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CHINESE HERBAL MEDICINE ALLEVIATING HYPERANDROGENISM OF PCOS RATS THROUGH REGULATING PPARG1 AND HDAC3 EXPRESSION IN THE OVARIES

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Abstract

Background: Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in women hence Chinese herbal medicine (CHM) has been chosen by many clinicians and patients as alternative treatment for PCOS. The present study was to explore the effects of CHM in alleviating hyperandrogenism of PCOS rats induced by testosterone propionate and the possible underlying mechanism.

Materials and methods: A total of forty female Sprague–Dawley rats were randomly divided into normal control group, PCOS model group, CHM1 group and CHM2 group, with ten rats in each group. The rat models with PCOS were established by single injection of testosterone propionate at the 9th day after birth. The status of estrous cyclicity for each rat was observed. After the treatment for 12 weeks ended, the serum levels of total testosterone (TT), sex hormone binding globulin (SHBG), androstenedione, follicle stimulating hormone (FSH) and luteinizing hormone (LH) of the rats were measured with ELISA and mRNA expression levels of peroxisome proliferator-activated receptor gamma 1(PPARG1) and histone deacetylase 3 (HDAC3) in the ovaries of the rats were detected with real-time quantitative PCR.

Results: The serum levels of LH/FSH, FAI and androstenedione of the PCOS model rats were the highest among all the groups ($P<0.05$) and no significant differences on the serum levels of LH/FSH and FAI between the rats from CHM1 and CHM2 groups were found ($P>0.05$). The serum levels of androstenedione of the rats from CHM1 group were significantly lower than those of CHM2 group ($P<0.05$). In the ovaries of PCOS model rats, PPARG1mRNA expression levels were significantly lower, and HDAC3 mRNA expression levels were significantly higher than the other three groups ($P<0.05$). There were no significant differences existed between CHM1 and CHM2 groups on mRNA expression levels of PPARG1 and HDAC3 in the ovaries of the rats($P>0.05$).

Conclusion: It is through regulating PPARG1 and HDAC3 expression levels in the ovaries that CHM significantly alleviates hyperandrogenism of PCOS rats induced by testosterone propionate.

Key words: Polycystic ovarian syndrome (PCOS); Chinese herbal medicine (CHM); hyperandrogenism

Introduction

Polycystic ovarian syndrome (PCOS) is the most common metabolic and endocrine disorder in women, and is characterized by oligo-ovulation/anovulation, hyperandrogenism and polycystic ovaries (Norman et al., 2007). Hyperandrogenism can be found in 60-80 % of PCOS women (Norman et al., 2007). Hyperandrogenism PCOS was found to be related to hepatic steatosis, independent of obesity and insulin resistance (Jones et al., 2012). Functional ovarian hyperandrogenism of PCOS could develop at a high rate in prepubertal girls with genetic polymorphisms and/or particular environmental factors (Siklar et al., 2007). Hyperandrogenism and its associated parameters are significantly associated with metabolic syndrome in non-obese PCOS subjects, and this association was not observed in obese PCOS women (Kim et al., 2014).

Although oral contraceptives and insulin sensitizers are widely used to improve the symptoms and signs for PCOS, Chinese herbal medicine (CHM) has been chosen by many clinicians and patients as an alternative treatment for PCOS (Song et al., 2006; Zhang et al., 2010). The

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spearmint herbal tea was found to possess the potential for use as a helpful and natural treatment for hyperandrogenism of PCOS (Grant, 2010). However, as the methodology of randomized clinical trials was not adequately reported by primary studies, there is limited evidence that the addition of CHM to clomiphene can improve the clinical pregnancy outcomes (Zhang et al., 2010). In the previous work, the corresponding author's team has created a patented formula, CHM1, which has been found to significantly alleviate hyperandrogenism of PCOS clinically. The present study was to compare the effects of a patented CHM with its refined version in treating hyperandrogenism of PCOS rats induced by testosterone propionate and the possible underlying mechanism.

