Original article

Estrogenic properties of genistein acting on FSHR and LHR in rats with PCOS

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Abstract

The purpose of the study was to study the activity of the phytoestrogen genistein (GEN) acting on FSHR and LHR in rat ovaries with polycystic ovary syndrome (PCOS). Sixty rats were divided into six groups. Rats in the dose group received genistein at a concentration of either 5 (low genistein dose group, L-gen), 10 (middle genistein dose group, M-Gen) or 20 (high genistein dose group, H-Gen) mg per kg of body weight per day. Estrogen group (EG, received 0.5 mg/kg Diethylstilbestrol). Concentration of sex hormones in serum was quantified by enzyme-linked immunosorbent assay (ELISA). Expressions of follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) protein were determined by immunohistochemistry. Treatment with genistein resulted in a strong stimulation of the concentration of sex hormone in serum. The concentration of progesterone and FSH was significantly higher in H-Gen when compared to the PCOS model control group (MG) ($P < 0.01$). In contrast, the concentration of testosterone, LH and the ratio of LH/FSH decreased in GEN treatment groups compared to MG, the effect was statistically significant, tested by the ANOVA test (p<0.01). For hormone receptor activity, treatment with genistein resulted in an improvement of ovarian function with LHR protein expression being enhanced and FSHR protein expression being suppressed. Our results demonstrate that Genistein played a significant role in regulating FSH and LH receptor expression to improve perimenopausal ovarian and uterine function.

Key words: genistein, polycystic ovary syndrome, follicle-stimulating hormone receptor, luteinizing hormone receptor, female rat

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Introduction

Polycystic ovary syndrome (PCOS) is the most common female endocrine disorder (Azziz et al. 2004), affecting approximately 7–10% childbearing age and menopausal women (Adams et al. 1986). Polycystic ovaries and hyperandrogenism are clinical characteristics (Azziz et al.2006). PCOS is associated with hyperandrogenism, anovulation, LH and abnormal insulin-like growth factor level. Phytoestrogens are known to play a role similar to estrogen in animals (Jahanfar et al. 1995). In addition, the duration of the menstrual cycle might be changed in women on a soy diet in some reports. So the dietary phytoestrogens affecting the progress of PCOS might be plausible (Eden et al. 1989).

Some plants contain steroids and isoflavones, which might be effective in the treatment of pituitary-ovarian dysfunction in young women (Ushiroyama et al. 2003). In particular, isoflavones, coming from Unkei-to, has been found to improve normal secretion of FSH and LH, resulting in an induction of ovulation. Another source of isoflavone-Unkeito has been confirmed that could raise the plasma LH level, the synthesis and release of LH and FSH in cultured rat pituitary cells (Taketani et al. 2003, Yasui et al. 2003). These findings suggest that phytoestrogens has direct regulatory effects on animal's ovary, although the effect of phytoestrogens on the human ovary remains to be clarified. Some scholars reported that postmenopausal women receiving high soy supplementation showed non-significant changes in serum FSH, LH and sex hormone-binding globulin (SHBG) levels (Adlercreutz et al. 1987, Key et al. 1990, Baird et al. 1995, Lu et al. 1996). On the contrary, other scholars reported that premenopausal women were fed different level of isoflavones, then follicular phase length was significantly increased and peak progesterone, FSH and LH levels were suppressed (Phipps et al. 1993, Cassidy et al. 1994). But in premenopausal women fed with daidzein and genistein, there was a significant decrease in estradiol (E_2) (Olsson et al. 1983), yet there was an endogeneous influence on the absorption and metabolism of the compounds (Adlerdreutz et al. 1981). Genistein, a principal ingredient for the beneficial effect of soy consumption, has structural similarity to estrogen and it binds to estrogen receptors, suggesting that it may exhibit estrogenic action (Clarkson et al. 1991, Shwaery et al. 1997, Murthy et al. 1998).

Up to now there have been no studies examining the action of genistein on patients with PCOS. The present study was designed to investigate the effects of genistein on gonadotropins and elucidate the action of genistein on their receptors such as FSHR and LHR in rats with PCOS. The results will help to elucidate the pathway and mechanism of actions of genistein in vivo as well as to provide information for the design of new synthetic structurally-modified derivatives of genistein that demonstrate optimal activities in PCOS.

