Bushe-Ditan Decoction, a Chinese medicine formula, attenuates polycystic ovarian syndrome in rat and promotes granulosa cell apoptosis

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Abstract: Polycystic ovarian syndrome (POCS) is a common endocrinopathy in women of reproductive age. Clinical use of Bushen-Ditan Decoction (BDD), a Chinese formula, has achieved favorable effects. The aim of study was to investigate the effect and molecular mechanism of BDD in PCOS rats. SD rats were intervened with estradiol valerate to induce POCS. Successful models rats were randomly divided into 3 groups and treated with BDD, metformin (as western medicine control), and PBS (as negative control). Before and at the end of observation period, blood samples were collected and tested for testosterone (T), estradiol (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH), fasting blood glucose (FBG), and fasting insulin (FINS). Primary granulose cells were isolated and treated with pharmacological sera. Cellular proliferation and apoptosis were measured by MTT and flow cytometry method, respectively. Activation of Akt signaling pathway was detected by Western blot. The results showed that BDD treatment reduced serum androgen levels and ameliorated homeostasis model assessment of insulin resistance (HOMA-IR). Culture with pharmacological sera inhibited proliferation and promoted...
apoptosis of granulosa cells. The pro-apoptosis role was associated with suppression of insulin-like growth factor (IGF)-1/Akt signaling pathway. In conclusion, BDD can effectively ameliorate the symptoms of PCOS rats. The molecular mechanism can be explained by inhibition of Akt signaling and regulation of cellular proliferation and apoptosis.

**Keywords**: Bushen-Ditan Decoction, polycystic ovarian syndrome, granulosa cells, apoptosis, insulin resistance, Akt
Introduction

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder in women of reproductive age. The prevalence of PCOS is about 7% in general population and 25%-30% infertile women suffer from PCOS (1). The etiology of PCOS is not fully clear. The disease is mainly manifested in amenorrhea, dilute ovulation, acne, hirsutism, and obesity caused by hyperandrogenism and insulin resistance (2). For women in period of childbearing age, the patients are troubled by infertility (3). As age increases, the risk of endometrial cancer, diabetes, and cardiovascular diseases increase in PCOS women (4). Early intervention for patients with puberty PCOS to relieve the clinical symptoms of PCOS after puberty is a serious issue that clinical gynecologists need to pay attention to.

For the treatment of PCOS, most of current therapies aim to regulate menstruation, promote ovulation, and increase pregnancy rate. Oral contraceptive pills are used to adjust menstrual cycle; cyproterone acetate/ethinyl estradiol tablets are used to reduce androgen levels; metformin is used to improve insulin sensitivity; clomiphene or letrozole are used to promote ovulation in patients with fertility requirements (5). These Western medications have limited clinical effects and are accompanied by many side effects. The theory of traditional Chinese medicine holds that PCOS is oriented in kidney deficiency and phlegm dampness is the main syndrome. Replenishing kidney-essence and removing phlegm is a common treatment strategy for PCOS. In our hospital, the prescription of Bushen-Ditan Decoction (BDD) has been used to treat PCOS for decades and has achieved equivalent curative effect as metformin (6). The aim of this study was to further investigate the therapeutic effect of BDD on PCOS rat and to reveal its molecular mechanism.

Materials and methods

Bushen-Ditan Decoction formula

Bushen-Ditan Decoction was provided by Jiangsu Province Hospital of
Traditional Chinese Medicine, which consists of *rehmanniae radix praeparata* (shudihuang, 12.5%), *radix angelicae sinensis* (danggui, 12.5%), pulp of *cornus officinalis* (shanyurou, 9.375%), *rhizome pinellinae praeparata* (fabanxia, 9.375%), *semen brassicae* (baijiezi, 9.375%), *rhizome atracylodis* (cangzhu, 12.5%), *rhizome cyperi* (xiangfu, 9.375%), and *poria cocos* (fuling, 12.5%). The dosage of BDD used in rats (9.6 g/kg body weight) was an equivalent to that for human (96 g/60 kg body weight).

**Animals and treatments**

Female Sprague-Dawley (SD) rats (8 weeks-old, weighing 220 g ± 20 g) were obtained from Shanghai SLAC experimental animal Co., Ltd (Shanghai, China, certificate number 2007000542991). The animal experiment protocols were approved by the Ethics Committee Board of Jiangsu Province Hospital of Traditional Chinese Medicine. The rats were randomly divided into 3 groups: 6 for negative control, 6 for sham group, and 30 for PCOS group. The rats of PCOS group received a single injection of 5 mg estradiol valerate dissolved in 0.4 ml sesame oil; the rats of sham group received intramuscular injection of 0.4 ml sesame oil only (7). After 30 days, the estrous cycle was determined daily by methylene blue staining of vaginal smears as described before (8). The smear primarily consisting of anucleated cornified cells for 10 days is considered as successful PCOS induction. The rats of model group were further randomly divided into 3 groups: BDD group received BDD by gavage (9.6 g/kg body weight), metformin group orally treated with metformin (100 mg/kg body weight), and control group subjected to 2 ml PBS by gavage. Metformin hydrochloride was manufactured by Peili Pharmaceutical Co., Ltd (Kunshan, China). The treatment was administered twice a day at a dose and lasted for 1 month.

