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Effect of microRNA-409 on the pathogenesis of polycystic ovary syndrome

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Abstract. – OBJECTIVE: To explore the expression level of microRNA-409 in PCOS (polycystic ovary syndrome) rats, as well as its potential effects on fertility of PCOS rats and phenotypes of offspring rats.

MATERIALS AND METHODS: PCOS model in rats was established by Letasazole administration. Follicular development of rats was evaluated by the percentages of the cystic follicle (FC) and corpus luteum (CL) of all follicles. The enzyme-linked immunosorbent assay (ELISA) was conducted to detect serum levels of hormones in rats, including LH, LH/FSH, T, INS, FSH, and E2. Subsequently, PCOS rats received a subcapsular injection of microRNA-409 mimics. The expression level of microRNA-409 in ovary was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Serum levels of LH, LH/FSH, T, INS, FSH, and E2 in PCOS rats with microRNA-409 overexpression were accessed by enzyme-linked immunosorbent assay (ELISA) as well. PCOS rats were mated with male rats for recording pregnancy rate. At 6-week-old of offspring, they were sacrificed for detecting microRNA-409 level, percentages of FC and CL, as well as serum levels of hormones.

RESULTS: PCOS rats showed irregular estrous cycle and they were mainly in the anestrum. Rats in the control group were in a regular estrous cycle. A higher percentage of FC and a lower percentage of CL were seen in PCOS rats compared with those of controls. ELISA data revealed higher serum levels of LH, LH/FSH, and T in PCOS rats compared with those of controls. However, levels of FSH and E2 were lower in PCOS rats. Although INS level increased in PCOS rats, we did not observe a significant difference in INS level between PCOS rats and control rats. MicroRNA-409 was lowly expressed in ovaries of PCOS rats than those of controls. After injection of microRNA-409 mimics into rat ovary, microRNA-409 expression remarkably upregulated than those PCOS rats without injection. Rats in PCOS+microRNA-409 mimics group showed the largest body weight compared with those in the PCOS group and control group. PCOS rats showed a lower pregnancy rate than those of controls, which was markedly increased after administration of microRNA-409 mimics. Rats in PCOS+microRNA-409 mimics group presented lower levels of LH, LH/FSH, T, and INS, but higher levels of FSH and E2 than those in PCOS group.

CONCLUSIONS: MicroRNA-409 is lowly expressed in the ovary of PCOS rats. Overexpression of microRNA-409 could improve hormone levels and pregnancy rate in PCOS rats, as well as affect clinical phenotypes of their offspring.

Key Words:
MicroRNA-409, PCOS, Hormone level, Follicular development

Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive endocrine syndrome in women of childbearing age. Typical clinical manifestations of PCOS include menstrual disorders, oligomenorrhea, hypertrichosis, obese, hemorrhoids, ovarian polycystic, and infertility[1]. Globally, about 5-20% of women of childbearing age suffer from PCOS[2]. It is generally believed that PCOS is the result of interaction between genetic polymorphisms and environmental factors, mainly involving genetic factors[3], high level of luteinizing hormone[4], hyperandrogenism[5], hyperinsulinemia and IR[6], low-level inflammatory response[7], obesity[8], environmental and other factors[9]. Although PCOS has been well studied, its specific pathogenesis still remains unclear.

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MicroRNAs are important regulators at post-transcriptional level. Mature microRNAs can completely or incompletely bind to the 3'UTR of target genes, thus forming an RNA-induced gene silencing complex. The mRNA of the target gene is then degraded or its translation is inhibited. MicroRNAs exert crucial roles in cell proliferation, differentiation, apoptosis, and hormone synthesis[10]. In recent years, a large number of studies have focused on the potential functions of microRNAs in follicular development and female reproductive disorders. Some certain microRNAs participate in the whole process of follicular development and are closely related to follicle formation, follicle recruitment, selection of dominant follicles, ovulation and corpus luteum formation, follicular atresia, etc.[10-14]. Expression levels of relative microRNAs are dynamically changed in different stages of follicular development. Since microRNAs are easily collected, stable in the circulatory system and not affected by the menstrual cycle, they have been applied as predictable biomarkers for the occurrence and development of female reproductive endocrine diseases. Therefore, studies on relative microRNAs in PCOS patients have been well concerned in recent years.

