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Original Article

An *atractylodes macrocephala* koidz extract alleviates hyperandrogenism of polycystic ovarian syndrome

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Abstract: Background: *Atractylodes macrocephala* Koidz (AMK) is a tonic herb widely prescribed in most Asian countries, which has been clinically used as the dominant herb in most of the Chinese medicinal formula of treating polycystic ovarian syndrome (PCOS). Objective: To explore the effects of a polar extract of AMK (EAMK) in a hyperandrogenic rat model of PCOS induced by testosterone propionate. Material and methods: Sixty rats were randomly allocated to five groups of 12: healthy controls; untreated PCOS; three groups of treated PCOS (high, medium and low-dose EAMK). PCOS was induced with a single injection of testosterone propionate on the ninth day after birth. The plasma levels of total testosterone (TT), sex hormone binding globulin (SHBG), androstenedione, luteinizing hormone (LH), follicle stimulating hormone (FSH), antimüllerian hormone (AMH) were measured by enzyme-linked immunosorbent assays. The expression of FSH receptors (FSHR) and aquaporin-9 (AQP-9) in the ovaries of the rats was measured by real-time quantitative PCR and immunohistochemistry. We also measured the expression of FSHR and AQP-9 in human ovarian granulosa-like KGN cells, control and EAMK-treated, in the absence and presence of dihydrotestosterone (DHT). Results: EMAK significantly improved the estrous cycles, decreased the plasma levels of TT, FAI and androstenedione of the PCOS rats in a dose-dependent manner ($P < 0.01$) and the EAMK groups displayed significantly higher plasma FSH levels and lower LH/FSH and AMH levels than the PCOS control group ($P < 0.001$). In a dose-dependent manner, the EAMK treatments significantly decreased the FSHR expression levels and increased AQP-9 expression both in the ovaries of PCOS rats ($P < 0.001$) and in the KGN cells treated with DHT ($P < 0.001$). Conclusions: EAMK alleviates the PCOS rats' hyperandrogenism and regulates the ovarian expression of FSHR and AQP-9.

Keywords: Atractylodes macrocephala Koidz, polycystic ovarian syndrome (PCOS), hyperandrogenism, follicle stimulating hormone receptor (FSHR), aquaporin-9 (AQP-9)

Introduction

Around 5 million women in the United States suffer from polycystic ovary syndrome (PCOS) with an economic burden conservatively estimated at $13.9 billion dollar [1] and the prevalence of PCOS in the Chinese community population was 5.6% [2]. Hyperandrogenism, a key feature of PCOS, induces epigenetic alterations of peroxisome proliferator-activated receptor gamma 1, nuclear corepressor 1, and histone deacetylase 3 in granulosa cells, thus leading to ovarian dysfunction [3] accompanied by hirsutism, ovulatory or menstrual dysfunction, and polycystic ovarian morphology [4]. Such a functional ovarian hyperandrogenism is more likely to develop in prepubertal girls at risk of developing PCOS (through genetic polymorphisms and/or environmental factors such as availability of high-calorie foods) [5].

As young women with PCOS often present elevated insulin levels and are more likely to develop diabetes, metformin, an insulin sensitizer, is often prescribed; however this treatment is not effective in alleviating hirsutism or acne which

result from hyperandrogenism and can be associated with gastro-intestinal disturbances [6]. Anti-androgens such as spironolactone or cyproterone are also associated with adverse effects (disturbed liver function and hyperkalemia, respectively) and, because of potential teratogenicity, cannot be used in patients trying to conceive. When there is such a paucity of therapeutic options, patients often tend to seek alternative or complementary treatments [7]. It is therefore not surprising that, in a survey of self-selected Australian PCOS patients, more than 70% used complementary medicine, usually nutritional and herbal supplements; 76.6% of these complementary medicine users reported consultation with a complementary practitioner [8]. A recent survey of published pre-clinical and clinical studies highlighted 6 herbal medicines, i.e. *Vitex agnus-castus*, *Cimicifuga racemosa*, *Cinnamomum cassia*, *Tribulus terrestris*, *Glycyrrhiza spp.* and *Paeonia lactiflora*, that present positive effects for women with oligo/amenorrhea, hyperandrogenism and PCOS [9].

