Chapter 3

In Vitro Toxicity Assessment of TiO$_2$ ENPs in Human Epidermal Cells
3.1. INTRODUCTION
The use of nanotechnology has seen an exponential growth in the areas of health care, consumer products, clothes, electronics, sporting goods etc. (PEN 2011) This is due to the unique properties of nanomaterials (e.g. chemical, mechanical, optical, magnetic, and biological) which make them desirable for commercial and medical applications (Oberdorster et al. 2005; Nel et al. 2006; Jin et al. 2008). According to a recent survey, the number of nanotechnology-based consumer products available in the world market has crossed the 1300 mark (PEN 2009). Titanium dioxide (TiO$_2$) engineered nanoparticles (ENPs) are among the most commonly used metal oxide NPs in industrial products, such as cosmetics, sunscreens, food products, paints and drugs. TiO$_2$ ENPs have a clear transparent appearance upon topical application compared to the micron-scale particles which leave a white residue on the skin. They also increase UV protection capability of sunscreens (Kiss et al. 2008; Barnard 2010). The increased use of NPs is a matter of a great concern among health and environmental scientists pertaining to human exposure and risk assessment (Colvin 2003; Warheit et al. 2004; Igarashi 2008; Bouwmeester et al. 2009; Gilbert 2009; Maurer-Jones et al. 2009; Song et al. 2009; Shi et al. 2010). Among the possible exposure routes, inhalation and skin contact are considered most important for NPs it is well known that the toxic effects of NPs can be attributed to the small size and hence large surface area, thereby increasing chemical reactivity and penetration in the living cells (Donaldson et al. 2000; Gojova et al. 2007; Medina et al. 2007; Pan et al. 2009). TiO$_2$ ENPs have also been shown to produce reactive oxygen species (ROS) leading the toxicity (Gurr et al. 2005; Wang et al. 2007; Kang et al. 2008; Barnard 2010).

Skin is the largest organ of the body and could serve an important portal route for entry of nanoparticles in the human body. Studies have shown that NPs penetrate the outermost layer of skin cells (Lademann et al. 1999; Bennat and Muller-Goymann 2000; Tinkle et al. 2003; Adachi et al. 2010; Kocbek et al. 2010; Tiano et al. 2010). TiO$_2$ NPs have been reported to elicit
various adverse cellular effects including oxidative stress and DNA damage (Gurr et al. 2005; Hussain et al. 2005; Jeng and Swanson 2006; Wang et al. 2007; Vamanu et al. 2008; Falck et al. 2009; Wang et al. 2009). Therefore, in the present study an attempt was made to assess the uptake and cellular toxicity including genotoxic potential of TiO$_2$ ENPs in human epidermal cells (A431) as well as to understand its possible mechanism.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals
Titanium (IV) oxide nanopowder (99.7%, anatase, CAS No.1317-70-0) was purchased from Sigma Aldrich Co. Ltd. The other chemicals used in the study are described previously in Chapter 2 (2.1.1).

3.2.2. Particle Preparation And Characterization
Nanoparticles tend to agglomerate upon addition of cell culture media if they are uncoated or functionalized (Ji et al. 2010). If the agglomerates are used directly for nanotoxicity studies, it would result in inaccurate dose estimation and the interpretation of the results would be flawed (Ji et al. 2010). Therefore, it is necessary to characterize the NP suspension prior to toxicity assessment.

3.2.2.1. Preparation
Samples were prepared freshly before giving exposure to the cells. TiO$_2$ ENPs were suspended in culture medium DMEM supplemented with 10% FBS at a concentration of 160µg/ml and probe sonicated (Sonics Vibra cell, Sonics & Material Inc., New Town, CT, USA) at 30 watt for 10 min (1.5 min pulse on and 1 min pulse off for 4 times).

3.2.2.2. Characterization
It is well known that nanoparticles have tendency to change their properties (size, stability, agglomeration tendency) in suspension. Therefore, in this study ENPs were characterized both in their powder form as well as in suspension using TEM and DLS, respectively. Stability of ENPs in dispersion medium was also assessed by DLS.
3.2.2.2.1. **Transmission electron microscopy**

Samples were prepared for transmission electron microscopy (TEM) analysis as described previously in Chapter 2 (2.2.1.1).