Materials and Methods

Chemicals and test compounds

Genistein (4′,5,7-trihydroxyisoflavone) was provided by Sigma company (America, the purity >99.9%). Diethylstilbestrol was provided by Hefei JIULIAN Pharmaceutical Company (China); HCG was provided by Sihuan pharmaceutical Company (China); Isophand insulin injection: Novo Nordisk (Brazil, drug license No.: H20091126); ELISA reagent kit was provided by Wuhan Boster Biological Company (China); immunohistochemical antibody was provided by Zhenjiang Biology Science and Technology Company, Rabbit polyclonal to FSHR (No. PR-1170), Rabbit polyclonal to LHR (No.PR-1318).

Animals and diets

Female Wistar rats (about 220 g, n=60) aged 2 months were obtained from Harbin (Harbin medical university breeding and research center, Harbin, China). Rats were housed one per cage and were maintained under controlled conditions of temperature (20°C±1), relative humidity (50–80%) and illumination (12h light, 12h darkness). Insulin (INS) was administered in combination with the HCG molding method (Bogovich,1987, Poretsky et al.1992): rats were given sufficient food and 5% glucose. During the first 10 days, the rats in MG, the dose groups (L-Gen, M-Gen and H-Gen) and EG, were gradually given increasing doses of intermediate-acting insulin injection; the dosage gradually increased from 0.5 IU / day to 6 IU / day. From the 11th to the 22nd day, the rats were given a fixed dose of 6 IU/day of HCG. Rats in CG were injected instead with the same dose of normal saline as a blank control. The selection standard used for this animal model was based on vaginal epithelial cell smears done for two consecutive sexual cycles (each cycle during 5 days). If vaginal keratinization of cells continued to appear this suggested that the model was successful. In addition, all the groups, excluding the CG, were given genistein by gastric gavage for 15 days.

All rats were divided into six groups of ten animals each, blank control group, MG, L-Gen (receive genistein at 5 mg/kg), M-Gen (receive genistein at 10 mg/kg), H-Gen (receive genistein at 20 mg/kg) and EG (receive Diethylstilbestrol at 0.5 mg/kg).

Table 1. SAFD feed formulation of the basic feed.

Feeding material	percentage
corn flour	30.56%
corn oil	2%
wheat flour	27.27%
yeast	2%
fish meal (60% protein)	10%
AIN mineral salt	3%
crude wheat	10%
vitamin premix	7%
casein	0.05%
choline chloride	0.12%
skim milk powder	5%
corn protein	3%

It is worth mentioning the rather different magnitudes of human exposure to those so-called environmental estrogens. Exposure to estrogenic isoflavones varies with dietary habits; estimates are between 1 and 100 mg per day for consumers on a typical western diet and those on a traditional soy-rich Asian style diet, respectively (Degen et al. 2002). As the content of genistein in isoflavone was about 2%~3%, the conversion factor between human (70 kg) and rat (250 g) was 7, the effective dose of genistein was about 0.14 mg~21mg, and we also read the other authors' articles for reference, so the doses of genistein (5, 10 and 20 mg/kg BW) were determined. Additionally, in order to compare the age of rats with women of childbearing age, we used 2 month old animals (Poretsky et al. 1992). The results of vaginal epithelial cell smears and sex hormone levels in this study proved that the rat models could simulate the PCOS patient's.

All animal experiments were approved by the Committee on Animal Care and Use of College of Animal Science and Veterinary Medicine of Heilongjiang Bayi Agricultural University and were performed according to accepted veterinary medical practice.

All procedures were carried out according to the Guide for the Care and Use of Laboratory Animals. All rats had free access to standard rat diet which did not contain genistein of soybean and alfalfa in rat feed (soy and alfalfa freediets, SAFD-Diet, Harbin, China) and water. The basic feed is SAFD feed formulation. The ingredients rich in genistein and alfalfa were replaced with corn, wheat and casein to highlight the effect of genistein on the test results.The main composition of feed is shown in Table 1.

