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Dysbiosis of gut microbiota by chronic coexposure to titanium dioxide nanoparticles and bisphenol A: Implications for host health in zebrafish

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ABSTRACT

Gut microbiota is of critical relevance to host health. However, toxicological understanding of environmental pollutants on gut microbiota is limited, not to mention their combined effects. In the present study, adult zebrafish (*Danio rerio*) were exposed to titanium dioxide nanoparticles (nano-TiO_2; 100 μg/L), bisphenol A (BPA; 0, 2, and 20 μg/L) or their binary mixtures for three months. Sequencing of 16S rRNA amplicons found that nano-TiO_2 and BPA coexposure shifted the intestinal microbial community, interacting in an antagonistic manner when the BPA concentration was low but in a synergistic manner at a higher BPA concentration. Sex- and concentration-dependent responses to the coexposure regime were also observed for zebrafish growth and intestinal health (e.g. neurotransmission, epithelial barrier permeability, inflammation, and oxidative stress). Correlation analysis showed that oxidative stress after nano-TiO_2 and BPA coexposure was tightly associated with the imbalanced ratio of pathogenic *Lawsonia* and normal metabolic *Hyphomicrobium*, where higher abundance of *Lawsonia* but lower abundance of *Hyphomicrobium* were induced concurrently. A positive relationship was observed between zebrafish body weight and the abundance of *Bacteroides* in the gut, which was also closely associated with the genera of *Anaerococcus*, *Finegoldia*, and *Peptoniphilus*. This study revealed, for the first time, the combined effects of nano-TiO_2 and BPA coexposure on the dynamics of the gut microbiome, which proved to have toxicological implications for zebrafish host health.

1. Introduction

Commensal microbes residing in animal guts are tremendously diverse and abundant. As an integral part of animal composition, gut microbiota is increasingly recognized to play crucial roles in the maintenance of host health (Holmes et al., 2011; Kinross et al., 2011). Because of the intricate involvement of microbes in many biological processes, such as energy metabolism, immune modulation, and neurotransmission (Tremaroli and Backhed, 2012), dysbiosis of gut microbiome composition may result in the onset of various diseases in host organisms, such as obesity and diabetes (Mathis and Benoist, 2012; Snedeker and Hay, 2012; Tilg and Kaser, 2011). Furthermore, gut microbiota dynamics are highly sensitive to exogenous stressors, environmental pollutants being of particular concern (Jin et al., 2017). A variety of pollutants have the potential to alter the composition of the gut microbiomic community, including nanoparticles (NPs) and persistent organic pollutants (POPs).

Since the advent of NPs synthesis, titanium dioxide NPs (nano-TiO_2) are one of the most promising engineered nanomaterials that have obtained widespread application on commercial, industrial, and environmental scales. This has led to their inevitable release into aquatic environments (predicted environmental concentration: 0.7–16 μg/L), which constitutes a potential threat to living organisms (Chen and Mao, 2007; Mueller and Nowack, 2008;

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Robichaud et al., 2009). Additionally, bisphenol A (BPA) is a widely used organic chemical in the industrial production of engineered plastics and food cans (Huang et al., 2012). Being listed as a representative POP, severe BPA pollution in aquatic environments has been reported to be as high as 4–28 μg/L (Heisterkamp et al., 2004; Huang et al., 2012).

The cytotoxic and antibacterial activity of nano-TiO_2, facilitated by the generation of reactive oxygen species, has been extensively documented (Sharifi et al., 2012; Brunet et al., 2009). In a model colon, nano-TiO_2 is reported to impact gut microbiota and disturb the production of short-chain fatty acids (Taylor et al., 2015; Pietroiusti et al., 2016; Waller et al., 2017). Though mainly known as a potent endocrine disruptor (Michalowicz, 2014; Bhandari et al., 2015), BPA dietary exposure also leads to changes in the gut microbiome of mice, where increased abundance of *Bacteroides* is associated with host metabolic disorders (Javurek et al., 2016). Considering the prevalence of both NPs and organic pollutants, they generally coexist in aquatic environments. As such, they will interact in the chemical mixture of the aquatic environment, which alters their innate bioavailability and toxicity. For instance, a previous study finds that nano-TiO_2 can adsorb BPA, which leads to elevated tissue burdens of both contaminants in zebrafish (*Danio rerio*) and synergistically enhances the endocrine disrupting effects (Fang et al., 2016). However, there is little or no information regarding the combined effects of nano-TiO_2 and BPA pollutants on gut microbiota.

In view of the susceptibility and importance of gut microbiota, this study sought to address this knowledge gap by conducting a chronic coexposure study of nano-TiO_2 and BPA using zebrafish as an ecotoxicological model, which metabolism is also closely regulated by the hosted microbes in gut. Adult zebrafish (*D. rerio*) were exposed to nano-TiO_2 (100 μg/L) or BPA (0, 2, and 20 μg/L), or to their binary mixtures for 3 months. Then, the intestinal microbial composition was assessed using high-throughput 16S rRNA amplicon sequencing, to elicit the interactive influence of the coexposure on gut microbiota. Host overall fitness and intestinal health were also determined and analyzed alongside the dysbiosis of the gut microbiome.

