Chapter 4

A Mechanistic Study of Cyto-Genotoxic Potential of TiO$_2$ ENPs
4.1. INTRODUCTION

Engineered nanoparticles (ENPs) are being widely used in electronics, engineering, therapeutics, diagnostic devices, pollutant remediation, personal care products and food/beverages. The increased use of ENPs has gained attention due to their adverse effects on the environment as well as on humans (Adachi et al. 2010). Titanium dioxide engineered nanoparticles (TiO₂ ENPs) are widely used in consumer products, paints, pharmaceutical preparations, food additives etc. and hence the likelihood of human exposure cannot be ignored. Toxicity assessment has shown that they can induce cytotoxic, genotoxic and carcinogenic responses both in vitro and in vivo (Johnston et al. 2009; Trouiller et al. 2009; Chen et al. 2010; Kim et al. 2010). Ultrafine TiO₂ have been shown to produce oxidative stress, DNA damage and inflammatory responses in human bronchial epithelial cells (BEAS-2B) without photo-activation (Gurr et al. 2005). TiO₂ ENPs have also been reported to cause mutations and genotoxic responses in human lymphoblastoid cells (Wang et al. 2007).

Previous studies have shown that interaction of free radicals (O₂⁻ production) with cellular components (nucleus, mitochondria, cytoplasm etc.) exhibit adverse effects (Xia et al. 2006). These interactions result in the structural modification of cysteine, methionine, histidine, tryptophan and other amino acids. ROS also attack DNA and produce chain breaks, modification of carbohydrate parts and nitro bases by oxidation, nitration, methylation or deamination reactions, finally leading to cell death/apoptosis (Song et al. 2005). Additionally, ROS plays a key role in ENPs induced toxicity in cultured mammalian cells but limited data are available for their mechanism of toxicity (Hussain et al. 2010; Sharma et al. 2011).

The use of TiO₂ ENPs as food additive could result in these ENPs reaching the non-target organs such as liver, spleen, lungs etc. Bio-distribution of TiO₂ ENPs leads to its accumulation in liver causing hepatic injury (Wang et al. 2007; Chen et al. 2009). TiO₂ ENPs have been shown to induce oxidative stress and changes in the mitochondrial membrane potential at higher concentrations (100-250 µg/ml) in rat liver cell line (BRL 3A) (Hussain et al. 2010; Sharma et al. 2011).
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2005). Recently, it has also been shown that TiO\(_2\) ENPs (anatase and rutile) induce a genotoxic response as evidenced by up-regulation of p53 and down-regulation of DNA damage genes in human hepatocellular (HepG2) cells (Petkovic et al. 2010). However the concentration of TiO\(_2\) ENPs at which the study was undertaken produces white precipitates. Also in this study the sonication of the particles was carried out in complete culture media which leads to degradation of FBS. It was therefore prudent to conduct a systematic study and investigate the mechanism of genotoxicity as well as to see if the DNA damage leads to apoptosis.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals
Titanium (IV) oxide nanopowder (99.7%, anatase, CAS No.1317-70-0) was purchased from Sigma Chemical Co. Ltd. (St Louis, MO, USA). The other chemicals and kits used in the study are described previously in Chapter 2 (2.1.1).

4.2.2. Size Determination of TiO\(_2\) ENPs using Transmission Electron Microscopy
Samples were prepared for transmission electron microscopy (TEM) analysis as described previously in Chapter 2 (2.2.1.1). The suspension on the grids was allowed to dry and TEM measurements were performed at an accelerating voltage of 120 kV (FEI; Technai G2 F30 S-Twin, Oregon, USA).

