

Research Article

Prenatal Exposure of Kisspeptin Antagonist on the Gonadotropin-Releasing Hormone (GnRH) Expression in Rat Model of Polycystic Ovary Syndrome

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Abstract

Introduction: Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age. Increased GnRH/luteinizing hormone (LH) pulse frequency is a characteristic of endocrine abnormalities in PCOS. Kisspeptin antagonists reduce LH pulse frequency and amplitude and are hence supposed to slow GnRH neuron activity that may adjust GnRH/LH levels in PCOS conditions.

Objective: To investigate the impact of kisspeptin antagonist P271 administration during prenatal life to reduce GnRH expression in adulthood in prenatally androgenized (PNA) rats as a model of PCOS.

Materials and methods: PNA rats (n=9) and controls (n=9) received P271 on day 20 of their prenatal life, and they were examined in adulthood (110-120 days). The ability of P271 to alter GnRH mRNA expression, and plasma levels of gonadotropins and steroid hormones were tested using reverse transcription q-Real-time PCR and ELISA methods, respectively.

Results: In this study, based on the result of Generalized Estimating Equation (GEE) model, we found that GnRH expression in PCOS+P271 rats decreased compared to PCOS rats in the diestrous phase. In addition, P271 administration reduced gonadal steroid and gonadotropin levels in both PCOS and non-PCOS rats.

Conclusion: In conclusion prenatal administration of kisspeptin antagonists can reduce GnRH expression and LH, FSH, T, P4 and E2 levels in PCOS rats in later life.

Introduction

Polycystic ovary syndrome (PCOS), one of the most common endocrine disorders in women during their reproductive age [1], is associated with reproductive, metabolic and public health disorders [2]. Although PCOS was recognized about 80 years ago, its etiology, pathophysiology and long-term health risks have not been clearly elucidated. Based on current scientific literature both genetic and environmental factors play an important role in the development of PCOS [3, 4]. Current treatments for PCOS rely on managing the symptoms of the syndrome rather than its cause; these treatments include lifestyle changes followed by regulating the menstrual cycle, reducing the androgen levels and promoting ovulation [5, 6]. The main endocrine abnormality of PCOS is increasing LH/GnRH pulse frequency, which none of the current treatments have yet targeted [7].

Kisspeptin, a principal activator of GnRH neurons and the target of endocrine and metabolic cues, is a prerequisite for the onset of puberty and maintenance of normal reproductive function [5,6,8]. Although PCOS symptoms generally become obvious during puberty, clinical and experimental evidence suggest an intrauterine origin for the syndrome [7]. Exposure to excess androgen during the critical period of early life leads to the development of a PCOS-like phenotype in adulthood, which is associated with reduced hypothalamic kiss1 (a male-typical kiss1 pattern) and unresponsive to sex steroid feedback actions; however there is enhanced GnRH neuron activity and LH secretion [9, 10]. Experimental studies demonstrate that kisspeptin antagonists decrease LH pulse frequency and amplitude but do not appear to affect basal LH secretion [11, 12]. As a result slowing GnRH neuron activity via kisspeptin antagonists may improve GnRH/LH pulse frequency in PCOS conditions [13]. In this study we investigated

whether prenatal exposure to a single dose of kisspeptin antagonist P271 during a critical period of fetal development can alter *gnrh* mRNA expression and hormonal profile in PCOS rats during adulthood.

Materials and Methods

Animals

All experimental procedures and protocols used in the present study were approved by the local ethics committee of the Research Institute for Endocrine Sciences (IR.SBMU.RIES. REC.1394.3). The details of production of prenatally androgenized rats model have previously been published [14]. In brief sexually mature female and male Wistar rats (n=20 each, body weight 170–190 g) were obtained from the RIES animal facility of Shahid Beheshti University of Medical

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Sciences (Tehran, Iran) and were housed under controlled photoperiod (12 h light/12 h dark), temperature ($22 \pm 3^\circ\text{C}$) and humidity (45–55%) conditions. Food and water were available ad libitum. One pair of male and female rats was caged for one night in standard animal housing conditions, and the day of vaginal plug observation was counted as the first day of gestation.

Experimental design

The flow diagram for the detailed experimental plan is described in Figure 1. Pregnant rats were randomly divided into two groups; group 1 received 5 mg of free testosterone (T1500; Sigma, Steinheim, Germany) dissolved in a 500 μl cocktail containing sesame oil (S3547; Sigma, Steinheim, Germany) and benzyl benzoate (B6630; Sigma, Steinheim, Germany) at a ratio of 4:1, by subcutaneous injection on the 20th day of pregnancy, while group 2 received only 500 μl of solvent [14]. These two groups were again divided into two subgroups; one subgroup received intraperitoneal injection (5 nmol) of P271 (EZBiolab Inc., Carmel, USA, cp7222) and the other received solvent, 4 hours after the first injection (according to Desroziers et al study, kisspeptin is developed between embryonic day 12.5 and 13.5) [15]. Female offspring of PCOS (n=9), control (n=8), PCOS+P271 (n=9) and P271 (n=9) group were kept under the standard animal housing conditions mentioned above.

