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Oral imazalil exposure induces gut microbiota dysbiosis and colonic inflammation in mice

Cuiyuan Jin, Zhaoyang Zeng, Zhengwei Fu, Yuanxiang Jin*

College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310032, China

HIGHLIGHTS

- Effects of imazalil on gut microbiota colonic inflammation in mice are evaluated.
- Imazalil exposure resulted in a significant reduction in the richness and diversity of gut microbiota.
- Imazalil exposure could also induce colonic inflammation in mice.

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ABSTRACT

The fungicide imazalil (IMZ) is used extensively in vegetable and fruit plantations and as a post-harvest treatment to avoid rot. Here, we revealed that ingestion of 25, 50 and 100 mg IMZ kg^{-1} body weight for 28 d induced gut microbiota dysbiosis and colonic inflammation in mice. The relative abundance of *Bacteroidetes*, *Firmicutes* and *Actinobacteria* in the cecal contents decreased significantly after exposure to 100 mg kg^{-1} IMZ for 28 d. In feces, the relative abundance in *Bacteroidetes*, *Firmicutes* and *Actinobacteria* decreased significantly after being exposed to 100 mg kg^{-1} IMZ for 1, 14 and 7 d, respectively. High throughput sequencing of the V3-V4 region of the bacterial 16S rRNA gene revealed a significant reduction in the richness and diversity of microbiota in cecal contents and feces of IMZ-treated mice. Operational taxonomic units (OTUs) analysis identified 49.3% of OTUs changed in cecal contents, while 55.6% of OTUs changed in the feces after IMZ exposure. Overall, at the phylum level, the relative abundance of *Firmicutes*, *Proteobacteria* and *Actinobacteria* increased and that of *Bacteroidetes* decreased in IMZ-treated groups. At the genus level, the abundance of *Lactobacillus* and *Bifidobacterium* decreased while those of *Deltaproteobacteria* and *Desulfovibrio* increased in response to IMZ exposure. In addition, it was observed that IMZ exposure could induce colonic inflammation characterized by infiltration of inflammatory cells, elevated levels of lipocalin-2 (lcn-2) in the feces, and increased mRNA levels of *Tnf-α*, *IL-1β*, *IL-22* and *IFN-γ* in the colon. Our findings strongly suggest that ingestion of IMZ has some risks to human health.

1. Introduction

The use of fungicides has been pivotal to maximizing the economic benefit in agriculture and industry. The main industrial applications of fungicides are in papermaking and cooling water recirculation systems (Williams, 2010). In agriculture, fungicides are extensively used to preserve freshness, prevent decay and control fungal infections of fruits, vegetables and ornamental plants. The extensive use of fungicides however comes at a cost as fungicides can enter the body via inhalation, ingestion or absorption and pose potential health risks to humans.

Imazalil (IMZ; 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl]-1*H*-imidazole; also known as enilconazole) is a highly effective, broad-spectrum fungicide widely used to prevent and treat fungal diseases in plants and animals (Leemput et al., 1989; Sepulveda et al., 2015). Alarmingly, several recent studies have demonstrated that IMZ can be detected in soil, vegetable, fruit, as well as fruit juice treated with this fungicide (Masiá et al., 2015; Ruiz-Rodríguez et al., 2015; Xu et al., 2014). Even though IMZ is currently classified as a compound with only medium toxicity, it can cause dermatitis and eczema if touched directly and endocrine and nervous system dysfunction if ingested in mice, rats and

* Corresponding author.
E-mail address: jinyx@zjut.edu.cn (Y. Jin).

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zebrafish (Tanaka, 1995; Nakagawa and Tayama, 1997; Jin et al., 2016c).

As a broad-spectrum fungicide, IMZ targets a wide range of microorganisms and thus may have detrimental effects on our intestinal flora, a complex microbial ecosystem formed by a large variety of microorganisms that have co-evolved with the human host. The gut microbiome is involved in many host functions including digestion, nutrient absorption, energy and fat metabolism, immune regulation and disease control (Bäckhed et al., 2004; Kelly et al., 2005; Pryde et al., 2002; Xu et al., 2003; Osamu et al., 2013). Several recent studies suggest that disorders of the microflora correlate with a variety of gastrointestinal diseases, such as colon cancer (Wang et al., 2011), inflammatory bowel disease (IBD) (Seksik et al., 2003; Zhang et al., 2007), obesity (Ley et al., 2006; Suárez-Zamorano et al., 2015) and diabetes (Dumas et al., 2006; Wen et al., 2008). Although the composition of the gut microbiota is relatively stable, it can be disrupted by environmental factors, food components, drugs and even stress (Jin et al., 2015c; Zhang et al., 2015b). Considering the wide range of potential targets of IMZ, it is therefore possible that exposure to IMZ can change the composition of the gut microbiota and cause subsequent adverse effects in animals.

