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Elevation of antimüllerian hormone in women with polycystic ovary syndrome undergoing assisted reproduction: effect of insulin

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Objective: To measure blood and follicular antimüllerian hormone (AMH) levels in women with polycystic ovary syndrome (PCOS) undergoing assisted reproductive technologies (ART) and to examine the direct action of insulin on AMH expression in human granulosa cells.
Design: Prospective clinical and experimental study.
Setting: University Hospital-based laboratory.
Patient(s): Women with (n = 86) and without (n = 172) PCOS in ART.
Intervention(s): Blood, follicular fluid, and luteinized granulosa cells were collected from PCOS and non-PCOS women in ART.
Main Outcome Measure(s): Hormone levels in blood and fluid, and gene expression in granulosa cells.
Result(s): Serum levels of AMH were elevated and inversely correlated with embryo cleavage rate in PCOS women in ART. Significant higher levels of AMH were also found in small and large follicles collected from PCOS women compared with non-PCOS women. Luteinized granulosa cells from PCOS women showed higher expression of AMH and its receptor AMHR2. Direct effect of insulin in increasing the expression of AMH in the isolated luteinized granulosa cells was observed, with the PCOS granulosa cells responding to a high dose of insulin. Cotreatment with AMH attenuated insulin-induced aromatase expression in the luteinized granulosa cells.
Conclusion(s): These results suggest that insulin may contribute to AMH elevation in PCOS and that AMH counteracts insulin-promoted aromatase expression in granulosa cells. (Fertil Steril® 2019;111:157–67. ©2018 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Polycystic ovarian syndrome (PCOS), assisted reproductive technologies (ART), antimüllerian hormone (AMH), insulin

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Polycystic ovary syndrome (PCOS), characterized by ovarian follicular arrest and hormonal disturbance, is a leading cause of female infertility, affecting 5%–10% women of reproductive age (1, 2). A variety of hormones are dysregulated in PCOS, including excessive androgens (3), hypersecreted LH (4), reduced FSH (4), and disturbed metabolic hormones (e.g., insulin and glucagon) (5). In particular, insulin resistance accompanied by compensatory hyperinsulinemia is commonly found in women with PCOS (up to 75% of lean and 95% of overweight) (6, 7).

Antimüllerian hormone (AMH), a key regulator of sex differentiation during embryonic development (8), was recently recognized to be elevated in PCOS, contributing to the ovarian dysfunction (9, 10). AMH is a homodimer glycoprotein belonging to the transforming growth factor β (TGF-β) family (11, 12). In addition to its role in inhibiting embryonic development of the müllerian duct and thus leading to sex differentiation to male (13), AMH is also known to be

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involved in folliculogenesis (14). In women, AMH is produced exclusively by granulosa cells (15), starting at the primary follicle stage, with its highest expression detected in preantral and small antral follicles, and declining afterward until absent in large (>8–10 mm diameter) follicles (16). Studies have shown that AMH inhibits FSH-stimulated expression of aromatase, a key enzyme for ovarian steroidogenesis (17), as well as the growth of preantral follicles (18). Therefore, the expression pattern of AMH during folliculogenesis is thought to preserve most antral follicles from being matured and ovulated, which is evidenced by the accelerated folliculogenesis leading to early depletion of ovarian follicles observed in AMH-null mice (19). Abnormally elevated AMH in PCOS, however, is thought to be detrimental to FSH-stimulated growth of selectable follicles, leading to anovulation (20).

Although its contribution to PCOS pathogenesis is generally accepted, the cause of AMH elevation in PCOS remains unknown. It was thought that the increased number of small antral follicles in PCOS might produce an environment of excessive AMH, but later studies have shown that AMH production is increased in individual follicles and granulosa cells from PCOS women (21–23), with the underlying mechanism largely unexplored. Interestingly, hyperinsulinemia is proposed as a reason for AMH elevation in PCOS, because AMH levels were found to be particularly higher in PCOS women with insulin resistance (24, 25) and long-term metformin treatment was reported to correct AMH levels in PCOS (26). However, contradictory findings were also reported (27, 28), and the direct effect of insulin on AMH production by granulosa cells is not clearly demonstrated.

PCOS women often seek in vitro fertilization (IVF)–based assisted reproductive technology treatment (ART). In ART, the AMH level has been associated with ovarian responses (29), implantation, pregnancy, and other qualitative ART outcomes (30, 31), although controversial results are also reported (32–35).

We undertook the present study to better characterize the association between AMH level and embryo quality as well as ART outcomes with PCOS. We also examined the possible action of insulin on AMH expression in human granulosa cells, comparing cells from PCOS and non-PCOS women.

MATERIALS AND METHODS

Human Subjects and Clinical Data Collection

A total of 258 women undergoing IVF-based ART at the Shanghai First Maternity and Infant Hospital from June 2014 to June 2017 were recruited for the present study. The Rotterdam criteria (36) were used for diagnosis of PCOS: 1) oligomenorrhea (cycle length >35 days) or amenorrhea (cycle length >3 months or no rise of progesterone before menstruation); 2) hyperandrogenism as defined by hirsutism, serum testosterone level >0.7 ng/mL, and/or androstenedione >2.2 ng/mL; and 3) the presence of more than 12 follicles of 2–9 mm in diameter in each ovary and/or unilateral ovarian volume >10 mL according to B-ultrasound. Eighty-six women showed the presence of Rotterdam criteria I and III and were diagnosed with PCOS. Androstenedione was not measured in them. Another 172 women met the following inclusion criteria and were included as non-PCOS for the present study: 1) age 20–40 years; 2) both ovaries present with no morphologic abnormalities and adequately visualized with the use of transvaginal ultrasound; 3) regular menstrual cycle; and 4) no current or past diseases affecting the ovaries or taking gonadotropin or sex steroids within 3 months before inclusion. The cause of infertility in the non-PCOS group was due to either tubal obstruct or male factor. The study was approved by the Ethics Committee of Tongji University, and all the subjects gave informed consents. All the procedures were performed in accordance with the relevant guidelines and regulations. The subjects' clinical data including age and body mass index (BMI) were documented. Patients with oligo- or amenorrhea were pretreated with progesterone to induce regular cycle before the blood test and ART treatment. A blood test was performed on the 3rd to 5th day of the menstrual cycle before the treatment to determine basal levels of hormones.