Monitoring the estrous cycle

Estrous cyclicity was monitored by daily observations of vaginal smears for all female offspring (age 70-90 days) between 08.00 and 12.00 h for 20 days. The vaginal secretion of each rat was collected with a plastic pipette filled with 50 μL of distilled water by inserting the pipette tip into the vagina. The pipette tip was rotated two or three times against the vaginal wall and then withdrawn and rolled on a clean glass slide; smears were fixed with 70% ethanol, stained with Giemsa and observed under a light microscope at 100x magnification. The estrous cycle was determined according to the cell types observed in the vaginal smear [14].

Animals were then divided to four subgroups, i.e. proestrous, estrous, metestrous and diestrous.

Blood sample collection

Since GnRH peaks occur only within a narrow temporal window,

typically late afternoon in nocturnal rodents, [16] the female offspring, 110-120 days of age, were weighed and anaesthetized by I.P. injection of pentobarbital sodium (P3761, Sigma, St Louis, MO, USA) dissolved in normal saline [60 mg (kg body weight) $^{-1}$] 1 to 2 h before darkness. Blood samples were collected from the abdominal aorta and centrifuged at 6000 g for 5 min, at 4°C . Sera were extracted, aliquoted and stored at -80°C until analysis.

Hypothalamus dissection

Following blood sampling, the rats were decapitated, brains were immediately dissected out, the entire hypothalamus were dissected out by an anterior coronal section, approximately 1 mm anterior to the optic chiasma, and a posterior coronal cut at the posterior border of the mammillary bodies was made. A small portion of the thalamus located above the hypothalamus was dissected out. The samples were snap frozen in liquid nitrogen and stored at -80°C . We were unable to extract mRNA from separate areas of the hypothalamus. Thus, all hypothalami were used.

Determination of the estrous cycle phases

Following hypothalamus isolation, the vaginal secretion of each rat was collected and the phase of the estrous cycle was determined according to the procedure mentioned above

Quantitation of GnRH mRNAs in the hypothalamus

Quantitative reverse transcription real-time PCR (qRT-PCR) reactions were performed in a Rotor Gene 6000 machine (Corbett Research, Sydney, Australia). Total hypothalamus RNA was extracted using the QIAGEN RNeasy Mini Kit (Cat No: 74104), according to the manufacturer's protocol. Quantity and purity of RNAs were measured using the NanoDrop 1000 (Thermo Scientific, Waltham, and Mass). Total RNA from each sample (250 ng) was used as template for reverse transcription (RT) reaction. The cDNA synthesis was performed using the QIAGEN QuantiNova Reverse Transcription Kit (Cat No/ID: 205411) in a 20 μl reaction mixture. Quantity and purity of cDNAs were measured using the NanoDrop 1000 (Thermo Scientific, Waltham, and Mass). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene (Table 1). qRT-PCR reactions

