Human Reproduction vol.13 no.2 pp.290–293, 1998

Polycystic ovary syndrome: anomalies in progesterone production

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The underlying cause of anovulation and miscarriage in polycystic ovary syndrome (PCOS) is unknown. Progesterone may play an important role in oocyte fertilization and embryo implantation. Therefore, in this study we analyse the endocrine function of luteinizing granulosa cells to synthesize progesterone *in vivo* and *in vitro* in PCOS and normal patients participating in an in-vitro fertilization programme. Human luteinizing granulosa cells were obtained from 10 patients with normal ovaries (controls) and 10 patients with PCOS by follicular aspiration of individual follicles of each patient and pooled in an attempt to obtain three groups: cells from follicle sizes ≤10, >10≤15 and ≥16. Serum concentrations of oestradiol and progesterone on the day of human chorionic gonadotrophin (HCG) injection were significantly higher ($P < 0.01$ and $P < 0.05$) in PCOS patients than in controls. After HCG stimulation, in-vitro progesterone production was enhanced in granulosa cells of the control group and concentrations increased with follicular size as expected. However, the concentration of progesterone of PCOS patients did not increase with follicular size and there was a significant difference between normal and PCOS groups in follicles >10≤15 mm ($P < 0.05$) and ≥16 mm ($P < 0.01$). Oestradiol production was increased in follicles ≥16 mm in both groups, although this did not reach significance. In summary, it seems that PCOS granulosa cells demonstrate an abnormal capacity to synthesize progesterone *in vivo* and *in vitro*. The understanding of granulosa cell function in PCOS may explain the anovulation and miscarriage that occurs in these patients.

Key words: granulosa cells/HCG/oestradiol/polycystic ovary syndrome/progesterone.

Introduction

Polycystic ovary syndrome (PCOS) is a reproductive endocrine abnormality characterized by anovulatory infertility and recurrent miscarriage (Erickson and Yen, 1993; Franks and White, 1993; Clifford *et al.*, 1994). The exact mechanism by which anovulation occurs in PCOS is unknown. Several theories have been proposed. The follicular fluid in PCOS appears to contain high concentrations of bioactive follicle stimulating hormone (FSH) and sufficient amounts of androstenedione substrate to saturate the aromatase enzyme, but the oestradiol concentration remains below the concentrations found in dominant follicles (Erickson *et al.*, 1992; San Roman and Magoffin, 1992). When the granulosa cells from PCOS are cultured *in vitro* and stimulated by FSH, they are able to produce normal or increased amounts of oestradiol (Erickson *et al.*, 1990; Mason *et al.*, 1994). These data support the hypothesis that PCOS follicular fluid contains one or more inhibitors of aromatase activity, for example 5α-androstane-3,17-dione (Agarwal, 1996). Anovulation in PCOS is associated with hyperinsulinaemia and insulin resistance. In-vitro preincubation with insulin of granulosa cells from PCOS increased basal and LH-induced, but not FSH-stimulated, steroid production (Willis *et al.*, 1996). In PCOS granulosa cells, growth hormone supplementation seems to enhance the ovarian response to gonadotrophins and significantly decreases follicular fluid androstenedione (Volpe *et al.*, 1992; Doldi *et al.*, 1996). Furthermore, PCOS granulosa cells have shown an abnormal capacity to synthesize progesterone *in vitro*. Erickson *et al.* (1992) demonstrated that, unlike normal granulosa cells, PCOS cells have a limited capacity to synthesize progesterone, either spontaneously or in response to FSH stimulation.

Little is known about PCOS and miscarriage. Indeed, the presence of PCOS does not predict miscarriage, but patients who miscarry have higher concentrations of total testosterone, free testosterone and dehydroepiandrosterone sulphate than women with continuing pregnancies (Tulppälä *et al.*, 1993). Furthermore, Donderwinkel *et al.* (1993) demonstrated that patients with PCOS have significantly lower luteinizing hormone (LH) concentrations during the luteal phase after ovulation induced by human menopausal gonadotrophin (HMG) and human chorionic gonadotrophin (HCG) in combination with gonadotrophin-releasing hormone analogue (GnRHa), and therefore may suffer from insufficient luteal phases.