Ovarian Hyperstimulation

All of the subjects were given diphereline (Ipsen Pharma Biotech; 1.25 mg) a GnRH agonist, at the mid-luteal phase of their menstrual cycles, to suppress pituitary secretion of gonadotropin hormones and prevent premature ovulation. After pituitary suppression was achieved, which was determined by E_2 ≤50 pg/mL, endometrial thickness ≤6 mm, and no follicles >10 mm according to transvaginal ultrasound, subjects were daily injected with Gonal-F, a recombinant human FSH (rhFSH; Merck-Serono), starting from day 5 of a normal menstrual cycle (initial day). Serum sex hormone levels were monitored, and transvaginal ultrasound (5 MHz; model SSD-620; Aloka Co) was used to evaluate the development of ovarian follicles and adjust the dose of rhFSH. When the lead follicle achieved 18 mm in diameter, the lead two 17 mm, or the lead three 16 mm, patients were subcutaneously injected with recombinant hCG (250 μg; Ovidrel; Merck Serono) to trigger oocyte maturation (hCG day). Thirty-six hours afterward, oocytes were retrieved by means of a transvaginal ultrasound–guided approach. Fertilization of the retrieved oocytes was done by means of either IVF or intracytoplasmic sperm injection, according to the sperm quality. Successful fertilization and cleavage were determined according to reported ART standards (37). Quality of the embryo was evaluated and graded from I to V: grade I: embryos with equal blastomeres and <5% cytoplasm fragmentation; grade II: embryos with equal blastomeres and 5%–25% cytoplasm fragmentation; grade III: embryos with unequal blastomeres and <5% cytoplasm fragmentation; grade IV: embryos with unequal blastomeres and 5%–25% cytoplasm fragmentation; and grade V: embryos with a few blastomeres and with strong or complete cytoplasm fragmentation. Embryos of grades I and II were considered to be good-, grade III intermediate-, and grades IV and V poor-quality embryos. Three to five days after cleavage was observed, up to two embryos were transferred in each patient per cycle. Grade I and II embryos were transferred as priority.

Grade III embryos were transferred in case no grade I or II embryo was available. In rare cases, grade IV and V embryos were cultured until blastocyst stage before the transfer. Patients were intramuscularly injected with progesterone (60 mg/d; Tongyong Pharmaceutical Co.) from oocyte retrieval day until 14 days after embryo transfer. Clinical pregnancy was defined as the identification of a gestational sac with fetal heart activity on ultrasound examination 4–5 weeks after embryo transfer. Implantation rate was calculated as the number of gestational sacs (observed at week 4 after transfer) divided by the number of embryos transferred in each patient. Other downstream parameters—pregnancy, miscarriage, ectopic pregnancy, and live birth rates—were calculated with the total number of patients in each group as the denominator.

Follicular Fluid and Granulosa Cell Collection

The ovarian follicles were collected after hCG injection from the patients as we previously reported (38). Follicles were classified into two groups, small (<10 mm diameter) and large (>18 mm), according to their size as measured by means of ultrasound. Under ultrasound guidance, a sterile needle, connected to a vaculum device, was transvaginally inserted. With gental suction, each follicle was individually collected through the needle to a collection vial. Large follicles were collected before the collection system was washed sufficiently, and small follicles were collected afterward. For each individually collected follicle, the oocyte was carefully dissected out under a dissecting microscope and the remaining granulosa cells with fluid were transferred into sterile tubes (Axygen Scientific) for centrifugation at 400*g* for 10 minutes. Afterwards, the supernate was used as follicular fluid. Cell pellets were washed with phosphate-buffered saline solution (PBS) and then a red blood cell lysis buffer and further centrifuged at 400*g* for 5 minutes. For ELISA measurements of the follicular fluid, a single large follicle with sufficient amount of fluid was used for one measurement, and follicular fluid gathered from three to four small follicles from a patient was used for another measurement. The collected granulosa cells and follicular fluid were stored at −80°C until mRNA analysis or resuspended for culture.

Hormonal Immunoassays

Blood was collected from subjects on the 3rd to 5th day of the menstrual cycle before the treatment (baseline), the day when FSH treatment started (initial day), and the day when hCG was given (hCG day) to determine testosterone, FSH, LH, and E_2 levels with the use of an automated chemiluminescence immunoassay analyzer (Siemens). On hCG injection day and before the injection, blood was collected to examine serum AMH levels with the use of an ELISA kit, Antimüllerian Hormone ELISA (Biomatik), according to the supplier's instructions. For follicular fluid samples, which are of small volume and have lower AMH levels, we used a more sensitive and specific chemiluminescence kit (Roche) according to the manufacturer's instructions. Insulin in follicular fluid was measured with the use of a specific ELISA kit (Mai Bio) according to the manufacturer's instruction. The plates were read with the use of an ELISA reader (Thermo Fisher Scientific).

