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Effect of anti-mullerian hormone on stem cell factor in serum, follicular fluid and ovarian granular cells of polycystic ovarian syndrome patients

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Abstract. – OBJECTIVE: Polycystic ovarian syndrome (PCOS) is a common disorder in gynecological practice. Anti-mullerian hormone (AMH) and ovarian granular stem cell factor (SCF) participate in the occurrence and progression of PCOS. This study aimed to investigate the expression of AMH and SCF in PCOS patients and attempt to analyze the effect of AMH on SCF.

PATIENTS AND METHODS: Both PCOS and non-PCOS patients who received *in vitro* fertilization (IVF) in our hospital were recruited for measuring AMH and SCF levels in serum, ovarian follicular fluid and granular cells by using enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry (IHC) and Real-time PCR were employed to quantify mRNA and protein levels of SCF in ovarian granular cells after treatment using different dosages of AMH.

RESULTS: AMH levels in serum, follicular fluid and granular cells in PCOS patients were significantly elevated, whilst SCF level was significantly decreased ($p<0.05$ in both cases). Therefore, there was a negative correlation between AMH and SCF level ($p<0.05$). In 5 ng/ml, 10 ng/ml and 15 ng/ml group, SCF protein positive rate was gradually decreased and was significantly lower compared to that of blank control ($p<0.05$). After treatment using AMH for 12, 24 and 48 h, SCF mRNA expression in the granular cell was significantly decreased ($p<0.05$). With higher dosage, SCF mRNA was gradually down-regulated in granular cells ($p<0.05$).

CONCLUSIONS: High level of AMH and low level of SCF existed in serum, follicular fluid, and granular cells in PCOS patients. AMH exhibited negative regulatory effects on SCF.

Key Words:
Polycystic ovarian syndrome, Anti-mullerian hormone, Ovarian granular stem cell factor, Ovarian granular cell.

Introduction

Anti-mullerian hormone (AMH) is mainly secreted from granular cells in middle to small follicles in ovary. It has a wide spectrum of functions including the inhibition of initiation and recruitment of primordial follicles, and the suppression of reactivity of preantral follicles and small antral follicles on (FSH). Moreover, it can also affect the cyclic recruitment of cells[1]. Recently, due to the environmental factor, infertility has become the research focus. Polycystic ovarian syndrome (PCOS) patients had increasing frequency of infertility. AMH abundantly expresses in both serum and follicular fluids of PCOS patients. PCOS mainly occurs in reproductive women, and is one common endocrine disorder in gynecology. Patients often present non-ovulation, abnormally high androgen level (hyperandrogenism), insulin resistance, and hyperinsulinemia, therefore drawing lots of research interests[2,3]. Researches showed more developmental arresting of ovarian antral follicles in PCOS patients, who also had significantly more non-advantageous follicles, all of which were probably related with AMH level[4]. AMH is the only endogenous cellular factor that could inhibit the growth of primordial follicles. In female ovarian tissues, type II receptor of AMH is only expressed by the granular cells with high specificity. AMH can also exert its biological effects via SMAD protein pathway, to affect the signal transduction pathway inside ovarian granular cells. As one important transcriptional factor, AMH has critical roles in regulating transcription of multiple genes and cytokine expressions[5,6]. Stem cell factor (SCF) is an important active fac-

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tor. Belonging to colony stimulating factor, SCF participates in proliferation and development of various body cells. It can also regulate the recruitment and development of primordial follicles via SCF/C-Kit signaling pathway[7,8]. Previous study indicated that both AMH and SCF have important roles regulating the whole process of follicle recruitment and growth[9]. In this study, we recruited PCOS patients from our hospital, and utilized different concentrations of AMH to interfere with SCF in ovarian granular cells, and to analyze the correlation between AMH and SCF. Finally, we investigated the alternation of these two factors and PCOS pathogenesis mechanism.

