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Polystyrene microplastics induce microbiota dysbiosis and inflammation in the gut of adult zebrafish☆

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ABSTRACT

Microplastic (MP) are environmental pollutants and have the potential to cause varying degrees of aquatic toxicity. In this study, the effects on gut microbiota of adult male zebrafish exposed for 14 days to 100 and 1000 μg/L of two sizes of polystyrene MP were evaluated. Both 0.5 and 50 μm-diameter spherical polystyrene MP increased the volume of mucus in the gut at a concentration of 1000 μg/L (about 1.456×10^{10} particles/L for 0.5 μm and 1.456×10^{4} particles/L for 50 μm). At the phylum level, the abundance of *Bacteroidetes* and *Proteobacteria* decreased significantly and the abundance of *Firmicutes* increased significantly in the gut after 14-day exposure to 1000 μg/L of both sizes of polystyrene MP. In addition, high throughput sequencing of the 16S rRNA gene V3-V4 region revealed a significant change in the richness and diversity of microbiota in the gut of polystyrene MP-exposed zebrafish. A more in depth analysis, at the genus level, revealed that a total of 29 gut microbes identified by operational taxonomic unit (OTU) analysis were significantly changed in both 0.5 and 50 μm-diameter polystyrene MP-treated groups. Moreover, it was observed that 0.5 μm polystyrene MP not only increased mRNA levels of *IL1α*, *IL1β* and *IFN* but also their protein levels in the gut, indicating that inflammation occurred after polystyrene MP exposure. Our findings suggest that polystyrene MP could induce microbiota dysbiosis and inflammation in the gut of adult zebrafish.

1. Introduction

Plastic production has increased steadily over the past 50 years, with global production reaching over 300 million tons. As previously reported, environmental microplastic (MP) particles, defined as plastic particles smaller than 5 mm (Arthur et al., 2009), are used in industrial production processes, cosmetics and toothpaste or generated through degradation of larger items (Andrady, 2011; Cole et al., 2011; Pedà et al., 2017). According to several recent studies, the concentrations of MP in the oceans and rivers around the world have reached even thousands of particles per cubic meter of water. For example, it has been reported that there were approximately 100,000 plastic particles/m^3 of seawater in a Swedish harbor area adjacent to a polyethylene production plant (Wright et al., 2013b). Recently, Sutton et al. (2016) found that the average MP abundance reached approximately 700,000 particles/km^2 in San Francisco Bay, California. The MP abundance of three sediment samples from a beach in the Chinese Bohai Sea were in the range of 102.9 ± 39.9 to 163.3 ± 37.7 items/kg (Yu et al., 2016). The concentrations of MP ranged from 178 ± 69 to 544 ± 107 items/kg in the surface sediments from Beijiang River (Wang et al., 2017b). Thus, the potential effects of MP to fish could not be ignored.

MP particles pose risks to aquatic organisms because they may be mistaken for food and ingested (Steer et al., 2017). MP particles can accumulate in the gut of aquatic organisms and even enter into the circulatory system (Avio et al., 2015; Grigorakis et al., 2017). Despite this, several studies have reported that MP have no adverse effects on several toxicity biomarkers in aquatic animals (Kaposi et al., 2014; Cauwenberghe et al., 2015). However, most of these studies reported that ingestion of MP by fish could not only cause physical and mechanical damages (Jovanović, 2017; Pedà et al., 2017) but also block the digestive tract, reduce growth rates, block enzyme production, induce oxidative stress and even affect reproduction (Wright et al., 2013b; Jeong et al., 2016; Sussarellu et al., 2016; Rodriguez-Seijo et al., 2017). Interestingly, some studies have indicated that MP could remain in the gut of fish and interact with microorganisms in the environment (Caruso, 2015;

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Grigorakis et al., 2017). However, little is known about the effects of MP on the gut microbiota in fish.

The gut microbiota, the large number of microbial species residing in the gastrointestinal tract, is essential for health and participates in regulating many physiological functions in the host organism (Hsiao et al., 2013; Yano et al., 2015; Jin et al., 2017a). These microbes reside in the intestinal mucus layer and participates in shaping the mucus layer (Jakobsson et al., 2015; Jin et al., 2018). Instability or imbalance of the physiologic gut microbial community is called "dysbiosis". Dysbiosis of the gut microbiota has generally been associated with several physiological changes such as colitis and abnormal behavior (Sartor, 2006; Garrett et al., 2010; Glenny et al., 2017). According to previous studies, different kinds of environmental chemicals, including antibiotics, pesticides and several heavy metals, can effectively induce gut microbiota dysbiosis, change the mucus layer and even result in inflammation in different experimental models, including fish (Lu et al., 2014; Jin et al., 2016a, 2016b; Carlson et al., 2017). Some pro-inflammatory biomarkers including expression of the cytokines used in this study had been used to assess biological effects of different environmental chemicals, however, the gut microbiota was the first time to evaluate the effects of MP to fish.

