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Impact of exposure of human osteoblast cells to titanium dioxide particles in-vitro

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ABSTRACT

Titanium Dental implant is the most successful treatment modality to replace missing teeth today. Although titanium is considered biologically biocompatible, strong, and corrosion-free, the risk of implant failure continues due to bone loss at the expense of optimum oral health. Current research points toward the presence of titanium dioxide (TiO_2) particles leached from dental implant surface, which occurred due to mechanical and chemical insults on the surface. This study aimed to investigate the influence of TiO_2 particles of different sizes leaching from implant surfaces on Human Osteoblast cells (HOB) in-vitro. Titanium dioxide particles in both nano (NPs) and micro (MPs) size and at different concentrations were introduced to human osteoblast cells with and without treatment with vitamin C. Production of ROS was measured using H2DCFDA cellular ROS Assay Kit and MCP-1 and IL-8 cytokines released were assayed at 24 h time point using ELISA technique. Results showed a dose dependent increase in ROS production following exposure of HOB to both nano and micro particles. MCP-1 and IL-8 were released and there was minimal difference between the amount generated by nano compared with micro size particles. Treatment of HOB with antioxidant vitamin C demonstrated a significant reduction in the generation of ROS. At the same time, MCP-1 release was reduced significantly for the 100 μg/mL TiO_2 NPs and MPs after Vitamin C treatment while IL-8 release increased significantly. This study suggests a positive role played by antioxidants in the control of ROS generation and chemokines production in the peri-implant tissue environment.

1. Introduction

Titanium dioxide (TiO_2) is a natural mineral that occurs in three different crystallographic structures and is widely used in paints, wastewater treatment, sterilization, cosmetics, foods, biomedical ceramics, and implanted biomaterials due to its physicochemical properties of good fatigue strength, machinability, biocompatibility, and whitening and photocatalytic effects.[1] The success of TiO_2 material in dental implant applications is due to its light weight, strong, malleable, corrosion resistance, and its surface oxidative process that convert to titanium dioxide making it biocompatible.[1,2] Upon exposure to air, an oxidative process forms an oxide layer that passivates the surface of the titanium implant making it resistant to corrosion, and ability to bond with bone, producing a phenomena known as osseointegration that is essential for implant success.[3]

Implantation of a foreign material into the human bone will always initiate a foreign body reaction with appropriate cellular responses. It has become evident that cellular response to the release of TiO_2 particles is affected by particle size, surface properties, crystal structure, and physical attributes. Therefore, it is necessary to investigate the association between the material characteristics of TiO_2 particles and their biological effects[2] following implantation. Previous studies also found that smaller particles size induce higher pro-inflammatory Interleukin-1β (IL-1β), IL-6 and TNF-alpha responses compared to larger particles, indicating the role of particles size having a direct effect on the upregulation of inflammatory mediators.[2]

Following the surgical placement of a dental implant, immune cells are immediately recruited to peri-implant area as a normal biological body response, corresponding to natural innate immunity. Generation and over production of reactive oxygen species (ROS) in cells around the peri-implant microenvironment results in the condition known as oxidative stress, which causes a variety of changes within the cell,

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including the activation of the transcription factor Nuclear Factor-Kappaβ (NF-kβ). Increased amount of ROS exceeding the local antioxidant capacity may directly contribute to stimulation of inflammatory mediators and decreased bone formation.

Recent evidence showed that leaching of titanium dioxide particles and ions, in the form of tribocorrosion may continue following implantation. The fate of these particles in the peri-implant environment is still poorly understood. The aim of this study was to investigate the impact of exposure of Human Osteoblast Cells (HOB) to TiO_2 particles and its influence in generation of ROS and release of chemokines.

2. Materials and methods

2.1. Human Osteoblast (HOB) cell culture

HOB cells were obtained from AddexBio (AddexBio P0004010, San Diego, CA), and maintained in a DMEM/F-12 culture medium (Gibco, Thermo Fisher Scientific, Inc., USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma-Aldrich, USA). The cells were maintained at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2.

