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Effect of a Traditional Chinese Medicine Formula (Bu Shen Zhu Yun) on Mifepristone-Induced Abnormal Pituitary Gonadotropin Secretion in Rats

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Authors' Contribution
HZ conceived and designed the work. ZL performed of experiment and wrote the article. BZ and BL helped perform the experiment. XJ helped in the data analysis. JX helped in designing the experiment.

Key words
Gonadotropin-releasing hormone receptor, Infertility, Luteal phase defect, Signaling pathway, Traditional Chinese medicine.

ABSTRACT

Mifepristone is effective in contraception and does not affect the menstrual cycle. However, the side-effects of mifepristone on sex hormones persist after drug discontinuation, possibly leading to luteal phase defect (LPD) and infertility. Recent studies revealed that mifepristone not only affects the ovary and uterus, but also the pituitary, resulting in LPD. Bu Shen Zhu Yun formula (BSZYF) was developed by Professor Guicheng Xia, a "great master in TCM." It effectively cures LPD, while its effect on the pituitary is unclear. In this study, we aimed to explore the effect of BSZYF on gonadotropins and gonadotropin-releasing hormone receptor (GnRHR) signaling transduction in mifepristone-induced LPD rats. To explore the effect of BSZYF on the pituitary in mifepristone-induced LPD rats, SD female rats were administered mifepristone suspension via oral gavage, followed by pharmaceutical intervention. Then, the serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were detected by ELISA, FSHβ and LHβ expression levels in the pituitary were determined by qPCR, and GnRHR expression levels were assessed by qPCR and western blot analysis. Simultaneously, transcription factors, such as c-Jun, Elk-1, Egr-1, Nur77, CREB, and transduction molecules in PKC-MAPK, Ca2+-CAM, and cAMP-PKA signaling pathways, such as PKC, p38mapk, ERK, CAM, and PKA were assessed by real-time PCR and western blot analysis. BSZYF increased the serum FSH levels suppressed by mifepristone ($P<0.05$). Moreover, the expression of c-Jun, Elk-1, Egr-1, CREB, PKA, p38mapk, JNK, ERK, and PKA was significantly up-regulated in the BSZYF group compared with that in the mifepristone group ($P<0.05$). However, there was no statistical difference in Nur77 and CAM expression between the blank, mifepristone, and BSZYF groups. BSZYF could regulate abnormal pituitary gonadotropin secretion and GnRHR signaling transduction systems induced by mifepristone.

INTRODUCTION

Mifepristone is a progesterone antagonist with high efficacy; it has been widely used in clinics for suppressing endometrial hyperplasia and terminating early pregnancy (Cameron *et al*., 1996; Boggavarapu *et al*., 2016). Mifepristone can effectively block the action of progesterone on the endometrium after ovulation. This induces a presentation resembling luteal phase defect (LPD) in the endometrium and prevents the normal implantation of a fertilized egg, thereby producing a contraceptive effect. However, mifepristone has limited effect on the development of the endometrium during menstrual cycle and does not induce menstrual cycle disturbances in females; therefore, mifepristone remains widely used as a contraceptive drug. However, contraception with mifepristone has been associated with amenorrhea, and patients treated with mifepristone are unable to become pregnant in a short time after discontinuing mifepristone (Lakha *et al*., 2007). Some studies have found that mifepristone affects follicle-stimulating hormone (FSH) and luteinizing hormone (LH)-both secreted by the pituitary, subsequently causing a series of reproductive endocrine changes-a side effect likely to contribute to pregnancy failure in females in a short time after discontinuing mifepristone treatment (Tamura *et al*., 2009; Weisberg *et al*., 2011). Reproductive endocrine function in most mammalian species is achieved primarily via the pituitary gonadotrophs, where gonadotropin-releasing

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hormone (GnRH) binds to GnRH receptors secreted by the hypothalamus to activate-via the three signaling transduction systems, protein kinase C/mitogen-activated protein kinase (PKC-MAPK), Ca^{2+}/calmodulin (Ca^{2+}-CAM), and cyclic adenosine monophosphate/protein kinase A (cAMP-PKA)-the key transcription factors AP-1, Elk-1, Egr-1, Nur77, and CREB, and subsequently regulate the production and secretion of FSH and LH (Escamilla-Hernandez *et al*., 2008; Kayampilly and Menon, 2008; Caunt *et al*., 2012). To date, through which of these three mechanisms does mifepristone influence the secretion of FSH and LH remains unclear.

Recently, we treated patients showing infertility after stopping mifepristone with traditional Chinese medicine (TCM) and observed an acceptable therapeutic efficacy. The Bu Shen Zhu Yun formula (BSZYF, Table I) was developed by Professor Guicheng Xia, a renowned TCM gynecologist and "great master in TCM," who developed this empirical formula by modifying Chinese medicinals based on two formulas-one named "Yu Lin Zhu," as reported in the TCM book Jing Yue Quan Shu, and the other named "Yang Jing Zhong Yu Decoction," reported in the TCM book *Fu Qing Zhu Nv Ke*. This empirical formula has been used for treating LPD-induced infertility and menstrual irregularities for more than 40 years. Our clinical investigations found that this formula exerted effects on gonadotropin levels, ovarian function, and endometrial receptivity in patients with LPD after discontinuing mifepristone. These effects have also been verified in animal experiments. In this study, we established an LPD animal model with mifepristone, performed intervention experiments with this TCM formula, and observed its effects on pituitary gonadotropin secretion and GnRH receptor signaling transduction systems. Finally, we explained the mechanism underlying mifepristone intervention in pituitary gonadotropin secretion and interpreted the regulating effects of BSZYF.

