Role of the Microbiota-Metabolite Axis in the Rotenone Model of Early-Stage Parkinson's Disease

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Research Article

Keywords: Parkinson's disease, gut microbiota, dysbiosis, serum amino acids, microbiota-metabolite axis

Posted Date: January 21st, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1191881/v1>

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Abstract

Gastrointestinal symptoms are common in the early-stage Parkinson's disease (PD), but its potential pathogenesis remains unclear. Therefore, in the present study, we used the 16S ribosomal RNA gene sequencing and gas chromatography coupled with mass spectrometry-based metabolomics investigated the gut microbiome alterations and the serum amino acid levels in early-stage PD mice model induced with rotenone and vehicle-induced control mice. The results demonstrated that the microbial taxa at phylum, family and genus levels remarkably altered in rotenone-induced mice relative to vehicle-induced mice. The rotenone-induced mice had higher relative abundances of Flavobacteriaceae, Staphylococcaceae, and Prevotellaceae as well as lower relative abundances of Lachnospiraceae_UCG-001, Ruminiclostridium, and Prevotellaceae_NK3B31_group than vehicle-induced mice. The evaluation of serum amino acids revealed the alterations in several classes of amino acids, including L-proline, L-alanine, L-serine, L-asparagine, L-threonine, L-glutamine, L-methionine, and L-4-hydroxyproline. Notably, the altered serum amino acid levels were significantly associated with the abundance of gut microbiota, especially Ruminococcaceae and Ruminiclostridium. Our study explored the possible role of the gut-microbiota-metabolite axis in the early-stage PD and provided the possibility of prevention and treatment of PD by gut microbiota-metabolite axis in the future.

Introduction

Parkinson's disease (PD) is known as a slowly progressive degenerative disease involving the peripheral and central nervous system (PNS and CNS), according to Braak's hypothesis (Braak and Del Tredici 2017). The full truncal vagotomy further proved that the misfolding α -synuclein could retrogradely spread from the gastrointestinal tract to the brain through vagal nerve, resulting in the loss of dopaminergic neurons in substantia nigra (SN) and the decrease of dopamine in striatum (Kim et al. 2019). Studies have shown that the motor symptoms didn't occurs apparently until the PD patients lost 60–80% dopaminergic neurons in SN (Deumens et al. 2002). Therefore, there recently remains a great need for diagnosis and therapy of early-stage PD.

Although evidences have indicated that the aggregation of pathological α -synuclein and the loss of dopaminergic neurons are the pathological hallmark of PD, the etiology of PD is still worthy of further study. There was an association between neuroinflammation and neurodegeneration of PD. For example, studies have found that in parkinsonian animal models, there were a large number of proinflammatory cytokines in the CNS (Dzamko et al. 2015; Lai et al 2018; Mamik and Power 2017). In addition, the inflammation in the gut was also the main cause of the misfolding and aggregation of α -synuclein. For example, patients with inflammatory bowel disease (IBD) have a higher risk of developing PD than non-IBD individuals (Brudek 2019). Recent studies confirmed that the gut microbiota exhibited more pro-inflammatory characteristics (e.g., the increase of lipopolysaccharide biosynthesis), which was considered to be one of the risk factors for PD (Keshavarzian et al. 2015). There is growing evidence that alterations of gut microbiota presented in patients and animal models of PD (Gerhardt and Mohajeri 2018; Petrov et al. 2017; Sun and Shen 2018; Qian et al. 2018). Remarkably, the alteration of the gut

microbiota with antibiotics could reduce the expression of proinflammatory markers, which could attenuate dopaminergic neuron loss in SN and prevent the development of PD (Koutzoumis 2020). The proposal of microbiota-gut-brain axis further indicated that the abnormality of gut microbiota and microbiota's metabolic products may be among potential candidates to trigger the formation of Lewy bodies in the enteric nervous system (ENS). To date, several studies investigated the metabolomics profile of PD, and found the alterations of amino acid level in vivo (Lu et al. 2014; Wuolikainen et al. 2016; Luan et al. 2015; Molina et al. 1997). And gut amino acids have also been shown to have potential neuromodulatory activities (Needham et al. 2020).