Human Luteinized Granulosa Cell Culture

The collected granulosa cells were cultured in DMEM/F12 (Gibco) with 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Life Technologies), 100 μg/mL streptomycin sulfate (Life Technologies), and 1× Glutamax (Life Technologies) at 37°C in a CO_2 incubator. Cells were deprived of serum for 24 hours before stimulated with 5, 10, or 100 ng/mL insulin (Sigma; bovine pancreatic insulin), GSK1904529A (5 μmol/L; Selleck), an insulin receptor inhibitoror anti-Mullerian hormone (20 ng/ml, R&D Systems, MN, USA).

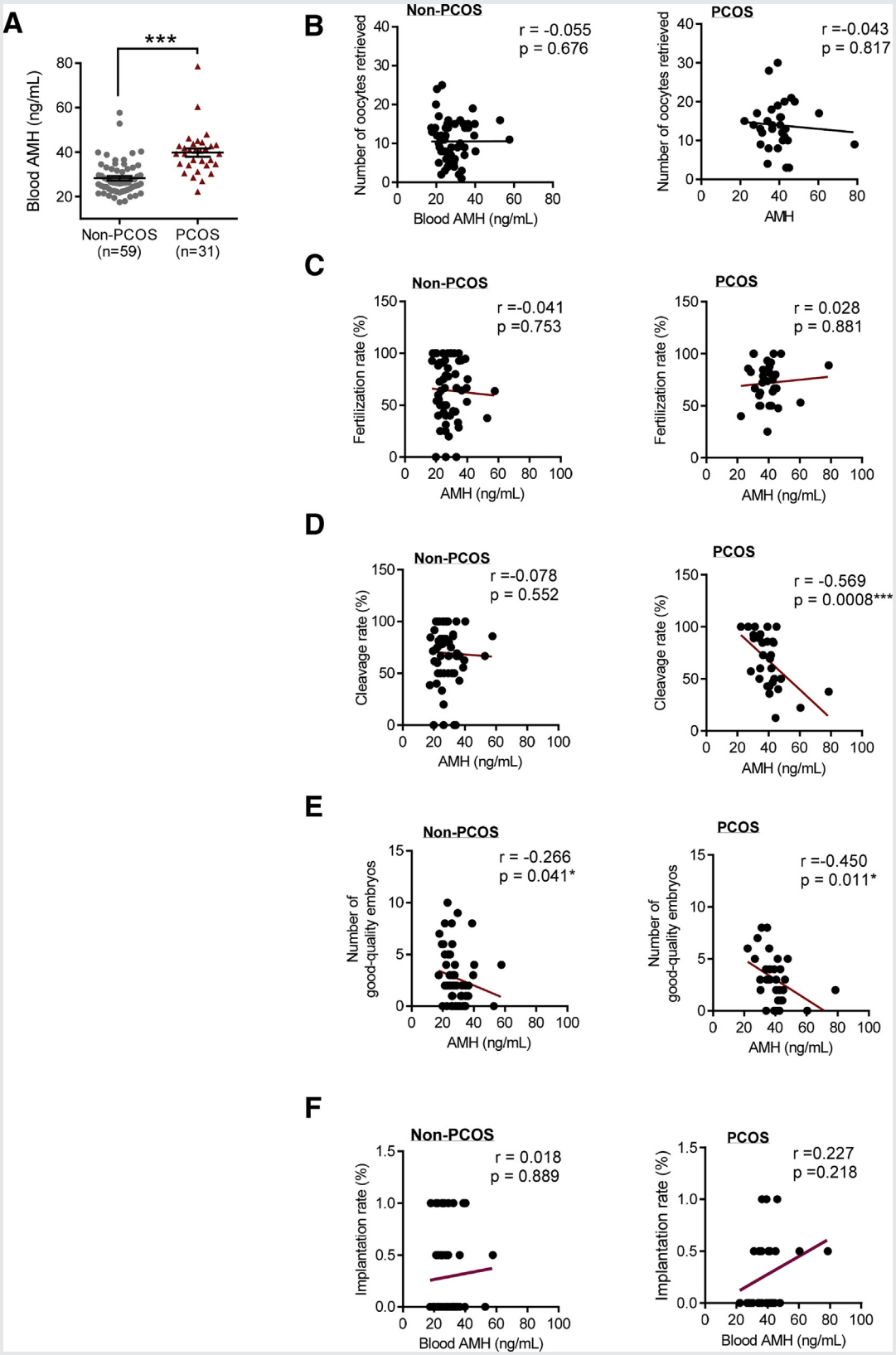
RNA Extraction, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction

Total RNAs from cells were extracted with the use of the Trizol reagent (Life Technologies) according to the manufacturer's protocol. The concentration of RNA was measured with the use of the Nanodrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific). One μg RNA was transcribed to cDNA with the use of the PrimeScript RT reagent kit (Takara). Briefly, a master mix containing 4 μL 5× Primescript Buffer, 1 μL Rimescript RT Enzyme Mix I, 1 μL Oligo dT Primer (50 μmol/L), 1 μL Random 6 mers (100 μmol/L), and RNase Free water was prepared for each 20-μL reaction, which was followed by 37°C for 30 minutes, 85°C for 5 seconds, and cooling at 4°C. Gene expression levels were evaluated by the manufacturer's protocol on the Steponeplus Real-Time PCR System (Thermo Fisher Scientific) with the use of the Sybr reagent (Tiangen). The primers were from Sangon Biotech, and the sequences are listed in Supplemental Table 1 (available online at www.fertstert.org). The general thermal profile of the reaction for all the tested genes was 10 seconds at 95°C to activate the enzyme followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds for each cycle. The dissociation stage was initiated at 95°C for 15 seconds, followed by one cycle at 60°C for 60 seconds and 95°C for 15 seconds. Each reaction was assayed in triplicate, and three separate experiments were performed on different cultures. A mean value (generated from the triplicate data of the target genes and reference gene of each sample) was used for the determination of mRNA levels by the comparative Ct ($2^{-\Delta\Delta Ct}$) method with β-actin as the housekeeping gene, where ΔΔCt = [Ct of target (sample A) − Ct of reference (sample A)] − [Ct of target (sample B) − Ct of reference (sample B)]. This equation can be used to compare the gene expression in two different samples (sample A and sample B); each sample is related to a housekeeping gene (β-actin).

