Titanium dioxide nanoparticles enhance production of superoxide anion and alter the antioxidant system in human osteoblast cells

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Abstract

Titanium dioxide (TiO_2) nanoparticles (NPs) are manufactured worldwide for a variety of engineering and bioengineering applications. TiO_2NPs are frequently used as a material for orthopaedic implants production. However, the biocompatibility of TiO_2NPs and their effects on osteoblast cells responsible for growth and remodeling of human skeleton has not been thoroughly investigated. We studied the effects of exposure of hFOB 1.19 human osteoblast cells to TiO_2 of NPs (5-15 nm) for 24 and 48 h. Cell viability, alkaline phosphatase activity (ALP), cellular uptake of NPs, cell morphology, superoxide anion ($O_2^{\bullet-}$) generation, superoxide dismutase (SOD) activity and protein level, sirtuin 3 (SIR3) protein level, correlation between MnSOD and SIR, total antioxidant capacity (TAC), malondialdehyde (MDA) were measured following exposure of hFOB 1.19 cells to TiO_2NPs. Exposure of hFOB 1.19 cells to TiO_2NPs

resulted in: (1) cellular uptake of NPs; (2) increased cytotoxicity and cell death in a time and concentration-dependent manner; (3) the ultrastructure changes; (4) decreased SOD and ALP activity; (5) decreased protein level of SOD1, SOD2 and SIR3; (6) decreased TAC; (7) increased $O_2^{\bullet-}$ generation; (8) enhanced lipid peroxidation (MDA level). The linear relationship between the protein level of MnSOD and SIR3 and between $O_2^{\bullet-}$ content and SIR3 protein level was observed. Importantly, cytotoxic effects of TiO_2NPs were attenuated by pre-treatment of hFOB 1.19 with SOD, indicating significant role of $O_2^{\bullet-}$ in observed cell damages and death. Thus, decreased expression of SOD leading to increased oxidizing stress may underlie nanotoxicity effects of TiO_2NPs on human osteoblasts.

Keywords: osteoblast cells, TiO_2 nanoparticles, superoxide anion, superoxide dismutase, sirtuin 3, nanotoxicity

Running header: TiO_2NPs alter the antioxidant system in osteoblast cells

Introduction

Titanium dioxide nanoparticles (TiO_2NPs) belong to the most widely manufactured NPs (1-100 nm) in the world and they are very often used in the production of bio-medical ceramic and orthopaedic implants.[1] TiO_2NPs, while added to composites, increase their durability for mechanical damages, specially cracking and breaking and minimise the risk of bacterial infections. Because of these unique properties TiO_2NPs are increasingly used in endoprosthesis and scaffolds for bone tissues reconstructions.[2,3] However, nano-toxicological studies are crucial for the safe and sustainable development of the emerging and established nanomaterials such as

TiO_2NPs. Indeed, TiO_2NPs may also cause harmful/cytotoxic effects. For example, the TiO_2NPs could induce DNA double strand breaks in bone marrow cells after oral administration.[4] Zhang et al[5] observed that TiO_2NPs stimulated pro-inflammatory gene expression in proosteoblast cells (MC3T3-E1). Wang et al[6] indicated that TiO_2NPs are potentially toxic to major organs and cause damage to the knee joints in rabbits.

A number of studies investigated mechanisms of this cytotoxicity and demonstrated that TiO_2NPs induce alterations in redox homeostasis, which includes both impairment of antioxidant defenses and increased production of ROS in different type of cells.[7-9] However, surprisingly little is known about the cellular mechanisms responsible for the effects of TiO_2NPs–induced oxidative stress on osteoblast functions in particular the role of superoxide anion ($O_2^{\bullet-}$).

