Berberine Improves the Symptoms of DHEA-Induced PCOS Rats by Regulating Gut Microbiotas and Metabolites

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Keywords

Polycystic ovary syndrome · Insulin resistance · Gut microbiota · Berberine

Abstract

Objectives: The aim of this study was to investigate the effects of berberine on polycystic ovary syndrome (PCOS) with insulin resistance (IR). **Design:** This study performed 16S rRNA sequencing and metabolomic analysis on dehydroepiandrosterone (DHEA)-induced PCOS rats treated with berberine, focusing on the improvement of PCOS-IR by modifying gut microbiota and metabolism. **Methods:** Forty-two female Sprague Dawley rats were randomly divided into 4 experimental groups of 8 rats each (PCOS + HFD, PCOS + HFD + BBR, NCD + PCOS, and NCD + PCOS + BBR groups). Homeostasis model assessment of insulin resistance (HOMA-IR) index-related indicators and hormone level in serum were analyzed. 16S rRNA sequencing and metabolomic analysis were performed on DHEA-induced PCOS rats treated with berberine. In addition, the differential microbiotas and metabolites were screened. Also, enrichment analysis was carried out on the differential metabolites. Finally, we constructed a correla-

tion network to analyze the correlation between differential microbiotas and metabolites. **Results:** Firmicutes and Bacteroidetes were changed at the phylum level, and *Romboutsia*, *Bacteroides*, and *Clostridium_sensu_stricto_1* were changed at the genus level after berberine treatment. In addition, a total of 26 differential operational taxonomic units and 3 metabolites (glutamine, unsaturated acids [CH = CH], and glucose) between 2 groups were obtained. Moreover, these metabolites were mainly involved in type 2 diabetes mellitus, 2-component system, and ABC transporter Kyoto Encyclopedia of Genes and Genomes pathways. And, 3 microbiotas (*Lachnospiraceae_NC2004_group*, *Flavonifractor*, and *Parasutterella*) were regulated by glucose and glutamine. **Limitations:** The sample size involved in this study is relatively small. In addition, relevant experiments need to be performed to verify the obtained results from this study, and in-depth functional studies are needed. **Conclusions:** Berberine is effective in improving the pathological condition in PCOS by regulating the gut microbiotas and metabolites. This study will provide evidence for therapeutic efforts to treat PCOS-IR using berberine.

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Introduction

Polycystic ovary syndrome (PCOS), also known as Stein-Leventhal syndrome, was first reported in 1935 [1]. PCOS is the most common endocrine and metabolic disorder in adolescent women of reproductive age, with an incidence of 5–10% [2–4]. Moreover, PCOS is a significant cause of infertility [5]. It is generally believed that insulin resistance (IR), which is an increased risk of glucose intolerance and type 2 diabetes, is the core of PCOS, and there are about 50–75% of PCOS patients with different degrees of IR [6, 7].

Insulin-sensitizing agents can improve the endocrine and clinical symptoms of PCOS patients in some way. Metformin is a widely used insulin-sensitizing agent, which was firstly administered in obese PCOS women in 1994 to reduce serum levels of insulin and androgen and regularize the cycle of menses [8], but some patients worry about the potential adverse effects [9]. Berberine is an isoquinoline alkaloid isolated from the traditional Chinese medicines coptis and phellodendron [10, 11], and it exerts beneficial effects that are hypoglycemic and lipid lowering, ultimately improving IR [12, 13]. Lublin-ski et al. [14] suggested that berberine can treat metabolic disorders such as IR by improving gut microbiota, and Wu et al. [15] found that berberine can be used to treat PCOS.

The gut microbiota entails microorganisms that exist in the human gastrointestinal tract, reaching approximately 10^{13} – 10^{14} , with about 5001,000 species [16]. It is interesting to explore the role of gut microbiota in PCOS, as the androgen level in PCOS women is always elevated [17]. Thus, we hypothesize that excess androgen biosynthesis in PCOS may result in the dysbiosis of host gut microbiota, and modulating of gut microbiota may be beneficial for PCOS treatment. In the present study, we performed 16S rRNA sequencing and metabolomic analysis on dehydroepiandrosterone (DHEA)-induced PCOS rats treated with berberine, focusing on the improvement of PCOS-IR by modifying gut microbiota and metabolism. Through this work, we hope to provide evidence that berberine has the therapeutic effect to treat PCOS-IR.