4.2.3. Preparation of TiO\(_2\) ENPs Suspension in Culture Media, Characterization and Exposure to HepG2 Cells
TiO\(_2\) ENPs (160 \(\mu\)g/ml) were suspended in IMEM (incomplete minimum essential medium; without FBS) and probe sonicated (Sonics Vibra cell, Sonics & Material Inc., New Town, CT, USA) for 10 min (1.5 min pulse on and 1 min pulse off for 4 times). After sonication, the suspension was diluted in CMEM (complete minimum essential medium; supplemented with 10% FBS) for cell exposure to ensure proper nutrition to the cells. The suspension sonicated did not contain FBS while the exposure media was supplemented with 10% FBS. The diluted TiO\(_2\) ENPs concentrations in CMEM were then characterized by dynamic light scattering (DLS) instrument (Zetasizer Nano-
ZS equipped with 4.0 mW, 633 nm laser (Model ZEN3600, Malvern instruments Ltd., Malvern, UK). Detail procedure described in chapter 2 (2.2.1.2). The size was also confirmed by TEM, detail procedure was described in Chapter 2 (2.2.1.1). The human hepatocellular liver carcinoma cell line (HepG2) was obtained from National Centre for Cell Sciences, Pune, India and cultured in MEM supplemented with 10% FBS, 0.2% sodium bicarbonate and 10 ml/L antibiotic and antifungal solution at 37ºC under a humidified atmosphere of 5% CO₂.

Stock suspension of TiO₂ ENPs (160 µg/ml) in IMEM was diluted to concentrations ranging from 1 - 80 µg/ml (corresponding to 0.31 to 25 µg/cm²) in CMEM respectively. HepG2 cells were exposed at varying concentrations for each experiment. Subsequent experiments involving cellular uptake assay; cytotoxicity assays; genotoxicity assays (Comet assay, micronucleus induction); oxidative stress marker (ROS generation, glutathione depletion and lipid peroxidation); apoptosis markers (mitochondrial membrane potential assay, Annexin-V and immunoblot assay) were conducted and analyzed. For different assays, 96, 12, 6 well cell culture plates and 75 cm² cell culture flasks were used having a treatment volume of 0.1 ml, 1.2 ml, 3 ml and 24 ml respectively. However, the concentration per cm² area remained the same in all treatment regimes.

4.2.4. Cellular Uptake of TiO₂ ENPs

4.2.4.1. Flow cytometric detection of TiO₂ ENPs

Flow cytometric detection of TiO₂ ENPs was carried out according to the method of (Suzuki et al. 2007) using light scattering principles. Cells were exposed to TiO₂ ENPs (1, 10, 20, 40 and to 80 µg/ml) for 6h. The cells were harvested and samples were prepared for flow cytometric analysis as described previously in Chapter 2. (2.2.2.1)

4.2.4.2. Transmission electron microscopy of TiO₂ ENPs treated HepG2 cells

Ultrathin sections of cells were analyzed using TEM to reveal the sub-cellular localization of TiO₂ ENPs in HepG2 cells. Samples were prepared and analyzed for TEM as described in Chapter 2 (2.2.2.2).
4.2.5. Cytotoxicity Assays
Cytotoxicity assessment of TiO$_2$ ENPs was determined by MTT and neutral red uptake (NRU) assay as described in Chapter 2 (2.2.4.1 and 2.2.4.2). Briefly, 1X10$^4$ cells/well were seeded in 96-well plates and kept for 24 h. They were then exposed to different concentrations of TiO$_2$ ENPs (1, 10, 20, 40 and 80 µg/ml) for varying time intervals (6, 24 and 48 h). Nanoparticle interference with the assay reagents was also checked, using a cell free system. The results were assessed by measuring the absorbance of end product at their respective wavelengths using a SYNERGY-HT multiwell plate reader, Bio-Tek (USA) using KC4 software. Detail procedure to check the interference of NPs with dyes is described previously in chapter 2 (2.2.4.3).

4.2.6. Genotoxicity Assessment
The genotoxic potential of TiO$_2$ ENPs was assessed by Fpg-modified Comet assay and cytokinesis-block micronucleus (CBMN) assay. The treatment scheme was same for both assays. Approximately, 7X10$^4$ cells in 1.2 ml of MEM were seeded in a 12-well cell culture plate. After 24 h, the cells were exposed to TiO$_2$ ENPs (1, 10, 20, 40 and 80 µg/ml) for 6 h.