**Detection of serum biochemical indicators**

Before and after the treatment, blood samples were collected to test for biochemical indicators including testosterone (T), estradiol (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH), fasting blood glucose (FBG), and fasting insulin (FINS). The tests were performed by appropriate ELISA kits for rat
(ThermoFisher Scientific, Carlsbad, USA) according to the manufacturer’s instruction. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as \( \text{FBG (μmol/ml)} \times \text{FINS (μIU/ml)} / 22.5 \).

**Preparation of pharmacological sera**

Thirty female SD rats were randomly divided into 3 groups: BDD group received BDD by gavage (9.6 g/kg body weight), metformin group received metformin (100 mg/kg body weight), and negative control (NC) group received 2 ml PBS. Two hours after the fourth day gavage, the rats were sacrificed. Blood samples were collected and centrifuged at low speed for 15 minutes to separate serum. Sera samples were inactivated at 56°C for 30 minutes and stored at -80°C before use.

**Isolation of primary rat granulosa cells**

Female SD rats were intraperitoneally injected with pregnant mare serum gonadotropin (0.4 IU/g) and sacrificed after 48 hours. The ovaries were excised, washed with pre-cold PBS, and removed off the surrounding tissue and surface membrane. Ovaries were placed in DMEM/F12 medium (ThermoFisher) and punctured with 26 gauge needles to release granulosa cells. Cell suspension was incubated with 2.5% trypsin and pipetted up and down to make single cell suspension. Cells were seeded in 3.5 cm petri dish and cultured with DMEM/F12 supplemented with 15% fetal bovine serum (FBS, ThermoFisher). The incubation conditions were at 37°C with 5% CO₂. After cells adhere, identification analysis was performed.

**Immunohistochemistry analysis**

Isolated granulosa cells were detected for FSH receptor (FSHR), which is specifically expressed on granulosa cells, by immunohistochemistry analysis. Cells were seeded in 24-well plates (1×10^5 cells/well) to prepare cell slides. After cells grew to 80% confluence, the slides were taken out, washed with PBS, and fixed with 4% paraformaldehyde. The immunohistochemical analysis was performed by using the SABC-AP (rabbit IgG) kit (Boster Biological Technology, Wuhan, China) as instructed. The slides were incubated with primary rabbit anti-FSHR antibody (1:1000, Boster) at 4°Covernight. The next day, the slides were incubated with goat
anti-rabbit IgG-biotin (1:1000, offered by the kit) at 37°C for 30 minutes. The slides were visualized by DAB method and observed under light microscope (Olympus BX51, Tokyo, Japan).

**Measurement of cellular proliferation and apoptosis**

Cells were seeded in 96-well plates (1×10^4 cells/well) and divided into 4 groups: BDD group cultured with DMEM/F12+15% BDD serum, metformin group cultured with DMEM/F12+15% metformin serum, negative control group cultured with DMEM/F12+15% NC serum. For measurement of cellular proliferation, the number of viable cells was detected by cell counting kit-8 (Boster) for 5 consecutive days. For measurement of cellular apoptosis, the cells were harvested after 5 days, washed with cold PBS, and stained with Annexin V-PE offered by apoptosis detection kit (Beyotime Biotechnology, Shanghai, China). The apoptotic signals were detected by flow cytometer (Beckman-Coutler Inc., Brea, USA).

**Western blot**

Granulosa cells were seeded in 24-well plates (1×10^5 cells/well) and treated with different pharmacological sera as described above. After culture for 4 days, cells were harvested, washed with cold PBS, and lysed with RIPA lysis buffer (Beyotime) on ice for 1 hour. Lysates were centrifuged and supernatants with total soluble proteins were used for Western blot. Thirty μg of total proteins were boiled for 5 minutes and loaded on 12% sodium dodecyl sulfate-polyacrylamide gel. Protein bands were separated by electrophoresis and transblotted onto PVDF membranes. After blocking with 5% skim milk at room temperature for 2 hours, membranes were probed at 4°C overnight with specific primary antibodies against IGF-1R (1:1000), p-Akt (ser473, 1:1000), Bcl-2 (1:1000), Bax (1:1000), and β-actin (1:1000, internal control). Next day, membranes were probed with mouse anti-rabbit IgG (horseradish peroxidase-conjugated, 1:2000) at room temperature for 2 hours. Probed bands were visualized by enhanced chemiluminescence method. β-actin was loaded as control. The antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

**Statistical analysis**
Data obtained were analyzed by SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean ± standard deviation. Comparisons were analyzed by t-test or one-way ANOVA followed by Turkey test. P value <0.05 was considered to be statistically significant.