Interestingly, the animal model of PD showed that chronic intragastric administration of rotenone (a complex-1-inhibitor) could result in a spatio-temporal distribution of α -synuclein corresponding to Braak's pathological stage (Pan-Montojo et al. 2012, 2010). In the present study, C57BL/6 mice were given oral low-dose rotenone administration for 3 weeks to confirm whether rotenone short-term exposure in the gut would first induce pathology and symptoms restricted to the gut. Then, in order to further understand the relevance of gut microbial ecosystem and PD, we used 16S rRNA gene-based amplicon sequencing and gas chromatography-mass spectrometry (GC-MS)-based metabolomics to explore the interactions between bacterial metabolisms, gut microbes, and disease.

Materials And Methods

Animals

The animal experiments in the present study were carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by Animal Care Committee of the First Affiliated Hospital of Harbin Medical University. Male C57BL/6 mice (23–25 g) were purchased from Changzhou Cavens Experimental Animal Co., Ltd. (Jiangsu, China). The mice were housed in a specific-pathogen-free (SPF) environment with 12-h light/dark cycle, $23 \pm 2^\circ\text{C}$ and $45 \pm 5\%$ humidity. And they were provided with food and water ad libitum. After one-week acclimation, all mice were randomly divided into two groups ($n = 10$ per group): control group and rotenone group. And a early-stage Parkinson's disease model was established by oral rotenone (Sigma, St Louis, MO, USA) solution (30 mg/kg suspended freshly in 4% carboxymethylcellulose and 1.25% chloroform vehicle) once a day for 21 days. Meanwhile, control animals received vehicle. After 21 days of rotenone treatment, all mice underwent behavioral tests and fecal samples were collected and immediately stored at -80°C for next analysis. All mice were sacrificed and then the acquired fresh distal colon and midbrain were fixed in 4% paraformaldehyde for immunofluorescence (IF).

Behavioral tests

In order to assess the motor function, the pole test was first performed according to the protocol previously described (Ogawa et al. 1985; Cao et al. 2017). Briefly, the mice were placed the top of the pole that was made 50-cm height, 1-cm diameter and with a rough surface. The time was recorded when the mice reached the the bottom from the top of the pole. Each mouse performed three successive trials, and

then the average value of the three trials was calculated for statistical analyses. The rotarod test was also performed as described before (Inden et al. 2007). And the mice were placed on an accelerating rod that was set the starting speed of 4 rpm and gradually increased to 40 rpm. The latency to fall was recorded for a maximum of 300 s. All mice received five trials, and the average latency was analyzed.

One hour stool collection

The one-hour stool frequency of mice was observed to assess the colonic motion function. Each mouse was placed in a clean plastic cage without food or water for one hour. Fecal pellets were collected immediately after expulsion and placed in sealed 1.5 ml tubes. Tubes were weighed to obtain the wet weight of stool, and the dry weight of stool were obtained by reweighing the tubes dried overnight at 65°C. The water content of stool was calculated from the difference between the wet and dry stool weights.

Immunofluorescence

The xylene and graded alcohols were applied to deparaffinise and hydrate the midbrain coronal paraffin slices (3 µm) and colon axial paraffin slices (3 µm). Antigen retrieval was performed in microwave EDTA buffer (pH 8.0). After blocking with serum for 1 h, the slides were firstly incubated with primary antibodies (anti- α -syn; anti-phospho- α -syn antibody; anti-TH; anti- β III antibody, Abcam) overnight at 4 °C, and washed with PBS for three times, then incubated with corresponding fluorescent secondary antibody. Finally, the images were taken with an epifluorescence microscope (Nikon eclipse C1, Nikon, Japan) and the relative expression levels of respective molecules in SN were analyzed by Image Pro-Plus software.

