Titanium Dioxide (TiO$_2$) Nanoparticles Induced ROS Generation and its Effect on Cellular Antioxidant Defense in WRL-68 Cell

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Abstract- The nanosized titanium dioxide (nano-TiO$_2$) is produced abundantly and used widely in the chemical, electrical/electronic and energy industries because of its special photovoltaic and photocatalytic activities. Previous reports have shown that the nano-TiO$_2$ can enter into the human body through different routes such as inhalation, ingestion, dermal penetration and injection. The effects of nano-TiO$_2$ on different organs are being investigated and the concerns on its large scale applications such as sunscreen, etc. In this study, the cytotoxicity of the nano-TiO$_2$ was investigated in WRL-68 cells. The human hepatic cell line (WRL-68) was used to evaluate molecular mechanism involved for toxic effect of TiO$_2$ NPs. The uptake of TiO$_2$ NPs in WRL-68 cells was monitored by measuring SSC intensity with maximum at 1000 μM. The ROS generated at concentration 1000μM of TiO$_2$ NPs in WRL-68 cells was 125.12 %. Moreover, ROS induced methylation of CpG island II on the catalase promoter and down regulated catalase expression at the transcriptional level in WRL-68 cell. Subsequently, proliferation of WRL-68 cells was increased on exposure to TiO$_2$ NPs as demonstrated by MTT and NRU assay. Conclusively, it is demonstrated that exposure of TiO$_2$ NPs at 1000 μM for 24h in WRL-68 cell induced methylation of CpG island II via ROS on the catalase promoter and down regulated catalase expression at the transcriptional level.

Keywords: TiO$_2$ NPs; ROS; WRL-68 cell; Catalase; MTT assay.

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1. Introduction

Titanium dioxide nanoparticles (TiO₂ NPs) have been widely used in manufacturing [4], in the environment to decontaminate air, soil, and water, and more recently in consumer products, car materials, and rubber [5,6]. Such widespread use and toxicological studies carried out in the last 10 years have shown that TiO₂ NPs caused oxidative stress-mediated toxicity in cells [7-10], alveolar macrophages [9,11], DNA [9,12], and neurological lesion [13]. However, the influences of TiO₂ NPs on human health are quite uncertain and less known. Recently, the potential impacts of nanoparticles on humans and the environment have greatly attracted the attention of scientists, industries, and regulatory issues of governments [8,14,15]. Numerous scientists’ work has given one main mechanism that the adverse health effects of TiO₂ NPs are caused by oxidative stress [8,16]. They revealed that oxidative stress occurs when reactive oxygen species (ROS) disrupt the balance between oxidative pressure and antioxidant defense. ROS (such as hydroxyl radical, superoxide, etc.) could be produced by photo-activated, some chemicals on the particle surface, or a consequence of the interaction between particles and cellular components [16,17]. The mitochondria are the target of TiO₂ NPs that have been phagocytosed by cells as well as a source for ROS production, and the disruption of mitochondria would also lead to the increase in ROS production, then the decrease of mitochondrial membrane potential and activation by apoptosis. Furthermore, ROS can also cause damage to protein, lipids, and DNA in cells. However, some studies have reported anatase–TiO₂ NPs to be more biologically active than rutile–TiO₂ NPs in terms of cytotoxicity [18]. It was demonstrated [19] that pure anatase– TiO₂ NPs induce cell necrosis and membrane leakage, but do not generate ROS. In contrast, rutile–TiO₂ NPs initiate apoptosis through the formation of ROS. In previous study we have reported that CuO nano particals can leads to hyper methylation of promoter sequence of catalase thus lead to epigentic gene silencing in WRL-68,human hepatic cell line.

