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Original Article

Inhibition of leptinleptinr-kisspeptinkiss1r signaling pathway promotes follicular development in polycystic ovarian rats

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Abstract: Polycystic ovary syndrome (PCOS) is a reproductive endocrine disease. It was shown that kisspeptin/kiss1R signaling pathway involves regulating ovarian function, but its specific upstream mechanism remains to be further studied. Therefore, this study aims to investigate the effect of leptin/leptinR-kisspeptin/kiss1R signaling pathway on follicular development in PCOS rats. The rats were divided into a normal control group, a PCOS model group established by RU486, and a leptinR antagonist group. The levels of serum leptin, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were tested by ELISA. Embryo implantation was used to detect ovarian function. Hematoxylin-eosin (H & E) staining was adopted to evaluate ovarian follicular development. Western blot was selected to detect kisspeptin, kiss1R, and leptinR protein levels. Leptin level was significantly increased in the PCOS model group compared with that in the control group ($P < 0.05$) and significantly reduced after leptinR antagonist treatment ($P < 0.05$). LH and FSH levels were significantly elevated in the PCOS group ($P < 0.05$), while leptinR antagonists significantly inhibited these changes ($P < 0.05$). The number of implanted embryos was significantly reduced in the PCOS group, whereas leptinR antagonists significantly increased the number of implanted embryos ($P < 0.05$). H & E staining revealed multiple early follicles and atresia follicles in the PCOS rats ovaries, which were improved to some extent after treatment with LeptinR antagonists. Meanwhile, leptinR antagonists reversed the up-regulation of leptinR levels and the down-regulation of kisspeptin and kiss1r in the PCOS model. In conclusion, inhibition of the Leptin/leptinR-kisspeptin/kiss1R signaling pathway promotes follicular development and improves ovarian function in PCOS rats.

Keywords: Polycystic ovary syndrome, leptin, kisspeptin/kiss1R signaling pathway, ovary development

Introduction

Polycystic ovary syndrome (PCOS) is a reproductive system, endocrine disease presenting in 5-10% of women of childbearing age [1]. Typical features of PCOS include ovarian polycystic changes, hyperandrogenism, and anovulation. The causes of polycystic ovaries are very complicated. PCOS patients are usually accompanied by hormonal disorders [2]. Progesterone is a steroid hormone that is mainly secreted by ovarian granulosa cells and corpus luteum secretory cells. The progesterone is related to follicles. It is associated with follicle maturation and development, embryonic development, endometrial receptivity, and embryo implantation. PCOS patients cannot form a corpus luteum, leading to low levels of progesterone and infertility [3]. Therefore, progesterone dysfunction is involved in the pathogenesis of PCOS.

RU486 is a progesterone receptor antagonist and used for contraception. Even with similar structure to progesterone, RU486's binding affinity to progesterone receptor is five times higher than that of progesterone [4]. In addition, RU486 inhibits the development and maturation of follicles, leading to delayed ovulation. Herington *et al.* [5] showed no ovulation in most women for 120 days after continuous administration of RU486 (2 or 5 mg/day) [6]. In addition, administration of RU486 destructed the formation of the corpus luteum and reduced

progesterone production [7]. Moreover, RU486 might also affect the development of the endometrium and endometrial receptivity *in vivo*, and pregnancy rate. So, we hypothesized that RU486 may be an etiological factor for PCOS and is associated with infertility. Therefore, RU486 was used in this study to induce the PCOS model.

Recent studies revealed that the kisspeptin/kiss1R system in the ovary is related to the secretion of ovarian autologous hormones and may be involved in the regulation of ovarian function [8]. It was suggested that the expression level of kisspeptin/kiss1R is significantly reduced in PCOS patients [9]. It is well-known that increased fat level is an important pathogenic factor that causes PCOS, and leptin was discovered in recent years as a protein hormone secreted by adipose tissue [10]. However, whether leptin/leptinR can affect the follicular development of PCOS patients through regulating the kisspeptin/kiss1R system has not been elucidated.

Therefore, we investigated the effect of leptin/leptinR-kisspeptin/kiss1R signaling pathway on follicular development in PCOS rats using leptinR antagonist.

