

Original Article

Effect of BushenZhuyun decoction on gonadal axis in mifepristone-induced luteal phase deficiency rat model via kisspeptin/GRP54 pathway

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Abstract: Kisspeptin regulates pituitary-gonad axis via binding to G-protein coupled receptor 54 (GPR54). This study used mifepristone to generate a luteal phase deficiency (LPD) rat model, on which the effect of BushenZhuyun Decoction on kisspeptin/GPR54 signal pathway was investigated along with related mechanisms. Female SD rats (6 weeks age) were randomly assigned into control, LPD model, treatment group and positive control group (N=10 each). After two sexual cycles, rats were mated and received 10 mg/kg mifepristone on E3 to generate LPD model. Animals were sacrificed at E5. Ovarian and uterus morphology were observed by HE staining. Chemiluminescence was used to test serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E₂), progesterone (P). Protein levels of kisspeptin, GRP54 and gonadotropin releasing hormone (GnRH) were measured by Western blot. Compared with control group, LPD model rats had lower follicle/luteal number, endometrium thickness, serum P, P/E₂ levels, hypothalamic expression of kisspeptin, GPR54 and GnRH, and elevated FSH and LH levels (P<0.05). Treatment group and positive control group had higher follicle/luteal number, endometrium thickness, serum P, P/E₂ levels, hypothalamic expression of kisspeptin, GPR54 and GnRH, and suppressed FSH and LH levels (P<0.05 compared with model group). However, no differences were observed between treatment and positive control group (P>0.05). BushenZhuyun Decoction improves luteal function of LPD rats and facilitates embryo implantation, probably via kisspeptin/GPR54 signal pathway.

Keywords: BushenZhuyun decoction, kisspeptin, GPR54, gonad axis, mifepristone

Introduction

Luteal phase deficiency (LPD) is probably related with insufficient secretion of FSH or LH, and immature follicular development before ovulation [1, 2]. Hypothalamus-pituitary-ovarian axis (HPOA) is correlated with luteal function, as intervention of this may lead to LPD onset [3, 4]. Kisspeptin can stimulate gonadotrophin releasing hormone (GnRH) secretion, induce positive/negative feedback of E₂, and regulate HPOA function via binding to G-protein coupled receptor 54 (GPR54) [5, 6]. Inactivation of GPR54 gene can cause idiopathic hypogonadotropic hypogonadism (IHH) or other disorders. Some studies believed that initiation of adolescence in animals was also related with expression of KISS-1/GPR54 gene in the brain [7, 8].

Therefore it is speculated that kisspeptin/GPR54 signal pathway might be the potential target on regulating HPOA function. Previous studies have shown that kisspeptin could reduce the occurrence of ovarian hyperstimulation syndrome (OHSS) [9, 10]. As a common disease, LPD is manifested as repeated miscarriage and infertility. In Chinese medicine, LPD can be classified into categories of “fetal leakage”, “infertile” and “habitual abortion”. BushenZhuyun Decoction was developed to have satisfactory effects on treating LPD-related infertility as it can elevate pregnant rate in clinics. Both *in vitro* and *in vivo* studies showed the effect of BushenZhuyun on improving gonad axis endocrine function, regulating body hormone level, facilitating angiogenesis and luteal development, improving uterus

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capacity and treating infertility, but leaving detailed functional mechanism unillustrated yet. This study constructed a rat LPD model through administration of mifepristone, to observe the effect of BushenZhuyun Decoction on kisspeptin/GPR54 signal pathway, thus elucidating improvement of this decoction on gonad axis function in LPD rats and related mechanisms.