MATERIALS AND METHODS

Medicines

Mifepristone (Shanghai New Hualian Pharmaceutical Company, Shanghai, China, 25 mg/tablet, Batch No. C019150101) and dydrogesterone tablets (Solvay Pharmaceuticals B.V., Netherlands, 10 mg/tablet, Batch No. 347870) were ground to prepare a uniform suspension with concentrations of 1.0 and 0.4 mg/L, respectively. The medicinals in BSZYF were obtained from TCM granules (Jiangyin Tianjiang Pharmaceutical Company, Jiangsu, China) and mixed according to the combination ratios mentioned (Table I). After mixing, distilled water at 70°C was added to generate a suspension with a concentration of 0.08 g (mixed granules)/mL (low-concentration) and 0.24 g (mixed granules)/mL (high-concentration), respectively. All medicines were stored at 4°C and allowed to warm to 25–28°C before use.

Feeding, grouping, and observation of experimental animals

All the following experimental procedures were performed in accordance with the National Institutes of Health Guidelines for Laboratory Animals, and approved by the Animal Ethics Committee of our institution [No. SYXK (Jiangsu) 2014-0001]. Fifty specific pathogen-free female Sprague-Dawley rats aged 8 weeks and weighing 200 ± 20 g were provided by the Experimental Animal Center, Nanjing University of Traditional Chinese Medicine [SYXK (Beijing) Certificate No. 2012-0001] and fed an SPF diet. The rats were maintained at a temperature of 22–25 °C, relative humidity 40–70%, and alternate conditions of light and dark (12h:12h). They had free access to food and water, and were maintained in a quiet environment. Fifty rats were adaptively fed for one week prior to the experiments. At 8:00 a.m. and 4:00 p.m. each day during the adaptive feeding period, vaginal exfoliated cell smears were collected from each rat and observed using an inverted microscope. The rats were allowed to fully adapt to the capture and smear procedure. Based on the regular patterns observed in the exfoliated cells, it was confirmed that the estrous cycles of all rats were normal (four days) (Caron *et al*., 2009). The 50 rats were divided into five groups comprising ten rats each, by means of a random number table. The five groups are as follows: Group A (blank), Group B (model),

Table I.- Composition of Bu Shen Zhu Yun formula.

Pharmaceutical	Botanical or zoological name	Family and part	Chinese name	Weight	Granule weight*
Cervi Cornu	*Cervus elaphus* Linnaeus	Cervidae; horn	Lu Jiao Pian	10 g	0.5 g; 3 g
Cuscutae Semen	*Cuscuta australis* R.Br.	Convolvulaceae; seed	Tu Si Zi	15 g	0.5 g; 10 g
Dioscoreae Rhizoma	*Dioscorea opposita* Thunb.	Dioscoreaceae; rhizome	Shan Yao	15 g	0.5 g; 10 g
Corni Fructus	*Cornus officinalis* Sieb. et Zucc.	Cornaceae; fruit	Shan Zhu Yu	10 g	1.5 g; 6 g
Paeoniae Radix Alba	*Paeonia lactiflora* Pall.	Ranunculaceae; root	Chao Bai Shao	10 g	1 g; 10 g
Radix Bupleuri	*Bupleurum chinense* DC.	Umbelliferae; root	Cu Chai Hu	6 g	1 g; 6 g

*Per bag and equivalent dose.

Group C (dydrogesterone), Group D (low-dose BSZYF), and Group E (high-dose BSZYF). There was no statistically significant difference in body weights among the groups.

Animal medication

Vaginal smears were collected for examination at 8:00 a.m. each day. Day 1 (referred to as D1) was identified as the day on which estrum (the early stage of corpus luteum after ovulation) was discerned. All rat groups except Group A were administered mifepristone suspension at a ratio of 1 mL/100 g on D1, D5, and D9 via oral gavage. From the fourth estrous cycle (D13), the rats received different solutions/medicine via oral gavage. Group B received isotonic saline (1 mL/100 g), Group C received dydrogesterone suspension (1 mL/100 g), Group D received low-concentration BSZYF (1 mL/100 g, equivalent to clinical dosage), and Group E received high-concentration BSZYF (1 mL/100 g, equivalent to 3 times the clinical dosage) at four time points (4:00 p.m. on D13, 8:00 a.m. on D14, 4:00 p.m. on D17, and 8:00 a.m. on D18). The blank group received isotonic saline (1 mL/100 g) at the above-mentioned medication time points from D1 to D18 (Zhou *et al*., 2009; Ghosh and Sengupta, 2014).

Specimen sampling methods

Blood (0.5–1 mL) was collected from the orbital cavity of each group at 4:00 p.m. on D2, D6, D10, and D14 during the treatment period, incubated at a temperature of 22-25 °C for 1–2 h, and centrifuged for 15 min at a centrifugation speed of 3000 rpm with a centrifugation radius of 18 cm. The upper layer of serum was collected and stored at −20 °C until use. All rats were subjected to intraperitoneal anesthesia with 10% chloral hydrate (0.35 mL/100 g) at 2:00 p.m. on D18. Following anesthesia, blood was collected from the carotid artery, the rats were sacrificed by cervical dislocation, and the pituitary was rapidly removed. The anterior pituitary was isolated on ice, quickly placed in liquid nitrogen, and stored at −80 °C until use. Serum was obtained from the collected blood samples as described previously. The feeding, medication, and sampling procedures are illustrated (Fig. 1).

