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Effect of nourishing "Yin"-removing "Fire" Chinese herbal mixture on hypothalamic kisspeptin expression in female precocious rats

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ABSTRACT

Aim: The present study aims to investigate the effect of nourishing "Yin"-removing "Fire" herbal mixture, a Chinese herb-based formulation, on hypothalamic kisspeptin expression in danazol-induced female precocious model rats.
Materials and methods: The female Sprague–Dawley rats were divided into intact normal (N), central precocious puberty (CPP) model (M), vehicle without CPP (V), CPP model exposed to herbal mixture (HM) and CPP model exposed to saline (S) groups. At postnatal day 5, a single subcutaneous injection of 300 μg of danazol was administered to induce CPP model rats. From P15, rats in the HM group were continuously gavaged with the 1 ml/50 g body weight mixture, until two consecutive regular estrous cycles were established. The hypothalamic Kiss-1 expression was detected by RT-PCR and immunohistochemistry.
Results: The day of vaginal opening and establishment of two regular estrous cycles were delayed in the HM group compared with M and S groups ($P < 0.05$, respectively). The level of hypothalamic Kiss-1 mRNA and the number of kisspeptin-immunoreactive (kisspeptin-ir) cells in the arcuate nucleus (ARC), preoptic area (POA) and periventricular nucleus (PeN), were decreased significantly in the HM group compared with the M and S groups ($P < 0.01$, respectively) on the day of onset-puberty. These results indicate that the kisspeptin signaling pathway might be involved in the effect of herbal mixture treatment on CPP.

1. Introduction

The neuroendocrine control of puberty onset relies on the concerted action of a complex network of regulatory signals. The decapeptide hypothalamic gonadotropin-releasing hormone (GnRH) operates as the driving signal for the pulsatile release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary (Divall and Radovick, 2009). A GnRH analogue has been recognized as an effective treatment for advance puberty onset in CPP (Kletter and Kelch, 1994; Lee, 1994; Shankar and Pescovitz, 1995).

The molecular determinant that control GnRH release was recently revolutionized by the identification of kisspeptins and their receptor, *Kiss-1r* (Gianetti and Seminara, 2008; Uenoyama et al., 2009; Colledge, 2009). Both male and female rats show an obvious increase in Kiss-1 mRNA levels that coincides with the onset of puberty (Navarro et al., 2004a; KuoHuang and Kaiser, 2006; Roseweir and Millar, 2009). Pharmacological analyses further set the contention that kisspeptin is likely the most potent elicitor of the GnRH/gonadotropic axis known so far (Navarro et al., 2004a; Navarro et al., 2005; Thompson et al., 2004). Repeated administration of kisspeptin-10 to female rats induces robust LH release, but the ability is completely abrogated when GnRH actions are blocked (Navarro et al., 2004b). As such, it is proposed that kisspeptin might play a potential role in puberty onset and GnRH/LH release. Interestingly enough, our previous study observed that hypothalamic Kiss-1 mRNA and kisspeptin were increased on the day of puberty onset in danazol-induced precocious rats (Sun et al., 2007). Central administration of kisspeptin-10 to immature female rats is able to induce GnRH-dependent LH release and earlier onset of puberty (Navarro et al., 2004b). From these studies, it is conceivable that kisspeptin might be an excitatory peptide involved in GnRH dysfunction and advanced puberty onset in precocious puberty. We hypothesized that abnormal kisspeptin expression might be regarded as a therapeutic target for advanced puberty onset in CPP.

Herbal medicines in China have conventionally been used in the treatment of pubertal abnormalities and improvement of clinical symptoms in precocious puberty for many years. The Chinese medical prescription, Nourishing "Yin"-removing "Fire" herbal

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mixture could markedly reduce the activity of GnRH neurons in the hypothalamus through by inhibiting central excitatory amino acid neurotransmitter release and by promoting central inhibitory amino acid neurotransmitter and beta-endorphin release (Cai et al., 2001). That results in an obvious decrease of the biosynthesis and secretion of GnRH from tonic and pulsatile secretory center of GnRH in the hypothalamus (Cai et al., 2001). Other studies have determined that the herbal mixture down-regulated ERα/IGF-1 mRNA and protein synthesis in female pubertal rats (Lu and Cai, 2008) and decreased the expression levels of GnRH, FSH and LH mRNAs in female adolescent rats (Cai et al., 2006). The present study evaluates the potential effect of the herbal mixture on hypothalamic kissspeptin expression in precocious rats, and further explores the possible therapeutic mechanism of the mixture on advance puberty onset in CPP.