Atractylodes macrocephala Koidz (AMK) is a tonic herb widely prescribed in most Asian countries [10], and it has been clinically used as the dominant herb in most of the Chinese medicinal formula of treating PCOS. The bioactive compounds of AMK, closely related to the ecological factors of the region they are cultivated in [11], comprise a toxic eudesmane-type sesquiterpenoids-based volatile oil. The drug is often processed to reduce the content in the most toxic constituent, atractylon [12]. The present study was designed to investigate the effects of *Atractylodes macrocephala* Koidz polar extracts (EAMK) in alleviating hyperandrogenism in PCOS rats. As this polar extract has been extensively dried, the presence of constituents from the volatile oil is negligible.

The follicle stimulating hormone receptor (FSHR) is thought to play a role in the genetic susceptibility to PCOS [13]. Aquaporins (AQPs) are membrane water-specific channel proteins; completely impermeable to charged species, they play critical roles in controlling the water contents of cells [14, 15]. The presence of AQPs in granulosa cells suggests that water permeability of antral follicles primarily occurs through transcellular mechanisms, which may be mediated by the granulosa cells AQP-9 [16]. The expression levels of AQP-9 are regulated by androgens [17, 18] and the expression of AQP-9 in granulosa cells may be involved in the follicle development [19]. We previously showed that, in PCOS patients follicular fluids, hyperandrogenism leads to the decreased expression levels of AQP-9 through the PI3K pathway, thus hampering the follicular development [20].

The present study was designated to explore the effects of EAMK on androgen levels of the model rats with PCOS and to investigate whether EMAK has any effects in regulating the ovarian expression of FSHR and AQP-9 of the PCOS rats.

Materials and methods

Materials

Roots of *Atractylodes macrocephala* Koidz were harvested from Tiantai, Zhejiang Province, China, and identified by a botanist according to the People's Republic of China Pharmacopoeia (Version 2010, volume I, page 95-96). Voucher specimens were deposited in herbarium of College of Food Science and Biotechnology, Zhejiang Gongshang University (Hangzhou, China) under the number 2014AMK. The plant material was thoroughly washed and dried at 40°C for 3 days before use. Preparation of the EAMK, was performed at a Good Manufacturing Practice facility. 1000 g of AMK powder were extracted 3 times with 2500 mL of 70% ethanol by sonication for 0.5 h, followed by rotary evaporation at 4°C under reduced pressure. The ethanol extract (97 g) was then loaded onto a Diaion® HP-20 open column (100 cm × 10 cm; the volume of the column was 7.8 L) (styrene-divinylbenzene resin, Sigma Aldrich, St. Louis, MO, USA), and eluted with a methanol gradient beginning with 100% water (Fraction 1) and then sequentially 30% (Fraction 2), 65% (Fraction 3), and finally 80% (Fraction 4) methanol.

The enriched EAMK fractions were obtained from 65% methanol fractions, which were evaporated to dryness at 4°C under reduced pressure.

Ethical approval

The study was performed according to the Care and Use of Laboratory Animals protocol of the

National Research Council of China, and was approved by the Zhejiang University Ethics Committee.

Animals, groups and administration

Sixty neonatal female Sprague-Dawley rats were provided by the Experimental Animal Centre, School of Medicine, Zhejiang University (Hangzhou, China). They were housed in a temperature-controlled room under a 12-hour light/dark cycle, at a constant 25°C temperature and 55% humidity, and fed standard pelleted food and plain tap water *ad libitum*. A randomization chart was used to assign rats into control group, PCOS model group and the high-dose, medium-dose and low-dose EAMK groups (n=12 in each group). On the ninth day after birth, PCOS was induced by subcutaneous injection of testosterone propionate (Jinyao Amino Acid Ltd., Tianjin, China) at the dose of 0.1 mg/0.004 mL olive oil per g of animal [3, 21]; the control group received olive oil only. All pups were weaned from their mothers at the age of 21 days. From the 22nd day, each group received individual treatments that were orally administered for 8 consecutive weeks: the control and PCOS model groups received water (10 ml/kg) once a day; the high-, medium- and low-dose EAMK groups received EAMK (0.9 g/kg, 0.3 g/kg and 0.1 g/kg, respectively) dissolved in water once a day. During the treatment period, one rat died in the PCOS model group due to severe inflammation.