Western Blot

Cells were washed with PBS and lysed in RIPA buffer (Beyotime) containing protease inhibitor cocktail and phenylmethylsulfonyl fluoride (Sigma-Aldrich) on ice. The extract was centrifuged at 15,000*g* for 30 minutes at 4°C to remove cellular debris, and the protein concentration was quantified by means of the BCA assay (Thermo Fisher Scientific). The same amount

FIGURE 1



Blood antimüllerian hormone (AMH) is elevated in women with polycystic ovary syndrome (PCOS). (**A**) ELISA measurement of AMH levels in the blood collected on hCG injection day (before the injection) from PCOS and non-PCOS women. n is shown in each column. ***$P<.001$ (*t* test). (**B–F**) Correlation analysis of the blood AMH level and (**B**) oocytes retrieved, (**C**) fertilization, (**D**) cleavage, (**E**) good-quality embryos, and (**F**) implantation in PCOS and non-PCOS women. Spearman coefficient correlation test.

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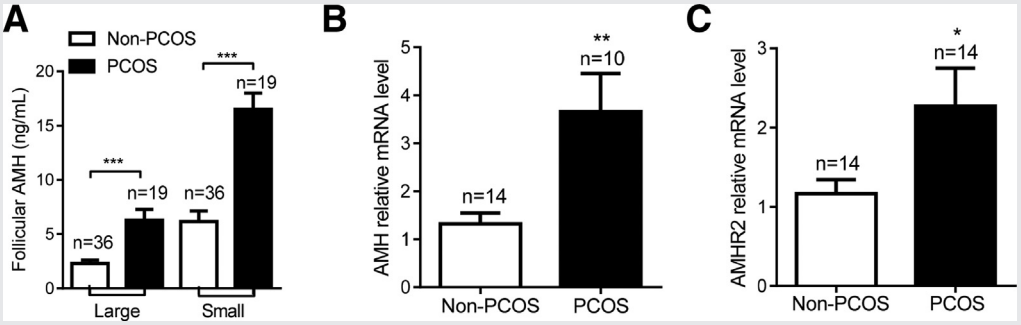
FIGURE 2



Antimüllerian hormone (AMH) is elevated in the follicular fluid and granulosa cells in women with polycystic ovary syndrome (PCOS). (**A**) ELISA measurement of AMH levels in follicular fluid from non-PCOS and PCOS women. Data are presented as mean ± SEM, n indicated at each column. ***$P<.001$ (t test). (**B–C**) Quantitative polymerase chain reaction (qPCR) analysis of (**B**) AMH and (**C**) AMHR2 mRNA levels in luteinized granulosa cells freshly isolated from non-PCOS and PCOS women. β-Actin was used as the internal control for relative mRNA level measurement by qPCR. Data are presented as mean ± SEM, n indicated at each column, *$P<.05$; **$P<.01$ (t test).

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of protein (20 μg) of each sample was separated with the use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Beyotime) with 5% stacking gel and 10% separating gel at 120 V for 2 hours and then transferred onto the polyvinyl difluoride membranes at 250 mA for 1 hour. Membrane-nonspecific binding was blocked with the use of 5% nonfat milk in TBST (0.01 mol/L Tris-HCl, 0.15 mol/L NaCl, and 0.1% Tween-20, pH 7.4; Beyotime), containing 0.05% Tween-20 for 1 hour at room temperature and then incubated with rabbit antihuman aromatase (Cell Signal Technolog; 1:1,000) and rabbit antihuman GAPDH (Abmart; 1:1,000) diluted in TBST with 5% milk overnight at 4°C. Next, the membranes were washed three times with TBST and then incubated for 1 hour with the horseradish peroxidase–conjugated secondary antibody (Abmart; 1:2,000). Immunoreactive bands were detected with the use of an enhanced chemiluminescent substrate (Millipore). The intensities of the bands were quantified with the use of Image-J software.

Statistical Analysis

Data are presented as mean ± SEM of at least three independent experiments. Comparisons of two independent groups were performed with the use of the Student's t test. One-way analysis of variance followed by Tukey post hoc tests was used to compare more than two groups. The correlation analysis was done with the use of the Spearman coefficient. $P<.05$ was considered to be statistically significant. All statistic procedures were run on SPSS 20.0 (IBM). Because there were differences in age, BMI, and duration of infertility between the PCOS and non-PCOS groups in cohorts I and III (see Results), we corrected a priori for these differences by means of analysis of covariance and multiple liner regression.

RESULTS

Clinical Characteristics of PCOS and non PCOS Women in ART

Blood, follicular fluid, and granulosa cells were collected from 3 cohorts of woman subjects respectively. Clinical characteristics are summarized in Supplemental Table 2 (available online at www.fertstert.org). In general, the PCOS women, when examined, were ~2 years younger with a higher average BMI compared with the non-PCOS women. The PCOS women were given a lower average initial dose of rhFSH with longer treatment duration than the non-PCOS women, although the total doses of rhFSH were similar, with no significant difference between the two groups.