Patients and Methods

Patients

A total of 30 PCOS patients who received *in vitro* fertilization (IVF) and embryo transplantation in Jinan Maternity and Child Health Care Hospital from January 2015 to January 2016 were recruited in this study. The average age of patient groups was 28.7±3.3 years (from 23 to 34 years old). Another cohort of 30 non-PCOS individuals (23 to 35 years old, average age = 29.1±3.5 years) who received IVF in our hospital were recruited as the control group. No significant difference has been detected in age, height and body weight between two groups (p>0.05). Therefore, the two groups are comparable for the data analysis. This study has been pre-approved by the Ethical Committee of Jinan Maternity and Child Health Care Hospital (Jinan, China). All subjects have signed the consent forms before recruitment in this study.

Inclusive Criteria

(1) With any three features of oligomenorrhea, hirsutism, infertility and obesity. (2) More than 10 small follicles (less than 10mm diameter) in bilateral ovaries. (3) Basal ratio of LH/FSH higher than 2.0, and T > 2.2 nmol/l. (4) Not taking hormone medicines in recent three months. (5) No benign/malignant lesion in ovary or uterus; (6) No severe liver/kidney disorder, autoimmune disease. (7) Normal blood, urine, liver function and kidney function examination.

Exclusive Criteria

(1) Pregnant or breast-feeding women. (2) With endometrial hypertrophy, inflammation in uterus or cervical tissues, or other endometrial diseases. (3) Long-term user of hormone replacement treatment. (4) Other malignant diseases of uterus, ovary and ovarian tubes.

Reagent and Equipment

Anti-mullerian hormone was purchased from Zizhu Pharma. (Shanghai, China). Dulbecco's Modified Eagle Meidum (DMEM) culture medium, streptomycin/penicillin and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kit for AMH and SCF, SCF blocking buffer and primary antibody, rabbit anti-mouse secondary antibody, diaminobenzidine (DAB) kit, xylene, absolute ethanol, paraffin, hematoxylin, resin were purchased from Shanfeng Chem Indust. Co. Ltd. (Changzhou, China). The polymerase chain reaction (PCR) kit was purchased from Sangon Biotech. (Shanghai, China). Ultrapure workstation was purchased from Formal (Shanghai, China). The inverted microscope was purchased from Olympus (Mode: IX70, Tokyo, Japan). Microplate reader was purchased from (Tecna, Farnborough, UK). Microtome was purchased from (Leica, Nussloch, Germany). Heated vibrator was purchased from Jinghong Co. Ltd. (Shanghai, China). Computer-assisted imaging analysis system was purchased form Hewlett-Packard (Palo Alto, CA, USA). Incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA). CO_2 incubator was purchased from Sanyo (Mode: MCO-18AIC, Tokyo, Japan). Biometra T1 PCR cycler was purchased from Biometra (Gottingen, Germany). Cold centrifuge was purchased from Beckman Coulter Inc. (Brea, CA, USA). The -80°C fridge was purchased from Sanyo (Tokyo, Japan).

Serum Sample Collection

Fasted venous blood samples were collected from the morning on the third day of menstrual cycle of all patients. Samples were centrifuged at 1500 r/min for 5 min. The upper phase serum was collected, liquated and stored in –20°C fridge.

Follicular Fluid Collection

After ovulation induction, and 36 h after HCG injection, ovarian follicular fluids were collected by bilateral puncture under ultrasonic guidance. Follicular fluids from the largest follicle were centrifuged at 1500 r/min for 5 min. The supernatant was collected and stored in –20°C fridge.

Cell Isolation and Culture

Eggs were collected via vagina by ultrasonic guidance from all patients. Granular cell colony

was detached under the microscope, and was kept in follicular fluids for 1500 r/min centrifugation for 15 min. Precipitation was placed in 50% v/v Percoll solution and were centrifuged at 1500 r/min for 10 min to remove red blood cells. Granular cells were then removed and placed in 2× volume of 0.2 g/l collagenase I solution for 30 min digestion. After re-centrifugation, Tryphan blue was used to measure cell survival rate. Cells were then seeded into culture plate at 1×10^5/ml density. In each well, there was 1mL culture medium containing 10% fetal bovine serum, 100 μg/mL streptomycin and 100 U/ml penicillin. Cells were cultured in a 5% CO_2 chamber under 37°C.