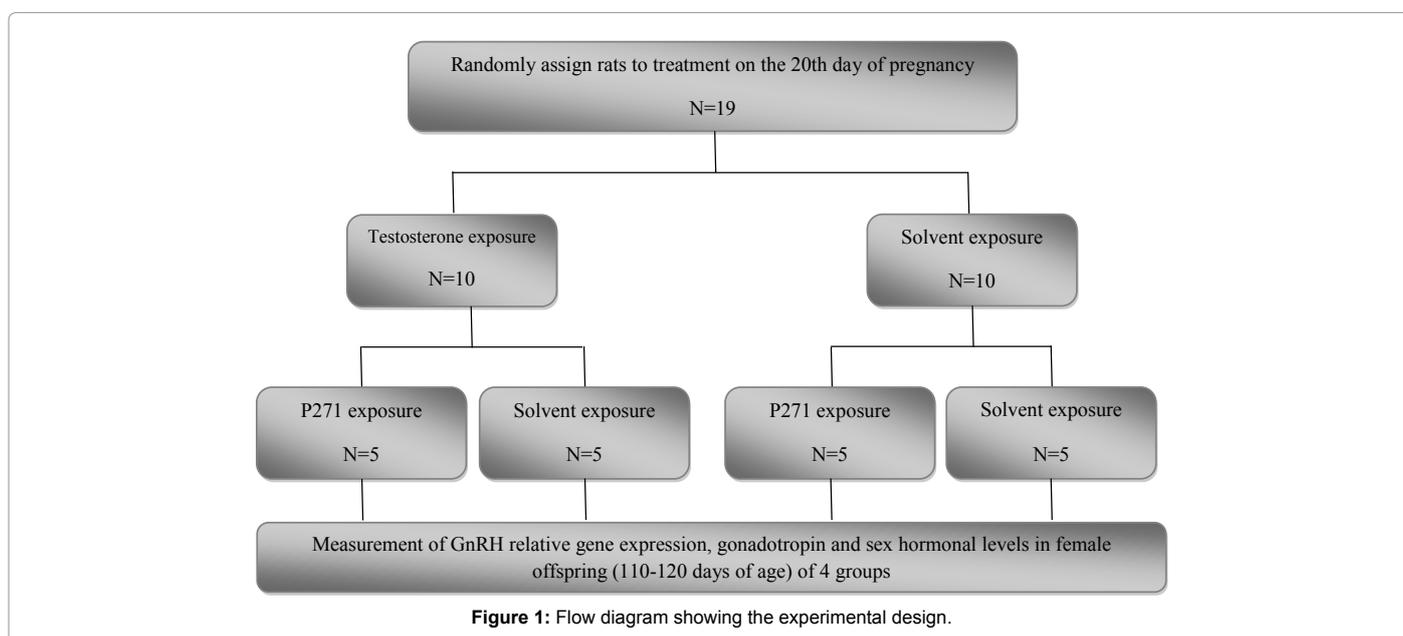


Figure 1: Flow diagram showing the experimental design.

Gene (accession no.)	Primer sequence	Annealing
	(5 to 3)	temperature (C)
GnRH1 (NM_012767.2)	Forward:	58.4
	GGCTTTCACATCCATACAGAATG	56.4
	Reverse: TAGGACCAGTGCTGGCTAGAG	
Gapdh (XM_017593963.1)	Forward: TGCCGCCTGGAGAAACCTGC	66.9
	Reverse TGAGAGCAATGCCAGCCCA	65.7

Table 1: Sequences of primers used for qReal-Time PCR.

were performed using the Thermo Scientific™ Maxima™ SYBR™ Green/ROX 2X qPCR Master Mix (Cat No.: K0221); PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 30 sec, 60°C for 60 sec and 72°C for 45 sec. Standard curve was used to estimate the PCR amplification efficiency. PCR reaction mixture (15 µl) contained a SYBR Green qPCR Master Mix 7.5 µL, 0.5 µl each of forward and reverse primer (Table 1), RNase-free water 5.5 µl, and 1 µl cDNA sample. For negative control, the Cdna sample was replaced with RNase-free water. Duplicate reactions were performed for the target and reference genes. The relative amount of mRNA in each sample (deltaCt) was calculated based on its threshold cycle (Ct) compared to the Ct of GAPDH (deltaCt= Ct_{GnRH}-Ct_{GAPDH}). So the lower the delta Ct the higher the gene is expressed. GnRH mRNA expression was evaluated in these 4 groups considering the estrous cycle.

Measurement of hormones

Serum concentrations of testosterone (T), progesterone (P4), estradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were quantified by the ELISA method. Rat specific ELISA kits were used to measure levels of T (Cat No. CSB-E05100r), E2 (Cat No. CSB-05110r), P4 (Cat No. CSB-E07282r), FSH (Cat No. CSB-E06869r) and LH (Cat No. CSB-E12654r); the sensitivity of these kits was 60 pg/ml, 40 pg/ml, 15 pg/ml, 0.07 mIU/ml and 0.15 mIU/ml, respectively. Intra-assay coefficients of variations for all hormones were <10%.

Data analysis and statistics

Data and design: This study was an *in vivo* experimental trial conducted on rats; we had two groups of PCOS and non-PCOS which had received intraperitoneal injections of either P271 or solvent, respectively. Vaginal sampling was not performed because of the possible risk of inducing stress and pseudo pregnancy, which may alter the criteria to be measured. We did not induce estrous synchronization, because hormonal changes were not our desirable. On the other hand, because of irregular estrous cycle in PCOS rats, the stage of the estrous cycle couldn't be predicted. We aimed to investigate the effect of a single dose of P271 during different phases of the estrous cycle, but we didn't want to kill many rats, so we used GEE.