4.2.6.1. Fpg-Comet assay for detection of oxidative DNA damage
Cells were harvested and processed as given below:

The protocol involving lesion specific DNA repair enzyme, formamidopyrimidine DNA glycosylase (Fpg) enzyme conjugated with Comet assay to identify the 8-oxoguanine and other damaged bases was performed according to (Collins et al. 1996). Briefly, cells were harvested and slides were prepared according to the method of (Singh et al. 1988) and as per the Comet assay guidelines (Tice et al. 2000). Protocol was followed as described in Chapter 2 (2.2.5.1).

4.2.6.2. Cytokinesis-block micronucleus (CBMN) assay
The cytokinesis-block micronucleus (CBMN) assay was carried out by a slightly modified method of (Fenech 2000). Detailed procedure is described in Chapter 2 (2.2.6.1).

Harvesting, slide preparation, staining and scoring were also conducted as described in Chapter 2 (2.2.6.1).
4.2.7. 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 (1, 10, 20, 40, 80 µg/ml) for 6 h. After harvesting they were washed twice with chilled PBS, centrifuged at 500Xg with pellet re-suspended in PBS and sonicated. Protein content was measured by Bradford’s method (Bradford 1976).

4.2.7.1. Glutathione estimation

Treated cell lysate was used for estimation of total glutathione (GSH) content expressed as micro moles/mg protein as described by (Ellman 1959)

4.2.7.2. 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 were performed as described in Chapter 2 (2.2.7.1 and 2.2.7.2).

4.2.8. Measurement of Intracellular Reactive Oxygen Species

The level of intracellular ROS generation was estimated by the method of Wan et al. (1993) as modified by Wilson et al. (2002) using 2, 7-dichlorofluorescein diacetate (DCFDA) dye.

The interference and auto fluorescence of TiO₂ ENPs with DCFDA was also monitored in a parallel experiment without cells. Percent ROS generation was calculated using the following formula, after correcting for background fluorescence:

\[
\% \text{ ROS generation} = \left( \frac{F_{485/528_{\text{sample}}} - F_{485/528_{\text{sample blank}}}}{F_{485/528_{\text{control}}} - F_{485/528_{\text{control blank}}}} \right) \times 100
\]

The detailed method was described in Chapter 2 (2.2.7.4).

4.2.9. Apoptosis Markers

4.2.9.1. Mitochondrial membrane potential

Mitochondrial membrane potential (Δψ) was measured using lipophilic cationic dye JC-1 which selectively enters mitochondria and changes its color reversibly from red to green if membrane potential decreases. Detailed procedure was described in Chapter 2 (2.2.8.1).
4.2.9.2. Annexin V binding assay
Actively undergoing apoptotic cells were identified by staining with fluorescein isothiocyanate-conjugated (FITC)-Annexin V and PI according to manufacturers’ protocol (BD Biosciences, San Jose, CA, USA). Protocol has been described in Chapter 2 (2.2.8.2).

4.2.10. Immunoblot Analysis
HepG2 cells were treated with TiO$_2$ ENPs at concentrations 20, 40 and 80 µg/ml for 48 h. After treatment removal, cells were harvested and followed as a standard procedure described in Chapter 2 (2.2.9.2) for immunoblot analysis.
Densitometric analysis was carried out using Quantity One Quantitation Software® version 4.3.1 (Bio-Rad, USA).
4.3. RESULTS
The study revealed the uptake and cellular internalization of TiO₂ ENPs in HepG2 cells. These particles upon internalization produced genotoxic effects, oxidative DNA damage and disruption of mitochondrial membrane, thereby leading to apoptosis. The results obtained are described in detail below:

4.3.1. Measurement of TiO₂ Engineered Nanoparticles
The size of TiO₂ ENPs as revealed by TEM ranged between 30-70 nm (Figure 4.1). DLS measurements showed a mean hydrodynamic diameter of TiO₂ ENPs in Milli Q and culture media to be 124.9 nm and 192.5 nm respectively (Table 4.1). However, the zeta potential changed from -17.6 mV in Milli Q to -11.4 mV in culture media (MEM supplemented with 10% FBS).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Dispersant</th>
<th>Hydrodynamic size (d.nm)</th>
<th>Poly dispersity index (PDI)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Culture medium (CMEM)</td>
<td>192.5±2.00</td>
<td>0.18±0.01</td>
<td>-11.4±0.25</td>
</tr>
<tr>
<td>2</td>
<td>Milli Q</td>
<td>124.9±3.20</td>
<td>0.12±0.01</td>
<td>-17.6±0.48</td>
</tr>
</tbody>
</table>

