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Original Article

Network pharmacology and transcriptomics analysis reveal the mechanism of BushenHuoxue formula attenuates premature ovarian failure via modulation PI3K/AKT pathway

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Abstract

This study aims to analyze the active components and mechanism of Bushen Huoxue (BSHX) formula on the autoimmune premature ovarian insufficiency (POI) by combining network pharmacology and Transcriptomics. The active components and targets of BSHXF were screened through Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP). POI-related targets were identified through Therapeutic Targets Database (TTD), DisGeNET and drugbank database. The Veen diagram was performed to obtain the action targets. The active compound-target network and Protein-Protein Interaction (PPI) network were built by using STRING database and Cytoscape software. Key targets and active compounds were further identified by topological analysis. Molecular docking shows that Kaempferol, Isorhamnetin and Anhydroicaritin have strong binding to AKT. Finally, a zp3-induced autoimmune ovarian function deficiency mouse model was used to explore the potential mechanism of POI. The potential pathways of BSHXF for the treatment of POI were identified by Transcriptomic analysis. PI3K-AKT and NF-kb pathways were the common pathways between network pharmacology and transcriptomics. Our results revealed that BSHXF could reduce the FSH expression levels and raise the E2, and AMH levels in the serum. Western bloting demonstrates that BSHXF could upregulate the expression of p-PI3K and p-AKT.

Keywords: BushenHuoxue formula, Autoimmune premature ovarian insufficiency, Network pharmacology, Transcriptomics, PI3K-AKT pathway

1. Introduction

Premature ovarian insufficiency (POI), which is also known as premature ovarian failure(POF), is a reversible syndrome that occurs under the age of 40 [1]. POI is a pathological condition of low serum estrogen levels that will increase the risk of osteoporosis, cardiovascular disease, dementia, cognitive decline, and Parkinsonism [2]. POI has a global incidence of 1-3%, 25%-30% of the causes of POI cases are identified while the causes of 50%-90% of POI cases are idiopathic [3]. Notably, autoimmunity accounts for approximately 4-30%, and immunosuppressive therapy fails to reverse the ovarian autoimmune process or enhance the responsiveness to gonadotropins [4]. The apoptosis of granulosa cells was thought to be the initiator of follicular atresia, which was demonstrated by the experiment [5]. The proliferation and differentiation of Granulosa cells were guided by oocytes, while the granulosa cells provided the corresponding key signals for the oocytes maturation [6]. In most cases, no effective therapeutic means is able to recover the POI patient fertility, and the life quality of women and reproduction face challenges seriously [7].

Hormone replacement therapy (HRT) could significantly relieve the symptom scores of hot flushes, vaginal dryness and sweating episodes [8]. However, the dose of HRT tends to be higher in the treatment of POI than that in the older age group which may increase the clotting cell activation clinically [9-11]. Meanwhile, an epidemiological study showed that the occurrence of breast cancer was positively correlated with HRT [12]. Moreover, a hospital-based multicenter study with propensity found that the risk of meningioma increased after hormone replacement therapy in Chinese women recently [13]. Tonofying Kidney (Bushen) and Activating Blood (Huoxue) formula (BSHXF) is a traditional Chinese herbal compound that has been applied in the clinical for 20 years. The function

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of tonifying the kidney, invigorating the blood and regulating the Chong-Ren axis are mainly the advantages of BSHXF for the POI. According to a meta-analysis study, the Chinese herbal medicine Tonofying Kidney (Bushen) and Activating Blood (Huoxue) formula could improve the hormone levels of POI patients and have lower related adverse events compared to hormone replacement therapy [14]. Previous studies indicated that BSHXF could protect the function of ovarian in ZP3-induced POI mouse model [15]. However, the mechanism of BSHX formula in the treatment of autoimmune POI is unclear. It is critical to uncover active ingredients of the formulae and identify the related target genes.

Network pharmacology connects compound prescriptions and diseases through "disease-phenotype-gene-drugs" and forms a multi-target and multi-component treatment model [16]. Transcriptomics may offer multiple genes which could shed light on the mechanism of traditional Chinese medicine [17]. In the present study, we selected the pathways shared by network pharmacology and transcriptomics to explore the mechanism of BSHXF in the treatment of immune POI mice models.

2. Materials and methods

2.1. Preparation of Chinese medicine

The total herbs were purchased from Jiangsu Province Hospital on integration of Chinese and Western Medicine (listed in Supplementary Table 1). All drugs were decocted in water (10 g/80 ml), and the supernatant was vortexed and mixed. The supernatant was taken after high-speed (12000 r/min, 4°C) centrifugation for 10 min and filtered by 0.22 um filter membrane. The dosage for the mice was calculated based on the surface area of a 60 kg human body. Low-dose: medium-dose: high-dose, according to 1:2:4.

2.2. Animal

20 female C57BL/6 mice and 10 male A/J mice (aged 6-8 weeks; weight 17.0-20.0 g) were purchased from Academy of Military Medical Sciences, China [certification NO. SCXK (JUN) 2007-004]. The total of mice were fed in the laboratory Animal Research Center of the Jiangsu Research Institute of Traditional Chinese Medicine [Nanjing, China; certification no. SYXK (SU) 2016-0018]. The animals were housed in a controlled environment (temperature of 23±2°C, relative humidity 42±5%, and 12:12 h light-dark natural cycle) and had adlib access to drinking water and food. Mice were allowed to be acclimated to the laboratory environment at least one week before treatment. The first generation (B6AF1) of hybrid mice was obtained by mating C57BL/6 and A/J mice. B6AF1 mice were studied at 6-8 weeks of age. The present study was ethically approved by the animal care ethics committee of Jiangsu Province Academy of Traditional Chinese Medicine (Nanjing, China).