Sample collection and measurement

From the age of 70 days, vaginal smears were examined daily for 9 consecutive days. On the day following the 8-week treatments, the body weight of each rat was measured, after fasting for 12 h. The rats were then anesthetized with intraperitoneal urethane (1.2 g/kg). Blood samples were drawn from the abdominal aorta into heparinized syringes, and then centrifuged at 550 g at 4°C for 10 min. The supernatant plasma was then transferred to clean Eppendorf tubes and stored at -80°C. Following the collection of blood samples, the animals were sacrificed, and the uterus and the ovaries were immediately removed, washed with physiological saline and weighed. After the left ovary was sliced, the tissue slices were fixed in 10% neutral-buffered formalin for 24 h in preparation for histological examination. The right ovary was frozen, stored at -80°C until dissection by a laser capture micro-dissection system (AS LMD, Leica, Germany). The plasma levels of total testosterone (TT), sex hormone binding globulin (SHBG), androstenedione, luteinizing hormone (LH), follicle stimulating hormone (FSH), antimüllerian hormone (AMH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) were measured with commercial enzyme-linked immunosorbent assay kits (R&D Systems, MN, USA for TT, SHBG, LH, FSH, AMH; Wuhan Boster Bio-Engineering Limited Company, Wuhan, China for ALT, AST, GGT). The free androgen index (FAI) was calculated as TT (nmol/L) divided by SHBG (nmol/L) × 100 [22]. All measurements were performed in duplicate and were conducted according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation were < 10%.

Measurement of FSHR and AQP-9 mRNA expression in ovarian tissues by real-time quantitative PCR

The total RNA was isolated with RNAiso™ reagent (Takara Biotechnology, Dalian, China) according to the instructions of the manufacturer. The purity and concentration of the RNAs were determined by OD260/280 readings with a NanoDrop® ND-100 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA. The cDNA was prepared from 500 ng total RNA by reverse transcription (RT) with the PrimeScript™ RT Reagent kit (Perfect Real Time; Takara Biotechnology). The cDNA samples were then diluted in DNase- and RNase-free water at a proportion of 1:3 prior to further analysis. PCR was performed using the iCycler iQ Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). The rat FSHR and AQP-9 gene-specific primers were provided by Sangon Biological Engineering Technology (Shanghai, China). The sequences of the primers were as follows: FSHR forward, 5'-CCTTGCTCCTGGTCTCCTT-3' and reverse, 5'-GGTCGGTCGGAATCTCTGT-3'; AQP-9 forward, 5'-ATCCAGCTGTCTGAGGAGAGAAGA-3' and reverse, 5'-CTACATGATGACACTGAGCTCG-3'; GAPDH forward, 5'-GCAAGTTCAACGGCACAG-3' and reverse, 5'-CGCCAGTAGA-

CTCCACGAC-3'. PCR reactions were performed using 2 μl cDNA, 10 μM each primer, and 2X SYBR® Premix Ex Taq™ (Takara Biotechnology) in 20-μl reactions. Thermal cycling conditions were as follows: 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. A final melting curve was used to verify single-product formation. Gene starting quantity was based on the cycle threshold (Ct) method. Each value was normalized to GAPDH, a housekeeping gene, to control the amount of input cDNA. The Ct value for GAPDH mRNA was subtracted from that of the target gene, and the mRNA levels of the target gene were expressed as 2^{-Ct}.

Measurement of FSHR and AQP-9 mRNA expression in ovarian tissues by immunohistochemistry

The fixed ovarian tissue slices were embedded in paraffin, sectioned, deparaffinized and rehydrated, sectioned and mounted on slides. For immunohistochemical staining of FSHR and AQP-9, a number of the sections were incubated with the monoclonal FSHR antibody (1:100 dilution; Abcam, Cambridge, UK), and monoclonal AQP-9 antibody (1:100 dilution; Bioss Inc., Massachusetts, U.S.A.) at 4°C overnight. Following washing of the slides with Tris-buffered saline (TBS) twice, biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin were applied to the ovarian sections, and the expression was visualized by adding 3,3'-diaminobenzidine substrate. Positive staining appeared as deep brown color. The stained sections were evaluated using an Olympus BX50 optical microscope equipped with Image-Pro Plus software (version 6.0, Media Cybernetics, Inc, MD, U.S.A.). The integrated optical density (IOD) was calculated by measuring 10 consecutive visual fields for each sample using a 200 × objective.

