Research Article

Therapeutic mechanisms of metformin and Bushen Ditan Decoction in polycystic ovary syndrome: Evidence from a rat model and miRNA sequencing

Jiayi Xu[1†], Qianchao Shao[1†], Hanqing Zhang[2], Dan Weng[1*], and Wei Lu[2*]

[1]Department of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing, Jiangsu 210094, China
[2]Department of Gynecology, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210029, China
[†]*These authors contributed equally to this work*

Abstract

Background: Polycystic ovary syndrome (PCOS) represents the most common endocrine disorder afflicting women of reproductive age. PCOS is characterized by hormonal imbalances, metabolic abnormalities, and infertility, yet only limited therapeutic options are available. Metformin and Bushen Ditan Decoction (BDD), a traditional Chinese medicine formula, are commonly used in Western and traditional Chinese medicine, respectively, for treating PCOS. Despite their demonstrated clinical efficacy, the mechanisms underlying their therapeutic effects remain incompletely understood. **Objective:** This study aimed to investigate the therapeutic mechanisms of metformin and BDD in a PCOS rat model. **Methods:** PCOS was induced in rats by using the Poretsky method. After model induction, the animals were given metformin (150 mg/kg/day) or BDD (8, 16, or 32 mg/kg/day) via intragastric gavage for 4 weeks. **Results:** Both metformin and BDD significantly reduced testosterone levels and improved lipid metabolism parameters. MicroRNA (miRNA) sequencing of ovarian tissues identified 18 miRNAs commonly regulated by both treatments, and functional enrichment analysis indicated that these differentially expressed miRNAs are involved in autophagy- and metabolism-related pathways implicated in the pathogenesis of PCOS. Conservation analysis across species highlighted the potential clinical relevance of these findings. **Conclusion:** This study demonstrated how mechanistically metformin and BDD work on PCOS, particularly through the regulation of autophagy- and metabolism-related pathways, providing insights for their future clinical applications.

Keywords: Polycystic ovary syndrome, Metformin, Traditional Chinese medicine, MicroRNAs

1. Introduction

As one of the most prevalent endocrine disorders affecting women of reproductive age, polycystic ovary syndrome (PCOS) is characterized by irregular menstrual cycles, distinct ovarian changes, hyperandrogenism, and polycystic ovarian morphology.[1,2] This clinical heterogeneity has complicated both diagnosis and treatment of the condition, leading to multiple diagnostic criteria over the years. Beyond its reproductive manifestations, PCOS is now recognized as a systemic metabolic disorder with profound implications for long-term health.[3] In addition to infertility, the complications of PCOS include obesity, type II diabetes, insulin resistance, hypertension, cardiovascular disease, and uterine cancer,[4,5-8] posing a significant threat to women's health. Moreover, the relationship between these conditions is typically bidirectional: insulin resistance exacerbates hyperandrogenism and ovulatory dysfunction, while PCOS *per se* increases the risk of impaired glucose tolerance and type II diabetes.[9] In recent years, the global incidence of PCOS has shown an alarming rise, with a 4.47% increase reported among women of reproductive age (15–49 years old) from 2007 to 2017.[10] This increasing prevalence highlights the urgent need for research into the pathogenesis of PCOS and

***Corresponding authors:**
Wei Lu (fsyy00604@njucm.edu.cn)
Dan Weng (danweng@njust.edu.cn)

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the development of effective therapeutic interventions. PCOS is intimately associated with numerous factors, including environmental influences, diet, and stress, among others. Environmental endocrine disruptors, such as bisphenol A and phthalates, elevate luteinizing hormone and progesterone levels, thereby increasing PCOS risk.[11] Excessive intake of advanced glycation end products is also a contributing factor to PCOS.[12] In addition, PCOS patients exhibit higher rates of depression and anxiety.[13]

PCOS is a multifactorial disease with an involved, not yet fully understood pathogenesis. PCOS patients tend to have an elevated pulse frequency of gonadotropin-releasing hormone, resulting in increased LH secretion without a corresponding rise in follicle-stimulating hormone. This hormonal imbalance leads to compromised follicular development, diminished aromatase activity, ovarian follicle atresia, and hyperandrogenism.[14] Elevated insulin levels due to insulin resistance contribute to increased LH expression and enhanced androgen secretion by the ovaries and adrenal glands. The interplay between elevated insulin and LH levels exacerbates ovarian follicle atresia, potentially driving PCOS progression.[15] Hyperandrogenemia, a hallmark of PCOS, is present in 70–80% of diagnosed women.[16,17] Current research suggests that PCOS onset is intricately linked to abnormalities in the hypothalamus–pituitary–ovary axis,[6] chronic inflammation, oxidative stress,[18,19] adrenal hyperactivity,[20,21] and other factors. However, despite this progress, knowledge gaps remain in the understanding of PCOS pathogenesis.

Due to the complexity of PCOS pathogenesis, clinical interventional options are limited. In addition to lifestyle modifications such as exercise and calorie-restrictive diets, Diane-35 and metformin are commonly used as the first-line therapies for PCOS. Diane-35, comprising 2 mg cyproterone acetate and 35 μg ethinyl estradiol (E2), is a widely employed androgen inhibitor for female androgen-dependent conditions. Metformin, known as an insulin sensitizer, is extensively used for treating type II diabetes[22] and PCOS.[23] It has demonstrated efficacy in promoting weight reduction and enhancing ovulation.[24,25] Mechanistically, metformin can lower androgens in ovarian theca cells and inhibit ovarian steroid production.[26] Moreover, metformin activates the AMP-activated protein kinase (AMPK) pathway, contributing to blood glucose reduction in type II diabetes.[27,28] There is no significant difference between Diane-35 and metformin in improving hirsutism. Diane-35 is superior to metformin in reducing androgen levels but inferior in lowering insulin levels. Concurrently, studies indicated that Diane-35 in combination with acupuncture significantly reduces LH, E2, and testosterone (T) levels, enhances ovulatory response, and shortens the reproductive cycle in patients with PCOS-related infertility.[29,30]