2.3. Pharmacology network construction

The total chemical ingredients of BSHXF were screened in the Traditional Chinese Medicines for Systems Pharmacology Database and Analysis Platform(TCMSP) [18] and related literature. The thresholds of active ingredients screening by the following criteria: Oral bioavailability (OB) ≥ 30%, Drug likeness ≥ 0.18. Among these factors, OB represents the percentage of unmodified drugs that enter the circulatory system after oral administration [19]. DL refers to “Drug-like” which assesses the ability of a compound to modulate its target [20].

The targets of these activated ingredients were obtained from the databases, including the OMIM database (*http://omim.org/)*, GeenCards database *(https://www.genecards.org/)* and DisGeNET database *(https://www.disgenet.org/web/DisGeNET/menu/home)*. Then the intersection genes were selected by the drug targets and disease targets. The Cytoscape 3.7.0 cluster cytoNCA and Bisogenet were used to analyze the hub genes. The hub genes were identified by the criteria: degree >61, betweenness > 6000. The SDF format of compound was downloaded from the PubChem database and then converted into the mol2 format file. The protein structures were downloaded from the RCSB PDB network. The hub genes GO and KEGG were analysised by the clusterprofile package.

The crystal structure of AKT (PDB, id: 2vuk) was obtained from Protein Data Bank (www.rcsb.org), one hub gene in the PPI network. Then the chemical structure of 52 BSHXF active ingredients was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) (listed in Supplementary Table 2). Then the 3D structure of the 52 active compounds were drawn by Chem3D 19.0 software. The MM2 algorithm is used for energy minimisation and the minimum RMS convergence parameter is set to 0.01. Then the total compounds of BSHXF were saved as “mol2” files. The target protein receptor molecule is hydrogenated and charge calculated by SYBYL X-2.0, and the ligand and target protein receptor are converted into a "pdbqt" parameter file and the appropriate box centre and box point parameters are set. Dehydration and removal of organic matter, hydrogenation and charge calculation of the target protein receptor molecules were carried out using SYBYL X-2.0 software.

2.4. Ovarain follicle count and apoptosis analysis

Ovaries were fixed with 4% formaldehyde solution at room temperature. Dehydrated in gradient ethanol and embedded in paraffin. Serial paraffin-embedded ovarian tissue sections (5 um) were prepared for HE staining and immunohistochemistry respectively. De-paraffinization by gradient xylene and ethanol solution method, hematoxylin staining for 2 min and then rinsed in distilled water. Then the sections were stained with eosin staining for 3 min.

IHC: Tissue sections for antigen repair, EDTA (pH 8.0) soaked at 56 degrees for 15min, naturally cooled and shaken 3 times for 5min each in PBS (pH 7.4). The primary antibody was incubated at 4°C overnight and the secondary antibody was incubated at room temperature for 50 min, followed by PBS washes 3 times. According to the manufacturer instructions, the slides were stained by DAB kit (Jiangsu Shitai Experimental Equipment Co, China). The primary antibodies used for IHC as follows: Caspase3 (221248, 1:200), BAX (14796, 1:500), and BEL-2 (196495, 1:500). The slides were then scanned and photographed using ortho-mounted fluorescent microscope imaging system (Nikon Japan). IHC staining was scored by two independent pathologists at the same time.Caspase3, BAX and Bcl-2 were detected in the cytoplasm. A semiquantitative scoring criterion was adopted to record the positive areas and the staining intensity of 3 proteins. The product of the intensity of positive staining (weak, 1; moderate, 2; strong, 3) and the proportion of positive cells

of interest(0%, 0; <25%, 1; 26-50%, 2; 51-75%, 3; >76%, 4) constitutes the staining index (values 0-12).

2.5. ELISA assay of sex hormone levels

Steroid hormone detection by ELISA. At room temperature, blood was clotted for 2 hours. Centrifuge for 20 minutes (3000 rpm/minutes) and collect the supernatant lipid. FSH, LH, E2 and AMH assays were performed according to the instructions of the enzyme-linked bioreagent kit respectively. The OD value was detected by 450nm wavelength light (American BioTek Enzyme Labeler). All ELISA kits were purchased from the mobio company: ml001910, ml063366, ml063198 and ml037597 respectively.

2.6. Transcriptome sequencing and analysis

Total RNA was isolated and purified using TRIzol reagent (Invitrogen, USA) following the manufacturer's procedure. The RNA amount and purity of each sample were quantified using NanoDrop 1000 (NanoDrop, USA). The cleaved RNA fragments were reverse-transcribed to create the cDNA by SuperScript II Reverse Transcriptase (Invitrogen, USA), which was next used to synthesise U-labeled second-stranded DNAs with E. coli DNA polymerase I (NEB, USA), RNase H (NEB, USA) and dUTP Solution (Thermo Fisher, USA). Meanwhile, an A-base is added to the blunt ends of each strand, preparing them for ligation to the indexed adapters. After the heat-labile UDG enzyme (NEB, USA) treatment of the U-labeled second-stranded DNAs, the ligated products are amplified with PCR by the following conditions: initial denaturation at 95°C for 3 min; 8 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 15 sec, and extension at 72°C for 30 sec; and then final extension at 72°C for 5 min. At last, we performed the 2×150 bp paired-end sequencing (PE150) on an Illumina Novaseq 6000.