The estrus cycle was determined by vaginal smear and the rats in the estrus phase were selected, for the follicles in this period were mature and ovulate. Rats were euthanised by pentobarbital sodium (0.2%, 0.5 ml/20 g) and their blood was collected from the abdominal aorta. Then ovaries were removed immediately for further analysis and adipose tissues on the ovarian surface were removed under the microscope. Bilateral ovaries were weighted with analytical balance. After being weighted, one side ovary was frozen in liquid nitrogen, another side was immediately placed in 10% formalin of (v/v) to be fixed and was stored for immunohistochemical determination.

Hormone levels determination

Concentration of testosterone (T) and progesterone (P), LH and FSH in serum were determined with enzyme-linked immunosorbent assay (ELISA) kits. 50 μl of standard diluent was added to the standard wells. The samples were diluted in a final ratio of 1:1 by mixing 50 μl of the sample with 50 μl of diluent and 50 μl of the diluted sample was added to the wells. 50 μl of diluted biotinylated anti- testosterone (progesterone, LH and FSH) was added to all the wells. Then the plate was covered and incubated for 1 hour at 37°C. After removing the cover the plate was washed three times. 80 μl of streptavidin-HRP solution were aliquoted into each well, including the blank wells. Next, the plate was covered and incubated for 30 min at 37°C. Then the solution was removed from all the wells, and the samples in the micro well plate were washed according to the corresponding washing step and then immediately proceeded to the next step. 50 μl of substrate A and substrate B were added to each well, and then incubated for 10 min at 37°C. The enzyme-substrate reaction was stopped by quickly

Table 2. Concentrations of progesterone, testosterone, LH, FSH and the ratio of LH/FSH in serum ($\bar{x}\pm$SD, n=10).

group	Progesterone (μg/L)	Testosterone (nmol/L)	LH (ng/L)	FSH (IU/L)	LH/FSH
CG	1.733±0.335	6.528±0.357b	3.695±0.245**	1.910±0.104	1.938±0.137*
MG	1.645±0.468	8.341±0.425	4.518±0.477	1.752±0.161	2.607±0.446
L-Gen	1.617±0.330	7.510±0.319*	3.776±0.131**	1.635±0.346	2.365±0.692
M-Gen	3.189±0.609**	7.137±0.277**	3.045±0.317**	1.831±0.147	1.678±0.282**
H-Gen	5.177±0.453**	7.269±0.625**	3.225±0.405**	2.336±0.186**	1.385±0.184**
EG	6.623±0.288**	7.138±0.879**	2.904±0.454**	2.588±0.149**	1.129±0.211**

The concentrations of sex hormones in serum after exposure to different concentrations (5, 10, 20 mg/kg) of genistein. Sex hormones levels were quantified using ELISA. All values represent means (n=10) ± SEM. Asterisk means significant against model group (*$p<0.05$;** $p<0.01$), ANOVA test. Control group (CG); PCOS model control group (MG); low dose genistein group (L-Gen); middle dose genistein group (M-Gen); high dose genistein group (H-Gen); estrogen group (EG).

Table 3. Average area and density of FSHR and LHR in mice' ovaries ($\bar{x}\pm$SD, n=10).

groups	Average area of FSHR(unit)	Average area of LHR (unit)
CG	11.44±1.21**	20.27±2.51**
MG	13.72±0.94	17.24±3.57
L-Gen	13.59±1.29	17.69±3.66
M-Gen	12.95±1.53	20.79±4.58**
H-Gen	11.61±1.43 **	21.21±4.37 **
EG	11.78±1.51**	20.76±5.37**

The average area and average density of follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) protein measured by image analysis. The immunohistochemical stained area was calculated as a percentage of total area, assessed by color segmentation analysis. **$p<0.01$ compared with MG. Control group (CG); PCOS model control group (MG); low dose genistein group (L-Gen); middle dose genistein group (M-Gen); high dose genistein group (H-Gen); estrogen group (EG).

adding 50 μL of H_2SO_4. The absorbance of each well was recorded by a spectrophotometer at 450nm as the primary wavelength and optionally at 620nm as the reference wavelength.