Differentially expressed microRNAs have been found in serum samples of PCOS patients. It is reported that expression levels of miR-21, miR-27b, and miR-155 are positively correlated to the serum level of free testosterone, suggesting that these microRNAs may be related to the hyperandrogenic phenotype of PCOS[15]. Also, miR-22 is highly expressed in plasma samples of PCOS patients, which is served as a diagnostic hallmark for PCOS. Single nucleotide polymorphisms of miR-146a (rs2910164) and miR-222 (rs2858060) are associated with susceptibility of PCOS. It is believed that miR-146a and miR-222 are involved in PCOS development by regulating their target genes[16]. In this study, we first constructed a PCOS model in rats and interfered microRNA-409 expression in PCOS rats. The potential effect of microRNA-409 on rat fertility and phenotypes of offspring rats were subsequently analyzed.

Materials and Methods

Construction of PCOS Model in Rats

Sprague Dawley (SD) rats were obtained from Wenzhou Medical University Animal Center and housed in a SPF level experimental room (temperature of 22-26ºC, humidity of 40%-70%, and light cycle of 12 h/12 h). Each rat was individually housed. Rat body weight was daily recorded from 6 weeks old. Rats in the PCOS group and control group received intragastric administration of 1 mg/kg Letrozole or 1 mg/kg CMC solution for consecutive 30 days, respectively. This study was approved by the Animal Ethics Committee of Wenzhou Medical University Animal Center.

Subcapsular Injection of Rat Ovary

On the 30th day of intragastric administration, rats were anesthetized and exposed for ovaries. Rat ovary was fixed using forceps and capsule covering the ovary surface was lifted with another forceps. MicroRNA-409 mimics was injected in the bilateral ovaries using the 10 μL microinjector, with 5 μL of microRNA-409 mimics in each side of the ovary. The incision was sutured layer by layer and antibiotics were applied on the incision. MicroRNA-409 mimics was injected every three days for three times.

Sample Collection

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.5 ml/100 g) and placed on the surgical table. A blood sample was harvested from abdominal aorta until it collapsed. Bilateral ovaries were quickly harvested by removing surrounding fat tissues and Fallopian tubes. One side of the ovary was fixed in 4% paraformaldehyde and the other side was preserved in -80ºC. A blood sample was centrifuged at 4ºC, 3000 rpm/min for 20 min for collecting serum sample.

Rat Vaginal Smear

Vaginal secretions were collected using a plastic pipette with normal saline by inserting the tip into the rat vagina at a fixed time point every day. After vaginal smear was air dried, it was fixed with methylene blue for 15 min and washed with clean water. Cell morphology was observed using a microscope to determine the estrous cycle. Round and nucleated cells were frequently seen in the proestrus, and leukocytes and keratinocytes were occasionally seen. Irregular keratinocytes were seen in the estrous. In the meta-estrus, the ratios of nucleated cells, leukocytes, and keratinocytes were similar. A large number of leukocytes were seen in the anestrum with a small number of nucleated cells. Vaginal smear was conducted for consecutive 3 estrous cycles (about 15 days) in each rat.

Rat Mate

After construction of the PCOS model in rats, female and male rats were mated at a ratio of 1:2 at 18:00 pm. Vaginal smear was prepared the next morning. Microscope observation of sperm was considered as the first day of pregnancy. Offspring rats were housed in a SPF level experimental room until 6 weeks old.