2.2. Titanium particles preparation

The preparation of particles was performed by following a methodology earlier reported.[4] Briefly, TiO_2 nanoparticles (NPs) of size <100 nm and TiO_2 microparticles (MPs) of size <5 μm in diameter were procured from Sigma (Sigma-Aldrich, USA) and the TiO_2 NPs and MPs were weighed and dispersed in Milli-Q water for stock preparation and was sonicated for 10 min with a probe sonicator (Qsonica sonicators, USA).

For cell culture studies, both TiO_2 NPs and MPs were suspended in a complete DMEM/F-12 medium to a final concentration of 1 mg/mL (stock suspension) and 5, 20, and 100 μg/mL were used as the treatment concentrations.

2.3. Measurement of intracellular reactive Oxygen species (ROS)

HOB cells were seeded at a density of 1×10^4 cells/well in a 96 well plate and were divided into two groups. The first group of HOB cells were treated with three different concentrations of 5 μg/mL, 20 μg/mL, and 100 μg/mL TiO_2 NPs while the second group were treated with 5 μg/mL, 20 μg/mL, and 100 μg/mL TiO_2 MPs; for 24h. Following incubation, the treated cells were trypsinized and centrifuged at 1500 rpm at room temperature for 5 min. The cell pellet was then incubated with 25-μM ROS dye (DCFDA/H2DCFDA - Cellular ROS Assay Kit, Abcam, UK) for 30 min. The cells were then washed with phosphate-buffered saline (PBS) and the cell suspension was transferred to 96 well black/-clear bottom plate and the signal intensity was read in a fluorescence microplate reader (Synergy HTX Multi-Mode Reader, Biotek Instruments, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Subsequently, the above experiment was repeated, whereby the HOB cells in both groups were treated with either NPs or MPs at similar concentrations for the first 24 h followed by treatment with 100 nM of ascorbic acid (Vitamin C, bio-world, USA) for another 24 h to see the antioxidant effect of vitamin C on the reactive oxygen species production. Following incubation, the treated cells were subjected to ROS dye treatment as described above and the signal intensity was read in a fluorescence microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The percentage change in the ROS production after antioxidant (AO) treatment for each TiO_2 concentration was compared to that of TiO_2 treated HOB cells without antioxidant treatment.

2.4. Enzyme-linked immunosorbent assay (ELISA)

TiO_2 treated HOB cells cultured for 24 h in the absence and presence of 100 nM of vitamin C were later analyzed for measurement of chemokine levels. The alterations in the secretion of IL-8 and MCP-1 by the HOB cells after preferred treatments in the culture supernatants were measured by ELISA. Human IL-8 and MCP-1 ELISA kits were purchased from Abcam (Abcam, UK), and ELISA was performed according to the manufacturer's instructions. Briefly, the culture supernatant collected after each treatment was added to anti-IL-8 and MCP-1 antibody-coated wells along with an antibody cocktail solution. After 1-h of incubation at 37 °C, the wells were washed three times with 1X wash buffer PT. Following this, 100 μl of TMB development solution was added and the plates were incubated with gentle shaking for 10 min in the dark, after which the wells were added with 100 μl stop solution, and the relative absorbance was quantified using an ELISA plate reader (Synergy HTX Multi-Mode Reader, Biotek Instruments, USA) at 450 nm. The concentrations of IL-8 and MCP-1 in the culture supernatant were calculated from concurrently plotted IL-8 and MCP-1 standard curves, respectively. All controls and experiments were done in triplicate.

2.5. Statistical analysis

Statistical significance for the test results was analyzed using One-Way ANOVA followed by Tukey's Post-hoc test. Data are represented as mean ± SD and a p-value < 0.05 was considered statistically significant. The statistical analysis was done using GraphPad Prism version 5.03 software (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Generation of ROS at different concentration of TiO_2 particles

There was increase in the production of ROS following the 24h exposure of HOB to different concentrations of TiO_2 NPs and MPs. The generation of ROS was highest in concentration of 100 μg/mL for both NPs and MPs compared to that of the control group. However, TiO_2 NPs at 100 μg/mL stimulated the highest amount of ROS. The amount of ROS generation was concentration dependent for both NPs and MPs (Fig. 1).