3.2.2.2.2. **Dynamic light scattering**

The average hydrodynamic size, size distribution and zeta potential of TiO₂ ENPs in suspension were determined by this instrument as described in Chapter 2 (2.2.1.2).

3.2.3. **Cell Culture and Exposure to NPs**

The human epidermal cell line (A431) was obtained from National Centre for Cell Sciences, Pune, India, and cultured in DMEM supplemented with 10% FBS, 0.2% sodium bicarbonate and 10ml/L antibiotic and antimycotic solution at 37°C under a humidified atmosphere of 5% CO₂/95% air.

Stock suspension of TiO₂ ENPs (160 µg/ml) in DMEM (supplemented with 10% FBS) was serially diluted to concentrations ranging from 0.008 µg/ml to 80 µg/ml (corresponding to 0.0025 to 25 µg/cm²) for cellular uptake, cytotoxicity assays, Comet assay, micronucleus assay, ROS generation and oxidative stress parameters (glutathione and lipid peroxidation). For each experiment, the particle suspension was freshly prepared, diluted to appropriate concentrations and immediately applied to the cells. For different assays, 96, 12, 6 well cell culture plates and 75 cm² cell culture flasks were used having treatment volumes of 100 µl, 1.2 ml, 3 ml and 24 ml respectively. However, the concentration per cm² area (e.g. 80 µg/ml corresponds to 25 µg/cm² for all the plates and flasks) remained same. Culture medium without TiO₂ ENPs served as the control in each experiment.

3.2.4. **Cellular Uptake**

NPs internalization into the cellular system is an important hallmark of nanotoxicology. In this study we have using flow cytometry method which is a fast rapid screening method and then confirmed it by Transmission electron microscopy image analysis.

3.2.4.1. **Flow cytometry**
The uptake of NPs using flow cytometry was carried out according to the method developed by Suzuki et al. (2007). Cells were exposed to TiO₂ ENPs (0.008, 0.08, 0.8, 8 and to 80 µg/ml) for 6h. Then the cells were harvested and samples were prepared for flow cytometric analysis as described previously in Chapter 2 (2.2.2.1).

3.2.4.2. Transmission electron microscopy (TEM)
Cells were exposed to TiO₂ ENPs (8 µg/ml) for 6 h. Thereafter, samples were prepared and analyzed in TEM as described in Chapter 2 (2.2.2.2).

3.2.5. Cytotoxicity Assays
Cytotoxicity of TiO₂ ENPs was measured by using MTT and NRU assay. Cells (1.0X10⁴ cells/well) in 100 µl of DMEM were seeded in 96-well plates. 24 h after seeding, the cells were exposed to different concentrations of TiO₂ ENPs (0.008, 0.08, 0.8, 8, 80 µg/ml) for three time periods i.e. 6, 24 and 48 h.

3.2.5.1. MTT Assay
Mitochondrial activity was assessed using the MTT assay according to a modified method of Mosmann et al. (1983) as described in Chapter 2 (2.2.4.1).

3.2.5.2. NRU Assay
Neutral red uptake assay was conducted according to the method of Borenfreund and Puerner (1985) as described in Chapter 2 (2.2.4.2).

3.2.5.3. Cell Viability
The cell viability using Trypan blue dye exclusion assay was conducted by the method of Phillips (1973) before the start and after the completion of the ENPs exposure in the Comet assay. This method is described previously in Chapter 2 (2.2.3.2).

3.2.6. Genotoxicity Assessment
The genotoxic potential of TiO₂ ENPs was assessed by the Comet assay as well as by cytokinesis-block micronucleus (CBMN) assay. The cells were exposed for 6 h to different concentrations of TiO₂ ENPs (0.008, 0.08, 0.8, 8, 80 µg/ml). Hydrogen peroxide (25 µM) and ethyl methanesulfonate (6 mM)
were used as positive control for Fpg-Comet assay and CBMN assay, respectively.

**3.2.6.1. Single cell gel electrophoresis / Comet assay**
After treatment, cells were washed with serum-free medium to remove the test compound and harvested with 0.005% trypsin. The cells were re-suspended in complete medium which was used for the Comet assay. Slides were prepared and the Comet assay was performed according to the method of Singh et al. (1988) and as per the Comet assay guidelines (Tice et al., 2000) described in Chapter 2 (2.2.5.1).