II. Materials and Methods

a) Cell culture

The WRL-68 cells (passage number 36) was procured from NCCS, Pune. WRL-68 cells were cultured at 37 °C in 5% CO₂ in Minimum Essential Medium (MEM) +10% FBS (Invitrogen, Carlsbad, CA, USA). For TiO₂ treatment, TiO₂ NPs are added in the medium at different doses for 24h as indicated in the legend.

b) Estimation of TiO₂ NPs uptake by flow cytometry

The cellular uptake of TiO₂ NPs in WRL-68 human hepatic cell line was carried out using flow Cytometry according to Suzuki et al., (2007) [45]. Cells were seeded in 6-well culture plates at a density of
1.0x10^6 cells/well, after about 12-15hrs, the cells were exposed at concentration 250, 500, 750 and 1000μM of TiO₂ NPs for 24 h respectively. The cells were then harvested and collected in a sterile centrifuge tube which was centrifuged at 800 rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in 500μl of 1xPBS. The uptake was determined by flow cytometer equipped with a 488nm laser.

c) **Measurement of intracellular reactive oxygen species generation**

The production of intracellular ROS was measured using 2,7-dichlorofluorescin diacetate (DCFH-DA) as described by Zhao et al., (2011) with some modifications [46]. In brief, WRL-68 cells were seeded in 96-well Black Bottom plate and allowed to adhere. The treated cells were washed with PBS and incubated for 30 min in dark in PBS containing DCFH-DA (10 mM). The control and treated cells were read at Excitation: 485, Emission:528, and Gain: 35,45,5,65,85 by use of a SYNERGY-HT multiwell plate reader, Bio-Tek (USA) using KC4 software.

d) **MTT assay**

MTT assay was done according to method of Mosmann (1983) [47]. Cells (10,000/well in 100μl medium) were seeded in 96 well plate and allowed to adhere overnight. Medium was aspirated and cells were incubated with TiO₂ NPs (250, 500, 750 and 1000μM) at 37 °C for 24 h, respectively and for 3h with MTT dye (5 mg /10ml pbs). The reaction mixture was carefully aspirated and the resulting formazan crystals were solubilized by adding 100 μl dimethylsulphoxide. After 10min, absorbance was read at 570 nm in a SYNERGY-HT multiwell plate reader, Bio-Tek (USA) using KC4 software.

e) **NRU ASSAY**

NRU assay was done according to method of Mosmann (1983) [47]. Cells (10,000/well in 100μl medium) were seeded in 96 well plate and allowed to adhere overnight. Medium was aspirated and cells were incubated with TiO₂ NPs (250, 500, 750 and 1000μM) at 37 °C for 24 h, respectively and for 2h with NRU dye (4 mg/ 10 ml in PBS). The reaction mixture was carefully aspirated and the cells were destained by adding 150 μl destaining solution. Absorbance was read at 540 nm in a SYNERGY-HT multiwell plate reader, Bio-Tek (USA) using KC4 software.

f) **Methylation-specific polymerase chain reaction (MSP)**

The methylation status of the catalase promoter was determined by primers designed for MSP using the Methyl Primer Express software (Applied Biosystems).

g) **Real-time quantitative PCR**

WRL-68 cells were cultured in 6-well plates and exposed to 1000μM of TiO₂ NPs for 24h. At the end of exposure, Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The integrity of RNA was visualized on 1% agarose gel using gel documentation system. The first strand cDNA was synthesized from 1 mg of total RNA by Reverse Transcriptase using M-MLV (Promega, Madison, WI) and oligo (dt) primers (Promega) according to the manufacturer’s protocol. Quantitative real-time PCR (RT-PCR) was performed by QuantiTect SYBR Green PCR kit (Qiagen) using ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) with specific primers designed using Primer Express software (Applied Biosystems). 1 microliter of template cDNA was added to the final volume of 10 ml of reaction mixture. Realtime PCR cycle parameters included 10 min at 95 °C followed by 40 cycles involving denaturation at 95°C for 15 s, annealing at 60 °C for 15 s and elongation at 72 °C for 15 s. All the realtime PCR experiments were performed in triplicate and data expressed as the mean of three independent experiments.

h) **Catalase activity assay**

The catalase activity was assayed by Aebi (1984) method [60]. Briefly, 0.1 ml of cell lysates was added to 1.5 ml of freshly prepared 13.2 mM H₂O₂ in 0.05 M K₂HPO₄ (pH 7.0) buffer. The rate of decomposition of H₂O₂ was monitored spectrophotometrically at 240 nm. Catalase activity is expressed as U/mg protein.