Results

BDD alleviates androgen release and insulin resistance in PCOS rats

First we tested the treatment effect of BDD on PCOS rats. The estrous cycle was prolonged from 4-5 days to 8-9 days by estradiol valerate exposure. Meanwhile, endocrinosity was also changed in estradiol valerate-exposed rats. As shown in Table 1, serum levels of T, LH and FSH were significantly increased while E2 was reduced. HOMA-IR index suggested that insulin resistance was increased. These features indicated that estradiol valerate could induce typical PCOS symptoms in rats. Administration of BDD ameliorated PCOS symptoms in rats as effectively as metformin did. The levels of T, LH, FSH, FBG, and FINS all decreased significantly when compared with the non-treated PCOS rats (Table 1). The levels of E2, LH, FSH, and FINS in the BDD-treated rats recovered to the same levels in normal control rats, although levels of T, FBG, and HOMA-IR were still higher compared with the controls. Nevertheless, BDD treatment achieved favorable therapeutic effects in PCOS rats.

BDD inhibits proliferation and promotes apoptosis of rat granulosa cells

Abnormal growth of follicles, which is associated with dysregulation of apoptosis in granulosa cells, is believed to be a pathological basis of ovulation and endocrine changes in PCOS. To further investigate the regulation mechanism of BDD, we tested its effect on proliferation and apoptosis of granulosa cells. Granulosa cells were isolated and identified by FSHR expression. Under light microscope, granulosa cells appeared irregular or spindle shape and FSHR positive cells showed dark brown in cytoplasm (Figure 1). The FSHR positive rate was > 90%, indicating the purity of separated granulosa cells reached 90%.
Table 1. Treatment effect of Bushen-Ditan Decoction on PCOS rats (X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>T (ng/ml)</th>
<th>E2 (pg/ml)</th>
<th>LH (mIU/ml)</th>
<th>FSH (mIU/ml)</th>
<th>FBG (μmol/ml)</th>
<th>FINS (μIU/ml)</th>
<th>HOMA-IR</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.29 ± 0.18</td>
<td>51.48 ± 3.21</td>
<td>4.77 ± 0.39</td>
<td>4.70 ± 0.24</td>
<td>4.11 ± 0.28</td>
<td>4.52 ± 0.71</td>
<td>0.83 ± 0.12</td>
</tr>
<tr>
<td>Sham</td>
<td>1.35 ± 0.24</td>
<td>55.33 ± 3.45</td>
<td>4.69 ± 0.38</td>
<td>4.80 ± 0.27</td>
<td>4.26 ± 0.23</td>
<td>4.75 ± 0.75</td>
<td>0.90 ± 0.15</td>
</tr>
<tr>
<td>PCOS</td>
<td>4.08 ± 0.63*</td>
<td>26.41 ± 1.39*</td>
<td>7.02 ± 0.48*</td>
<td>5.78 ± 0.30*</td>
<td>6.65 ± 0.36*</td>
<td>10.25 ± 0.87*</td>
<td>3.03 ± 0.35*</td>
</tr>
<tr>
<td>PCOS+BDD</td>
<td>2.53 ± 0.32*#</td>
<td>46.97 ± 2.84*#</td>
<td>4.95 ± 0.31*#</td>
<td>4.93 ± 0.21*#</td>
<td>5.20 ± 0.28*#</td>
<td>5.72 ± 0.79*#</td>
<td>1.32 ± 0.34*#</td>
</tr>
<tr>
<td>PCOS+metformin</td>
<td>1.41 ± 0.25*#</td>
<td>45.68 ± 2.79*#</td>
<td>4.86 ± 0.26*#</td>
<td>4.86 ± 0.18*#</td>
<td>4.78 ± 0.31*#</td>
<td>5.12 ± 0.68*#</td>
<td>1.09 ± 0.29*#</td>
</tr>
</tbody>
</table>