**3.2.6.2. Cytokinesis -block micronucleus (CBMN) assay**
The cytokinesis block micronucleus (CBMN) assay was carried out by a slightly modified method of Fenech et al. (2000). Detailed procedure has been described in Chapter 2 (2.2.6.1). Harvesting, slide preparation, staining and scoring were conducted as described in Chapter 2 (2.2.6.1).

**3.2.7. Measurement of Intracellular Reactive Oxygen Species (ROS)**
The level of intracellular ROS generation was estimated by the method of Wan et al. (1993) and modified by Wilson et al. (2002) using 2, 7-dichlorofluorescein diacetate (DCFDA) dye. The details of the method are described in Chapter 2 (2.2.7.4).

**3.2.8. Oxidative Stress Markers**
Cells at a final density of ~6x10^6 in a 75 cm² culture flask were exposed to different concentrations of TiO₂ ENPs (0.008, 0.08, 0.8, 8, 80 µg/ml) for 6 h and different oxidative stress markers have assessed as described below:

**3.2.8.1. Glutathione estimation**
Glutathione content was assayed in the cell lysate according to the method of Ellman (1959). This is a colorimetric method in which 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman’s Reagent) reacts with GSH to form chromophore, [5-thionitrobenzoic acid (TNB)] that gives yellow color.
3.2.8.2. Lipid peroxidation (LPO) assay

Lipid peroxidation levels were estimated by lipid hydroperoxide assay kit™ (Cayman Chemical Company, MI, USA) according to manufacturers’ protocol. Both the assays are done according to the procedure described in Chapter 2 (2.2.7.1 and 2.2.7.2).
3.3. RESULTS

3.3.1. TiO₂ Nanoparticles Characterization

The mean hydrodynamic diameter of TiO₂ ENPs in Milli Q water as measured by DLS was 124.9 nm (Figure 3.1 A) and the zeta potential was -17.6 mV (Figure 3.1 B). The characterization of TiO₂ ENPs was also done in DMEM supplemented with 10% FBS. In culture medium NPs showed a slight increase in the hydrodynamic size (171.4 nm) with a concomitant decrease in the zeta potential (-11.5 mV) (Figure 3.1 C and 3.1 D). The average size observed from TEM analysis was 30-50 nm (Figure 3.2).

![Figure 3.1](image1.png)

**Figure 3.1:** Measurement of size and zeta potential of TiO₂ ENPs by dynamic light scattering: (A-B) in Milli Q water; (C-D) in DMEM supplemented with 10% FBS.

![Figure 3.2](image2.png)

**Figure 3.2:** Transmission electron microscopic (TEM) microphotograph of TiO₂ ENPs
3.3.2. Cellular Uptake

3.3.2.1. Flow Cytometry

The uptake of TiO$_2$ ENPs in the epidermal cells was assessed using a flow cytometer. The side scatter (SSC) intensity which represents granularity of a cell and forward scatter (FSC) representing the size of cell were analyzed as shown in Table 3.1. The percentage intensity of SSC of cells treated with TiO$_2$ ENPs (8 and 80 µg/ml) were significantly (p<0.05) increased (157, and 885% respectively) as compared to the granularity of control cells (100%) (p<0.05).

Table 3.1 – Cellular uptake by TiO$_2$ ENPs after 6 h exposure in human epidermal cells using flow cytometry

<table>
<thead>
<tr>
<th>Concentration</th>
<th>% Forward scatter (FSC)</th>
<th>% Side scatter (SSC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.00±0.00</td>
<td>100.00±0.00</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (0.008 µg/ml)</td>
<td>100.49±3.00</td>
<td>102.46±0.48</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (0.08 µg/ml)</td>
<td>101.25±2.63</td>
<td>105.80±2.48</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (0.8 µg/ml)</td>
<td>96.90±0.97</td>
<td>110.67±1.78</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (8 µg/ml)</td>
<td>93.75±0.18</td>
<td>257.28±9.25*</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (80 µg/ml)</td>
<td>93.07±1.09</td>
<td>985.38±10.21*</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E. of three experiments.