### III. Results

a) **Estimation of TiO₂ NPs uptake in WRL-68 cells**

The uptake of TiO₂ NPs in WRL-68 cells was determined by using flow cytometry. The SSC intensity represents granularity of a cell and FSC represents size of the cell. Nanoparticle uptake is considered to increase proportionally with increase in side scatter intensity of the cell. Table 1 shows concentration dependent increase in uptake of TiO₂ NPs attributed by increase in intensity of SSC 30.99%, 97.30%, 100.15%, 125.12% respectively. Result shows increase in granularity but size of cells remains constant.

b) **Effect of TiO₂ NPs exposure on proliferation of WRL-68 cells**

The epigenetic regulation of an antioxidant enzyme may subsequently result in increase in cell proliferation. The proliferation of WRL-68 cells was found to be increased with increase in concentration of TiO₂ NPs as attributed by fig 1 and fig 2.
c) Intracellular reactive oxygen species (ROS) measurement

ROS generation plays a key role in toxicity of NPs in mammalian cells [41]. Fig. 3 shows an increase in % ROS generation in WRL-68 cells on treatment with increase in concentration of TiO$_2$ NPs in WRL-68 cells.

d) MSP analysis and RT-PCR

WRL-68 cells were treated with TiO$_2$ NPs at 750 μM and 1000 μM for 24 h. The effect of ROS generated by TiO$_2$ NPs on the methylation status of the catalase promoter was analyzed using MSP analysis (fig. 4). We specifically selected the CpG island II region located in the catalase promoter. The methylation of CpG island II was observed at 24 h. RT-PCR was performed within the same cells, demonstrated that messenger RNA (mRNA) (fig. 5) expression was also found to be reduced as compared to control. The catalase enzyme activity was determined in order to validate the results obtained from RT-PCR. Result show decrease in catalase enzyme activity with increase in Concentration of nano-TiO$_2$ as showed in Fig. 6.

IV. Discussion

Nanotechnology involves the creation and manipulation of materials at nanoscale to create products that exhibit novel properties [48]. Nanomaterials, which range from 1 to 100 nm, have been used to create unique nanosized devices possessing novel physical and chemical properties [49]. Because of these special properties, nanomaterials are widely used in many fields. nano-TiO$_2$, a kind of nanomaterials, is widely used because of its unusual properties. Therefore the potential risk of nanoparticles to biological systems is needed to be investigated. Several studies had reported that the nano-TiO$_2$ can cause the damage of different cells. The Nano TiO$_2$ particles could be incorporated into cellular membranes, and might be endocytosed from the extracellular fluid and made fused with lysosomes, then led to the damage and destruction of organelles [48-49]. In this study the cytotoxicity of the nano-TiO$_2$ was assessed in WRL-68 cells cultured with different nanoparticle concentrations (250μM, 500μM, 750μM, 1000μM). We worked on commercial form which is already been characterized by the manufacture. In this study, the viability of WRL-68 cells incubated with the nano- TiO$_2$ was tested. The MTT test showed that cell viability was greatly increased in a concentration dependent and time-dependent manner (Fig. 1). The results indicated that the nano-TiO$_2$ increased the cell viability and reduced an apoptosis of the cells. The cell apoptosis usually occurs when there is a destruction of the internal environment. ROS is an important factor in the apoptotic process. Excessive generation of ROS induces mitochondrial membrane permeability and damages the respiratory chain to trigger the apoptotic process [49]. It was reported that when the cells exposed to the nano-TiO$_2$, there was an alteration in the antioxidant enzymes activity, but the cells still showed an enhancement of lipid peroxidation and increased the rate of hydrogen peroxide generation, which suggested that the nano-TiO$_2$ may lead to the oxidative stress. This might not be sufficient enough to cope in against the toxic action of the nano-TiO$_2$ [41]. In this study, we measured the ROS generation using DCFH-DA-treated WRL-68 cells (Fig. 3). The result indicated that when the cells were incubated with the different concentrations of nano-TiO$_2$, oxidative stress was occurred in response to the treatment of the nano-TiO$_2$, and the contents of the ROS was increased significantly. We thus confirmed that exposure to ROS generated by TiO$_2$ NPs is significantly associated with catalase downregulation and methylation of the catalase promoter in WRL-68 cell. Here, we showed that ROS may downregulate the expression of catalase at the transcriptional level in WRL-68 cells. We observed that DNA methylation abolished the transcriptional activity of the catalase promoter. These findings suggest that catalase downregulation by ROS occurred in TiO$_2$-treated cells. From the data of WRL-68 cells we thus confirmed that exposure to nano-TiO$_2$ generate ROS that is significantly associated with catalase downregulation and methylation of the catalase promoter during the development of WRL-68 cells. In this study, we did not investigate whether ROS directly cause methylation of the catalase promoter in normal cells. Further studies should focus on identifying the putative ROS-mediated pathway that affects DNA methylation. Nevertheless, our results strongly suggest that ROS affect the methylation status of the catalase promoter. Thus, we propose the presence of a functional pathway involving ROS-induced epigenetic changes in which persistently elevated ROS induce methylation of CpG island II of the catalase promoter in WRL-68 cells.