ELISA

A double antibody sandwich ELISA was performed using a kit (Elabscience Biotechnology Co., Ltd., Wuhan, China) to detect FSH and LH in rats. Serum samples were mixed well and diluted to prepare standard samples. The assay was performed in a 96-well plate according to the manufacturer's protocol, and data were collected and analyzed using a multifunction ELISA detection system (TECAN Company, Switzerland).

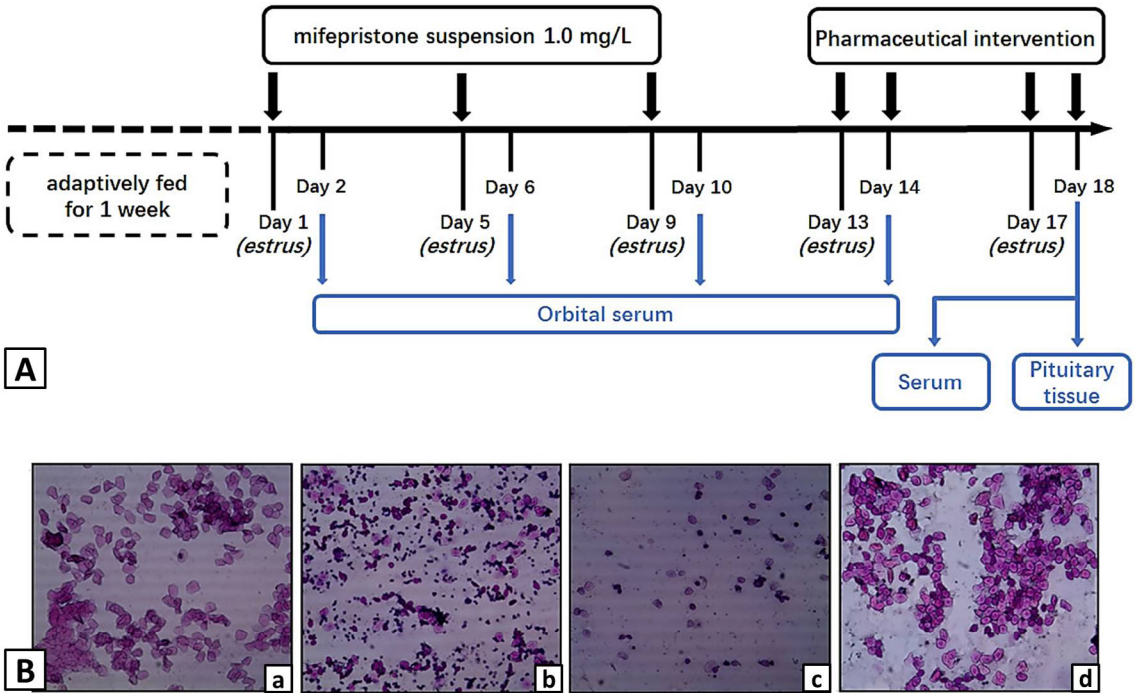

Fig. 1. The feeding, medication, and sampling procedures of Rats. A, experimental protocol for model establishment and challenge as described in Section 2. B, differences in rat vaginal smears during four periods of the rat sexual cycle (HE, X100). a, estrus; b, metestrus; c, diestrus; d, proestrus.

Real-time PCR

We extracted whole RNA from the rat anterior pituitary tissue using TRIzol lysis solution (Takara Company, Japan) according to the manufacturer's instructions, and synthesized cDNA using a PrimeScript® RT kit (Takara Company, Japan). Real-time PCR was performed using SYBR® Premix Ex TapTM II kit (Takara Company, Japan) and the primers are listed (Table II). The reaction conditions were as follows: samples were maintained at 95 °C for 30 s, and then subjected to 40 cycles of degeneration at 95 °C for 5 s and amplification at 60°C for 34 s. Each sample was analyzed in triplicate. Data collection and analysis were performed using QuantStudio 7 Flex (Thermo Fisher Scientific Company, USA) and the relative quantization method (ΔΔCt). Using HPRT1 as an internal reference parameter, all data were converted for calculation ($2^{-\Delta\Delta Ct}$) and statistical analysis.

Western blotting

Rat anterior pituitary tissues were lysed in phenylmethylsulfonyl fluoride (PMSF)-containing RIPA (100 mg/mL; Boster Company, Wuhan, China) and centrifuged at a speed of 12,000 rpm with a radius of 18 cm for 30 min at 4°C. Protein content was determined using a bicinchoninic acid (BCA) protein concentration kit (Thermo Fisher Scientific Company, USA) and the samples were denatured in 2X electrophoresis buffer (BioRad Company, USA) at 100 °C for 5 min, followed by storage at −80 °C. Total proteins were isolated using 8–12% SDS-PAGE in a vertical electrophoresis apparatus, transferred to semi-dry polyvinylidene fluoride (PVDF) membrane (Millipore, USA), blocked with 5% bovine serum albumin (BSA) solution [with tris-buffered saline (TBS) Tween 20, TBST, 25 mM Tris [pH 7.5], 150 mM NaCl and 0.1% Tween 20] for 2 h. The membranes were incubated with primary antibodies, including GnRHR, c-Jun, Elk-1, Egr-1, Nur77, CREB, PKC, ERK1/2, p38MAPK, JNK1/2, PKA, CAM, and tubulin (Abcam Ltd., USA) overnight at 4 °C. Goat anti-rabbit horseradish peroxidase-conjugated IgG (Cell Signaling Technology Inc., USA) was used to detect antibody binding. A ChemiDOCTM MP (BioRad Company, USA) western blot multifunction imaging system was used to develop blot images, and image data were collected and analyzed using ImageLab software (BioRad Company, USA).