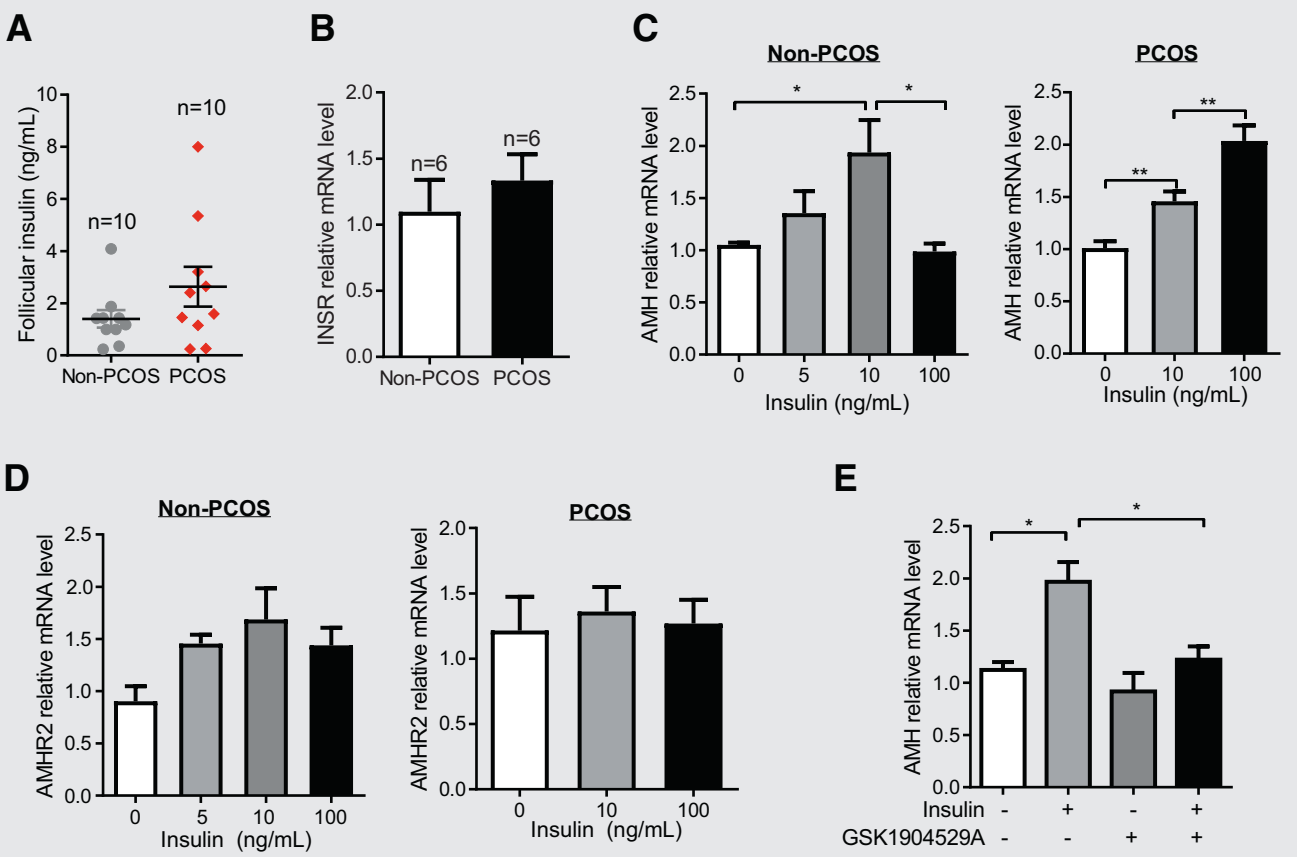
Hormonal profiles of the two groups are presented in Supplemental Table 2. Before the treatment (baseline), the PCOS group exhibited lower FSH and higher LH levels than the non-PCOS group. The serum FSH, LH, and E_2 in both PCOS and non-PCOS subjects were largely decreased to a low level before rhFSH treatment started (initial day), indicating successful pituitary suppression. After rhFSH treatment and right before hCG injection (hCG day), the LH level remained low and E_2 level was highly elevated in both groups, with no significant difference. Progesterone levels on hCG day also were found to be similar between PCOS and non-PCOS women.

Clinical outcomes also are presented in Supplemental Table 2. More PCOS patients were considered to have risk of ovarian hyperstimulation syndrome (OHSS) and therefore chose to cancel embryo transfer within the same cycle and freeze the formed embryos for later treatment. Among the assessed parameters, including fertilization, cleavage, embryo quality, implantation, and pregnancy, PCOS and non-PCOS women exhibited similar ART outcomes.

AMH is Elevated and Negatively Correlated with Oocyte/Embryo Quality in PCOS Women

We collected blood samples on hCG injection day from 31 PCOS and 59 non-PCOS (cohort I; Supplemental Table 2) women to determine AMH blood levels. Results showed that the average blood level of AMH in PCOS women (39.77 ± 1.84) was significantly higher than in non-PCOS women (28.26 ± 1.00; Fig. 1A). To explore possible effects of AMH on embryo quality, correlation analyses

FIGURE 3



Effect of insulin on AMH expression in human luteinized granulosa cells. Data are presented as mean ± SEM. (**A**) ELISA measurement of insulin levels in follicular fluid from women without and with PCOS. (**B**) qPCR analysis of mRNA levels of insulin receptor (INSR) in cultured luteinized granulosa cells isolated from non-PCOS and PCOS women., n indicated in each column. (**C, D**) qPCR analysis of mRNA levels of (**C**) AMH and (**D**) AMHR2 in cultured luteinized granulosa cells isolated from non-PCOS and PCOS women, after incubation with insulin (0–100 ng/mL) for 24 hours. Cells isolated from 14 non-PCOS women and six PCOS women were pooled together for the experiments. n = 3 (number of independent experiments). *$P<.05$; **$P<.01$ (one-way analysis of variance [ANOVA] with post tests). (**E**) qPCR analysis of mRNA levels of AMH in non-PCOS granulosa cells pretreated with or without INSR antagonist GSK1904529A (5 μmol/L) for 8 hours before incubation with or without insulin (10 ng/mL) for 24 hours. Cells isolated from ten women were pooled together for the experiments. n = 3 (number of independent experiments). *$P<.05$ (one-way ANOVA). Abbreviations as in Figure 2.