Apart from modern medicine, traditional Chinese herbal medicine is frequently utilized for managing PCOS in China. Bushen Ditan Decoction (BDD), a time-honored recipe, has shown promising clinical effectiveness in ameliorating manifestations of PCOS-related symptoms and is frequently employed in clinical practice in China. The main components of BDD include *Rehmanniae Radix Praeparata*, *Atractylodis Rhizoma*, *Angelicae Sinensis Radix*, *Sinapis Semen*, *Corni Fructus*, *Cuscutae Semen*, *Poria*, and *Gleditsiae Spina*. Having been put into clinical application for over 30 years, BDD has demonstrated efficacy in relieving symptoms in women with PCOS.[31,32] However, despite its clinical efficacy, the underlying mechanism by which BDD ameliorates PCOS symptoms remains elusive.

This study aimed to explore the therapeutic mechanisms of metformin and BDD and to gain insights into the pathogenesis of PCOS. A rat model of PCOS was first established and was then treated with metformin and varying doses of BDD. The efficacy of these treatments was evaluated by measuring blood markers related to hormone and lipid metabolism. MicroRNAs (miRNAs), small non-coding RNA molecules that play critical roles in post-transcriptional gene regulation, are implicated in various physiological processes and diseases, including PCOS. Understanding the regulatory roles of miRNAs in PCOS can provide valuable insights into the pathogenesis of the syndrome and help identify potential therapeutic targets. Therefore, we conducted an analysis of miRNA expression in rat ovaries to elucidate the therapeutic mechanisms of traditional Chinese medicine (TCM) and metformin. We sought to identify the common miRNAs regulated by both treatments and explore their involvement in autophagy and metabolism-related pathways. This integrated approach, combining physiological measurements with high-throughput sequencing technologies, was designed to provide more in-depth insight into the pathogenesis of PCOS. Furthermore, by identifying conserved miRNAs across species, this study aimed to lay a stronger bedrock for translating these findings to the understanding of pathophysiology and treatment of PCOS.

2. Materials and methods

2.1. Chemicals and reagents

Metformin was purchased from Peili Pharmaceutical Co. (China). Human chorionic gonadotropin (HCG) was procured from Aladdin Biotechnology Ltd. (China). *R. Radix Praeparata* (Shudihuang), *C. Semen* (Tusizi), *C. Fructus* (Shanzhuyu), *G. Spina* (Zaojiaoci), *S. Semen* (Jiezi), *A. Rhizoma* (Cangshu), *A. Sinensis Radix* (Danggui), and *Poria* (Fuling) were provided by Jiangsu Provincial Hospital of TCM. For the preparation of the decoction, 12 g of *R. Radix Praeparata*, 12 g of *C. Semen*, 9 g of *C. Fructus*, 9 g of *G. Spina*, 9 g of *S. Semen*, 12 g of *A. Rhizoma*, 12 g

of *A. Sinensis Radix*, and 12 g of Poria were mixed, boiled, and concentrated to obtain decoctions with concentrations of 0.8 g/mL, 1.6 g/mL, and 3.2 g/mL, respectively, corresponding to BDD at different dosages.

2.2. Animals

A total of 60 female Sprague–Dawley rats (6–7 weeks old, specific-pathogen-free grade) were obtained from the Experimental Animal Research Center of Nanjing University of TCM, Nanjing, China. Rats were housed at 23 ± 2℃ with a 12-h light/dark cycle and given free access to food and water. After a 2-week acclimation period, estrous cycles were determined by vaginal smear. Rats (240–260 g, 8–9 weeks old) with regular estrous cycles were randomly assigned to a normal control group (n = 10) and a model group (n = 50). The normal control group was fed a normal chow (NC) diet (Trophic Animal Feed, China), while the model group received a high-fat diet (HFD; basic diet 39%, lard 23%, sucrose 24%, egg 9%; Trophic Animal Feed, China) according to the Poretsky modeling protocol[33] and 5% glucose was added to the drinking water. Model group rats were injected subcutaneously with insulin once daily from day 1 to day 10, starting at 0.5 IU and increasing by 0.5 IU each day until 5 IU, followed by 5 IU insulin daily from day 11 to day 22. In addition, 1.5 IU HCG (dissolved in 0.2 mL normal saline) was injected subcutaneously twice daily. After 22 days, serum T levels (≥2-fold higher than normal controls) confirmed successful establishment of the PCOS model. The normal control group stayed on the NC diet, while the model group remained on the HFD and was randomized into five groups over a 4-week treatment period.

Following model establishment, the model blank group was given normal saline (10 mL/kg) by daily intragastric gavage at 8:00 a.m.; the metformin group received metformin suspension (150 mg/kg, 10 mL/kg) by daily intragastric gavage at 8:00 a.m.; BDD low-, medium-, and high-dose groups received BDD at 8 g/kg, 16 g/kg, and 32 g/kg, respectively, dissolved in 10 mL/kg normal saline, administered daily at 8:00 a.m. These doses corresponded to 5, 10, and 20 times the human clinical dose; the normal control group received normal saline (10 mL/kg) daily at 8:00 a.m. After 4 weeks of treatments, all rats were euthanized, and blood and ovarian tissue were collected (Figure 1).