When the cells were treated with 100 nM of AO (Vitamin C) for another 24h, the results showed that the amount of ROS production was reduced in all concentrations of TiO_2 NPs and MPs used, while the reduction was significant in the concentration of 100 μg/mL in both NPs and MPs (Fig. 1).

3.2. Effect of antioxidant (AO) on IL-8 release

The IL-8 levels after TiO_2 treatments were comparatively the same except for the 100 μg/mL MPs treated group (Fig. 2a). There was a significant increase in the IL-8 production by the 100 μg/mL MPs treated HOB without AO cell group compared to the non-treated cells ($p < 0.05$). However, upon treatment with AO, the IL-8 production was found to be high in all groups but significantly lower compared to the control cells ($p < 0.5$).

3.3. Effect of antioxidant (AO) on MCP-1 release

The expression levels of MCP-1 cytokine after 24 h TiO_2 treatment without AO were comparable with the control for all the groups except for the 100 μg/mL MPs treated group (Fig. 2b). Following treatment with AO, there was a significant decrease observed in the expression levels of MCP-1, in the 100 μg/mL MPs treated HOB cell group compared to the non-treated cells ($p < 0.05$). Meanwhile, the treatment with AO did not demonstrate a significant change in the level of MCP-1 at concentrations of 5 and 20 μg/mL, but there was a significant drop in MCP-1 level release from the HOB cell line at a concentration of 100 μg/mL. The

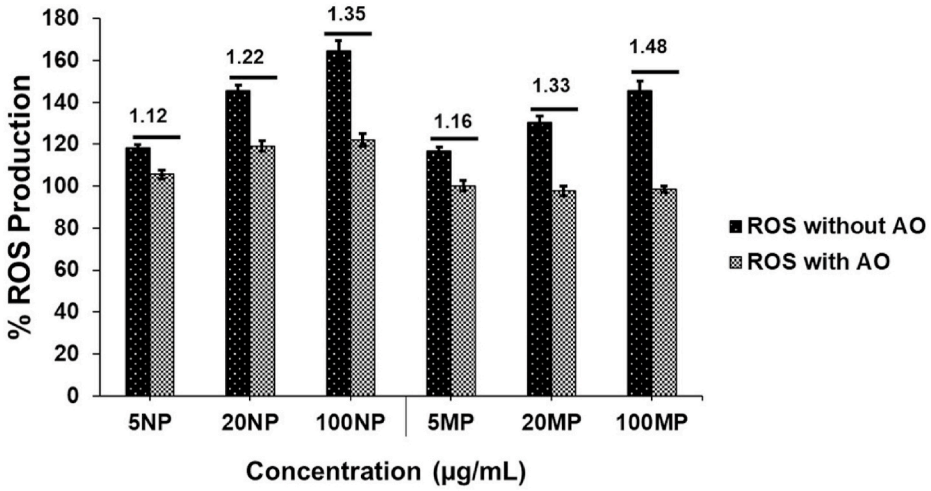

Fig. 1. The effect of antioxidant (AO) Vitamin C on reactive oxygen species (ROS) production in human osteoblast cells exposed to TiO_2 particles. Significant reduction in ROS production was observed after treatment with AO mainly, with higher concentration of TiO_2 NPs and MPs.

