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Kisspeptin regulates gonadotropin-releasing hormone secretion in gonadotropin-releasing hormone/enhanced green fluorescent protein transgenic rats☆

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Abstract
Kisspeptin is essential for activation of the hypothalamo-pituitary-gonadal axis. In this study, we established gonadotropin-releasing hormone/enhanced green fluorescent protein transgenic rats. Rats were injected with 1, 10, or 100 pM kisspeptin-10, a peptide derived from full-length kisspeptin, into the arcuate nucleus and medial preoptic area, and with the kisspeptin antagonist peptide 234 into the lateral cerebral ventricle. The results of immunohistochemical staining revealed that pulsatile luteinizing hormone secretion was suppressed after injection of antagonist peptide 234 into the lateral cerebral ventricle, and a significant increase in luteinizing hormone level was observed after kisspeptin-10 injection into the arcuate nucleus and medial preoptic area. The results of an enzyme-linked immunosorbent assay showed that luteinizing hormone levels during the first hour of kisspeptin-10 infusion into the arcuate nucleus were significantly greater in the 100 pM kisspeptin-10 group than in the 10 pM kisspeptin-10 group. These findings indicate that kisspeptin directly promotes gonadotropin-releasing hormone secretion and luteinizing hormone release in gonadotropin-releasing hormone/enhanced green fluorescent protein transgenic rats. The arcuate nucleus is a key component of the kisspeptin-G protein-coupled receptor 54 signaling pathway underlying regulating luteinizing hormone pulse secretion.

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Key Words
neural regeneration; basic research; gonadotropin-releasing hormone; enhanced green fluorescent protein; transgenic; luteinizing hormone; G protein-coupled receptor 54; medial preoptic area; arcuate nucleus; anteroventral periventricular nucleus; organum vasculosum of the lamina terminalis; photographs-containing paper; neuroregeneration

Research Highlights
(1) Kisspeptin directly promotes gonadotropin-releasing hormone secretion in gonadotropin-releasing hormone/enhanced green fluorescent protein transgenic rats.
(2) Kisspeptin is a gonadotropin-releasing hormone pulse generator.
(3) The arcuate nucleus plays an important role in regulation of pulsatile luteinizing hormone secretion.
(4) The regulatory effect of kisspeptin on gonadotropin-releasing hormone secretion can be directly observed by localization of gonadotropin-releasing hormone gene expression using a green fluorescent protein reporter gene.

Abbreviations
GnRH, gonadotropin-releasing hormone; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay

INTRODUCTION

Kisspeptins are a family of peptides encoded by the KiSS-1 gene[1]. The receptor for kisspeptin can activate G protein-coupled receptor 54[2-4]. Kisspeptin-G protein-coupled receptor 54 signaling plays an important role in initiating secretion of gonadotropin-releasing hormone (GnRH) at puberty[5]. Numerous studies have confirmed that kisspeptin administration stimulates GnRH or luteinizing hormone secretion in various species including mice[6], rats[7-8], sheep[9], monkeys[10] and humans[11]. Kisspeptin perikarya are located in two discrete hypothalamic regions in rodents: the anteroventral periventricular nucleus and the arcuate nucleus. Kisspeptin's effects on GnRH expression have been determined by immunohistochemistry and enzyme linked immunosorbent assay (ELISA), but there have been few studies that have used a reporter gene.

In this study, we performed microinjection of fertilized eggs and embryo transfer to establish the GnRH/enhanced green fluorescent protein (EGFP) transgenic rats, and then used this model to study pulsatile luteinizing hormone secretion following kisspeptin injection of GnRH/EGFP transgenic rats by immunohistochemistry and ELISA.

RESULTS

Quantitative analysis of experimental animals

A total of 100 Wistar-Imamich rats were used to establish GnRH/EGFP transgenic rats. Fifty successful rat models were randomly divided into three groups: an intracerebroventricular group (n = 10), an intra-arcuate nucleus group (n = 20) and an intra-medial preoptic area group (n = 20). Five rats from each group were injected with artificial cerebrospinal fluid (control group). The remaining five rats from the intracerebroventricular group were injected with the kisspeptin antagonist peptide 234. The remaining 15 rats from each of the intra-arcuate nucleus and intra-medial preoptic area groups were randomly assigned to three subgroups, and separately injected with 1, 10 or 100 pM kisspeptin-10, a peptide derived from full-length kisspeptin. All 50 rats were included in the final analysis, with no drop outs.