2.3. MiRNA sequencing

For miRNA sequencing, two rats were randomly selected from each group, and ovarian tissues were used for miRNA profiling. Total RNA was extracted from approximately 30 mg of frozen ovarian tissue using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA quality and quantity were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, United States of America [USA]), and only samples with an RNA Integrity Number >7.0 were used for library construction. Small RNA libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, USA). Briefly, 1 μg of total RNA was ligated with 3' and 5' adapters, reverse transcribed, and amplified by polymerase chain reaction (PCR) for 12 cycles. The resulting cDNA libraries were purified and size-selected (140–160 bp) using polyacrylamide gel electrophoresis. Library quality was assessed using the Agilent 2100 Bioanalyzer, and quantification was performed by quantitative PCR. Sequencing was conducted on an Illumina NovaSeq 6000 platform (Illumina, USA) with 50 bp single-end reads at Beisipai Biotechnology (China), generating approximately 10 million raw reads per sample.

Quality control of raw data was performed using FastQC (v0.11.9, Babraham Bioinformatics, UK). Cutadapt (v3.4, Marcel Martin, Germany) was employed to filter out low-quality reads (quality score <20), 3' connectors, 5' adapters, and poly-A fragments. High-quality clean reads were then mapped to the rat genome (Rn6) using miRDeep2 (v2.0.1.2, Friedrich Miescher Laboratory of the Max Planck Society, Germany) to remove ribosomal RNAs, transfer RNAs, small nuclear RNAs, and small nucleolar RNAs. The remaining sequences were aligned with the MiRBase database (v22.1, https://www.mirbase.org/) to identify known miRNAs. Novel miRNA prediction was performed using miRDeep2 based on the secondary structure, Dicer cleavage site, and minimum free energy. Sequencing was conducted by Beisipai Biotechnology (China).

2.4. Differential MiRNA expression analysis

To identify differentially expressed miRNAs among groups, expression abundance was calculated based on the genomic mapping positions of reads. Reads mapped to the hairpin

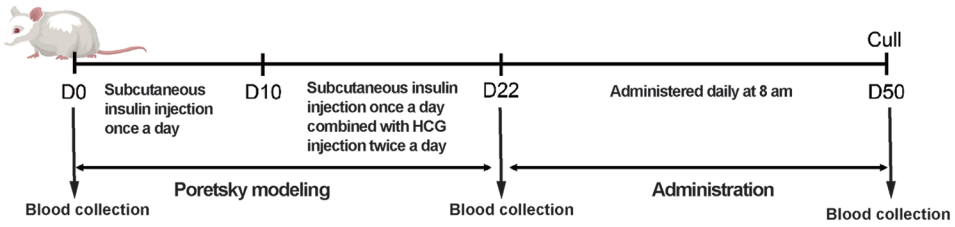

Figure 1. Establishment of the polycystic ovary syndrome rat model and subsequent treatment strategies

sequence region of known rat miRNAs were validated, and only miRNAs with consistent mapping positions were counted. Expression levels were compared between groups, and miRNAs significantly up- or down-regulated were identified using a threshold of fold change ≥2 ($\log_2$FC ≥ 1 or ≤ −1) and p≤0.05.

2.5. Target gene prediction and enrichment analysis

Target genes of miRNAs were predicted by matching miRNA seed regions with the 3'-untranslated region or full sequence of potential targets, while considering free energy and prediction scores. Two databases, MiRDB (https://mirdb.org/) and MiRWalk (http://mirwalk.umm.uni-heidelberg.de/), were used to increase prediction accuracy. Overlapping results from both databases were employed as the final predicted target mRNAs. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using Webgestalt (http://www.webgestalt.org/). A p-value threshold of ≤0.05 was used to determine significance.

2.6. Physiological parameter analysis

Serum cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), T, LH, and E2 were measured using commercial kits by following the manufacturer's protocols. Blood samples were harvested at 3 time points: before modeling (baseline), immediately after model establishment (day 22), and after the 4-week treatment period (day 50). Serum was separated by centrifugation at 3,000 rpm for 15 min and stored at −80°C until analysis.

2.7. Statistical analysis

Changes in serum indicators were analyzed by GraphPad Prism 9 software (GraphPad Software, Inc., USA) and expressed as mean ± standard error of the mean. An unpaired Student's t-test or one-way analysis of variance (ANOVA) was performed to assess differences in mean values, with ANOVA used for comparisons involving more than two groups. A p<0.05 was considered statistically significant. For miRNA analysis, counts were normalized using a modified global normalization method. Based on the normalized counts, expression differences were assessed using a t-test. The EdgeR package (v3.18.1, Developer, Country) was used for differential expression analysis. miRNAs with fold change ≥ 2 ($\log_2$FC≥ 1 or ≤ −1) and p≤0.05 were considered significant.

3. Results

3.1. Evaluation of the therapeutic effects of metformin and BDD on the PCOS rat model

To assess the therapeutic effects of metformin and BDD in PCOS rats, a PCOS rat model based on the Poretsky method was first made through combined injections of insulin and HCG, followed by treatment with metformin (150 mg/kg) or varying doses of BDD (8, 16, and 32 g/kg) for 28 days. Serum hormone levels were then measured to confirm the success of model establishment. Measurements were taken at baseline, immediately after model induction, and after the 4-week treatment period. As shown in Figure 2A, serum T levels were significantly elevated in the PCOS group, confirming successful model establishment. Treatment with both metformin and BDD significantly reduced T concentrations, with BDD demonstrating a more pronounced effect than metformin, suggesting the therapeutic efficacy of both treatments (Figure 2A). E2 levels remained unchanged throughout the experiment (Figure 2B), consistent with previous studies using the Poretsky model.[33] Although LH levels in the PCOS group were elevated compared with the control group, the difference did not reach statistical significance (p=0.0569). Treatment with metformin and BDD showed a trend toward reduced LH levels in PCOS rats (p=0.0754 between the PCOS and high-dose BDD groups) (Figure 2C).