Statistical analysis: The Generalized Estimating Equation (GEE) method was used to estimate coefficient of interest in a generalized linear model (GLM), where each phase was considered as a repeated measure for a PCOS (non-PCOS) case which had/had not received P271. The independent working correlation matrix was assumed according to the design of the study. Compared to GLM methods, the GEE approach is more consistent when data has not fulfilled normality assumption [17]. To assess the mean effect of P271 administration on the outcomes of interest (gene expression and hormonal profiles) was adjusted for group (PCOS and non-PCOS) and phase (proestrous, estrous, metestrous and diestrous) of study; we designed a model in which the main effects of the P271 administration [18], PCOS and phase of study were estimated (table 2). In addition, two-and-three-

way effect modifier variables (interaction effect) of P271 administration *phase and P271 administration *PCOS*phase were estimated to check the effect of P271 administration, adjusted by PCOS and phase during four levels of phase of the study on the outcome of interest. Model equation was defined as the following:

$$\text{Mean of (deltaCt)} = \beta_0 + \beta_1 (\text{PCOS}) + \beta_2 (\text{P271.A}) + \beta_3 (\text{phase} = \text{Metestrous}) + \beta_4 (\text{phase} = \text{Diestrous}) + \beta_5 (\text{phase} = \text{Estrous}) + \beta_6 (\text{P271.A*Diestrous}) + \beta_7 (\text{P271.A*Metestrous}) + \beta_8 (\text{P271.A*Estrous}) + \beta_9 (\text{P271.A*Diestrous*PCOS}) + \beta_{10} (\text{P271.A*Diestrous*PCOS}) + \beta_{11} (\text{P271.A*Metestrous*PCOS}) + \beta_{12} (\text{P271.A*Estrous*PCOS}) + \beta_{13} (\text{P271.A*proestrous*PCOS}) + \beta_{14} (\text{No-P271.A*Diestrous*PCOS}) + \beta_{15} (\text{No P271.A*Metestrous*PCOS})$$

Results

Estrous cycle

Observation of vaginal smears on a daily basis for 20 days showed that PCOS rats had longer and irregular estrous cycles compared with controls.

Gene expression results

Using the GEE model we calculated the mean gene expression (deltaCt) in various condition using the mentioned formula; for instance deltaCt in metestrous phase for those PCOS cases who had received P271 was calculated to be 5.47, considering the coefficients of various items as bellows:

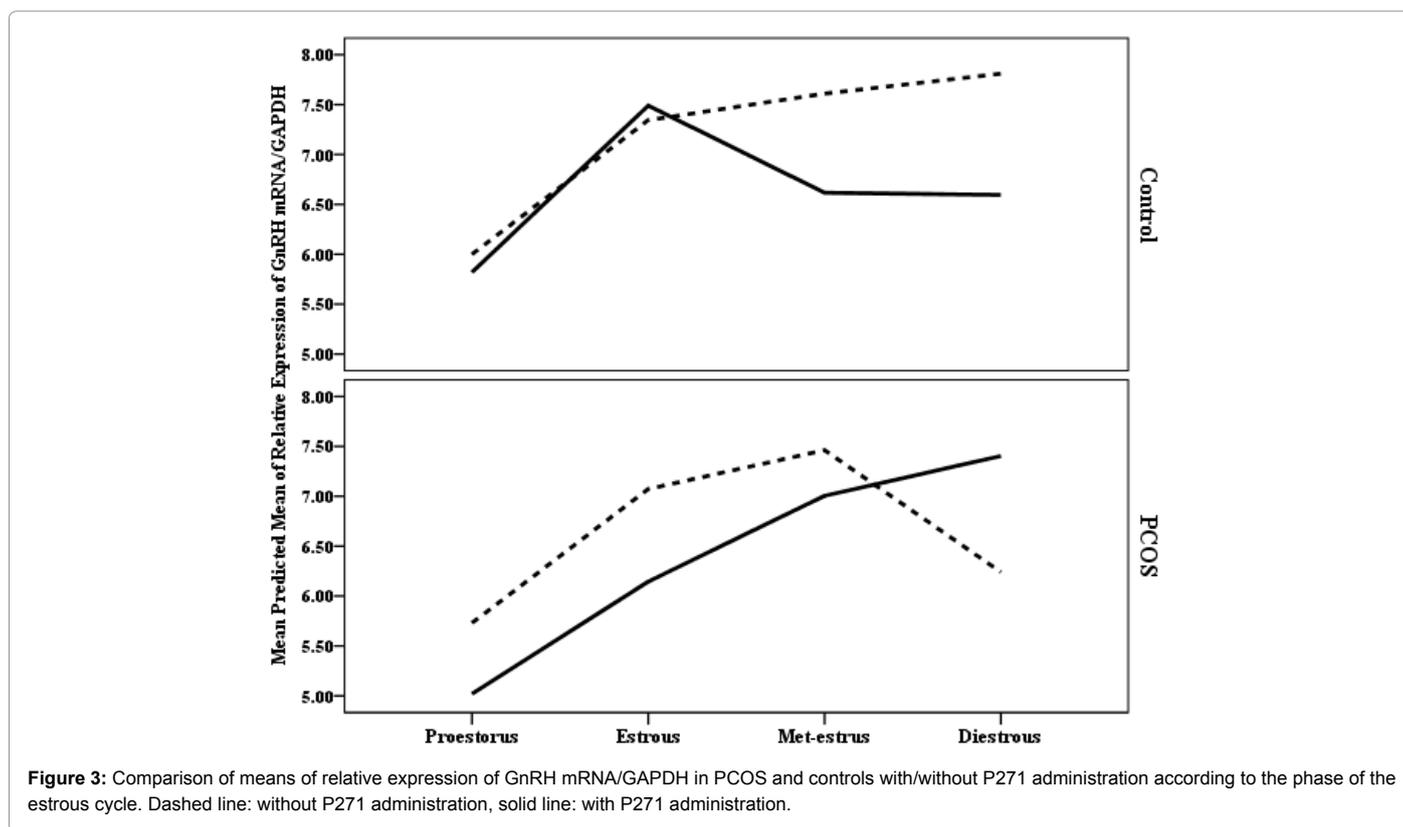
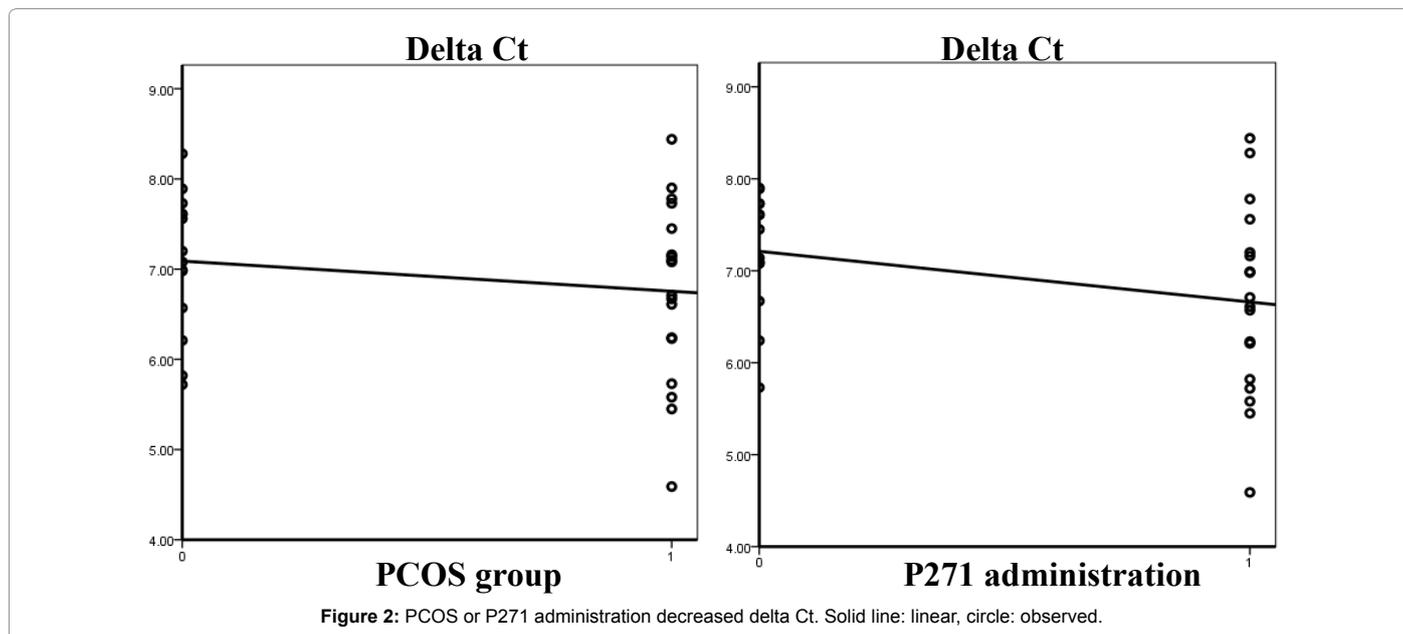
$$\text{Mean of (deltaCt)} = 6.0 - 0.27 (\text{PCOS} = 1) - 0.18 (\text{P271.A} = 1) + 1.8 (\text{phase} = \text{Metestrous}) - 0.81 (\text{P271.A*Metestrous}) - 1.07 (\text{P271.A*Metestrous*PCOS}) = 5.47$$

Figure 2 shows the results of the GEE model, illustrating that regardless of other conditions, the PCOS status or P271 administration decreased the mean of deltaCt by 0.27 (95%CI: 0.79-0.24, p=0.301) and 0.18 (95%CI: 0.70-0.33, p=0.489), respectively, although these changes were not statistically significant.