Statistical analysis

Statistical analysis was conducted using Prism 6.0 software (GraphPad Software, San Diego, CA, USA). All quantitative data were subjected to a normal distribution test. Data conforming to a normal distribution were presented as mean ± standard deviation (X ± SD), and one-way ANOVA was used to compare the means among different groups of data that conformed to homogeneity of variances. A least significant difference test was used for paired comparison. The Dunnett T3 method was used for analysis of data not conforming to homogeneity of variances. Data not following a normal distribution were expressed as interquartile ranges, and a non-parametric test (Kruskal-Wallis) was employed for data analysis. Values of $P<0.05$ were deemed statistically significant.

Table II.- Real-time PCR primer sequences.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product (bp)
FSHβ	GGACCCAGCTAGACCAAACA	ACAGTGGCATTCAGTGGCTA	133
LHβ	CTGAGCCCAAGTGTGGTGT	ATGCTGGTGGTGAAGGTGAT	122
GnRHR	GTGGTGATTAGCCTGGATCG	ATAACTGTGGTCCCGCAAAG	130
c-Jun	TGACTGCAAAGATGGAAACG	CCAGGTTCAAGGTCATGCTC	126
Elk-1	TCCACCTCCACCACTGAGAT	CCAGAGCTTGTTCCTGATCC	131
Egr-1	AACACTTTGTGGCCTGAACC	AGGCAGAGGAAGACGATGAA	118
Nur77	GGCATGGTGAAGGAAGTTGT	GAATGAGGGACGTGAGGAGA	118
CREB	GATTCTAGTGCCCAGCAACC	GGAGGACGCCATAACAACTC	118
PKC	AATGGCCTGTCTGATCCCTA	ACACGAAGGTCTCGTTCCAC	121
ERK1	CTCTCCAACCGGCCTATCTT	AGGTAGTTTCGGGCCTTCAT	128
ERK2	CCTACGGCATGGTTTGTTCT	TCTCATGTCTGAAGCGCAGT	141
P38mapk	GGCACACTGATGACGAAATG	CCACGGACCAAATATCCACT	111
Jnk	ATGATGACGCCTTACGTGGT	GGCAAACCATTTCTCCCATA	121
CAM	GCACCATTGACTTCCCAGAG	GATGTAGCCATTGCCATCCT	119
PKA	TACCTCCATTCCCTCGACCT	CCTTGACTCTCTTGGCGAAC	112
HPRT1	AGCAGACGTTCTAGTCCTGTG	GGCTGCCTACAGGCTCATAG	136