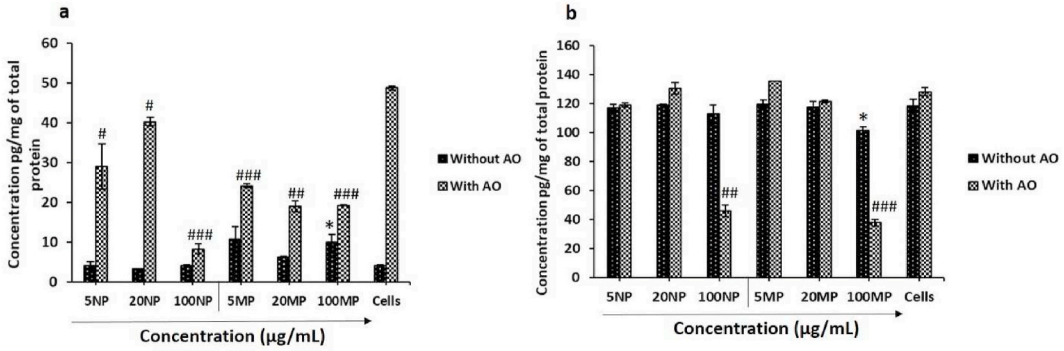

Fig. 2. The effect of antioxidant vitamin C on (a) IL-8 and (b) MCP-1 production. TiO_2 particles stimulated the release of IL-8 from HOB cells. Treatment with antioxidant caused further increase in the level of IL-8 for the control cells as well as the TiO_2 NPs and MPs treatment. Treatment with 100 MP TiO_2 particles without further AO treatment showed an increase in IL-8 production with respect to control HOB cells (* represents $p < 0.05$). When compared with control cells with AO treatment, TiO_2 NPs and MPs showed significant decrease in IL-8 production (#, ## and ### represents p values < 0.5, 0.01 and 0.001 respectively). MCP-1 release was found to be diminished in 100NP and 100 MP TiO_2 administered cells (## and ### represents p value < 0.01 and 0.001 respectively). Without AO treatment groups were comparable with that of control cells except for 100 MP TiO_2 treated cells (* represents $p < 0.05$).

effect of AO treatment reduced the release of MCP-1 for the 100 µg/mL TiO_2 NPs and MPs treated HOB cells compared to the control group.

4. Discussion

Success in dental implant therapy and orthopedic implant surgery has been attributed to tremendous improvement in material composition and implant design over the last two decades. Good patient selection, more refined surgical techniques, maintenance of optimum oral hygiene, and engagement in inpatient health education have further enhanced the success of dental implant therapy. Unfortunately, poor osseointegration at the bone-implant interphase may still occur due to continuous leaching of titanium particles from the implant surface into the peri-implant tissue environment, leading to local pro-inflammatory conditions and may progress towards implant failure.

In the present study, we investigated the effects of TiO_2 NPs and MPs on their immediate response with regard to ROS generation and proinflammatory chemokine release. This study demonstrated the possible link between TiO_2 particle size and their different concentrations on the generation of ROS, and subsequent cytokine production, in addition to the influence of antioxidants on the generation of ROS and release of MCP-1 and IL-8.

The results found that TiO_2 particles caused an increase in the production of ROS at all concentrations, and the highest increase was with the concentration of 100 µg/mL for both NPs and MPs. These results were in agreement with Niska K et al., 2015; Du H et al., 2012; Park EJ et al., 2008; Saquib Q et al., 2012[5–8] who found that excessive generation of ROS and oxidative damage impaired the antioxidant system due to the toxicity induced by TiO_2 NPs in human osteoblast cells.

This study found that the expression levels of MCP-1 cytokine after 24 h TiO_2 treatment were comparable with the control for all the groups except for the 100 µg/mL MPs treated HOB cells. Other workers showed that MCP-1 production is enhanced by an increase in oxidative stress,[9] while additionally, Chen XL et al., 2004 found that the oxidation-reduction mechanism could affect the activation of MCP-1 gene expression.[10]

Many cell types, including non-immune cells, are capable of producing MCP-1. Indeed, this study has shown the ability of HOB to release MCP-1 upon stimulation by TiO_2 particles.[11] Oxidative stress-related induction of MCP-1 by osteoblasts in-vivo may have important implications in driving bone regeneration or resorption.