Gut microbiota dysbiosis always linked to the occurrence of inflammation in the gut (Ganz et al., 2011). Here, we hypothesized that MP might interact with the microbes in the gut, resulting in inflammation in gut of zebrafish. Different sizes (ranged from nanoscale to millimeter scale) of polystyrene MP can be detected in aquatic systems (Yu et al., 2016; Frère et al., 2017). We sought to analyze the effects of different sizes and concentrations of polystyrene MP. For this purpose, we exposed male adult zebrafish to 0.5 and 50 μm diameter spherical polystyrene MP in water and determined whether or not they could induce microbiota dysbiosis and inflammation in the gut. The results acquired in the present study provide new information regarding MP-induced aquatic toxicity.

2. Materials and methods

2.1. Polystyrene MP

Both sizes of polystyrene MP were purchased from Microspheres-Nanospheres (New York, USA) and used as received. We also analyzed the morphology of the different sizes of polystyrene MP in water by scanning electron microscopy. The shape and size of polystyrene MP were in accordance with the data provided by the supplier (Fig. S1).

2.2. Experimental fish

Healthy six-month-old male adult zebrafish (AB strain) were used in the present study. The average body weights and lengths of 10 fish selected randomly were 323.12 ± 27.42 mg and 2.97 ± 0.18 cm for male fish. All fish were maintained at an ambient temperature (26 ± 1 °C) with a photoperiod consisting of 14 h light/10 h dark in a zebrafish aquarium facility. The fish were fed twice a day with brine shrimp in the morning and a commercial diet in the afternoon.

2.3. Fish exposure and sample collection

The selected zebrafish were exposed to 0.5 and 50 μm-diameter polystyrene MP at concentrations of 100 and 1000 μg/L in tap water (pH 7.0 to 7.5, hardness between 40 and 60 mg $CaCO_3$/L). In each group, a total of 14 fish were equally reared in two separately glass aquaria with 3 L of the indicated solutions. Control fish were reared in water alone. Zebrafish were exposed to the polystyrene MP-containing water under semi-static conditions for 14 days. Every day, we prepared new exposure solutions in clean glass aquaria and the exposed fish were moved into it. During the exposure, all the fish were fed twice daily with brine shrimp in the morning and a commercial diet in the afternoon.

After exposure, the fish were anesthetized on ice and killed by cutting off the spine before being dissected. The whole gut was excised from each fish and collected as one sample. Five whole guts from each group were used for DNA extraction (3 fish from one aquarium, 2 fish from another one), five whole guts from each group were used for RNA extraction (2 fish from one aquarium, 3 fish from another one), and the remaining four whole guts (2 fish from each aquarium) in each group were used for ELISA analysis. After dissection, the guts were stored at −80 °C until analysis. All experiments were performed in accordance with the Guiding Principles for the Use of Animals of Zhejiang University of Technology, and all efforts were made to minimize animal suffering.

MP could be ingested and retained in the intestine in fish. For example, in a study examining 504 fish from the English Channel that included benthic and pelagic species, 36.50% of specimens had microplastics in their gastrointestinal tracts (Lusher et al., 2013). More recently, Grigorakis et al. (2017) observed that MP particles were retained in goldfish gastrointestinal tracts after 6 days. In addition, exposure to MPs in water can interfere with normal digestive processes due to intestinal blockage, causing histopathological alteration to intestinal of fish (Pedà et al., 2016). Thus, polystyrene MP ingestion and retention was not measured in the present study.

2.4. Histopathological analysis of the gut

Segments of middle intestine from three fish in each group were selected and fixed in freshly prepared 4% paraformaldehyde solution. Subsequently, the fixed gut tissues were dehydrated in a rising series of ethanol, hyalinized in xylene, and embedded in paraffin wax at 56 °C. Then, three middle guts samples from each group were cut into 5 μm-thick sections. Each gut was cut into at least 4 sections for hematoxylin & eosin (H&E) and Alcian Blue-Periodic Acid Schiff (AB-PAS), respectively. All slides were stained with H&E or AB-PAS before examination with a microscope (Olympus). The mucus coverage ratio from 9 sections in each group was calculated by the pixels in the mucus area to the total pixels area of the gut section. And the pixels were determined using an image analyzer (Image-pro Plus 6.0).

2.5. DNA extraction, PCR amplification, quantification, and 16S rRNA gene sequencing

The microbial genomic DNA (gDNA) in zebrafish guts was isolated with a magnetic bead DNA isolation kit (Hangzhou Foreal Nanotechnology, China). After DNA quality analysis by electrophoresis, the gDNA was amplified using primers (Forward primer: 5′-ACTCCTACGGGAGGCAGCAG-3'; Reverse primer: 5′-GGACTACHVGGGTWTCTAAT-3′) to target the V3 and V4 regions of the bacterial 16S rRNA gene. The PCR amplification was performed with the following protocol: 94 °C for 3 min; 94 °C for 5 s, 57 °C for 90 s, and 72 °C for 10 s, repeated for 24 cycles; and 72 °C for 5 min. 5 samples in each group were used for PCR amplification. The compositions of the gut microbiota were analyzed using dual-indexing amplification and sequencing on an Illumina MiSeq platform followed by QIIME (vision 1.6.0) bioinformatics analysis. Briefly, sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH(1.9.6) against the Silva 119 database, pre-clustered at 97% sequence identity. The Ribosomal