Materials and Methods

Rats, treatment and collection of samples

A total of forty neonatal female Sprague-Dawley rats at 9 days of age were provided by the Experimental Animal Centre, Zhejiang University (Hangzhou, China). The rats were then grouped and housed together in a temperature-controlled room with a 12-hour light: dark cycle and maintained at a constant temperature of 25℃ and humidity of 55%. They were randomly divided into four groups: normal control group, PCOS model group, CHM1 group and CHM2 group, with ten rats in each group. All of the rats were fed with standard pelleted food and plain tap water *ad libitum*. To establish the models with PCOS, the rats from PCOS model group, CHM1 group and CHM2 group were once injected with 1.25 milligram of testosterone propionate (Jinyao amino acid Ltd., Tianjin, China) dissolved in vehicle at the 9th day after birth (Tamura et al., 2005). All of the experiments were conducted according to the National Research Council's protocol for the care and use of laboratory animals and were approved by the Institutional Review Board. From the 21st day after birth, the rats in each of the groups received the following treatments respectively. The normal control and PCOS model rats were orally administered with 2ml of water once daily for 12 consecutive weeks. The rats from CHM1 and CHM2 groups were orally administrated with the extracts of CHM1 and/or CHM2 respectively dissolved in 0.5% of carboxymethyl cellulose with the dosage of 0.54g/kg (CHM1) or 0.24g/kg (CHM2) once daily for 12 consecutive weeks. The observation of vaginal smears was conducted daily for ten consecutive days from 70 days of age. In the tenth week following injection of testosterone propionate, PCOS model rats were successfully established. None of the rats died during the above treatment period and on the next day following the above 12-week treatment, the rats were anaesthetized with urethane (1.2 g/kg, intraperitoneally) after fasting for 12 h. The samples of blood were then taken from the hepatic portal vein into heparinized injectors, which were then centrifuged at 3000 rpm in 4℃ for 10 min. The supernatant serums were transferred to clean EP tubes and stored at −80°C until assay. After the blood samples were collected, the rats were sacrificed and the ovaries were rapidly removed. They were washed with physiological saline, snap frozen in 2-methylbutane/dry-ice baths, and stored at -80°C.

Preparation of CHM extracts

In the present study, two CHM formulas (CHM1 and CHM2) were respectively used in CHM1 and CHM2 groups. CHM1 is a patented formula owned by the corresponding author's team (China Patent: No. ZL 201110030846.6; International patent PCT: Publication No. WO 2012/100471, Appication No. PCT/CN2011/073938). CHM1 is composed with 20g of Radix Astragali, 35g of Radix Rehmanniae Preparata, 30g of Cuscuta chinensis Lam, 35g of Fructus Ligustri Lucidi, 30g of Fructus Psoraleae, 20g of Radix Salviae Miltiorrhizae and 20g of Rubus idaeus Linn. CHM2 is the refined version of CHM1, composing of 30g of Cuscuta chinensis Lam, 35g of Fructus Ligustri Lucidi and 20g of Rubus idaeus Linn. The crude herbs of the above formulas were respectively extracted by 10x boiling water for 60 min. The extraction procedure was repeated once with 8x boiling water after filtrate. All the filtrates were combined and condensed under reduced pressure and freeze dried.

Detection of sex hormones in the serum

The serum levels of total testosterone (TT), sex hormone binding globulin (SHBG), androstenedione, follicle stimulating hormone (FSH) and luteinizing hormone(LH) of the rats were measured with ELISA (R&D Systems, MN, USA). FAI was calculated as TT (nmol/l) divided by SHBG (nmol/l)×100 (Doi et al., 2006). All the measurements were conducted in duplicate according to the manufacturer's instruction. Both intra- and inter assay coefficients of variation were less than 10%.

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Measurement of mRNA expression levels of peroxisome proliferator-activated receptor gamma 1(PPARG1) and histone deacetylase 3 (HDAC3) in the ovaries of rats

We applied RNAiso^{TM} Reagent (TAKARA, Dalian, China) to perform the isolation of the total RNA, based on the manufacturer's instructions. The purity and concentration of RNA were measured by NanoDrop$^{®}$ND-100 Spectrophotometer (Thermo Fisher Scientific Inc, USA). Then cDNA was prepared from 500ng of total RNA by reverse transcription, with the PrimeScript^{TM} RT reagent Kit (Perfect Real Time, TAKARA, Dalian, China). The samples of cDNA were diluted in DNase- and RNase-free water at a proportion of 1:3. Quantitative real-time PCR was performed with the iCycler iQ Real-Time Detection System (Bio-Rad). The PPARG1, HDAC3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene specific primers for rats were provided by Sangon, Shanghai, China. PCR reactions were conducted with 2μL of cDNA, 10 μM of each primer, and 2×SYBR$^{®}$ Premix Ex Taq^{TM} (TAKARA) in 25-μL reactions. Thermal cycling conditions were 95°C for 4 minutes, followed by 40 cycles of 95°C for 15 seconds and 60.0°C for 1 minute. The mRNA levels of the target gene were expressed as $2^{-\Delta Ct}$.

Statistical analysis

The data were analyzed with Statistical Package for Social Sciences (SPSS 15.0 for Windows). The comparison of the indicators was performed with analysis of variance (ANOVA). A 5% significance level ($P<0.05$) and two-tailed tests were applied for all hypothesis tests.