Materials and methods

Main materials and reagents

The whole protein extraction kit was purchased from KGI Biotechnology Development Co., Ltd. (Nanjing, China). Western blot lysate and BCA protein assay kit were provided by Beyotime (Suzhou, China). Anti-leptinR monoclonal antibody was purchased from Abcam. Anti-kisspeptin and anti-kiss1R polyclonal antibodies were purchased from Proteintech Co., Ltd. (Wuhan, China). Horseradish peroxidase-labeled goat anti-rabbit IgG (H+L) was purchased from ZSbio (Beijing, China). Leptin antagonists and other reagents were purchased from Sigma (New York, USA). Serum leptin, LH, and FSH ELISA kits were purchased from Elabscience (Wuhan, China).

Main instruments

UVP Multispectral Imaging System (California, USA) and PS-9 type semi-dry transfer electrophoresis instrument were purchased from JM Co., Ltd. (Dalian, China). Thermo-354 microplate reader was purchased from Thermo Fisher Scientific. Company (New York, USA).

Experimental animals and grouping

Female rats 6-8 weeks-old weighing 180-220 g were purchased from the Experimental Animal Center of Zhejiang University. The rats were housed in a clean animal room with a temperature of 24°C, the relative humidity of 60%, and 12 h light/dark cycle. The rats were free to eat and drink and the litter was changed every day to avoid infection. Animal experiments were divided into three groups: normal control group, polycystic ovary model group, and leptinR antagonist group, with 10 rats in each group.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Lishui Hospital of Traditional Chinese Medicine.

PCOS rat modeling

Vaginal smear method was applied to observe the rats for at least two consecutive estrus cycles. The rats in the model group were given daily subcutaneous injections of RU486 olive oil solution (2 mg/0.1 mL/100 g/Day) from the first day of the estrus cycle. The rats in the normal control group were injected with the same dose of olive oil. The leptinR antagonist and the kiss1R antagonist were administered intraperitoneally, and were given two hours after RU486 injection, at a dose of 2 μf/kg. After model establishment, the blood and ovarian tissues were collected for further analysis.

Serum leptin level detection

Serum obtained from each group was used to test leptin level by ELISA according to the instructions. The absorbance (A) was measured at a wavelength of 450 nm using a microplate reader. Each test was repeated 3 times.

Serum LH and FSH levels detection

LH and FSH levels were detected by ELISA according to the instructions. The absorbance (A) was measured at a wavelength of 450 nm using a microplate reader. Each test was repeated 3 times.

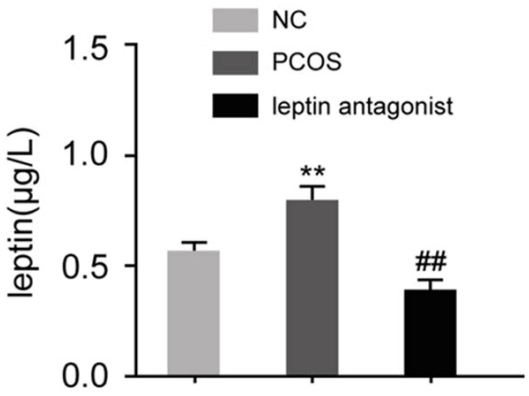

Figure 1. Serum leptin levels in each group. Serum was obtained from each group for analysis of the leptin level by ELISA. $^{**}P < 0.05$, compared with normal control; $^{\#\#}P < 0.05$, compared with PCOS group.

Fertility assessment

Rats in each group were caged with male rats after modeling, and vaginal embolism was found on the next day to indicate successful mating. Ten days later, the female rats were sacrificed to confirm pregnancy and the number of embryos implanted was examined.

H & E staining

The rat ovaries were immersed and fixed in 4% paraformaldehyde for 24 hours, then dehydrated with 100%, 95%, 85%, and 75% ethanol. Next, the sample was hyalinized by turpentine oil and embedded in wax. The placental tissue in paraffin was sectioned to a thickness of approximately 4 μm. After conventional dewaxing, the sample underwent hydration, hematoxylin staining, 1% hydrochloric acid alcohol differentiation, eosin staining, gradient alcohol dehydration, and sealing.

Western blot

The ovary tissues of each group were washed with PBS followed by addition of 10 μL of 100 mM PMSF. Next, 100 mg of tissue were mechanically homogenized after addition of 1000 μl lysate and lysed on ice for 5-10 min. After being centrifuged at 4°C at 12000 rpm for 5 min, the supernatant was collected for the following experiment.