Materials and Methods

Animals and Model

Forty-two female Sprague Dawley rats (21 days of age) were randomly divided into 4 experimental groups of 8 rats each (PCOS + HFD, PCOS + HFD + BBR, NCD + PCOS, and NCD + PCOS + BBR groups). Two control groups were composed of 5 rats each

(NCD and NCD + BBR groups). The experimental groups were continuously injected with 6 mg/100 g/day of body weight of DHEA (source leaf organisms, S24516) in 0.2 mL of soybean oil for 21 days, while the control groups were only injected with 0.2 mL of soybean oil. Partial PCOS model rats were fed with a high-fat diet (HFD) to induce IR, and the control groups were fed with a normal chow diet (NCD) (the nutrient ratios in the HFD included 10% lard, 20% sucrose, 2.5% cholesterol, 1% porcine bile salt, 1% egg, 30% sprouts, and 35.5% basic feed, obtained from the Shanghai Laboratory Animal Center [SLAC], Shanghai, China). Then, the rats in NCD + BBR, PCOS + HFD + BBR, and NCD + PCOS + BBR groups were treated with berberine of 99% purity (150 mg/kg) (Sigma-Aldrich, Louis, MO, USA) once daily for 6 weeks.

Homeostasis Model Assessment of Insulin Resistance Index-Related Indicators

Blood samples from 3 rats in every group were taken from the tail after an overnight fasting (8 h), to measure fasting plasma glucose by using an ACCU-CHEK Performa glucose [18] meter (Roche Diabetes Care, Indianapolis, IN, USA) and fasting insulin (FINS) by using a Rat Insulin ELISA Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The HOMA-IR was calculated as (fasting plasma glucose [mmol/L] × FINS [μIU/mL])/22.5, and higher HOMA-IR values indicated lower insulin sensitivity [18].

Detection of the Hormone Level in Serum

A total of 3 rats in every group were used to detect the hormone level in serum. The level of testosterone in rats was determined by ELISA, and the Testosterone Parameter Assay Kit (KGE010; R&D systems) was used to measure the testosterone level according to the manufacturer's instruction.

Bacterial Sequencing and Analysis

This study aimed to evaluate the effects of berberine on PCOS-IR, since HFD induces significant metabolic disorders and gut microbiome dysbiosis in PCOS [19, 20]. Thus, 16S rRNA sequencing and metabolomic analysis were performed on the PCOS + HFD + BBR and PCOS + HFD groups to exclude the influence factor of HFD. Fecal samples were collected from the rats in the 2 groups, and the DNeasy PowerSoil Kit (Qiagen, Germantown, MD, USA) was used to extract the bacterial genomic DNA. The V3–V4 region of the 16S rRNA gene was selected for subsequent pyrosequencing, and the acquired gene sequences were PCR amplified with bar-coded universal primers. Then, the PCR products were visualized on a 2% agarose gel and quantified with a Qubit fluorometer (Promega, Madison, WI, USA).

Data of 16S sequencing have been uploaded to the NCBI database (SRA accession: PRJNA594263, <https://www.ncbi.nlm.nih.gov/sra/PRJNA594263>). The sequencing data were preprocessed to generate high-quality sequences, and the Vsearch software was used to combine the sequences according to their similarity. The remaining sequences with a similarity ≥97% were grouped into the same operational taxonomic unit (OTU). Then, differential expression analysis was performed using the quasi-likelihood *F* test of the edgeR package with the cutoff value of *p* value <0.05. And, the Pearson correlation coefficient of the differential gut bacterial microbiome was calculated with a threshold of *p* value <0.05 and $|r| > 0.8$.

Fig. 1. Berberine treatment altered IR-related indices and testosterone level in the PCOS rats according to our previous study [21]. Body weight (**a**); level of fasting blood glucose (**b**); level of fasting insulin (**c**); HOMA-IR (**d**); level of testosterone (**e**). * p value <0.05. IR, insulin resistance; PCOS, polycystic ovary syndrome; HOMA-IR, homeostasis model assessment of insulin resistance.