Determination of FSHR and LHR by immunohistochemical method

For this study we used a rabbit polyclonal antibody specific for FSHR and LHR, as the primary antibody, and the DAKO anti-rabbit, as the secondary antibody. The antibodies were purified by the affinity chromatography using the Protein A/G Column. After deparaffinization, the antigens were retrieved by incubating the slices in 10 mM citrate buffer, pH 6.0, for 10 - 15 mins, at 20°C. The tissue sections were incubated in 1% BSA solution (0.01 M PBS, pH 7.4, 0.05% NaN_3), containing primary antibody (1:200), at 20°C overnight. Following washes with PBS, the slices were transferred into 1% BSA buffer, containing secondary antibody (1:200), and incubated at 20°C for 60 min. After three times rinsing with PBS, the slices were dehydrated and mounted for imaging.

Since tissue samples contained ovarian follicles of different developmental stages, the number and type of cells were significantly variable between different slides. Different types of follicle were divided into two developmental periods. The primordial follicles, the primary follicles and the secondary follicles were ascribed to the early follicular period, while the antral follicles and mature follicles belonged to late follicular period. At least 20 follicles were observed in each period of ovarian follicles.

Immunohistochemically stained slides were visualized by using the X microscope (Company name). The images of the stained slides were processed by the Image-Pro Plus 6 software and color segmentation analysis was performed to detect the positively stained areas (brown staining). The average optical density of the selected area was quantified and the immunohistochemical area (IHCSA) was defined as the calculated percentage of the total area. The background area (black area) was calculated from at least 25 images of each area (granulosa, theca externa, and theca interna) in each slide. The positive immunohistochemical staining intensity can represent the protein expression levels of the positive stained cells.
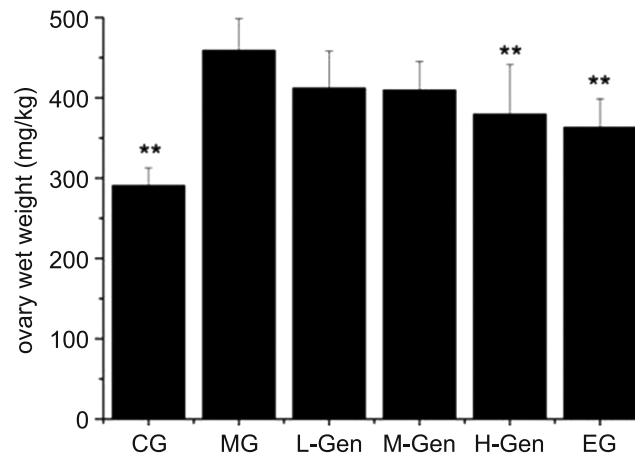
Fig. 1. Ovary wet weight of rats after 15 days of treatment with genistein. Asterisk means significant difference against MG (* p<0.05; ** p<0.01), ANOVA test. PCOS model control group (MG); low dose genistein group (L-Gen); middle dose genistein group (M-Gen); high dose genistein group (H-Gen); estrogen group (EG).

Statistical analysis

The data were expressed as mean ±standard deviation ($\bar{x}\pm$SD). All data were analyzed by two-way analysis of variance (ANOVA) followed by pair-wise comparison of selected means using the pooled within-group variance comparison, and differences were considered statistically significant for p<0.05.

Results

The result of ovary weight

The result of ovary weight are shown in Fig.1. To confirm that genistein has effect on reproductive tissues of animals with PCOS, we examined ovary weight at the end of the experiment. After administration of 20 mg/kg genistein, the average ovary wet weight was 379 mg/kg, which was lower compared to 459 mg/kg in MG. As expected, the higher dose of genistein (20 mg/kg) led to a stronger ovary weight loss response (p<0.01). There was only a small and non-significant effect after treatment with lower dose of genistein (5 mg/kg and 10 mg/kg).

Concentrations of sex hormones in serum

Concentrations of sex hormones in serum showed in Table 2. Treatment with genistein resulted in a rising tendency of progesterone and FSH levels in M-Gen and H-Gen than in the model group animals. As expected, genistein with higher dose increased the concentration of progesterone. In contrary, the concentrations of testosterone, LH and the ratio of LH/FSH was reduced significantly in GEN treatment groups, the effect was statistically significant, as tested by the ANOVA test (p<0.01).