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

4.3.2. Cellular Uptake of TiO₂ ENPs
Flow cytometric analysis revealed a significant (p<0.05) concentration-dependent increase in the cellular internalization of TiO₂ ENPs after 6 h exposure (Figure 4.2). This was evident by an increase in the side scatter intensity (granularity) of TiO₂ ENP treated cells in a concentration-dependent manner (Figure 4.3). Further, sub-cellular localization of TiO₂ ENPs inside cytoplasm and nucleus was confirmed using transmission electron microscopy (Figure 4.4).
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Figure 4.1: TEM microphotograph of TiO$_2$ ENPs

Figure 4.2: Analysis of internalization of ENPs by flow cytometric parameter viz, side scatter intensity. HepG2 cells exposed to TiO$_2$ ENPs (0–80 µg/ml) for 6 h. Results expressed as mean ± SE from three independent experiments (*P<0.05).
Figure 4.3: Dot plot showing internalization of TiO₂ ENPs in HepG2 cells using flow cytometer. HepG2 cells exposed to TiO₂ ENPs (0–80 µg/ml) for 6 h.

Figure 4.4: Transmission Electron photomicrographs of HepG2 cells showing internalization of TiO₂ ENPs in (A-B) control, (C) cytoplasm and (D) nucleus. Arrows indicate the presence of TiO₂ ENPs.
4.3.3. Evaluation of TiO₂ ENPs Induced Cytotoxicity

Cytotoxicity of TiO₂ ENPs was assayed for 6, 24 and 48 h respectively. In the MTT assay, mitochondrial succinate dehydrogenase activity in HepG2 cells was reduced to 82% and 75% (relative to 100% of control) at 40 and 80 µg/ml after 24 h exposure which further reduced up to 79% and 68% respectively after 48 h exposure (Figure 4.4 A). NRU uptake assay showed that the dye uptake was reduced to 82% and 81% at 40 µg/ml treatment of TiO₂ ENPs whereas it further reduced to 79% and 73% at 80 µg/ml at 24 and 48 h exposure when compared to control (Figure 4.4 B). However, no significant cytotoxicity of TiO₂ ENPs was observed at 6 h exposure in both the assays.

Figure 4.5: Concentration and time dependent cytotoxicity of TiO₂ ENPs in HepG2 cells. (A) % MTT reduction, (B) % Neutral red uptake. The viability of the control cells was considered as 100%. The data are expressed as means ± SEM from three independent experiments. * p<0.05, when compared to control.
4.3.4. Genotoxic Potential of TiO₂ ENPs

4.3.4.1. Oxidative DNA damage using Fpg-Comet assay

The Fpg-modified Comet assay revealed a significant (p<0.05) concentration-dependent increase in oxidative DNA damage in response to TiO₂ ENPs exposure as analyzed using qualitative and quantitative parameters of the Comet assay viz. Olive tail moment (OTM) and % Tail DNA respectively (Table 4.2). A statistically significant (P<0.05) induction in DNA damage was observed at the different concentration of TiO₂ ENPs (10-80 µg/ml) after 6 h exposure as compared to the respective control cells in standard Comet assay. However, TiO₂ ENPs induced significant oxidative DNA damage even at 1 µg/ml concentration as evident by the Fpg treatment (Table 4.2). When compared among the groups, Fpg elicited a significantly greater response at all the concentrations of TiO₂ ENPs (1, 10, 20, 40 and 80 µg/ml) as evident by the Comet assay parameter.

