicological report of TiO$_2$-np in vitro has been elegantly reviewed by Iavicoli et al., (2011). Recently, Thevenot et al., (2008) had also demonstrated that the surface chemistry of the TiO$_2$ nanoparticles play a major role in increasing or decreasing its toxicity. A significant decrease in MMP was observed also in lymphocyte cells stained with Rhodamine123 at all the concentrations tested. The result indicated significant damage to mitochondria, which confirmed our results obtained from MTT and WST-1 cytotoxicity assays. AnnexinV-FITC–PI staining was used to study induction of apoptosis. Experimental results indicate of induction of apoptosis at all treatment concentrations compared to control. Significant increases in percentage of apoptotic cells were observed. Only a small percentage of cells were found to undergo necrosis at
concentrations of 500 µM and above. The result from annexinV-FITC-PI staining could be correlated to results of cytotoxicity assays. Results of trypan blue assay could be correlated to the absence of necrotic (propidium iodide negative) cells analysed by flow cytometry, indicating membrane integrity. The results of cytotoxicity assays (MTT and WST-1) suggest that TiO₂ nanoparticle induced mitochondrial dysfunction which could result in apoptosis. A recent study (Zhao et al., 2009) has suggested role of mitochondrial signalling in TiO₂ nanoparticle-induced apoptosis.

ZnO-np: Cytotoxicity was evaluated simultaneously by multiple assay endpoints that detected cell viability as a marker of cell membrane integrity, mitochondrial dehydrogenase activity, metabolic activity and lysosomal integrity. In a number of studies it has been reported that classical dye-based assays could produce false positive/negative results and hence in the present study several cytotoxicity assays were used to eliminate variability arising from any one of the assays. Trypan blue dye exclusion method was used to study the effect on membrane integrity. Trypan blue dye exclusion method did not reveal any significant alteration in cell viability, indicating that ZnO-np did not alter membrane integrity. Decrease in mitochondrial dehydrogenase activity could be attributed to decrease in reduction of tetrazolium salts (WST-1) to formazan dyes (Mosmann, 1983); however was not observed in cells treated with ZnO-np. Alterations of the cell surface or the sensitive lysosomal membrane leads to lysosomal fragility and other changes that gradually becomes irreversible. Changes produced by toxic substances result in decreased uptake and binding of neutral red, making it possible to distinguish between viable, damaged, or dead cells via spectrophotometric measurements. In the present study the assay did not reveal any significant changes. Resazurin or alamar blue is a water-soluble dye that has been previously used for quantifying in vitro viability of various cells (Fields and Lancaster,
1993; Ahmed et al., 1994). ZnO-np was found to be no cytotoxic to human lymphocyte cells at the concentrations. The number of viable cells correlates with the magnitude of dye reduction giving an overview of cell’s metabolic condition. ZnO-np treatment did not induce significant alterations in metabolic activity. Using flow cytometry, the effect of ZnO-np on mitochondrial membrane potential (MMP) were assessed under control and exposed conditions. Results showed that mitochondrial function decreased significantly in cells exposed to ZnO-np. The Rhodamine 123 fluorescence intensity was reduced in cells exposed to ZnO-np by 45-50% compared to control set. Previous cytotoxicity study on BEAS-2B and RAW264 cells have shown induction of the intracellular Ca²⁺ flux, lowering of the mitochondrial membrane potential, and loss of membrane integrity after exposure to 20 nm ZnO NPs (George et al., 2009). Studies of ZnO-np (32-95 nm, with a TiO₂ shell) on A549 lung cells have revealed similar results (Hsiao and Huang 2011; Vandebriel and Jong 2012). Genotoxic potential of ZnO-np (<20 nm) and their ability to perturb the mitochondrial membrane potential, possibly through oxidative stress, in human PBMCs have been reported by Mussarat et al., 2009. A decrease in mitochondrial membrane potential triggered by reactive oxygen species have also been reported by Sharma et al., (2012) in human liver cells (HepG2). Hence in the present study the role of reactive oxygen species was investigated using flow cytometry. Dichlorofluorescin diacetate (DCFDA) is first deacetylated by endogenous esterases to dichlorofluorescein (DCFH), which when reacts with ROS, forms the fluorophore DCF. In the present study, increase in fluorescence intensity of DCF measured by flow cytometric method revealed induction of ROS in cells exposed to ZnO-np. Results of comet assay, in vitro on human PBMCs revealed of an increase in % tail DNA in cells exposed to ZnO-np concentrations. However responses were not statistically
significant (P<0.05). Despite evidences of toxicology of ZnO-np certain researchers have also reported otherwise (Colon et al., 2006; Qi et al., 2008).

EVALUATION OF IN VIVO CYTOTOXICITY AND GENOTOXICITY IN SWISS ALBINO MALE MICE

In large part, indications for potential DNA damaging effects of nanoparticles are based on inconsistent in vitro studies. To clarify these effects, the implementation of in vivo studies has been emphasised.