Gut microbiota analysis

As described in our previous studies (Yan et al. 2021), 16S rRNA gene sequencing was employed to analyse the gut microbiota. The microbial DNA was extracted from the fecal samples using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The V3-V4 variable regions of 16S rRNA gene were amplified with universal primers 343F/798R (343F: 5'-TACGGRAGGCAGCAG-3'; 798R: 5'-AGGGTATCTAATCCT-3'). Then, 16S rRNA gene amplicons was sequenced on the Illumina MiSeq (200-bp paired-end reads) platform (Illumina Inc., San Diego, USA). The final effective reads were obtained by removing the chimeric sequences using QIIME software (Caporaso et al. 2010) (version 1.8.0). Subsequently, Operational taxonomic units (OTUs) were clustered using Vsearch software (Rognes et al. 2016) with 97% similarity threshold. All representative reads of the OTUs were annotated and blasted against Silva database Version 123 using Ribosomal Database Project classifier (Wang et al. 2007) (confidence threshold was 70%).

Metabolites sample preparation

For metabolomics, frozen serum samples were mixed in succinic acid-2,2,3,3-d₄ (25 µmol/L) and methanol-acetonitrile (2:1, vol/vol, containing 0.1% formic acid) and sonicated. After centrifugation, the supernatants were dried and derivatized with methoxyamine hydrochloride dissolved in anhydrous pyridine (15 mg/mL). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (with 1% trimethylchlorosilane)

and n-hexane were added, and then the samples were resuspended and derivatized. A quality control (QC) sample was created by mixing aliquots of all samples (a pooled sample).

GC-MS analysis parameters

For GC-MS analysis, 1 µl of the derivatized sample was injected splitless into an Agilent 7890B GC system coupled to an Agilent 5977A mass selective detector (Santa Clara, CA, USA), with a DB-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J&W Scientific, Folsom, CA, USA). Helium (>99.999%), as carrier gas, was injected into the column at a constant flow rate of 1.2 ml/min. The temperatures of injector, transfer line and electron impact ion source were set to 300°C, 280°C and 330°C, respectively. Temperature programming: the initial oven temperature was 60 ° C, increased to 125°C at a rate of 15°C/min, to 210°C at 8°C/min, to 270°C at 11°C/min, to 305°C at 25°C/min and finally held at 305°C for 3 min. MS data were acquired in a full-scan mode (m/z 40–600), and the solvent delay time was set to 4 min. QC samples were detected at regular intervals (every 10 samples) throughout the analysis.

Statistical Analysis

The alpha diversity indices of gut microbiota, including observed_species, Chao, Simpson, Shannon and PD_whole_tree were calculated by QIIME software (Version 1.8.0). The characterizations of microbial communities at different levels were identified by linear discriminant analysis (LDA) effect size (LEfSe), and LDA was used to estimate the effect size of each feature. The metabolic analyses: the unsupervised principal component analysis (PCA) and supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) were performed using online resources (<http://www.metaboanalyst.ca/>). Continuous variables were expressed as the mean ± standard deviation (SD), and compared by parametric Student's t-test and non-parametric Wilcoxon rank-sum test. Heatmap of correlations between the microbiome and the metabolome were presented by Spearman rank correlation. Analyses were conducted with R software, version 4.0.4 (Foundation for Statistical Computing, Vienna, Austria). The P-values were modified to q-values by false discovery rates (FDR). The P value < 0.05 was considered as statistical significance.

Results

Neurotoxicity of gut and brain in early-stage PD mice

To definite the effects of oral rotenone administration on colon motility, the one-hour stool frequency were observed. We found that stool frequency (Fig. 1a), stool weight (Fig. 1b), and water content (Fig. 1c) were significantly decreased in rotenone-induced mice, compared with vehicle-induced mice. In addition, the pole and rotarod tests were used to asses the motor deficits and bradykinesia in rotenone-induced mice. And as shown in the Fig. 1d and 1e, there was no significant difference between rotenone-induced mice and vehicle-induced mice.