Enzyme-Linked Immunosorbent Assay (ELISA)

50 μL of enzyme conjugate and 50 μL of antibodies were added in the serum sample of each well. After incubation for 1 h, the plate was washed with the washing buffer for three times with 10 s for each time. Substrate buffer was then added for color development, followed by adding termination buffer. The optical density was determined at the wavelength of 450 nm.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract total RNA from ovary tissues for reverse transcription according to the instructions of PrimeScript RT reagent Kit. 1 μL of complementary Deoxyribose Nucleic Acid (cDNA) was collected for PCR using SYBR Green method (TaKaRa, Otsu, Shiga, Japan). MicroRNA-409 primer was: forward, 5'-GTGGATAATGCGAGATATTTT-3'; reverse, 5'-AAATCTCGCTAAATTATCACC-3'.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was utilized for data analyzed. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Normal distribution data between the two groups were analyzed by the *t*-test. $p < 0.05$ was considered statistically significant.

Results

Construction of PCOS Model in Rats

From the 12th day of PCOS model construction, consecutive 3 estrous cycles were observed by vaginal smear. The estrous cycle of PCOS rats was markedly irregular, and they were mainly in the anestrum. By comparison, rats in control group showed regular estrous cycle (Figure 1A). Follicular development of rats was evaluated by calculating the percentages of the cystic follicle (FC) and corpus luteum (CL) of all follicles. A higher percentage of FC and a lower percentage of CL were found in ovaries of PCOS rats compared with those of control group (Figure 1B). Furthermore, ELISA was conducted to detect serum levels of hormones in rats. Compared with rats in control group, PCOS rats showed higher serum levels of LH (luteinizing hormone), LH/FSH (luteinizing hormone/follicle-stimulating hormone) and T (testosterone). However, levels of FSH and E_2 (estradiol) were lower in PCOS rats. Although INS (insulin) level increased in PCOS rats, we did not observe a significant difference between PCOS rats and control rats (Figure 1C-1H). The above data demonstrated the successful construction of PCOS model in rats by intragastric administration of Letrozole. Compared with rats in control group, PCOS rats showed evident alterations in ovary morphology and hormone levels.

Downregulated MicroRNA-409 Improved Hormone Levels and Increased Pregnancy Rate in PCOS Rats

We found that microRNA-409 is lowly expressed in ovaries of PCOS rats than those of controls. After injection of microRNA-409 mimics into rat ovary, microRNA-409 expression remarkably upregulated than those PCOS rats without injection (Figure 2A). We subsequently compared body weight of rats in control group, PCOS group, and PCOS+microRNA-409 mimics group. PCOS rats had higher body weight compared with those of controls, which may be resulted from PCOS-induced metabolic disturbance. Rats in PCOS+microRNA-409 mimics group showed the highest body weight among the three group, suggesting the effect of microRNA-409 on body weight gain (Figure 2B). To explore the potential effect of microRNA-409 on pregnancy rate of PCOS rats, we recorded the pregnancies after rats in three groups successfully mated with male rats. PCOS rats showed a lower pregnancy rate than those of controls, whereas administration of microRNA-409 mimics markedly increased pregnancy rate (Figure 2C). The serum levels of hormones in rats were then detected by ELISA. Rats in PCOS+microRNA-409 mimics group presented lower levels of LH, LH/FSH, T, and INS, but higher levels of FSH and E_2 than those in the PCOS group (Figure 2D-2I).