Silver nanoparticle: We demonstrated in vivo genotoxicity of Ag-np in mouse bone marrow cells. Compared to control the percentage of aberrant cells induced by Ag-np (20-80 mg/kg body weight) was significantly higher. The aberrations scored were mainly found to be of chromatid breaks. Therefore, Ag-np can be classified as a clastogen. In addition, comet assay revealed an increase in DNA strand breaks. The comet parameter (% tail DNA) showed no further increase in DNA damage beyond the concentration of 20 mg/kg. Results of ROS quantification in bone marrow cells by flow cytometry revealed comparable results. Kim et al., (2008) reported negative genotoxic response in bone marrow cells of Sprague-Dawley rats exposed to Ag-np. The frequency of micronucleated polychromatic erythrocytes were not significantly altered. The discrepancy can be best explained on the difference in route of administration and the sampling time. Ag-np have been found to induce micronuclei in CHO cells (Kim et al., 2008) and BRL 3A rat liver cells (Husain et al., 2005). While alteration in mitochondrial membrane potential was not observed in our study, significant increase in ROS production was observed at all concentrations tested.
MWCNT: *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 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. MWCNT treated bone marrow cells were characterized by a decrease in mitochondrial membrane potential and a significant increase in ROS production. The *in vivo* results were supportive of the *in vitro* genotoxicity analysis performed in human lymphocyte cells.

TiO$_2$-np: Following intra peritoneal injection of TiO$_2$-np, genotoxicity was studied in bone marrow and liver cells using comet assay. A significant increase in level of DNA damage was observed in both liver and bone marrow cells. Oxidative stress has been attributed to nanoparticle toxicity (Nel et al., 2006) and hence ROS generation was studied in bone marrow cells. Flow cytometric studies on bone marrow cells revealed a significant increase in ROS generation. A significant decrease in mitochondrial membrane potential was also observed using Rhodamine 123 flow cytometric assay. The results thus obtained in bone marrow cells were in line with that obtained in human PBMCs, *in vitro*. Cell cycle progression analysis was performed in bone marrow cells that revealed an accumulation of cells in sub G0 fraction. Previous reports of TiO$_2$-np exposure on mice (Trouiller et al., 2009; Sycheva et al., 2011), suggested induction of DNA strand breaks, increase in micronuclei, and inflammatory reactions. Saber et al., (2012) reported that TiO$_2$-np induced significant inflammatory response in broncho-alveolar fluid cells and increased DNA damage in liver cells of intratracheally instilled mice. Landsiedel et al., (2010) and Naya et al., (2012) reported negative findings from *in vivo* genotoxicity assays.
ZnO-np: The *in vitro* study result was validated using *in vivo* toxicity study in Swiss albino male mice. Followed by a schedule of intra peritoneal injection of ZnO-np, genotoxicity was studied in peripheral blood cells, bone marrow cells and liver cells using comet assay. An elevated level of DNA damage was observed in the liver cells, however DNA strand breaks were not observed in peripheral blood cells and bone marrow cells. Investigating further, bone marrow cells were studied for chromosomal aberration and induction of micronuclei to confirm presence/ absence of genotoxicity. The results revealed a significant dose dependent increase in number chromosomal aberrations and micronucleated polychromatic erythrocytes, indicating genotoxic response. The differences in the result of genotoxicity assays cannot be explained for at the moment. However it would be important to understand from the results that it is extremely important to study a number of endpoints to provide conclusive evidence in nanotoxicological studies. Oxidative stress has been attributed to nanoparticle toxicity (Nel *et al.*, 2006) and hence ROS generation was studied in bone marrow cells. Flow cytometric studies on bone marrow cells revealed an increase in ROS generation. A decrease in mitochondrial membrane potential was also observed using Rhodamine 123 flow cytometric assay. The results thus obtained in bone marrow cells were in line with that obtained in human PBMCs, *in vitro*. Cell cycle progression analysis was performed in bone marrow cells that showed cells were arrested strongly by ZnO-np in a dose-dependent manner. Approximately 56% of cells exposed to 100 mg/kg of ZnO-np were arrested in G1/G0, and 14 % cells were in the sub G0 section. A G0/G1 arrest and apoptosis (Sub G0) was evident from the cell cycle analysis of bone marrow cells.

**NANOPARTICLE UPTAKE**

Silver nanoparticle: TEM images of cells treated with Ag-np revealed gross morphological alterations and vacuolation in both *A. cepa* and human blood cells. Extensive vacuolation, loss of
nuclear organization, ruptured plasma membrane and shrinkage of the protoplast could be associated with apoptotic/necrotic (in human blood cells) and necrotic/vacuolar (in *A. cepa*) (Van Doroon et al., 2011) cell death. The ultra structural alterations in human blood cells could be correlated to the necrotic mode of cell death studied using flow cytometry.