Superoxide is formed when oxygen (O_2) acquires an additional electron. $O_2^{\bullet-}$ is converted by the antioxidant enzyme superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2) and singlet oxygen.[10] SOD is the first enzymatic line of antioxidant defense system, which scavenges superoxide radical and prevents the lipid peroxidation of cellular membrane. Antioxidant enzymes are supported by non-enzymatic antioxidants and the total antioxidant capacity (TAC) is often used to estimate the overall antioxidative status in cells. Recently, particular attention has been paid to link between mitochondrial SOD (SOD2/MnSOD) and SIR3. SIR3 is a member of the sirtuin family and deacetylates SOD2 to increase its catalytic activity. It is localized in mitochondria and regulates mitochondrial function; homeostasis and oxidative metabolism, as well as oxidative stress and cellular injury.[11,12]

Therefore, the main objective of the present study was to study the role of the $O_2^{\bullet-}$/SOD/SIR3 system on TAC and human osteoblast hFOB 1.19 cytotoxicity following challenge with TiO_2NPs.

Materials and methods

2.1. Chemicals

TiO_2NPs (5-15 nm) were purchased from US Research Nanomaterials. The concentrations of TiO_2NPs used in experiments were carefully selected according to the results obtained from a preliminary concentration-response study (S1).

Characterization of NPs

TiO_2NPs in were investigated using TEM JEM 1200 EXII transmission electron microscope (JEOL, Japan) to determine the size and shape.

Cell line and procedures

Human fetal osteoblast cell line (hFOB 1.19) was obtained from the American Type Culture Collection (ATCC number: CRL-11372) and maintained as a monolayer culture in T-75 cm^2 tissue culture flasks. The cells were grown in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham F12 medium (1:1 ratio) containing also sodium pyruvate 110 mg/l and supplemented with 10 % fetal bovine serum, 6 µg/ml penicillin-G and 10 µg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 95 % O_2 - 5 % CO_2. When confluent cells were detached enzymatically with trypsin-EDTA and sub-cultured into a new cell culture flask. The medium was replaced every 2 days. These hFOB cells have been indicated to be an excellent model system for the study of osteoblast biology in vitro.

Treatments

hFOB 1.19 cells were treated with TiO_2NPs (5 - 100 µg/ml) for 24 and/or 48 h. TiO_2NPs were suspended in serum-free (SF) cell culture and diluted to appropriate

concentrations *ex tempore* every time before adding the cells. The dilutions of TiO_2NPs were filtered through a 0.22 μm membrane filter, then sonicated using a sonicator bath at room temperature for 20 min to reduce nanoparticle agglomeration. For some experiments, SOD: 50 U/ml (Sigma-Aldrich, Poland) was added 30 min before TiO_2NPs incubation with cells.

Cytotoxicity

WST-1

Cell viability was measured by WST-1 assay (Roche Applied Science). hFOB 1.19 cells were seeded in triplicate at a density of 10^4 cells/100 μl of cell culture medium into 96-well. The following day, hFOB 1.19 cells were treated with TiO_2NPs under SF conditions at the concentrations: 5, 25, 50 and 100 μg/ml for 24 and 48 h. Mitochondrial activity assay (an index of cell growth and cell death) was performed by adding a premixed optimised dye reagent WST-1 to culture wells. Absorbance was read at 450 nm, reference: 630 nm (ASYS Hitech GmbH microplate reader, Biogenet). Absorbance values were also corrected with a NPs blank.

TEM analysis

hFOB 1.19 cells were fixed in 2.5 % glutaraldehyde (GA) in 0.1 M Na-cacodylate buffer at pH 7.4. Fixation was carried out at 4 °C for 1 h and then cells were rinsed three times in the same buffer. Following fixation in GA the cells were post fixed in 1% osmium tetroxide in 0.1 M Na-cacodylate buffer for 1 h. After washing with the same buffer, they were dehydrated in graded series of ethanol, immersed in propylene oxide, embedded in Epon 812 and polymerized. Sectioning was performed with a diamond knife on an OmU2 ultramicrotome (Reichert, Austria). Semi-thin sections (1.5 μm)

were stained with toluidine-blue and examined under a light microscope. Ultrathin sections were placed on form war-covered cooper grids, and double-stained with lead citrate and uranyl acetate. Observations were carried out with a JEM 1200 EXII TEM at an accelerating voltage of 80 kV.