Human ovarian granulosa-like KGN cells culture and treatments in vitro

To further explore the effects of EAMK on the ovarian expression of FSHR and AQP-9, we measured the expression of FSHR and AQP-9 in human ovarian granulosa-like KGN cells, control and EAMK-treated, in the absence and presence of DHT. Cells were cultured in RPMI 1640 with 5% (v/v) fetal bovine serum in 12-well plates in duplicates overnight. The KGN cells were untreated (control) or treated for 24 h with either 10^{-9} mol/L DHT (DHT), 10^{-9} mol/L DHT with 800 μg/mL EAMK (DHT+High-EAMK), 10^{-9} mol/L DHT with 200 μg/mL EAMK (DHT+Medium-EAMK), or 10^{-9} mol/L DHT with 50 μg/mL EAMK (DHT+Low-EAMK) [9, 10]. RPMI 1640 and DHT were purchased from Sigma Chemical Co.

Indirect immunofluorescence detection of FSHR and AQP-9 in human KGN cells

The KGN cells, washed and fixed as previously described [3], were blocked in 2% BSA and 30% inactivated goat serum in 1 × PBS for 40 min, followed by incubation with rabbit monoclonal primary antibody of FSHR and AQP-9 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a 1:100 dilution in antibody dilution/wash buffer (2% BSA + 30% inactivated goat serum in 1 × PBS) overnight. After the KGN cells were rinsed three times with the wash buffer, they were incubated for 30 min in the dark with anti-rabbit immunoglobulin G/DyLight594 secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at 1:400 in the dilution buffer and then washed 3 times. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). The fluorescent images were recorded with an Inversion Fluorescence Microscope (Olympus IX71S1F-3, Japan). The negative control experiments were performed by omitting primary antibodies to FSHR or AQP-9.

Detection of FSHR and AQP-9 protein in KGN cells by western blotting

The KGN cells, washed and lysed as previously described [3], were centrifuged at 10,000 g for 12 min. Bicinchoninic acid protein assay (Santa Cruz Biotechnology) was used to determine the protein concentration. Gel electrophoresis was performed on a Protean III mini-gel apparatus (Bio-Rad, Hercules, CA, USA) using 8% gel with 0.1% (w/v) SDS under a constant current of 35 mA; proteins were then electro-transferred to nitrocellulose membranes (Dingguo Biotechnology Company, Beijing, China) for 2.0 h. The membranes were blocked for 2.5 h at room temperature with 5% dried milk in Tris-Buffered Saline Tween (10 mM Tris, pH 7.6, 150 mM NaCl and 0.05% Tween-20) and incubated with primary antibody (FSHR monoclonal antibody from Santa Cruz Biotechnology, 1:600; AQP-9

Table 1. Estrous cycles and weights of body, uterus and ovaries after 8 weeks of treatment

Items	Normal group (n=12)	PCOS model group (n=11)	High-dose EAMK group (n=12)	Medium-dose EAMK group (n=12)	Low-dose EAMK group (n=12)
estrous cycles (n)	1.9 ± 0.3	0.0 ± 0.0***	1.4 ± 0.7###	1.1 ± 0.5###	0.8 ± 0.6##
body weight (g)	193.8 ± 21.4	254.4 ± 25.3***	223.3 ± 26.1#	218.9 ± 29.3#	238.0 ± 27.2
uterus weight (mg)	309.3 ± 38.5	307.6 ± 48.9	292.4 ± 44.4	322.9 ± 63.5	323.2 ± 31.1
ovaries weight (mg)	94.6 ± 15.9	170.7 ± 24.7***	115.4 ± 15.6###	146.5 ± 22.2	149.8 ± 45.0

Note: PCOS, polycystic ovarian syndrome; EMAK, extract of *Atractylodes macrocephala* Koidz. Data were presented as the mean ± standard deviation. ***$P < 0.001$, PCOS model group vs. normal group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, EAMK groups vs. PCOS model group.