Different expression (DE) genes analysis was performed with R package edgeR negative binomial distribution [21]. The DE mRNAs were screened based on the criteria: |fold change|> 1.2 and *P*-value < 0.05 between BSHXF vs Model and Model vs Control groups separately. Then the overlapping genes were visualized by the Veen diagrams in the Lianchuan platporm. The total of genes was used for the GO and KEGG pathway analysis in the LianChuan Biowebsite platform (https://www.omicstudio.cn/tool).

2.7. Western Blotting

The total protein of ovary tissue was extracted from RIPA lysate (R0010) containing 1% protease inhibitor (HY-K 0010,1:100) and 1% phosphorylated protease inhibitor (HY-K0020, 1:100). The tissue lysate was clarified by centrifugation at 12000 rpm for 10 min at 4°C, and then collected the supernatant. Collected proteins were quantified by a BCA protein assay. The concentration of protein samples was diluted to 5 ug/ml. All protein samples were stored at -80°C for vertical electrophoresis. For 20-110 kDa proteins,30 ug protein(about 6 ul) were loaded on 10% polyacrylamide SDS gel, and vertical electrophoresis was run at 80-V for 1.5 h at room temperature (Bio-Rad Laboratories). Under ice bath conditions, proteins were transferred to polyvinylidene fluoride membranes (PVDF) at 230 mA for 2 h. Then PVDF membranes were blocked with 5% BSA for 1 h.

All primary antibodies were incubated at 4°C for 12 h followed by secondary antibodies for 2.5 h at room temperature. The membranes were washed 3 times and visualized by ECL luminescent. The concentration of Primary anti-protein antibodies were listed: p-PI3K (AF3242, 1:1000), PI3K (4257, 1:1000), p-AKT (CST, 1:1000), AKT (4685, 1:1000), β-action (200068-8F10, 1:10000). The secondary antibody were list: Goat against rabbits (511203, 1:3000).

2.8. Statistical analysis

Three biological replicates were required for each experiment, $P<0.05$ was considered statistically different, and $P<0.001$ was considered significantly different. Statistical comparison between groups was determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test. All data are presented as the mean ± SEM with GraphPad PRISM 8.0 (GraphPad, LA Jolla, CA, USA).

3. Results

3.1. BSHXF regulates sex hormone levels in a mouse model of immune ovarian premature failure

E2, FSH, LH, and AMH were the diagnostic markers according to the ESHRE Guideline: management of women with premature ovarian insufficiency [22]. According to the ESHRE Guideline, amenorrhea or oligomenorrhea with high FSH and low E_2 are the main features of POI. In the present study, the total serum concentration of FSH was higher in the model group than in the control group. FSH was lower significantly in the BSHXF-H, BSHXF-M and Estrogen groups (Figure 1F). Nevertheless, the serum concentration of LH has no statistically significant difference (Figure 1E). The serum concentrations of E_2 and AMH were lower in the model group than in the control group. The concentration of E_2 was elevated in the BSHXF-H group (Figure 1D). The serum concentration of AMH was significantly elevated in the BSHXF-H, BSHXF-M and Estrogen group (Figure 1G). Besides, the body weight loss was reduced after BSHXF-H and BSHXF-M compared to the control and BSHXF-L groups (Figure 1B). Meanwhile, mouse ovarian index was higher in the BSHXF-H and BSHXF-M groups than in the model group (Figure 1C). Thus, high-dose and middle-dose BSHXF could regulate the sex hormone levels in the B6AF1 mouse.

3.2. BSHXF regulates the number of growing follicles and ovarian index in different groups

BSHXF-H and BSHXF-M could upregulate the number of primordial, primary and secondary follicles in vivo of B6AF1 mice. In the B6AF1 model group, the number of primordial, primary and secondary follicles was significantly lower than that of control group (Figure 2A-C). BSHXF-H and BSHXF-M could improve the number of primordial, primary and secondary follicles. BSHXF-H, BSHXF-M and Estrogen group had a higher level of secondary follicle numbers compared to control group (Figure 2C). Besides, BSHXF-H and BSHXF-M have a lower number of atretic follicles compared to model group. Therefore, we conclude that both medium and high doses of BSHXF are functional to improve primordial follicle activation in mice.