V. Acknowledgement

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Conflict of Interest: Author declare there is no conflict of Interest

References Références Referencias

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### Tables and Figures

**Table 1:** The cellular uptake of TiO₂ NPs in WRL-68 cells

<table>
<thead>
<tr>
<th>Concentration</th>
<th>% Gated(SSC)</th>
<th>% Total(SSC)</th>
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<tr>
<td>Control</td>
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<td>0.10</td>
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<td>1000μM</td>
<td>125.12</td>
<td>125.12</td>
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**Figure 1:** Effect of TiO₂ NPs on Cell proliferation

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**Figure 2**: Effect of TiO₂ NPs on Cell proliferation

**Figure 3**: Estimation of ROS generated by TiO₂ NPs in WRL-68 cells

**Figure 4**: Methylation status of the catalase promoter in WRL-68 human hepatic cell lines
Fig 1 and Fig 2: Effect of TiO2 NPs on Cell proliferation

The WRL-68 cells were exposed at concentration 250 μM, 500 μM, 750 μM, 1000 μM of TiO2 NPs for 24h, respectively. Experiments were performed in triplicate and data expressed as the mean of three independent experiments.

Figure 3: Estimation of ROS generated by CuO NPs in WRL-68 cells

The WRL-68 cells were exposed at concentration 250 μM, 500 μM, 750 μM, 1000 μM of TiO2 NPs for 24h.

Figure 4: Methylation status of the catalase promoter in WRL-68 human hepatic cell lines

WRL-68 cells were treated with 1000 μM of TiO2 for 24h, after which MSP was performed using genomic DNA isolated from these cells. Genomic DNA was isolated after serum-starvation from WRL-68 human hepatic cell lines. PCR was performed with primers specifically designed to amplify the DNA sequence of the catalase promoter CpG island II; SM, size marker; U, unmethylated (control) DNA; M, methylated DNA.

**Figure Legends**
Figure 5: Effect of TiO₂ NPs on mRNA expression in WRL-68 cell lines

mRNA expression was assessed by real-time RT-PCR. Experiments were performed in triplicate and data expressed as the mean of three independent experiments. P value is calculated which was >0.05.

Figure 6: Catalase activity in WRL-68 cell lines on exposure to TiO₂ NPs

The Catalase activity was measured using cell lysates of the cells. The WRL-68 cells were exposed at concentration 250 μM, 500 μM, 750 μM, 1000 μM of TiO₂ NPs for 24h. Experiments were performed in triplicate and data expressed as the mean of three independent experiments.

Table 1: The cellular uptake of TiO₂ NPs in WRL-68 cells

WRL-68 cells were treated at concentration 250 μM, 500 μM, 750 μM, 1000 μM of TiO₂ NPs for 24h, respectively. Experiments were performed in triplicate and data expressed as the mean of three independent experiments.