Table 4.2 - DNA damage in HepG2 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>0.94±0.06</td>
<td>0.96±0.04</td>
</tr>
<tr>
<td>H₂O₂ (25 µM)</td>
<td>3.14±0.26*</td>
<td>5.04±0.18*</td>
</tr>
<tr>
<td>TiO₂ ENPs (1 µg/ml)</td>
<td>1.13±0.06</td>
<td>1.35±0.13*</td>
</tr>
<tr>
<td>TiO₂ ENPs (10 µg/ml)</td>
<td>1.20±0.05*</td>
<td>1.58±0.08*</td>
</tr>
<tr>
<td>TiO₂ ENPs (20 µg/ml)</td>
<td>1.40±0.02*</td>
<td>1.95±0.17*</td>
</tr>
<tr>
<td>TiO₂ ENPs (40 µg/ml)</td>
<td>1.55±0.07*</td>
<td>2.18±0.10*</td>
</tr>
<tr>
<td>TiO₂ ENPs (80 µg/ml)</td>
<td>1.76±0.09*</td>
<td>2.81±0.12*</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E. of three experiments. * H₂O₂- Hydrogen peroxide (Positive control). * p < 0.05 when compared to control using one way ANOVA; α p < 0.05 using when compared to Fpg (-) at the same concentration using Student ‘t’ test.

4.3.4.2. Micronucleus induction

TiO₂ ENPs induced a statistically significant (p<0.05) increase in the number of micronucleated cells at 20 µg/ml (15.00 MN/1000 BNCs) when compared to the control (7.00 MN/1000 BNCs) after 6 h exposure. However, on further
increasing the concentrations (40 and 80 µg/ml), the micronucleus formation decreased (Table 4.3). A large number of TiO$_2$ ENPs were also seen at these concentrations (Figure 4.6).

![Figure 4.6: Photomicrographs of HepG2 Cells showing CBMN assay. (A) Field view of control cells, (B) Field view of TiO$_2$ ENPs exposed cells. Magnification X 400. (C) Control binucleate cell, (D) TiO$_2$ ENPs exposed binucleate cell showing micronucleus indicated by black arrow. (E-F) TiO$_2$ ENPs exposed binucleate cells showing presence of nanoparticles at the position of micronucleus indicated by white arrow.]

**Table 4.3 - Effect of TiO$_2$ ENPs on micronucleus formation in HepG2 cells**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of MN / 1000 binucleated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.00 ± 0.58</td>
</tr>
<tr>
<td>EMS* (6 mM)</td>
<td>23.33 ± 1.45***</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (1 µg/ml)</td>
<td>8.00 ± 1.15</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (10 µg/ml)</td>
<td>11.00 ± 1.53*</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (20 µg/ml)</td>
<td>15.00 ± 0.58**</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (40 µg/ml)</td>
<td>12.33 ± 0.33**</td>
</tr>
<tr>
<td>TiO$_2$ ENPs (80 µg/ml)</td>
<td>10.67 ± 0.88*</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E. of three experiments for each concentration.

* EMS- ethyl methanesulfonate-positive control. *p < 0.05; **p < 0.01; ***p < 0.001; when compared to control.
4.3.5. Evaluation of Oxidative Stress

TiO$_2$ ENPs caused a significant (p<0.05) concentration-dependent increase in intracellular ROS (77.78%, 114.83%, 131.59%, 143.65% at concentration of 10, 20, 40 and 80 µg/ml respectively) as evident by increase in the fluorescence intensity of DCFDA dye (Figure 4.7 A-D; Figure 4.8 A).

![Figure 4.7](image)

**Figure 4.7:** Photomicrographs showing the generation of intracellular reactive oxygen species (ROS) using DCFDA dye in human hepatocellular cells (A) Control cells; (B-D) Cells exposed to TiO$_2$ ENPs (20 µg/ml, 40 µg/ml and 80 µg/ml respectively) for 6 h showing increase in fluorescence (Magnification-X200).

A similar effect was observed on the antioxidant defense system where significant (p<0.05) decrease in intracellular GSH levels (19.39%, 25.07% and 29.51%) of HepG2 cells was observed (20, 40 and 80 µg/ml) at 6 h exposure of TiO$_2$ ENPs (Figure 4.8 B). TiO$_2$ ENPs also caused lipid peroxidation as a concentration-dependent statistically significant (p<0.05) increase in hydroperoxide concentration was observed at 20 µg/ml (54.06%), 40 µg/ml (59.05%) and 80 µg/ml (87.35%; Figure 4.8 C).
Figure 4.8: Effects of TiO$_2$ ENPs on (A) ROS, (B) GSH and (C) LPO levels in HepG2 cells. t-BOOH (200 µM) was used as positive control. Data represents means ± SEM from three independent experiments. *p<0.05, when compared to control.
4.3.6. Extent and Mode of Apoptosis