Table 2. Plasma levels of hormones

Items	Normal group (n=12)	PCOS model group (n=11)	High-dose EAMK group (n=12)	Medium-dose EAMK group (n=12)	Low-dose EAMK group (n=12)
TT (nmol/L)	7.6 ± 1.0	24.1 ± 5.7***	11.4 ± 2.1###	12.8 ± 1.9###	18.0 ± 2.8###
SHBG (nmol/L)	22.7 ± 3.1	19.7 ± 2.1	16.5 ± 2.5	16.8 ± 2.9	21.9 ± 4.5
FAI	34.4 ± 6.5	122.7 ± 27.3***	70.0 ± 12.4###	77.3 ± 13.1###	86.0 ± 23.1###
Androstenedione (ng/mL)	0.7 ± 0.1	1.4 ± 0.2***	0.8 ± 0.3###	0.8 ± 0.1###	1.0 ± 0.4##
LH (IU/L)	4.1 ± 0.5	7.1 ± 1.3***	6.6 ± 0.8	5.1 ± 1.5###	5.9 ± 0.5#
FSH (IU/L)	7.2 ± 0.8	3.5 ± 0.7***	5.9 ± 1.0###	5.4 ± 0.7###	5.5 ± 0.8###
LH/FSH	0.6 ± 0.1	2.1 ± 0.4***	1.2 ± 0.35###	1.0 ± 0.3###	1.1 ± 0.2###
AMH (pg/mL)	21.5 ± 6.5	103.9 ± 17.2***	37.2 ± 9.4###	28.2 ± 6.9###	32. 9 ± 10.8###

Note: PCOS, polycystic ovarian syndrome; EMAK, extract of *Atractylodes macrocephala* Koidz; TT, total testosterone; SHBG, sex hormone binding globulin; FAI, free androgen index; LH, luteinizing hormone; FSH, follicle stimulating hormone; AMH, antimüllerian hormone. Data were presented as the mean ± standard deviation. ***$P < 0.001$, PCOS model group vs. normal group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, EAMK groups vs. PCOS model group.

monoclonal antibody from Santa Cruz Biotechnology, 1:200; beta-actin antibody from Sigma, 1:2000) overnight. The membranes were incubated with their corresponding secondary antibody at room temperature for 2.0 h. The proteins were detected with the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA). Densitometric intensity was measured with a GS-800 densitometer (Bio-Rad) and normalized against a beta-actin internal control.

Statistical analysis

Data are presented as mean ± standard deviation. Analysis of group differences was conducted with analysis of variance (ANOVA) and Tukey's post hoc tests. The results were considered statistically significant when $P < 0.05$, two-tailed. Statistical analyses were performed using GraphPad Prism 6.0c (GraphPad Software Inc., San Diego, CA).

Results

Estrous cycles

As shown in **Table 1**, based on the vaginal smears, the PCOS model rats had persistently keratinized vaginal cells. EMAK significantly improved the estrous cycles of the PCOS rats in a dose-dependent manner ($P < 0.01$).

Weights of body, uterus and ovaries

As shown in **Table 1**, the average body and ovaries weights of the PCOS model rats were significantly higher than those of the normal rats ($P < 0.001$). Compared with the model group, the high-dose and medium-dose EAMK groups displayed significantly lower average body weight ($P < 0.05$). The ovaries weight of the high-dose EAMK group was significantly lower than that of the model group ($P < 0.001$). There were no significant differences in the uterus weight among all the groups ($P > 0.05$).

Plasma levels of hormones

As shown in **Table 2**, the plasma levels of TT, FAI and androstenedione of the PCOS model group were significantly higher than those of the normal control group ($P < 0.001$). High-dose, medium-dose and low-dose EAMK treatment significantly decreased the plasma levels of TT, FAI and androstenedione of the PCOS