2. Materials and methods

2.1. Preparation of herbal mixture

The nourishing "Yin"-removing "Fire" Chinese herbal mixture was kindly provided by the Department of Integrative Medicine, Children's Hospital of Fudan University, 60 ml/bottle (1 ml containing 3 g of natural medicament power), stored at 4 °C. The mixture prescription is mainly composed of 10 medicinal plants: 15 g of *Rehmannia glutinosa* (*Sheng di*), 9 g each of *Scrophularia buergeriana* (*Xuan shen*), *Anemarrhena asphodeloides* (*Zhi mu*), *Cortex Phellodendri* (*Huang bai*), *Paeonia suffruticosa Andr.* (*Dan pi*), *Alisma plantago-aquatica* L. *var. orientala Sam* (*Ze xie*), *Prunella vulgaris* L. (*Xia ku cao*), 12 g of *Carapax et Plastrum Testudinis* (*Gui jia*), 30 g of *Fructus hordei germinate* (*Mai ya*) and 6 g of *Gentiana scabra* Bge. (*Long dan cao*). The family of the herbs is listed in Table 1. An extract was manufactured by boiling the above crude drugs gently in 1000 ml water for 40 min followed by concentration under reduced pressure to leave a brown residue.

2.2. Animals

Female Sprague–Dawley rats at 3 days of age in company with their mothers were purchased from Medical Experimental Animals Center of BK Co. (Shanghai, China). Animals were housed under laminar flow in an isolated room with controlled temperature at a 12/12 (light/dark) schedule. The animals were weaned on day 21 and were examined daily for vaginal opening after which daily vaginal smears were examined. All experimental procedures involving the use of animals were conducted in accordance with NIH Guidelines and were reviewed and approved by Animal Use and Care Committee for Fudan University.

2.3. Experimental design

The model litters at postnatal day 5 were given a single subcutaneous injection of 300 μg of danazol (Hualian Pharm Ltd., Shanghai, China) dissolved in 25 μl vehicle of propylene glycol–ethanol (1:1, v/v), and allowed to grow without further treatment (Sun et al., 2007; Tian et al., 2004; Morishita et al., 1993). The day of vaginal opening and establishment of two regular estrous cycles were observed in the normal (N), CPP model (M), vehicle without CPP (V), CPP model gavaged with herbal mixture (HM) and CPP model gavaged with saline (S) ($n = 6$, respectively). From P15, rats in the HM and S groups were continuously gavaged with nourishing "Yin"-removing "Fire" Chinese herbal mixture or saline 1 ml/50 g body weight, until two consecutive regular estrous cycles were established. After two consecutive regular estrous cycles, all animals were killed by decapitation on diestrum. The uterus and ovaries were dissected out of the surrounding fat and weighed to evaluate the organ coefficients (mg/100 g).

2.4. Immunohistochemistry analysis

The effects of herbal mixtures on the hypothalamic kisspeptin and GnRH expression in rats were studied. The GnRH expression was observed as described previously (Tian et al., 2004, 2005). For kisspeptin analysis, hypothalamic samples from the same five groups were obtained on the pre-puberty period (postnatal 20 days, $n = 6$), onset-puberty (the day of vaginal opening, $n = 6$), and post-puberty period (establishment of two regular estrous cycles, $n = 6$). The animals were exsanguinated with normal saline following anesthetisia with an intraperitoneal injection of chloral hydrate (400 mg/kg). Perfusion was done and the brains were removed and post-fixed for >48 h in 4% paraformaldehyde in 0.1 M PB (PH 7.4) with 30% sucrose. Specimens were sliced at 35 μm on a vibratome microslicer and stored at 4 °C in tissue culture wells containing 0.1 M PBS (PH 7.4) plus 0.02% sodium azide until further processed.