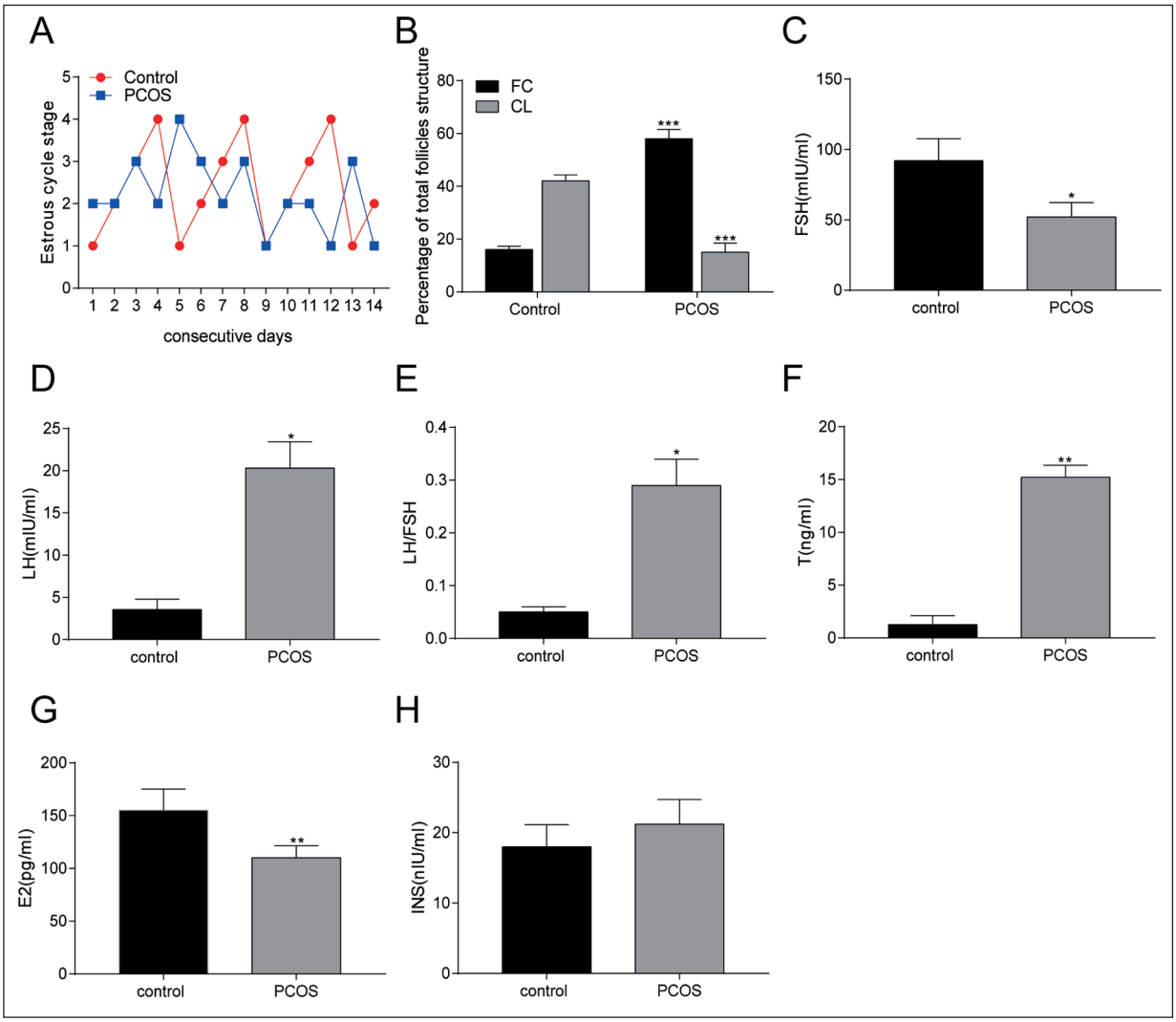

Figure 1. onstruction of PCOS model in rats. ***A***, The estrous cycle of PCOS rats was obviously irregular, and they were mainly in the anestrum. By comparison, rats in control group showed regular estrous cycle. B, Percentages of FC and CL of total follicles in rats of control group and PCOS group. ***C-H,*** Serum levels of LH, LH/FSH, T, INS, FSH, and E_2 in rats of control group and PCOS group.

MicroRNA-409 Expression in Rat Maternal Ovaries Affected Its Expression in Ovaries of Offspring Rats and Their Phenotypes

To elucidate whether the microRNA-409 expression in maternal rat ovaries could affect its expression in ovaries of offspring rats, we detected microRNA-409 expression in offspring of the three groups. MicroRNA-409 expression was downregulated in offspring of PCOS+microRNA-409 mimics group compared with those in PCOS group (Figure 3A). To further reveal the effect of microRNA-409 on the follicular development of offspring, percentages of FC and CL of all follicles were accessed. The data showed higher percentages of FC and CL in offspring of PCOS group than those in PCOS+microRNA-409 mimics group (Figure 3B). It is suggested that PCOS has a genetic predisposition and microRNA-409 is capable of improving follicular development in offspring of PCOS rats. Furthermore, serum levels of hormones were detected in offspring rats. Serum level of T was markedly higher in control group than that of PCOS group. However, no significant differences in serum levels of LH, LH/FSH, INS, FSH, and E_2 were found in offspring between PCOS group and PCOS+microRNA-409 mimics group (Figure 3C-3H).