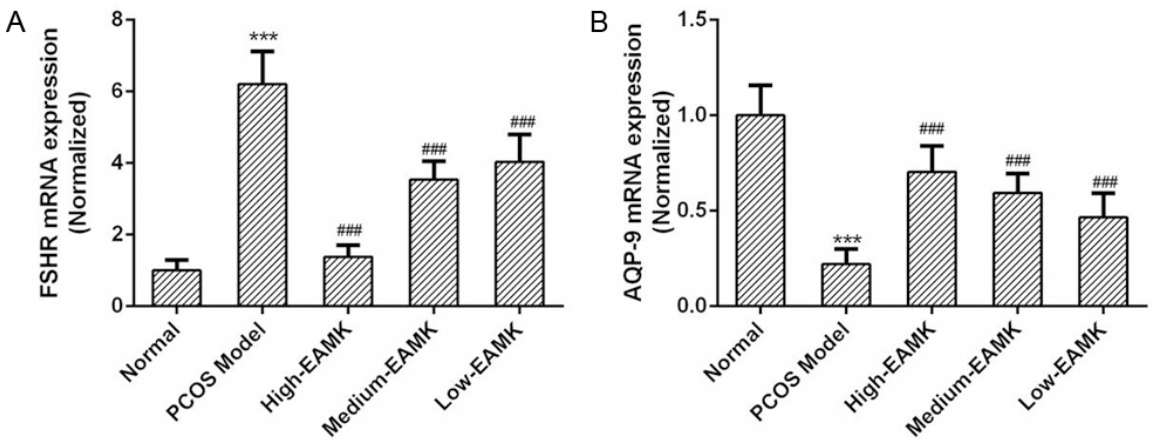

Figure 1. (A) Follicle stimulating hormone receptor (FSHR) and (B) aquaporin-9 (AQP-9) mRNA expression levels in the rat ovaries. PCOS, polycystic ovarian syndrome; EMAK, polar extract of *Atractylodes macrocephala* Koidz. Data are presented as the mean ± standard deviation. ***$P < 0.001$, PCOS model group vs. normal group; ###$P < 0.001$, EAMK groups vs. PCOS model group.

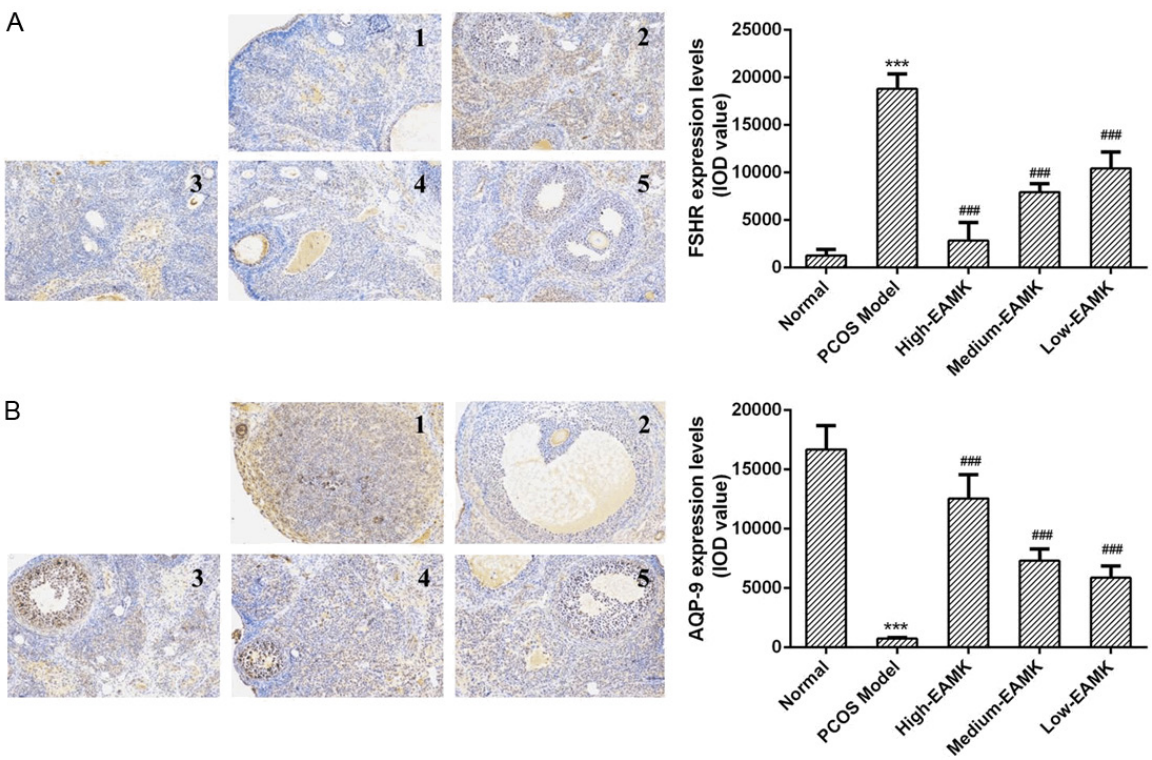

Figure 2. (A) Follicle stimulating hormone receptor (FSHR) and (B) aquaporin-9 (AQP-9) protein expression levels in the rat ovaries. PCOS, polycystic ovarian syndrome; EMAK, extract of *Atractylodes macrocephala* Koidz. 1. Normal group; 2. PCOS Model group; 3. High-EAMK group; 4. Medium-EAMK group; 5. Low-EAMK group. Data were presented as the mean ± standard deviation. ***$P < 0.001$, PCOS model group vs. normal group; ###$P < 0.001$, EAMK groups vs. PCOS model group.