2. Materials and methods

2.1. Chemicals

BPA at a purity of >99% was purchased from Acros Organics (Belgium). Stock solutions of BPA were prepared in dimethyl sulfoxide (DMSO; purity >99%; Sigma-Aldrich, St. Louis, MO, USA). Nano-TiO_2 particles were obtained from Hangzhou Wan Jing New Material Company (China; purity >99.9%) and previously characterized (diameter, 9.7 nm; ζ potential, −21.4 mV; surface area, 123.7 m^2/g) (Fang et al., 2016). Stock solutions of nano-TiO_2 were prepared by homogeneously dispersing particles in ultrapure water with sonication (50 W/L, 40 kHz) for 20 min; the average particle size in suspension was measured to be 240.7 nm by dynamic light scattering using a Zetasizer Nano ZS (Malvern instruments, Worcestershire, UK) due to aggregation. All other reagents used were of analytic grade.

2.2. Fish maintenance and exposure

Adult zebrafish (4-months old) were cultured in a semi-static system containing charcoal-filtered fully-aerated tap water with a constant ambient temperature of 28 ± 0.5 °C, under a switchable light: dark cycle of 14 h: 10 h (Chen et al., 2012). The fish were fed twice daily with pellet food (Zeigler Brothers, Gardners, PA, USA) and freshly hatched *Artemia* nauplii. After two weeks acclimation, the zebrafish were exposed to nano-TiO_2 (100 μg/L) or BPA (0, 2, and 20 μg/L) individually, or in their binary combinations (2Ti: 2 μg/L BPA and nano-TiO_2; 20Ti: 20 μg/L BPA and nano-TiO_2). The exact concentrations of BPA in the exposure media (2 μg/L, 2Ti, 20 μg/L, and 20Ti groups) were previously determined to be 1.4, 1.4, 20.2, and 19.6 μg/L, respectively, while no BPA was detected in the control group (Fang et al., 2016). Therefore, concentrations of waterborne BPA are environmentally realistic. The environmentally relevant concentration (100 μg/L) of nano-TiO_2 was selected based on its predicted environmental concentrations and also with reference to previous reports, which verify that this concentration is acutely non-toxic but chronically toxic to zebrafish (Clemente et al., 2014; Fang et al., 2015; Wang et al., 2011, 2014; Zhu et al., 2008). Three replicate tanks were included in each exposure group, with 10 males and 10 females per tank in 20 L of media. Each tank received an equal volume of DMSO, with a final concentration of <0.001%. The water was renewed daily, to maintain the appropriate concentrations of the pollutants. After 3 months of exposure, zebrafish were anesthetized in 0.03% MS-222 (Sigma-Aldrich) and dissected for intestine tissues, which were then snap-frozen in liquid nitrogen and stored at −80 °C for microbiota and physiological analyses.

2.3. 16S rRNA amplicon sequencing and bioinformatic analyses

Five intestines of the same sex were pooled together as a biological replicate (n = 3). Genomic DNA was extracted using a DNeasy Blood & Tissue Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The quality and concentration of DNA extraction were determined by electrophoresis on 1% agarose gels and Nanodrop 2000 (Thermo Scientific, DE, USA). Genomic DNA were then used for the amplification of 16S rRNA using the primer pair 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′), targeting the V3V4 hypervariable regions. The amplicons were sequenced on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) to generate paired-end raw reads of 250 base pairs (bp). The raw reads were merged by use of FALSH and then filtered to remove low-quality reads and chimera sequences by use of QIIME (v1.7.0) and UCHIME (v5.1), respectively. The effective tags were then clustered by UPARSE to Operational Taxonomic Units (OTUs) according to 97% similarity. After taxonomic annotation based on the RDP classifier (version 2.2) and the Green Genes Database, the number of sequences was calculated and summed for each classification level (e.g. phylum and genus) to compare the gut microbial abundance and diversity among exposure groups.

2.4. Physiological analyses of the intestines

Five intestines of the same sex were pooled together as a biological replicate (n = 3). The intestines were homogenized in 0.5 mL normal saline (0.9% sodium chloride) on ice using a tissue tearer (BioSpec Products, Bartlesville, OK, USA). After centrifuging at 6000 × g for 10 min at 4 °C, the supernatant was transferred and used for physiological analyses.

The enzyme-linked immunosorbent assay (ELISA) kit for fish (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to determine the intestinal levels of serotonin, according to the manufacturer's instructions. The expression levels of tight junction protein 2 (TJP2) and interleukin 1β (IL1β) were also measured using fish-specific ELISA assay kits (Mybiosource, San Diego, CA).

In addition, oxidative stress in the exposed intestines was assessed through the measurement of a number of sensitive biomarkers (Chen et al., 2015). To this end, we followed the protocols