Titanium dioxide induced inflammation in the small intestine

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Abstract

AIM: To investigate the effects of titanium dioxide (TiO$_2$) nanoparticles (NPTiO$_2$) and microparticles (MPTiO$_2$) on the inflammatory response in the small intestine of mice.

METHODS: Bl 57/6 male mice received distilled water suspensions containing TiO$_2$ (100 mg/kg body weight) as NPTiO$_2$ (66 nm), or MPTiO$_2$ (260 nm) by gavage for 10 d, once a day; the control group received only distilled water. At the end of the treatment the duodenum, jejunum and ileum were extracted for assessment of cytokines, inflammatory cells and titanium content. The cytokines interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IL-23, tumor necrosis factor-α (TNF-α), intracellular interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) were evaluated by enzyme-linked immunosorbent assay in segments of jejunum and ileum (mucosa and underlying muscular tissue). CD4$^+$ and CD8$^+$ T cells, natural killer cells, and dendritic cells were evaluated in duodenum, jejunum and ileum samples fixed in 10% formalin by immunohistochemistry. The titanium content was determined by inductively coupled plasma atomic emission spectrometry.

RESULTS: We found increased levels of T CD4$^+$ cells (cells/mm$^3$) in duodenum: NP 1240 ± 139.4, MP 1070 ± 154.7 vs 458 ± 50.39 (< 0.01); jejunum: NP 908.4 ± 130.3, MP 813.8 ± 103.8 vs 526.6 ± 61.43 (< 0.05); and ileum: NP 818.60 ± 123.0, MP 640.1 ± 32.75 vs 466.9 ± 22.4 (< 0.05). In comparison to the control group, the groups receiving TiO$_2$ showed a statistically significant increase in the levels of the inflammatory cytokines IL-12, IL-4, IL-23, TNF-α, IFN-γ and TGF-β. The cytokine production was more pronounced in the ileum (mean ± SE): IL-12: NP 33.98 ± 11.76, MP 74.11 ± 22.34 vs 25.65 (< 0.01); IL-4: NP 17.36 ± 9.96, MP 22.94 ± 7.47 vs 2.19 (< 0.05); IL-23: NP 526.6 ± 61.43, MP 640.1 ± 32.75 vs 22.4 (< 0.05); TNFα: NP 3.71 ± 1.33, MP 5.44 ± 1.67 vs 0.99
INTRODUCTION

We are exposed daily through inhalation, ingestion or contact to many environmental and engineered particles. The gastrointestinal tract is continuously exposed to particles that are ingested per person per day in the United Kingdom. Besides the amount of exogenous particles that are in the diet, such as TiO$_2$ nanoparticles (NP), there are other sources of exposure to TiO$_2$ particles in terms of their inflammatory potential within the gastrointestinal tract.

Many studies have revealed that exposure to TiO$_2$ can cause adverse effects such as the generation of reactive oxygen species$^{[5-7]}$, inflammatory responses$^{[5,6,11-13]}$, tumors$^{[14]}$, cytotoxicity$^{[15]}$ and apoptosis$^{[16]}$. In vitro studies showed that NP can be accumulated in many organs such as the liver, kidney, spleen, lung, heart and brain$^{[7,18]}$, thus generating a number of adverse effects. Previous investigations have found that TiO$_2$ accumulates in the intestine in rats$^{[19]}$ and fish$^{[20]}$ and migrates to other organs. Accumulation of TiO$_2$ inside the intestinal cells, especially in lymphoid-rich areas (Peyer's patch), might lead to damaging outcomes such as inflammation and could be involved in the pathogenesis of inflammatory bowel disease$^{[21,22]}$. However, little is known about the influence of either micro- or NP on the gut, which is potentially exposed to particles in the diet, such as TiO$_2$. To date, most of the studies regarding the adverse effects of TiO$_2$: particles on human health have involved the pulmonary tract. No available in vivo work has evaluated the impacts of TiO$_2$: particles in terms of their inflammatory potential in the small intestine of mice. We aimed to evaluate cytokine production and inflammatory cell proliferation in the small intestine of mice after oral exposure to TiO$_2$.