Figure 3 compares the P271 administration to the control group, adjusted by PCOS status in four phases of the estrous cycle. This figure demonstrates a significant increasing trend of deltaCt in the estrous cycle in controls; it was 6, 7.30, 7.60 and 7.80 in the proestrous, estrous, metestrous and diestrous phases respectively in controls. This model shows an increasing trend of expected deltaCt in the PCOS group from proestrous to metestrous (proestrous (5.73), estrous (7.03), metestrous (7.45)), while deltaCt decreased in the diestrous phase (6.23) in these rats. In the P271 group, deltaCt increased from proestrous (5.82) to estrous (7.45), while it decreased from estrous to metestrous and reached a plateau from metestrous through diestrous (6.60). In the PCOS+P271 group, an increasing trend of expected deltaCt in estrous cycle (proestrous (5.20), estrous (6.11), metestrous (7) and diestrous (7.40)) was observed. This figure shows that relative expression is increased approximately by 1.17 in PCOS+P271 cases in the diestrous phase compared to the PCOS ones with no P271 administration at the same phase.

	Intervention															
	No PCOS Group						Yes PCOS Group									
	Control Phase of estrous cycle		PCOS Phase of estrous cycle		Diestrus		Proestrus		Estrus		Metestrus					
	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean				
Predicted Value of	138.77	69.56	55.58	59.3	141.8	72.59	93.74	131.4	63.57	64.93	22.98	61.13	77.56	75.31	76.99	104.01
Mean of Response																
FSH																
Predicted Value of	1.4967	1.72	0.44	1.075	0.08	0.3033	0.945	0.69	0.055	0.125	0.2933	0.115	0.19	0.245	0.21	0.3767
Mean of Response LH																
Predicted Value of	62.33	32.27	38.32	34.82	68.75	38.68	49.45	65.08	46.93	42.98	28.39	35.88	57.62	24.87	43.65	42.95
Mean of Response																
Progesterone																
Predicted Value of	100.35	61.75	11.06	37	59.8	21.19	24.28	7.4	18.78	11.91	11.5	10.68	10.87	12.15	10.24	10.79
Mean of Response																
Estrogen																
Predicted Value of	3.6	1.74	1.07	1.75	2.85	0.99	1.3	1.84	1.08	0.67	1.39	0.77	0.58	1.08	1.17	1.39
Mean of Response																
Testosterone																
Predicted Value of	0.022	0.025	0.008	0.018	0.001	0.004	0.01	0.005	0.001	0.002	0.016	0.003	0.002	0.003	0.002	0.004
Mean of Response LH																
To FSH ratio																

Table 2: Results of the generalized estimating equation (GEE) approach on hormonal profile of study groups.



Hormonal profiles

Hormonal profiles of various conditions have been reported in table 2; as shown, in both the proestrous and estrous phases, mean differences of LH, E2 and T in PCOS rats, compared to controls, are -1.42 (mIU/ml), -40.56 (pg/ml) and -0.75 (pg/ml), respectively. These differences are 0.51 (mIU/ml), 13.22 (pg/ml) and 0.24 (pg/ml) in the metestrous phase, respectively (figure 4). Compared to controls, mean differences of FSH and P4 in PCOS rats, are 38.16 (mIU/ml) and 11.14

(pg/ml) in the metestrous, and 72.10 (mIU/ml) and 30.26 (pg/ml) in the diestrous phase, respectively (figure 5). This table demonstrates that in both PCOS and control groups, administration of P271 resulted in a decrease in LH, FSH, E2, T, P4 and LH/FSH ratio levels by 1.44 (mIU/ml) (95%CI: 1.85-1.04), 75.21 (mIU/ml) (95% CI:93.31-57.11), 81.58 (pg/ml) (95%CI:106.58-56.58), 2.53 (pg/ml) (95%CI: 3.54-1.51), 15.40 (pg/ml) (95% CI:34.28-3.48) and 0.02 (95% CI:0.03-0.01) respectively. All of these predicted changes are statistically significant, except for that related to P4.

