The Loadings of Titanium Dioxide on Multi-Walled Carbon Nanotubes

Determine Different Oxidative Stress in vitro

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Abstract
Titanium dioxide (TiO$_2$) has been engineered with multi-walled carbon nanotubes (MWCNT) to optimize their performance with different loading ratios. With their expanded industrial and commercial utilization, there is a parallel concern for their potential health risks. Evidence has shown that MWCNT can induce oxidative stress and cause pulmonary injury. However, little is known if different loadings of TiO$_2$ on MWCNT can modulate their toxicity. We hypothesize that higher loading MWCNT-TiO$_2$ hybrids will lead to increased reactive oxygen species (ROS) production, impaired mitochondrial respiration, and increased antioxidant gene expression in small airway epithelial cells (SAEC). MWCNT with varied loading of TiO$_2$, 10:1 MWCNT-TiO$_2$ (high), 20:1 MWCNT-TiO$_2$ (medium), and 30:1 MWCNT-TiO$_2$ (low) were tested for cytotoxicity, ROS production, oxygen consumption rates, and antioxidant gene expression. Cytotoxicity assays indicate that these nanohybrids are not acutely toxic to SAEC at the tested doses. Bioenergetics were insignificantly affected by the tested doses based on the Seahorse assay data. All treatments, except TiO$_2$ alone, significantly induced ROS production with a dose-dependent trend. The qPCR results demonstrated that expression of antioxidant genes was altered by the treatment of MWCNT-TiO$_2$ hybrids. Loading of TiO$_2$ to MWCNT can lead to oxidative stress, distinct from that induced by MWCNT alone.
Introduction

With the growing development of nanotechnology, carbon nanotubes (CNTs) are widely used in various applications with seemingly unlimited potential. CNTs are small-sized, hollow cylinders of one or multiple sheets of rolled graphene, called single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), respectively. By manipulating CNTs, there are countless functions in drug delivery, renewable energy, cancer treatment, and more (Gannon et al., 2007; Jackson et al., 2013; Wu et al., 2005). However, there are parallel concerns of their implicating environmental and health risks from unintended exposure.

Nanoparticles (NPs) are recognized inducers of reactive oxygen species (ROS) production. With an excess of ROS, the body's biological system is unable to detoxify and repair the consequential damage properly. This imbalance can lead to a progression of detrimental oxidative stress within cells, prompting DNA damage, mitochondrial dysfunction, and activation of antioxidant signaling pathways, associated with cell death. Upon inhalation, the nanoparticles provoke the production of free radicals, driving oxidative stress and its inflammatory responses. The biopersistant nature of nanoparticles and its consequent excessive inflammatory reactions can be attributed to the atypical tissue remodeling and fibrosis observed in alveolar epithelial cells (Ravichandran et al., 2011).

Particularly, titanium dioxide (TiO$_2$) nanoparticles, a white, fine crystalline powder, widely used for its stability and anticorrosive and photocatalytic properties (Shi et al., 2013), have been engineered with multi-walled carbon nanotubes (MWCNT) to optimize their microbial fuel cell performance. Fuel cells are sources of sustainable power that convert natural gases into electrical energy (Abdullah et al., 2017). Traditionally, platinum (Pt) is the catalyst for fuel cells; however, Pt can be poisoned by carbon-containing species, diminishing the capacity of the fuel cells. By substituting Pt with TiO$_2$, fuel cells gain superior electro-catalytic performance (Ito et al., 2013; Ercelik et al., 2017) with the unique benefits of TiO$_2$, such as its high surface-to-volume ratio (Bhattacharya et al., 2009).
The large-scale production and use of TiO$_2$ nanoparticles warrant questions of its toxicity. Conventionally believed to have low toxicity, TiO$_2$ has also been linked to unfavorable ROS production and pulmonary reactions (Afaq et al., 1999; Wang & Li, 2012). A great deal of attention has been focused on the benefits of CNTs and their capabilities when functionalized with other nanoparticles, like TiO$_2$. While there is a number of research on the toxic effects of MWCNT or TiO$_2$ alone, little to no research has been conducted on if, and how, the different loadings of TiO$_2$ to MWCNT would modulate the toxicity of cells.