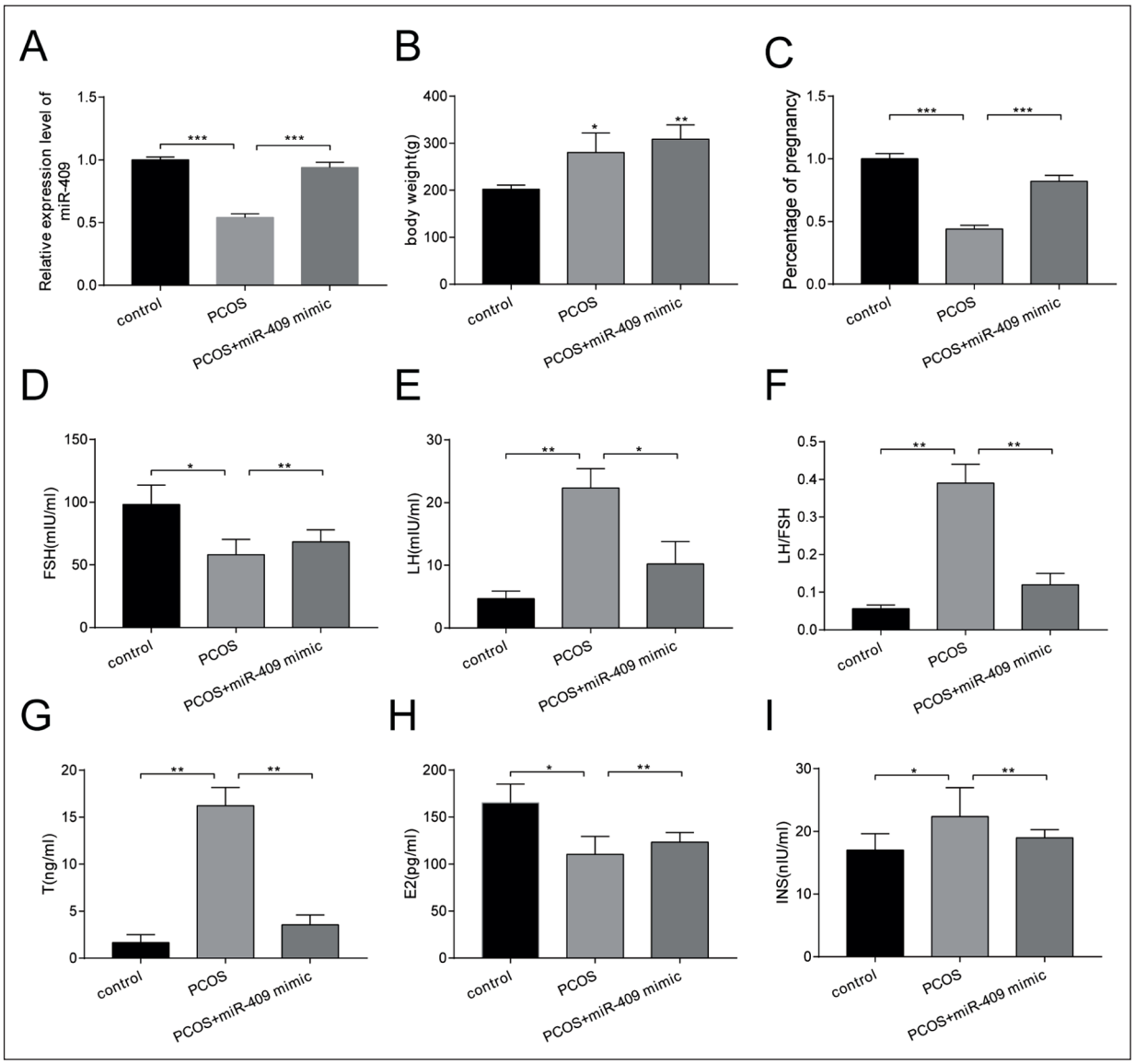

Figure 2. Downregulated microRNA-409 improved hormone levels and increased pregnancy rate in PCOS rats. ***A,*** MicroRNA-409 expression in rat ovaries of PCOS group and PCOS+microRNA-409 mimics group. ***B,*** Body weight of rats in PCOS group and PCOS+microRNA-409 mimics group. ***C,*** Pregnancy rate in rats of PCOS group and PCOS+microRNA-409 mimics group. ***D-I,*** Serum levels of LH, LH/FSH, T, INS, FSH, and E_2 in rats of PCOS group and PCOS+microRNA-409 mimics group.

Discussion

PCOS is a common reproductive endocrine disease in women of childbearing age with a high level of heterogeneous. Clinical manifestations and biochemical characteristics in PCOS patients vary a lot. In this study, the PCOS model in rats was established by Letasazole administration. The data showed a higher ratio of cystic follicles and lower number of corpus luteum in PCOS rats compared with those of controls. Besides, PCOS rats showed higher serum levels of LH, LH/FSH, and T, whereas lower levels of FSH and E_2 than those of controls, which were consistent with clinical characteristics of PCOS. It is considered that our PCOS model in rats could simulate the similar endocrine disorders and ovarian pathology of PCOS patients.

Some microRNAs are differentially expressed in blood, follicular fluid, granulosa cells, and ovarian follicular cells of PCOS patients compared with healthy controls. We speculated that