To investigate the impairments of intestinal transit and motor, we detected the the expression of phosphorylated α-syn in gut and SN, and TH in SN. We observed a significant increase in the

phosphorylated α -syn in the colon of the rotenone-induced animals compared to those of vehicle-induced animals (Fig. 1f and g), whereas there was no significant difference in the phosphorylated α -syn in SN between rotenone-induced mice and vehicle-induced mice (Fig. 1h and i). Furthermore, the decrease was observed in the TH of rotenone-induced mice compared to vehicle-induced mice (Fig. 1h and j). Since the decrease of TH was less than 50%, this may explain that the mice treated with rotenone for 21 days did not display the motor deficits and bradykinesia. These results showed that the manifestations of intestinal dysfunction rather than motor deficits appeared in early-stage PD mice.

Gut microbiota dysbiosis in early-stage PD mice

The gut microbiota of fecal samples was characterized by 16S rRNA sequencing and bioinformatic analysis. We found that there were no significant differences of alpha diversity indices including observed_species, Chao, Simpson, Shannon and PD_whole_tree indices between rotenone-induced mice and vehicle-induced mice (Fig. 2a). To identify the compositions of gut microbial community in rotenone-induced and vehicle-induced mice, we revealed the alterations of microbial compositions at phylum, family and genus levels, respectively (Fig. 2b and Supplementary Table 1). And as shown in Fig. 2c, the relative abundance of Flavobacteriaceae, Staphylococcaceae, and Prevotellaceae in the rotenone-induced mice at the level of family were significantly more abundant than that in the vehicle-induced mice (Wilcoxon rank-sum test, $q < 0.05$). At the level of genus, the relative abundance of Lachnospiraceae_UCG-001, Ruminiclostridium, and Prevotellaceae_NK3B31_group in the rotenone-induced mice were increased, compared with the vehicle-induced mice (Wilcoxon rank-sum test, $q < 0.05$). Subsequently, LEfSe was employed to identify the specific bacterial taxa related to PD (Fig. 2d). These findings suggested that the dysbiosis of gut microbiota has occurred in the early-stage PD.

Evaluation of serum amino acids in early-stage PD mice

According to targeted GC-MS metabolomics, a total of 19 amino acids were identified (Supplementary Table 2). We found that there was unobvious separation between the rotenone-induced mice and vehicle-induced mice in the score plot of unsupervised PCA (Fig. 3a). Meanwhile, the supervised OPLS-DA analysis presented a clear separation from two different regions in rotenone-induced and vehicle-induced mice ($R^2X = 0.241$, $R^2Y = 0.487$, and $Q^2 = 0.400$) (Fig. 3b), and the differential amino acids according to the corresponding S-plot (Fig. 3c and Supplementary Table 3). And the respective permutation test ($R^2Y = 0.840$, and $Q^2 = 0.444$, $P < 0.01$) are shown in Fig. S1. Furthermore, the serum amino acid concentrations in rotenone-induced mice showed a significant decrease of several metabolites, including L-proline, L-alanine, L-serine, L-asparagine, L-threonine, L-glutamine, L-methionine, and L-4-hydroxyproline, by independent Student's t-test or Mann-Whitney U test (Fig. 4).

The correlations of gut microbiota and serum amino acids in early-stage PD mice

The Spearman correlation analysis showed that there were several significant associations of gut bacteria with amino acids in the two groups (Fig. 5 and Supplementary Table 4). Ruminococcaceae and Ruminiclostridium showed negative correlations with L-proline, L-alanine, L-serine, L-asparagine, L-threonine, and L-methionine. And L-proline and L-methionine was negatively correlated with Firmicutes as well as positively associated with Bacteroidetes. Additionally, negative correlation was observed between Prevotellaceae and L-threonine. On the other hand, there were positive correlations between Muribaculaceae and L-methionine as well as uncultured_Bacteroidales_bacterium and L-glutamine.