* p < 0.05 when compared to control

3.3.2.2. Transmission electron microscopy

Human epidermal cells exposed to TiO$_2$ ENPs showed a significant cellular uptake of the NPs as evident from the TEM microphotographs (Figure 3.3 A-B). ENPs were found to be present in the cytoplasm as well as nucleus (Figure 3.3 A). TEM images showed that ENPs were found to be distributed inside the cells mostly in cytoplasm, but some of them were also found in the nucleus (Figure 3.3 B).
In Vitro Toxicity Assessment of TiO₂ ENPs in Human Epidermal Cells

Figure 3.3: TEM photomicrographs of human epidermal cells showing internalization of TiO₂ ENPs (8 µg/ml): (A-B) ENPs in cytoplasm and nucleus. Arrows indicate the presence of TiO₂ ENPs inside different cellular organelles.

3.3.3. Cytotoxicity

A statistically significant (p<0.05) cytotoxic effect of TiO₂ ENPs in human epidermal cells (A431) was observed at the two higher concentrations (8 and 80 µg/ml) after 48 h exposure in MTT and NRU assays (Figure 3.4 A-B). The MTT results demonstrated 73% and 68% MTT reduction (relative to control) while the NR uptake was reduced to 75% and 65.4% at 8 and 80 µg/ml respectively demonstrating a mild cytotoxic effect of TiO₂ ENPs. Reduction in % cell viability was also observed after 6 and 24 h exposure, however it was not statistically significant. There was no major interaction observed between dyes and ENPs as seen by the experiment conducted in the cell free system.
Figure 3.4: Cytotoxicity of TiO₂ ENPs with different concentrations (0.008, 0.08, 0.8, 8 and 80 µg/ml) exposed to A431 cells for 6, 24 and 48 h. (A) % MTT reduction. (B) % Neutral red uptake. The viability of the control cells was considered 100%. The data are expressed as means ±SEM from three independent experiments. *p<0.05; when compared to control.

3.3.4. Genotoxicity Assessment

3.3.4.1. DNA damage

The cell viability in the Comet assay exceeded 90% for all experimental groups (data not shown) before and after the treatment as assessed by Trypan blue dye exclusion assay. A significant (p<0.05) increase in the qualitative and quantitative DNA damage in cells exposed to TiO₂ ENPs was observed, as evident by the Comet assay parameters viz. Olive tail moment (OTM) and % Tail DNA
respectively, with and without Fpg treatment at 0.8 µg/ml, 8 µg/ml and 80 µg/ml (Table 3.2).

A statistically significant (P<0.05) induction in DNA damage was observed after 6 h exposure to 8 and 80 µg/ml concentration of TiO₂ ENPs as compared to the respective control cells in the conventional Comet assay. However, the DNA damage was significantly enhanced even at 0.8 µg/ml and higher concentrations of TiO₂ ENPs with Fpg treatment. When compared among the groups, Fpg elicited a significantly greater response at the two higher concentrations of TiO₂ ENPs (8 and 80 µg/ml) as evident by the Comet assay parameters (Table 3.2). The data was also analyzed in terms of percentage distribution of cells on the basis of OTM. With the increasing concentration of TiO₂ ENPs, a shift from a low DNA damage category

Figure 3.5: Effect of TiO₂ ENPs on percentage distribution of human epidermal cells with respect to OTM (A) Fpg (-) and (B) Fpg (+).
(OTM<1.5), in control, to high DNA damage category (OTM>2.5) in TiO₂ ENPs exposed cells, was observed (Figure 3.5 A-B).

3.3.4.2. Micronucleus induction

Human epidermal cells (A431) showed a statistically significant (p<0.05) induction in micronucleus formation after 6 h exposure to TiO₂ ENPs at 0.8 µg/ml (14.67 MN/1000 BNCs), 8 µg/ml (15.67 MN/1000 BNCs) and 80 µg/ml (16.00 MN/1000 BNCs) with respect to control (9.33 MN/1000 BNCs) (Table 3.3).

