Polycystic ovary syndrome (PCOS) is a complex, multifaceted, heterogeneous disorder, affecting 4% to 18% of reproductive-aged women and is associated with reproductive, metabolic and psychological dysfunction. Evidences from studies on women with PCOS and on an experimental rat PCO model suggest that the sympathetic regulatory drive to the ovary may be unbalanced (disturbance of ovarian noradrenaline homeostasis). The present study aimed to evaluate the sympathetic innervation in central and peripheral pathways in PCO rats. Our objectives in this study were (1) to estimate LC activity in rats with estradiol valerate (EV)-induced PCO; (2) to antagonize alpha adrenoceptor in systemic conditions with yohimbine and clonidine during modeling. Forty-eight rats were divided into two groups: LC and drug groups. Every group subdivided in two groups: modeling and control. In modeling groups, eighteen rats were treated with estradiol valerate for induction of follicular cysts and remained on sesame oil for control groups. Estradiol concentration was significantly augmented by the LC lesion in PCO rats (P < 0.001), while LC lesion could not alter serum concentrations of LH and FSH, like yohimbine and clonidine. The morphological observations of ovaries of LC lesion rats showed follicles with hyperthecosis, but Yohimbine reduced the cysts, increased corpus lutea and developed follicles like clonidine. In this study the biochemical and histological findings show that simultaneous administration of yohimbine and clonidine with estradiol valerate can prevent induction of PCO modeling in rat.

Keywords: Polycystic ovary syndrome (PCOS), Locus coeruleus, Yohimbin, Clonidine, Estradiol, rat

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women. Its prevalence among fertile women is 15%-20%. Being overweight worsens all clinical features of PCOS. These clinical features include reproductive manifestations such as reduced frequency of ovulation and irregular menstrual cycles, reduced fertility, polycystic ovaries on ultrasound, and high male hormones such as testosterone which can cause excess facial or body hair growth and acne. PCOS is also associated with metabolic features and diabetes and cardiovascular disease risk factors including high levels of insulin or insulin resistance and abnormal cholesterol levels. Autonomic and central nervous systems play an important role in the regulation of ovarian physiology. The autonomic nervous system has been suggested to contribute to PCOS. In the rat, a substantial fraction of the sympathetic innervation, targeting the ovary, is provided by neurons of the celiac ganglion and travels through the superior ovarian nerve (SON). Sympathetic innervation represents almost 90% of the noradrenaline (NA) present in the ovary, and yet, after surgical denervation, NA levels still remain over 10-15%, suggesting the existence of intraovarian cells participating in ovarian NA homeostasis. Furthermore, oocytes could be in part responsible for this because at least in monkeys they showed to take up dopamine (DA), through membrane DA transporter (DAT) and synthesize NA (via DA, -hydroxylase). Feature of PCOS is associated with hyperandrogenemia, hyperinsulinemia and insulin resistance, as well as abdominal obesity, cardiovascular disease and obstructive sleep apnea—all factors hypothesized to be associated with increased activity of the sympathetic nervous system. PCOS is associated with an increase in ovarian catecholaminergic nerve fibers and altered catecholamine metabolism, suggesting increased sympathetic nervous system activity. It is well known that one of the major neurotransmitters that control LH secretion is NA. The NA turnover in the preoptic area (POA) parallels which effects changes in circulating LH concentrations. Infusion of noradrenaline either intracerebroventricularly of the rat suppresses LH pulses, as does electrical stimulation of the brain stem ascending noradrenergic pathways, thus suggesting that increases in noradrenergic activity results in suppression of the GnRH pulse generator. The peripheral administration of adrenergic receptor antagonists also decreases the frequency of the GnRH pulse generator. The finding that both a reduction and increase in adrenergic receptor activity have the same effect on pulsatile LH secretion led Lang and colleagues to propose that fluctuating patterns of adrenergic receptor activity are essential for pulsatile GnRH release, a postulate substantiated by theoretical modeling experiments. Thus, the sympathetic nervous system may be an important factor in the development and maintenance of PCOS. Women with PCOS have significantly higher sympathetic nerve activity than their matched controls and the increased sympathetic outflow is related to hormonal and metabolic features. LC is the primary source of NA in the brain and has been implicated in the regulation of female reproductive functions. The stimulatory role of LC in LH release is suggested by previous studies demonstrating that its activation increases LH release after preoptic area (POA) electrochemical stimulation, whereas LC lesion decreases NA concentrations in the hypothalamus and POA, and prevents the occurrence of the LH surge. During metestrus, diestrus and estrus, LC lesion did not modify either LH plasma concentrations or LH RH content. LC lesion decreases NA concentrations and LC lesion can disturb the positive feedback action of estrogen receptor (ER) on LH secretion. Electrolytic lesions of the LC block the preovulatory surge of LH this blockade of LH surge is accompanied by a decrease in the NA content in the medial preoptic area (MPOA) and medial basal hypothalamus (MBH). Oestradiol has been reported to regulate the gene expression of NA biosynthetic enzymes in the LC. Experimental induction of a PCOS in rodents by administration of a single dose of estradiol valerate (EV) results in activation of the peripheral sympathetic neurons that innervate the ovary. This activation is evidenced by an increased capacity of ovarian nerve terminals to incorporate and release NA, an increase in ovarian NA content, and a decrease in ovarian beta-adrenergic receptor number (down regulation) in the ovarian compartments receiving catecholaminergic innervation. This increased ovarian sympathetic outflow suggested by these alterations in catecholamine homeostasis was accompanied by a thalamic cell-interstitial tissue selective down-regulation of beta-adrenergic receptors; the beta-adrenergic receptor concentration in these sympathetically innervated ovarian compartments was significantly lower in PCO than during the estrous phase of the estrous cycle, a time at which the beta-adrenergic receptor concentration reaches its lowest levels in normal cycling ovaries. The sympathetic ovary nerve (SON) transsection also reduced the elevated levels of ovarian NA resulting from EV treatment and caused up-regulation of beta-adrenoceptors. Most importantly, SON transsection restored estrous cyclicity and ovulatory capacity. The results indicate that the
increased output of ovarian steroids in PCOS is at least in part due to an enhanced responsiveness of the gland to both catecholamnergic and gonadotropin stimulation, but the effects of ovarian steroids on the activity of LC neurones and the relevance of such actions for LH secretion remain poorly understood. The present study aimed to evaluate the effect of LC lesion and α2 receptor inhibitors in the processing of polycystic ovary syndrome modeling in rat.