Cell necrosis

Cell viability was measured by lactate dehydrogenase (LDH) assay (PROMEGA, Poland). hFOB 1.19 cells were seeded in triplicate at a density of 10^4 cells/100 µl of cell culture medium into 96-well. The following day, hFOB 1.19 cells were treated with TiO_2NPs under SF conditions at the concentrations: 5, 25, 50 and 100 µg/ml for 48 h. LDH release into the surrounding medium was determined according to the manufacturer's instructions. Absorbance values were also corrected with a NPs blank.

Cell metabolism

The alkaline phosphatase (ALP) activity was determined according to manufacturer instructions using commercially available kit (Abcam Inc., MA, USA). Briefly, hFOB 1.19 osteoblast cells were cultured until they reached 80-90% confluence and treated as described in Section *Treatments* for 48 h. After that, the cells were washed with PBS and lysed in buffer containing 10 mM Tris-HCl (pH 7.5), 0.5 mM $MgCl_2$, and 0.1% Triton X-100. The cell lysates were centrifuged at 2,000 g and the soluble portion was used for enzymatic assay. An aqueous solution of 2 mg/ml of p-nitrophenyl phosphate was mixed with 0.1 M amino propanol in 2 mM $MgCl_2$ with a pH of 10.4 being prepared. Next, 200 µl of the substrate was added to the 96-well plates and incubated in the dark for about 30 minutes. The enzymatic reaction was stopped by the addition of 50 mM NaOH. The final product (p-nitrophenol) was quantified at 405 nm using a microplate reader (ASYS Hitech GmbH microplate reader, Biogenet). The results were

normalized by the amount of cells and by specific activity (nmol p-nitrophenol/min/mg/ of protein). Absorbance values were also corrected with a NPs blank.

Superoxide anion production

Superoxide generation was measured using flow cytometry. Briefly, the cells were cultured in 6-well plates until they reached 80-90% confluence and treated as specified in Section *Treatments* for 48 h. Cells were then split and cultured on cover slips and incubated with 5 mM dihydroethidium (DHE) at 37°C for 30 min. The DHE staining detecting superoxide anion production was quantified by Flow cytometer (BD FACSCalibur; US).

Total Antioxidant Capacity

The TAC was determined according to manufacturer instructions with modifications using the commercially available kit (Sigma Aldrich, Poland). The cells were cultured in 10 cm Petri dish until they reached 80-90% confluence and treated as indicated in Section *Treatments*. For determining cellular total antioxidant capacity, post-treatment cells were washed with PBS and suspended in 200 µl of ice-cold lysis buffer and sonicated. The lysate was centrifuged at 10,000 x g for 10 min, and protein concentration of the supernatant fraction was determined by the Bradford method. The Trolox equivalent antioxidant activity was measured by measuring the ability of hydrogen-donating antioxidants to scavenge the radical cation generated by 2,2V-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid. Absorbance was recorded at 570 nm (ASYS Hitech GmbH microplate reader, Biogenet). Absorbance values were also corrected with a NPs blank.

Total SOD activity

The SOD activity was measured using the Superoxide Dismutase Assay Kit (Cayman Chemical, US). Briefly, 10 µl of cell supernatants that were obtained by scraping, sonicating, and centrifugation (15 000 ×g, 5 min) of cells monolayer (2×10^6 cells) in cold environment (4°C) was added to 200 µl radical detector (50 µl tetrazolium mixed with 19.95 ml assay buffer, i.e., 50 mM Tris-HCl, pH 8.0 contained 0.1 mM DTPA and 0.1 mM hypoxanthine). The reaction was initiated by adding 20 µl of xanthine oxide in a 96-well plate. The plate was shaken and incubated for 20 min at room temperature before the absorbance (450 nm) recorded by using microplate reader (ASYS Hitech GmbH microplate reader, Biogenet). The SOD activities in the samples were calculated by using the formula according to company's manufacturing kit.

