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Manganese-induced sex-specific gut microbiome perturbations in C57BL/6 mice

Liang Chi [b], Bei Gao [a], Xiaoming Bian [a], Pengcheng Tu [b], Hongyu Ru [c], Kun Lu [b,*]

[a] Department of Environmental Health Science, University of Georgia, Athens, GA 30602, United States
[b] Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, 27599, United States
[c] Department of Population Health and Pathobiology, North Carolina State University, Raleigh, NC 27607, United States

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ABSTRACT

Overexposure to manganese (Mn) leads to toxic effects, such as promoting the development of Parkinson's-like neurological disorders. The gut microbiome is deeply involved in immune development, host metabolism, and xenobiotics biotransformation, and significantly influences central nervous system (CNS) via the gut-brain axis, i.e. the biochemical signaling between the gastrointestinal tract and the CNS. However, it remains unclear whether Mn can affect the gut microbiome and its metabolic functions, particularly those linked to neurotoxicity. In addition, sex-specific effects of Mn have been reported, with no mechanism being identified yet. Recently, we have shown that the gut microbiome is largely different between males and females, raising the possibility that differential gut microbiome responses may contribute to sex-selective toxicity of Mn. Here, we applied high-throughput sequencing and gas chromatography–mass spectrometry (GC–MS) metabolomics to explore how Mn^{2+} exposure affects the gut microbiome and its metabolism in C57BL/6 mice. Mn^{2+} exposure perturbed the gut bacterial compositions, functional genes and fecal metabolomes in a highly sex-specific manner. In particular, bacterial genes and/or key metabolites of neurotransmitter synthesis and pro-inflammatory mediators are significantly altered by Mn^{2+} exposure, which can potentially affect chemical signaling of gut-brain interactions. Likewise, functional genes involved in iron homeostasis, flagellar motility, quorum sensing, and Mn transportation/oxidation are also widely changed by Mn^{2+} exposure. Taken together, this study has demonstrated that Mn^{2+} exposure perturbs the gut microbiome and its metabolic functions, which highlights the potential role of the gut microbiome in Mn^{2+} toxicity, particularly its sex-specific toxic effects.

1. Introduction

Trillions of bacteria reside in human gastrointestinal tract and they are deeply involved in human metabolism and health (Ley et al., 2006). Besides food digestion and energy harvest, the gut microbiome plays a crucial role in neurodevelopment, immune response, inflammation and xenobiotic biotransformation (Guarner and Malagelada, 2003; Bäckhed et al., 2005). The gut microbiome is a highly dynamic system and can be influenced by environmental factors, such as heavy metals and antibiotics (Jakobsson et al., 2010; Lu et al., 2014). Multiple xenobiotics can alter bacteria community compositions and disturb the production of key metabolites, which can largely influence the interactions between the gut microbiome and host (Maurice et al., 2013; Lu et al., 2014). On the other hand, gut bacteria can modulate the effects of xenobiotics on the host. For example, gut bacteria transform Hg^{2+} and Cr (VI) to Hg^0 and Cr(III) to reduce their toxicity, while other xenobiotics, such as nitrazepam, are converted to more toxic species by gut bacteria (Takeno and Sakai, 1991; Upreti et al., 2004; Monachese et al., 2012; Younan et al., 2016). Therefore, bi-directional interactions between the gut microbiome and exposure actually exist.

Manganese (Mn) is an essential trace element for mammals and many microorganisms (Jakubovics and Jenkinson, 2001; Aschner and Aschner, 2005). Mn is necessary for normal brain function and amino acid, lipid, and carbohydrate metabolism (Greger, 1998; Aschner and Aschner, 2005). Mn also functions as the cofactor of numerous key enzymes, such as arginase, glutamine synthetase, manganese catalase, and manganese superoxide dismutase (Greger, 1998; Jakubovics and Jenkinson, 2001). However, Mn overload is toxic and associated with a series of diseases, including chronic liver failure, cardiovascular diseases, bone loss and neurodegeneration (Roth and Garrick, 2003; Crossgrove and Zheng, 2004; Milatovic et al., 2009). Mn can cross the blood-brain barrier, accumulate in the brain and cause neurodegenerative disorders, such as Parkinson's disease (PD) (Crossgrove and Zheng, 2004; Reaney et al., 2006). Mn-induced tissues and neuron damages involve multiple mechanisms, including mitochondrial dysfunction,

* Corresponding author at: Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States.
E-mail address: kunlu@unc.edu (K. Lu).

