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Bu-shen-zhu-yun decoction promotes synthesis and secretion of FSHβ and LHβ in anterior pituitary cells in vitro

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ABSTRACT

Luteal phase defects (LPD) are an important etiology of infertility which has increased in recent years. Studies have shown that bu-shen-zhu-yun decoction (BSZY-D) can lower the expression of estrogen receptor and progesterone receptor, in rats endometrium of embryonic implantation period, which upregulated by mifepristone, and improve uterine receptivity. The aim of present study was to determine the effect of BSZY-D on the synthesis and secretion of gonadotropic hormones in the anterior pituitary cells of rats. Rats were treated with saline (control) or BSZY-D two times/day for three estrous cycles by gavage. The cerebrospinal fluid (CSF) were collected for further cell treatment. The components in BSZY-D, serum and CSF were analysed by High Performance Liquid Chromatography (HPLC). Cells were either pretreated with normal CSF or BSZY-D/CSF before being stimulated with or without cetrorelix. The mRNA and proteins levels of receptors, hormones, and transcription factors were detected by RT-PCR, western blot analysis and immunostaining. We show that non-toxic concentrations of cetrorelix, a GnRH antagonist, can reduce the mRNA and protein levels of GnRHR, LH, and FSH. This effect could be reversed by the addition of BSZY-D/CSF. We also show decreased mRNA and protein expression of transcription factors, such as CREB, and Egr-1 and secretory vescicles, including SNAP-25 and Munc-18 upon treatment with cetrorelix could be reversed post co-treatment with BSZY-D/CSF. These results indicate that BSZY-D/CSF treatment led to increased levels of GnRHR, transcription factors, and secretory vesicles leading to increased secretion of FSH and LH. Thus, BSZY-D presents a promising candidate to treat luteal phase defects and infertility.

1. Introduction

Several factors determine infertility, including lifestyle, nutritional factors, epidemics, sexually transmitted diseases, chronic stress, and environmental pollutants [1]. Women with uterine fibroids, endometriosis, polycystic ovary syndrome, and chronic anovulation are also at a higher risk for infertility [1]. Luteal phase defects (LPD) and anovulatory endometrium were found to be the major causes of infertility, and LPD was present in 13.5% of the cases with infertility and in 32.5% of cases with recurrent miscarriage [2–5]. LPD results from abnormal corpus luteum function due to low progesterone levels. LPD is characterized by follicular defects, lack of progesterone secretion, endometrial secretion, and irregular menstruation which may lead to primary infertility and recurrent early pregnancy abortion [6].

Studies have shown that diseases or factors affecting the hypothalamic-pituitary-ovarian reproductive axis can lead to LPD [7,8]. Therefore, infertility caused by LPD may not only be due to abnormal ovarian or uterine function, but could be related to uncoordinated hypothalamic-pituitary-ovarian reproductive axis. GnRH synthesised in the hypothalamus regulates the secretion of pituitary gonadotropins, including FSH and LH to further regulate the ovarian endocrine function, improve the uterine receptivity, and to promote cultivation of the embryos [9–12]. The GnRH secretion is pulsed which is important for the normal regulation of menstrual cycles. Several reproductive disorders, including hypogonadotropic hypogonadism, hypothalamic amenorrhea, hyperprolactinemia, and polycystic ovary syndrome (PCOS) are associated with disrupted pulsatile secretion of GnRH [13]. About 50% of the LPD were found to be due to improper generation of

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pulses in the hypothalamus [14]. Anomalous secretion of LH and FSH in the anterior pituitary cells is one of the pathology of LPD. Currently, the conventional treatment for LPD primarily includes clomiphene citrate, progesterone, human menopausal gonadotropin, and bromocriptine; however, the treatment effects are not very satisfactory and are associated with several adverse effects [15–17].

Chinese medicine suggests the pathogenesis of this disease as kidney-based, involving deficiency of liver, spleen, qi, xue, yin, and yang [18,19]. Meta-analysis of cohort studies show a pregnancy rate of 50% using Chinese Traditional Medicine compared to pregnancy rate of 30% using in vitro fertilization [20]. Bu-shen-zhu-yun decoction (BSZY-D) containing cervi cornu, cuscutaesemen, paeoniae radix alba, dioscoreae rhizoma, corni fructus, bupleuri radix has been previously shown to increase uterine receptivity and pregnancy rate in mice [21,22]. Additionally, in our previous study we show that BSZY-D can reduce the estrogen and progesterone receptor levels, improve the expression of integrin α 5 and β3, and improve the endometrial receptivity of LPD during embryo implantation, thereby enhancing the pregnancy rates [23]. BSZYD can also regulate gonadotropin secretion through the PKC-MAPK, Ca^{2+} -CAM, cAMP-PKA signal pathway in SD rats in vivo (unpublished results).

In this study, we investigate the effect of cerebrospinal fluids containing BSZY-D on anterior pituitary gonadotropin cells. We find that BSZY-D can promote the synthesis and secretion of gonadotropin releasing hormones in anterior pituitary gonadotropin cells in vitro.