Discussion

Studies found that the mice treated with rotenone for three weeks presented gastrointestinal dysfunctions, which was characterized by the decrease of intestinal transit, colon length and colon motility, and the mice treated with rotenone for four weeks showed the onset of motor deficits (Yang et al. 2018). Similarly, in this study, we found that the rotenone, as a parkinsonian neurotoxin, could result in pathological α -synuclein aggregation in gut in early-stage PD mice model to disrupt the function of gut. In addition, the loss of dopaminergic neurons also is closely related the development of PD. Our results showed that oral rotenone administration for three weeks could lead to the loss of dopaminergic neurons, but not enough to cause motor deficits in early-stage PD mice. Combined with the previous research findings, we could speculate that the gut is the origin of the pathological process of PD, and. Gut microbiota and their metabolic products may be the eventual cause of pathological α -synuclein accumulation in gut. A growing number of evidences supported that the gut microbiota could contribute to neurological disorders and neurodegenerative diseases. For example, the ameliorated PD-like symptoms were exhibited in germ-free mice and antibiotic-treated mice, rather than in the specific pathogen-free mice (Sampson et al. 2016).

Our 16S rRNA gene sequencing data revealed the abnormal microbial composition at different taxa levels. We found that Firmicutes, Epsilonbacteraeota, Ruminococcaceae, Ruminiclostridium, Alloprevotella, Helicobacteraceae, Helicobacter, Prevotellaceae, Muribaculaceae, Lachnospiraceae_UCG-001 enriched in rotenone-induced mice, while Bacteroidetes, uncultured_organism, uncultured_Bacteroidales_bacterium enriched in vehicle-induced mice. Research showed that the increase in the Firmicutes/Bacteroidetes ratio is associated with IBDs (Frank et al. 2007). Therefore, in our results, the increase of Firmicutes and decrease of Bacteroidetes in rotenone-induced mice may contribute to the aggregation of α -synuclein in gut. Moreover, consistent with our results, Perez-Pardo et al. found an increase in Ruminococcaceae in the cecum of a rotenone-treated mice (Perez-Pardo et al. 2018). The increase in Ruminococcaceae was found to correlate with longer duration of PD disease (Hill-Burns et al. 2017).

The communication between gut and brain could be realized via microbiota-metabolite axis, because the metabolites produced by gut microbiota could promote the pathogenesis of PD through a variety of ways (e.g. inflammatory cascades, energy metabolism and potential neuromodulatory activities) (Needham et al. 2020). Therefore, in the present study, we evaluated the alterations of serum amino acid

concentrations in rotenone-treated mice. Our data showed that the metabolism of several amino acids, such as L-proline, L-alanine, L-serine, L-asparagine, L-threonine, L-glutamine, L-methionine, and L-4-hydroxyproline, was significantly decreased in rotenone-treated mice. On the one hand, the distribution of amino acids in the gastrointestinal tract could be influenced by amino acid-fermenting bacteria (Dai et al. 2011). On the other hand, gut amino acid levels were also identified to have potential neuromodulatory activities (Needham et al. 2020). For example, glutamate, as excitatory neurotransmitter, was affected by gut microorganisms and metabolized to become the major inhibitory neurotransmitter GABA (Matsumoto et al. 2013; Zhu et al. 2010; O'Byrne et al. 2008). And alanine was also related to inhibitory neurotransmitter (Mori et al. 2002). And there were also some literatures have proved that arginine could be metabolized by the microbiota, and then play an important role in CNS via glutamate receptors (Williams et al. 1994). In addition, previous studies suggested that the alterations of amino acid concentrations could reveal changes in energy metabolism (Trupp et al. 2014). For example, the decrease of glutamic acid may be related to the increase of oxidative stress in the disease progression (Lei et al. 2014). Interestingly, we herein identified that the levels of L-serine, L-proline, L-asparagine, L-alanine, L-threonine and L-methionine were negatively correlated with the shifts of Ruminococcaceae and Ruminiclostridium. Overall, our results revealed that the alterations of gut microbiota correlated with serum amino acid levels.