HepG2 cells treated with TiO$_2$ ENPs (20, 40 and 80 µg/ml) for 24 h demonstrated alteration in the mitochondrial membrane integrity as evidenced by JC-1 dye analyzed using flow cytometry (Figure 4.9 A).
Figure 4.9: TiO$_2$ ENPs induced loss of mitochondrial membrane potential (MMP) in HepG2 cells (A) distribution of JC-1 aggregates and monomers after 24 h exposure (B) bar graph shows the percentage of JC-1 monomer positive cells (%MMP loss). Camptothecin (1 µM) was used as positive control. Data of % MMP loss are expressed as mean ± SEM from three independent experiments.*p<0.05, when compared to control.

An increase in the green fluorescence intensity (7.6±0.21%, 14.8±1.4% and 16.9±0.82%) was observed when compared to control (4.8±0.27%; Figure 4.9 A-B). Further, early and late apoptotic cells were detected using Annexin V-PI dual staining assay. The data revealed the presence of apoptotic and necrotic cells upon TiO$_2$ ENPs exposure at concentrations of 20, 40 and 80 µg/ml after a 48 h exposure. Early apoptotic cells increased from 4.2% (control) to 14.5% (20 µg/ml), 22.6% (40 µg/ml), 21.7% (80 µg/ml) whereas late apoptotic cells increased 1.5% (control) to 8.9% (20 µg/ml), 8.9% (40 µg/ml), 10% (80 µg/ml). Similarly, necrosis was also observed in a dose dependent manner with maximum (11%) at 80 µg/ml (Figure 4.10 A-B). To evaluate the mode of action of apoptosis by TiO$_2$ ENPs, induction of key apoptotic marker proteins were examined.
Immunoblot analysis of HepG2 cells treated with TiO$_2$ ENPs (40 and 80 µg/ml) for 6 h exposure showed significant increase (p<0.05) in the expression of stress proteins hsp60 (54%, 80%), hsp70 (30%, 62%) and for 48 h exposure showed significant up-regulation in the expression of tumor suppressor protein p53 (43%, 60%), cytochrome c (35%, 52%), Bax (18%, 24%), caspase-9 (37%, 49%), caspase-3 (21%, 29%), Apaf1 (22%, 34%) with down-regulation of anti-apoptotic mitochondrial protein Bcl-2 (28%, 37%) as shown in Figure 4.11 B and C.
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**A**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Hsp70</th>
<th>Hsp60</th>
<th>β-Actin</th>
</tr>
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<tbody>
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<td>Control</td>
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<td><img src="image3" alt="Image of β-Actin" /></td>
</tr>
<tr>
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<td><img src="image6" alt="Image of β-Actin" /></td>
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<tr>
<td>40µg/ml</td>
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<td><img src="image8" alt="Image of Hsp60" /></td>
<td><img src="image9" alt="Image of β-Actin" /></td>
</tr>
<tr>
<td>80µg/ml</td>
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<td><img src="image11" alt="Image of Hsp60" /></td>
<td><img src="image12" alt="Image of β-Actin" /></td>
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</tbody>
</table>

**A'**

![Bar chart](image13)

Fold change relative to control

*P < 0.05

**B**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>p53</th>
<th>Bcl-2</th>
<th>Bax</th>
<th>β-Actin</th>
</tr>
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<td><img src="image16" alt="Image of Bax" /></td>
<td><img src="image17" alt="Image of β-Actin" /></td>
</tr>
<tr>
<td>20µg/ml</td>
<td><img src="image18" alt="Image of p53" /></td>
<td><img src="image19" alt="Image of Bcl-2" /></td>
<td><img src="image20" alt="Image of Bax" /></td>
<td><img src="image21" alt="Image of β-Actin" /></td>
</tr>
<tr>
<td>40µg/ml</td>
<td><img src="image22" alt="Image of p53" /></td>
<td><img src="image23" alt="Image of Bcl-2" /></td>
<td><img src="image24" alt="Image of Bax" /></td>
<td><img src="image25" alt="Image of β-Actin" /></td>
</tr>
<tr>
<td>80µg/ml</td>
<td><img src="image26" alt="Image of p53" /></td>
<td><img src="image27" alt="Image of Bcl-2" /></td>
<td><img src="image28" alt="Image of Bax" /></td>
<td><img src="image29" alt="Image of β-Actin" /></td>
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</tbody>
</table>