Table 3.2 - DNA damage in human epidermal cells after 6 h exposure to TiO₂ ENPs as evident by the Comet parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>OTM (arbitrary unit)</th>
<th>Tail DNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fpg (-)</td>
<td>Fpg (+)</td>
</tr>
<tr>
<td>Control</td>
<td>1.20±01</td>
<td>1.24±04</td>
</tr>
<tr>
<td>H₂O₂ a (25 µM)</td>
<td>4.42±20*</td>
<td>6.31±51**</td>
</tr>
<tr>
<td>TiO₂ ENPs (0.008 µg/ml)</td>
<td>1.27±05</td>
<td>1.44±07</td>
</tr>
<tr>
<td>TiO₂ ENPs (0.08 µg/ml)</td>
<td>1.30±03</td>
<td>1.80±11</td>
</tr>
<tr>
<td>TiO₂ ENPs (0.8 µg/ml)</td>
<td>1.43±09</td>
<td>2.20±26*</td>
</tr>
<tr>
<td>TiO₂ ENPs (8 µg/ml)</td>
<td>1.79±08*</td>
<td>2.56±20**</td>
</tr>
<tr>
<td>TiO₂ ENPs (80 µg/ml)</td>
<td>1.91±04*</td>
<td>2.95±20**</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E. of three experiments. a H₂O₂ - Hydrogen peroxide (Positive control). * p < 0.05 when compared to control using one way ANOVA, a” p < 0.05 using when compared to Fpg (-) at the same concentration using Student ‘t’ test.
Table 3.3 - Effect of TiO₂ ENPs on micronucleus formation in human epidermal cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of MN / 1000 binucleated cells</th>
<th>Cytokinesis block proliferation index (CBPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.33±1.00</td>
<td>1.64</td>
</tr>
<tr>
<td>EMSa (6 mM)</td>
<td>26.67±0.88***</td>
<td>1.70</td>
</tr>
<tr>
<td>TiO₂ ENPs (0.008 µg/ml)</td>
<td>11.67±1.20</td>
<td>1.70</td>
</tr>
<tr>
<td>TiO₂ ENPs (0.08 µg/ml)</td>
<td>12.67±0.88</td>
<td>1.72</td>
</tr>
<tr>
<td>TiO₂ ENPs (0.8 µg/ml)</td>
<td>14.67±1.20*</td>
<td>1.70</td>
</tr>
<tr>
<td>TiO₂ ENPs (8 µg/ml)</td>
<td>15.67±0.88**</td>
<td>1.77</td>
</tr>
<tr>
<td>TiO₂ ENPs (80 µg/ml)</td>
<td>16.00±0.58**</td>
<td>1.78</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E. of three experiments for each concentration. a EMS- ethyl methanesulfonate -positive control. * p < 0.05; ** p < 0.01; *** p < 0.001 when compared to control.

3.3.5. Measurement of Intracellular ROS

A significant (p<0.05) qualitative and quantitative dose dependent increase in % ROS generation was observed in the form of fluorescence on treatment with TiO₂ ENPs (49.2%, 66.7%, 78.1% at 0.8, 8 and 80 µg/ml respectively; Figure 3.6-3.7).
Figure 3.6: Effect of TiO$_2$ ENPs on the generation of reactive oxygen species (ROS) in human epidermal cells. The % ROS generation of the control cells was considered 100%. Data represents mean ± SEM of three experiments.*p<0.05; when compared to control.

![Figure 3.6](image)

Figure 3.7: Photomicrographs showing the generation of intracellular reactive oxygen species (ROS) using DCFDA dye in human epidermal cells (A) Control cells; (B-D) Cells exposed to TiO$_2$ ENPs (0.08 µg/ml, 8 µg/ml and 80 µg/ml respectively) for 6 h showing increase in fluorescence (Magnification-X200).

3.3.6. Oxidative Stress Markers

3.3.6.1. Effect of TiO$_2$ ENPs on glutathione (GSH)

Cellular GSH level in human epidermal cells was significantly (p<0.05) reduced after 6 h exposure to TiO$_2$ ENPs at the concentrations of 8 µg/ml (15.76%) and 80 µg/ml (23.56%), when compared to control cells (Figure 3.8 A).

3.3.6.2. Effect of TiO$_2$ ENPs on lipid peroxidation (LPO)

Cells exposed to TiO$_2$ ENPs showed a concentration-dependent statistically significant (p<0.05) increase in hydroperoxide concentration at 8 µg/ml (60.51%) and 80 µg/ml (82.4%). (Figure 3.8 B).
Figure 3.8: Effect of TiO$_2$ ENPs on the oxidative stress markers in human epidermal cells. (A) Glutathione, (B) Lipid peroxidation. Data represents mean ± SEM of three experiments. *p<0.05; when compared to control.
3.4. DISCUSSION