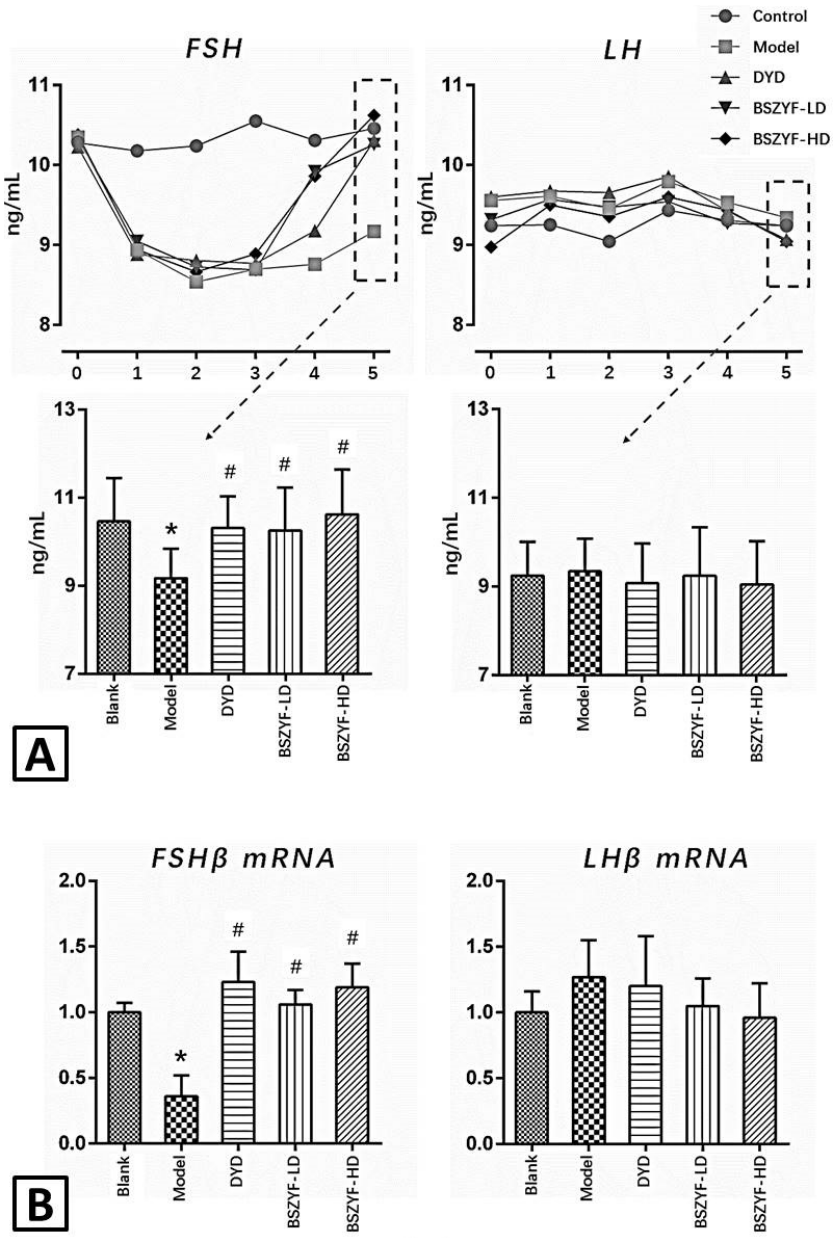

Fig. 2. FSH and LH levels in the serum and mRNA expression in pituitary. A, serum samples were collected from five groups during the treatment phase (D2, D6, D10, D14, D18) and once before medication as a baseline value. All sampling times were during the metestrus stage of each cycle. Mifepristone downregulated the level of FSH. However, following treatment with Bu Shen Zhu Yun formula (BSZYF), FSH levels ìncreased to the level of the blank group. There were no significant differences in LH levels between the groups. The levels of FSH and LH in the serum were measured by ELISA; B, differences in FSHβ and LHβ mRNA expression in the anterior pituitary tissue were similar to differences in serum levels. Values are represented as mean ± SD (n=6 per group), *P<0.05 compared with the blank group, #P<0.05 compared with the model group.

RESULTS

BSZYF improved suppression of FSH secretion induced by mifepristone

ELISA results obtained in serum collected at 5 different times showed that mifepristone effectively suppressed the secretion of FSH from the start of treatment to two weeks after the end of treatment. All differences were statistically significant. Following BSZYF treatment, the FSH levels recovered rapidly. At D14 and D18, there

was no statistically significant difference in FSH levels between the BSZYF groups and the blank group. The BSZYF groups showed better efficacy to recover the FSH levels than the dydrogesterone group. Consistent with the FSH level results, the BSZYF group improved the suppression of FSHβ mRNA expression induced by mifepristone in the anterior pituitary tissue samples. However, both mifepristone and BSZYF groups did not show a significant effect on LH secretion, and there was no statistically significant difference in LHβ mRNA levels between the groups (Fig. 2).

BSZYF increased GnRH receptor expression following its suppression by mifepristone

GnRH receptors in the anterior pituitary gonadotrophs are the primary regulation nodes for the secretion of FSH and LH. GnRH binds to GnRH receptor resulting in the synthesis and release of FSH and LH (Yu *et al*., 2011). The expression levels of GnRH receptor in the rat anterior pituitary following mifepristone intervention for three estrous cycles and two estrous cycles post-intervention were significantly lower than those in the blank group, indicating that mifepristone might influence the secretion of FSH and LH by reducing GnRH receptor activity in the pituitary. However, GnRH expression levels in Groups D and E increased to some extent, indicating that BSZYF may be involved in regulating gonadotropin levels (Fig. 3).

Effect of mifepristone and BSZYF on the expression of key transcription factors in the rat anterior pituitary tissues

Among the signal transduction pathways in pituitary gonadotrophs, the PKC-MAPK pathway primarily activates c-Jun, Elk-1, and Egr-1, and the cAMP-PKA pathway primarily activates CREB and Egr-1, while the Ca2+-mediated signaling pathway primarily activates Nur77, c-Jun, and Egr-1 (Naor *et al*., 1997; Melamed *et al*., 2012; Thompson *et al*., 2013). Mifepristone significantly decreased both mRNA and protein expression of c-Jun, Egr-1, Elk-1, and CREB in the rat anterior pituitary tissues but did not influence the expression of Nur77. BSZYF treatment increased the mRNA and protein expression of c-Jun, Elk-1, Egr-1 and CREB, which had decreased following mifepristone treatment; however, the expression of c-Jun, Elk-1, Egr-1, and CREB was not significantly different from that in the blank group (Fig. 4).