**B'**

![Bar chart](image30)

Fold change relative to control

*P < 0.05
Figure 4.11: Immunoblot analyses of proteins of HepG2 cells treated with different concentrations (20, 40 and 80 µg/ml) of TiO$_2$ ENPs. β-actin was used as an internal control. (A) stress proteins (Hsp60, Hsp70; 6 h exposure); (B-C) apoptotic proteins (P53, Bax, and Bcl-2, Apaf1, caspase-9, caspase-3 and cyto c; 48 h exposure) and the corresponding (A’, B’ and C’) bar graphs exhibiting their densitometric analysis.
4.4. DISCUSSION
The present study has systematically examined the effects of TiO$_2$ ENPs in human liver cells (HepG2 cells). It has been shown that TiO$_2$ ENPs induce oxidative DNA damage leading to apoptosis in HepG2 cells. Characterization of ENPs is essential due to the fact that shape, size, surface area, surface charge, monodispersity etc. affects physiochemical properties responsible for differential responses observed within biological systems (Dhawan et al. 2009; Taurozzi et al. 2010; Zhang et al. 2010). Studies so far have used a variety of methods for size determination of nanoparticles in dry powder form using TEM and in liquid suspension or culture media using DLS respectively ((Sharma et al. 2009; Hsiao and Huang 2011; Kumar et al. 2011; Sharma et al. 2011)). Our TEM measurements and DLS analysis showed that TiO$_2$ ENPs were stable and mono-dispersed in culture media, making them suitable for toxicity studies.

Since these ENPs have smaller distribution size range, they can easily enter and localize inside cells. The present study through TEM measurements demonstrates that TiO$_2$ ENPs get internalized into the cell and localize in the cytoplasm as well as the nucleus. This could help in explaining the oxidative stress and DNA damage observed. These findings were also consistent with our flow cytometry analysis where concentration-dependent increase in the intensity of side scatter (due to particle uptake) was observed. Similar studies have been carried out using CHO, primary culture and human lung epithelial cells (BEAS 2B) (Suzuki et al. 2007; Xu et al. 2009; Zucker et al. 2010).Distribution of these TiO$_2$ ENPs inside cells would therefore enable interactions with biological macromolecules, including lipids, proteins and nucleic acids thereby eliciting toxic responses.

Studies so far have focused on cytotoxic, genotoxic effects mediated by oxidative stress (Di Virgilio et al. 2010; Ghosh et al. 2010; Kim et al. 2010; Pujalte et al. 2011). Recently, it has been reported in HepG2 cells (Petkovic et al. 2010) that exposure to TiO$_2$ ENPs at 250 µg/ml concentration leads to two fold increase in ROS levels at 5 h exposure duration thereby causing DNA damage. Since no data on the stability of the particles was provided, it is
difficult to interpret the results from such a study using high concentrations. Our earlier studies with TiO$_2$ (Anatase) purchased from Sigma Aldrich (USA) company have shown that the particles tend to agglomerate and even precipitate at concentrations above 80 µg/ml in the absence of a stabilizer such as propylene glycol (Gurbani et al. 2011).

In the present study we used a range of TiO$_2$ ENPs concentrations (1, 10, 20, 40 and 80 µg/ml) for different time points (6, 24 and 48 h). Initially the cytotoxicity experiments were performed for all the concentrations and time points. The cells were more than 90% viable after 6 h exposure of TiO$_2$ ENPs. Hence, these non-cytotoxic concentrations and time point were used for genotoxicity studies and their mechanistic studies (oxidative stress).