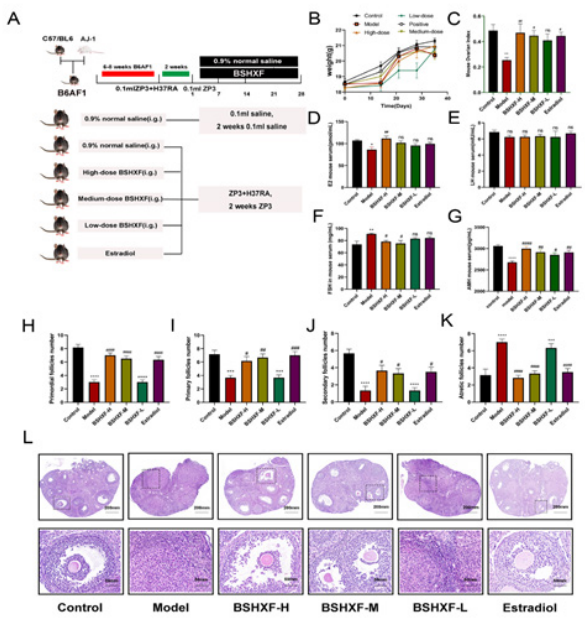

Fig. 1. BSHXF regulated the serum sex levels, body weight and the number of follicles in the B6AF1 mouse. (A) The schedule of the model construction and BSHXF treatment in the POI mouse. (B) Time course of the body weight of the B6AF1 mice in response to the BSHXF treatment. (C) High-dose and middle-dose of BSHXF could elevate the ovarian index. (D) The serum E2 concentration was higher in the high-dose BSHXF group. (E) No statistical differences in LH levels among the groups. Higher FSH (F) and AMH (G) serum levels in the High-dose and middle-dose groups were significantly compared to the model group. Haematoxylin and eosin (HE) staining analysis of mouse ovarian tissue (HE staining ×5, ×40). (L) the number of primordial follicles (H), the number of primary follicles (I), the number of secondary follicles (J), and the number of atretic follicles (K). BSHXF-H: 63.07 g/kg/d, BSHXF-M: 31.53 g/kg/d, BSHXF-L: 15.77 g/kg/d, Estradiol: 0.183 mg/kg/d; *P<0.05, **P<0.01, ***P<0.001, vs control. #P<0.05, ##P<0.01, ###P<0.001, vs. model (ANOVA).

3.3. Combining Network Pharmacology and Transcriptome sequencing to predict the drug target of BSHXF

In order to study the effect of compound BSHXF in the treatment of POI, we combine network pharmacology and transcriptomics to screen the potential targets. In this study, a total of 51 active ingredients and 145 active targets (shared by POI-targets and drug-related targets) were identified based on the network pharmacology approach (Figure 3A). To further research the molecular mechanism of the effect of BSHXF on POI, a network between 51 active components and the shared targets was constructed using Ctyoscape 3.7.0 (Figure 3B). These 145 shared genes were performed with Gene Ontology (GO) and KEGG pathway analysis (Figure 3C and D). KEGG analysis showed that TNF-a, Apoptosis, Toll-like receptor signaling pathway, Estrogen signaling pathway, PI3K-Akt signaling pathway et.al were the critical pathways.

To further identify the targets of system network pharmacology analysis, transcriptomics was conducted. Different expression genes (DEGs) analysis by the P-value<0.05 and |log2(fold change)| >1 genes were identified in the Model VS Control. DOWN group; 520 genes were selected in the Model VS Control. UP group; 608 genes were screened in the BSHXF VS Model. DOWN group; 667 genes were identified in the BSHXF VS Model. UP group (Figure 3A).144 common genes were screened between BSHXF VS Model. UP and Model VS Control. DOWN groups. 251 common genes were identified between BSHXF VS Model. DOWN and Model VS Control. UP groups (Figure 3B). The heatmap is presented by the FPKM of 395 DEGs (Figure 3C). GO and KEGG pathway enrichment analysis of 391 DEGs were performed by DAVID and visualized by Omicshare (https://www.omicshare.com/tools/). Totally, we found that apoptosis, NF-kb pathway and PI3K-AKT pathway were enriched in system network pharmacology and transcriptomic analysis.

3.4. BSHXF attenuates the apoptosis of ovarian granulosa cell via the PI3K/AKT pathway

To further identify the target of BSHXF in the immune premature ovarian insufficiency, PI3K and AKT were examined by western bloting. Tunel staining demonstrated that more apoptosis occurred in the granulosa cell layer of ovary in the model group compared to the control group (Figure 4 A-C). BSHXF-H and BSHXF-M have a lower number of TUNEL-positive cells in the area of granulosa cell layer (Figure 4 C). Compared to the normal group, the expression of Bax and Caspase3 was higher in the model group and the low-dose BSHXF group (*P<0.05*) (Figure 4A-B). High-dose and medium-dose BSHXF