The primary objective of this study was to analyse the endocrine properties of luteinizing granulosa cells to synthesize progesterone from size-matched follicles in PCOS and normal patients participating in an in-vitro fertilization (IVF) programme.

Materials and methods

Patients

This study includes 10 patients with normal ovaries (control) and 10 patients with PCOS participating in an IVF programme in the San Raffaele Scientific Institute, University of Milan. All patients had a tubal factor for infertility and all of the male partners had normal

semen quality according to World Health Organization (WHO) criteria. Normal and PCOS patients were classified according to menstrual history and ultrasound examination. PCOS was diagnosed according to the following criteria: a history of anovulatory infertility and/or oligomenorrhoea or amenorrhoea, increased ovarian volume (>9 ml), and ≥10 follicles of 2–8 mm in diameter. Normal patients had a history of normal menstrual cycles, normal ovarian size and no more than five follicles >2 mm in diameter. Patients were treated with GnRHa, buserelin (Suprefact; Hoechst, L'Aquila, Italy), beginning in the mid-luteal phase of the prior menstrual cycle for 1 week and then continuing until the day of HCG administration, at a dose of 0.6 mg/day. In all cycles, three ampoules of FSH (urofollitrophin, 75 IU; Metrodin; Serono, Rome, Italy) were administered i.m. from cycle day 3 onwards. The dosage of gonadotrophin was maintained or increased appropriately until an adequate oestradiol response was achieved. Ovarian response was monitored by measurements of serum oestradiol concentrations and by follicular growth, using transvaginal ultrasonography.

Human chorionic gonadotrophin (Profasi, 5000 IU; Serono, Rome, Italy) was administered i.m. when sonography revealed at least two follicles measuring ≥16 mm in diameter, in association with adequate serum oestradiol concentrations.

Granulosa cell cultures and hormone measurements

Human luteinizing granulosa cells were obtained by ultrasound-guided transvaginal follicular aspiration of individual follicles, which was carried out 35 h after HCG injection. After removing the oocytes, the remaining cells from follicles of similar size of each patient were pooled in an attempt to obtain three groups: cells from follicle sizes ≤10, >10≤15, and ≥16 mm. Then cells were washed twice with medium 199 (Flow Laboratories, Milan, Italy). Granulosa cells and red blood cells were transferred to a 12 ml tube containing 3.5 ml Lymphocyte Separation Medium (Flow Laboratories) and separated by centrifugation at 600 *g* for 5 min. Granulosa cells were dispersed by gentle shaking at 37°C for 30 min in 5 ml culture medium containing 0.1% collagenase and 20 mg DNase/ml. The dispersed cells were washed in culture medium, counted and plated at a density of $4–5\times10^5$ cells/10 cm plastic culture dish (Falcon) in serum-free medium 199 containing 2 mM glutamine and 50 mg/ml gentamycin. Cells were cultured at 37°C in a 95% air–5% CO_2 humidified environment. After 2 days, the cells had attached to the wells. At this time, the medium was removed and 24-h incubations with 50 ng/ml of HCG in serum-free medium 199 were initiated.

Cultured media were stored at –20°C until assayed for oestradiol and progesterone. Oestradiol and progesterone were measured by radioimmunoassay (Serono Diagnostics, Milan, Italy).

Statistical analysis

All results are reported as the mean ± SE. Differences in the mean values for individual hormone measurements were assessed by using ANOVA and two-tailed group *t*-test. Statistical significance was considered to be $P < 0.05$.

Results

Hormone measurement in serum

Body mass index (BMI), LH and androgen (testosterone and androstenedione) concentrations were, as expected, significantly raised ($P < 0.05$ and $P < 0.01$) in the PCOS patients compared with those in the controls (Table I). There were no significant differences between groups in age and FSH concentrations. All patients underwent follicular stimulation by FSH and final maturation of the oocytes by HCG for an IVF programme. Despite receiving significantly fewer ampoules of FSH, there were more follicles on the day of oocyte retrieval in the patients with PCOS (Table II). Serum concentrations of oestradiol and progesterone on the day of HCG injection (35 h before follicular aspiration) were significantly higher ($P < 0.01$ and $P < 0.05$) in PCOS than in control patients (Table II).