rats in a dose-dependent manner ($P < 0.01$). In the normal control group, the plasma levels of LH, LH/FSH and AMH were significantly lower, and the plasma FSH levels were significantly higher than PCOS model group ($P < 0.001$, **Table 2**). The EAMK groups displayed significantly higher plasma FSH levels and lower LH/FSH and AMH levels than the PCOS model group ($P < 0.001$). The plasma LH levels of the medium-dose and low-dose EAMK groups were

Table 3. Liver function indices

Items	Normal group (n=12)	PCOS model group (n=11)	High-dose EAMK group (n=12)	Medium-dose EAMK group (n=12)	Low-dose EAMK group (n=12)
ALT (U/L)	90.5 ± 17.5	88.1 ± 22.3	86.6 ± 15.9	78.7 ± 15.2	80.7 ± 14.2
AST (U/L)	183.6 ± 35.1	190.3 ± 40.9	157.8 ± 39.6	162.8 ± 32.9	163.8 ± 38.2
GGT (U/L)	2.6 ± 0.3	2.6 ± 0.4	2.4 ± 0.4	2.9 ± 0.4	2.4 ± 0.2

Note: Data are presented as the mean ± standard deviation. PCOS, polycystic ovarian syndrome; EMAK, extract of *Atractylodes macrocephala* Koidz; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase.