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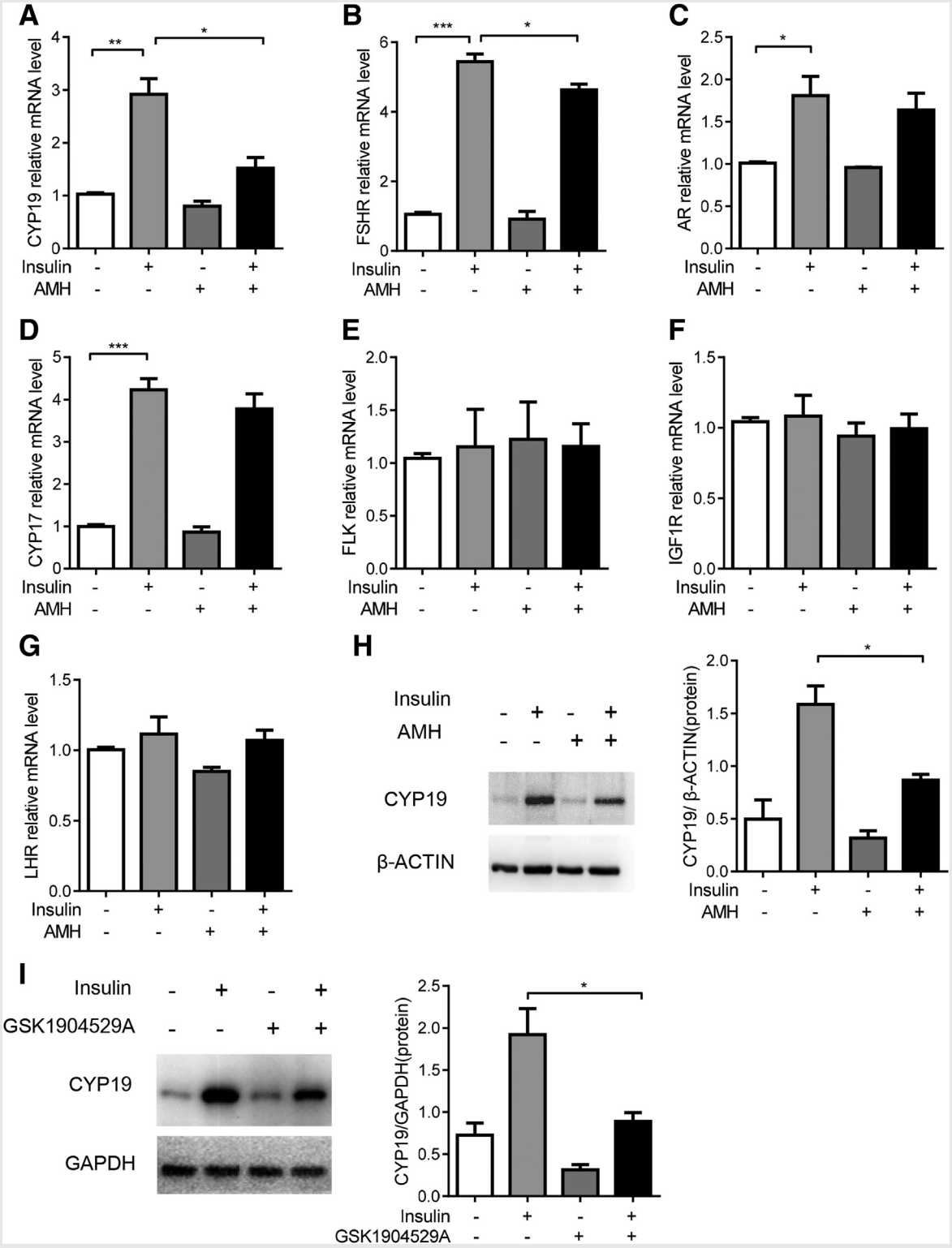
were performed between the AMH blood level and the number of oocytes retrieved (Fig. 1B), fertilization rate (Fig. 1C), cleavage rate (Fig. 1D), number of good-quality embryos (Fig. 1E), and implantation rate (Fig. 1F) in PCOS and non-PCOS subjects. Results showed significantly negative correlation of the AMH blood levels with the cleavage rate (Fig. 1D) and number of good-quality embryos (Fig. 1E) in PCOS women. In non-PCOS women, only a weak inverse correlation of AMH with number of good-quality embryos was found.

AMH Is Elevated in Follicular Fluid and Granulosa Cells in PCOS Women

Because the follicular environment is particularly important to oocyte development and maturation, we further examined the AMH level in the follicular fluid in another cohort of PCOS (n = 19) and non-PCOS (n = 36) women (cohort II; Supplemental Table 2). Both small (<10 mm) and large (>18 mm) follicles were obtained under ultrasound during oocyte pick-up and the follicular fluid was collected. In both PCOS and non-PCOS groups, the small follicles contained higher concentrations of AMH compared with the large follicles, which is consistent with a role of AMH during folliculogenesis (Fig. 2A). Importantly, PCOS follicles exhibited higher AMH levels (large: 6.15 ± 0.98 ng/mL; small: 16.51 ± 1.48 ng/mL) compared with those in the non-PCOS group (large: 2.29 ± 0.32 ng/mL; small: 6.29 ± 1.00 ng/mL; Fig. 2A). Because AMH is known to be exclusively produced by granulosa cells in women (8), we tested whether the elevated AMH in follicular fluid could be due to up-regulation of AMH expression in PCOS granulosa cells. Indeed, in collected luteinized granulosa cells from large follicles, significantly higher mRNA levels of AMH were observed in PCOS women compared with non-PCOS women (Fig. 2B). Moreover, the AMH receptor AMHR2 was also found