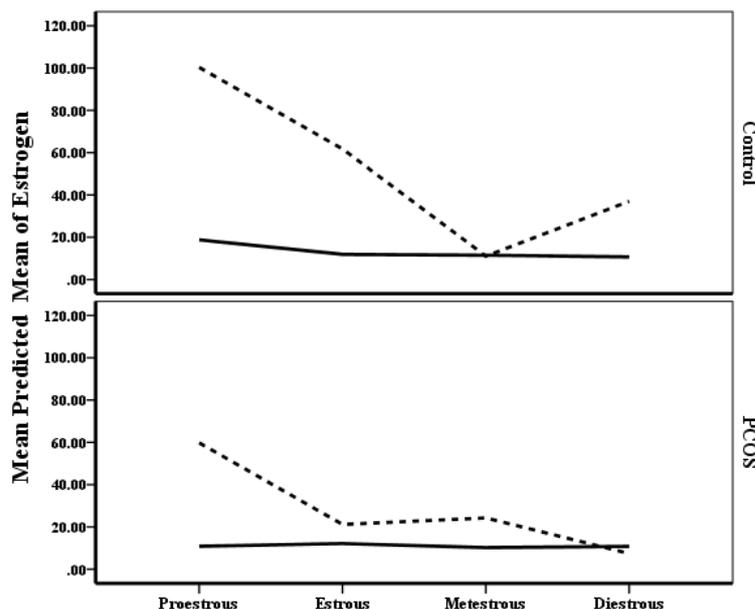


Figure 4: Comparisons of means of LH (luteinizing hormone), testosterone and estrogen levels in PCOS and controls with/without P271 administration according to the phase of the estrous cycle. Dashed line: without P271 administration, solid line: with P271 administration.

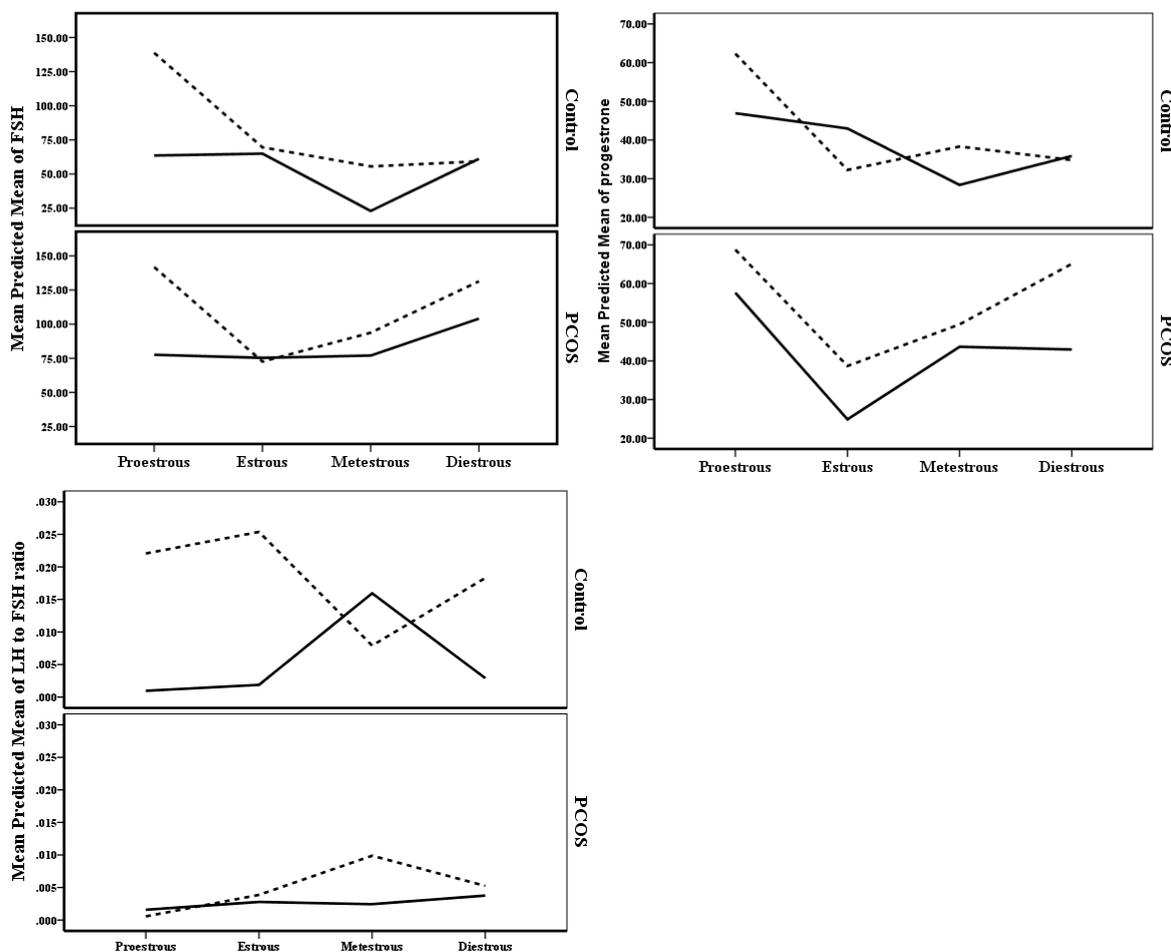


Figure 5: Comparisons of means of FSH (follicle stimulating hormone) and progesterone levels and LH/FSH ratio in PCOS and controls with/without P271 administration according to the phase of the estrous cycle. Dashed line: without P271 administration, solid line: With P271 administration.