Given the association between PCOS and increased risk of metabolic disorders such as obesity and insulin resistance, serum lipid levels were measured to assess metabolic status. PCOS rats exhibited significantly elevated serum cholesterol and LDL levels, indicating hyperlipidemia (Figure 2D and F). Both metformin and BDD effectively reduced cholesterol and LDL levels in PCOS rats (Figure 2D and F). In addition, HDL levels were notably lower in PCOS rats than in controls, and a slight, although statistically non-significant, increase was observed following metformin and BDD treatment (Figure 2E). Collectively, these results confirm the successful establishment of the PCOS rat model and demonstrate that both metformin and BDD influence hormone levels and metabolic parameters in PCOS rats.

3.2. MiRNA sequencing analysis

To elucidate the potential pharmacological mechanisms of metformin and BDD, miRNA sequencing was performed using ovarian tissue from all six experimental groups. After filtering raw data using Cutadapt, miRNA prediction based on clean reads identified a total of 724 known miRNAs. Differential expression analysis against a threshold of p<0.05 and $|\log_2(\text{fold change})| \geq 1$ identified 90 differentially expressed miRNAs between the PCOS model and control groups. Compared with the PCOS model group, 224 miRNAs were differentially expressed in the metformin-treated group. In the BDD-treated groups, the high-, medium-, and low-dose treatments resulted in differential expression of 95, 131, and 33 miRNAs, respectively (Figure 3).

Subsequently, we analyzed the differentially expressed miRNAs and identified the top 10 from each comparison.

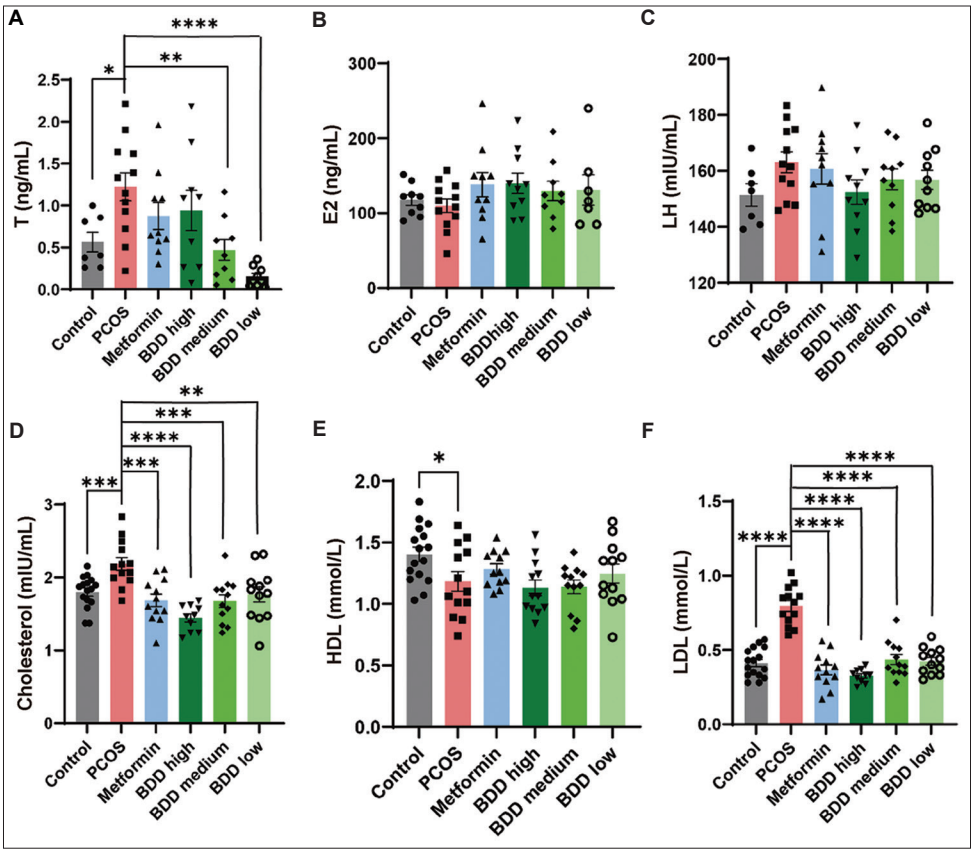

Figure 2. Hormone- and lipid metabolism-related parameters in rat serum. The polycystic ovary syndrome (PCOS) rat model was established and then treated with metformin (150 mg/kg) or BDD (8, 16, 32 g/kg) by intragastric gavage. After 28 days, changes in (A) androgen, (B) estrogen, (C) luteinizing hormone, (D) cholesterol, (E) HDL, and (F) LDH were measured.
Notes: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; one-way analysis of variance.
Abbreviations: E2: Estradiol; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; LH: Luteinizing hormone; T: Testosterone.