Granulosa cell cultures

The influence of follicle size on the response of luteinizing granulosa cells to HCG in normal and PCOS ovaries is illustrated in Figures 1 and 2. These data are from experiments that were performed on cells of pooled follicles of similar size of each patient in order to obtain three groups: follicle size ≤10, >10≤15, and ≥16 mm. Progesterone production, as expected, was enhanced in granulosa cells of the control group and concentrations increased with follicle size (Figure 1).

However, the concentration of progesterone of PCOS patients did not increase with follicle size and there was a significant difference between normal and PCOS groups in follicles >10≤15 mm ($P < 0.05$) and ≥16 mm ($P < 0.01$). Oestradiol production was increased in follicles ≥16 mm in both groups, although this did not reach significance (Figure 2).

Discussion

In the present study, we have demonstrated that in-vivo and in-vitro oestradiol and progesterone production of PCOS granulosa cells is abnormal. The data have shown that: (1) serum concentrations of oestradiol and progesterone on the day of HCG injection are significantly higher in PCOS patients; (2) luteinizing granulosa cells of PCOS ovaries do not enhance progesterone production as in normal ovaries after HCG stimulus *in vitro*.

It is already known that PCOS patients have a different response to gonadotrophins compared to normal subjects. Despite receiving significantly less HMG, PCOS patients have significantly higher serum oestradiol concentrations on the day of HCG administration, developed more follicles and produce more oocytes. However, fertilization rate is reduced in PCOS patients (MacDougall *et al.*, 1992, 1993). In agreement with these studies, we found significantly higher serum oestradiol concentrations on the day of HCG administration. Nothing is known about serum progesterone concentrations on the day of HCG administration in PCOS patients. Elevated progesterone during ovulation induction in IVF cycles seems to be a poor prognostic factor for achieving pregnancy. Randall *et al.* (1996) showed that premature luteinization in luteal leuprolide acetate down-regulated patients and progesterone values >0.8 ng/ml are associated with significantly lower pregnancy rates. Furthermore, they found that the oestradiol concentration and the total number of oocytes transferred are higher in patients with high progesterone concentrations on the day of HCG administration, but the pregnancy rate is significantly lower.

On the other hand, Huang *et al.* (1996) demonstrated that serum progesterone concentration >0.31 ng/ml during ovulation induction reflects good follicular recruitment and is

Table I. Clinical and hormonal data from normal and polycystic ovary syndrome (PCOS) patients. Values are means ± SEM

	Age (years)	BMI	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (nmol/l)	Androstenedione (nmol/l)
Normal	31.9 ± 0.2	20.3 ± 0.7	5.9 ± 0.4	5.1 ± 0.4	1.8 ± 0.3	5.8 ± 0.6
PCOS	31.2 ± 0.5	21.1 ± 0.8*	5.8 ± 0.4	7.8 ± 0.8**	4.3 ± 0.6**	12.1 ± 1.5**

BMI = body mass index; FSH = follicle stimulating hormone; LH = luteinizing hormone.
*$P < 0.05$, **$P < 0.01$.

Table II. Total amount of FSH used, serum oestradiol and progesterone concentrations on the day of HCG induction and number of follicles on the day of oocyte recovery

	Total FSH (ampoules)	Oestradiol (nmol/l)	Progesterone (nmol/l)	No. of follicles of specified diameter (mm)		
				≤10	>10≤15	≥16
Normal	33.5 ± 2.1	1.36 ± 0.04	1.12 ± 0.07	2.1 ± 0.2	4.5 ± 1.2	3.4 ± 0.6
PCOS	25.4 ± 1.9*	2.12 ± 0.11**	1.61 ± 0.13*	4.1 ± 0.4**	6.3 ± 0.7**	5.3 ± 0.5**

FSH = follicle stimulating hormone.
*$P < 0.05$, **$P < 0.01$.