MATERIALS AND METHODS

Animals and care

Adult female Wistar rats weighing 220–230 g (7–8 wk of age) from the animal house of the Pasteur Institute were kept in a central animal care facility under a 12-h light, 12-h dark cycle and controlled temperature (24 ± 0.5 C). Food and water were provided ad libitum. Vaginal smears were taken daily and only rats showing at least three consecutive 4-d regular estrous cycles were used in the experiment. All of the animal studies were also approved by a group from the Ethics Committee of Tehran University of Medical Sciences and experiments were performed in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication No. 85-23, revised 2007).

Experimental design

Vaginal Smears

The stage of cyclicity was determined by microscopic analysis of the predominant cell type in vaginal smears. Estrous cyclicity was monitored by vaginal smears obtained between 0800 and 1200 hours, and it was assessed by analysis at the light microscopy level of the relative proportion of leukocytes, epithelial and cornified cells found in daily vaginal lavages, which characteristically change during different stages of the estrous cycle. The rat estrous cycle (estrus, diestrus I, diestrus II, and proestrus) usually lasts about 4 days, in controls or PCO rats.23

Hormonal treatment and study procedure

After 1 week of acclimatization, 7-8 week-old rats (n=24) and lasts about 4 days each received an i.m. injection of Estradiol valerate (EV) (Abaraihan Co., Iran), 2mg in 0.2 ml of sesame oil, to induce PCOS.25 All experiments treated with EV were performed 60 days after the injection, when follicular cysts are first detected. Then PCO rats and sesame oil groups were subdivided in two groups: LC normal and LC lesion groups. Drug groups: yohimbine and clonidine groups subdivided in two groups: Se-yoh, Est-yoh and Se-cl, Est-cl. According to previous dose response trials drugs were prescribed as below: clonidine (0.1 mg/kg i.p.), yohimbine (0.4 mg/kg i.p.). PCO modeling was down simultaneously with drug medication (60 days).

Study groups

A LC lesion group

A1 Injection of estradiol valerate for PCO modeling
A2 Injection of sesame oil control for PCO modeling

B Control (without LC lesion)

B1 Injection of estradiol valerate for PCO modeling
B2 Injection of sesame oil control for PCO modeling

C Yohimbine group

C1 Injection of estradiol valerate for PCO modeling
C2 Injection of sesame oil control for PCO modeling

D Clonidine group

D1 Injection of estradiol valerate for PCO modeling
D2 Injection of sesame oil control for PCO modeling

LC neurochemical lesion

Under ketamine 100 mg/kg body weight, ip and xylazine 14 mg/kg body weight, ip anesthesia, rats were positioned in a stereotaxic instrument with the incisor bar set at the zero point. The dorsal surface of the skull was exposed, and holes, 2 mm in diameter, were drilled bilaterally. Bilateral LC lesions were made using an SEG 5 gl syringe. The coordinates were: AP = -0.8 mm (interaural), L = +0.9mm (bregma) and V = +3.0mm (interaural). (Paxinos and Watson, 1986) under an angle of 30 ~ in rostro-caudal direction.