Results

Estrous cyclicity

We found 4-5-day estrous cycles in the normal control rats. However, for the PCOS model rats, no significant estrous cycle was presented. After the treatment for 12 weeks ended, nine from the ten rats in CHM1 group and eight from the ten rats in CHM2 group exhibited epithelial keratinocytes.

The serum levels of LH/FSH, FAI and androstenedione

The serum levels of LH/FSH, FAI and androstenedione of the normal control rats were significantly lower than those of the rats from the other three groups ($P<0.05$) and the serum levels of LH/FSH, FAI and androstenedione of the PCOS model rats were the highest ($P<0.05$) (Figure 1), indicating PCOS model rats were successfully established through single injection of testosterone propionate. No significant differences on the serum levels of LH/FSH and FAI between the rats from CHM1 and CHM2 groups were found ($P>0.05$) (Figure 1A and B). However, the serum levels of androstenedione of the rats from CHM1 group were significantly lower than those of CHM2 group ($P<0.05$) (Figure 1C).

The mRNA expression levels of PPARG1 and HDAC3 in the rats ovaries

As shown in Figure 2A, the mRNA expression levels of PPARG1 in the ovaries of PCOS model rats were significantly lower than those of the other three groups ($P<0.05$) and its mRNA expression levels in the ovaries of the normal control rats were the highest among all the rats ($P<0.05$). HDAC3 mRNA expression levels in the ovaries of the normal control rats were the lowest, and in the PCOS model rats, the gene's mRNA expression levels were the highest ($P<0.05$) (Figure 2B). There were no significant differences existed between CHM1 and CHM2 groups on the mRNA expression levels of PPARG1 and HDAC3 in the ovaries of the rats($P>0.05$) (Figure 2).

Discussion

As we know, the frequency and amplitude of LH pulse increased with the rapid GnRH secretion, thus resulting in an elevation of the plasma LH concentrations and LH to FSH ratio (Taylor et al., 1997). The excessive androgen in the circulation can lead to the hyper-secretion of LH

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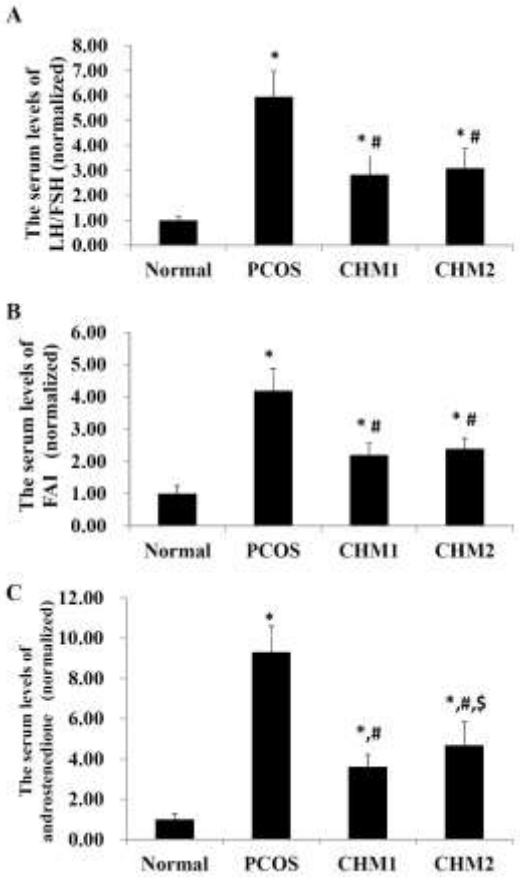

Figure 1: The serum levels of LH/FSH, FAI and androstenedione

A: the serum levels of LH/FSH levels; B: the serum levels of FAI; C: the serum levels of androstenedione. Data were shown as mean ± SD. (n=10 in each group). The significant difference was set at * P<0.05, compared with normal group; # P<0.05, compared with PCOS model group; $ P<0.05, CHM1 group compared with CHM2 group (ANOVA).