Slices were treated with 3% H_2O_2 to quench endogenous peroxidase firstly, washed in PBS for 30 min at room temperature (RT) and blocked with 10% normal goat serum, then incubated in a rabbit polyclonal antibody against kisspeptin-10 [(Kiss-1 (112–121)/metastin (45–54) (human) 1:1000, Phoenix Pharmaceuticals Ltd., USA)] dilution in PBS containing 1% BSA, 0.02% sodium azide and 0.03% Triton X-100 for 2 h at room temperature (RT), and then at 4 °C for 48 h. After rinsing, sections were incubated in secondary antibody solutions (goat anti-rabbit IgG conjugated to peroxidase 1:200, Sino-American Technology Company, China) for 2 h at 37 °C, washed (3×, 30 min), and diaminobenzidine (DAB) was used as chromogen (Vectastain Elite kits, Vector Labs).

Specificity of kisspeptin staining was determined by preincubation of antiserums for 48 h at 4 °C with varying concentrations, with primary antibody omitted to identify non-specific staining as well.

2.5. RT-PCR analysis of Kiss-1 mRNA expression in hypothalamus

The effects of the herbal mixture on the Kiss-1 and GnRH mRNA expression in the hypothalami of rats were detected. The GnRH mRNA expression was observed as described previously (Tian et al.,

Table 1
The composition of the herb mixture.

Botanical name of the herb	Family	Common name	Used part
Rehmannia glutinosa	Scrophulariaceae	Rehmannia root	Dried root tuber
Scrophularia buergeriana	Scrophulariaceae	Buerger's Figwort	Dried root tuber
Anemarrhena asphodeloides	Liliaceae	Zhimu	Dried rhizome
Cortex phellodendri	Rutaceae	Phellodendron bark	Dried bark
Paeonia suffruticosa Andr.	Ranunculaceae	Moutan	Bark dried root
Alisma plantago-aquatica L. *var. orientala Sam*	Alismataceae	Oriental water-plantain rhizome	Dried root tuber
Prunella vulgaris L.	Lamiaceae	Common self-heal	Dried aerial parts and flowers
Carapax et Plastrum Testudinis	Testudinidae	Plastron of fresh-water tortoise	Carapace and plastron of the turtle *Chinemys reevesii*
Fructus hordei germinates	Gramineae	Germinated barley	Germinant fruit of *Hordeum vulgare* L.
Gentiana scabra Bge.	Gentianaceae	Chinese gentian	Dried root and rhizome

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2004, 2005). For kisspeptin analysis, the target regions, including mediobasal hypothalamus and the suprachiasmatic-preoptic area, were dissected (limited anteriorly by the optic chiasma, laterally by the hypothalamic fissures, posteriorly by the mammilary bodies and in depth by the subthalamic sulcus) on the pre-puberty, onset-puberty and post-puberty period in the five groups of rats. Total hypothalamic RNA was extracted using 'Trizol Regent' (Invitrogen Inc., America) according to the manufacturer's instructions. The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures.