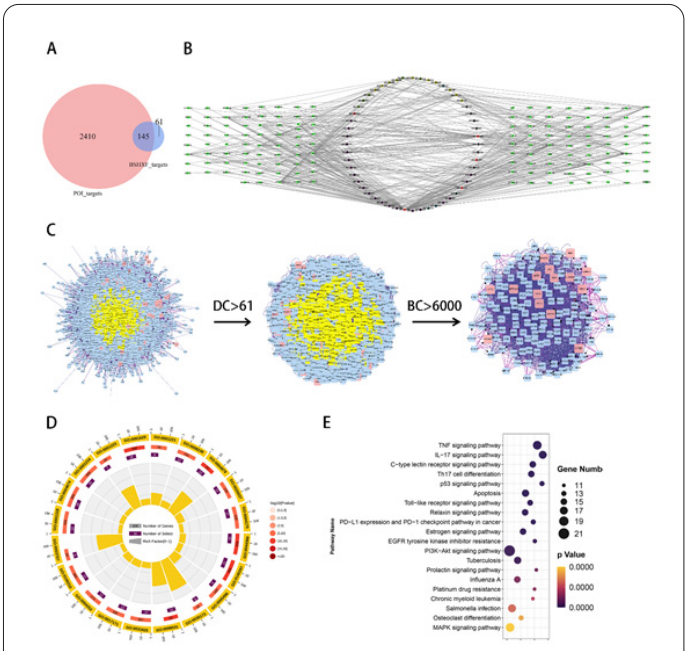

Fig. 2. Network pharmacology reveals the potential targets of BSHXF for POI mice. (A) A total of 145 common genes were screened between TCMSP database (BSHXF_targets) and genecards database (POI_targets), red circles represent TCMSP database, and blue circles represent genecards database. (B) Relationship between BSHXF targets and common genes, dark green: the common genes, light green: Phellodendri Chinrnsis Cortex, dark red: Radix Bupleuri, light pink: multiDrugs, dark pink: Anemarrhenae Rhizoma, grey: Epimrdii Herba, yellow: Cuscutae Semen, dark blue: Radix Bupleuri. (C) The hub genes were identified by the Cytoscape cytoNCA and Bisogenet, with the criteria: DC>61, BC>6000, and the pink box represented the seed genes. (D) For the top 20 GO pathways, red represents greater P-value, and blue represents smaller p-value. (E) The top 20 KEGG pathways. All pathways were sorted by P-value. dark blue represents smaller P-value, and yellow represents greater P-value.

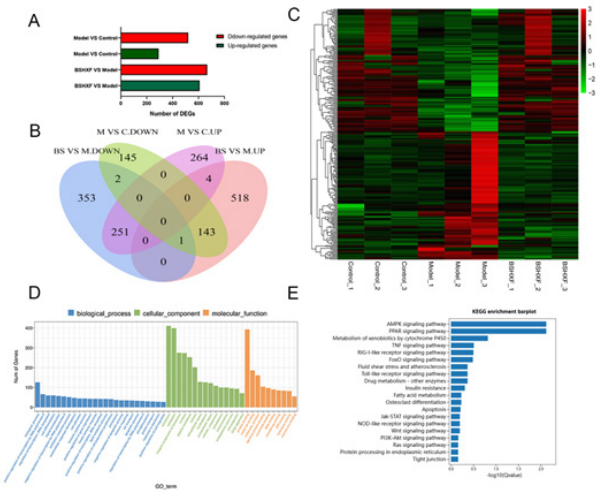

Fig. 3. Transcriptomic analysis of the potential mechanism of BuShenHuoXue formula for the treatment of POI. (A) Different expression genes (DEGs) analysis by the *P*-value<0.05 and |log2(fold change)|>1, red box represents upregulated genes, green box represents downregulated genes. (B) Veen diagram shows the DEGs genes in different groups. (C) Heatmap of mRNA expression profile in the 3 different groups. Red squares represent upregulated genes; Green squares represent downregulated genes. (D) GO analysis of DEGs. Blue box, biological process; green box, cellular component; orange box, molecular function. (E) The top 20 KEGG analysis pathways.

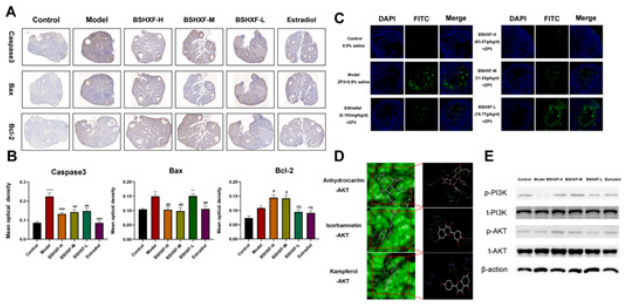

Fig. 4. BSHXF could decrease apoptosis of ovarian granulosa cells related to PI3K-AKT pathway. (A) Effect of BuShen-HuoXue formula (BSHXF) on the expression of caspase3, Bax, BCL-2 proteins analysised by Immunohistochemistry staining. (B) Mean optical density of Caspase3, Bax and BCL-2. (C) BSHXF decreases the positivity of Tunel staining cells, apoptosis occurs mainly in the granulosa cell area of ovary (green). Green fluorescence represents TUNEL-positive cells under microscope. Nuclear was stained by the DAPI (×400). (D) 3D molecular docking diagrams of active ingredients and AKT. Anhydroicaritin and AKT. Isorhamnetin and AKT. Kampferol and AKT.E. The expression of p-PI3K, PI3K, p-AKT, and AKT, were detected by Western Blot. β-action protein was used as internal reference (n=3).

could decrease the expression of Bax and Caspase3 levels. Meanwhile, immunohistochemical staining showed that the expression of BCL-2 levels were lower in the model and BSHXF-L groups compared to the other groups (Figure 4C).

The expression of p-PI3K and p-AKT were significantly higher in the high-dose and medium-dose BSHXF than in the model group. While the expression of t-PI3K and t-AKT proteins did not differ among the 6 groups (Figure 4E-F).

4. Discussion

Increasing numbers of studies showed that Bushenhuoxue Formula is a promising treatment for POI [15, 23-25]. Recently, a mouse model of exogenous of ZP3-induced premature ovarian insufficiency was constructed to explore the mechanism of autoimmune premature ovarian failure usually [26, 27]. Presently, we found that BSHXF could increase the body weight, mouse ovarian index, and ovarian follicle numbers in the ZP3-induced POI mice model. Moreover, BSHXF could reduce the serum FSH level and elevate serum E2 and AMH levels. The expression of caspase3, BAX and TUNEL-positive staining were lower in the BSHXF-treatment POI group compared to the ZP3-induced model group. Besides, combining the system network pharmacology and transcriptomics, we found that PI3K-AKT was the common gene. Meanwhile, the ratio of p-PI3K/PI3K, p-AKT/AKT were higher in high-does and medium-dose BSHXF treatment. All data suggested that PI3K-AKT may be the main pathway of BSHXF for the treatment of autoimmune POI.