Discussion

This study, for the first time, evaluated the effect of one dose of prenatally administered P271 on GnRH expression and hormonal levels in various phases of estrus cycles in PCOS rats and controls during adulthood. However, our study does have its limitations, including simultaneous assessment of GnRH expression and gonadotropin secretion; this could be partly explained by the inconsistency observed on GnRH expression and gonadotropin secretion; the highest hypothalamic GnRH expression occurs 1 to 2 h before darkness, whereas gonadotropins secrete during later times. Since this was the first study investigating the effect of prenatal administered P271 on GnRH expression and hormonal profiles in PCOS rats, it cannot provide a comprehensive explanation in terms of causality, indicating the need for further prospective comprehensive studies to confirm our findings.

Using animal models, it has been shown that exposure of the female fetus to androgens, during the critical period of their early life, results in the defeminization of the GnRH surge-generating system, limiting their ability to produce a GnRH/LH surge [19]. These animals exhibit elevated LH pulse frequency and irregular reproductive cycles, which may result from a number of mechanisms, including alterations in synaptic input to GnRH neurons, 2004, desensitization of GnRH neurons to steroid feedback, increased amplitude/frequency of GnRH pulses, and increased gonadotropic sensitivity to the GnRH stimulation [20-24]. Kisspeptin, an essential gatekeeper of puberty onset and GnRH secretion, mediates steroid feedback and metabolic cues during different developmental stages throughout the lifespan [9, 25]. In addition, kisspeptin is involved in the pathophysiology of the HPG axis; e.g., in PCOS animal models, the hypothalamic expression of kiss 1 and GnRH is abnormal, which might lead to multiple tissue abnormalities, observed in this syndrome [26]. In line with this evidence our results revealed that administration of P271 to prenatally androgen exposed rats altered the pattern of hypothalamic GnRH expression during the estrous cycle, in such a way that this pattern was similar to that seen in controls.

It has been shown that hypothalamic GnRH mRNA expression varies during the estrous cycle, with the highest expression levels in the late afternoon of the proestrous phase [27-28]. Our study, also, showed a peak in hypothalamic GnRH mRNA levels during the proestrous phase in each group. Prenatal androgen exposure led to higher GnRH levels compared to controls, especially during the diestrous phase. This result is in accordance with an earlier study, in which postnatally androgenized rats were constantly in diestrous phase and showed an increase in the number of GnRH-immunoreactive cells in adulthood [29]. Although some other studies have reported that pre/postnatal exposure to androgen excess did not alter hypothalamic GnRH mRNA expression [30,31], the difference may be due to various causes, e.g. dose of androgen, timing of androgen exposure, species of animals, phase of estrous cycle, or time of sampling.

Our previous study showed that the effect of androgen on the female reproductive system is highly influenced by the time of exposure and the hormone must be present during the appropriate time to have its effect; if it is present too early or too late, the impact will not be the same. Prenatal exposure of female fetuses to androgen during embryonic days 16-19, produced developmental and morphological disorders in the reproductive system and androgen-sensitive tissues in female offspring. On the other hand, exposure to excess androgen on embryonic day 20, had little effect on the morphology, but did induce a PCOS like phenotype [32]. Thus, in this study, we administered P271

during this critical period of fetal development, which could reverse some of the disrupted feedback actions/endocrine abnormalities, induced by androgen.

In the current study, PCOS rats showed disrupted hormonal profiles compared to controls. In the PCOS rats, serum LH, E2 and T levels reduced during proestrous and estrous phases, whereas they increased during metestrous phase, compared to controls. In addition, PCOS rats exhibited higher serum FSH and P4 levels during metestrous and diestrous phases than controls. In agreement with our results, previous studies have reported disrupted hormonal profile in PCOS animal models, compared to controls [33]. Evidence for endocrine changes in PCOS animal models is inconsistent, indicating the differences may be due to various factors, including type of hormone administered, time of hormone exposure, age of animal during the time of study, different PCOS phenotypes in animal models and time of sampling [21, 33-35].

Conclusion

Kisspeptin antagonist during the critical period of fetal life reduced GnRH mRNA expression, sex steroid hormone and gonadotropin levels. Kisspeptin antagonist may amend the disrupted HPO axis of PCOS cases and may have a potential role in the treatment of affected women.

Since this was the first study investigating the effect of prenatal administered P271 on GnRH expression and hormonal profiles in PCOS rats, it cannot provide a comprehensive explanation in terms of causality, indicating the need for further prospective comprehensive studies to confirm our findings.

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