Expressions of FSH and LH receptor protein in ovarian granulosa cells

Expressions of FSH and LH receptor protein in ovarian granulosa cells are shown in Table 3, Fig. 2 and Fig. 3. Immunohistochemical results showed that the positive signals of FSHR and LHR protein expression were mainly located in the cytoplasm of mature ovarian follicular and luteal tissue, and there was almost no expression in early ovarian. Brown granules in the images represent positive expression. The results showed that positive staining was observed in the genistein obtaining group, especially in the M-Gen and H-Gen groups. According to imaging analysis, the protein expression levels of FSHR in the H-Gen (average IHCSA: 11.61) groups was stronger than in the MG (average IHCSA: 13.72) group, and this difference was statistically significant (p<0.01, Fig. 2, panels A-E). LHR was mainly distributed in the cytoplasm and the corpus luteum of the mature follicle. According to imaging analysis, the protein expression levels of LHR in the M-Gen (average IHCSA: 20.79) and the H-Gen (average IHCSA: 21.21) groups was stronger than in the MG (average IHCSA: 17.24) group, and this difference was statistically significant (p<0.01). With GEN dose increased, more and more positive staining for LHR was observed (Fig. 3, panels A-E). The expression profile of LHR in the ovarian tissues collected from the Gen treatment group was similar to the estrogen treated mice. The higher expression of LHR protein with the increase of genistein dose might indicate slowing of the aging process in the ovaries.