Western blotting of SOD and SIR3

Western blotting was used in order to investigate the SOD1 (Cu/ZnSOD), SOD2 (MnSOD) and SIR3. Briefly, hFOB 1.19 cells were cultured in 10 cm Petri dishes until they reached about 90% confluence and treated with TiO_2NPs under SF conditions at the concentrations: 25, 50 and 100 µg/ml for 48 h. Afterwards, conditioned media were discharged and attached cells rinsed with phosphate buffered saline (PBS), detached, and homogenized. Following electrophoresis, proteins were transferred onto nitrocellulose membrane (Protran, Schleicher and Schuell; Dassel, Germany) and detected using antibodies: anti-Sirt3, anti-SOD1 and anti-SOD2 antibodies (Cell Signaling, Hitchin, UK). Protein bands were quantified using densitometry software (Bio-Rad), and normalized using β-actin (Sigma Aldrich) as a loading control.

Lipid peroxidation

hFOB 1.19 cells were plated into 12-well plates at a density of 2.5 x 10^6 cells per well in complete medium. Pre-confluent cells were exposed to TiO_2NPs asindicated in

Section *Treatments* for 48 h. Malondialdehyde (MDA), a marker of lipid peroxidation, was measured using an OxiselectTM TBARS Assay kit (Cell Biolabs, Inc. Cambridge, UK) following the manufacturer's protocol. Spectrophotometric measurements were recorded on a plate reader (ASYS Hitech GmbH microplate reader, Biogenet) at 532 nm. The concentration of MDA in samples was calculated using MDA standards as reference. The concentration of MDA in TiO_2NPs-treated cells is presented as the fold increase of MDA production over the untreated cell control. Absorbance values were also corrected with a NPs blank.

Protein content

Protein content determination was measured by the method of Bradford.[13]

Statistical analysis

All data are presented as the mean ± standard error of 3-4 independent experiments. Statistical analysis was determined by one-way ANOVA and Tukey's post hoc test and p value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism. Pearson correlation analysis was conducted to investigate the relationship between SIR3 and MnSOD; SIR3 and $O_2^{\bullet -}$.

Results and discussion

In the present study, we studied the effects of TiO_2NPs on viability, cell metabolism and oxidative stress of human fetal osteoblast cells (hFOB 1.19). As far as we are concerned, it is the first report to show the link between TiO_2NPs-induced osteoblast toxicity and impairment of antioxidant system, including changes of SIR3 at protein levels in hFOB 1.19 cells.

TiO_2NPs size and shape

The TEM morphological study demonstrated that single particle size of TiO_2NPs was larger (about 30 nm in diameter) than that indicated by manufacturer (5-15 nm). Most particles were round with a smooth surface (Figure 1).

(Please insert Figures 1 here)

TiO_2NPs exposure decreases viability of hFOB 1.19 cells

The TiO_2NPs-mediated cytotoxicity was evaluated using the WST-1 assay, which is indicative of mitochondrial damage. This test is based on water soluble dye and it does not interfere with TiO_2 NPs measurement compared to MTT assay.[14] Using WST-1 assay we found that the viability of cells exposed to TiO_2NPs significantly decreased in a time- and concentration-dependent manner compared to the control, see Figure 2.

(Please insert Figures 2 here)

TiO_2NPs when incubated with cells for 24 h not cause statistically significant changes in mitochondrial activity. In contrast, the exposure of cells to TiO_2NPs for 48 h resulted in a significant decrease in osteoblast cell viability (Figure 2). Our results are consistent with others, who demonstrated cytotoxic effect of TiO_2NPs in different cell lines and in vivo. Indeed, a significant decreases in cell viability were noticed in hamster lung fibroblasts (V79 cells) exposed to TiO_2NPs for 24 and 48 h.[4] Hussain et al[15] have detected that TiO_2NPs did not induce significant decrease in cell viability at concentration at 50 and 100 μg/ml in rat liver cells, but they did observed a significant cytotoxic effect at higher concentration (100-250 μg/ml). In vivo experiments showed also TiO_2NPs-induced injury. Liu et al[16] observed that intra-tracheal instillation with

TiO_2NPs into rats leads to a dose-dependent inflammatory injury in lungs. Furthermore, intraperitoneal administration of 5 nm TiO_2NPs to mice for 14 days caused serious damage to the liver, kidneys, and myocardium at the higher concentrations.[17]

Based on all these data, the 48 h of incubation was chosen for further biochemical study.