MATERIALS AND METHODS

Particles

Uncoated anatase TiO$_2$: microparticles (MPTiO$_2$) (260 nm) that are commercially available for use in food, pharmaceuticals, and cosmetics were obtained from Evonik Degussa (Kronos® 1171). Uncoated TiO$_2$: nanoparticles (NPTiO$_2$) (mean diameter of 66 nm), consisting mostly of anatase, were synthesized by Professor de Azevedo WM from the Department of Fundamental Chemistry of the Federal University of Pernambuco (Recife, Brazil) at pH = 2.0, followed by centrifugation. Particle size was determined by dynamic light scattering Nanotrac$^\text{®}$ (Microtrac Inc., United States) by Professor Toma SH from the Laboratory of Supramolecular Chemistry and Nanotechnology of the Chemistry Institute of the University of São Paulo (São Paulo, Brazil). Particle phase was characterized using an X-ray diffractometer Rigaku MiniFlex$^\text{®}$ (Rigaku Corporation, Japan) under monochromatic radiation, Cu Kα (1.541 Å, 30 kV, 15 mA, 0.02°, 2° to 61° range), also by Professor Toma SH.

Animals and treatment

B1 57/6 male mice (20 to 25 g) were obtained from the Center of Bioterism of the School of Medicine, University of São Paulo (São Paulo, Brazil). Animals were housed in cages in a ventilated room in a 12-h light/dark cycle. Food and water were available ad libitum. They were acclimated to this environment for 1 wk before treatment. All animal experimental procedures were in...
compliance with the School of Medicine, University of São Paulo Ethics Committee. Mice were randomly divided into three groups of 12 animals, and received either distilled water suspensions containing TiO$_2$ (100 mg/kg body weight) as MP, or as NP, or distilled water as a control. The suspension was given by gavage for 10 d, once a day. TiO$_2$ particles were suspended in 500 μL of distilled water. The suspension was mixed and sonicated immediately before being administered to animals to minimize particle aggregation. At the end of the treatment the animals were weighed and killed in a CO$_2$ chamber, and had their duodenum, jejunum and ileum extracted for assessment of cytokines, inflammatory cells and titanium content.

**Assessment of cytokines**
Segments of jejunum and ileum - mucosa and underlying muscular tissue - were extracted from animals, stored at -80°C and subsequently homogenized with Tris-buffer (10 mmol)-ethylenediamine tetraacetic acid (1 mmol)-Triton (1%) containing protease, aprotinin, chymostatin and leupeptin inhibitors (1 μg/mL of solution) and phenylmethylsulfonyl fluoride (1 μL/mL of solution). After homogenization, the sample was centrifuged at 14 000 g for 10 min at 4°C and the supernatant was stored at -80°C until the cytokines were analyzed using an enzyme-linked immunosorbent assay (ELISA). Interleukin-1β (IL-1β), IL-2, IL-6, IL-8 (Keratoocyte Chemotactrant), IL-10, IL-12, IL-13, IL-17, IL-23, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) were evaluated using ELISA kits (IL-1β, IL-2, IL-6, IL-12, IL-13, IL-17, IL-23, TNF-α, IFN-γ, and TGF-β from eBioscience; and IL-10 and IL-8 from R and D systems) according to the manufacturer’s recommendations.

**Quantification of inflammatory cells**
Duodenum, jejunum and ileum samples were fixed in 10% formalin and were embedded in paraffin. Five-mm thick sections were cut, placed onto glass slides and then stained with HE. The slides were examined by optical microscopy (E800, Nikon, United States) by Professor Dagli MLZ from the Department of Pathology of the School of Veterinary Medicine, University of São Paulo (São Paulo, Brazil).

**Histopathological evaluation of small intestine**
Duodenum, jejunum and ileum samples were fixed in 10% formalin and embedded in paraffin. Five-mm thick sections were cut, placed onto glass slides and then stained with HE. The slides were examined by optical microscopy (E800, Nikon, United States) by Professor Dagli MLZ from the Department of Pathology of the School of Veterinary Medicine, University of São Paulo (São Paulo, Brazil).