AMH Treatment on Ovarian Granular Cells in Non-PCOS Patients

Ovarian granular cells at log-phase were counted and seeded into culture plate for routine attachment growth overnight, using DMEM containing 10% fetal bovine serum. AMH at 10 ng/ml, 15 ng/ml and 20 ng/ml were added for 12 h, 24 h or 48 h treatment. Ovarian granular cells from untreated non-PCOS patients were recruited in the blank control group.

ELISA for Measuring AMH and SCF Levels

The test kit was placed under room temperature for 30 min. A total of five standard samples were serially diluted. Samples were added into duplicated wells. After adding reaction buffer, washing and development buffer, absorbance values at 450 nm were measured in each well. Linear regression function was plotted for deducing sample concentrations.

SCF Protein Expression in Ovarian Granular Cells by IHC Staining

Tissues were fixed, dehydrated and immersed in paraffin for preparing tissue blocks, which were then sectioned and mounted onto glass slides. Next, tissue sections were dewaxed, rehydrated and processed in antigen retrieval. Blocking reagent was added, followed by primary antibody at room temperature for 1 h incubation, and secondary antibody (10 min). Streptavidin-peroxidase was added for 10 min development. After quenching and hematoxylin counter-staining, images were taken under the microscope for analysis in computers. Criteria: SCF positive was deduced as brown-yellow granules on the membrane or cytoplasm but not in the nucleus. SCF negative (-): less than 10% of positive cells in a field. Weak positive (+): 11%-25% positive staining cells. Positive (++): 26-50% of positive staining cells. Strong positive (+++): more than 50% positive cells.

RT-PCR for SCF mRNA Expression in Ovarian Granular Cells from PCOS and non-PCOS Individuals After AMH Intervention

Ovarian granular cells at log-phase were cultured in Dulbecco's Modified Eagle Medium (DMEM). 10 ng/ml, 15 ng/ml and 20 ng/ml AMH were added for 12 h, 24 h or 36 h intervention. Cellular mRNA was extracted and measured for total RNA concentration. D260 nm/D280 nm was calculated. 200 nm total RNA were extracted. Using polyA tail of miRNA, cNDA was synthesized. Using complementary DNA (cDNA) as the template, PCR amplification was performed using primers (SCF-F, 5'- CACCA TGAAG CCTAC ACTGT GTTTC C-3'; SCF-R, 5'- TTAAA CCATT CGGCA GCAGC GG-3'; GAPDH-F, 5'- GCCAA GGTCA TCCAT GACAA CTTTG G-3'; GAPDH-R, 5'-GCCTG CTTCA CCACC TTCTT GATGT C-3') under the following conditions: 95°C for 30 s, followed by 40 cycles each containing 95°C 5 s, 60°C 30 s.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for data processing, which were presented as mean ± standard deviation (SD). The comparison of enumeration data was performed by x^2-test, while measurement data were compared by Student *t*-test. A statistical significance was defined when $p<0.05$.

Results

AMH and SCF Levels in Patient Serum, Follicular Fluids and Granular Cells

Assays for AMH and SCF levels in patient serum, follicular fluids and granular cells found significantly higher AMH level and lower SCF level in PCOS patients compared to those in non-PCOS people ($p<0.05$ in both cases, Table I). AMH and SCF levels were negatively correlated ($r=-0.39$, $p<0.05$).

SCF Protein Expression in Non-PCOS Patients After AMH Intervention

We further measured SCF protein expression levels in AMH-treated cells from non-PCOS patients. Results showed 4 cases of strong positive and 5 cases of positive individuals in non-PCOS

patients who received 5 ng/ml AMH intervention (positive rate = 60%). In 10 ng/ml treated group, the positive rate was as high as 46.67% (2 strong positive and 10 positive cases). In 15 ng/ml treatment group, the positive rate was 33.3% (1 strong positive and 4 positive cases). Therefore, 15 ng/ml AMH significantly depressed SCF protein expression ($p<0.05$). All three groups (5 ng/ml, 10 ng/ml and 15 ng/ml) had significantly lower SCF positive rates compared to the blank control group ($p<0.05$, Table II and Figure 1).