Several previous studies have reported that IMZ exposure can result in a variety of toxic effects in mice (Tanaka, 1995; Tanaka et al., 2013). However, it is still unclear whether and how IMZ impacts the gut microbiota or colonic inflammation. Because of the low mammalian toxicity and the actual concentrations of IMZ may be higher than the reported concentrations. Thus, relative high doses of IMZ were selected in this study. Here, we orally administered IMZ to Institute of Cancer Research (ICR) mice for 28 d and determined the effects of IMZ ingestion by evaluating molecular hallmarks of the gut microbiota and colonic inflammation. We hope that these results can bring new insight into the potential risks of IMZ exposure to human health.

2. Materials and methods

2.1. Reagents

Imazalil (IMZ; 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl]-1*H*-imidazole) was purchased from Adamas (CAS: 35554-44-0, purity: ≥97%). Acetone (CAS: 67-64-1, purity: ≥99.5%) was purchased from Shuanglinhuagong (Hangzhou, China).

2.2. Animals care and experimental scheme

Five week-old male Institute of Cancer Research (ICR) mice (n = 42) were purchased from the China National Laboratory Animal Resource Center (Shanghai, China) and individually housed at 22 ± 1 °C on a 12 h: 12 h light:dark cycle (200 lux at cage level) for 1 week prior to the start of treatments. IMZ was dissolved in acetone and mixed with mouse chow, and all acetone was completely evaporated prior to feeding. The mice were divided into 4 groups at random. Three groups of mice were orally administered 25 mg kg^{-1} (n = 8), 50 mg kg^{-1} (n = 8) or 100 mg kg^{-1} (n = 13) IMZ body $weight^{-1}$ daily for 4 weeks. The control group (n = 13) was fed untreated mouse chow. Water was available *ad libitum* while food was available only at night. During IMZ exposure, feces from each mouse were collected every other day and stored at −80 °C until processing. Eight mice from each group were sacrificed after 28 d of IMZ exposure. The remaining 5 mice in the control and 100 mg kg^{-1} IMZ-treated groups were then maintained for an additional 35 d without IMZ treatment while feces were collected weekly to determine the recovery of gut microbiota.

All mice were fasted for 8 h and anesthetized by ether before killing. Whole blood was collected, livers quickly removed and weighed, and colon and cecum contents dissected. All tissues were flash-frozen in liquid nitrogen and stored at −80 °C until processing.

2.3. Histopathological analysis of liver and colon

Liver and colon were cut into 5 μm-thick sections embedded in paraffin, fixed in 10% (vol/vol) formaldehyde and then hematoxylin & eosin (H&E) stained. The ratio of ballooning degeneration area to the percent coverage of inflammatory cells was determined using an image analyzer (Image pro-Plus 6.0).

2.4. Determination of serum and hepatic indices

Serum glucose, free fatty acid (FFA), total cholesterol (TC), triglyceride (TG), total bile acid (TBA), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were quantified using kits purchased from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China) according to the manufacturer's instructions.

Hepatic triglycerides were extracted by first homogenizing liver in three volumes methanol, then adding six volumes of chloroform and incubating for 16 h at room temperature. The chloroform layer was collected after separation by centrifugation at 3000×*g* for 10 min. Protein concentration was quantified using a commercial BCA protein kit provided by Sangon Biotech (Shanghai, China) according to the manufacturer's instructions.

Lipopolysaccharide (LPS) levels were quantified in 10 μL serum per sample by mouse ELISA kits (RapidBio, USA) according to the manufacturer's instructions.

2.5. Gene expression analysis

Total RNA was isolated from liver and colon using Trizol reagent (Takara Biochemicals, Dalian, China). Quantitative RT-PCR was performed using a reverse transcriptase kit with SYBR Green (Toyobo, Tokyo, Japan) in an Eppendorf MasterCycler ep RealPlex^2 thermocycler (Wesseling-Berzdorf, Germany). Gene-specific primer sequences are listed in Table S1. All transcript levels were normalized to 18S rRNA levels. The following cycling conditions were used to amplify cDNA: denaturation for 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR and quantification of relative gene expression were performed as previously described (Jin et al., 2010a, 2016a).

2.6. Microbial analysis

Microbial genomic DNA (gDNA) was extracted from freeze-dried cecal contents and fecal samples using a commercial magnetic bead DNA isolation kit provided by Hangzhou Foreal Nanotechnology (Hangzhou, China) following the manufacturer's instructions, and then quantified by UV spectroscopy. The V3-V4 region of the 16S rRNA gene was PCR-amplified from microbial gDNA using primers (forward primer: 5′-ACTCCTACGGGAGGCAGCAG-3′; reverse primer: 5′-GGACTACHVGGGTWTCTAAT-3′). The composition of the gut microbiome was determined by dual-indexing amplification and sequencing on the Illumina MiSeq platform followed by QIIME (vision 1.6.0) bioinformatic analysis. In addition, part of microbial gDNA was amplified by a RT-qPCR with bacterial phyla specific primers (Table S2) and the following cycling conditions: 50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min, repeated for 40 cycles; and 72 °C for 10 min.