**Titanium content analysis**
The titanium content in intestinal samples from each group was analyzed with the purpose of verifying the efficacy of the treatment, and to make sure that the animals of the control group did not contain titanium (Ti) in their tissues. To determine the presence of Ti in the small intestine of the animals receiving TiO$_2$ particles, the experiment was repeated including two animals in each group. At the end of the treatment, the small intestine was extracted for analysis of the titanium content by inductively coupled plasma atomic emission spectrometry (ICP-AES). The small intestine was removed in its entirety (from the pylorus to the ileocecal valve), preserving the mucosa and muscle tissue, stored at -80°C and taken to the Basic Analysis Laboratory of the Analytical Center, Chemistry Institute, University of São Paulo (São Paulo, Brazil) where it was homogenized and processed by the local team following their standard protocol.

**Statistical analysis**
Data for each parameter evaluated are shown as the mean ± SE. Data were analyzed assuming a gamma distribution (identity link) and using generalized linear models. Pairwise comparisons and Fisher’s least significant difference post-hoc test were applied to evaluate the differences between the groups. $P < 0.05$ was considered significant. Commercialedge available software was used for analysis (PASW Statistics Version 18, United States).

**RESULTS**

**Titanium content**
The titanium content in the small intestine from each group was determined by ICP-AES. The control group showed no detectable levels of titanium in the intestine. The titanium content (mg/kg of tissue) in the intestine was (animal 1/animal 2): 1.43/11.68 in the NP group, and 0.39/0.22 in the MP group.

**Cytokine concentration in jejunum and ileum**
In comparison to the control group, MPTiO$_2$ generated a statistically significant increase in inflammatory cytokines. The group receiving MPTiO$_2$ showed an enhanced concentration of IFN-γ and IL-23 in the jejunum, and IL-12, TNF-α, IFN-γ, IL-2, IL-23, and TGF-β in the ileum (Figure 1). The group receiving NPTiO$_2$ showed increased TNF-α, IFN-γ, IL-2, IL-23, and TGF-β, but only in the ileum. There was no significant difference between the groups receiving MPTiO$_2$ and NPTiO$_2$, as both showed statistically similar levels of cytokine production. A more important cytokine production was found in the ileum compared to the jejunum.

**Inflammatory cell infiltration in small intestine**
We found a statistically significant increase in T CD4$^+$ cells in the duodenum, jejunum and ileum of mice treated with MPTiO$_2$ and NPTiO$_2$, compared to the control group. There was no significant difference between the
Figure 1  Mean and SE of cytokine concentration in the small intestine of mice according to treatment groups. A: Pro-inflammatory cytokines in the jejunum; B: Pro-inflammatory cytokines in the ileum; C: T-helper (Th) 1 type cytokines in the jejunum; D: Th1 type cytokines in the ileum; E: Th2 type cytokines in the jejunum; F: Th2 type cytokines in the ileum; G: Th17 type cytokines in the jejunum; H: Th17 type cytokines in the ileum; I: Transforming growth factor-β in the jejunum and ileum. *P < 0.05 for pairwise comparison test. NPTiO: Titanium dioxide nanoparticles; MPTiO: Titanium dioxide microparticles; IL: Interleukin; IFN: Intracellular interferon.
MPTiO₂ and NPTiO₂ groups. The results are illustrated as the mean and SE in Figure 2. Mice treated with MP-TiO₂ or NPTiO₂ showed no increase in T CD8⁺, natural killers, or dendritic cells in the small intestine (data not shown).

**Histopathological evaluation of the small intestine**

No major histological changes were found in small intestine samples of experimental animals, except for hypertrophy and hyperplasia of the mucosal epithelium of mice receiving TiO₂ particles, which were not seen in the control group. These findings were observed in all three regions of the small intestine (duodenum, jejunum and ileum) of mice treated with NPTiO₂, while animals treated with MPTiO₂ showed these effects only in the ileum.