SCF mRNA Expression in Ovarian Granular Cells after AMH Intervention in Non-POCS Individuals

We further employed different concentrations of AMH to treat ovarian granular cells from non-POCS patients for different times. Results showed that using the same concentration of AMH for 12 h, 24 h and 48 h treatment, the expression of SCF mRNA in granular cells was gradually decreased ($p<0.05$). At the same time point, with elevated AMH concentration, SCF mRNA expression in granular cells was gradually decreased ($p<0.05$, Table III).

Discussion

Ovarian is the basic unit of female reproductive system. In normal women body, the recruitment, induction and growth of oocytes were under the tight control of both positive and negative cellular factors. With the occurrence of imbalance of positive/negative regulatory homeostasis, ovarian function will be disrupted, thus severely affecting fertility[10]. Previous study[11] indicated that follicles in PCOS patients did not completely die, and were just at growth arresting. AMH is an important member of transforming growth factor β super-family. Basic study has found the decrease of primordial follicle bank in AMH-deficient female mice, suggesting the close correlation between AMH level and reserve function of ovaries[12]. AMH was positively expressed across female life from teenagers to sexual maturation. AMH concentration can reflect the size of primordial follicle bank, thus evaluating the reserve potency of ovary[13]. SCF belongs to colony stimulating factor, and is secreted by ovarian granular cells. It can bind with Kit ligand on the oocyte membrane, and stimulate recruitment of primordial follicles

Table I. AMH and SCF level in patient serum, follicular fluids and granular cells.

Index	No.	PCOS	non-PCOS
AMH	30		
Serum		16.37±6.92*	10.38±3.18
Follicular fluid		7.21±1.03*	5.31±0.52
AMH mRNA		11.86±2.08*	5.09±1.31
SCF	30		
Serum		0.56±0.13*	1.14±0.15
Follicular fluid		0.22±0.04*	0.30±0.02
SCF mRNA		0.17±0.16*	0.31±0.13

Note: *$p<0.05$ compared to non-PCOS patients.

Table II. SCF protein expression in non-PCOS patients after AMH intervention.

Group	n	SCF protein expression intensity			Positive rate (%)
		-	+-++	+++	
Non-POCS group	60				
5 ng/ml	15	6	5	4	60*
10 ng/ml	15	8	5	2	46.67*&
15 ng/ml	15	10	4	1	33.33*&#
Blank control	15	2	7	6	86.67

Note: *$p<0.05$ compared to blank control group. &$p<0.05$ compared to 5 ng/ml group. #$p<0.05$ compared to 10 ng/ml group.

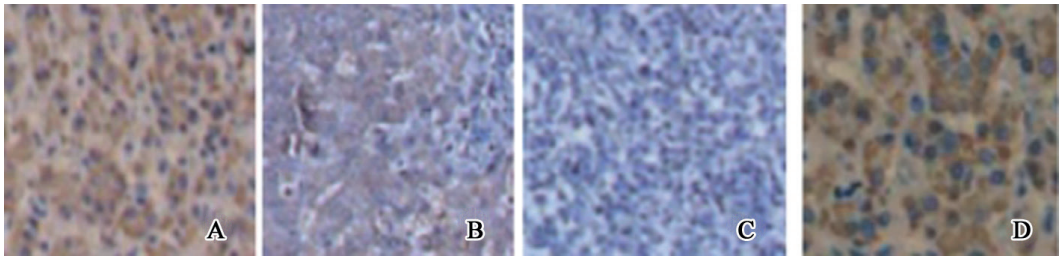

Figure 1. SCF protein expression. ***A,*** 5 ng/ml AMH treatment, SCF positive (++). ***B,*** 10 ng/ml AMH treatment, SCF strong positive (+++). C. 15 ng/ml AMH treatment, SCF negative (-). ***D***, Control group, SCF strong positive (+++).

Table III. SCF mRNA expression in ovarian granular cells after AMH treatment.

