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Titanium dioxide (TiO_2) nanoparticles induced ROS generation and its effect on cellular antioxidant defense in WRL–68 cell

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 1000µM. 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 1000µM for 24h in WRL–68 cell induced methylation of CpG island II via ROS on the catalase promoter and downregulated catalase expression at the transcriptional level.

Keywords: TIO_2 NPs, ROS, WRL–68 cell, catalase, MTT assay

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Komal Agarwal,[1] Sandesh Chibber[2]
[1]Ahmedabad University, India
[2]Department of Applied Sciences, Rai University, India

Correspondence: Sandesh Chibber, Associate Professor, School of Arts, Science and commerce, Department of Applied Sciences, Rai University, Ahmdabad–382260, Gujarat, India, Email sandesh.chibber@raiuniversity.edu

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Abbreviations: ROS, reactive oxygen species; DNA, deoxyribonucleic acid, NRU, national research universal; MSP, methylation specific polymerase chain reaction

Introduction

Titanium dioxide nanoparticles (TIO_2 NPs) have been widely used in manufacturing,[1–4] 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 TIO_2 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 TIO_2 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 TIO_2 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 TIO_2 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–TIO_2 NPs to be more biologically active than rutile–TIO_2 NPs in terms of cytotoxicity.[18] It was demonstrated[19] that pure anatase–TIO_2 NPs induce cell necrosis and membrane leakage, but do not generate ROS. In contrast, rutile–TIO_2 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 sequencing. In the present study we evaluate adverse consequence of TIO_2 Nps on epigenetic gene silencing of catalase enzyme in WRL–68, human hepatic cell line.

Materials and methods

Cell culture

The WRL–68 cells (passage number 36) was procured from NCCS, Pune.WRL–68 cells were Cultured at 37°C in 5% CO_2 in Minimum Essential Medium (MEM) +10% FBS (Invitrogen, Carlsbad, CA, USA). For TIO_2 treatment, TIO_2 NPs are added in the medium at different doses for 24h as indicated in the legend.

Estimation of TIO2 NPs uptake by flow cytometry

The cellular uptake of TIO_2 NPs in WRL–68 human hepatic cell line was carried out using flow Cytometry according to Suzuki et al.[20] Cells were seeded in 6–well culture plates at a density of $1.0x10^5$ cells/well, after about 12–15hrs, the cells were exposed at concentration 250, 500, 750 and 1000µM of TIO_2 NPs for 24h respectively. The cells were then harvested and collected in a sterile centrifuge tube which was centrifuged at 800rpm 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.

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.[21] with some modifications.[21] 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 (10mM). 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 multi well plate reader, Bio–Tek (USA) using KC4 software.

MTT assay

MTT assay was done according to method of Mosmann.[22] 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 TIO_2 NPs (250, 500, 750 and 1000µM) at 37°C for 24h, respectively and for 3h with MTT dye (5mg /10ml pbs). The reaction mixture was carefully aspirated and the resulting formazan crystals were solubilized by adding 100µl dimethylsulphoxide. After 10min, absorbance was read at 570nm in a SYNERGY–HT multiwell plate reader, Bio–Tek (USA) using KC4 software.

NRU assay

NRU assay was done according to method of Mosmann.[22] 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 TIO_2 NPs (250, 500, 750 and 1000µM) at 37°C for 24h, respectively and for 2h with NRU dye (4mg/ 10ml in PBS). The reaction mixture was carefully aspirated and the cells were destained by adding 150µl destaining solution. Absorbance was read at 540nm in a SYNERGY–HT multiwell plate reader, Bio–Tek (USA) using KC4 software.

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).

Real–time quantitative PCR: WRL–68 cells were cultured in 6–well plates and exposed to 1000µM of TIO_2 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–PCRq) 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 10ml of reaction mixture. Realtime PCR cycle parameters included 10 min at 95°C followed by 40 cycles involving denaturation at 95°C for 15s, annealing at 60°C for 15s and elongation at 72°C for 15s. All the realtime PCR experiments were performed in triplicate and data expressed as the mean of three independent experiments.

Catalase activity assay

The catalase activity was assayed by Aebi[23] method. Breifly, 0.1ml of cell lysates was added to 1.5ml of freshly prepared 13.2mM $H2O_2$ in 0.05M $K2HPO_4$ (pH 7.0) buffer. The rate of decomposition of $H2O_2$ was monitored spectrophotometrically at 240nm. Catalase activity is expressed as U/mg protein.