FIGURE 4



Effect of insulin and AMH on genes expression in human luteinized granulosa cells. Data are presented as mean ± SEM; n = 3 (number of independent experiments). *$P<.05$; **$P<.001$; ***$P<.001$ (one-way ANOVA). qPCR analysis of mRNA levels of (**A**) CYP19, (**B**) FSHR, (**C**) AR, (**D**) CYP17, (**E**) FLK, (**F**) IGF1R, and (**G**) LHR in luteinized granulosa cells from non-PCOS women, treated with or without insulin (10 ng/mL) and AMH (20 ng/mL) for 24 hours. β-Actin was used as the internal control for relative mRNA level measurement by means of qPCR. Cells isolated from nine women were pooled together for the experiments. (**H**) Western blotting for CYP19 in luteinized granulosa cells isolated from non-PCOS women, treated with or without insulin (10 ng/mL) or AMH (20 ng/mL). β-Actin was used as a loading control for Western blot. (**I**) Western blotting for CYP19 in luteinized granulosa cells isolated from non-PCOS women, treated with or without INSR antagonist GSK1904529A (5 μmol/L) for 8 hours before incubation with or without insulin (10 ng/mL) for 24 hours. Cells isolated from 10 women were pooled together for the experiments. Abbreviations as in Figures 2 and 3.

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to be increased at the mRNA level in PCOS compared with non-PCOS granulosa cells (Fig. 2C).

Insulin Induces Up-Regulation of AMH in PCOS Granulosa Cells

We next explored possible reasons for AMH up-regulation in granulosa cells in PCOS. Hyperinsulinemia was proposed to be responsible for AMH elevation in PCOS (25). We detected the presence of insulin in both PCOS and non-PCOS follicular fluid, with a relatively higher level detected in the PCOS group (Fig. 3A). We next examined the direct effect of insulin on AMH expression in cultured human granulosa cells. Luteinized granulosa cells from 24 non-PCOS and 6 PCOS women (cohort III; Supplemental Table 2) were isolated and cultured as previously reported (38). The expression of insulin receptor showed no significant difference between PCOS and non-PCOS granulosa cells (Fig. 3B). After 24-hour insulin treatment, the mRNA levels of AMH in granulosa cells were significantly increased by in an insulin dose–dependent manner in both non-PCOS and PCOS groups (Fig. 3C). At 100 ng/mL, insulin no longer increased AMH expression in non-PCOS cells, suggesting desensitization to a high dose of insulin in these cells (Fig. 3C). In contrast, PCOS cells continued to respond to insulin at 100 ng/mL, resulting in further increases in AMH mRNA levels compared with those at 10 ng/mL, suggesting their loss of desensitization to high-dose insulin (Fig. 3C). In addition, pretreatment with the antagonist of insulin receptor GSK1904529A (5 μmol/L) for 8 hours, blocked the insulin-induced AMH up-regulation in non-PCOS granulosa cells (Fig. 3E). No significant changes in AMHR2 mRNA were observed after the insulin treatment in either non-PCOS or PCOS cells (Fig. 3D).

AMH Inhibits Insulin-Stimulated Aromatase Expression in Granulosa Cells

Granulosa cell function is essential to oocyte development and maturation. Given the presence of both insulin and AMH in follicular fluid and the direct effect of insulin in increasing AMH expression as suggested above, we wondered whether the expression of genes essential to granulosa cell function would be subject to regulation by either the follicular insulin or AMH. Aromatase (CYP19), CYP17, FSH receptor (FSHR), androgen receptor (AR), vascular endothelial growth factor receptor (FLK), insulin-like growth factor 1 receptor (IGF1R), and LH receptor (LHR) were examined in luteinized granulosa cells from non-PCOS women after treatment with insulin (10 ng/mL), AMH (20 ng/mL), or their combination for 24 hours. Results showed that insulin (10 ng/mL) alone significantly increased mRNA levels of CYP19 (Fig. 4A), FSHR (Fig. 4B), AR (Fig. 4C), and CYP17 (Fig. 4D), compared with control cells. AMH (20 ng/mL) alone did not alter any of these genes (Fig. 4). Cotreatment with AMH, however, substantially attenuated the effect of insulin (10 ng/mL) in increasing CYP19 mRNA expression (Fig. 4A). A slight effect of AMH cotreatment in inhibiting insulin-induced FSHR expression was also observed (Fig. 4B). No obvious changes in FLK, IGF1R, and LHR with either insulin or AMH were observed in these cells (Figs. 4E–4G). To confirm the inhibitory effect of AMH on insulin-induced CYP19, we also examined the protein level of CYP19, which consistently showed, that compared with cells treated with insulin (10 ng/mL) alone, cells treated with combination of AMH (20 ng/mL) and insulin (10 ng/mL) exhibited significantly lower CYP19 protein levels (Fig. 4H). Antagonizing insulin receptor with the use of GSK1904529A (5 μmol/L; Fig. 4I) also inhibited insulin-induced up-regulation of CYP19 in these cells.

DISCUSSION

Altogether, the present study indicated a higher blood AMH level in PCOS than in non-PCOS women during ART. The blood AMH levels were in negative correlation with the cleavage rate and number of good-quality embryos in PCOS women. AMH was also elevated in the collected follicular fluid and up-regulated in isolated luteinized granulosa cells from PCOS women. Direct effect of insulin in increasing the expression of AMH in the isolated granulosa cells was observed, with the PCOS cells responding to a high dose of insulin. The presence of AMH exerts an inhibitory effect on insulin-induced aromatase expression in isolated luteinized granulosa cells.