Western blot was performed according to previous literature [11]. The extracted total protein solution was quantified by BCA and denatured in boiling water for 5 minutes. Samples were separated on 10% SDS-PAGE, transferred to PVDF membrane at 300 mA for 1 h and incubated with NOX4 antibody (1:1000) at 4°C overnight and washed with TTBS. Next, the secondary antibody (1:1000) was added and incubated with the membrane at 37°C for 2 h. At last, the bands were visualized after addition of enhanced chemiluminescence reagent. The data were analyzed using image J software.

Statistical analysis

Data were analyzed by SPSS 19.0 software. Measurement data conforming to normal distribution were presented as mean ± standard deviation (SD) and analyzed by one-way ANOVA and student t test. Kaplan-Meier method was used to analyze the survival curve. $P < 0.05$ was treated as statistical significance.

Results

Serum leptin level changes

The level of serum leptin in each group are shown in **Figure 1**. Compared with the normal group, serum leptin levels in the PCOS group was significantly increased, while administration of leptinR antagonists significantly reduced serum leptin levels.

Serum LH and FSH level changes

Serum LH and FSH levels were important indicators for assessing follicular function and they are shown in **Figure 2**. In the PCOS model group, both FSH and LH levels were significantly increased compared with normal controls. Administration of leptinR antagonists significantly inhibited the levels of FSH and LH.

Embryo implantation

To investigate the impact of leptin/leptinR signaling on embryo implantation, we tested changes in the number of embryos implanted on day 10 of pregnancy (**Figure 3**). In the PCOS rat group, the number of implanted embryos was significantly reduced, whereas leptinR antagonist treatment significantly increased the number of implanted embryos.

Ovarian tissue morphology changes

In the normal group, the ovary contained multiple corpus luteum and follicles of different

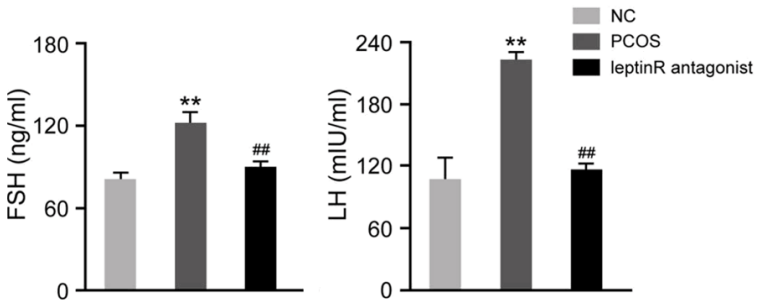

Figure 2. Serum LH and FSH levels in each group. Serum was isolated from each group followed by analysis of the levels of LH and FSH by ELISA. $^{**}P < 0.05$, compared with normal control; $^{\#\#}P < 0.05$, compared with PCOS group.

developmental stages, and part of the follicles exhibited oocytes and radiation crowns. In the PCOS model group, the rat ovaries contained small follicles and atresia follicles that developed in early stage, and the number of cystic expansion follicles was increased significantly, whereas the number of granular cell layers was decreased to 2-3 layers, and the number of corpus luteum was significantly reduced. Most follicles did not show oocytes and radiating crowns. After administration of leptinR antagonists, ovarian morphology was improved to some extent. The leptinR antagonist may be an effective drug for relieving polycystic ovaries (**Figure 4**).

The protein levels of kisspeptin, kiss1R, and leptinR

Compared with normal control group, kisspeptin and kiss1R protein levels were significantly reduced, while leptinR expression was significantly elevated in the ovaries of PCOS rats. However, in the antagonist group, kisspeptin and kiss1R protein expressions were up-regulated compared to the PCOS model group. On the contrary, the expression level of leptinR was inhibited. This indicated that the leptin/leptinR signal has a negative regulatory effect on kisspeptin/kiss1R (**Figure 5**).

Discussion

PCOS keeps plaguing the reproductive health of women of reproductive age and even adolescent females. In clinical practice, menstrual sparsity and infertility caused by follicular developmental disorders are the major causes of PCOS. From the current study, the abuse of RU486 is an important cause of PCOS [12].