IL-8 is the other pro-inflammatory cytokine that was investigated in this study. IL-8 is a chemokine that has recently been associated with tumor formation and proangiogenic properties, including its ability to cause directed migration of neutrophils and which is critical in the wound healing process.[12] IL-8 is normally low or undetected in normal

tissues, but it can be upregulated by oxidative stress.[13]

In this study, there was a significant increase in the IL-8 production by 100 μg/mL MPs of HOB treated cells compared to the non-treated cells. However, the TiO_2 NP_S treated cell groups showed a similar trend in IL-8 production as that of the control group. This finding was in consistent with another study that exposed A549 cells to TiO_2 for 24 h and found that the level of IL-8 increased significantly compared to the control group and that the release of IL-8 by TiO_2 was dose-dependent,[14] reflecting the inflammatory potency of TiO_2 particles on tissues.[15,16] Herein, from the results, we could ascertain that the pro-inflammatory response by the TiO_2 particles is size and concentration-dependent.

It was reported in many studies that the treatment with antioxidants can reduce the basal ROS generation and support osteoblastic activity.[17] Our findings also showed that the treatment with AO Vitamin C lead to a reduction in the generation of ROS at all the concentrations and the reduction was significant in the concentration of 100 μg/mL for both NPs and MPs TiO_2. Antioxidants suppressed the activation of the transcription factor NF-kβ and that in turn downregulate the chemokines stimulated MCP-1 (Xing L et al., 2007). On the contrary, this study showed that the level of IL-8 increased in all HOB treated with NPs and MPs following additional treatment with AO vitamin C, demonstrating the special role of IL-8 in the tissue healing process by exhibiting an anti-inflammatory property.[18] IL-8 has an interesting relationship with oxidative reaction[19] although it is still unclear about the effect of IL-8 on the fate of bone regeneration induced by the oxidative reaction. Yang A. et al., 2018 recently showed that IL-8 stimulates the potency of bone marrow mesenchymal stem cells towards osteoblastic lineage and differentiation via CXCR2-Mediated PI3k/Akt Signaling Pathway.[20]

The data in this study suggest that although IL-8 is a known pro-inflammatory chemokine, it tends to possess protective and defensive effects against oxidative stress when HOB is exposed to TiO_2 particles. It also highlighted the less known aspect of IL-8 in gene expressions related to oxidant generation.[21] IL-8 expression is highly sensitive to oxidative stress and stimulants, and antioxidants would ideally reduce IL-8 gene expression as confirmed by other workers.[22] However, results in this study showed that the IL-8 level increased in the presence of TiO_2, especially 100 μg/mL MPs, and was significantly further enhanced with AO vitamin C treatment. It is known that oxidative inactivation of the proteasome and the related activation of the p38 MAPK pathway provides a potential link between oxidative stress and overproduction of proinflammatory cytokines such as IL-8.[23] In other studies, Zhang X et al., 2005 noted that oxidative stress could initiate or propagate inflammatory responses through the activation of redox-sensitive chemokines following tissue damage. Since transcription factor Nrf2 is responsive to oxidative stress and upregulates the expression of cytoprotective and antioxidant genes that mitigate tissue injury, they postulated that Nrf2 might regulate chemokine expression. Based on the evidence that Nrf2 activation is largely cytoprotective, their findings raise the possibility that IL-8 may serve an anti-inflammatory role and further support the healing process.[18]

This study demonstrated an intense generation of oxidative stress when HOB cells were exposed to TiO_2 NPs and MPs in-vitro and leading to the release of MCP-1 and IL-8, in a dose-dependent manner. Treatment with Vitamin C repressed the release of MCP-1 while it further enhance the production of IL-8. However the ROS and cytokine response of HOB in this study was measured after 24 h only reflecting the acute cellular response that occurred following early exposure of titanium particles to the peri-implant environment that usually occur during the surgical implant placement phase. These findings may not represent the cellular response following chronic exposure and leaching of titanium particles into peri-implant environment.[24] Although Vitamin C has been employed as the antioxidant to mitigate ROS in this study, there is a growing body of evidence demonstrating other strategies in combating reactive oxygen species and development of oxidative stress in biomaterial implantation studies.[25,26] The findings may suggest future strategies in mitigating the impact of leaching of titanium dioxide particles from the dental implant surface into the peri-implant tissue environment.

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Declaration of competing interest

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. The revised manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We confirm that the order of authors listed in the manuscript has been approved by all of us. All authors have been involved in writing the manuscript and have read the manuscript before submission.

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