Each side was injected with 2gl 6-OHDA hydrobromide (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) (6gg/gl dissolved in 0.1 mg/ml ascorbic acid; injection time: 0.4gl/min) 26.

Measurement of circulating levels of gonadotropins and gonadal steroid

Blood was collected from heart and serum Lutein hormone (LH); Follicular stimulation hormone (FSH) and Estradiol levels were determined by ELISA. Kits were used to measure LH (Kit CA-92627 Cod.No.EIA-4K2G5, from American Co. Monobind, Inc. Costa Mesa), FSH (Kit CA-92627 Cod .No. EIA-6K2G5 from American Co. Monobind, Inc. Costa Mesa) and Estradiol (Kit DRG Cod. No. EIA-2693 from American Co. DRG International GmbH).

Ovarian morphology

The ovaries from controls (EV-treated), LC lesion, yohimbine, clonidine and sesame oil groups treated were removed, cleaned of adherent connective fat tissue, and fixed in 10% formaldehyde buffer for at least 24 hours. Ovaries were imbedded in paraffin, cut in 8-μm sections, and stained with hematoxylin and eosin.

Statistical Analysis

Data are presented as Mean ± Standard deviation. Serum level of hormones was compared between groups by ANOVA. In this analysis both modeling and treatment groups were used as fix factor effect and their interaction was calculated. Pairwise comparison was done by Bonferroni post hoc test. P-value less than 0.05 considered as significant level. SPSS 13 (SPSS Inc. Chicago USA) was used for statistical analysis.

RESULTS

LC lesion, Yohimbine and Clonidine administration effects on serum levels of ovarian steroids and gonadotropins

The serum level of Estradiol was significantly different between modeling (F = 16.6, df1 = 1, df2 = 40, P-value = 0.001) and study group (F = 21.8, df1 = 3, df2 = 40, P-value < 0.001). Interaction between modeling and study groups also was significant (F = 7.6, df1 = 3, df2 = 40, P-value < 0.001) (Table 1). The serum level of Estradiol was significantly increased between PCO groups (estriadiol valerate) against non PCO groups (sesame oil) (P=0.030), the same as LC damaged group (P=0.001). In yohimbine and clonidine groups serum level of estradiol had not statistically significant difference between PCO and non PCO groups (Figure 1).

The serum level of FSH was significantly different between modeling (F = 7.1, df1 = 1, df2 = 40, P-value = 0.001) but it was not any difference between study group (F = 1.9, df1 = 3, df2 = 40, P-value = 0.177). Pair wise comparison was showing any considerable difference between groups (Table 2). The serum level of LH was significantly different between modeling (F = 3.2, df1 = 1, df2 = 40, P-value = 0.032) but it was not any difference between study groups (F = 0.1, df1 = 3, df2 = 40, P-value = 0.898). Pair wise comparison was showing any considerable difference between groups (Table 3).

Histological findings

LC lesion, yohimbine and clonidine administration upon ovarian morphology

The morphological analyses of ovaries have been showed from control rats on estrus revealed the presence of numerous corpora lutea in different stages of development and regression, many of which clearly resulted from recent ovulation, as well as some atretic antral follicles (Fig.2A). Ovarian morphology on estrus of rats exposed to (EV) showed numerous small cysts in cortex of ovary and severe hemorrhage with oedematosis in medulla and so observed to be the presence of numerous healthy antral follicles with small size and follicles with enlarged theca cell layer (hyperthecosis) and cyst (Figs. 2B). As shown in Fig. 2F, corpora lutea and growing atretic follicles observed in histological findings after yohimbine
administration, which appears to be similar effect of clonidine (Fig 2G).