**MWCNT:** Light microscope images of MWCNT treated cells showed the presence of a large number of vacuoles. 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 (Magrez et al., 2006). TEM images of cells treated with MWCNT revealed gross morphological alterations and vacuolation in both *A. cepa*. Extensive vacuolation, loss of nuclear organization, ruptured plasma membrane and shrinkage of the protoplast confirmed the results obtained from DNA laddering and flow cytometric assay suggesting apoptotic cell death.

**TiO$_2$-np and ZnO-np:** TiO$_2$-np and ZnO-np uptake in root cells was studied using TEM. TEM images of treated cells revealed gross morphological alterations and vacuolation in *A. cepa* root cells. Extensive vacuolation, loss of nuclear organization, ruptured plasma membrane and shrinkage of the protoplast could be associated with apoptotic/necrotic necrotic/vacuolar (Van Doroon et al., 2011) cell death.
### SUMMARY TABLE OF MAJOR FINDINGS

**Table 14:** Summary of results obtained from the present study

<table>
<thead>
<tr>
<th>CHARACTERIZATION</th>
<th>TEM</th>
<th>SEM</th>
<th>EDX</th>
<th>XRD</th>
<th>DLS/ Zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag-np 1.25 nm</td>
<td>-128 nm</td>
<td>3</td>
<td>✓</td>
<td>✓</td>
<td>-0.94 mV</td>
</tr>
<tr>
<td>MWCNT 2.15 nm</td>
<td>-23.8 nm</td>
<td>np</td>
<td>np</td>
<td>np</td>
<td>-1895 mm</td>
</tr>
<tr>
<td>TiO2-np 50 nm</td>
<td>-48 nm</td>
<td>np</td>
<td>✓</td>
<td>✓</td>
<td>-52.36 mm</td>
</tr>
<tr>
<td>ZnO-np 80 nm</td>
<td>-85 nm</td>
<td>3</td>
<td>✓</td>
<td>np</td>
<td>-5000 mm</td>
</tr>
</tbody>
</table>

**TOXICITY IN PLANT SYSTEM**

<table>
<thead>
<tr>
<th>CA/MN</th>
<th>Comet assay</th>
<th>DNA laddering</th>
<th>Cell Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag-np</td>
<td>+ve (A. cepa)</td>
<td>np</td>
<td>Sub G0 accumulation</td>
</tr>
<tr>
<td>MWCNT</td>
<td>+ve (A. cepa)</td>
<td>+ve</td>
<td>Sub G0 accumulation, Decrease in G2/M and S phase</td>
</tr>
<tr>
<td>TiO2-np</td>
<td>+ve (A. cepa)</td>
<td>+ve</td>
<td>np</td>
</tr>
<tr>
<td>ZnO-np</td>
<td>+ve (A. cepa &amp; V. faba)</td>
<td>np</td>
<td>Sub G0 accumulation, G2/M arrest</td>
</tr>
</tbody>
</table>

**TOXICITY IN HUMAN LYMPHOCYTE IN VITRO**

<table>
<thead>
<tr>
<th>Characterized</th>
<th>Comet assay</th>
<th>MMP</th>
<th>ROS generation</th>
<th>Apoptosis/Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag-np Trypan blue</td>
<td>+ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>MTT</td>
<td>+ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>WST-1</td>
<td>+ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>MWCNT Trypan blue</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>MTT</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>WST-1</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>TiO2-np Trypan blue</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>MTT</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>WST-1</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>ZnO-np Trypan blue</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>MTT</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>WST-1</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
<tr>
<td>Neutral Red</td>
<td>-ve</td>
<td>+ve</td>
<td>Significant decrease</td>
<td>Mostly necrotic</td>
</tr>
</tbody>
</table>

**TOXICITY IN SWISS ALBINO MALE MICE IN VIVO**

<table>
<thead>
<tr>
<th>CA</th>
<th>MN</th>
<th>Comet assay</th>
<th>MMP</th>
<th>ROS generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag-np</td>
<td>+ve</td>
<td>np</td>
<td>+ve</td>
<td>No change</td>
</tr>
<tr>
<td>MWCNT</td>
<td>np</td>
<td>np</td>
<td>+ve</td>
<td>No change</td>
</tr>
<tr>
<td>TiO2-np</td>
<td>np</td>
<td>np</td>
<td>+ve</td>
<td>No change</td>
</tr>
<tr>
<td>ZnO-np</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
</tbody>
</table>

up: not performed; CA: Chromosomal aberration; MN: Micronucleus assay; MMP: Mitochondrial membrane potential; ROS: Reactive oxygen species; TEM: Transmission electron microscopy; SEM: Scanning electron microscopy; EDX: Energy-dispersive X-ray spectroscopy; XRD: X-ray Diffraction; DLS: Dynamic light scattering; +ve indicates positively toxic result from the assay.