EGFP expression level in GnRH neurons of GnRH/EGFP transgenic rats

Green fluorescent cells were confined to areas in which GnRH neurons are known to be distributed (Figures 1A–D). These areas included the olfactory bulb, terminal ganglia, medial and lateral septal nuclei, the diagonal band of Broca and the preoptic area, which contained the largest number of green fluorescent cells, and the following caudal hypothalamic regions: the retrochiasmatic area, the retrochiasmatic part of the supraoptic nucleus, the lateral hypothalamic area, and the arcuate nucleus. EGFP fluorescence was also detected in axons and dendrites in these areas, as well as in axon terminals in the organum vasculosum of the lamina terminalis and median eminence.

All green fluorescent neurons in brain slices from GnRH/EGFP transgenic rats contained GnRH. We observed green fluorescence in only 65% of GnRH-positive neurons, perhaps because the fluorescence in the remaining GnRH neurons could not be detected (Figure 2). The number of GnRH-immunopositive neurons was greater than the number of fluorescent neurons.

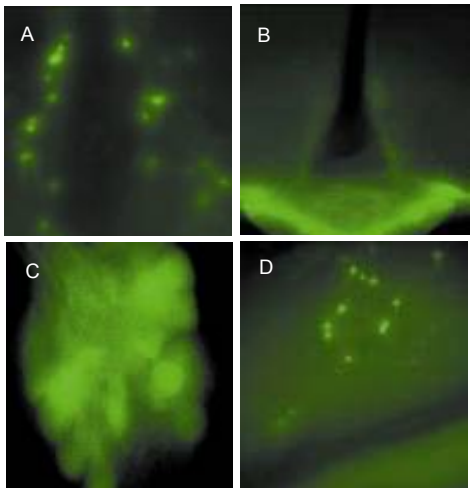

Figure 1 Green fluorescent protein (GFP) reporter gene and GFP-expressing neurons in brain tissue from gonadotropin-releasing hormone/enhanced green fluorescent protein (GnRH/EGFP) transgenic rats (fluorescence microscope).

Green fluorescent cells were confined to areas in which GnRH neurons are known to be distributed. The fluorescent neurons in C are the GnRH-positive neurons in B; the number of GnRH-positive neurons is larger than the number of fluorescent neurons.

(A) Preoptic area (× 80); (B) the third ventricle (× 30); (C) diagonal band of Broca (× 40); (D) terminal ganglion (× 400).

Effect of intracerebroventricular administration of kisspeptin antagonist on pulsatile luteinizing hormone secretion

Pulsatile luteinizing hormone secretion was effectively suppressed immediately after infusion of the kisspeptin

antagonist peptide 234 (2.5 nmol/h). Luteinizing hormone pulse frequency was significantly lower in the kisspeptin antagonist group compared with the artificial cerebrospinal fluid infusion group (Figure 3C; $P < 0.05$).

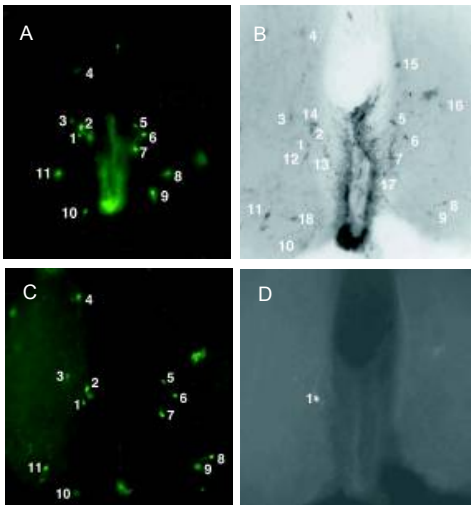

Figure 2 Green fluorescent protein (GFP) and gonadotropin-releasing hormone (GnRH) expression in brain tissue from GnRH/enhanced green fluorescent protein (EGFP) transgenic rats (fluorescence microscope, × 100).