Materials and methods

Animals and grouping

Health female unpregnant SD rats (6 weeks age, body weight 200~220 g) and age-matched SD male rats (body weight 240~260 g) were provided by Laboratory Animal Center, Nanjing University of Traditional Chinese Medicine (Certificate No.: SYXK-2013-0025) Animals were separately housed in an SPF grade facility with standard chow and water provided *ad libitum*. Female rats were randomly divided into control (A), LPD model (B), which received 10 mg/kg mifepristone, treatment group (C), which received BushenZhuyun Decoction for two sexual cycles, and positive group (D) which received 2 mg/kg dydrogesterone. Control or model group received equal volume of saline.

This study has been pre-approved by the ethical committee of Jiangsu Province Hospital of Traditional Chinese Medicine.

Test drugs and reagents

BushenZhuyun Decoction (containing Yam, radix bupleuri and radix paeoniaealba) was purchased from Tongrentang Pharm (Beijing, China) and was prepared for 2 g/ml suspensions using traditional decoction method. Briefly, 300 ml water was added into BushenZhuyun Decoction for soaking about half an hour followed by big fire boiling and subsequent small fire for decoction about 20-30 minutes to obtain about 100 ml decoction liquid. After that, 300 ml water was added into the container for decoction about 20 min to obtain another 100 ml decoction liquid. The two 100 ml decoction liquid were combined and mixed, which was then concentrated into 2 g/ml suspensions and was stored at 4°C. Mifepristone (25 mg pill) was purchased from Zizhu Pharm (Qinhuangdao, Hebei, China) and was prepared

for 1 mg/ml suspensions in 0.5% carboxymethylcellulose sodium, which was stored at 4°C. Dydrogesterone (10 mg/kg) was purchased from the Dutch solvay pharmaceuticals co., LTD. (Shenyang, Liaoning, China). Hydrate chloral, paraformaldehyde and carboxymethylcellulosesodium were purchased from KemiouChem (Tianjin, China). Goat anti-rabbit horseradish peroxidase labelled secondary antibody, rabbit anti-kisspeptin, anti-GPR54 and GnRH polyclonal antibody were purchased from Santa Cruz (Dallas, Texas, USA). Test kit for progesterone (P), follicular stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E_2) were purchased from ZhongshanJinqiao (Beijing, China). Microscope model BX41 was purchased from Olympus (Shinjuku, Tokyo, Japan). Imaging analysis was performed by Quantity One software.

Animal model preparation

Animals were acclimated for 3 days. Their sexual cycle was observed by vaginal smear assay for 10 consecutive days. In brief, sterile cotton was dipped in saline and collected vaginal secretion from un-pregnant female rats. The secretion was paved evenly on the slide, which was observed after HE staining. Those rats with major difference in sexual cycles were excluded. Treatment group received drugs daily for 2 sexual cycles at a dose of 1 mg/100 g and 19.6 g/kg, which was pre-determined based on pilot study. Group D received 2 mg/kg dydrogesterone as a positive control. The other two groups received equal volume of saline. At the end of second sexual cycle, rats were mated at 2:1 female/male ratio. In the next morning, vaginal smear was collected from female rats to record the existence of sperms. On pregnant day 3, LPD model was prepared by 10 mg/kg mifepristone by gavage in both model and treatment group. On pregnant day 5, rats were sacrificed and collected for brain, ovary and uterus tissue samples. Morphology was observed under the microscope.

Serum P, FSH, LH and E_2 assay

5 ml artery blood samples were collected from each rat. Serum was collected by 1500 g centrifugation for 10 min. Chemiluminescence assay was performed for quantifying the serum levels of sex hormones.