Results

Estimation of TIO2 NPs uptake in WRL–68 cells

The potential risk of nanoparticles to biological systems has to be understood more strongly. Several studies had reported that the Nano–TiO_2 can cause the damage to different cells.[24] 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.[24,25] The uptake of TIO_2 NPs in WRL–68 cells was determined by using flow cytometery. 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 TIO_2 NPs attributed by an 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. WRL–68 cells were treated at concentration 250µM, 500µM, 750µM, 1000µM of TiO_2 NPs for 24h, respectively. Experiments were performed in triplicate and data expressed as the mean of three independent experiments.

Table 1 The cellular uptake of TiO_2 NPs in WRL–68 cells

Concentration	% Gated(SSC)	%Total(SSC)
Control	0.1	0.1
250µM	30.99	30.99
500µM	97.3	97.3
750µM	100.15	100.15
1000µM	125.12	125.12

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.[26] The proliferation of WRL–68 cells was found to be increased with increase in concentration of TIO_2 NPs as attributed by (Figure 1) (Figure 2). 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.[27] The WRL–68 cells were exposed at concentration 250µM, 500µM, 750µM, 1000µM of TiO_2 NPs for 24h, respectively. Experiments were performed in triplicate and data expressed as the mean of three independent experiments. The WRL–68 cells were exposed at concentration 250µM, 500µM, 750µM, 1000µM of TiO_2 NPs for 24h, respectively. Experiments were performed in triplicate and data expressed as the mean of three independent experiments.

Intracellular reactive oxygen species (ROS) measurement

ROS generation plays a key role in toxicity of NPs in mammalian

cells.[28] 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.[29] Figure 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. 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 down regulation and methylation of the catalase promoter in WRL–68 cell. The WRL–68 cells were exposed at concentration 250µM, 500µM, 750µM, 1000µM of TiO_2 NPs for 24h.

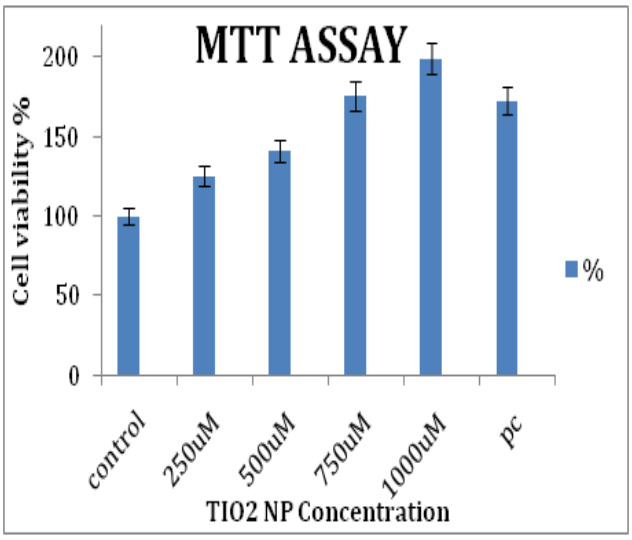

Figure 1 Effect of TIO_2 NPs on Cell proliferation

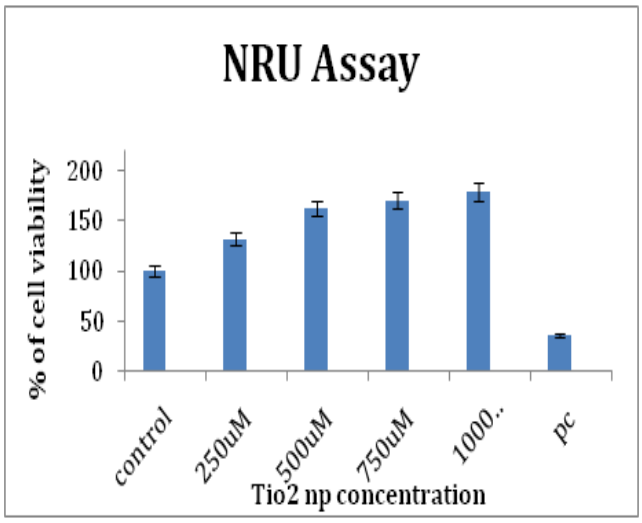

Figure 2 Effect of TIO_2 NPs on Cell proliferation

MSP analysis and RT–PCR

WRL–68 cells were treated with TIO_2 NPs at 750µM and 1000µM for 24h. The effect of ROS generated by TIO_2 NPs on the methylation status of the catalase promoter was analyzed using MSP analysis (Figure 4). We specifically selected the CpG island II region located in the catalase promoter. The methylation of CpG island II was observed at 24h. RT–PCR was performed within the same cells, demonstrated that messenger RNA (mRNA) (Figure 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 Figure 6. It was reported that when the cells were 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.[29] We thus confirmed that exposure to ROS generated by TiO_2 NPs is significantly associated with catalase down regulation and methylation of the catalase promoter in WRL–68 cell. Here, we showed that ROS may down regulate 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 down regulation 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 down regulation and methylation of the catalase promoter during the development of WRL–68 cells. We observed that DNA methylation abolished the transcriptional activity of the catalase promoter. These findings support that catalase down regulation may occur in WRL–68 cells via ROS when treated with TiO_2 NPs. WRL–68 cells were treated with 1000µM of TiO_2 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. 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. The Catalase activity was measured using cell lysates of the cells. The WRL–68 cells were exposed at concentration 250µM, 500µM, 750µM, 1000µM of TiO_2 NPs for 24h. Experiments were performed in triplicate and data expressed as the mean of three independent experiments.