(A) GFP-expressing neurons, numbered 1–11, near the diagonal band of Broca-preoptic area border at the level of the organum vasculosum of the lamina terminalis from the same mouse. (C) Same slice as in A after fixation. Same slice as in C after immunostaining for GnRH. GnRH-immunopositive neurons are numbered 1–19 (B–D).

Effects of kisspeptin-10 administration on luteinizing hormone secretion in the rat arcuate nucleus and medial preoptic area

Both intra-arcuate nucleus and intra-medial preoptic area administration of kisspeptin-10 resulted in a dose-dependent increase in circulating levels of luteinizing hormone that lasted approximately 1 hour before recovering to a normal pulsatile pattern of luteinizing hormone secretion (Figures 4B, D; $P < 0.05$). The mean area under the luteinizing hormone profile during the first hour of kisspeptin-10 infusion was significantly greater in the 100 pM kisspeptin-10 group than in the 10 pM kisspeptin-10 group (Figures 4E, F; $P < 0.05$). Control intra-nuclear injection of artificial cerebrospinal fluid had no effect on luteinizing hormone secretion ($P > 0.05$).

DISCUSSION

The recent development of selective kisspeptin antagonists has facilitated investigations of the role of endogenous kisspeptin in the control of the hypothalamo-pituitary-gonadal axis. Central administration of the kisspeptin antagonist peptide 234 inhibited the post-castration rise in luteinizing hormone secretion in rats, blunted the luteinizing hormone response to exogenous kisspeptin in rats, suppressed luteinizing hormone pulse frequency and amplitude in ewes, and suppressed GnRH pulses in monkeys[12-14].

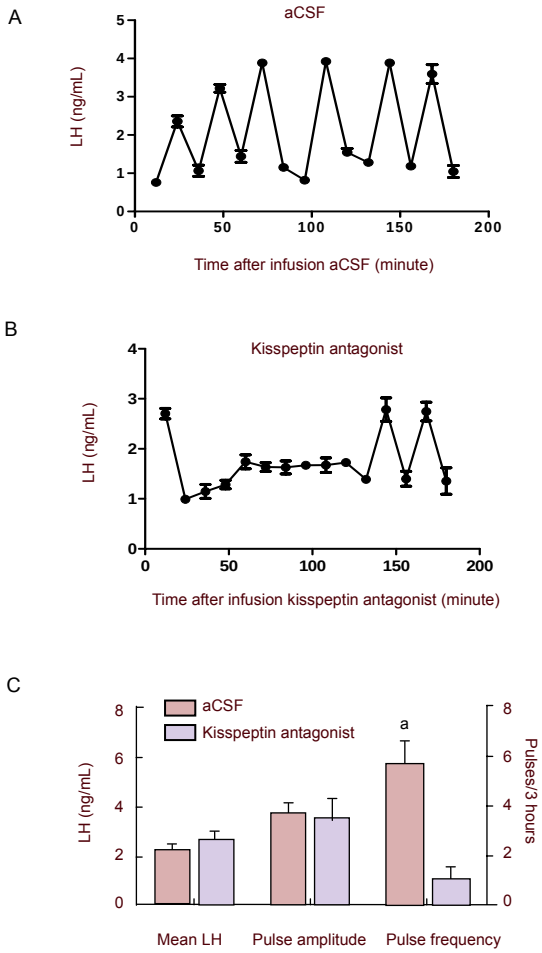

Figure 3 Effect of intracerebroventricular administration of kisspeptin antagonist on luteinizing hormone (LH) pulse frequency.

(A) Injection of artificial cerebrospinal fluid (aCSF) (4 μL/h) into the cerebral ventricular zone.

(B) Injection of kisspeptin antagonist (205 nmol/h) into the cerebral ventricular zone; these animals showed a complete absence of LH pulses during kisspeptin antagonist infusion (12–120 minutes).

(C) Comparison of mean LH levels (left vertical axis), pulse amplitudes and LH pulse frequencies (right vertical axis, times) in the cerebral ventricular zone between aCSF and kisspeptin antagonist groups.

[a]$P < 0.05$, *vs*. kisspeptin antagonist peptide 234 group (mean ± SEM, $n = 5$; one-way analysis of variance, paired samples *t*-test).