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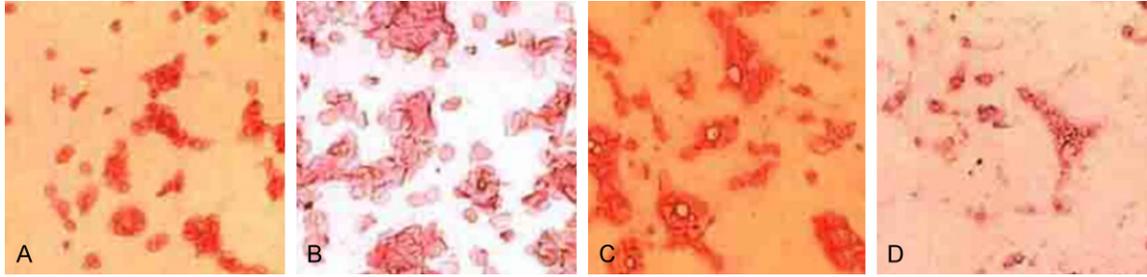


Figure 1. Observation of vaginal smear in female rats (HE staining, $\times 100$). A. Proestrus (oval nucleus epithelial cells); B. Estrus (platelet and keratinized epithelium); C. Metestrus (keratinized leukocytes, epithelium and nuclear cells); D. Anestrus (leukocyte only).

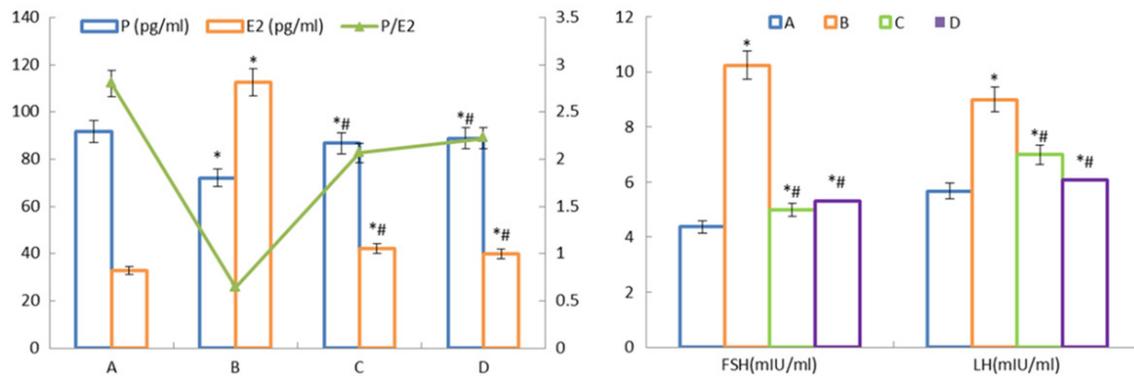


Figure 2. Effects of BushenZhuoyun Decoction on serum sex hormones of LPD rats. * $P < 0.05$ compared to control group; # $P < 0.05$ compared to model group. A. Control group; B. Model group; C. Treatment group; D. Positive control group. Left Y axis indicated the value of P (pg/ml) and E₂ (pg/ml) as well as FSH (mIU/ml) and LH (mIU/ml) Right Y axis represented the ratio of P/E₂.

Hematoxylin-eosin (HE) staining for ovary and uterus morphology

After sacrifice, ovary and uterus samples were collected. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin to generate 5 μ l slices, which were stained by HE method and observed in a light field microscope. Imaging analysis was performed to count number of follicles and luteal, thickness of endometrium and gland.

Western blot for kisspeptin, GPR54 and GnRH protein level

Rat brain tissues were lysed and collected the supernatant by centrifugation. BCA method was used to quantify proteins, which were separated by SDS-PAGE and transferred to PVDF membrane. Followed by 1 h blocking, primary antibody (kisspeptin at 1:100, GPR54 at 1:100 and GnRH at 1:200) was added for 4°C overnight incubation. After TBST washing, second-

ary antibody (1:1000) was added for 1 h incubation. After TBST rinsing, the membrane was developed and exposed. Protein bands were analyzed by Quantity One software and calculated for relative expression level, which was the ratio of target protein band's optical density to that of internal reference.

Statistics

SPSS20.0 software was used for statistical analysis. Measurement data were tested for normality firstly. Those fitted normal distribution were presented as mean \pm standard deviation (SD). One-way analysis of variance and LSD test was used for comparison of means. A statistical significance was defined when $P < 0.05$.