Item	SCF mRNA		
	12 h	24 h	48 h
Non-POCS			
5 ng/ml	0.327±0.013[a]	0.298±0.011[ad]	0.247±0.009[ade]
10 ng/ml	0.246±0.015[ab]	0.223±0.010[abd]	0.117±0.008[abde]
15 ng/ml	0.114±0.012[abc]	0.101±0.006[abcd]	0.008±0.003[abcde]
Blank control	0.437±0.917	0.452±0.875	0.477±0.912

Note: [a]$p<0.05$ compared to blank control group. [b]$p<0.05$ compared to 5 ng/ml group. [c]$p<0.05$ compared to 10 ng/ml group. [d]$p<0.05$ compared to 12 h group. [e]$p<0.05$ compared to 24 h group.

via PI3-AKT signal pathway[14]. We tested AMH and SCF levels in ovarian granular cells, in order to analyze their correlation. In this study, we collected serum, follicular fluid, and ovarian granular cells from included patients. By comparing with non-PCOS patients, we found elevated AMH level and decreased SCF level in serum follicular fluid and granular cells in PCOS patients. These results suggested that high level of AMH and low level of SCF existed in PCOS patients. The elevation of serum or follicular fluid AMH level in PCOS patients probably contributed to abnormal growth and ovulation in PCOS patients. Pellatt et al[15] suggested that *in vitro* culture of follicular granular cells can stimulate the production of follicular fluid, which had significantly higher AMH level compared to normal people. Das et al[16] pointed that the AMH level in non-ovulation PCOS patients was as high as 5-fold of those patients with ovulation. La Marca et al[17] suggested the AMH level in PCOS patients might be regulated by antral follicles number. The higher number of antral follicles is accompanied with higher AMH level, which was positive correlated with PCOS. The treatment of mice ovarian tissues by SCF facilitated more development of primordial oocytes. SCF expression level was known to be related with depletion, growth and maturation Meanwhile, AMH can inhibit SCF, and facilitate growth and maturation[18]. Granular cell exert its function via autocrine or paracrine manner, thus benefiting the maturing of oocytes, maintaining stability of local microenvironment and facilitating normal development of follicles. To further study the effect of AMH on SCF, we separated ovarian granular cells from non-PCOS patients, and treated then with 5 ng/ml, 10 ng/ml and 15 mg/ml for 12 h, 24 h or 48 h. After cell fixation and mounted, IHC method was used to detect SCF protein expression after AMH treatment in non-PCOS group. The positive rate was 60% in 5 ng/ml intervention group, while positive rates for 10 ng/ml and 15 ng/ml group were only 46.67% and 33.3%, respectively. In all three groups, SCF positive rates were significantly lower than blank control group. These results suggested that AMH treatment on ovarian granular cells in non-PCOS patients, and can inhibit SCF protein expression, especially at 15 ng/ml concentration. SCF is mainly expressed in cytoplasm and membrane of ovarian follicles, and regulate related functions during the whole process of follicular recruitment and development. Dong et al[19] suggested that the FH/LSH antagonist can interfere with ovarian follicular cells, decreasing AMH expression and potentiating SCF expression. In this work, we treated granular cells with the same concentration of AMH for 12 h, 24 h and 48 h. We found SCF mRNA expression was gradually decreased in granular cells. At the same time, with elevated treatment concentration, SCF mRNA expression was gradually decreased. These results showed that AMH could inhibit SCF mRNA expression in ovarian granular cells in non-PCOS patients. The most potent inhibitory effect occurred at 15 ng/ml for 24 h incubation. In ovarian granular cells, AMH inhibits CREB phosphorylation via cAMP pathway, to inhibit the binding on SCF promoter, thus down-regulating SCF transcription, leading to difficulty of oocyte recruitment, and exerting stimulatory effect of PCOS and development[20-22].

Conclusions

AMH is abundantly expressed in serum, follicular fluid and granular cells. The decrease of SCF expression indicated the negative control on SCF by AMH, which may affect growth and recruitment of follicles via inhibiting SCF expression,

and suppresses occurrence and progression of PCOS. However, the detailed mechanism between AMH and SCF still requires further studies in basic field. In clinical, the present conclusion of this study could be used to predict the progression and prognosis of the PCOS, which is very important for the doctors to intervene the patients in the early stage of the PCOS. Therefore, this conclusion could be applied in the clinical to diagnose the severe of the PCOS patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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