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oxidative stress (Milatovic et al., 2009), activation of pro-inflammatory mediators and neuroinflammation (Chen et al., 2006; Milatovic et al., 2009), and alterations of ion homeostasis (Klaassen and Amdur, 1996; Zheng et al., 1999; Roth and Garrick, 2003; Zhang et al., 2003; Crossgrove and Zheng, 2004). Manganese toxicity is also species-dependent. Mn^{3+} is more reactive and toxic than Mn^{2+} (Crossgrove and Zheng, 2004; Reaney et al., 2006). A previous study revealed that Mn^{3+} exposure caused significantly higher blood manganese levels than Mn^{2+}, and Mn^{3+} accumulated in brain more efficiently than Mn^{2+} (Reaney et al., 2006). Since a considerable amount of Mn comes from food and water, gut bacteria are being exposed to Mn before it is absorbed to the body. However, it is largely unknown whether Mn exposure can perturb the gut microbiome and its functions. It is also unclear whether gut bacteria can influence the toxicity and physiological effects of Mn.

In particular, a compelling body of evidence demonstrates that the gut microbiome significantly affects central nervous system (CNS) via the gut-brain axis, i.e. the bidirectional biochemical signaling between the gastrointestinal tract and the CNS. The gut microbiome can largely influence behaviors and diseases in the host, such as depression and schizophrenia (Collins et al., 2012; Cryan and Dinan, 2012; Foster and Neufeld, 2013; Dinan et al., 2014). Animals with depression and anxiety were generally associated with alterations of gut bacteria (O'Mahony et al., 2009; Park et al., 2013). Oral administration of *Lactobacilli rhamnosus* to mice could alter the GABA receptor expression in key CNS stress-related brain regions and influence anxiety-like behaviors (Foster and Neufeld, 2013). Gut microbiome perturbation has been proposed to play a role in neurodegenerative disorders such as PD (Ghaisas et al., 2016). Previous studies clearly showed that the gut produced a large amount of neurotransmitters and related compounds (O'Mahony et al., 2015; Yano et al., 2015). For example, intestinal cells, but not brain cells, generate >90% of serotonin in the body (Gershon and Tack, 2007; Yano et al., 2015). Gut compounds play key roles in the crosstalk of microbiome-gut-brain. Inflammatory signaling is another important type of interaction in the gut-brain axis (Bercik et al., 2010; Hanamsagar and Bilbo, 2016; Rea et al., 2016). It has been shown that chronic gastrointestinal inflammation induces anxiety-like behaviors and alters central nervous system biochemistry (Bercik et al., 2010). However, it remains unknown whether Mn exposure perturbs the gut microbiome, which leads to altered chemical signaling involved in the gut-brain axis.

It has been reported that Mn has sex-selective toxicity (Zhang et al., 2003; Madison et al., 2011; Mergler, 2012). For example, $MnCl_2$ exposure had reverse effects on the body weight of male and female SD rats (Zhang et al., 2003). Another study found that Mn^{2+}-exposed female mice had long-lasting effects in neuronal morphology, which was absent in male mice (Madison et al., 2011). However, the mechanism underlying sex-specific effects of Mn is poorly understood. Recently, we and others have shown that the gut microbiome is largely different between male and female animals (Chi et al., 2016; Cong et al., 2016), raising the possibility that differential gut microbiome responses may contribute to sex-selective toxicity. In fact, toxicants, such as arsenic and organophosphate pesticides, cause sex-specific perturbations of the gut bacteria (Chi et al., 2016; Gao et al., 2017), which may further affect toxicity and disease susceptibility in males and females when exposed to these toxicants.