Results

General conditions of rats

Control group had normal motility and shiny furs. Model group had abnormal furs, with spi-

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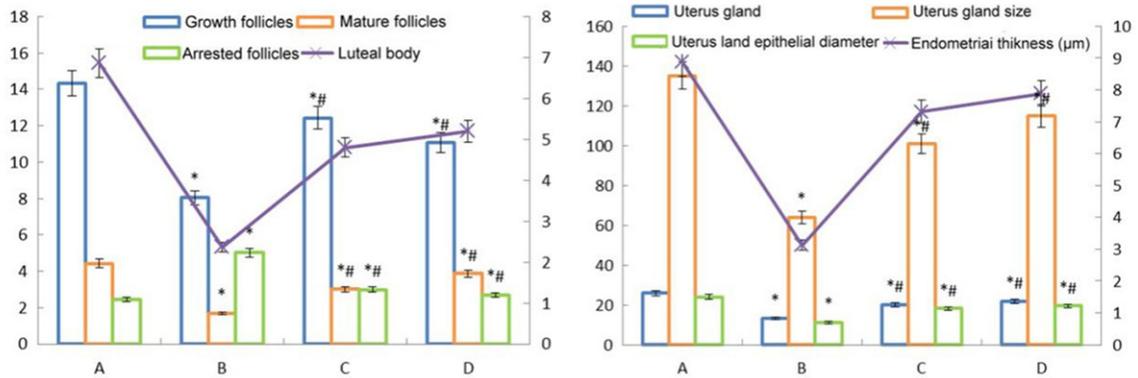


Figure 3. Effects of BushenZhuyun decoction on uterus and ovary of LPD rats. * $P < 0.05$ compared to control group; # $P < 0.05$ compared to model group. A. Control group; B. Model group; C. Treatment group; D. Positive control group. Left Y axis indicated the number of Growth, Mature and Arrested follicles in the left panel and the number of Uterus gland, Uterus gland size and Uterus gland epithelial diameter in the right panel. Right Y axis represented the number of Luteal body in the left panel and endometrial thickness in the right panel.