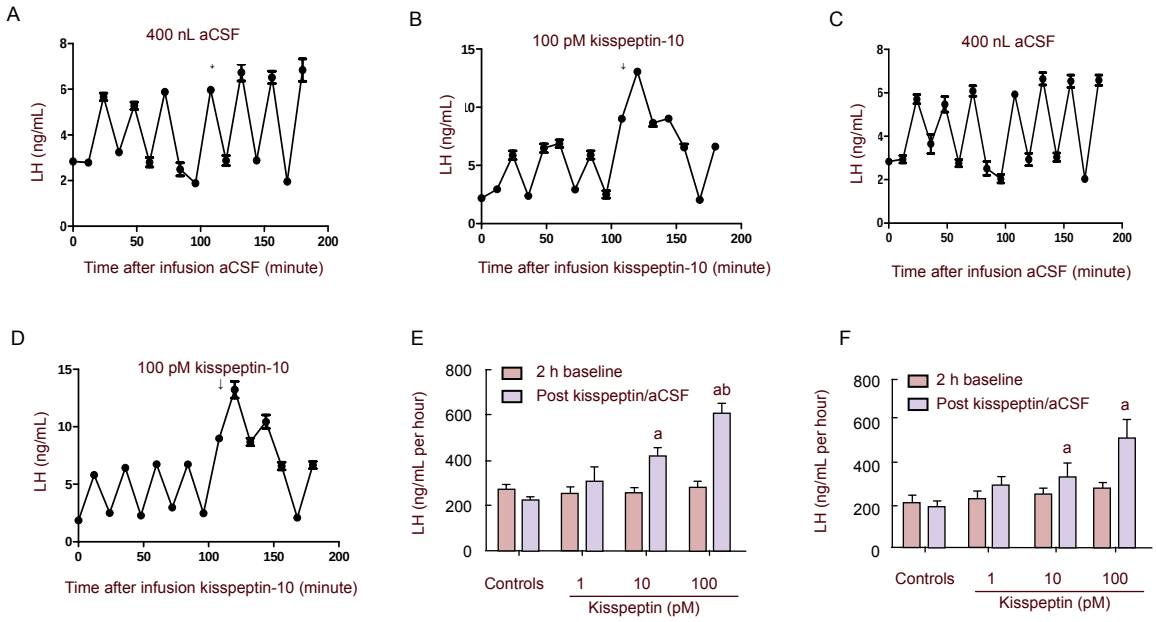

Figure 4 Effects of administration of kisspeptin-10 into the arcuate nucleus (ARC) and medial preoptic area (mPOA) on luteinizing hormone (LH) secretion.

Representative examples illustrating the effects of intra-ARC infusion of 400 nL of artificial cerebrospinal fluid (aCSF) (A) or 100 pM kisspeptin-10 (B) in gonadotropin-releasing hormone/enhanced green fluorescent protein (GnRH/EGFP) rats. Representative examples illustrating the effects of intra-mPOA infusion of 400 nL aCSF (C) or 100 pM kisspeptin-10 (D) in the rats. Effect of intra-ARC (E) and intra-mPOA (F) infusion of kisspeptin-10 on LH secretion. Two-hour baseline: Base background value.

[a]$P < 0.05$, *vs*. 1 pM kisspeptin-10 group; [b]$P < 0.05$, *vs*. 10 pM kisspeptin-10 group (mean ± SEM, $n = 5$; one-way analysis of variance, paired samples *t*-test).

However, the precise neural site of action of the antagonist remains to be established. The results of this study reveal that administration of a selective kisspeptin antagonist profoundly suppresses pulsatile luteinizing hormone secretion in rats.