RU486 can eliminate progesterone functions [13, 14]. Marions *et al.* [15] demonstrated that RU486 influenced the ovulation process. In addition, RU-486 can prevent endometrial maturation and proliferation when used in a dose exceeding 10 mg or in repeated low doses. Gemzell-Danielsson *et al.* [16] showed that injection of high doses of RU486 after ovulation can inhibit uterine secretory phase progression and embryo implantation through direct action on the endometrium. Currently, women of childbearing age often used RU486 as a contraceptive drug. However, long-term usage of RU486 may cause infertility. In this study, we established rat PCOS model which was induced by RU486, and observed body weight, serum hormone levels, estrus cycle and ovarian morphology. It was confirmed that RU486 can induce similar characteristics of human PCOS, such as anovulation, expansion of the ovary containing atresia follicles or follicular cysts, elevated testosterone, estrogen, and LH levels. Moreover, Lakhani *et al.* [17] demonstrated that the RU486-induced PCOS rat model was successful.

FSH and LH can promote ovarian development, and the regulation of FSH on follicular growth and development is much greater than LH [18, 19]. In this study, leptinR antagonists can effectively reverse the up-regulation of FSH and LH levels, the number and morphology of ovarian follicles in PCOS rats, indicating that it has a role in the improvement of PCOS.

Early studies suggested that the expression of kisspeptin/kiss1R in the ovary may regulate the secretion and function of ovarian hormones. Shahed *et al.* used Siberian hamsters to test that the immunoreaction of kisspeptin in the ovaries was increased during the ovulation period and was positively regulated by gonadotropins [20]. It was also revealed that kisspeptin antagonists may be a method for the treatment of PCOS patients with excessive secretion of LH [21]. Therefore, according to other evidence and the results of this study, it

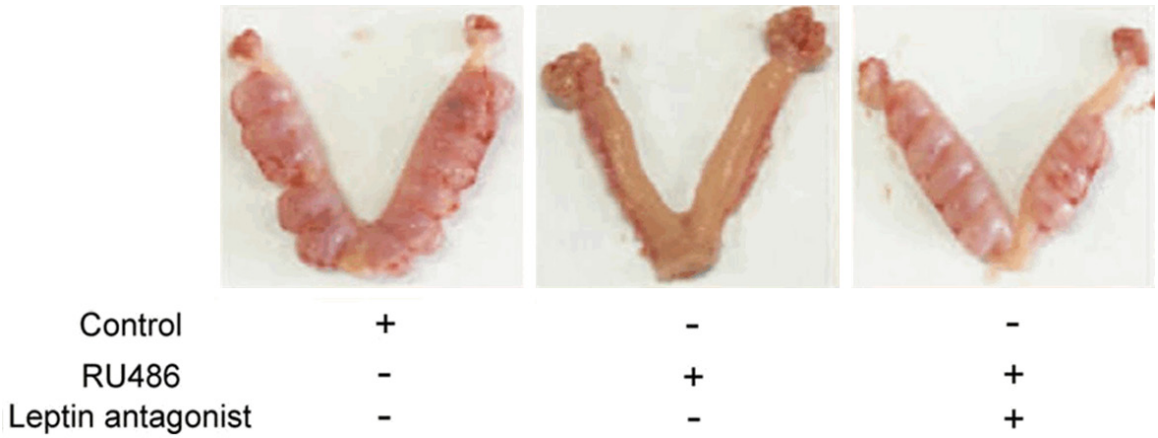

Figure 3. The number of implanted embryos. Rats in each group were caged with male rats after modeling. Ten days after pregnancy, the female rats were sacrificed to calculate the number of implanted embryos.