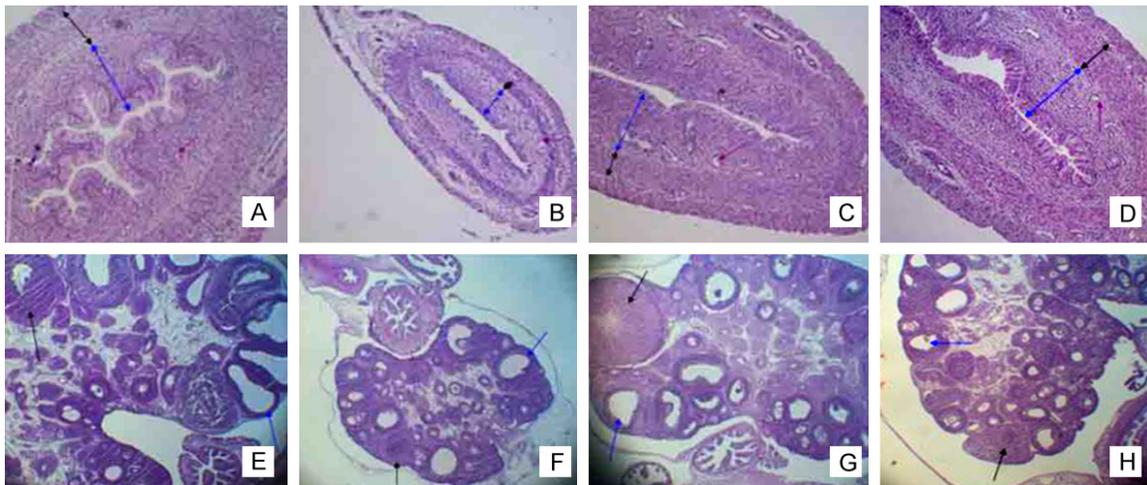


Figure 4. Ovary and uterus tissue morphology (HE staining, $\times 200$). A. Uterus in control group (Decidua-like endometrial mesenchymal tissues, with synchronized development of mesenchyme and gland body, plus visible nuclear vacuoles); B. Uterus in model group (Edema in mesenchymal, loosening and secretory like change, unsynchronized development of endometrial gland and mesenchymal, plus proliferative change in the gland with sub-nuclear vacuoles); C. Uterus in treatment group; D. Uterus in the positive control group. Blow arrow, thickness of endometrium; black arrow, thickness of uterus muscular layer; purple arrow, gland body. E. Ovary in control group (less mesenchymal, visible primordial, primary and secondary follicles); F. Ovary in model group (less luteal body, more mesenchymal gland, reduced follicle size); G. Ovary in treatment group; H. Ovary in positive control group. Blue arrow, follicle; black arrow, luteal body.

nal curvature. Treatment group had improvement of fur condition and motility compared to model group. Fertility was found when sperms existed in vaginal smear of normal female rats (**Figure 1**).

Serum sex hormone levels in LPD rats

Compared with control group, model rats had lower serum P and P/E_2 values, plus higher FSH and LH ($P < 0.05$). Treatment group and positive

control group had significantly improved P, P/E_2 , FSH and LH levels ($P < 0.05$ compared with model group, **Figure 2**). No differences were observed between treatment and positive control group ($P > 0.05$).

Effect of BushenZhuyun decoction on ovary and uterus morphology of LPD rats

Compared with control group, model rats had lower ovary follicle and luteal number, plus

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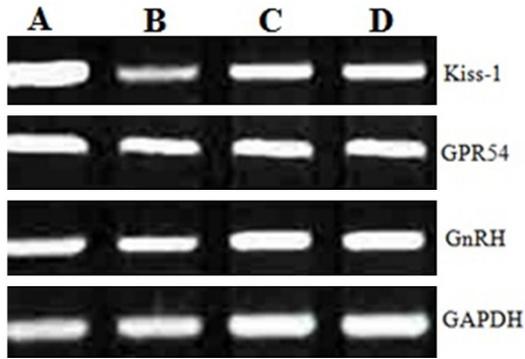


Figure 5. Western blot for hypothalamic expression of kisspeptin, GPR54 and GnRH. A. Control group; B. Model group; C. Treatment group; D. Positive control group.

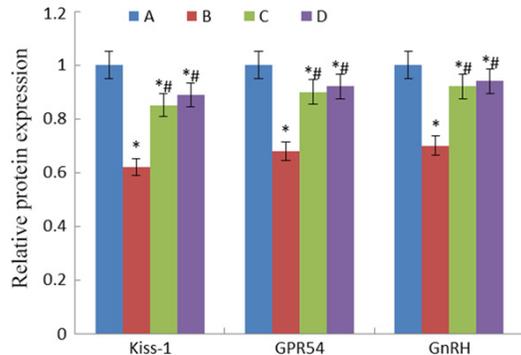


Figure 6. Effects of BushenZhuyun Decoction on hypothalamic expression of kisspeptin, GPR54 and GnRH in LPD rats. *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to model group. A. Control group; B. Model group; C. Treatment group; D. Positive control group.

lower endometrial thickness or glands. Treatment group and positive control group had increased ovarian follicle or luteal number, thickening of endometrium and more glands ($P < 0.05$, **Figure 3**). Under light field microscope, tissue morphology of ovary and uterus were significantly improved (**Figure 4**). However, no differences were found between treatment and positive control group.