Uptake of TiO_2NPs by hFOB 1.19 cells and ultrastructural changes in cell

We have also evaluated TiO_2NPs toxicity using TEM. Images of ultrathin sections provided information on cell death, survival, and metabolic activities.[18] The TEM analysis revealed that TiO_2NPs were engulfed and internalized within the osteoblast cytoplasm (Figure 3).

(Please insert Figures 3 here)

The invaginations in the cell membrane before nanoparticle uptake and vacuolization are indicative of nonspecified internalization.[19] On the other hand, Zhang at al[5] indicated that the uptake of TiO_2NPs by murine MC3T3-E1 proosteoblasts cells occurred via receptor-mediated endocytosis. In osteoblasts, TiO_2NPs have been deposited over 24 and 48 h inside multiple vacuoles sometimes forming aggregates. The TEM analysis indicates that incubation of TiO_2NPs with osteoblasts for 48 h led to alterations in mitochondria organization known as the condensed configurations. (Figures 3C and Figures 4A).

(Please insert Figures 4 here)

This configuration was identified by dark, condensed matrix and expanded, translucent cristae. Intensive vacuolization of mitochondria was also noted (Figure 3C). Such morphological changes observed in mitochondria in TiO_2NPs treated osteoblast culture were accompanied by described here biochemical changes (WST-1 assay). The TEM

examination revealed also single-membrane vacuoles called autophagolysosomes. These vacuoles contain remnants of organelles, especially mitochondria. Autophagolysosomes are able to degrade sequestered autophagosomal components (Figure 4B). It is likely that this autophagy process constitutes a stress adaptation pathway that promotes cell survival during osteoblast exposure to TiO_2NPs.[20] We also observed features of necrotic lysis, such as advanced swelling of intracellular organelles associated with cell membrane disruption and intensive vacuolization (Figure 3B). Such changes are typical for necrotic cell death without signs of massive cell death or apoptosis.

TiO_2NPs induce necrosis in hFOB 1.19 cells

TiO_2NPs-induced necrosis was further confirmed by the measurement of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released early in necrosis, and only in late stage apoptosis.[21] Incubation of osteoblast cells with TiO_2NPs for 48 h led to increased release of LDH (Figure 5).

(Please insert Figures 5 here)

Our results are in keeping with data by Zhang et al[5] who indicated that TiO_2NPs of 5 and 32 nm in diameter increased LDH release in size-, concentration- and time-dependent manner in proosteoblast cells (MC3T3-E1). Interestingly, Lai et al[22] indicated that apoptosis, necrosis, and well as possibly apoptosis-like and necrosis-like was the cell death mechanisms underlying the effects of TiO_2 micro- and nanoparticles on human astrocytoma U87 (astrocytes-like) cells.

TiO_2NPs decrease metabolism of hFOB 1.19 cells

As expected, TiO_2NPs-induced reduction of osteoblast survival led to decreased function. Indeed, incubation of hFOB 1.19 cells with 25, 50 and 100 µg/ml TiO_2NPs for 48 h decreased the activity of ALP (Figure 3), an enzyme participating in bone tissue mineralization and commonly used as a marker of osteoblastic differentiation in *in vitro* studies.[23,24]

(Please insert Figures 6 here)

These results are consistent with those by Hou et al[25] who showed that the ALP activity was decreased in mesenchymal stem cells incubated with TiO_2NPs.

TiO_2NPs induce generation of $O_2^{\bullet-}$ in hFOB 1.19 cells

Recently, it has been proposed that the cytotoxicity of TiO_2NPs is related to the induction of oxidative damage.[26-28] However, the exact molecular mechanism by which TiO_2NPs affects oxidative metabolism, especially in the context of the cellular antioxidant defense system in osteoblast cells is unknown. Therefore, we investigated the effects of incubation of osteoblasts with TiO_2NPs at 5, 25, 50 and 100 µg/ml TiO_2NPs for 48 h on $O_2^{\bullet-}$ generation, TAC, SOD1, SOD2, SIR3.