2. Materials and methods

2.1. Preparation of BSZY-D extracts

BSZY-D is composed of cornu-cervi, *Semen Cuscutae*, Radix Paeoniae Alba, Dioscoreae Rhizoma, Corni Fructus, Radix Bupleuri, and two other Chinese medicines (confidential) which were purchased from Jiangsu Province Hospital of Chinese Medicine. The dose (measured as dry weight) of each component is listed in Suppl. Table 1. First, the cornu-cervi was decocted with water (800 mL) for 0.5 h, while the other herbs were soaked in eight aliquots of distilled water (6.08 L, v/w). All the herbs were then mixed together and boiled for 0.5 h. After the first decoction was prepared and filtered, the residues were sequentially extracted with water twice (5.16 L, 3.44 L) for another 0.5 h. The above filtrates were merged and vacuum evaporated at 62 °C by rotavapor (EYELA; Shanghai, China) to obtain a final concentrate (2 g/mL). It was used for the experiment and stored at 4 °C.

2.2. Animal treatment and sample collection

Eight-week-old specific-pathogen-free female Sprague-Dawley rats weighing 180–200 g were purchased from the Zhejiang Laboratory Animal Center Co. Ltd. (Zhejiang, China). The animal experiment was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The animals were housed in the experimental animal center of the Nanjing University of Chinese Medicine, with a 12 h/12 h light/dark cycle at a constant temperature of 22 ± 2 °C. Cages, food, and water were sterilized before use. The animals were acclimatized for 1 week prior to initiating the experiments. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for Laboratory Animals and approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine (license No. SYXK (Su) 2014–0001). There were no significant differences in the weights of animals prior to starting the experiment. Rats were examined by daily vaginal smears to verify the regularity of estrous cycle and they presented a regular 4-day estrual activity. A total of 80 rats were divided into two groups (n = 40 in each group): normal control rats treated with saline (10 mL/kg); rats treated with BSZY-D (4 mL/kg) at 10:00 a.m. and 16:00 p.m. of estrus for three estrous cycles by gavage.

After treatment for three cycles, the rats were anesthetized with 10% chloral hydrate (3 ml/kg) at the metestrus phase of the estrous cycle. Cerebrospinal fluid (CSF) was collected at the foramen magnum and supernatant was collected by centrifugation (850 g, 10 min). Blood samples were collected and serum were separated by centrifugation (850 g, 10 min). All samples were stored at −80 °C.

2.3. High performance liquid chromatography-mass spectrometry (HPLC-MS) analysis

The authentic standards of Albiflorin (batch number: 150516), Paeoniflorin (batch number: 150726), Kaempferol (batch number: 141228), Hyperoside (batch number: 150411), Quercetin (batch number: 150522), Chinese yam (batch number: 150412, Saikosaponin A (batch number: 150316), Saikosaponin B2 (batch number: 150207), Saikosaponin C (batch number: 150608) and Saikosaponin D (batch number: 150113) were purchased from Shanghai Haling Bio-technology (Shanghai, China), and the purities of all these compounds were > 98% based on HPLC-UV. Formic acid (> 99%) and acetonitrile (> 99%) were obtained from ROWE Scientific Inc. (Newark, USA) and Merck (Billerica, USA).

The content of Albiflorin, Paeoniflorin, Kaempferol, Hyperoside, Quercetin, Chinese yam, Saikosaponin A, Saikosaponin B2, Saikosaponin C, and Saikosaponin D in the extract of BSZY-D, serum sample, and CSF samples were determined with LTQ Orbitrap-MS (Thermo Fisher Scientific, San Jose, CA) coupled with a HPLC model U3000 apparatus (Dionex, San Jose, CA).

Chromatographic separation was performed with BDS HYPERSIL C18 (2.1 mm × mm, 2.4 μm) column at 40 °C using a gradient mobile phase containing 0.01% formic acid in water (solvent A) and 0.5% acetonitrile in water (solvent B). The linear gradients were the following: 5% B for 0–5 min; 50% B for 4–20 min; 90% B for 20–23 min, 25% B for 20–22 min; 5% B for 23–26 min. Samples were analyzed at a resolution of 30,000. Full scan mass spectra were acquired in negative ion mode in the mass range *m/z* 100–1000. The voltage on the electrospray needle was set to 3.0 kV, and the capillary temperature was set at 300 °C. The sheath and auxiliary gases were set at 45 and 6 arbitrary units. The mobile phases were eluted at 0.20 mL/min, and the total injection volume was 10 μL.

2.4. CCK8 cell viability assay

R1200 cells were cultured in 96-well plates at a density of 1000 cells/well. The culture medium was removed after 24 h and 100 μl of medium containing different drug concentrations (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} mol/L for cetrorelix; 5%, 10%, and 20% for CSF) were added and cultured overnight. Then, 10μl of CCK8 was added and cultured for 2 h. Plate with no cell culture was set as the blank and culture medium without the drug was set as the control group. The sample had four replicates and the experiment was repeated thrice. Absorbance at 450 nm was measured using a microplate reader. Cell viability = (experimental group OD − blank group OD)/(control group OD − blank group OD) * 100%.

2.5. Cell culture and treatment

R1200 rat adenopharyngeal cells were purchased from Sciencell (Carlsbad, CA, USA; Cat No: 7209) and cultured in neuronal medium (Sciencell, Carlsbad, CA, USA; Cat No: 1521) or high glucose DMEM (Yuanpei, Shanghai, China; batch number: B40403) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA; batch number: 1647565) and penicillin-streptomycin (Yuanpei, Shanghai, China; batch number: G40403). Luteinizing hormone releasing hormone (LHRH) was purchased from Sigma (Steinheim, Germany; batch number: 91K1133) and cetrorelix was from Merck Serono (Idron,