The ovarian granulosa cell apoptosis could decrease the production of estrogen17-β required for maturation and development in the ovary [28]. The hypo-estrogen level may trigger mitochondria-mediated caspase pathway in the ovarian microenvironment [29]. In our present study, we found that the level of E2 was significantly elevated after BSHXF treatment in the serum. Elevated E2 levels could induce positive feedback to decrease the FSH level [30]. For a long time, FSH has been considered to be a candidate biomarker to describe the reproduction that stimulates folliculogenesis [31]. Compared with younger ovulatory women, higher FSH levels were observed in the older ovulatory women parallel with the decreased oocyte quantity and quality [32, 33]. Moreover, the rise in FSH levels precedes the fall in steroid hormone levels [34]. Presently, FSH levels were elevated in the autoimmune B6AF1 mice model while the expression were decreased in the high-dose BSHXF and medium-dose BSHXF groups. We speculate that BSHXF could inhibit the apoptosis of the ovarian granulosa cell via decreasing the FSH levels and elevating the E2 levels.

Some studies have shown that Chinese traditional treatment modalities for anti-apoptosis improve the expression of PI3K-AKT-mTOR pathway [35, 36]. The PI3K/AKT/mTOR signaling pathway could inhibit the activation of BCL-2 family members, as well as inactivating caspase3 [37, 38]. Additionally, a recent study showed that upregulation of PI3K/AKT could improve number of primordial follicles, primary follicles and secondary follicles in autoimmune premature ovarian insufficiency mice model [39]. Kaempferol, Isorhamnetin and Anhydroicaritin were identified as representative compounds of BSHXF and molecular docking showed good binding between these compounds and AKT. Kaempferol was found in Tusizi, Yinyanghuo, Baishao, Chaihu and zhimu. One study showed that Kaempferol could enhance the phosphorylation of PI3K and Akt, which may stimulate the anti-apoptotic signals and anti-oxidation in liver cells [40]. We surmise that Kaempferol may be one potential drug compound for the treatment of POI, which relieves the production of ROS via activing PI3K-Akt pathway.

According to the TCMSP database, Isorhamnetin is a flavonoid that is widely distributed in Tusizi and Chaihu. Meanwhile, Anhydroicaritin was mainly found in Yinyan-

ghuo and zhimu. Recent study found that Isorhamnetin could inhibit GC apoptosis via activating PI3K/AKT which regulates steroidogenesis [41]. Anhydroicaritin had a very poor binding affinity with estrogen receptors [42]. Our study found that Isorhamnetin and Anhydroicaritin showed strong binding affinities to AKT. Anhydroicaritin could suppress the SREBPs activation which alleviates insulin resistance [43]. SREBP-2 was identified as an indirect target of AKT in neurons [44]. Therefore, Anhydroicaritin and Isorhamnetin bind to AKT and thus regulate SREBP-2, which has important research value in the treatment of immune premature ovarian failure.

However, there are some deficiencies in our study at present. Inflammation-related cell clarity experiments need further implementation.PI3K inhibitor should be added to further validate our suspicions. Moreover, Kaempferol, Isorhamnetin and Anhydroicaritin were further identified in the treatment of autoimmune POI.

5. Conclusion

Our results demonstrated that BSHXF could inhibit the granulosa cell apoptosis via upregulating the PI3K/AKT pathway to increase ovarian reverse. Meanwhile, Kaempferol, Isorhamnetin and Anhydroicaritin may be the potential active compounds of BSHXF for the treatment of immune premature ovarian insufficiency via regulating the PI3K-AKT pathway.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine Animal Center.

Informed Consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Weibo Zhao: Conceptualization, Investigation, Experimental implementation, Origal draft writing. Yue Chen: Investigation, Funding support, Data analysis, Validation. Jianing He: Experimental implementation, Origal draft writing, Data analysis. Si Chen: Formal analysis, validation. Jiayun Shen: Data analysis, Data visualition. Beibei Jiao: Experimental implementation, Writing-review. Tian Li: Writing-review & editing. Tianjiao Mao: Experimental implementation, Data analysis. Peijuan Wang: Supervision, Funding acquisition, Resource, Project administration.