This study was designed to address three questions: Will Mn exposure alter the gut microbiome and its metabolic functions? Are there any changes of chemical signaling involved in the gut-brain interactions? Are these changes sex-specific? Therefore, both male and female C57BL/6 mice were exposed to $MnCl_2$ in drinking water for 13 weeks, followed by the assessment with multi-omics, including 16S rRNA gene sequencings, metagenomics and GC–MS metabolomics. 16S rRNA sequencing and metagenomics sequencing were used to define the alterations of bacterial compositions and functional pathways of gut bacteria. GC–MS metabolomics was employed to analyze the metabolic changes related to the gut microbiome. To the best of our knowledge, this is the first study to examine the sex-specific effects of Mn exposure on the gut microbiome and associated metabolic functions.

2. Materials and methods

2.1. Animals and manganese exposure

C57BL/6 mice (7 weeks old, Jackson Laboratory, Bar Harbor, ME) were housed in the University of Georgia animal facility for a week before exposure, as well as throughout the duration of the experiment in static microisolator cages with Bed-O-Cob combination bedding under environmental conditions of 22 °C, 40–70% humidity, and a 12:12 h light:dark cycle. Before experimentation, all mice were allowed to consume tap water ad libitum, and were provided with standard pelleted rodent diet before and during experimentation. At the experimental period, mice were randomly assigned into either the control group, or 100 ppm $MnCl_2$ treatment group (consumption of Mn is ~20 mg/kg body weight/day) ($n = 20$, with 5 male mice and 5 female mice per group). The Mn dose used in this study was modeled according to several previous studies that demonstrated neurotoxicity of Mn at similar concentrations (Moreno et al., 2009; Avila et al., 2010; Krishna et al., 2014). The animals were treated humanely and every effort was made to alleviate suffering. The animal protocol was approved by the University of Georgia Institutional Animal Care and Use Committee. At the start of experiment, $MnCl_2$ (Pfaltz & Bauer, Inc., Waterbury, CT) was dissolved in tap water and was administered to individual animal (~8 weeks of age) in drinking water for 13 weeks. The mice were allowed to consume ad libitum. Drinking water with $MnCl_2$ was made fresh every week. Control mice (~8 weeks of age) continued to receive tap water in their drinking water bottles which they were allowed to consume ad libitum.

2.2. 16S rRNA sequencing

Mice fecal pellets from individual mouse were collected for 16S rRNA analysis at 0 and 13 weeks, and stored under liquid nitrogen before being transferred to −80 °C until further analysis. DNA was isolated from fecal pellets using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The resultant DNA was quantified by Nanodrop® and stored at −80 °C until further analysis. Purified DNA (1 ng) was used to amplify the V4 region of 16S rRNA of bacteria using universal primers of 515 (5′-GTGCCAGCMGCCGCGGTAA) and 806 (5′-GGACTACHVGGGTWTCTAAT). The resultant DNA products were barcoded and quantified by Qubit 2.0 Fluorometer using Qubit dsDNA HS Assay kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions and pooled to be sequenced. Sequencing was performed on an Illumina Miseq at the Georgia Genomics Facility to generate pair-end 250 × 250 (PE250, v2 kit) reads. The raw mate-paired fastq files were merged and quality-filtered using Geneious 8.0.5 (Biomatters, Auckland, New Zealand) with error probability limit set as 0.01. The data then were analyzed using quantitative insights into microbial ecology (QIIME, version 1.9.1). UCLUST was used to get the operational taxonomic units (OTUs) with 97% sequence similarity. The data was assigned at five different levels: phylum, class, order, family and genus.

2.3. Metagenomics sequencing

For metagenomics sequencing, DNA (10 ng/μL) was fragmented using the Bioruptor UCD-300 sonication device, followed by sequencing library construction using the Kapa Hyper Prep Kit according to the manufacturer's instructions. The resulting DNA was pooled, quantified, and sequenced at the Georgia Genomics Facility using an Illumina