However, our data also exhibit that only at 20, 40 and 80 µg/ml treatment concentration induces significant cytotoxicity after 24 and 48 h exposure. Hence, these concentrations were further used for mitochondrial membrane potential and annexin V binding assay to demonstrate apoptosis. Our data exhibit a significant MMP loss after 24 h exposure but the annexin V binding was not significant at the same time. Hence the annexin V binding assay and immunoblot analysis were performed after 48 h exposure at concentrations 20, 40 and 80 µg/ml.

Our study showed that TiO$_2$ ENPs induce stress in HepG2 cells as evident by an induction of Hsp 60 and Hsp 70. Hsp family of proteins is the first tier of cellular defense that induces the protein folding to minimize degradation and cellular stress. Further, >2fold induction in ROS generation, depletion in GSH levels with a concomitant increase in LPO revealed that TiO$_2$ ENPs induce oxidative stress. Our findings are in accordance with the previous studies in different cell types where TiO$_2$ has been shown to produce ROS even without photo-activation (Park et al. 2008; Xue et al. 2010).

Further oxidative DNA damage was observed in the HepG2 cells after treatment with TiO$_2$ ENPs. This could be due to the ROS generation even at lower concentration 1µg/ml of TiO$_2$ ENPs. 8-Hydroxy- deoxyguanosine (8-OHdG), is the major oxidative DNA-damage product that can produce mutations - A: T to G: C or G: C to T: A transversion mutations- since it base pairs with adenine as well as cytosine (Valko et al. 2004). The data from Fpg-
Comet strengthened our assumption that TiO$_2$ ENPs induces ROS mediated oxidative DNA damage. Further, TiO$_2$ ENPs significantly induced the formation of micronucleus at lower concentrations (1-20 µg/ml). However, at higher concentrations (40 and 80 µg/ml), number of micronucleus were slightly decrease than the lower concentration (20 µg/ml) but was statistically significant ($p<0.05$) as compared to control. This may be due to the deposition of TiO$_2$ ENPs on the slides during the slide preparation which hinders the counting of micronucleus as reported earlier (Falck et al. 2009; Di Virgilio et al. 2010).

The present study also investigated the induction of apoptosis by TiO$_2$ ENPs in HepG2 cells. Our data revealed the presence of early apoptotic cells as evident by a decrease in the mitochondrial membrane potential at 24 h. TiO$_2$

**Figure 4.12:** A schematic showing possible mechanisms of TiO$_2$ ENPs induced cellular toxicity in HepG2 cells.
ENPs have also been shown to induce oxidative stress leading to apoptosis in cultured rat neuronal cells (PC-12), human lung epithelial cells (BEAS-2B cells) and mouse epidermal cells (JB-6) (Park et al. 2008; Zhao et al. 2009; Liu et al. 2010; Shi et al. 2010). However at 48 h, cells undergoing late apoptosis and necrosis were observed by Annexin V/PI assay. To decipher the mechanism behind mitochondrial mediated apoptosis, p53 levels were measured in HepG2 cells after exposure to TiO₂ ENPs.

The immunoblot analysis showed a concentration-dependent increase in the expression of p53 in manner. Our data exhibited a concentration-dependent increase in the expression of BAX (pro-apoptotic) and decrease in levels of Bcl2 (anti-apoptotic). This could be due to increased p53 levels which results in the modulation in the Bax/Bcl2 ratio. This leads to the release of cytochrome c which binds to the apoptotic protease activating factor (Apaf-1) resulting in the formation of an apoptosome. Our data also suggest that caspase-9 and caspase-3 activation leads to cascade of events that trigger cell death in TiO₂ ENPs treated HepG2 cells. A pathway for TiO₂ ENP induced apoptosis in HepG2 cells is depicted in Figure 4.12.

These findings suggest that induction of apoptosis by TiO₂ ENPs triggered by the activation of mitochondrial intrinsic pathway, whereas some of the studies have demonstrated that TiO₂ ENPs can induce apoptosis via caspase 8 dependent Bid pathway (Kang et al. 2009; Hussain et al. 2010).

The present study systematically demonstrates the role of mitochondrial intrinsic pathway for TiO₂ ENP induced apoptosis in HepG2 cells which could be attributed to ROS mediated DNA damage.