TiO$_2$ ENPs are widely used in consumer products especially sunscreen and cosmetics. Although several studies have been done to assess their toxicity in mammalian cells, to best of our knowledge this is the first study showing genotoxicity in human epidermal cells. Since the skin serves as the first portal of entry of NPs used in cosmetics, we selected human epidermal cells (A431) as an in vitro model for assessing genotoxicity of TiO$_2$ ENPs. Concentrations used in this study are far less than those used in the cosmetics. In the commercial sunscreens being sold in the market, the concentration of TiO$_2$ ENPs is between 3-15%. Therefore a person applying 5 ml lotion/cream is exposed to 150-750 mg NPs (depending on the concentration in the cream). In our study the highest concentration of TiO$_2$ ENPs was 80µg/ml (0.080 mg/ml), which is far less than the concentrations used in sunscreens (30 mg/ml at the lowest concentration). However considering the fact that some NPs may persist on skin even after the cream is washed off and the fact that TiO$_2$ ENPs could gain entry inside the skin in case it is damaged, the concentrations used in the present study are very relevant.

The findings of the present study demonstrate that TiO$_2$ ENPs possess DNA damaging potential in human epidermal cells (A431). These ENPs also induce significant GSH depletion, LPO induction and ROS generation in a concentration dependent manner. The direct correlation between ROS generation and oxidative DNA damage further suggests that oxidative stress could act as an important route by which TiO$_2$ ENPs induce DNA damage in human epidermal cells.

Prior to studying genotoxicity and oxidative stress potential of TiO$_2$ ENPs, they were characterized for their size and zeta potential. The Brunauer Emmett Teller (BET) size of TiO$_2$ ENPs claimed by its commercial supplier was 25 nm. However, the size of the ENPs was further confirmed by two independent methods: DLS and TEM. The mean hydrodynamic size obtained from DLS was found to be more in culture medium (171.4 nm) as compared to milli Q water (124.9 nm), which might be due to slight increase in
agglomeration. However, the size observed by TEM was 30-50 nm. This difference in size can be attributed to the different principles employed for measurement (i.e. BET, DLS and TEM). TEM gives the direct measurement of particle size, distribution and morphology by image analysis, while DLS measures the size distribution of particles in aqueous state which is usually larger than BET or TEM diameter. DLS measurement gives the idea of stability of nanoparticles in aqueous medium which resembles more to the exposure condition as compared to TEM and BET (Lin et al. 2009; Sharma et al. 2009). Agglomeration commonly occurs when nanoparticles are introduced in to the aqueous medium; it was observed that the nanoparticles were stable for longer period in serum than without it (Ji et al. 2010; Casanova et al. 2011. This may be due to the coating of ENPs with proteins in serum. Suzuki et al. (2007) have suggested that the determination of granularity of cells through flow cytometry is a good way to evaluate the uptake potential of ENPs in cells. Our results show that human epidermal cells (A431) when exposed to TiO$_2$ ENPs incorporate that in a concentration-dependent manner. Our results are consistent with Suzuki et al. (2007) and Xu et al. (2009), who have reported that higher concentrations of TiO$_2$ ENPs resulted in higher granularity in the cells.

To confirm our cytometry data, we performed TEM to investigate whether TiO$_2$ ENPs are internalized into the human skin epidermal cells or adhere to the cell membrane. We therefore, observed that small particles (30-100 nm) were internalized into the cell cytoplasm, vesicles and also in the nucleus, whereas larger particles (>500 nm) remained outside the cells. Our data demonstrates that TiO$_2$ ENPs is a mild cytotoxic on human epidermal cells (A431). The results from the MTT assay, used for studying the expression of mitochondrial enzymes, showed that there were significant reductions in the viability of human skin epidermal cells when exposed to TiO$_2$ ENPs at 8 and 80 µg/ml for 48 h. Earlier studies pertaining to cytotoxic evaluation using MTT have been inconclusive with some of them showing a positive response (Jin et al. 2008; Simon-Deckers et al. 2008; Di Virgilio et al. 2010) while others showing negative response (Park et al. 2007; Wang et al.
In Vitro Toxicity Assessment of TiO\textsubscript{2} ENPs in Human Epidermal Cells

2007) in different mammalian cell types. To further confirm, we performed the NRU assay and observed similar kind of response. The variability in the results of above mentioned studies may be attributed to the different cell types involved. In addition, the source of ENPs and method of treatment preparation may also contribute to the differential response of cellular systems.