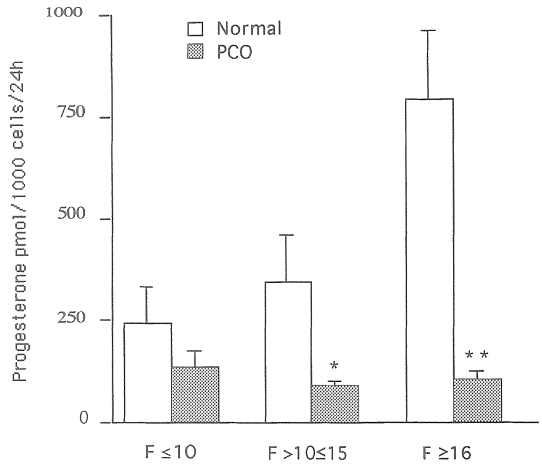

Figure 1. Progesterone response to human chorionic gonadotrophin (HCG) (50 ng/ml) by cultured human luteinizing granulosa cells for 24 h from follicles of increasing size (≤10, >10≤15 and ≥16 mm diameter) of normal (open bars) and polycystic ovaries (shaded bars). The results are mean and SE of triplicate experiments. Cells from polycystic ovaries were significantly less responsive to HCG in follicles >10 and ≤15 mm (*$P < 0.05$) and in follicles ≥16 mm (**$P < 0.01$) than cells from normal ovaries.

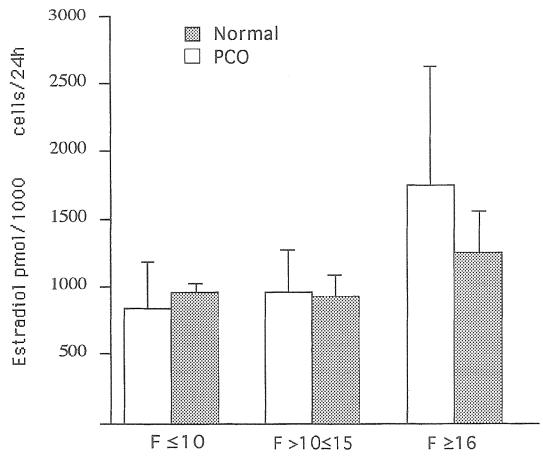

Figure 2. Oestradiol response to human chorionic gonadotrophin (HCG) (50 ng/ml) by cultured human luteinizing granulosa cells for 24 h from follicles of increasing size (≤10, >10≤15 and ≥16 mm diameter) of normal (open bars) and polycystic ovaries (shaded bars). The results are mean and SE of triplicate experiments. There were no significant differences in either size group between follicles from normal and polycystic ovaries.

not a predictor of IVF outcome. In our study the serum progesterone concentrations on the day of HCG injection are significantly higher in PCOS patients than in normal controls.

The most interesting observation of this study is the suppressed capacity of the luteinizing granulosa cells of PCOS ovaries to secrete progesterone after HCG stimulus. Erickson *et al.* (1992) have demonstrated that freshly isolated granulosa cells from untreated PCOS patients have a limited capacity to synthesize progesterone, either spontaneously or in response to FSH stimulation. Furthermore, Andreani *et al.* (1996) found that human granulosa luteal cells from PCOS ovaries, when incubated with follicular fluid from PCOS patients, showed a lower increase of progesterone production with respect to normal ovaries. In our study, we found that the luteinizing granulosa cells from follicles of increasing sizes lose the capacity to synthesize progesterone. Gilling-Smith *et al.* (1994) showed that in PCOS patients, progesterone production is increased in theca cells under LH-stimulated conditions, but the androstenedione to progesterone ratio is significantly higher, suggesting increased conversion of progesterone to androstenedione. The increase in androgens may affect oocyte and embryo quality (Brzynski *et al.*, 1995) and patients with a history of recurrent miscarriage have higher androgen concentrations (Tulppala *et al.*, 1993).

The suppressed capacity of the luteinizing granulosa cells of PCOS ovaries to secrete progesterone after HCG stimulus and the increased androgen production may be possible mechanisms to explain anovulation and miscarriage in PCOS women.

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Received on July 14, 1997; accepted on October 17, 1997