Tissue RNA (2 μg) was reversed transcribed, in a final volume of 20 μl, using 200 IU M-MLV reverse transcriptase in the presence of 25 pmol Kiss-1 antisense primer, 0.5 mM deoxy-NTP and 20 IU Rnasin (from Progma) for 60 min at 42 °C, then heat denatured for 5 min at 95 °C. 5 μl cDNAs were further amplified by PCR using 25 pmol of primer (Sangon Inc.) for Kiss-1 [13,19]: sense 5′-TGG CAC CTG TGG TGA ACC CTG AAC-3′; antisense 5′-ATC AGG CGA CTG CGG GTG GCA CAC-3′. We first determined the linear range of amplification of cDNA using each of the primer sets, and then chose an appropriate amplification cycle within this range for each cDNA species. We used 30 PCR amplification cycles for Kiss-1 and 20 cycles for β-actin gene expression. Each PCR reaction underwent an amplification regimen characterized by (Kiss-1: 96 °C for 30 s, 62.5 °C for 30 s, 72 °C for 1 min) with Taq DNA polymerase (3 U per tube) and 2.2 mM magnesium chloride (from Promega) in a final volume of 50 μl. A final extension cycle of 72 °C for 15 min was included. In additional, 2 μg of total RNA without M-MLV reverse transcriptase was performed to check for the presence of DNA contamination RT-PCR. An internal control (β-actin, sense, 5′-AAG CAG GAG TAT GAC GAG TCC G-3′; antisense, 5′-GCC TTC ATA CAT CTC AAG TTG G-3′) for each RT-PCR procedure was performed to account for procedural variations. For each sample, 5 μl of PCR amplification products were analyzed on 1.5% agarose gels and visualized by ethidium bromide staining. Standard DNA (100 bp DNA ladder Promega) was run to provide the appropriate size marker. The intensities of the bands were evaluated using the Image Master Software (SYDR-1990, SYNGENE, USA). The RT-PCR products were extracted and purified from agarose gel by Golden Beads Gel Extraction kit (Sangon Inc. China) and sequenced using radioactive dideoxychain terminating method (Sangon Inc. China). Quantification of the intensity of the bands was evaluated by Image Master Software (SYDR-1990, SYNGENE, USA).

2.6. Statistical analysis

All data are expressed as mean ± SEM. Comparisons among groups were made using the Student's *t*-test. Statistical analysis was performed on raw data using one-way analysis of variance (ANOVA), with the significance concentrations of $P < 0.05$ in two-tailed testing chosen.

3. Results

3.1. Effects of herbal mixture on the day of vaginal opening and establishment of two regular estrous cycles

The day of vaginal opening and establishment of two regular estrous cycles were significantly advanced in Model rats compared to Normal and Vehicle groups ($P < 0.05$, Table 2). There was no difference between Normal and Vehicle groups. The day of vaginal opening and establishment of two regular estrous cycles in HM were significantly delayed compared to Model ($P < 0.05$, Table 2) and Saline groups.

Table 2
Effects of herb mixture on the day of vaginal opening, two regular estrous cycles and organ coefficient of uterus and ovary of the rats ($\bar{x} \pm s$, days; mg/100 g, respectively).

	Group		
	Normal ($n = 6$)	Model ($n = 6$)	Herb ($n = 6$)
Day of vaginal opening	42.50 ± 5.71	35.00 ± 3.21*	39.50 ± 4.65
Two regular estrous cycles	56.23 ± 3.28	46.50 ± 2.62*	52.75 ± 3.76
Organ coefficient of uterus	67.27 ± 6.18	74.67 ± 3.32*	70.13 ± 2.80
Organ coefficient of ovary	58.65 ± 4.27	71.45 ± 4.26*	63.27 ± 3.60#

Data of Vehicle and Saline groups were not showed.
* $P < 0.05$, Model vs. Normal and Vehicle, respectively.
# $P < 0.05$, Herb vs. Model and Saline, respectively.

3.2. Effects of herbal mixture on organ coefficients of ovary and uterus

The organ coefficients of uteri and ovaries of the Model ($n = 6$) which were 74.67 ± 3.32 mg/100 g and 71.45 ± 4.26 mg/100 g, respectively, were increased significantly comparing to the ones of Normal (Table 1) and Vehicle groups. The organ coefficients of uteri and ovaries in the Herbal Mixture group were 70.13 ± 2.80 mg/100 g and 63.27 ± 3.60 mg/100 g. These were decreased significantly compared to those of the Model and Saline groups.