Nanoparticles can interfere with the various dyes and their products used in colorimetric and fluorometric assays due to their physical and chemical properties (Doak et al. 2009; Monteiro-Riviere et al. 2009; Stone et al. 2009; Baer et al. 2010; Howard 2010). Hence, this could lead to misinterpretations of the results. In the present study, this was obviated by the parallel set of experiments conducted to check the interaction of ENPs with MTT, NR and DCFDA dyes. Our data demonstrate that TiO\textsubscript{2} ENPs do not interact with the dyes used for cytotoxicity assessment as well as for ROS measurement.

ROS generation has been proposed as a possible mechanism involved in the toxicity of NPs (Gurr et al. 2005; Long et al. 2006; Nel et al. 2006; Eom and Choi 2009). NPs in aqueous suspension produce free radicals (Hirakawa et al. 2004) which may damage DNA by oxidation, nitration, methylation or deamination reactions (Schins and Knaapen 2007).

The data of the present study demonstrate that TiO\textsubscript{2} ENPs induce oxidative DNA damage as evident by the results of the Fpg-modified Comet assay. This was also corroborated by the fact that ROS generation was significantly enhanced on exposure to TiO\textsubscript{2} ENPs. These results are in accordance with Kang et al. (2008) who reported that TiO\textsubscript{2} ENPs induced ROS generation in human lymphocytes. A statistically significant dose-dependent increase was observed in the MN frequency at 0.8, 8 and 80 µg/ml concentration of TiO\textsubscript{2} ENPs, further confirming their genotoxic potential. Our data, though in accordance with that reported by Gurr et al. (2005) who have shown that 10 µg/ml of TiO\textsubscript{2} induced genotoxicity in BEAS 2B cells. However the concentrations at which DNA damage was observed are lower than those reported by Wang et al. (2007) and Falck et al. (2009). These differences
might be due to the difference in cell type and culture condition (Lanone et al. 2009).

In this study we also observed a strong correlation between ROS and genotoxic endpoints (DNA damage and micronucleus formation). The correlation between these genotoxic endpoints with ROS indicate that ROS might be responsible for the DNA damage observed in the Comet assay \((r^2=0.99)\) and MN formation observed in the CBMN assay \((r^2=0.96)\) of TiO\(_2\) ENPs (Figure 3.9 A-B).

Glutathione depletion and lipid peroxidation are other important hallmarks of oxidative stress. We assayed the glutathione and lipid peroxidation levels after exposure to TiO\(_2\) ENPs and observed the condition of oxidative stress indicated by elevated hydroperoxide and depleted glutathione levels. Previously, the same observations were made by Gurr et al. (2005) who measured MDA level in human bronchial epithelial cells while Jin et al. (2008) and Reeves et al. (2008) have shown that TiO\(_2\) ENPs induce oxidative stress in mouse fibroblast and fish cells respectively. In the present study the oxidative stress markers (GSH and LPO) were assessed in human epidermal cells and their correlation with ROS was also evaluated. Our data also showed a good correlation between ROS and oxidative stress markers [ROS vs GSH \((r^2=0.93)\) and ROS vs LPO \((r^2=0.85)\)] (Figure 3.9 C-D).

Schins and Knaapen (2007) reported that Reactive oxygen species (ROS) are believed to play a major role in primary genotoxicity mechanism of nanoparticles, which may derive from their surface properties, the presence of transition metals and lipid peroxidation. The data from the present study demonstrated that TiO\(_2\) ENPs induce ROS and oxidative stress leading to genotoxicity in human epidermal cells. This probable primary genotoxicity mechanism may further trigger signal transduction pathways leading to apoptosis or cause interferences with normal cellular processes thereby causing cell death. The possible schematic mechanism of TiO\(_2\) ENPs induced cellular toxicity in human epidermal cells under \textit{in vitro} condition is depicted in Figure 3.10.
Figure 3.9: Regression analyses of % ROS generation with respect to different oxidative stress parameters and DNA damage. Correlation after 6 h exposure to TiO$_2$ ENPs with (A) Olive tail moment (OTM) (B) micronucleus (MN) formation (C) glutathione and (D) hydroperoxide levels.

Figure 3.10: Possible mechanism of TiO$_2$ ENPs induced genotoxicity in human epidermal cells.
The DNA damage, micronucleus formation and oxidative stress markers raise concern about the safety associated with applications of TiO$_2$ ENPs in consumer products. However more studies need to be conducted *in vivo* to fully understand the mechanism of TiO$_2$ ENPs toxicity.