Effects of BushenZhuyun decoction on kisspeptin, GPR54 and GnRH expression in hypothalamus of LPD rats

Compared with control group, model group had decreased expression of hypothalamic kisspeptin, GPR54 or GnRH. Treatment and positive control group had elevated expression lev-

els ($P < 0.05$ compared to model group, **Figures 5 and 6**) without differences between treatment and positive control.

Discussion

Female infertility is frequently correlated with luteal dysfunction. Mifepristone can bind to P receptor with high affinity, thus directly or indirectly mediating HPOA axis function. High dosage of mifepristone can inhibit follicular development, lyse luteal body, suppress P and E_2 level, and shorten luteal phase, probably via inhibiting granular cell synthesis, which further stimulate plasminogen activator activity in luteal cells, leading to endometrium detachment and luteal function inhibition [11, 12]. Embryo implantation can only occur during acceptance or sensitive period of uterus, which is featured by endometrial change [13]. This study applied mifepristone to inhibit rat endometrial or luteal development on pregnant day 3, to generate LPD-induced rat infertile model, on which endometrial and ovarian tissue morphology were observed. Results showed lower follicular/luteal number, endometrial thickness and gland in model group. BushenZhuyun Decoction treatment increased follicular/luteal number, endometrial thickness and gland number compared with model group. Mifepristone arrested transition of endometrium toward secretory phase, thus blocking embryo implantation. BushenZhuyun Decoction can improve luteal function, facilitate endometrial development, thus benefiting embryo implantation.

Progesterone negatively mediates HPOA axis function, inhibits FSH or LH secretion, and facilitates decidual-like change of uterus mesenchymal and transition of endometrium from growth phase into secretory phase, thus preparing for embryo implantation and further development. Insufficient secretion of progesterone can lead to underdevelopment of endometrium. Progesterone produced by ovary during luteal phase has major effects on endometrial development. E_2 level can reflect ovarian function to certain extents, as underdevelopment of ovary is correlated with insufficient E_2 secretion, which can impede embryo implantation [14, 15]. Ovary progesterone/ E_2 level affects uterus compatibility, as individuals with luteal dysfunction usually have imbalance of P/ E_2 ratio. Lower P/ E_2 ratio suppresses fertilization and pregnancy.

This study showed alternation of reproductive hormones in rats with luteal dysfunction. BushenZhuyun Decoction improved reproductive hormone levels, indicating that mifepristone affected ovarian function. High body FSH level inhibits FSH receptor in primordial follicles. BushenZhuyun Decoction facilitated progesterone secretion and induced granular cell genesis, thus facilitating follicular development and improving maturity of ovarian luteal development and embryo compatibility, benefiting pregnancy maintenance.

During puberty of primates, activation of GPR54 can facilitate GnRH release, during which KISS-1 gene expression and brain release of kisspeptin polypeptide play important roles in driving HPOA axis into sexual maturation via kisspeptin/GPR54 signal pathway [16, 17]. The functional mechanism is believed to be related with elevated intracellular calcium level in GnRH positive neurons for action potential generation. Previous study showed the close correlation between kisspeptin and infertile disease such as polycystic ovarian syndrome (PCOS), as demonstrated by significantly elevated kisspeptin level in the serum of PCOS patients. Moreover, kisspeptin has certain correlations with other reproductive endocrine disorders [18, 19]. Injection of kisspeptin into lateral ventricles can stimulate GnRH release in mice, and GnRH antagonist can block kisspeptin-induced stimulatory effects [19-21]. In this study, model rats had decreased expression of hypothalamic kisspeptin, GPR54 and GnRH. Compared with model group, BushenZhuyun Decoction treatment elevated these gene expressions, indicating that this formula might stimulate GnRH and sex hormone release via kisspeptin/GPR54 signal pathway, thus improving luteal function of LPD rats.

Conclusion

BushenZhuyun decoction can improve luteal function of LPD rats and facilitate embryo implantation, probably via kisspeptin/GPR54 signal pathway in LPD rats.

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Disclosure of conflict of interest

None.

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