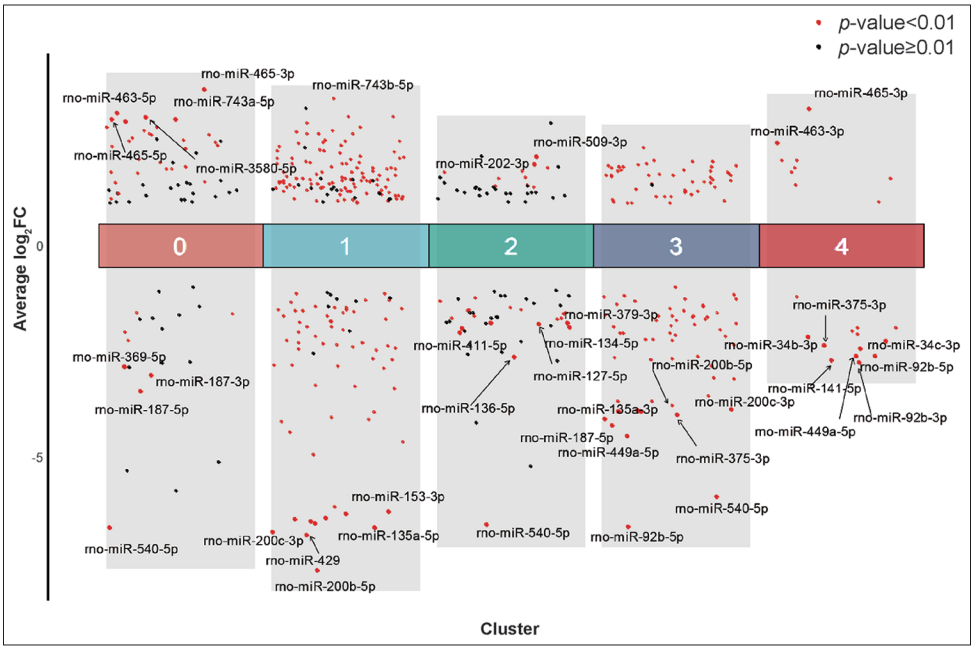

Figure 3. Differential expression of microRNAs (miRNAs) in ovaries from different groups compared with the model group. Comparisons include 0 = normal control versus model; 1 = metformin *versus* model; 2–4: high-, medium-, and low-dose BDD versus model. Selected miRNAs with significant expression differences were labeled (some points not labeled due to high density).

Notably, some miRNAs were shared across treatments. For example, miR-141-3p and miR-503-5p appeared repeatedly among the differentially expressed miRNAs induced by metformin and various BDD doses (data not shown), suggesting that both treatments may exert therapeutic effects through overlapping pathways. The comprehensive visualization of differential miRNA expression across all treatment groups (Figure 3) provides an overview of both distinct and shared regulatory patterns induced by the interventions. The medium-dose BDD group exhibited the most extensive miRNA modulation.

3.3. Analysis of differentially expressed MiRNA profiles in metformin and BDD Therapy

To further investigate the pharmacological mechanisms underlying metformin and BDD therapy, a comprehensive analysis and comparison of differentially expressed miRNA profiles in both treatment groups were performed. A Venn diagram constructed from the data revealed that 18 miRNAs were commonly regulated by both BDD and metformin treatments (Figure 4A). Among these, five miRNAs were upregulated, while the remaining 13 were downregulated. Notably, all 18 miRNAs showed significant changes in expression in PCOS rats compared with the control group, as depicted in the heatmap in Figure 4B.

Importantly, both BDD and metformin treatments were found to reverse the PCOS-induced alterations in miRNA expression, indicating a shared mechanism underlying their therapeutic effects. These 18 miRNAs therefore represent potential candidates for further investigation into the common pharmacological mechanisms of BDD and metformin in treating PCOS.

3.4. KEGG and GO analysis of target genes and related pathways of common MiRNAs

To explore the underlying mechanisms further, target genes of the common miRNAs were predicted using the MiRDB and MiRWalk databases. Subsequent GO enrichment and KEGG pathway analyses were conducted to determine the biological functions associated with these genes (Figure 5A and B).

KEGG pathway analysis revealed that the target genes of the five upregulated miRNAs were significantly enriched in autophagy-related pathways (Figure 5A). Furthermore, the phosphoinositide 3-kinase–protein kinase B signaling pathway, Forkhead box O signaling pathway, and AMPK signaling pathway—well-established regulators of autophagy—were highlighted in the enrichment analysis.[34-37] Conversely, the target genes of the 13 downregulated miRNAs were primarily involved in cancer- and metabolism-related pathways (Figure 5B).

To gain additional insight into the physiological roles of these common miRNAs, GO analysis of their target genes was performed. In the biological process category, numerous targets were associated with "biological regulation," "metabolic process," and "response to stimulus." In the cellular component category, predicted target genes were enriched in "membrane," "nucleus," and "protein-containing