Metabolomic Analysis

Metabolomic analysis was performed on the PCOS + HFD + BBR and PCOS + HFD groups. Blood samples were collected from rats in the 2 groups, and the serum was separated by centrifugation (13,000 rpm, 10 min, and 4°C) and stored at -80°C. Then, the LC-MS analysis was conducted by ProfLeader Biotech Co., Ltd. (Shanghai, China).

Data of metabolomic analysis have been uploaded to a public online database (<https://figshare.com/s/1463481d11efafc88b97>, DOI: 10.6084/m9.figshare.11662473). LC-MS raw data were collected using the UNIFI software and processed using Progenesis QI software. The Progenesis QI analysis provided a matrix including peak picking, peak grouping, retention time (RT) correction, second peak grouping, and annotation of isotopes for every sample. Any peak detected in <50% of quality control samples or 80% of biological samples was removed; the combination of the RT and m/z data was used to identify each ion. Then, the data were pre-processed by normalization, and the principal component analysis was performed by prcomp function to assess the outlier detection and batch effect. The ropls in R package (<http://bioconductor.org/packages/release/bioc/html/ropls.html>, Version 1.6.2) was used to carry out orthogonal partial least-squares discriminant analysis (OPLS-DA) and PLS-DA to visualize the metabolic alterations

among the 2 groups. And, the variable importance in the projection (VIP) value was calculated. Moreover, the differential metabolites were screened with the cutoff value of p value <0.05 and VIP > 1. Besides, enrichment analysis was conducted using MBROLE (<http://csbg.cnib.csic.es/mbrole2/analysis.php>) with the threshold of p value <0.05.

Correlation Analysis between Differential Microbiotas and Metabolites

The Pearson correlation coefficients of differential microbiotas and differential metabolites were calculated, and the correlation network was constructed through Cytoscape software (<https://cytoscape.org/>).

Statistical Analysis

All statistical analyses were performed using SPSS statistical software package standard version 16.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad prism 5 (GraphPad Software, San Diego, CA, USA). The data are presented as mean $\pm$ standard deviation. Student's t test was used to analyze the differences between 2 groups, and the differences between individual OTUs were analyzed by the nonparametric Kruskal-Wallis test. p value <0.05 was considered statistically significant.

Fig. 2. Berberine alters microbiota composition in PCOS rats. At the phylum level (a); at the genus level (b). PCOS, polycystic ovary syndrome.

Results

Berberine Treatment Altered IR-Related Indices and Testosterone Level in the PCOS Rats

Herein, the IR-related indices, testosterone level, and ovarian morphology changes were evaluated according to our previous study [21]. As shown in Figure 1a–d, the body weight, fasting blood glucose, fasting insulin, and HOMA-IR index in the HFD group were higher than those in NCD groups, indicating that the rats in the HFD group had IR. However, the HOMA-IR index of the PCOS + HFD + BBR group was significantly lower than that of the PCOS + HFD group (p value <0.05), and the other 3 indicators also showed a downward trend, although there was no significant difference. In addition, the level of testosterone in NCD + PCOS + BBR and HFD + PCOS + BBR groups was significantly decreased than that of HFD + PCOS and NCD + PCOS groups, respectively (p value <0.05) (Fig. 1e). Taken together, these data demonstrate that berberine has the potential to alter PCOS testosterone level and IR values in PCOS model rats.

Berberine-Induced Changes in the Gut Bacterial Microbiome

Furthermore, the taxon summary indicated distinct changes in the gut microbial composition in response to berberine administration. In terms of the assignment at the phylum level, the dominant phyla were Firmicutes and Bacteroidetes (Fig. 2a). At the genus level, *Romboutsia*, *Bacteroides*, and *Clostridium_sensu_stricto_1* relative abundance values were significantly affected by berberine (Fig. 2b).

In addition, to find out the key discriminatory OTUs, the quasi-likelihood F test was used here. A total of 26 differential OTUs between PCOS + HFD + BBR and PCOS + HFD groups were obtained with the cutoff value of p value <0.05 . Of these differential OTUs, a total of 10 OTUs were upregulated and 16 were downregulated. The differential bacteria taxa on phylum and genus levels between the 2 groups are displayed in Figure 3.