Consistent with others (10, 39), the present study demonstrates that AMH is abnormally up-regulated in PCOS. First, protein levels of AMH are increased in the blood and follicular fluid of either small (presumably preantral/antral) or large (matured) follicles. Second, we were only able to collect analyzable granulosa cells from large but not small follicles, which are usually thought to be luteinized cells and supposed to have reduced AMH expression. Nevertheless, in these cells, significantly increased mRNA expression of both AMH and its receptor AMHR2 was observed in PCOS compared with non-PCOS. It should also be noted that the blood AMH level was examined on hCG day, when pituitary suppression and exogenous FSH was already applied and other hormones became similar between PCOS and non-PCOS. AMH is elevated in PCOS even after hormonal correction in ART, which suggests intrinsic changes in PCOS granulosa cells resulting in abnormally up-regulated AMH and its downstream signaling.

Whether AMH can affect outcomes of ART is controversial (40–44). The present study found negative correlations of AMH blood level with the cleavage rate and number of good-quality embryos in PCOS women. Thus, a negative impact on embryo quality, particularly at early stages of embryo development, by the elevated AMH levels in PCOS is suggested. However, because only cleaved or good-quality embryos are selected to proceed with embryo transfer and implantation in ART, the later stages and ultimate ART outcomes seem not to be influenced by the elevated AMH levels in PCOS. Interestingly, a recent study has shown that prenatal AMH exposure may account for the pathogenesis of PCOS in adulthood (45), also suggesting the influence of AMH on embryo development in PCOS. Of note, it was previously reported in a general population that no relationship between AMH and embryo quality was found (46). Others showed that low maternal AMH level could be a predicting marker for fetal aneuploidy (47), suggesting that a low AMH level also is not good for embryos. In the present study,

although a high AMH level correlated with poor embryo quality in PCOS women, the maternal AMH levels in non-PCOS women had no or weak correlation with embryo quality parameters. It seems consistent that AMH level can hardly predict embryo quality within a general population. Nevertheless, detailed mechanisms underlying the effect of AMH on embryo quality in PCOS await further investigation.

An association between the environmental insulin and granulosa cell production of AMH is suggested. A direct effect of exogenous insulin, mediated by insulin receptor, in increasing AMH expression was observed in both PCOS and non-PCOS luteinized granulosa cells. Interestingly, luteinized granulosa cells isolated from PCOS women seemed to be quite sensitive to insulin, rather than insulin-resistant, in terms of AMH expression (Fig. 3C). PCOS cells responded to insulin even at a high dose that already desensitized non-PCOS cells (Fig. 3C). Given that the expression level of the insulin receptor in PCOS cells showed no difference from that of non-PCOS cells (Fig. 3B), the difference observed might be due to functional change of the insulin receptor or alterations in its regulatory pathways, which is possibly a cause for the observed elevation of AMH in PCOS. However, the detailed mechanisms underlying the discrepancy in responses to insulin between PCOS and non-PCOS granulosa cells await further investigation. Of note, it was suggested that the overexpression of AMH and AMHR2 in PCOS women could be due to increased LH levels, especially in PCOS women with oligo/anovulation (48). Because insulin and LH are thought to have synergy effects (49), hyperinsulinemia may enhance the effect of LH in increasing AMH expression in PCOS women.

It should be noted that insulin is known to increase aromatase activity in granulosa cells from either normal and PCOS women (50, 51). Similar effect of insulin in increasing aromatase expression is also observed presently. Whereas, aromatase is known to be dysfunctional or down-regulated (38) contributing to a hyperandrogenic environment in PCOS. In addition to the previously reported effect of AMH in reducing FSH-induced aromatase expression (52), the present study has shown the effect of AMH in counteracting the action of insulin on aromatase expression. Together with the finding of insulin's role in promoting AMH production, it may provide an explanation to the reduced aromatase activity despite a high-insulin environment in PCOS.

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Elevación de la hormona antimülleriana en mujeres con síndrome de ovario poliquístico sometidas a reproducción asistida: efecto de la insulina.

Objetivo: Medir los niveles sanguíneos y foliculares de hormona antimülleriana (AMH) en mujeres con síndrome de ovario poliquístico (SOP) sometidas a técnicas de reproducción asistida (TRA) y examinar la acción directa de la insulina sobre la expresión de AMH en células de la granulosa humanas.

Diseño: Estudio prospectivo clínico y experimental.

Entorno: Laboratorio de hospital universitario.

Paciente(s): Mujeres con (n = 86) y sin (n = 172) SOP en TRA.

Intervención(es): Se tomaron muestras de sangre, líquido folicular y células de la granulosa luteinizadas en mujeres en TRA con SOP y sin SOP.

Principales medidas de resultado: Niveles hormonales en sangre y líquido folicular, y expresión génica en las células de la granulosa.

Resultado(s): Los niveles séricos de AMH fueron elevados y se correlacionaron inversamente con la tasa de división del embrión en mujeres con SOP en TRA. También se encontraron niveles significativamente más altos de AMH en los folículos pequeños y grandes de mujeres con SOP en comparación con mujeres sin SOP. Las células de la granulosa luteinizadas de las mujeres con SOP mostraron una mayor expresión de la AMH y de su receptor AMHR2. Se observó un efecto directo de la insulina incrementando la expresión de la AMH en las células de la granulosa luteinizadas, con respuesta de las células de la granulosa del SOP a una dosis alta de insulina. El tratamiento con AMH atenuó la expresión de la aromatasa inducida por la insulina en las células de la granulosa luteinizadas.

Conclusión(es): Estos resultados sugieren que la insulina puede contribuir a la elevación de la AMH en el SOP, y que la AMH contrarresta la expresión de la aromatasa estimulada por la insulina en células de la granulosa.