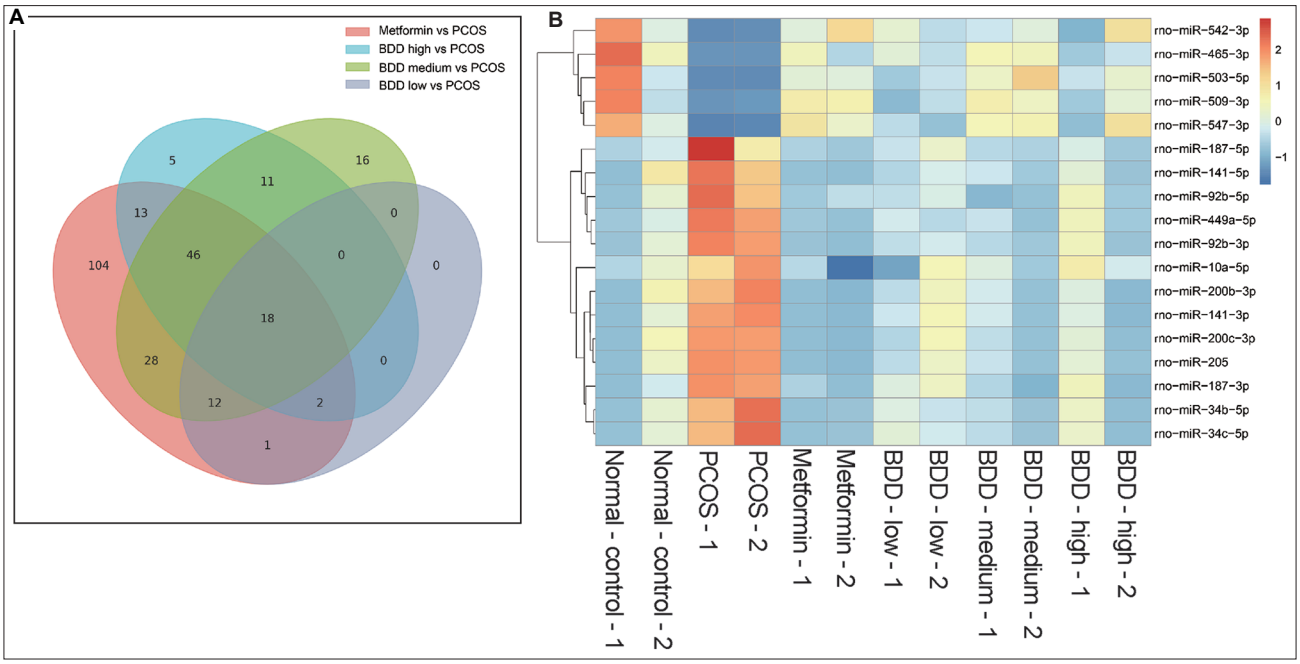

Figure 4. Differentially expressed microRNAs (miRNAs) in different treatment groups. Differentially expressed miRNAs in all treatment groups compared with the PCOS group are summarized in (A) a Venn diagram, and expression changes of 18 common miRNAs are shown in (B) a heatmap. Abbreviations: BDD: Bushen Ditan Decoction; PCOS: Polycystic ovary syndrome.

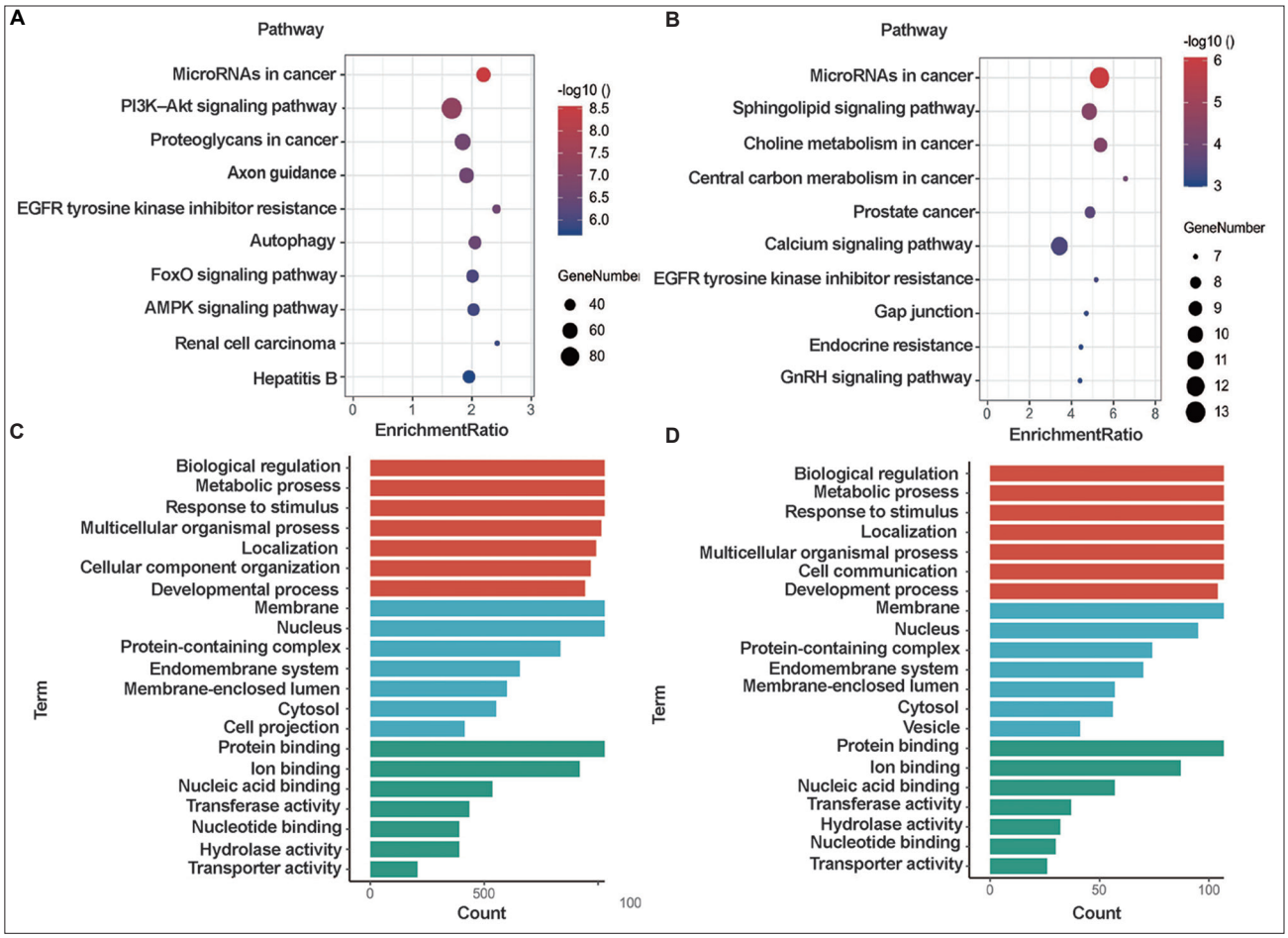

Figure 5. Kyoto encyclopedia of genes and genomes (KEGG) and gene ontology (GO) pathway analyses of common miRNA target genes. KEGG and GO pathway analyses of target genes of (A and C) five upregulated miRNAs and (B and D) 13 downregulated miRNAs.
Abbreviations: Akt: Protein kinase B; AMPK: AMP-activated protein kinase; EGFR: Epidermal growth factor receptor; FoxO: Forkhead box O; GnRH: Gonadotropin-releasing hormone; PI3K: Phosphoinositide 3-kinase.

complex." In the molecular function category, most genes were annotated under "protein binding," "ion binding," and "nucleic acid binding" (Figure 5C and D). These findings suggest that metformin and BDD treatments may alleviate inflammation and metabolic dysfunction associated with PCOS and may help reduce the cancer risk linked to PCOS, primarily through the regulation of metabolism- and autophagy-related pathways.