Besides, in order to infer the possible cooperative or competitive relationships between these differential gut bacterial microbiomes, correlation analysis was carried out. As shown in Figure 4, a total of 123 relational interactions were obtained with the threshold of p value <0.05 and $|r| > 0.8$. Among these, a cooperative relationship was found between *Ruminiclostridium_6* and *Tyzzelerella*, and a competitive relationship existed between *Alistipes* and *Family_XIII_AD3011_group*.

Berberine-Induced Metabolic Profiling Analysis

After data preprocessing (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000518040 for all online suppl. material), PCA, OPLS-DA, and PLS-DA were subsequently employed to reveal the clustering trends of each group. As shown in Figure 5, 2 samples (S4 and S5) showed serious deviation (Fig. 5a), and thus the 2 samples were removed. Then, the samples were divided into blocks suggesting that the 2 clusters were clearly divided (Fig. 5b), indicating 2 groups with different metabolic profiles. And, the results of OPLS-DA and PLS-DA show that samples were clustered closely together, which indicates that the experiment had good stability and repeat-

Fig. 3. The differential bacteria taxa between 2 groups. At the phylum level (a); at the genus level (b).

Fig. 4. The relationships between the differential gut microbiotas.

Fig. 5. Berberine alters metabolites in PCOS rats. PCA of all samples (**a**) and after removing 2 samples (S4 and S5) (**b**); OPLS-DA (**c**) and PLS-DA (**d**). PCOS, polycystic ovary syndrome; PCA, principal component analysis; OPLS-DA, orthogonal partial least-squares discriminant analysis.

ability (Fig. 5c, d). Three metabolites with the cutoff value p value <0.05 and VIP > 1 were screened between the 2 groups, including 1 upregulated (glutamine) and 2 down-regulated (unsaturated acids, UFA [$\text{CH} = \text{CH}$], and glucose) (Fig. 6a). The enrichment analysis showed that 22 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, including type 2 diabetes mellitus, 2-component system, and ABC transporters, were screened with the threshold of p value <0.05 (Fig. 6b).

Correlation Analysis

As mentioned before, a total of 22 relationships were obtained, including 2 metabolites and 19 differential microbiotas (Fig. 6c). And, 3 microbiotas (*Lachnospiraceae_NC2004_group*, *Flavonifractor*, and *Parasutterella*) were regulated by glucose and glutamine.

Fig. 6. The differential metabolites between 2 groups and correlation analysis between differential microbiotas and metabolites. Heatmap of differential metabolites. Red indicates upregulated, and green indicates downregulated (**a**); pathway enrichment analysis of differential metabolites. The size of the ball represents the metabolite ratio enriched in each term, and the color of the ball

represents the number of the p value (**b**); correlation analysis between differential microbiotas and metabolites. The red nodes indicate upregulated microbiotas, and green nodes indicate downregulated microbiotas. The pink diamond indicates upregulated metabolite, and blue diamond indicates downregulated metabolite (**c**).

Discussion

PCOS is an endocrine disease with complex and diverse manifestations of metabolic disorders [22, 23]. At present, drugs constitute the principal treatment for PCOS, and lifestyle adjustment is advocated to control diet, exercise, and weight [24, 25]. Berberine, a major active component of the Chinese herbal medicines *Rhizoma Coptidis*, *Cortex Phellodendri*, and *Cortex Berberidis*, has

been prescribed for the treatment of diarrhea, metabolic disorders, and infertility [26]. There is recent evidence indicating that berberine offers promise for treating PCOS-IR [27]. In this study, Firmicutes and Bacteroidetes were changed at the phylum level, and *Romboutsia*, *Bacteroides*, and *Clostridium_sensu_stricto_1* were changed at the genus level. In addition, a total of 26 differential OTUs and 3 metabolites (glutamine, unsaturated acids, UFA [CH = CH], and glucose) between 2 groups were obtained. More-

over, these metabolites were mainly involved in the type 2 diabetes mellitus, 2-component system, and ABC transporter KEGG pathways. And, 3 microbiotas (*Lachnospiraceae_NC2004_group*, *Flavonifractor*, and *Parasutterella*) were regulated by glucose and glutamine.