**Aims and Hypotheses**

The aim of this study is to investigate the potential role of different loadings of TiO$_2$ to MWCNT in the modulation of small airway epithelial cells’ (SAEC) toxicity. We hypothesize that higher loading MWCNT-TiO$_2$ hybrids exposed to SAEC will lead to:

1. Increased reactive oxygen species (ROS) production,
2. Impaired mitochondrial respiration, and
3. Increased antioxidant gene expression.

**Methods**

**Materials and Procedure**

Human SAEC cultures were maintained in Advanced RPMI (Roswell Park Medium Institute) 1640 medium. MWCNT, TiO$_2$, and MWCNT-TiO$_2$ hybrid nanoparticles were provided by Navid Saleh (University of Texas, Austin). For the suspensions, glass tubes were weighed and prepared with equal volumes of MWCNT or MWCNT-TiO$_2$ hybrids and 1% Pluronic F68 in deionized water. TiO$_2$ alone was prepared volume-for-volume in Advanced RPMI 1640 medium. The tubes were then sonicated at 20-50-watt power for 25 minutes (SonifierTM S-450, Branson Ultrasonics) in an ice bath. Before use in the experiments, all stocks were re-sonicated at 20-watt power for 1 minute. SAEC were exposed to concentrations of 2 μg/mL, 20 μg/mL, and 50 μg/mL of each of the following suspensions: MWCNT, TiO$_2$, 10:1 MWCNT-TiO$_2$ hybrid (highest loading of TiO$_2$), 20:1 MWCNT-TiO$_2$ hybrid, 30:1 MWCNT-TiO$_2$ hybrid (lowest loading of TiO$_2$).
Cytotoxicity Assays

SAEC were prepared with exposure media (RPMI 1640 + 1% PSN + 1% L-Glutamax) in 12-well plates. After removing the media and washing the cells with PBS, the control media and the various suspensions (50 μg/mL) were added to the designated wells and incubated for 24 hours. Following the exposure period, cytotoxicity was determined by a standard trypan blue dye staining technique, which allowed the differentiation of the number of live and dead (blue) cells under a light microscope with a hemocytometer. The assays were run in triplicates.

ROS Assays

Reactive oxygen species (ROS) production was measured using a DCFDA (2',7'-dichlorofluorescin diacetate) method (Abcam). SAEC were plated in a 96-well plate at 20,000 cells/well to adhere overnight. After 24 hours, the media was removed and washed with phosphate buffered saline (PBS). The cells were then stained with 100 μL/well DCFDA dye and incubated for 45 minutes in the dark. After washing with PBS, the cells were treated with the various suspensions with a positive control, H₂O₂ (50 μM). Fluorescence activity was read, and the relative fold change of fluorescence activity from the control was calculated. The ROS assays were run in duplicates for each treatment.

Seahorse Assays

Oxygen consumption rates were measured using the Seahorse XF24 instrument (Agilent Technologies). SAEC were plated in a 24-well plate at 20,000 cells/well overnight, then exposed to 50 μg/mL of the CNT suspensions or 1% Pluronic F68 (control) for 24 hours. Following exposure, the media was removed, and the cells were washed twice before being treated with XF mito stress test media (Agilent Technologies). Basal respiration rates were measured thrice, then SAEC was injected with oligomycin (1 μM) to inhibit ATP synthase and measure the mitochondrial respiration associated with ATP production. Three respiration rates were measured again, with a subsequent injection of Carbonyl cyanide-4(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 μM). FCCP impedes the mitochondrial proton gradient, allowing free
electron flow, ensuing maximal oxygen consumption. Maximal respiration rates were measured thrice. The cells were then injected with antimycin A (1 μM), which halts mitochondrial respiration to allow the measurement of the cell’s non-mitochondrial respiration. Spare respiratory capacity was calculated by subtracting the basal respiratory rate from the maximal respiratory rate. The Seahorse assay was run in duplicates.