Discussion

In order to determine the pathway of GEN acting on ovaries of rats with PCOS, we investigated the influence
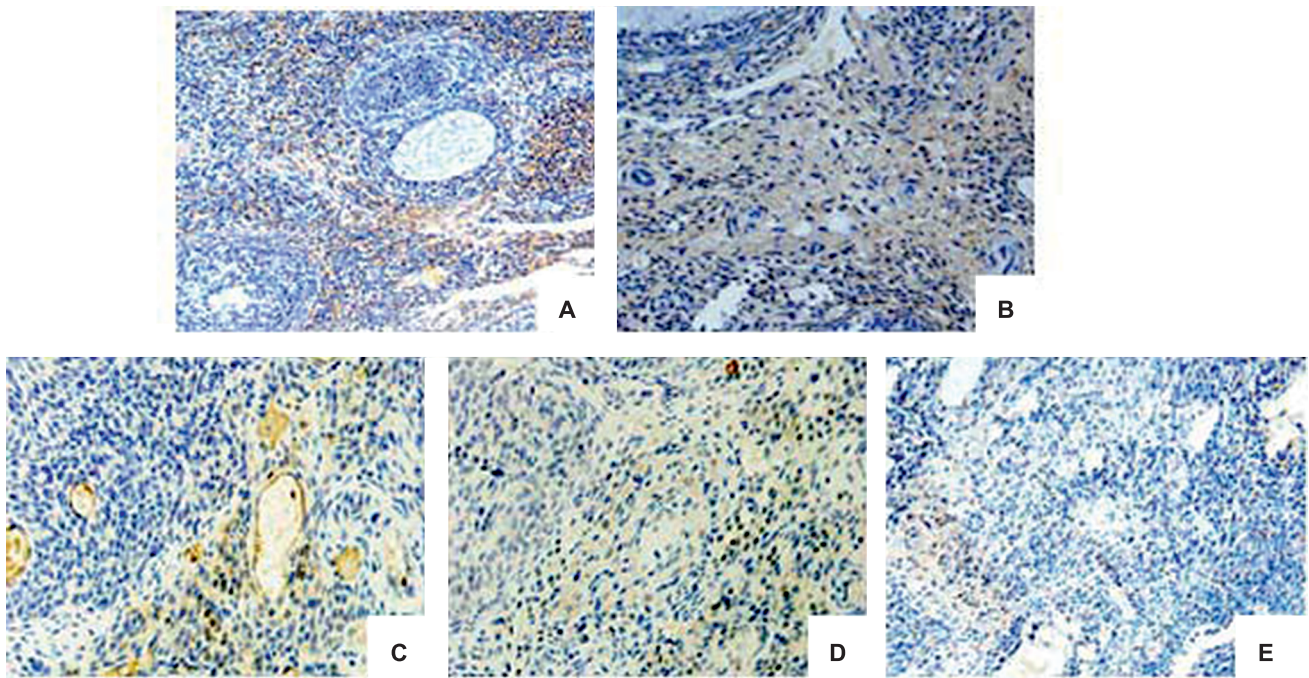
Fig. 2. Expression of follicle stimulating hormone receptor (FSHR) protein in ovarian granulosa cells detected by immuno-histochemistry. Brown-staining located in cytoplasmic was regarded as positive signal (diaminobenzidine staining and hematoxylin counterstaining, ×400). A: PCOS model control group (MG); B low dose genistein group (L-Gen); C: middle dose genistein group (M-Gen); D: high dose genistein group (H-Gen) E: estrogen group (EG).
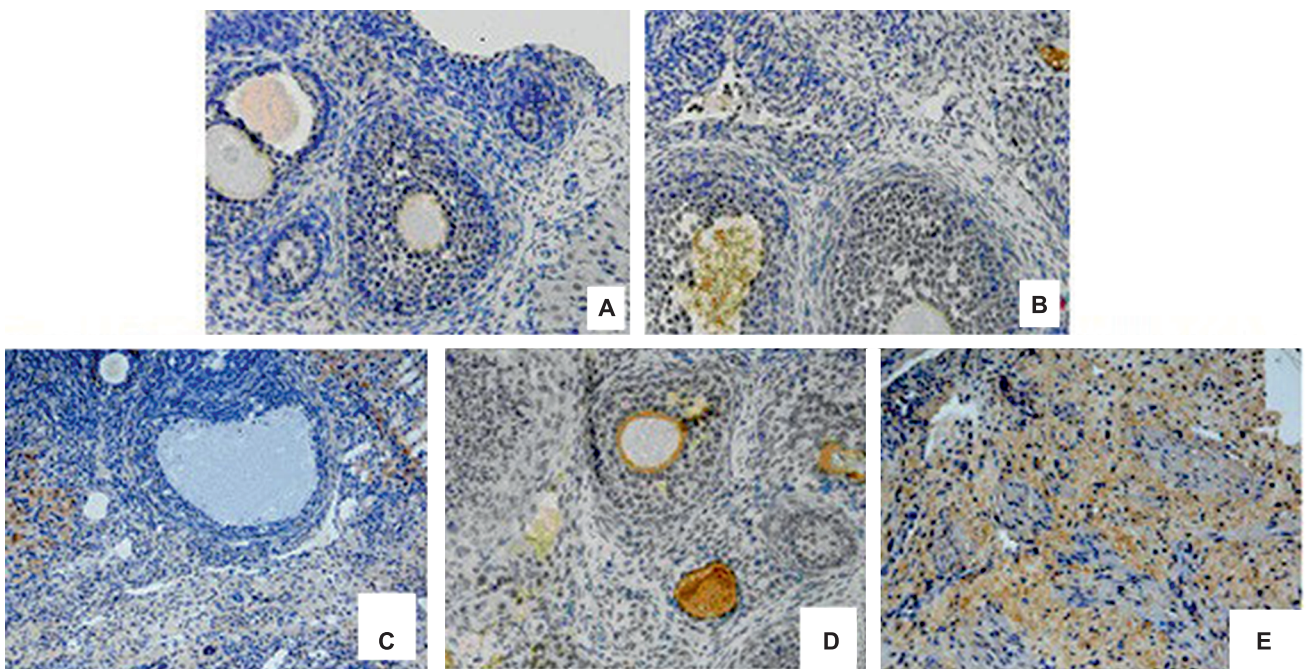

Fig. 3. Expression of luteinizing hormone receptor (LHR) protein in ovarian granulosa cells detected by immunohisto-chemistry. Brown-staining located in cytoplasmic was regarded as positive signal (diaminobenzidine staining and hema-toxylin counterstaining,×400). A: PCOS model control group (MG); B low dose genistein group (L-Gen); C: middle dose genistein group (M-Gen); D: high dose genistein group (H-Gen) E: estrogen group (EG).

of GEN on gonadotropins and their receptors which are involved in hormone secretion (i.e. LHR and FSHR). To the best of our knowledge, for the first time, this study identified that our findings may provide new insights into the effect of genistein on rats with PCOS, which may be based on the direct or indirect action on the levels of the hormones and their receptors. These effects are worth of attention regarding solving

ovulation disorders and hormone dependent diseases in female.