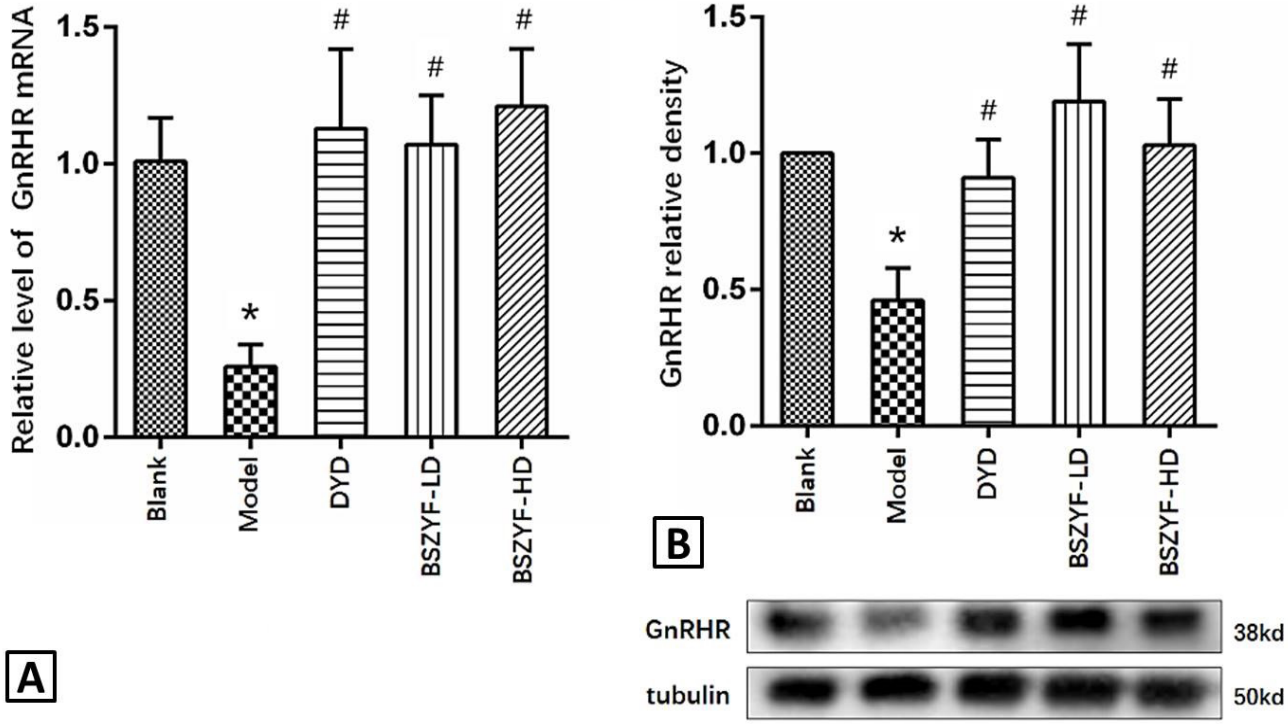

Fig. 3. Treatment with BSZYF upregulated the expression of GnRHR in the anterior pituitary tissue. A, mRNA expression of GnRHR was analyzed by qPCR. B, Western blotting to measure the protein expression of GnRHR. The relative density was quantified relative to tubulin. Values are represented as mean±SD (n=6 per group), **P*<0.05 compared with the blank group, #*P*<0.05 compared with the model group.

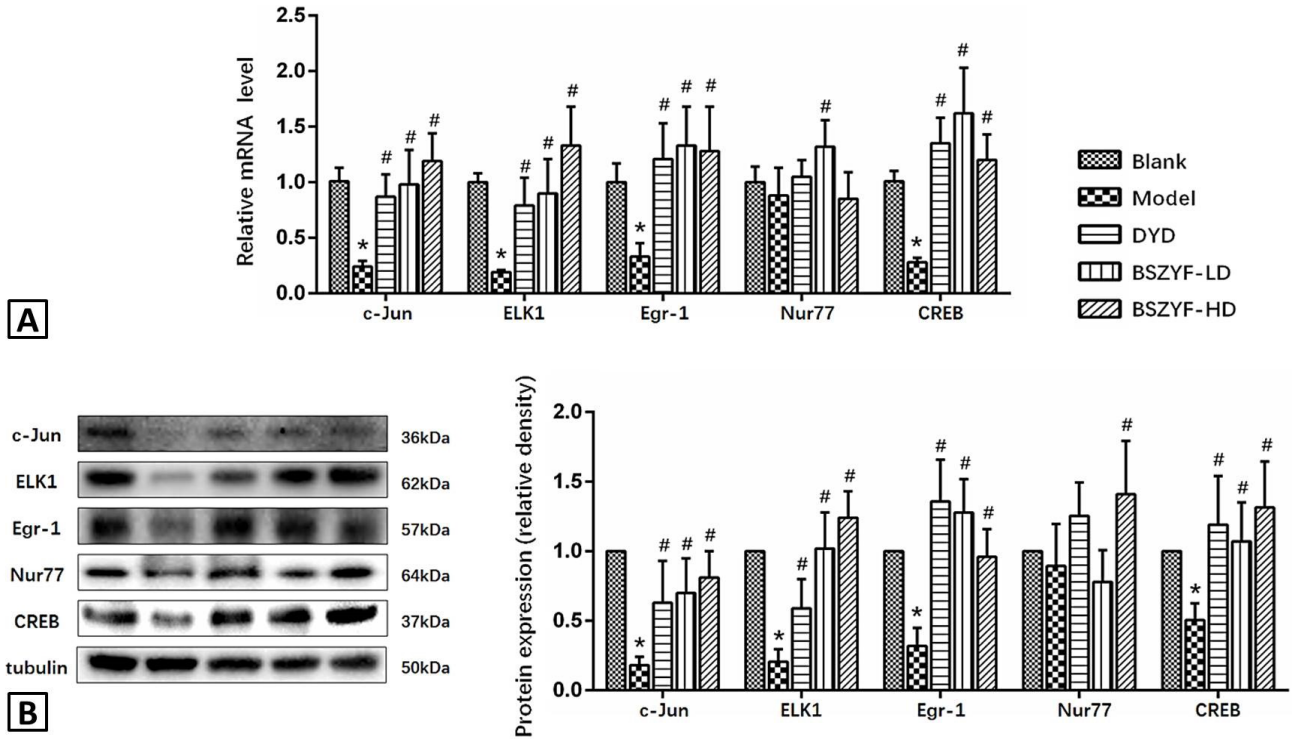

Fig. 4. BSZYF treatment promoted the activation of transcription factors c-Jun, ELK-1, Egr-1, and CREB. A, the mRNA expression of transcription factors was analyzed by qPCR. B, Western blotting to measure the protein expression of transcription factors. The relative density was quantified relative to tubulin. Values are represented as mean±SD (n=6 per group), *P<0.05 compared with the blank group, #P<0.05 compared with the model group.