**Antioxidant Gene Expression**

RNA from SAEC was isolated by use of STAT-60 and chloroform, precipitated by glycol blue, isopropanol and ethanol, reconstituted with RNAsecure (Ambion), then quantified by the Synergy H1 plate reader (BioTek Instruments). The isolated RNA was further treated with DNase I (PerfeCta, Quanta Bioscience), then reverse transcribed to cDNA (qScript cDNA Synthesis Kit, Quanta Bioscience). For MWCNT alone, the RNA was first isolated using the RNeasy Mini Kit (Qiagen), then reverse transcribed to cDNA with the previously stated process. The mRNA expression of oxidative stress genes (superoxide dismutase 2 [SOD2], glutathione peroxidase 1 [GPX1], heme oxygenase 1 [HMOX1], surfactant-associated protein D [SFPD], mitofusin 1 [MFN1], and mitofusin 2 [MFN2]) was measured by quantitative polymerase chain reaction (qPCR) assays. Expression assays were run in duplicate for each gene, normalized to the housekeeping gene, GAPDH. Relative fold change was analyzed to the control.

**Statistical Analysis**

Statistical significance was determined by either one-way ANOVA or two-way ANOVA, followed by a post-hoc Tukey’s multiple comparisons test, using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). The null hypothesis was rejected at a p-value < 0.05.

**Results**

**Cytotoxicity of SAEC**

With a cell viability of 80-90% for all treatments, cytotoxicity assays indicated that the suite of nanomaterials tested was not acutely toxic to SAEC at the doses applied. However, all
treatment groups presented statistically significant modulated viabilities against that of the control group (p < 0.0001), see Figure 1.

**ROS Production by Loadings of MWCNT-TiO$_2$**

All treatment groups, except TiO$_2$ alone, induced ROS production in a dose-dependent manner with significance shown at 50 μg/mL (p < 0.05) (see Figure 2). Interestingly, when comparing the concentrations of one treatment groups to that of another, lower loading of TiO$_2$ on MWCNT significantly induced more ROS production compared to that of MWCNT alone, TiO$_2$ alone, and hybrids at 50 μg/mL (p < 0.0001). Other doses did not show statistical significance, except at the dose of 20 μg/mL, in which ROS production of TiO$_2$ alone was significantly different from that of 10:1 MWCNT-TiO$_2$ (p < 0.01).

**MWCNT Do Not Alter Mitochondrial Function in SAEC**

While we had predicted that exposure to MWCNT-TiO$_2$ suspensions would lead to impaired mitochondrial function, the Seahorse assay determined that the NP treatments (50 μg/mL) insignificantly affected the bioenergetics of the cell. Oxygen consumption rates (OCR) of all treatments varied little from the control (see Figure 3).

**Gene Expression Levels of Antioxidant Genes in SAEC**

Under the assumption that oxidative stress would amplify the expression of antioxidant genes, the qPCR results demonstrated that treatment of MWCNT-TiO$_2$ hybrids variably altered gene expression in SAEC (see Figure 4). While SOD2 activity of MWCNT-treated SAEC at 50 μg/mL was significantly decreased, the activity of 10:1 MWCNT-TiO$_2$ cells at 20 μg/mL was significantly increased. GPX1 activity was significantly lowered in the treatment of MWCNT at 50 μg/mL, but insignificantly affected in the other treatment groups. Within the 50 μg/mL dosage, the 10:1 MWCNT-TiO$_2$-treated SAEC showed significant differences in GPX1 activity from MWCNT alone and TiO$_2$ alone. HMOX1 did not show significance between treatments, but within the 50 μg/mL dose, TiO$_2$ alone was significantly different from MWCNT alone, 10:1 MWCNT-TiO$_2$, and 30:1 MWCNT-TiO$_2$, and MWCNT alone was significantly different from 20:1
MWCNT-TiO₂. The activity of SFPD only showed a significant difference between the control and 50 μg/mL of MWCNT alone. Within the 50 μg/mL dose, each treatment’s SFPD activity was significant against MWCNT alone. MFN1 showed a significant decrease in activity between the control and 50 μg/mL of MWCNT. Within the 50 μg/mL dose, activity from MWCNT-treated SAEC was significantly lower than that in TiO₂ alone and 30:1 MWCNT-TiO₂. Activity due to TiO₂ alone was significantly higher than that in 10:1 MWCNT-TiO₂ and 20:1 MWCNT-TiO₂. For MFN2, there was no observed significance between treatment groups, but within the 50 μg/mL dose, the expression of MWCNT was significantly lower than that in TiO₂ alone and 30:1 MWCNT-TiO₂. TiO₂ alone was significantly higher than that in 10:1 MWCNT-TiO₂.