**DISCUSSION**

**Inflammatory response**

To date, there have been few data regarding the inflammatory potential of TiO₂ particles in the intestine. Here, we sought to evaluate inflammatory responses induced by TiO₂ in the small intestine in mice. We found that TiO₂ as micro- and nano-sized particles produced a pro-inflammatory response in the small intestine by generating increased inflammatory cytokine production and T CD4⁺ cell proliferation. The generation of pro-inflammatory cytokines and T CD4⁺ cells induced by NPTiO₂ was also described by Schanen et al. in an in vitro immune construct study.

The main cytokines enhanced in our study were IL-12, IFN-γ, TNF-α, IL-4, IL-23 and TGF-β. IL-12, TNF-α and IFN-γ are Th1-type cytokines, whereas IL-23 and TGF-β are both involved in the Th17 pathway. In murine models, TGF-β together with IL-6 promotes differentiation of naïve T cells to Th17 cells. Here we found no significant increase in IL-6 or IL-17, which is produced by Th17 cells. Taken together, these data suggest that TiO₂ particles provoke a pro-inflammatory response mainly through the Th1-mediated pathway in the small bowel in mice. Other authors found a Th-2 mediated immune response in the lungs, induced by nano-sized TiO₂ exposure in mice. However, inflammatory responses differ depending on the organ, and thus the immune response caused by exposure to TiO₂ may diverge between the respiratory tract and the gut.

Cytokine production was more pronounced in the ileum. These findings might be related to differences concerning particle uptake throughout the gut. It is known that the ileum presents the greatest concentration of M cells (Peyer’s patch) in the intestine, which are believed to represent the main pathway of particle uptake across the gastrointestinal tract. Li et al. observed greater absorption of lipid NP in the ileum and colon of rats when compared to other segments of the intestine, reinforcing the importance of M cells as a pathway of particle uptake. Given that the ileum represents the major site of particle uptake, we would expect to find a more substantial inflammatory response in this area.

We also evaluated histopathological changes in the small intestine of mice after exposure to TiO₂. We observed hypertrophy and hyperplasia of the mucosal epithelium in both groups receiving TiO₂ particles. These findings were also described by other authors in TiO₂-related studies. Alveolar epithelium hypertrophy was observed in the lungs of rats exposed to nano-sized TiO₂. Mice, rats and hamsters showed histopathological changes consistent with alveolar epithelial cell hypertrophy and hyperplasia after long-term inhalation of fine TiO₂.

Taken together our data provide evidence that micro- and nano-sized TiO₂ particles induce a pro-inflammatory response in the small intestine in mice, after a short period of oral exposure.

The titanium content of the small intestine samples was determined by ICP-AES at the end of the experiments to guarantee the efficacy of the treatment with TiO₂ and to ensure that the control group had no detectable levels of titanium in their tissues. Our results demonstrated that TiO₂ particles were absorbed by the small intestine in mice after a short period of oral exposure, as the animals that received TiO₂ particles had titanium in their tissues at the end of the experiment. The control group showed no detectable levels of titanium in any sample. We found greater amounts of titanium in the small intestine of the animals receiving NPTiO₂ in comparison to those receiving MPTiO₂. These findings indicate that smaller particles may be absorbed to a greater extent than larger ones in the gut.

**Nanoparticles vs microparticles**

Previous investigations of TiO₂ particles found that TiO₂ as nano-sized particles is more toxic than similarly composed, but larger sized particles. Thus, we aimed to compare the effects of micro- and nano-sized TiO₂ on the small intestine. However, we found no statistically significant difference in cytokine secretion or T CD4⁺ cell proliferation between the groups who received MPTiO₂...
and NPTiO. Other authors have already reported the lack of significant differences in the pulmonary effects in rats exposed to TiO\(_2\) particles of different size[13,32-35].

In conclusion, We demonstrated that over a short period TiO\(_2\) as micro- and nano-particles induced a Th1-mediated inflammatory response in the small intestine of mice, especially in the ileum. These findings provide evidence of the inflammatory potential of TiO\(_2\) particles in the gastrointestinal tract. Since we are exposed to TiO\(_2\) particles on a daily basis, as well as to many other engineered particles, these data should be taken into consideration when evaluating the safety of biomaterials.

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