3.5. Cross-species conservation analysis of identified MiRNAs

To explore the functional conservation of the 18 identified miRNAs across different species, including mice, humans, rats, and macaques, a comprehensive sequence conservation analysis was conducted. Among the 18 miRNAs, 10 demonstrated substantial sequence conservation across all four species (Figure 6). These included eight downregulated miRNAs and two upregulated miRNAs. The alignment analysis indicates that these miRNAs may have similar functions in humans. Moreover, the results suggest that the pharmacological mechanisms underlying metformin and BDD treatment in PCOS patients may parallel those observed in rats, particularly regarding the regulation of autophagy- and metabolism-related pathways. Collectively, these findings provide added support for the potential clinical relevance of the identified miRNAs in PCOS treatment across multiple species.

4. Discussion

PCOS affects 6–21% of women of reproductive age worldwide, presenting a significant global health concern.[38] Although available clinical therapies provide partial relief, a comprehensive understanding of PCOS pathogenesis remains elusive, underscoring the need for more effective treatment strategies. In this study, we employed an animal model and miRNA sequencing analysis to investigate the pharmacological mechanisms of two clinically effective therapies—metformin from Western medicine and BDD from TCM—and identified autophagy-related pathways as a common mechanistic target.

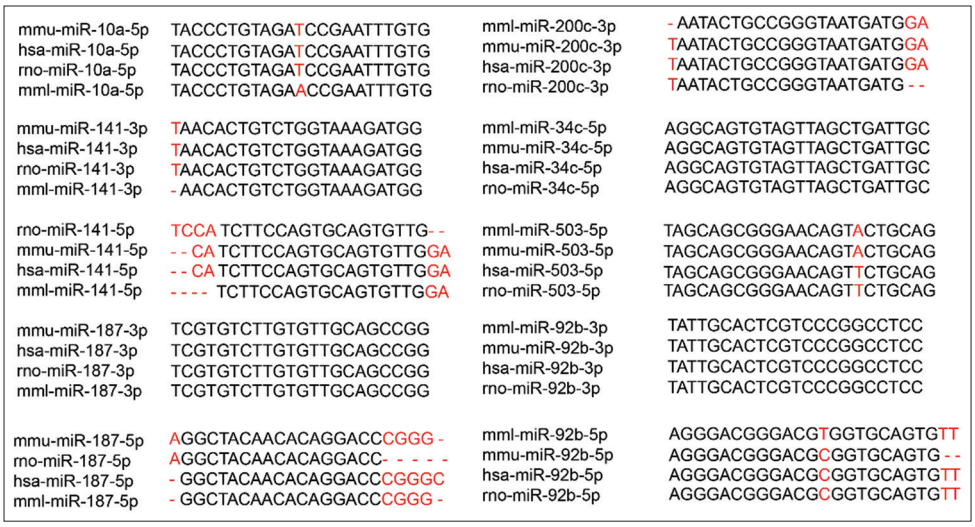

Figure 6. Sequence conservation analysis of selected microRNAs (miRNAs). Ten miRNAs were found to be highly conserved in mice (*Mus musculus* [mmu]), humans (*Homo sapiens*, [hsa]), rats (*Rattus norvegicus* [rno]), and macaques (*Macaca mulatta* [mml]).

In Western medicine, PCOS is characterized by hyperandrogenism, insulin resistance, and polycystic ovarian morphology. Common clinical treatments include drugs such as Diane-35 and metformin, which are designed to alleviate hyperandrogenism and insulin resistance.[23,39,40] In TCM, PCOS corresponds to conditions such as "metrorrhagia," "amenorrhea," and "infertility," and is attributed primarily to kidney deficiency and phlegm-dampness. BDD, derived from Guishao Dihuang Decoction and Ditan pill, has demonstrated promising clinical efficacy by tonifying the kidney and resolving phlegm, in accordance with TCM theory.[41-43]

BDD consists of *R. Radix Praeparata*, *A. Rhizoma*, *A. Sinensis Radix*, *S. Semen*, *C. Fructus*, *C. Semen*, *Poria*, and *G. Spina*. Among these herbal components, *C. Fructus* and *R. Radix Praeparata* have been reported to nourish the kidney, while *R. Radix Praeparata* and *A. Sinensis Radix* have been shown to regulate lipid metabolism, insulin resistance, and androgen levels.[44,45] Considering that PCOS is a multifactorial disorder involving dysregulation of metabolic, hormonal, and reproductive systems, our results suggest that combination therapies targeting multiple pathways simultaneously may represent a promising strategy for PCOS management. *R. Radix Praeparata* is a processed form of raw *Radix Rehmanniae*. It is sweet in taste, slightly "warm" in nature, and is believed in TCM to enter the liver and kidney meridians. According to TCM, it functions to tonify the blood, enrich "yin," strengthen essence, and "nourish the 'yin' of the five viscera." Therefore, it is an important herb for treating conditions related to blood deficiency, kidney "yin" deficiency, and depletion of essence and blood in the liver and kidney. Wang *et al*.[46] hypothesized that *R. Radix Praeparata* may regulate energy metabolism, peripheral blood production, and oxidative injury of hemocytes to exert its blood-tonifying effects, as suggested by metabolomic profiling. As both *R. Radix Praeparata* and *A. Sinensis* are components of BDD, this may partially explain the observed metabolic regulatory and insulin-sensitizing effects of BDD.