3.3. Effects of herbal mixture on hypothalamic kisspeptin and GnRH expression by immunohistochemistry

The kisspeptin-ir cells distributed in the arcuate nucleus (ARC), preoptic area (POA) and periventricular nucleus (PeN) were calculated. The characteristic of kisspeptin-ir cells expression is the same as our previous study (Sun et al., 2007). There were few kisspeptin-ir cells found in the hypothalamus of Normal, Model, Vehicle, Saline and Herbal Mixture groups on the pre-puberty period. On the day of onset-puberty, the number of kisspeptin-ir cells in ARC, POA and PeN in Model rats were increased compared to Normal and Vehicle groups ($P < 0.01$, $P < 0.01$, $P < 0.01$). There was no difference between Normal and Vehicle groups. The number of positive kisspeptin-ir cells was significantly decreased in the Herbal Mixture group compared to the Saline ($P < 0.01$) group. There was no statistical difference between Model and Saline groups. On the post-puberty period, the kisspeptin-ir cell numbers were significantly higher in Model than in Vehicle groups, and decreased in the Herbal Mixture group. There was no difference between Herbal Mixture and Normal group (Fig. 1).

GnRH-ir cells bodies, which were abundant in the MS, DBB and MPOA, were calculated. On the day of onset-puberty, the number of GnRH-ir cells in Model rats was less than in Normal ($P < 0.05$, Fig. 3) and Vehicle groups. The number of GnRH-ir cell was significantly increased in Herbal Mixture group than that in Saline administered rats, and there was no difference between Herbal Mixture and Normal group.

3.4. Effects of herbal mixture on hypothalamic Kiss-1 and GnRH mRNA expression by RT-PCR

Comparison of the amplified PCR fragments with rat Kiss-1 and GnRH sequences revealed 100% homology (data not shown). Densitometric analysis of the Kiss-1 mRNA concentration using Kiss-1/β-actin expressed as the mean with SEM. The ratio of Kiss-1 to β-actin in Model rats increased significantly compared with those in Normal ($P < 0.05$) and Vehicle groups on the day of onset-puberty. The ratio in the Herbal Mixture group decreased significantly when compared to the Model and Saline groups ($P < 0.01$) (Fig. 2). There was no significant difference between Herbal Mix-

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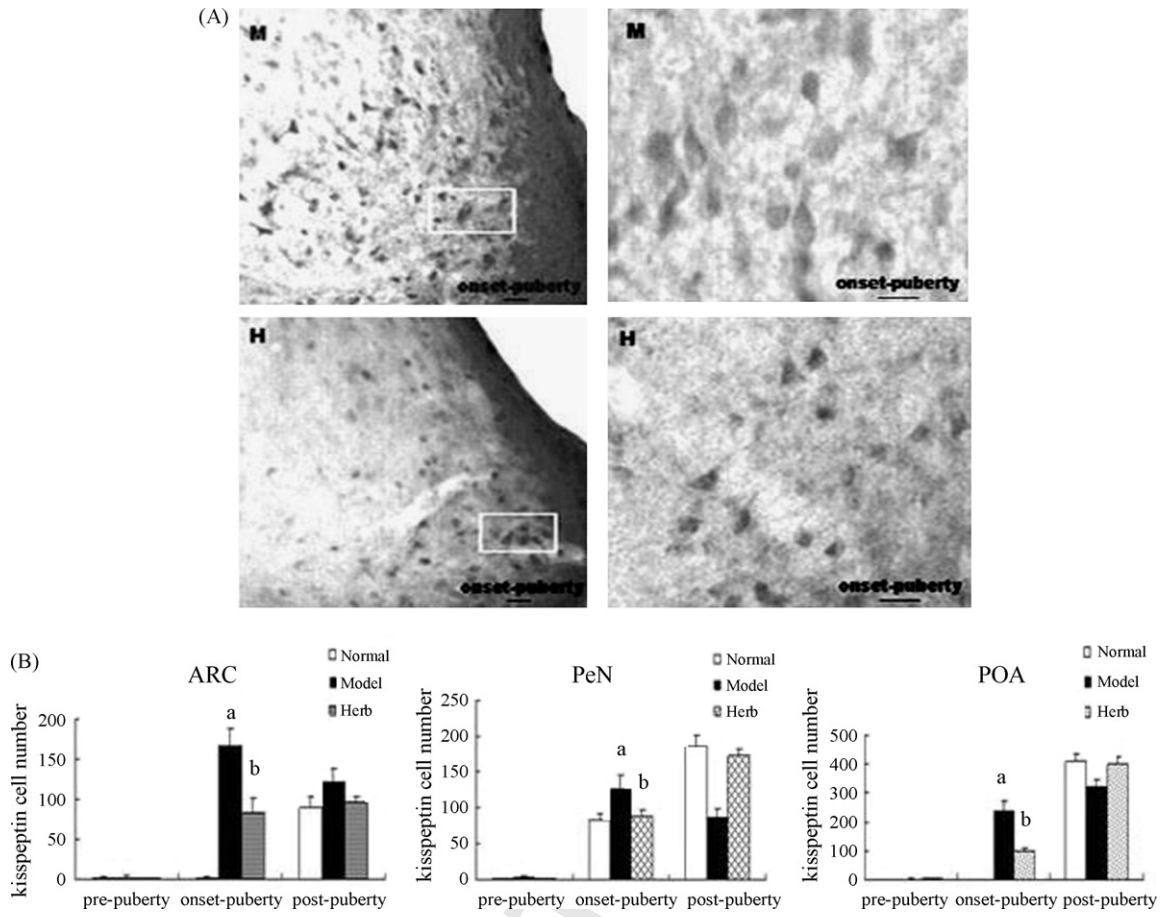