This finding supports findings in the rhesus monkey where perfusion of the antagonist into the stalk-median eminence apparently suppressed GnRH pulses, and findings in the ewe where intracerebroventricular infusion of the antagonist apparently suppressed luteinizing hormone pulse amplitude[15-16]. However, as in these previous studies, it is sometimes difficult to differentiate between amplitude and frequency effects as pulses become undetectable if the amplitude is strongly suppressed. In the present study, there was an immediate and complete absence of luteinizing hormone pulses following intracerebroventricular infusion of the antagonist. In addition, there was a rapid restoration of luteinizing hormone pulses with normal pulse amplitude but reduced frequency post-treatment. These findings suggested that kisspeptin affects the GnRH pulse generator[17-18]. However, the most definitive evidence that the kisspeptin antagonist modulates the frequency of GnRH pulses was derived from our demonstration that intra-arcuate nucleus administration reduced luteinizing hormone pulse frequency in a dose-dependent manner without affecting pulse amplitude. In summary, the present study shows that administration of a selective kisspeptin antagonist into the arcuate nucleus, but not into the medial preoptic area, reduced luteinizing hormone pulse frequency in a dose-dependent manner without affecting pulse amplitude in GnRH/EGFP transgenic rats. These data are the first to identify the arcuate nucleus as a key site for kisspeptin modulation of luteinizing hormone pulse frequency in GnRH/EGFP transgenic rats, supporting the notion that kisspeptin-G protein-coupled receptor 54 signaling in this region of the mediobasal hypothalamus is a critical neural component of the hypothalamic GnRH pulse generator.

The GnRH/EGFP transgenic rats generated in this study will facilitate studies of the cellular basis of pulsatile and neurotransmitter-modulated GnRH release, including further studies into the manner in which GnRH neuronal physiology varies with brain region, age, sex, reproductive state, and environmental factors[19-21].

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed in the School of Medicine Laboratory, Beihua University, China from October 2009 to October 2011.

Materials

All clean Wistar-Imamich rats obtained from Shanghai SLAC Laboratory Animal Co., Ltd., (License No. SCXK (Hu) 2010-008) were housed under controlled conditions (12-hour light/dark cycle with lights on at 19:00; temperature at 22 ± 2°C) and provided with free access to food and water. Rats ranged from 8 to 13 months of age, weighing 250–350 g, with equal numbers of male and female rats. The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of the People's Republic of China[22].

Methods

Preparation of GnRH/EGFP transgenic rats

Wistar-Imamich rat fertilized eggs were used for microinjection and embryo transfer was performed to establish GnRH/EGFP transgenic rats as follows. High-quality single-cell fertilized embryos in batches of 30–40 were placed under an Olympus inverted microscope for male pronuclear microinjection[23-24]. To ensure maximum consistency, the impact of the needle was excluded. Injection needles of the same specifications and from the same batch were used. An IM-300 automatic injection device (Narishige, Sea Cliff, NY, USA) was used to control injection pressure and dose. After injection, embryos were transferred in drops of fresh M16 culture medium at 37°C, in a 5% CO_2 incubator, for 60 minutes. The numbers of living embryos were examined under an inverted microscope and recorded. Egg cytoplasm with a complete egg plasma membrane and a normal perivitelline space was taken as the indication for a living embryo. Those showing cytoplasmic diffusion were considered to represent embryonic death. Surviving single-cell embryos were bilaterally transplanted through the fallopian tubes of Wistar-Imamich pseudopregnant rats in the same estrus period, with 8–12 embryos being implanted on each side. At 19–22 days after transplantation, the number of births was recorded[25].

Integration of PCR detection of the first established rats

DNA was extracted from rat tail tissue. A pair of specific primers was designed based on the microinjection sequence (Table 1). PCR was used to amplify a 278-bp DNA fragment (Figure 5), the presence of which indicated successful model establishment. Finally, a total of 50 GnRH/EGFP transgenic rats of both genders were obtained (aged 11.7 ± 0.6 months).

Table 1 Gonadotropin-releasing hormone gene primer

Primer	Sequence (5'–3')	Product size (bp)
Gonadotropin-releasing hormone	Sense: CCG CTG TTG TTC TGT TGA CT Antisense: GCA GAT CCC TAA GAG GTG AA	20

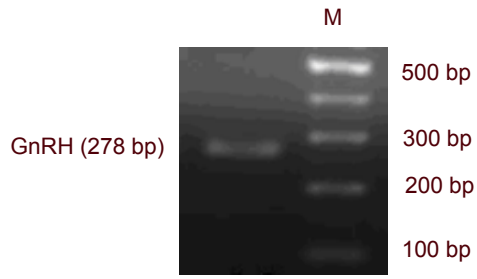

Figure 5 PCR detection of the first established rats.
M: 100 bp DNA marker; GnRH: gonadotropin-releasing hormone.