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References

1. Kokcu A (2010) Premature ovarian failure from current perspective. Gynecol Endocrinol 26:555-562. doi: 10.3109/09513590.2010.488773
2. Vujovic S, Brincat M, Erel T, Gambacciani M, Lambrinoudaki I, Moen MH et al (2010) EMAS position statement: Managing women with premature ovarian failure. Maturitas 67:91-93. doi: 10.1016/j.maturitas.2010.04.011
3. Chen Q, Ke H, Luo X, Wang L, Wu Y, Tang S et al (2020) Rare deleterious BUB1B variants induce premature ovarian insufficiency and early menopause. Hum Mol Genet 29:2698-2707. doi: 10.1093/hmg/ddaa153
4. Novosad JA, Kalantaridou SN, Tong ZB, Nelson LM (2003) Ovarian antibodies as detected by indirect immunofluorescence are unreliable in the diagnosis of autoimmune premature ovarian failure: a controlled evaluation. Bmc Womens Health 3:2. doi: 10.1186/1472-6874-3-2
5. Matsuda-Minehata F, Inoue N, Goto Y, Manabe N (2006) The regulation of ovarian granulosa cell death by pro- and anti-apoptotic molecules. J Reprod Develop 52:695-705. doi: 10.1262/jrd.18069
6. Hsueh AJ, Eisenhauer K, Chun SY, Hsu SY, Billig H (1996) Gonadal cell apoptosis. Recent Prog Horm Res 51:433-455, 455-456.
7. Igboeli P, El AA, Sheikh U, Takala H, ElSharoud A, McHugh A et al (2020) Intraovarian injection of autologous human mesenchymal stem cells increases estrogen production and reduces menopausal symptoms in women with premature ovarian failure: two case reports and a review of the literature. J Med Case Rep 14:108. doi: 10.1186/s13256-020-02426-5
8. Hammar M, Christau S, Nathorst-Boos J, Rud T, Garre K (1998) A double-blind, randomised trial comparing the effects of tibolone and continuous combined hormone replacement therapy in postmenopausal women with menopausal symptoms. Br J Obstet Gynaecol 105:904-911. doi: 10.1111/j.1471-0528.1998.tb10237.x
9. Birkhauser MH, Panay N, Archer DF, Barlow D, Burger H, Gambacciani M et al (2008) Updated practical recommendations for hormone replacement therapy in the peri- and postmenopause. Climacteric 11:108-123. doi: 10.1080/13697130801983921
10. Dragojevic-Dikic S, Rakic S, Nikolic B, Popovac S (2009) Hormone replacement therapy and successful pregnancy in a patient with premature ovarian failure. Gynecol Endocrinol 25:769-772. doi: 10.3109/09513590903004126
11. Holroyd CR, Edwards CJ (2009) The effects of hormone replacement therapy on autoimmune disease: rheumatoid arthritis and systemic lupus erythematosus. Climacteric 12:378-386. doi: 10.1080/13697130903025449
12. Coombs NJ, Taylor R, Wilcken N, Fiorica J, Boyages J (2005) Hormone replacement therapy and breast cancer risk in California. Breast J 11:410-415. doi: 10.1111/j.1075-122X.2005.00132.x
13. Shu X, Jiang Y, Wen T, Lu S, Yao L, Meng F (2019) Association of hormone replacement therapy with increased risk of meningioma in women: A hospital-based multicenter study with propensity score matching. Asia-Pac J Clin Onco 15:e147-e153. doi: 10.1111/ajco.13138
14. Li HF, Shen QH, Chen WJ, Chen WM, Feng ZF, Yu LY (2020) Efficacy of Traditional Chinese Medicine Tonifying Kidney (Bushen) and Activating Blood (Huoxue) Prescription for Premature Ovarian Insufficiency: A Systematic Review and Meta-Analysis. Evid-