**DISCUSSION**

It is clear that the key to understand the neural control of the ovary includes the knowledge of which spinal cord and brain nuclei are neurally linked to this organ. It is an autonomic nervous system innervated by autonomic nerves composed of both sympathetic and parasympathetic efferent fibers. It seems to be very likely that the neural signals transmitted via the autonomic nerves to the ovary are integrated signals that include neuronal impulses from very different sites of the CNS. Apart from the most recent years, very little structure may be connected and probably involved in rat neuronal control of the peripheral endocrine glands including the ovary. Some structures of the supraspinal brain, such as hypothalamus, amygdala, locus coeruleus, which are transneuronally connected with the ovary, have already been reported previously to be involved in the control of ovarian functions. The first observation suggesting the existence of a neural pathway between a paired peripheral endocrine gland and the hypothalamus was reported more than 40 years ago. The significant overlap of CNS structures transneuronally connected with the ovary and other organs of the female reproductive tract strongly suggest a common representation of viscera in the CNS and similar neuronal circuits of the autonomic nervous system innervating the ovaries. Now it is well known that one of the major neurotransmitters that control LH secretion is NA. LC is the primary source of NA in the brain and has been implicated in the regulation of female reproductive functions. Neurons arising in the LC project directly to the GnRHa rich regions of the hypothalamic area (POA) and selective lesioning of the LC results in a decrease in NA levels within the POA, hypothalamus and prevents the occurrence of the LH surge. LC noradrenergic neurons may directly alter the activity of the GnRH neural system. Numerous studies implicated NA as an important regulator of GnRH/LH release. Infusion of NA either intracisternally or directly into the POA of adult female rats suppresses LH pulses. It is interesting to note that near complete interruption of ascending noradrenergic pathways did not alter LH pulse frequency whereas discrete lesions of the LC, destroying 50% or more of the nucleus, resulted in a permanent inhibition of pulsatile LH release. Administration of α-adrenergic receptor antagonists also decreases the frequency of the GnRH neural generator. The stimulatory role of LC in LH release is suggested by previous studies demonstrating that its activation increases LH release after POA electrochemical stimulation, through the activation of LHRR neurons. Both oestrogen (ER) and progesterone (PR) receptors have been demonstrated to exist in LC neurones, suggesting that these cells are possibly responsive to variations in circulating levels of ovarian steroids. LC can express ER and regulate the activity of GnRH neurons through both pre-synaptic and postsynaptic mechanisms. E2 significantly modulates the mRNA expression of numerous ion channels (K and Ca) in GnRH neurons. Quesada et al. in 2001 showed that E2-induced activation of IGF-1 receptors augments α1-adrenergic receptor signaling. E2 females have a higher number of LC neurones than males, and this difference appears to depend on estrogen receptors. E2 imparts a multifaceted influence over synaptic transmission in the mammalian central nervous system. Not only can E2 alter synaptic responses via genomic mechanisms, but there exists a wealth of information that indicates the steroid can also modulate cell-to-cell communication much more rapidly. Estrogen modulates the excitability of a number of neurons that are involved in the control of homeostasis, including reproduction, stress responses, feeding and motivated behaviors. E2 is essential for cyclical GnRH neuronal activity and secretion and to prime the neurons that control reproductive behavior for subsequent action of progesterone and other molecules. In this respect, brain insulin-like growth factor-1 (IGF-1) receptor activity is required for E2 priming of the female reproductive axis. Systemic administration of E2 in ovariectomized rats IGF-1 receptors and induces the association between IGF-1 receptors and ERα in the hypothalamus. The E2-induced activation of IGF-1 receptors augments α1-adrenergic receptor signaling, which is important for reproductive functions. On the other hand, blockade of IGF-1 receptors during E2 priming prevents E2-induced increases in α1-adrenergic receptor binding density as well as IGF-1 enhancement of noradrenergic receptor signaling. Collectively, these findings support functional interactions between IGF-1 and E2: 1) E2 may have a critical role in the actions of E2 on the IGF-1 receptor signaling pathway may be a key mechanism by which E2 affects synaptic remodeling and neuronal plasticity during the estrous cycle and help to understand the etiology of PCOS. The rat model for experimentally induced polycystic ovaries (PCO) produced by a single injection of estradiol valerate (estrous cycle) has similarities with human PCOS. Lara in 1993 reported that PCO induced by the administration of a single dose of EV to rats (estrous cycle) results in profound changes in ovarian catecholamine homeostasis, which were initiated before the development of cysts and persist after the cysts were formed. These changes include an increased ovarian NAC content, enhanced Na release from ovarian nerve terminals and down-regulation of β-adrenergic receptors in theca-interstitial cells and in granulosa cells. In rat increased ovarian sympathetic tone with EV-induced PCO has been evidenced by elevations in tyrosine hydroxylase activity and NE concentration, downregulation of the β-adrenoceptors and increased production of ovarian nerve growth factor, a target-derived neurotrophin. In this study the comparison of significant increment of estradiol in LC lesion of PCO rats with its control group shows that positive feedback system have been disturbed between E2 and LH in LC and confirmed the study of Helena et al., 2002. This can be the cause for LH not to increase significantly. Histological data shows that LC lesion has induced hyperthecosis confirming Bernuci's report in 2008. Hyperthecosis can be interpreted as the cause of latency in the processing of rat modeling of PCO in LC lesion group. These results suggest that an abnormality in the regulation of hypothalamic GnRH secretion is present in PCOS and LC lesion seems to alter the feedback regulation of the hypothalamic-pituitary-gonadal axis, because the increase in serum levels of estradiol was not accompanied by an alteration in gonadotropin secretion. This may be explained by a reduced sensitivity of GnRH neurons to ovarian steroid inhibition, which is a frequent feature of PCO disease. In yohimbine and clonidine groups serum level of estradiol was not changed significantly. Histological findings of yohimbine shows an increase in corpus lutea and developed follicles in PCO rats and it is interesting to note that data of yohimbine control group also shows increasing of corpus lutea, that resembles data of PCO group and seems to be the direct effect yohimbine in rat ovary in PCO and non PCO groups without the role of EV. Clonidine is a selective drug because it is direct-acting α2 adrenergic agonist and has specificity towards the presynaptic α2 receptors, which this binding decreases presynaptic calcium levels and inhibits the release of NA. The net effect of this drug is a decrease in sympathetic tone. These receptors close a negative feedback loop that begins with descending sympathetic nerves from the brain that controls the production of catecholamines in the adrenergic medulla. By fooling the brain into believing that catecholamine levels are higher than they really are, clonidine causes the brain to reduce its signals to the adrenal medulla, which in turn lowers catecholamine production and blood levels. This is the mechanism for opiate withdrawal, by decreasing adrenergic neurotransmission from the locus coeruleus. In this study we showed that administration of clonidine also decreased cysts and increased corpus lutea and dominant follicles in PCO rat. We did not find changes in plasma LH and FSH levels in either groups of LC damage, yohimbine and clonidine.