GnRH protein expression in rat brain tissues, as determined by immunohistochemical staining

Briefly, brain tissue sections were fixed for 1 hour in 4% paraformaldehyde (Sigma, Poole, UK), washed twice with PBS, and incubated for 1 hour in 2% (v/v) normal goat serum (Invitrogen, Paisley, Scotland) in PBS supplemented with 1% (w/v) bovine serum albumin (Invitrogen) and 0.3% (v/v) Triton X-100 (Invitrogen). Slices were then placed overnight in buffer containing mouse anti-human polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which recognizes amino acids 6–10 of GnRH in pro-GnRH and GnRH, at a dilution of 1:10 000. On the following day, slices were washed twice with PBS supplemented with 0.3% bovine serum albumin and 0.1%Triton X-100, incubated for 1 hour in 7-amino-4-methylcoumarin-3-acetic acid-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at a dilution of 1:100 in buffer, washed twice with buffer, three times in PBS followed by H_2O, and then mounted on poly-L-lysine-coated glass slides

using an aqueous medium. Immunostained slices were observed using an upright fluorescence microscope (Olympus BX51, excitation filter G365, dichroic mirror FT395, and emission filter LP420, Tokyo, Japan)[26-27].

Kisspeptin or antagonist infusion into the lateral ventricle

On the morning of experiment, a single internal injection cannula with extension tubing, preloaded with the selective kisspeptin antagonist peptide 234 (7.5 nM in 12 μL of artificial cerebrospinal fluid) or artificial cerebrospinal fluid (12 μL) was inserted into the guide cannula, extending 1.0 mm beyond its tip to reach the left ventricle. After 2 hours of venous blood sampling at the elbow, kisspeptin antagonist peptide 234 or artificial cerebrospinal fluid was infused into the relevant animals over a period of 3 hours. Blood sampling continued throughout the experiment. Blood samples were frozen at –20°C for later assay to determine luteinizing hormone concentrations by ELISA[26]. The method of injecting 1 pM, 10 pM and 100 pM kisspeptin-10 + 400 nL of artificial cerebrospinal fluid into the arcuate nucleus or medial preoptic area was as described above.

Intra-arcuate nucleus or intra-medial preoptic area infusion of kisspeptin-10

For administration of kisspeptin-10, bilateral injection cannulae (Plastics One) with extension tubing preloaded with different doses of kisspeptin-10 (Sigma, St. Louis, MO, USA) in 400 nL of artificial cerebrospinal fluid per brain locus (arcuate nucleus or medial preoptic area), or artificial cerebrospinal fluid, were inserted into the guide cannulae, extending 1.0 mm beyond its tip to reach the site of the brain nuclei. The distal end of the tubing was extended outside of the animal cage to allow remote infusion without disturbing the rats. The automated blood sampling system was set up in the same way as described above for the collection of 25-mL blood samples every 5 minutes, but only for 5 hours. All treatments were given by injection over 5 minutes after 2 hours of blood sampling.

Pulsatile GnRH release level, as detected by ELISA

In rats, a strong temporal relationship exists between the onset of a GnRH pulse in hypothalamic perfusates and the secretion of luteinizing hormone into the plasma, indicating that release of luteinizing hormone is a reasonable index of episodic GnRH release[28]. Accordingly, we used repetitive sampling of venous blood in castrated rats to determine the pattern of luteinizing hormone secretion. On the following day, sequential blood samples were taken between 800 and 1 200, an interval during which episodic luteinizing hormone secretion has been demonstrated in rats. Blood samples were collected in 1-mL syringes at 12-minute intervals for 3 hours[29-31]. The luteinizing hormone level was detected using an ELISA kit (Santa Cruz Biotechnology).

Statistical analysis

Data are expressed as mean ± SEM. Differences in luteinizing hormone pulse frequency were compared using one-way analysis of variance (α = 0.05). In the case of steroid-induced and mating-induced responses, differences in plasma luteinizing hormone concentrations were also analyzed by one-way analysis of variance (α = 0.05). Differences between two groups were analyzed utilizing paired samples *t*-tests (α = 0.05).

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Author contributions: Haogang Xue and Chunying Yang conceived and designed the experiments. Xiaodong Ge performed the experiments. Weiqi Sun analyzed the data. Chun Li and Mingyu Qi provided reagents/materials/analysis tools. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Experimental Animal Ethics Committee, Beihua University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

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