Based Compl Alt 2020:1789304. doi: 10.1155/2020/1789304
15. Wang P, Lu Y, Chen S, Chen Y, Hu C, Zuo Y (2018) Protective function of Bu Shen Huo Xue formula on the immunity of B6AF1 mice with experimental autoimmune premature ovarian failure. Exp Ther Med 15:3302-3310. doi: 10.3892/etm.2018.5804
16. Zhou Z, Chen B, Chen S, Lin M, Chen Y, Jin S et al (2020) Applications of Network Pharmacology in Traditional Chinese Medicine Research. Evid-Based Compl Alt 2020:1646905. doi: 10.1155/2020/1646905
17. Liu Y, Ai N, Liao J, Fan X (2015) Transcriptomics: a sword to cut the Gordian knot of traditional Chinese medicine. Biomark Med 9:1201-1213. doi: 10.2217/bmm.15.91
18. Ru J, Li P, Wang J, Zhou W, Li B, Huang C et al (2014) TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. J Cheminformatics 6:13. doi: 10.1186/1758-2946-6-13
19. Xu X, Zhang W, Huang C, Li Y, Yu H, Wang Y et al (2012) A novel chemometric method for the prediction of human oral bioavailability. Int J Mol Sci 13:6964-6982. doi: 10.3390/ijms13066964
20. Casas AI, Hassan AA, Larsen SJ, Gomez-Rangel V, Elbatreek M, Kleikers P et al (2019) From single drug targets to synergistic network pharmacology in ischemic stroke. P Natl Acad Sci Usa 116:7129-7136. doi: 10.1073/pnas.1820799116
21. Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139-140. doi: 10.1093/bioinformatics/btp616
22. Webber L, Davies M, Anderson R, Bartlett J, Braat D, Cartwright B et al (2016) ESHRE Guideline: management of women with premature ovarian insufficiency. Hum Reprod 31:926-937. doi: 10.1093/humrep/dew027
23. Song KK, Ma WW, Huang C, Ding JH, Cui DD, Tan XJ et al (2016) Effect and mechanism of Bushen Huoxue recipe on ovarian reserve in mice with premature ovarian failure. J Huazhong Univ Sci Technolog Med Sci 36:571-575. doi: 10.1007/s11596-016-1627-2
24. Chen S, Lu Y, Chen Y, Xu J, Chen L, Zhao W et al (2021) The effect of Bu Shen Huo Xue Tang on autoimmune premature ovarian insufficiency via Modulation of the Nrf2/Keap1 signaling pathway in mice. J Ethnopharmacol 273:113996. doi: 10.1016/j.jep.2021.113996
25. Cao Y, Peijuan-Wang, Lu Y, Chen Y, Chen S, Weibo-Zhao (2021) Effectiveness and safety of Bushen Huoxue granules in treatment of premature ovarian insufficiency: study protocol for a randomized, double-blinded, placebo-controlled, and multicenter clinical trial. Trials 22:877. doi: 10.1186/s13063-021-05838-w
26. Bagavant H, Adams S, Terranova P, Chang A, Kraemer FW, Lou Y et al (1999) Autoimmune ovarian inflammation triggered by proinflammatory (Th1) T cells is compatible with normal ovarian function in mice. Biol Reprod 61:635-642. doi: 10.1095/biolreprod61.3.635
27. Zhang XL, Li J, Zhou XL, Chen QL (2021) Effect of Er-Xian decoction on autoimmune premature ovarian failure in mice. J Tradit Chin Med 41:725-731. doi: 10.19852/j.cnki.jtcm.2021.05.007
28. Tripathi A, Shrivastav TG, Chaube SK (2013) An increase of granulosa cell apoptosis mediates aqueous neem (Azadirachta indica) leaf extract-induced oocyte apoptosis in rat. Int J Appl Basic Med 3:27-36. doi: 10.4103/2229-516X.112238
29. Chaube SK, Prasad PV, Khillare B, Shrivastav TG (2006) Extract of Azadirachta indica (neem) leaf induces apoptosis in rat oocytes cultured in vitro. Fertil Steril 85 Suppl 1:1223-1231. doi: 10.1016/j.fertnstert.2005.11.034
30. Day FR, Ruth KS, Thompson DJ, Lunetta KL, Pervjakova N, Chasman DI et al (2015) Large-scale genomic analyses link reproductive aging to hypothalamic signaling, breast cancer susceptibility and BRCA1-mediated DNA repair. Nat Genet 47:1294-1303. doi: 10.1038/ng.3412
31. Mohamed A, Viveiros A, Williams K, Posse DCE (2018) Abeta inhibits SREBP-2 activation through Akt inhibition. J Lipid Res 59:1-13. doi: 10.1194/jlr.M076703
32. Burger HG (1994) Diagnostic role of follicle-stimulating hormone (FSH) measurements during the menopausal transition--an analysis of FSH, oestradiol and inhibin. Eur J Endocrinol 130:38-42. doi: 10.1530/eje.0.1300038
33. Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF (1992) Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum Reprod 7:1342-1346. doi: 10.1093/oxfordjournals.humrep.a137570
34. Klein NA, Houmard BS, Hansen KR, Woodruff TK, Sluss PM, Bremner WJ et al (2004) Age-related analysis of inhibin A, inhibin B, and activin a relative to the intercycle monotropic follicle-stimulating hormone rise in normal ovulatory women. J Clin Endocr Metab 89:2977-2981. doi: 10.1210/jc.2003-031515
35. Lee SJ, Lenton EA, Sexton L, Cooke ID (1988) The effect of age on the cyclical patterns of plasma LH, FSH, oestradiol and progesterone in women with regular menstrual cycles. Hum Reprod 3:851-855. doi: 10.1093/oxfordjournals.humrep.a136796
36. Zhang H, Qin F, Liu A, Sun Q, Wang Q, Li Q et al (2019) Kuntai capsule attenuates premature ovarian failure through the PI3K/AKT/mTOR pathway. J Ethnopharmacol 239:111885. doi: 10.1016/j.jep.2019.111885
37. Zhang H, Qin F, Liu A, Sun Q, Wang Q, Xie S et al (2019) Electro-acupuncture attenuates the mice premature ovarian failure via mediating PI3K/AKT/mTOR pathway. Life Sci 217:169-175. doi: 10.1016/j.lfs.2018.11.059
38. Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C (2003) PI3K/Akt and apoptosis: size matters. Oncogene 22:8983-8998. doi: 10.1038/sj.onc.1207115
39. Zhu JJ, Cui Y, Cui K, Li X, Zhang ZY (2016) Distinct roles of parafibromin in the extracellular environment, cytoplasm and nucleus of osteosarcoma cells. Am J Transl Res 8:2426-2431. doi:
40. Yin N, Wang Y, Lu X, Liu R, Zhang L, Zhao W et al (2018) hPMSC transplantation restoring ovarian function in premature ovarian failure mice is associated with change of Th17/Tc17 and Th17/Treg cell ratios through the PI3K/Akt signal pathway. Stem Cell Res Ther 9:37. doi: 10.1186/s13287-018-0772-x
41. Rajendran P, Ammar RB, Al-Saeedi FJ, Mohamed ME, ElNaggar MA, Al-Ramadan SY et al (2020) Kaempferol Inhibits Zearalenone-Induced Oxidative Stress and Apoptosis via the PI3K/Akt-Mediated Nrf2 Signaling Pathway: In Vitro and In Vivo Studies. Int J Mol Sci 22doi: 10.3390/ijms22010217
42. Li X, Chen H, Zhang Z, Xu D, Duan J, Li X et al (2021) Isorhamnetin Promotes Estrogen Biosynthesis and Proliferation in Porcine Granulosa Cells via the PI3K/Akt Signaling Pathway. J Agr Food Chem 69:6535-6542. doi: 10.1021/acs.jafc.1c01543
43. Tao Z, Liu J, Jiang Y, Gong L, Yang B (2017) Synthesis of prenylated flavonols and their potents as estrogen receptor modulator. Sci Rep-Uk 7:12445. doi: 10.1038/s41598-017-12640-9
44. Zheng ZG, Zhou YP, Zhang X, Thu PM, Xie ZS, Lu C et al (2016) Anhydroicaritin improves diet-induced obesity and hyperlipidemia and alleviates insulin resistance by suppressing SREBPs activation. Biochem Pharmacol 122:42-61. doi: 10.1016/j.bcp.2016.10.016