Our results suggest that both metformin and BDD may regulate lipid metabolism and autophagy through the modulation of miRNA expression, thereby contributing to the amelioration of PCOS-related metabolic and hormonal disturbances. Abnormalities in glucose and lipid metabolism are hallmark features of PCOS. Epidemiological studies indicate that the prevalence of insulin resistance, obesity, type II diabetes, and dyslipidemia in PCOS patients is significantly higher than in the general population.[8,47-49] Therefore, numerous treatment strategies for PCOS focus on improving metabolic dysfunction. Both metformin and BDD exhibited beneficial effects in ameliorating PCOS-associated metabolic disturbances, with substantial reductions in serum cholesterol and LDH. MiRNA sequencing of ovarian tissues identified 18 common miRNAs regulated by both treatments, and these differentially expressed miRNAs were enriched in autophagy- and metabolism-related pathways. Autophagy is a "self-engulfing" process by which cells degrade and recycle misfolded or aggregated proteins and damaged organelles to maintain intracellular homeostasis.[50] It is a tightly regulated process initiated by the formation of an isolation membrane called the phagophore. Dysregulated autophagy has been linked to various diseases such as neurodegenerative diseases,[51] cardiomyopathy,[52] infectious diseases,[53] type II diabetes,[54] fatty liver,[55] PCOS,[56] and cancer.[57] Autophagy plays a crucial role in maintaining ovarian homeostasis, influencing the ovarian life cycle and supporting reproductive health.[58] Nakashima *et al*.[59] demonstrated that autophagy, autophagic cell death, and autophagy-induced apoptosis are important for the ovarian life cycle. Thus, autophagy is essential for maintaining female ovarian homeostasis. Depending on

the specific ovarian cell types affected, either activation or inhibition of autophagy may improve PCOS symptoms.[56] Our findings suggest that both metformin and BDD may alleviate PCOS symptoms by regulating the autophagy-associated pathways in the ovary, indicating that autophagy should be considered when selecting targets for PCOS treatment.[60]

Analysis of miRNA expression patterns across the three BDD dosage groups revealed a dose–response, but nonlinear, relationship. In the low-dose BDD group (8 g/kg) 33 miRNAs were modulated, while in the medium- (16 g/kg) and high-dose (32 g/kg) groups, 131 and 95 miRNAs were involved, respectively. This nonlinear response suggests that lower doses may activate specific pathways, while higher doses impact a broader spectrum of regulatory network. Notably, the high-dose BDD group shared the greatest number of commonly regulated miRNAs with the metformin group (15 miRNAs), indicating potential mechanistic convergence at therapeutic doses. The distinct clusters of miRNA expression presented in Figure 3 further support the notion that BDD exerts multi-target regulatory effects on ovarian function in PCOS.

The 10 highly conserved miRNAs identified across species strengthen the potential clinical relevance of our findings. The conservation of these regulatory elements suggests that disruption of fundamental biological processes in PCOS may be a shared event across mammalian species. This evolutionary conservation enhances the value of our findings at the translational level and supports further investigation of these miRNAs as potential biomarkers for the diagnosis of PCOS or monitoring of treatment response in clinical settings.

While our study provides valuable insights, several limitations should be acknowledged. The PCOS rat model, although well established, cannot fully recapitulate the complexity of human PCOS, particularly the psychological and long-term metabolic consequences. In addition, the miRNA sequencing was performed on whole ovarian tissue, which may mask cell-type-specific expression patterns. Future studies should employ single-cell sequencing approaches to delineate miRNA expression in distinct ovarian cell populations. Furthermore, functional validation of the identified miRNAs through gain- and loss-of-function experiments in relevant cells and animal models is necessary to establish causal relationships. Translation of these findings to human PCOS will require validation in clinical samples and confirmation of the correlation between patient phenotypes and treatment responses.

5. Conclusion

Our comprehensive analysis of miRNA sequence data indicates that both metformin and BDD hold promise in ameliorating the PCOS phenotype, potentially through the modulation of metabolic and autophagy-related pathways. Specifically, the common miRNAs regulated by both treatments were enriched in pathways such as autophagy and metabolism, which are crucial for maintaining ovarian homeostasis and metabolic balance. Moreover, our findings highlight a potential link between PCOS pathogenesis and hormonal imbalances driven by disruptions in ovarian autophagy. These disruptions may significantly impact women's metabolic homeostasis, ultimately contributing to the development of insulin resistance. This investigation not only deepens our understanding of PCOS pathophysiology but also underscores the therapeutic potential of interventions targeting metabolic and autophagy pathways for managing this complex gynecological endocrine disorder. Further studies are warranted to elucidate the precise molecular mechanisms underpinning these effects, particularly the causal relationships between specific miRNA alterations and phenotypic improvements. Clinical validation in human subjects will be essential to translating these insights into more targeted and effective therapeutic strategies for PCOS. The conserved miRNAs identified in this study represent promising candidates for further development as diagnostic biomarkers or therapeutic targets. Combined with the growing understanding of autophagy regulation in reproductive health, these findings open new avenues for developing integrated treatment approaches that address both metabolic and reproductive aspects of PCOS.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Conceptualization: Dan Weng, Wei Lu
Formal analysis: Jiayi Xu, Qianchao Shao
Investigation: Jiayi Xu, Qianchao Shao
Methodology: Qianchao Shao, Hanqing Zhang
Writing–original draft: Qianchao Shao, Hanqing Zhang
Writing–review & editing: Jiayi Xu, Dan Weng, Wei Lu

Ethics approval and consent to participate

All animal experiments conducted in this study were approved by the Ethics Committee of Nanjing University of Science and Technology (approval no. 20210228054).

Consent for publication

Not applicable.

Data availability statement

Data are available from the corresponding author upon reasonable request.

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