Symbols: *, P< 0.05 vs. control group; #, P<0.05 vs. PCOS group.
Abbreviations: testosterone (T), estradiol (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH), fasting blood glucose (FBG), fasting insulin (FINS), homeostasis model assessment of insulin resistance (HOMA-IR), polycystic ovarian syndrome (PCOS), Bushen-Ditan Decoction (BDD).
We then investigate the effect of BDD on proliferation and apoptosis on granulosa cells. As shown in Figure 2, both BDD pharmacological sera and metformin pharmacological sera inhibited proliferation of granulosa cells compared with the control. Meanwhile, cellular apoptosis was significantly promoted by BDD pharmacological sera or metformin pharmacological sera (Figure 3). The apoptosis rate raised from 8.87% ± 0.59% in the control group to 10.86% ± 1.01% in the BDD group and 10.65% ± 0.86% in the metformin group. The results indicated that BDD can regulate proliferation and apoptosis of granulosa cells.
IGF-1/Akt pathway is involved in the alleviative effect of BDD on PCOS rats

Insulin regulates metabolic processes via several intracellular signaling pathways, among which the insulin-like growth factor-1 (IGF-1) signaling pathway is a highly conserved regulatory module and has been implicated in PCOS (9). Next, we investigated the whether BDD could affect the activity of IGF-1 pathway. As shown in Figure 4, expression of IGF-1 receptor (IGF-1R) was suppressed in granulosa cells cultured with BDD pharmacological sera compared with the control. Sequentially, phosphorylation of Akt was also inhibited in the BDD group. Bcl-2 and Bax are downstream executors of IGF-1 pathway. Western blot analysis showed that proliferation-associated factor Bcl-2 was down-regulated by BDD pharmacological sera or metformin sera. However, apoptosis-associated factor Bax only significantly decreased in the metformin group; while Bax expression was not changed in the BDD
group. In summary, BDD treatment changed activities of IGF-1/Akt pathway and further influenced growth of granulosa cells.

![Image of Western blot results]

**Figure 4. Effect of Bushen-Ditan Decoction (BDD) on activities of insulin-like growth factor-1 (IGF-1)/Akt pathway**

Granulosa cells were treated with BDD pharmacological sera or metformin pharmacological sera. Expression of IGF-1 receptor (IGF-1R), phosphorylated Akt (p-Akt), Bcl-2, and Bax were detected by Western blot. β-actin was used as internal control.

**Discussion**

PCOS is an endocrine and metabolic disorder affected by the interaction of environmental and genetic factors. The pathogenesis of PCOS is complex and the pathophysiological characteristics of individual patients are quite different, mainly characterized by insulin resistance, hyperinsulinemia, hyperandrogenemia, and follicular development disorder. The present study demonstrated that BDD, a Chinese traditional medicine formula, could attenuate symptoms of PCOS rats by reducing androgen secretion and insulin resistance. The ameliorate effect could be explained by the inhibition of cellular growth and promotion of apoptosis in granulosa cells.

Abnormal development of follicles is a typical symptom characterized by dilute ovulation or anovulation. During the stages of follicle development, atresia is initiated by oocyte apoptosis followed by death of the surrounding granulosa cells; while atresia of maturing follicles is demarcated by granulosa cell apoptosis (10). There was no significant difference in the number of primordial follicles in PCOS women compared with normal healthy women; however, there was significant difference in the rate of cell death and proliferation (11). Obesity or high insulin levels can promote
proliferation of granulosa cells in primary follicles and fail to develop atresia in time, causing polycystic changes in ovary (12). Contraceptive RU486 could inhibit proliferation and promote apoptosis of human KGN granulosa cells by down-regulating progesterone receptor membrane component 1 to restore ovarian functions (13). Heqi San, also a traditional Chinese medicine, has beneficial effects on PCOS via PI3K/Akt pathway (14). Metformin treatment reduces androgen levels, improves insulin sensitivity, and corrects reproductive cycle through PI3K/Akt network. Similar results were also observed in our study. These studies indicated that regulating follicle growth is a promising treatment strategy for PCOS.

Akt pathway and Bcl-2 family members are commonly recognized as regulators of apoptosis and mediate abnormalities in the growth of granulosa cells in PCOS (1,15). Our study showed that BDD could reduce androgen release via inhibiting Akt pathway. There is a reciprocal feedback between Akt and androgen during proliferation and folliculogenesis of granulosa-lutein cells (16). Therefore, BDD treatment could help to build a positive feedback and continuously ameliorating PCOS symptoms. In our research, BDD pharmacological sera could influence expression of Bcl-2 while Bax expression was not changes. Bas et al. reported that Bax expression level was not different between PCOS or normal rats (15). Therefore, balance of Bax/Bcl-2 ratio may be more important than expression of single protein in PCOS.

In conclusion, the present study investigated the ameliorated effect of BDD on LPS-induced polycystic ovary and follicle development abnormalities. The inhibition of androgen and IGF-1/Akt pathway contributed to the therapeutic effect of BDD. These findings may provide new insights into the mechanism and treatment of PCOS.

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References