It is known that ROS play physiological and pathological roles in bone metabolism.[29] They modulate bone cell function and they are also implicated in the pathophysiology of skeletal tissues. ROS represent a family of molecules and free radicals. They are generated during mitochondrial oxidative metabolism as well as in cellular response to cytokines, bacterial invasion, environmental factors and different xenobiotics, including nanoparticles.[27,29] The reduction of oxygen by one electron at a time produces relatively stable intermediates. In our study, we have focused on the role of $O_2^{\bullet-}$ in TiO_2NPs-induced cytotoxicity in osteoblast cells. $O_2^{\bullet-}$ is the product of a one-electron reduction of oxygen, the precursor of potent oxidizing species such as H_2O_2, hydroxyl radical

($^{\bullet}OH$), peroxynitrite ($ONOO^{\bullet-}$) and a mediator in oxidative chain reactions.[30] We have observed a significant increase in $O_2^{\bullet-}$ levels in cells incubated with 25, 50 and 100 µg/ml TiO_2NPs for 48 h (Figure 7).

(Please insert Figures 7 here)

Wang et al[31] noticed that TiO_2NPs induce the oxidative stress in rat synovial cell line by increased generation of free radicals. It has been previously demonstrated that metal, half-metal and metal oxide NPs induce production of ROS in different cell lines. We have shown an elevation of ROS levels in human gingival fibroblast cells exposed to AgNPs and human lung submucosal cells exposed to SiO_2NPs.[32,33] Moreover, it has been reported that nanoscale TiO_2 could be phagocytized by neurons and microglia, which then released ROS: $^{\bullet}OH$ and $O_2^{\bullet-}$.[34-36]

TiO_2NPs exposure impairs the antioxidant defense system in hFOB 1.19 cells

It is likely that during evolution constant exposure of cells to free radicals resulted in development of complex defense mechanisms involving enzymatic and non-enzymatic antioxidants. Major enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Therefore, we studied the effects of TiO_2NPs on the activity and protein level of SOD in hFOB 1.19 cells. Superoxide dismutases are a family of enzymes that function to efficiently catalyze the dismutation of superoxide anion. SOD1, or Cu/ZnSOD, is a copper and zinc-containing homodimer localized in intracellular cytoplasmic spaces. SOD2, or MnSOD is a manganese-containing tetramer of 22 kDa subunits, which is one of the primary mitochondrial antioxidants. The extracellular SOD (ECSOD) is expressed in significant amounts in lung, kidney, and fat tissue and is regulated mainly by cytokines.[37] We have found that incubation of osteoblast cells with TiO_2NPs leads to significant reduction in activity of total i.e. cytosolic and mitochondria SOD - (Figure 8) and it is associated with

decreased protein levels of SOD1 and SOD2 (Figure 9A and Figure 9B, respectively). To the best of our knowledge, this is the first report characterizing changes of SOD activity and protein levels in osteoblast hFOB 1.19 cells.

(Please insert Figures 8 here)

(Please insert Figures 9 here)

Recently, the sirtuin enzyme family has attracted increasing attention due to their involvement in many cellular processes. Sirtuins important protect cells against stress and control a number of metabolic pathways. In our study we have focused on SIR3, which deacetylates and enhances the activity of various proteins that play a key role in the antioxidative defense system of mitochondria such as MnSOD.[11,12] Interestingly, we have observed that TiO_2NPs significantly reduces the levels of SIR3 protein in osteoblast cells (Figure 10).

(Please insert Figures 10 here)

Furthermore, we have found a significant positive correlation between SIR3 and MnSOD at protein level and a significant negative correlation between decreased SIR3 protein level and increased $O_2^{\bullet-}$ level (Table 1). These results demonstrate that SIR3 contributes to regulation of antioxidant defense system in osteoblast cells via its effects on MnSOD and generation of $O_2^{\bullet-}$.

(Please insert Table 1 here)

It is important to emphasize, that some antioxidants can interact with each other regenerating their properties, and this mechanism is often referred to as the "antioxidant network".[38] Therefore, the measurement of a panel of antioxidants as well as the total antioxidant capacity (TAC) may provide more relevant biological information than the

measurement of single factor of antioxidant system.[39] Interestingly, we have found a decrease in TAC in osteoblasts after 48 h exposure to TiO_2NPs (Figure 11).