Effect of mifepristone and BSZYF on the expression of nodal molecules in the signaling pathways

PKC in PKC-MAPK is a pivotal node in post-GnRH receptor signaling pathways, mediating a cascade amplification of MAPK, and it includes three branches: ERK, p38MAPK, and JNK (Sharma *et al*., 2013). PKA and CAM are pivotal nodes in the cAMP-PKA and Ca^{2+}-CAM signaling pathways. Mifepristone significantly suppressed both mRNA and protein expression of PKC, ERK1/2, p38MAPK, JNK, and PKA in PKC-MAPK signaling pathway, while there was no significant change in the expression of CAM. BSZYF increased the expression of PKC, ERK1/2, p38MAPK, JNK, and PKA, which was suppressed by mifepristone treatment; however, the differences were not significant compared with the blank group (Fig. 5).

DISCUSSION

Based on the experimental results, we were able to draw some preliminary conclusions. First, after ovulation, mifepristone decreased the secretion levels of gonadotropin at the late stage of the rat estrous cycle (similar to the human luteal phase (McClintock, 1983)), and the secretion levels do not automatically recover for at least two estrous cycles after discontinuing mifepristone treatment. This effect may be attributed to the suppression of GnRH receptor expression in the pituitary and downstream transcription factor c-Jun, Egr-1, Elk-1, and CREB expression in PKC-MAPK and cAMP-PKA signaling transduction pathways by mifepristone. Second, BSZYF effectively restores GnRH receptor expression in the rat anterior pituitary and post-receptor signaling transduction systems suppressed by mifepristone, and increases FSH secretion levels back to the normal level of the blank group. Finally, mifepristone and BSZYF did not have a significant effect on the expression of CAM or downstream Nur77 in the Ca^{2+}-CAM signaling pathway, indicating that these medications do not affect Ca^{2+} binding to CAM under normal conditions.

Mifepristone acts as a competitive antagonist of progesterone, blocking its binding to the progesterone receptor. It has a four- or five-fold greater affinity for the progesterone receptor than progesterone (Bouchard *et al*., 2011), thereby significantly suppressing the normal biological effects of progesterone as well as the function

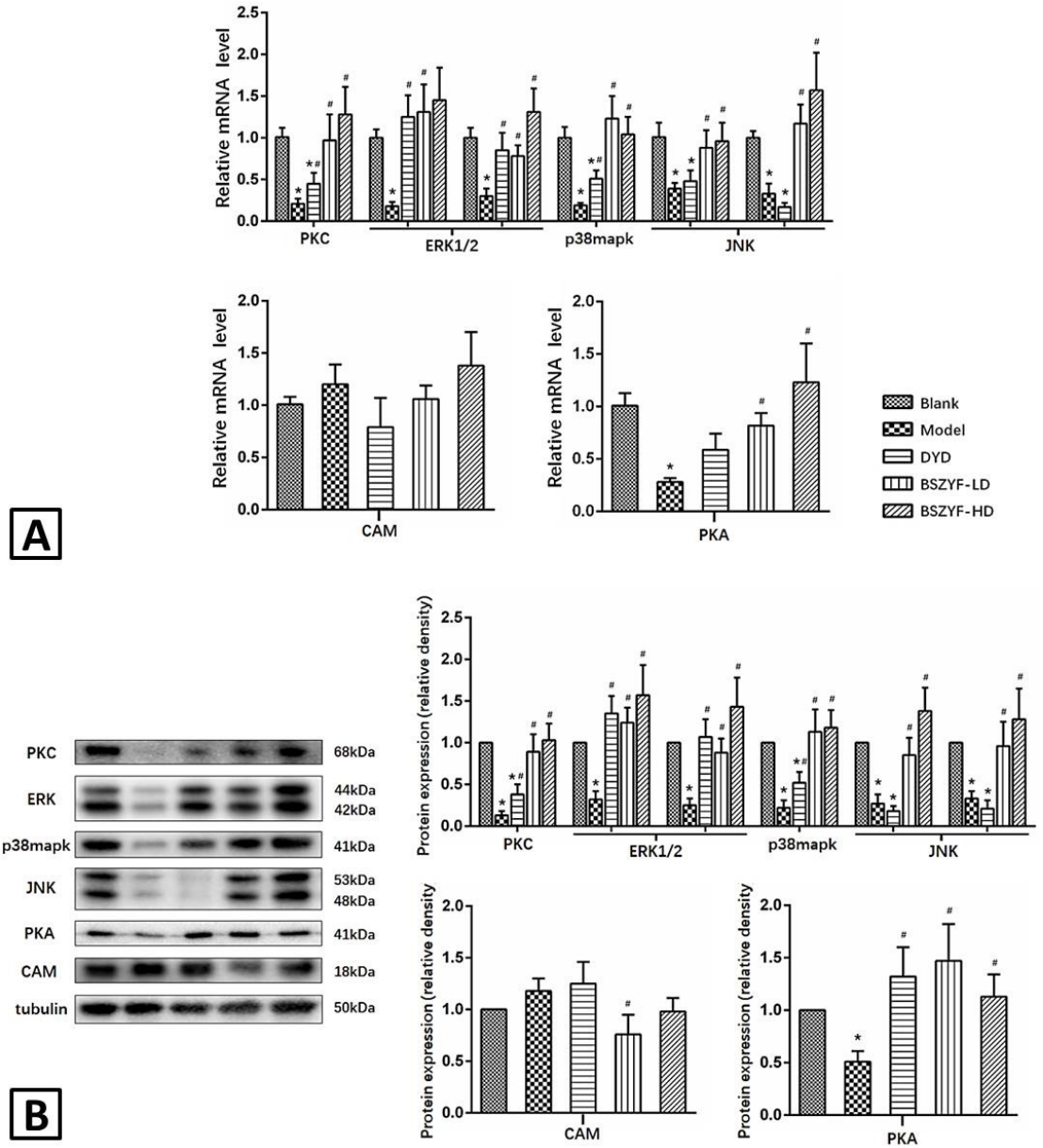

Fig. 5. BSZYF treatment promoted the activation of PKC-MAPK and cAMP-PKA signaling pathways. A, the mRNA expression of signal transducers was analyzed by qPCR. B, Western blotting to measure the protein expression of signal transducers. The relative density was quantified relative to tubulin. Values are represented as mean±SD (n=6 per group), **P*<0.05 compared with the blank group, #*P*<0.05 compared with the model group.