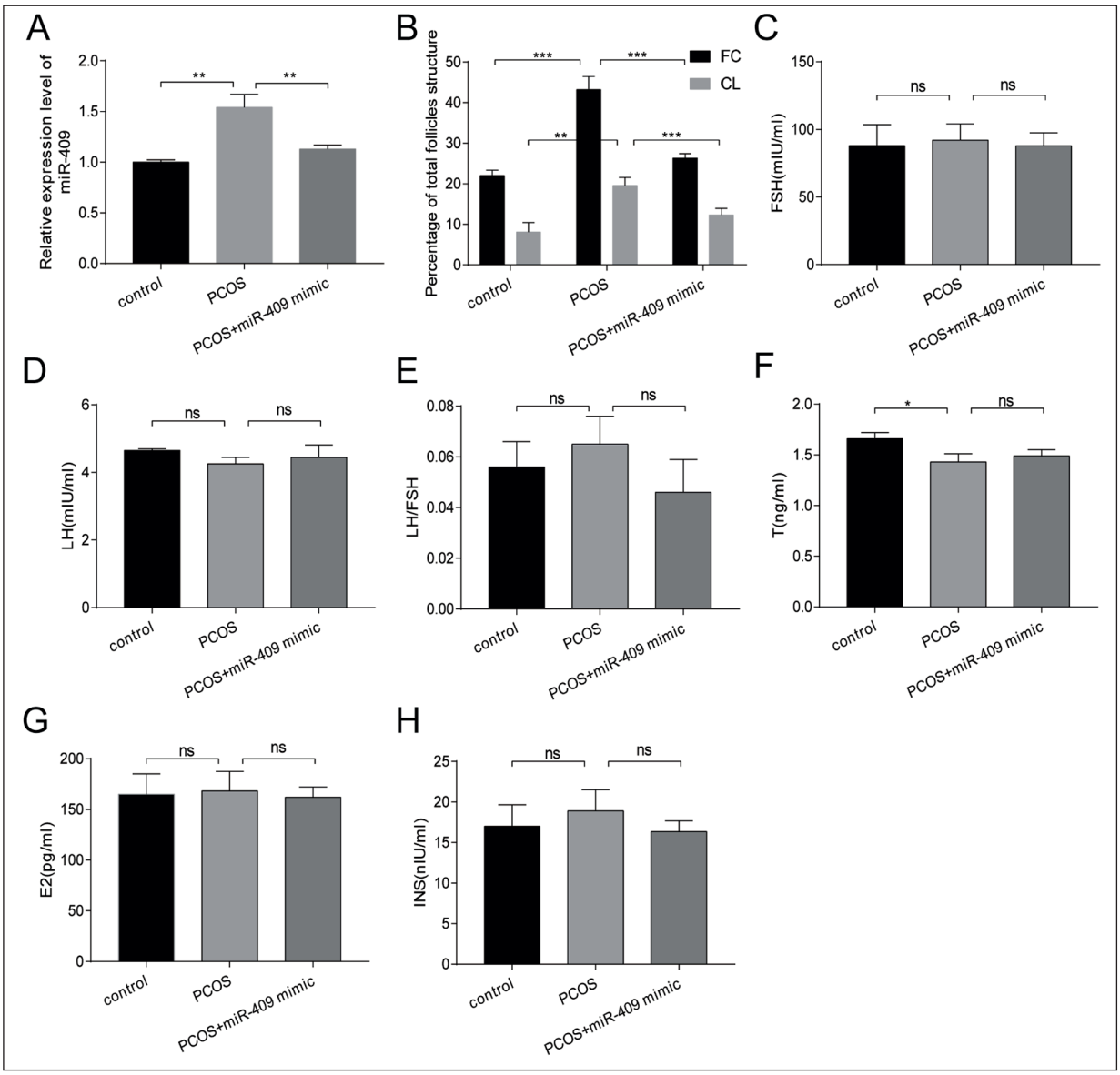

Figure 3. Effect of microRNA-409 on offspring of PCOS rats. ***A,*** MicroRNA-409 expression in offspring rat ovaries of PCOS group and PCOS+microRNA-409 mimics group. ***B,*** Percentages of FC and CL of total follicles in offspring rats of PCOS group and PCOS+microRNA-409 mimics group. ***C-H,*** Serum levels of LH, LH/FSH, T, INS, FSH, and E_2 in offspring rats of PCOS group and PCOS+microRNA-409 mimics group.

these microRNAs may participate in the pathogenesis of PCOS[17]. Our study found that microRNA-409 is lowly expressed in ovary tissues of PCOS rats, suggesting the potential role of microRNA-409 in PCOS development. Researches have shown that the synthesis and secretion of hormones in ovarian cells of PCOS patients are abnormal, and microRNAs may exert a vital role during the process. For example, miR-135a, miR-155, miR-132, and miR-146a have been proved to inhibit testosterone release[18-20]. In addition, miR-145, miR-143, and miR-376a are crucial in the formation and maintenance of primordial follicles[21].

To further investigate whether microRNA-409 could affect hormone levels in PCOS, microRNA-409 mimics was injected into rat ovary through the subcapsular ovary. Injection of microRNA-409 mimics in PCOS rats remarkably decreased serum levels of T, LH, and LH/FSH, whereas increased rat pregnancy rate compared with those PCOS rats without injection. It is

suggested that microRNA-409 can significantly improve hormone levels and pregnancy rate of PCOS rats, providing a new direction for future researches on PCOS. To elucidate whether microRNA-409 in maternal ovarian of PCOS rats could affect their offspring, we examined the expression level of microRNA-409 in the ovaries of offspring rats. The data showed a higher level of microRNA-409 in ovaries of offspring rats from those PCOS rats injected with microRNA-409 mimics compared with those without microRNA-409 administration. Follicular development was improved as well, but hormone levels did not change significantly.

Conclusions

We found that microRNA-409 is lowly expressed in the ovary of PCOS rats. Overexpression of microRNA-409 could improve hormone levels and pregnancy rate in PCOS rats, as well as affect clinical phenotypes of their offspring. MicroRNA-409 may serve as a new target for PCOS treatment and improve the success rate of assisted reproductive technology.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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