Fig. 1. Effect of herb mixture on hypothalamic kisspeptin expression by immunohistochemistry. (A) Representative microscopic images showing the kisspeptin-ir positive neurons located in ARC on the day of onset-puberty decreased in the herb mixture rats compared with model ones (low magnification 10×, high magnification 40×). M: model, H: herb mixture. (B) Calculated number of the kisspeptin neurons in the ARC, PeN and POA in the normal, model and herb mixture rats. Twelve observations per animal and six animals per group, all observations from individual animal averaged for that animal, and then collapsed into a single value for that animal. These single numbers of each animal used to calculate the group mean. Vehicle and saline groups were no shown in the figure. N: normal, M: model and H: herb mixture. [a] $P < 0.01$, onset-puberty in M vs. onset-puberty in N; [b] $P < 0.01$, onset-puberty in H vs. onset-puberty in M.

ture and Normal groups on the post-puberty period and there was no difference between Normal and Saline groups.

The ratio of GnRH to β-actin in Model rats increased significantly compared with those in Normal and Vehicle groups ($P < 0.05$) on the day of onset of puberty (Fig. 3). The ratio in the Herbal Mixture group decreased significantly compared to the ratio in the Model and Saline groups ($P < 0.05$) on the day of onset of puberty. There was no difference between Herbal Mixture and Normal groups. There was no significant difference between Model and Saline groups.

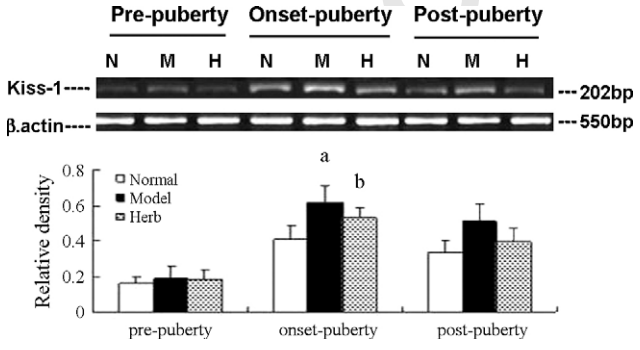

Fig. 2. Effect of herb mixture on hypothalamic Kiss-1 expression by RT-PCR. The upper picture shows the gel electrophoresis of the RT-PCR products for the hypothalamic Kiss-1 mRNA expression of different pubertal development periods in Model, Normal and Herb rats. Kiss-1 mRNA expression in vehicle and saline groups was no shown in the figure. Densitometric analysis used the ratio of Kiss-1/β-actin ($n = 6$ per group) expressed as the mean with SEM. N: normal, M: model and H: herb mixture. [a] $P < 0.05$, onset-puberty in M vs. onset-puberty in N, [b] $P < 0.01$, onset-puberty in H vs. onset-puberty in M.