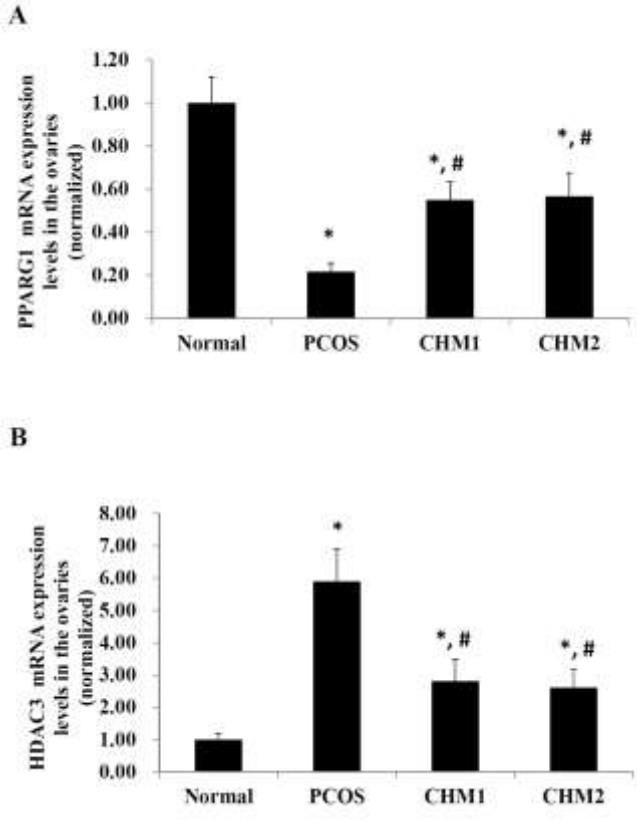

Figure 2: The mRNA expression levels of PPARG1 and HDAC3 in the rats ovaries

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A: the mRNA expression levels of PPARG1 in the rats ovaries; B: the mRNA expression levels of HDAC3 in the rats ovaries. Data were shown as mean ± SD. (n=10 in each group). The significant difference was set at * P<0.05, compared with normal group; # P<0.05, compared with PCOS model group; $ P<0.05, CHM1 group compared with CHM2 group (ANOVA).

through affecting the hypothalamic-pituitary axis and compromising the feedback inhibition on LH secretion by ovarian steroids (Eagleson et al., 2000). However, androgen excess did not reduce hypothalamic feedback inhibition in all adolescent girls with PCOS due to the genetic basis or the adequate duration of androgen excess (Chhabra et al., 2005). In concurrence with excessive androgens, the FSH fails to stimulate an adequate amount of aromatase to convert androgens into estrogens, thus resulting in the ovarian hyperandrogenism (Diamanti-Kandarakis, 2008). As an "androgen-sensitive period" existed during the first ten days of life in rats and mice, especially on the 9th day following birth (Sun and Yu, 2000; Tamura et al., 2005), in the present study, the model rats with PCOS were successfully established with single subcutaneous injection of testosterone propionate on the 9th day after birth (Tamura et al., 2005). FAI and androstendione were selected to reflect hyperandrogenism of PCOS in the study, as the two indicators have the highest clinical utility to evaluate PCOS-associated hyperandrogenism (Escobar-Morreale et al., 2001; Koskinen et al., 1996). We found in the study that the serum levels of LH/FSH, FAI and androstenedione of the PCOS model rats were the highest among all the groups and no significant differences on the serum levels of LH/FSH and FAI between the rats from CHM1 and CHM2 groups were found. The serum levels of androstenedione of the rats from CHM1 group were significantly lower than those of CHM2 group. We found that the patented CHM (CHM1) has similar effects with its refined version (CHM2) in treating hyperandrogenism of PCOS rats induced by testosterone propionate.

We found in the present study that in the ovaries of PCOS model rats, PPARG1mRNA expression levels were significantly lower, and HDAC3 mRNA expression levels were significantly higher than the other three groups. There were no significant differences existed between CHM1 and CHM2 groups on mRNA expression levels of PPARG1 and HDAC3 in the ovaries of the rats. As early as 2003, it was ever suggested that even mild forms of hyperandrogenism must be considered seriously and dysregulations of the steroidogenic pathway and ovarian abnormalities must be evaluated carefully to determine the risk of functional ovarian hyperandrogenism of PCOS (Cetinkaya et al., 2003). The follicular hyperandrogenism may induce the epigenetic alterations of PPARG1, NCOR1 and HDAC3 in the ovarian granulosa cells, which may be involved in the underlying mechanism of the ovarian dysfunction in hyperandrogenism PCOS women (Qu et al., 2012). These results indicate that it is through regulating PPARG1 and HDAC3 expression levels in the ovaries that CHM significantly alleviates hyperandrogenism of PCOS rats induced by testosterone propionate. Future studies are supposed to focus on the epigenetic and transcriptional regulation mechanism underlying CHM alleviating hyperandrogenism of PCOS. As the present study showed that the refined version of the patented formula had similar effects in alleviating hyperandrogenism of the model rats with the original one, it should be emphasized in the future study design.

Conclusion

It is through regulating PPARG1 and HDAC3 expression levels in the ovaries that CHM significantly alleviates hyperandrogenism of PCOS rats induced by testosterone propionate.

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