In conclusion, our findings demonstrated that the α -synuclein aggregation occurred first in the gut rather than in SN in early-stage PD. In addition, we observed the alterations of the gut microbiota and serum amino acids in early-stage PD mice. Our data provided the new insights that the changes in gut microbial communities may deprive the normal functions of gut microbiota to result in the progression of neurodegeneration of PD. Therefore, it's necessary to maintain the normal symbiotic relationship between host and gut microbiota to intervent the development of PD.

Abbreviations

BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; CNS, central nervous system; ENS, enteric nervous system; FDR, false discovery rates; GC-MS, gas chromatography-mass spectrometry; IBD, inflammatory bowel disease; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; OPLS-DA, orthogonal partial least-squares discriminant analysis; OTUs, operational taxonomic units; PCA, principal component analysis; PD, Parkinson's disease; PNS, peripheral nervous system; QC, quality control; SD, standard deviation; SN, substantia nigra; SPF, specific-pathogen-free.

Declarations

Competing Interests The authors declare no conflict of interest.

Acknowledge We thank the Shanghai Luming biological technology co., LTD (Shanghai, China) for providing metabolomics services.

Funding This work was supported by the National Natural Science Foundation of China [grant numbers 62072143 and 81271208].

Authors' contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Zhenzhen Yan, Ruihua Li and Wanying Shi. The first draft of the manuscript was written by Zhenzhen Yan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Availability of data and material The data used or analyzed during this study are available from the corresponding author on reasonable request.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University (No 201985).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Figures

Figure 1

The alterations of gastrointestinal transit time, motor functions and pathology. **a-c** The stool frequency (**a**), stool weight (**b**) and stool water (**c**) were decreased in rotenone-induced mice. **d-e** Total time to descend (**d**) and latency to fall (**e**) were no significant difference between rotenone-induced mice and vehicle-induced mice. **f** Representative pictures of colon sections immunostained for p- α -syn and β III-tubulin in vehicle-induced mice (left) and rotenone-induced mice (right). Dapi (blue); p- α -syn (red); β III-tubulin (green). Scale bar = 200 μ m. **g** Quantitative analyses of mean fluorescence intensity of p- α -syn in colon. **h** Representative pictures of mesencephalic sections immunostained for p- α -syn and TH in vehicle-induced mice (left) and rotenone-induced mice (right). Dapi (blue); p- α -syn (red); TH (green). Scale bar = 100 μ m. **i-j** Quantitative analyses of mean fluorescence intensity of p- α -syn (**i**) and TH (**j**) in SNpc. Data were presented as the mean $\pm$ SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001

Figure 2

The dysbiosis of gut microbiota in rotenone-induced mice. **a** Alpha diversity analysis of the gut microbiota showed no significant differences between rotenone-induced mice and vehicle-induced mice. **b** The relative abundances of gut microbial compositions at phylum, family and genus levels. **c** The relative abundance of f_Flavobacteriaceae, f_Staphylococcaceae, f_Prevotellaceae, g_Lachnospiraceae_UCG-001, g_Ruminiclostridium, and g_Prevotellaceae_NK3B31_group between rotenone-induced mice and vehicle-induced mice. *, P < 0.05. **d** The significant bacterial taxa identified by LEfSe analysis. LDA scores (log10) > 2 and P < 0.05 are listed. The enriched taxa in vehicle-induced mice and rotenone-induced mice are indicated with LDA score (Red and Green, respectively).

Figure 3

The multivariate statistical analysis of serum amino acid metabolites between vehicle-induced and rotenone-induced groups. **a** PCA score plot. **b** OPLS-DA score plot. **c** S-plot from OPLS-DA analysis.

Figure 4

Statistically significant amino acids in serum samples of rotenone-induced versus vehicle-induced groups comparison. Data were presented as the mean $\pm$ SD. *, P < 0.05; **, P < 0.01.

Figure 5

The relationships between gut microbiota at genus level and serum amino acid concentrations were presented using Spearman's correlation analysis. Red, positive correlations; blue, negative correlations. *, $P < 0.05$, **, $P < 0.01$