As we all know, the ratio of Firmicutes to Bacteroidetes is considered as a key indicator to measure the state of gut microbiota [28, 29]. In this study, Firmicutes were decreased and Bacteroidetes were increased at the phylum level after berberine treatment, which means that the berberine improved the structure of gut microbiota. Besides, Kelley et al. [30] suggested that Bacteroidales and Clostridiales were changed in a letrozole-induced PCOS rat model, which were consistent with our results. In addition, a total of 26 differential OTUs were dysregulated; one possible explanation for the changes we observed in the gut microbiome is that berberine had a direct effect on the gut microbiome.

PCOS is a complex endocrinopathy, which includes numerous abnormalities and influences some metabolic pathways. Thus, the metabolomic analysis was performed, and 3 metabolites, including glutamine, unsaturated acids, UFA [CH = CH], and glucose, were changed after berberine treatment. Glutamine is the most abundant amino acid in the body, with antioxidant and anti-inflammatory properties [31, 32]. Numerous studies indicated that oxidative stress and chronic inflammation might have an important effect on the pathophysiology of PCOS [33–35]. In addition, glucose is capable of inciting oxidative stress and an inflammatory response from mononuclear cells of women with PCOS [7]. Herein, glutamine was increased, and glucose and unsaturated acids, UFA [CH = CH], were decreased after berberine treatment, illustrating that berberine might be effective in improving the pathological condition in PCOS. Besides, the enrichment analysis showed that these metabolites were mainly involved in the type 2 diabetes mellitus, 2-component system, and ABC transporter KEGG pathways. Due to the high prevalence of type 2 diabetes in women with this syndrome and their relatives, PCOS should be considered a marker of family pathology, a pathway to type 2 diabetes [36]. ABC transporters are primary active membrane proteins that translocate solutes across lipid bilayers [37]. Members of the ABC transporter family, such as ABCA1, have been shown to control cellular lipid metabolism. Changes happening in the ATP binding cassette transporter 1 (ABC1) gene encoding a protein regulating entry and exit from the cell membrane may contribute to dyslipidemia in patients with PCOS [38]. However, few studies of the 2-component system pathway on the PCOS have been reported. Taken to-

gether, we suspected that berberine might be effective in improving the pathological condition in PCOS by regulating these metabolites via type 2 diabetes mellitus, 2-component system, and ABC transporter KEGG pathways.

Besides, in this study, the correlation analysis showed that glutamine was regulated by 12 microbiotas, and glucose was regulated by 10 microbiotas. Increasing evidence implied that gut microbiome functions by regulating metabolites that can directly or indirectly affect host physiology [39, 40]. In addition, dysbiosis of gut microbiota theory revealed that gut microbiome dysbiosis causes a breakdown in gut mucosal permeability, passage of gut microbiome endotoxin into the systemic circulation, inflammation initiating IR, and an increase in testosterone production – PCOS phenotype [19]. Moreover, glutamine is an important metabolite involved in maintaining gut wall barrier integrity [41], and glutamine was increased after berberine treatment in this study. Therefore, we speculated that one possible mechanism for berberine action might be via modulating gut wall barrier integrity to prevent gut microbiome endotoxin into the systemic circulation.

However, this study has some limitations. The sample size involved in this study is relatively small, and a large sample size should be used to carry out further study. In addition, relevant experiments need to be performed to verify the obtained results from this study, and in-depth functional studies are needed.

Conclusion

These results confirmed that the gut microbiome is altered and contributes to performance by interacting with metabolism under berberine application. Collectively, our results provide a guide for future research in the development of medical treatments for PCOS.

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Statement of Ethics

All the animal experiments were conducted in strict accordance with the animal ethics standards and with full ethical approval of the Ethics Committee of Obstetrics and Gynecology Hospital of Fudan University (No. [2015] 45).

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

X.L. contributed to conception and design of the research; H.S. was involved in acquisition of data; H.S. and X.X. analyzed and interpreted the data; D.Y. performed statistical analysis; X.L. obtained funding; H.S. and X.X. drafted the manuscript; X.L. revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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