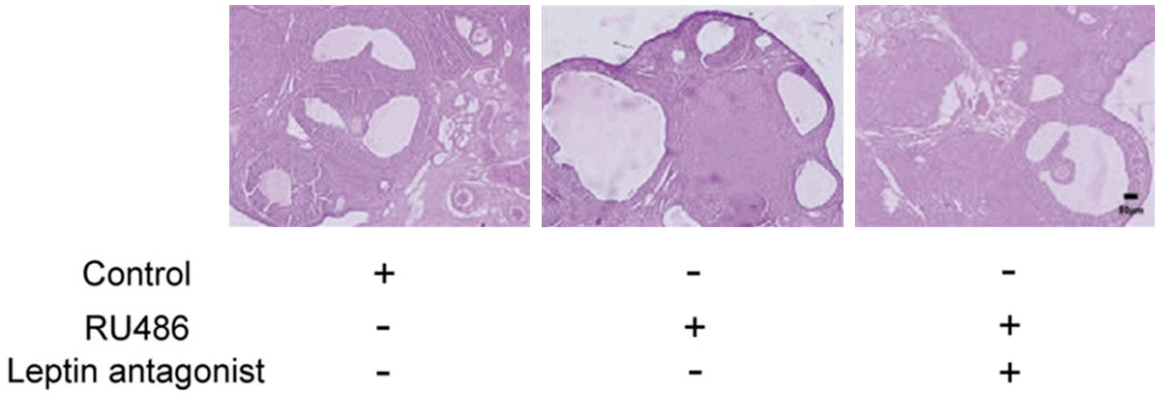

Figure 4. Ovary H & E staining in each group (40×). The rat ovaries were isolated, fixed in 4% paraformaldehyde, then dehydrated with 100%, 95%, 85%, and 75% ethanol. Followed by embedding and dewaxing and subsequent hematoxylin staining and eosin staining.

can be inferred that kisspeptin in the ovary can bind to its specific receptor kiss1R and exert potential direct or indirect regulatory effects on ovarian secretion and follicular development. In this study, we detected the expressions of kisspeptin and kiss1R in ovarian follicles. Moreover, the expressions of kisspeptin and kiss1R in the ovaries of PCOS model rats were significantly reduced, which was consistent with the results in previous models of ovulatory disorders which were constructed by indomethacin [22]. Thus, we speculated that the kisspeptin/kiss1R system plays an important role in PCOS.

Leptin is a protein hormone secreted by adipose tissue and its main function is to regulate energy balance and body weight. In recent years, it was found that decreased estrogen in females leads to an increase of fat and leptin levels. It is well known that fat levels are closely related to ovarian function, and it is also reported that administration of exogenous leptin to obese dysplasia mice lacking endogenous leptin increased the uterus muscle, relative ovarian mass, and follicle count to restore fertility [23]. It was suggested that the expression of kisspeptin/kiss1R in the arcuate nucleus of rats was increased after puberty, and that leptin and leptinR showed corresponding changes [24]. This study indicated that there may be interactions between the two proteins, but there is still a lack of effective experimental evidence. In this study, leptinR antagonists were administered to PCOS rats for the first time to investigate the regulatory role of Leptin/leptinR-kisspeptin/kiss1R signaling. However, whether Leptin/leptinR-kisspeptin/kiss1R signaling also plays a similar role in regulating other

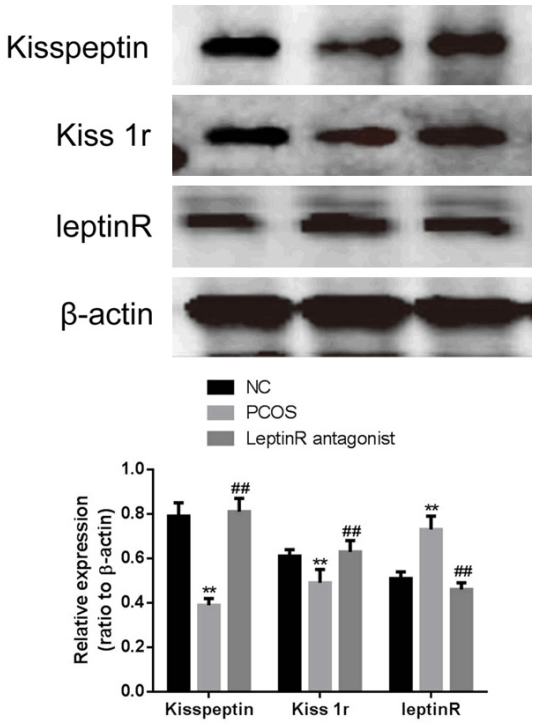

Figure 5. Kisspeptin, kiss1R, and leptinR protein levels in ovary tissue. Total protein was extracted from ovary tissues followed by measurement of the expression of kisspeptin, kiss1R, and leptinR protein by western blot. $^{**}P < 0.05$, compared with normal control; $^{\#\#}P < 0.05$, compared with PCOS group.

molecules caused by PCOS, and what the specific regulatory relationship exists between Leptin/leptinR-kisspeptin/kiss1R signals remains to be elucidated in the future. More animal experiments and cell experiments are needed in the future.

Conclusion

Inhibition of Leptin/leptinR-kisspeptin/kiss1R signaling pathway promotes follicular development and improves ovarian function in PCOS rats.

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Disclosure of conflict of interest

None.

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