4. Discussion

The nourishing "Yin"-removing "Fire" Chinese herbal mixture, a potent combination in complementary medicine, is considered a natural and efficacious in modulating the course of puberty development (Cai et al., 2001, 2006; Lu and Cai, 2008). But the mechanism of the herbal compound is still not clear. To our knowledge, the present study is the first experiment that investigated the influence of the herbal mixture on kisspeptin expression in CPP.

One of the most interesting findings in our current data was that the herbal mixture could significantly down-regulate hypothalamic Kiss-1 mRNA expression and decrease the weights of uteri and ovaries on the day of onset of puberty in precocious rats. As indicated in previous studies, the maximum expression of Kiss-1 mRNA in onset of puberty contributes to the activation of the gonadotropic axis development in normal female rats (Navarro et al., 2005). Kiss-1 mRNA was clearly up-regulated on the day of onset-puberty in precocious rats (Sun et al., 2007), along with a simultaneous advance in vaginal opening and with an increase in weights of both uteri and ovaries. Functional analyses further

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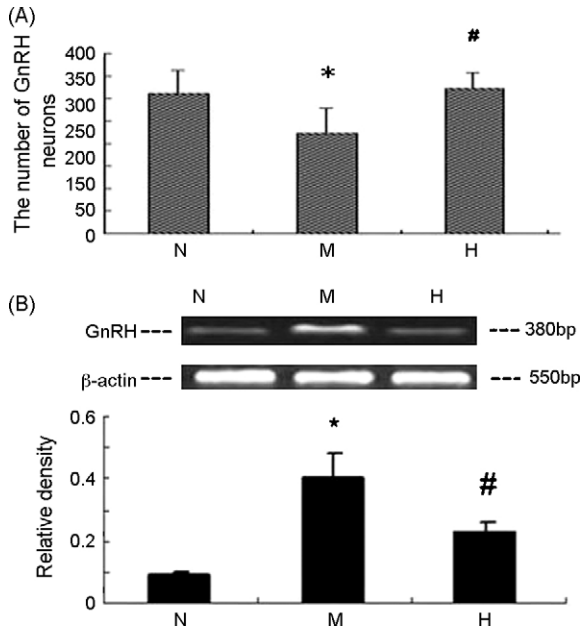

Fig. 3. Effect of herb mixture on hypothalamic GnRH and GnRH mRNA expression by immunohistochemistry and RT-PCR. (A) Total GnRH cells in the MS, DBB, and MPOA of the rats. Twelve observations per animal and six animals per group, all observations from individual animal averaged for that animal, and then collapsed into a single value for that animal. These single numbers of each animal used to calculate the group mean. The number of GnRH cells on the day of onset-puberty in model ones was less than those in normal group ($n = 6$ per group), the number in herb mixture rats increased compared with those in model. GnRH cells in vehicle and saline groups were not shown in the figure. N: normal, M: model, and H: herb mixture. $^{*}P < 0.05$, M vs. N; $^{\#}P < 0.05$, H compared with M. (B) The upper picture shows the gel electrophoresis of the RT-PCR products for the GnRH. Densitometric analysis of the mRNA concentration using GnRH/β-actin expressed as the mean with SEM bar ($n = 6$) in each column indicated in the lower panel. The ratio of GnRH to β-actin in the model increased significantly compared with those in normal, it decreased in herb mixture rats compared with the model rats. GnRH mRNA in vehicle and saline groups were not shown in the figure. N: normal, M: model, and H: herb mixture. $^{*}P < 0.05$, M vs. N; $^{\#}P < 0.05$, H compared with M.