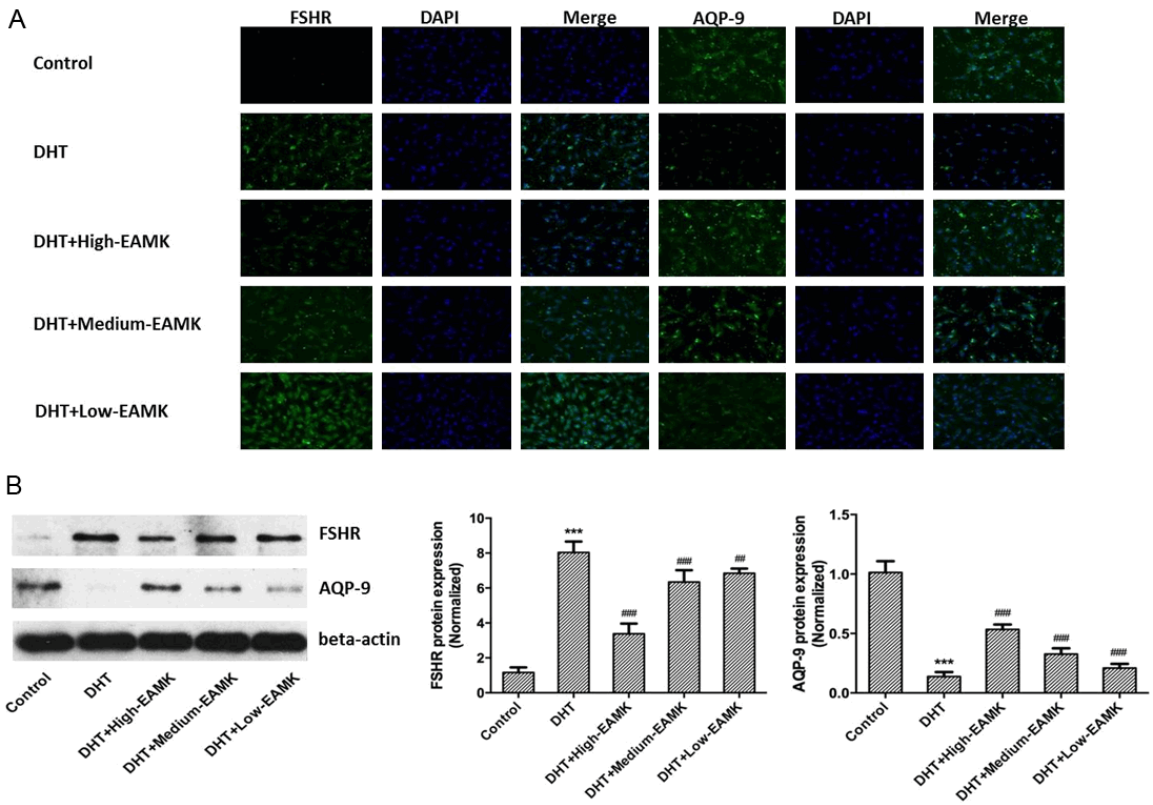

Figure 3. Follicle stimulating hormone receptor (FSHR) and aquaporin-9 (AQP-9) protein expression in the KGN cells control and treated with extract of *Atractylodes macrocephala* Koidz (EAMK) in the absence and presence of dihydrotestosterone (DHT). A. Representative images of indirect immunofluorescence. All the images of each group were recorded at the same magnification (× 200). No immunofluorescence staining was detected in the negative control (data not shown). B. Detection of FSHR and AQP-9 protein in KGN cells by western blotting. Data are presented as the mean ± standard deviation. ***$P < 0.001$, DHT group vs. control group; ##$P < 0.01$, DHT+EAMK groups vs. DHT group; ###$P < 0.001$, DHT+EAMK groups vs. DHT group.

significantly lower than that of the PCOS model group ($P < 0.05$).

FSHR and AQP-9 mRNA expression levels in the ovaries

As shown in **Figure 1**, the PCOS model group displayed significantly higher FSHR mRNA expression levels and lower AQP-9 mRNA expression levels than the normal group ($P < 0.001$). The EAMK treatments significantly decreased the FSHR mRNA expression levels and increased the AQP-9 mRNA expression levels in the ovaries of the PCOS rats in a dose-dependent manner ($P < 0.001$).

FSHR and AQP-9 protein expression in the ovaries

As shown in **Figure 2**, the protein expression was significantly higher for FSHR and lower for AQP-9 protein in the PCOS group compared to

the normal group ($P < 0.001$). EAMK significantly decreased the FSHR protein expression and significantly increased the AQP-9 protein expression in the ovaries of the PCOS rats in a dose-dependent manner ($P < 0.001$).

Liver function indices

As shown in **Table 3**, the plasma levels of ALT, AST and GGT were not significantly different between any of the five groups ($P > 0.05$).

FSHR and AQP-9 protein expression in the KGN cells control and EAMK-treated in the absence or presence of DHT

As shown in **Figure 3**, the DHT treatment significantly increased the FSHR protein expression and significantly decreased the AQP-9 protein in the KGN cells ($P < 0.001$). EAMK significantly decreased the FSHR protein expression and significantly increased the AQP-9 protein expression in the KGN cells treated with DHT in a dose-dependent maner ($P < 0.001$).

Discussion

This is the first demonstration that a polar extract of the traditional Chinese medicine *Atractylodes macrocephala* (EAMK) significantly lowers plasma TT and androstenedione levels in PCOS. The EAMK treatment also significantly reduced LH and increased FSH levels. No adverse effects were found on liver function. These results raise the possibility that this medication might have potential as a novel therapy for symptoms of PCOS, a condition which affects around 10% of women. The extracts of AMK have been previously proposed as potential aromatase inhibitors [23] but their effects on PCOS in humans or animal models have not been investigated so far.

TT, FAI and androstenedione are the most widely used indicators of hyperandrogenism in PCOS [24] and intrarovarian androgen excess is thought to play an important role in arresting follicular development in PCOS. So our finding that EAMK reduces TT and androstenedione may explain the improved estrous cycles in our animal model. AMH has been proposed as a useful marker of the severity and prognosis of PCOS [25]; it is therefore encouraging that the EAMK treatment significantly decreased the plasma AMH levels in PCOS rats. The results indicate that EAMK both decreases the ovarian FSHR expression and increases that of AQP-9 in a dose-dependent manner. It is possible that the phytochemicals of flavonoid glycosides might be responsible for the observed effects. The effects observed in rats (medium-dose of 0.3 g/kg) are observed at a dosage compatible with the one applied for traditional use in humans.

The results of this study in a rat model of PCOS suggest that EAMK can significantly reduce hyperandrogenism in this condition, and EAMK can also significantly decrease the ovarian expression of FSHR and increase the ovarian expression of AQP-9. Moreover, we also showed that EAMK significantly decreased the FSHR protein expression and significantly increased the AQP-9 protein expression in the human ovarian granulosa-like KGN cells treated with DHT in a dose-dependent manner. If these findings are replicated in the human, this would provide a valuable therapeutic intervention for the distressing features of androgen excess which affects around 10% of women. Our results also suggest a positive effect on fertility although meticulous attention to teratogenicity would be required before *Atractylodes macrocephala* could be incorporated into clinical practice for this indication.

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Disclosure of conflict of interest

None.

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