Discussion

The increased use of CNTs needs to be met with proportionate research of its toxicity. Though the cytotoxicity of the SAEC and its mitochondrial function were found to be insignificantly affected, decreased cell viability and mitochondrial dysfunction were typically detected in other similar studies (Huerta-García et al., 2014; Tang et al., 2013). The dose-dependent upward trend of ROS production in SAEC was similarly seen in a study with dose-dependence from MWCNT alone (Yu et al., 2016). Notably, there is an apparent contradiction in the mitochondrial function data and ROS production data. Dysfunctional mitochondria produce ROS as by-products of the electron transport chain, in which electrons leak and react with oxygen. The observed mitochondrial function due to treatments at 50 μg/mL were insignificantly different from the control, yet ROS production of treatments at 50 μg/mL were close to 8-fold of the control. Normal mitochondrial function would have shown ROS levels closer to the control.

The genes studied (SOD2, GPX1, HMOX1, SFPD, MFN1, MFN2) are all antioxidant and mitochondrial function genes that encode for proteins and enzymes to relieve oxidative stress in the cells. SOD2 translates for a mitochondrial protein that binds to superoxides and converts them into H₂O₂, which is then further reduced into H₂O by GPX1 (Hosoki et al., 2012). Previous studies have found up-regulation of SOD2 expression in MWCNT and down-regulation of
expression in metal-exposed cells, conflicting with our findings (Sellamuthu et al., 2015; Wang et al., 2015; Yu et al., 2016). The inhibition of GPX1 activity due to higher loading of MWCNT-TiO$_2$ was similarly found in another study (Wang et al., 2015). A study also observed increased expression of HMOX1 activity in the presence of metal-exposed cells compared to the control (Sellamuthu et al., 2015), which relates to our findings that HMOX1 activity increases with TiO$_2$ alone and the loading of TiO$_2$ onto MWCNT. SFPD is a surfactant protein that modulates inflammatory responses to cell injury. The resulting increase in SFPD activity for respiratory protection in MWCNT-exposed cells is consistent with prior studies (Han et al., 2009). MFN1 and MFN2 act to support mitochondrial fusion, protecting the organelle against respiratory harm (Chen et al., 2003). TiO$_2$-exposed cells showed the highest level of MFN1 and MFN2 expression, but also showed increasing expression as loading of MWCNT-TiO$_2$ decreased. The expression of SOD2, GPX1, HMOX1, MFN1, and MFN2 in the MWCNT-TiO$_2$ hybrids seemed to all fall approximately half way between that of MWCNT and TiO$_2$ alone. The expression of SFPD did not follow this trend; instead showing a decline in expression as hybrid loading decreased. Unfortunately, the gene expression results were limited in their lack of treatments for MWCNT at the concentration of 2 and 20 μg/mL, which could have given way to possible gaps in significant gene interaction.

Contrary to our hypotheses, we can conclude that lower loading of TiO$_2$ onto MWCNT at higher concentrations is the most likely to induce oxidative stress and antioxidant gene modulation. These contradictory results prove need for further investigation of the effects of complex MWCNT-TiO$_2$ hybrids. The next steps of this study would be to increase replicates of treatments for stronger statistical power, explore other antioxidant genes that affect oxidative stress pathways and find interactions between those genes, as well as investigate the effects of MWCNT-TiO$_2$ hybrids on other environmentally exposed organisms. Oxidative damage is a key indicator of potential nanomaterial toxicity. Future research should prioritize the interactions between MWCNT and TiO$_2$ and study their combined toxicity and cellular damage effects.
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**Figure 1.** Cytotoxicity levels indicated by percentage of live cells from each treatment group.

**Figure 2.** Fold change of ROS production compared to the control. Statistical significance ($p < 0.05$) is shown between treatment groups (a, b, c, d, e) and within concentrations of treatments (* indicates significance to MWCNT, + indicates significance to TiO$_2$, • indicates significance to 10:1 MWCNT-TiO$_2$, and • indicates significance to 20:1 MWCNT-TiO$_2$). Refer to the legend from Figure 1.
Figure 3. Mitochondrial function indicated by oxygen consumption rates in SAEC. No statistical significance was observed.
Figure 4. Fold change of antioxidant gene expression compared to the control. Significance (p < 0.05) is shown between treatment groups (a, b, c) and within concentrations of treatments (* indicates significance to MWCNT), + [indicates significance to TiO$_2$]). Refer to the legend of Figure 1.