observed that chronic central administration of Kiss-1 peptide to female rats at the juvenile period elicited a clear-cut advancement of the age of vaginal opening, together with a significant increase in uteri weights (Navarro et al., 2004b). Thus, it is likely that hypothalamic Kiss-1 mRNA increases and the change of pubertal maturation parameters (such as uteri and ovarian weight, vaginal opening) supports the hypothesis that Kiss-1 plays a major role in advancement of puberty onset of precocious rats. Interestingly, our results provide evidence that the herbal mixture decreased hypothalamic Kiss-1 mRNA and GnRH mRNA expression in precocious model rats, decreased the weights of uteri and ovaries and delayed the vaginal opening. This phenomenon suggests that the precocious activation of the reproductive axis and the Kiss-1/GnRH mRNA was inhibited after administration with the herbal mixture. It is noteworthy that central administration of kisspeptin induced *c-fos* expression (as early marker of activation) in more than 86% of GnRH neurons, more than 77% of GnRH neurons in the rat hypothalamus coexpress *Kiss-1r* mRNA (Irwig et al., 2004; Han et al., 2008), the available information makes it tempting to propose that the herb mixture might have an effect on Kiss-1 mRNA transcription. Kiss-1 mRNA down-regulation subsequently altered Kiss-1r mRNA expression in GnRH neurons, which is directly regulate hypothalamic GnRH mRNA expression. The precise mechanism of whether *Kiss-1r* mRNA, participates in the signaling pathway is currently under investigation in our laboratory.

To further understand the mechanism of how kisspeptin might be involved in the process of the herbal mixture regulating GnRH activity and precocious puberty onset in rats, the morphological differences of hypothalamic kisspeptin immunohistochemistry cells in herbal mixture-administered precocious rats was taken into consideration. The number of kisspeptin-ir cells in ARC, PeN and POA on the puberty was observed by a commercially available antibody against human Kp10. The number of kisspeptin-ir cells in those three nuclei was decreased on the day of onset of puberty in herbal mixture group rats. It is known that the AVPV sends ascending projections to the ventral part of the septal nucleus and the region adjacent to the organum vasculosum lamina terminalis. From Herbison's definition, a functional anatomical construct of RP3V (RP3V = MEPO + AVPV + PVpo) neurons such as kisspeptin neurons, might have an important influence on GnRH neurons (Steiner and Herbison, 2005). A significant increase in kisspeptin-54 release occurred in association with the pubertal increase in GnRH release in monkeys (Keen et al., 2008). It is likely that the herbal mixture decreased kisspeptin neuron expression in the PeN and POA which, in turn, reduced kisspeptin stimulation of GnRH release directly. Alternatively, the GnRH pulse generator may be located within the ARC in the rat so we also evaluated that the herbal mixture associated decrease in kisspeptin neurons in ARC might alter GnRH release pulsatility and delay the sexual development in precocious rats. The fact that hypothalamic kisspeptin-ir cells and Kiss-1 mRNA expression were restored to a normal level in the post-pubertal period might be due to the effect of herbs is chronic but consecutive.

Another interesting phenomenon discovered in this study is that although the herbal mixture down-regulated high levels of GnRH mRNA in the period of onset of puberty in precocious model rats the number of GnRH neurons was increased. The possible mechanism of this inconsistent change might have resulted from the herbal mixture inhibiting GnRH release thereby inducing GnRH-containing neurons that were increased in the rats that received the herbal mixture.

The distribution of kisspeptin neurons in the rat by immunocytochemistry has been assisted by the development of several anti-kisspeptin antibodies in previous experiments. Some studies indicated that those antibodies might also cross-react with other RF-amide peptides. If these antibodies cross-react with other RF-amide peptides, the kisspeptin-ir neurons should have been detected prior to puberty in our present study. Antiserum preincubated with the peptides RFRP-1 and RFRP-3 might help validate the anibody specificity (Goodman et al., 2007; Greives et al., 2007). Better antibodies to kisspeptin will be required to clarify this matter.

5. Conclusions

The Nourishing"Yin"-removing "Fire" Chinese herbal mixture down-regulate Kiss-1 mRNA and kisspeptin expression in ARC, PeN and POA on the onset-puberty in CPP rats. The kisspeptin signaling pathway might be involved in the efficacy of this herbal mixture on CPP. The potential mechanism of the herb mixture was further revealed so as to bring evidence to clinical exercise.

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Contributions. Yan Sun and Zhanzhuang Tian designed the study. Yan Sun performed the animal and molecular genetic studies, and drafted the manuscript. Genevieve Neal Perry gave good suggestion and advice for manuscript writing and revising. Boying Chen and

Jian Yu conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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