**CONCLUSIONS**

Our results indicate that PCO modeling in rat had been prevented by simultaneous systemic administration of yohimbine or clonidine. These findings support two theories: 1) LC have a critical role in the positive feedback system of estradiol and 2) the increasing sympathetic activity contributes to the development and maintenance of PCOS because the simultaneously administration of clonidine and yohimbine prevents PCO modeling in rat. These data suggest new alternatives for etiology and treatment of PCOS.
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**Fig. 1:** Comparison of serum level of estradiol (pg/ml) between study groups.

*: Significant difference in comparison between PCO (estradiol) and non PCO (sesame oil) groups, **x:** Significant difference in comparison of non PCO groups treated with Yohimbine and non PCO control groups.

**Table 1:** Plasma level of Estradiol (pg/ml) in PCO and non-PCO group.

<table>
<thead>
<tr>
<th>groups</th>
<th>PCO Modeling</th>
<th>n</th>
<th>Mean±SD</th>
<th>P-value¹</th>
<th>P-value²</th>
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<td>Estradiol Valerate (PCO)</td>
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<td>19.0±4.12</td>
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<td>Control</td>
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P-value¹: Comparison between PCO and non PCO groups (Bonferroni Post hoc).  
P-value²: Comparison with control groups (Bonferroni Post hoc).  

**Table 2:** Plasma level of FSH (IU/ml) in PCO and non-PCO group.

<table>
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<th>groups</th>
<th>PCO Modeling</th>
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P-value¹: Comparison between PCO and non PCO groups (Bonferroni Post hoc).  
P-value²: Comparison with control groups (Bonferroni Post hoc).  

**Table 3:** Plasma level of LH (IU/ml) in PCO and non-PCO group.

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<th>Mean±SD</th>
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P-value¹: Comparison between PCO and non PCO groups (Bonferroni Post hoc).  
P-value²: Comparison with control groups (Bonferroni Post hoc).
Fig. 2: The histological findings of LC lesion, yohimbine and clonidine effects upon ovarian morphology: A, Ovarian morphology on estrus of rat maintained at ambient temperature (intact/control). B, ovarian morphology on EV treated rats (PCO). C and D, on sesame oil groups as control of EV and yohimbine. E, ovarian morphology on LC lesion on PCO rats. F, Yohimbine effects upon ovarian morphology of PCO rat. G, clonidine.
ACKNOWLEDGEMENTS

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