of corpus luteum. Pharmacokinetic studies have shown that a one-time oral administration of 100 mg mifepristone at the non-pregnant luteal phase leads to a peak plasma-drug concentration within 1 h, with an elimination half-life of 12–24 h. Therefore, a 100–800 mg dose would not cause a significant change in plasma-drug concentration and would less likely result in the accumulation of the drug in the blood (Sarkar, 2005). However, until recently, the adverse effects of mifepristone on reproductive endocrine function have received little attention and remain insufficiently studied. Some studies have shown that in normal conditions, the ratio between FSH and LH in the luteal metaphase is relatively stable. If the ratio of LH:FSH is too high, it can lead to early atrophy of the corpus luteum and affect the normal development of the ovarian follicle in the next menstrual cycle, which in turn imposes further

adverse effects on the function of corpus luteum (Cook *et al*., 1983). Moreover, mifepristone can disturb the normal secretion of inhibin (Spitz, 2003) and testosterone (Wu *et al*., 2014), aggravate the ratio imbalance of LH/FSH, and cause LPD or even infertility after the medication is discontinued.

Pituitary gonadotropin secretion is not only regulated by GnRH secreted by the hypothalamus, but is also associated with the signal transduction of the GnRH receptor. GnRHR is expressed in the anterior pituitary (Mertani *et al*., 1996) and is a member of the rhodopsin-like G-protein coupled receptor family (Naor and Huhtaniemi, 2013). Following GnRH binding to GnRHR in the pituitary, GnRH enables signaling transduction via a variety of G-protein coupled signaling pathways and ultimately affects the secretion of FSH and LH (Naor *et al*., 1998; Oh *et al*., 2003; Melamed *et al*., 2012). In normal conditions, an intervention of PKA and PKC in the cAMP-PKA and PKC-MAPK signaling pathways of pituitary GTH cells affect gene expression and secretion levels of FSH and LH in the pituitary, implying that the transduction functions of these two signaling pathways are pivotal factors influencing the secretion of FSH and LH in the pituitary (Naor *et al*., 1998; Garrido-Gracia *et al*., 2006; Zhao *et al*., 2011). CREB, a key protein that regulates gene transcription function, contains multiple activation sites that can be activated by a number of protein kinases including PKA, and it is one of the key nodes in the signaling transduction pathway of cAMP-PKA (Kasper *et al*., 2010). CREB can also be activated by the influx of Ca^{2+}. It can competitively bind to the AP-1 site in the MAPK signaling transduction pathway and reduce the production of c-Jun subunit of AP-1 (Cheng and Leung, 2001), thereby suppressing MAPK signaling transduction and affecting PKC-MAPK signaling pathway. In this study, both CREB and AP-1 were suppressed by mifepristone, but their expression levels returned to normal levels following BSZYF treatment and no competitive effect was observed. We suggest that the decreased expression levels of CREB following suppression of the cAMP-PKA signaling pathway also suppressed the PKC-MAPK signaling pathway, which plays a pivotal role in AP-1 expression, thereby decreasing the expression of AP-1. Similarly, after the suppression of cAMP-PKA and PKC-MAPK signaling pathways was ameliorated, the expression of CREB and AP-1 increased significantly. However, in this study, we found that although mifepristone affected the expression of PKA and PKC as well as a series of downstream key nodes, the levels of LH did not change significantly when FSH secretion decreased. It is unclear whether this observation was attributed to the signaling transduction pathway of Ca^{2+}-CAM or other underlying mechanisms. Therefore, more research is required to address this issue.

When administered in the premenstrual period (after ovulation), BSZYF can tonify the kidney yang, move the Qi in heart and liver, and tranquilize the heart and liver. Clinical research and previous studies have also shown that this formula can improve ovarian cell function and endometrial receptivity, regulate menstrual function, and promote pregnancy (Zhou *et al*., 2009). Given the difference in the theoretical system and the expression approach between Western medicine and TCM, the "hypothalamus-pituitary-ovary reproduction axis" (H-P-O axis) in Western medicines does not exactly correspond to the "heart (brain)-kidney-uterus reproduction axis' in TCM, although both axes represent the same basic process. Mifepristone not only has an antagonistic effect on progesterone in the endometrium, but can also cause the dysfunction of reproduction axis. Although it is unlikely to lead to severe menstrual disorders, mifepristone withdrawal is likely to result in a certain degree of dysfunction and LPD-like presentation (Lakha *et al*., 2007; Sun *et al*., 2014). Following BSZYF administration, the secretory function in the pituitary, which is at the center of the reproduction axis, is likely to recover.

CONCLUSION

Based on our experimental results, we speculate that the modulation of corpus luteal function by BSZYF not only affects the uterus and ovaries, which are downstream of the reproduction axis, but also regulates gonadotropin secretion in the pituitary, which is in the center of the reproduction axis.

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Statement of conflict of interest

We declare no conflicts of interest in this study.

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