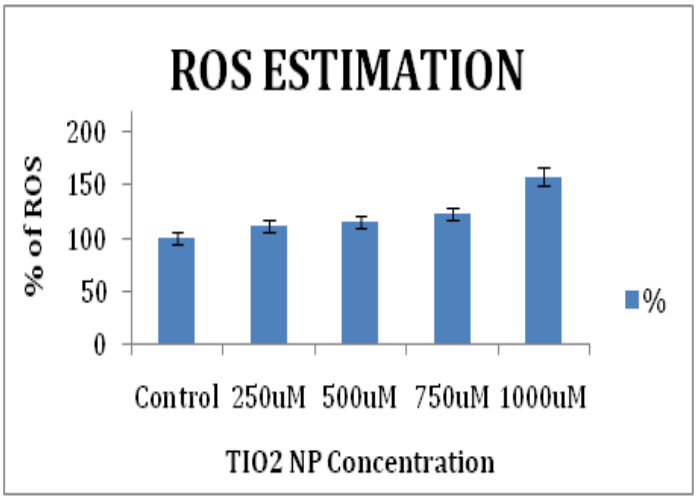

Figure 3 Estimation of ROS generated by TIO_2 NPs in WRL–68 cells.

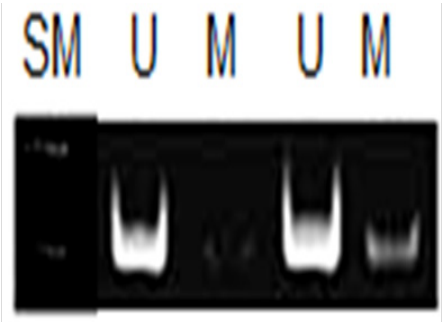

Figure 4 Methylation status of the catalase promoter in WRL–68 human hepatic cell lines

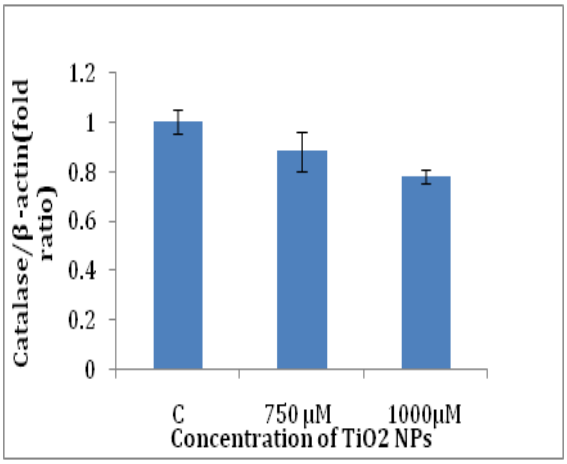

Figure 5 Effect of TIO_2 NPs on mRNA expression in WRL–68 cell lines.

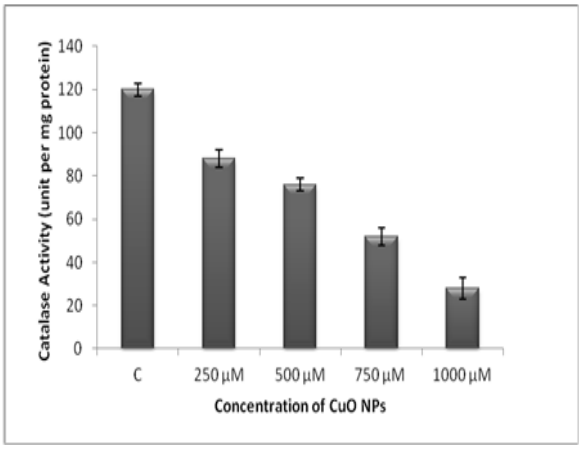

Figure 6 Catalase activity in WRL–68 cell lines on exposure to TiO_2 NPs.

Conclusion

Nanotechnology involves the creation and manipulation of materials at nanoscale to create products that exhibit novel properties. We worked on commercial form which is already been characterized by the manufacture. 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.

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Conflict of interest

The author declares no conflict of interest.

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