Over the years, increasing LH concentration is considered the main endocrine changes of PCOS. LH stimulation is insufficient, FSH is relatively insufficient, androgen cannot be converted into estrogen in time, all of the above can lead to PCOS. LH and FSH are directly involved in stimulating follicular development, ovulation, luteinization and synthesis of steroid hormones. Our paper describes that GEN at 20 mg/kg BW can regulate the levels of LH and FSH in vivo in rats with PCOS, which has the similar trend with that in estrogen group. GEN can make the concentration of free testosterone decreased, while at the same time increases progesterone level. These suggest that GEN can reduce the hormone levels of gonadotropins in hyperandrogenism female and play the similar therapeutic role in the clinical medicine.

FSH is an important endocrine hormone in regulating ovarian function, but it must bind to special receptor distributed in the ovary, to play physiological function (Hirakawa et al. 1999). It directly participates in the stimulation of follicular development and sex hormone synthesis. The expression of FSHR in ovarian granular cell is an important link of gonadotropin sensitivity. The production of estrogens is limited by the content of FSHR in the follicle. The interaction of FSH and FSHR is the key of follicle development during the process of follicle development. Expression of FSHR plays a decisive role in the gonads as regards the gonadotropin response. However, the binding of FSHR and biochemical characteristics in human are still not clear, because of the low level of FSH receptor and the limited number of human follicular granulosa cells. So, many studies are limited to animal experimental stage at present. Combination of luteinizing hormone and LHR has effect on the synthesis of androgen and the development of follicle. Patients with PCOS with high LH level could promote the granule cell generation by inducing follicular theca cells to enhance the expression of LHR. The increasing level of LH in the blood leads to persistent anovulation. There are few reports regarding the differentiation of granulosa cells and the increasing of progesterone secretion which leads to the development of follicular stagnation. The exactly reason and mechanism needs to be studied in future. Many studies have shown that the high concentration of LH, high estrogenic hormones and hyperandrogenism are important clinical feature of PCOS (Pekhlivanov et al. 2007). Hyperandrogenism is an important anovulation factor in patients with PCOS (Mifsud et al. 2000). The content of FSHR in follicular can restrict estrogen formation. Although the pathological mechanism of PCOS remains unclear, as long

as the concentration of FSH in serum increased, the follicles could grow normally and become mature. It indicated that the pathogenesis of PCOS is in relationship with defect of FSH function (Vegetti et al. 2006). Our results show that relative to the MG, the expression of FSHR protein decreased and LHR protein increased in rat ovary both in the H-Gen and the EG group, which suggests that GEN could regulate the expression of FSHR in vivo in rat and also showed that the regulatory effect of GEN was similar to estrogen. The results suggest that GEN can promote estrogen formation and adjust reproductive endocrine disorders by inhibiting FSHR protein expression and promoting LHR protein expression. GEN can regulate ovarian function by enhancing the function of FSH and LH acting on ovary and enhance the ovarian reactivity to FSH and LH. GEN can promote the growth of ovarian granulosa cells and adjust female reproductive endocrinology, thus promoting the development and discharge of mature follicles and alleviate the symptoms of PCOS.

Our results demonstrate that GEN exhibits estrogenic properties which had the effect on the expression of LHR and FSHR in ovaries and concentrations of sex hormone in the female rats with PCOS. The therapeutic effect of GEN on rats with PCOS is similar to that of clinical estrogen medication. The relationship between phytoestrogen and hormone biology has been reviewed. Phytoestrogens occur in plant diets and traditional medicines, especially genistein in soy food, plausibly exerting the beneficial effects on female reproduction and reducing the level of androgen in female with PCOS. They showed direct and indirect effects on the sex hormones and their receptors. Those effects might be shown in the improvement of ovulatory disorders and in the decrease of the risk of hormone-dependent cancers. The similarity of genistein to estrogen, observed by the ovarian responses, suggests that genistein may have the potential to be used as an effective drug for menopause therapy, and may prevent ovarian aging.

Acknowledgements

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