(Please insert Figures 11 here)

In addition to SOD and SIR3 the levels of TAC were significantly decreased in rat synovial cell line (RSC-364) after exposure to nano-TiO_2.[31] Thus, TiO_2NPs exert very strong inhibitory effects on the cellular defense systems.

Of particular significance is the fact that TiO_2NPs induce the oxidizing stress not only in mammalian cells but also in lower animals. Hao et al[26] found a significant decrease in SOD and catalase (CAT) activities in liver, gill and brain tissues of carp (*Cyprinus carpio*) during sub-acute exposure to 100 and 200 mg/l TiO_2NPs. However, Zhu et al[28] observed that the activity of SOD significantly increased in mature marine abalone (*Haliotis diversicolor supertexta*) exposed to 1.0 mg/l TiO_2NPs. This increase could result from the mobilization of antioxidant enzymes activities during rapid increase of ROS generation in cells exposed to high concentrations of xenobiotic. Indeed, in the same experiment, the decrease of reduced glutathione was observed. Therefore, the inadvertent presence of TiO_2NPs in an ecosystem is likely to have profound environmental repercussions.

TiO_2NPs induce lipid peroxidation in hFOB 1.19 cells

When antioxidant defenses fail to restore redox equilibrium, an elevated level of oxidative stress could lead to cellular damage and death.[40,41] In the next step of our study, we have demonstrated that TiO_2NPs-induced overproduction of $O_2^{\bullet-}$ and an impairment of antioxidant defense system is accompanied by enhanced lipid

peroxidation. The cell membrane peroxidation and damage was reflected by the elevated MDA levels (Figure 12) and was also observed using TEM (Figure 4B)

(Please insert Figures 12 here)

Lipid peroxidation reactions can alter the structure and function of membrane lipids, thus resulting in cell injury and cell death.[41] Indeed, oxidized lipids stimulated bone resorption by increasing recruitment and differentiation of osteoclast precursors and decreasing osteoblast differentiation.[40] Carré et al[42] also suggested that TiO_2NPs led to excessive generation of $O_2^{\bullet-}$ resulting in enhanced lipid peroxidation and oxidative stress. Moreover, Ma et al[27] showed that lipid peroxidation increased in brain of mice injected into abdominal cavity with TiO_2NPs every day for 14 days. Significantly, the impact of TiO_2NPs has also been shown in invertebrates. Valant et al[43] found in an invertebrate model organism (*Porcellio scaber, Isopoda, Crustacea*), fed with food containing TiO_2NPs after 3 days exposure, destabilized cell membranes, but lipid peroxidation was not detected. However, the levels of lipid peroxidation increased in liver, gill and brain tissues of carps exposed to TiO_2NPs for up to 8 days.[26]

Pre-incubation of hFOB 1.19 cells with SOD attenuates TiO_2NPs-induced toxicity

Finally, to provide a conclusive evidence for the involvement of $O_2^{\bullet-}$ in TiO_2NPs-induced osteoblast toxicity, the cells were pre-incubated with purified SOD before adding NPs. Following incubation the osteoblast viability, the activity of ALP and the levels of MDA were measured. Figure 13 A-C shows that the treatment with SOD significantly attenuated the cytotoxic effect of TiO_2NPs, showing that $O_2^{\cdot-}$ radicals participate in TiO_2NPs-induced cell death, decrease osteoblast metabolism and increase lipid peroxidation.

(Please insert Figures 13 here)

In summary, we have demonstrated that excessive production of $O_2^{\bullet -}$ and impairment of antioxidant system resulted in oxidative damage is likely to constitute a major mechanism implicated in TiO_2NPs-induced toxicity in osteoblast cells. Moreover, our study highlights, for the first time, that TiO_2NPs cause the impairment of SOD, at the protein and enzyme activity levels and of SIR3 at protein levels in these cells.

Conflict of Interest

The authors declare no conflicts of interest.

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