Retrieval, Maturation, and Fertilization of Immature Oocytes Obtained from Unstimulated Patients with Polycystic Ovary Syndrome

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Purpose: Our purpose was to determine whether immature oocytes could be retrieved under local anesthesia, whether these oocytes would mature and fertilize in vitro, and whether adequate endometrium development could be obtained after hormonal supplementation.

Methods: Ovum pick-up was performed under local anesthesia. Immature oocytes were cultured and inseminated. To prepare the endometrium, estradiolvalerate was administered in combination with micronized progesterone.

Results: Immature oocytes were obtained in all cases. Fifty-six percent ($n = 30$) of the oocytes developed into metaphase II (MII) after 48 hr of culture, and another 20% reached the MII stage by 72 hr. Normal fertilization was observed in only 10% of oocytes inseminated. No embryonic development occurred, and therefore embryo transfer was not performed in any of the patients. Endometrial microbiopsy was performed in all subjects and endometrial development was considered sufficient in eight patients.

Conclusions: We collected immature oocytes from patients with polycystic ovary syndrome without general anesthesia. In vitro maturation of these oocytes seemed adequate but fertilization rates were poor. Sufficient endometrial quality was obtained after hormonal substitution.

KEY WORDS: endometrium; immature oocytes; in vitro maturation; oocyte maturation; polycystic ovary syndrome.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a heterogeneous disorder, with different clinical symptoms. Hyperan-

drogenicity, high luteinizing hormone (LH) levels, and polycystic ovaries on ultrasound are often mentioned as important diagnostic parameters. Ovarian abnormalities in patients with PCOS are characterized by follicle maturation arrest at the early antral stage (1). Besides atretic follicles, these ovaries also contain multiple healthy antral follicles (2). In diagnosing PCOS, a large overlap has been demonstrated between endocrine and sonographic criteria (3). Because of this heterogeneity, major variability in therapy outcome has been reported. In induction of ovulation "low-dose step-up" (4) or "step-down" (5) gonadotropin protocols often result in monofollicular development, although in some patients development of multiple preovulatory follicles cannot be avoided. Following hyperstimulation for in vitro fertilization (IVF) a significant increase in the incidence of ovarian hyperstimulation syndrome (OHSS) is observed in patients with PCOS (6,7). Moreover, in IVF, cycle cancellation rates in patients with PCOS are also higher due to imminent OHSS (8).

The ability to perform IVF in unstimulated patients with PCOS could, therefore, exhibit several potential advantages. A method to retrieve immature oocytes from unstimulated patients with PCOS has been developed (9). Human immature oocytes can be matured and fertilized in vitro (9–11). Pregnancies and the birth of healthy children after these procedures have been reported in patients without (12–15) as in patients with PCOS (9,16).

A pilot study was initiated to investigate whether in unstimulated women with PCOS, immature oocytes could be retrieved, matured, and fertilized. Furthermore, hormonal supplementation was given to obtain a sufficient endometrial quality—to enable us to decide whether a "fresh" embryo transfer (ET) could be performed or if an ET after cryopreservation and thawing had to be done.

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MATERIALS AND METHODS

Patients and Study Design

The local ethics review committee approved this study, and informed consent was obtained from all patients. Ten women agreed to take part in the study. Inclusion criteria were PCOS and a medical reason for IVF, age younger than 38 years, absence of any male factor, and no hormonal therapy for the last 2 months prior to the study. Furthermore, easy transvaginal access for ovarian puncture was required. PCOS was defined as oligo- or amenorrhea (intervals between menses >40 days), elevated LH (>8.0 IU/L), and/or elevated serum androgen concentrations and polycystic ovaries at ultrasound. Elevated androgens were defined as a free androgen index (FAI) >5 [= Testosterone (T)/ sex hormone binding globulin (SHBG) $\times$ 100] and/or dehydroepiandrosterone-sulfate (DHEAS) >10 μ Mol/L. Using transvaginal ultrasound, polycystic ovaries were diagnosed if two of the three following criteria were met: volume >10 ml per ovary, markedly increased stroma density, or more than 10 follicles per ovary (17). The patients' characteristics are depicted in Table I.

The day of ovum pick-up (OPU) was randomly chosen, the only criterion being no vaginal blood loss. All egg collections took place in our outpatient clinic. Some patients elected to receive diazepam, 10 mg (orally), 1 hr before OPU. Atropine, 0.5 mg, and fentanyl, 0.1 mg, were administered intramuscularly 15 min before OPU, and a paracervical block was obtained using lidocaine/epinephrine (100 mg/0.1 mg). An

immature oocyte aspiration needle (Cook Australia Ltd) was used as described by Trounson *et al.* (9). Aspiration pressure was reduced from 180 to 90 mm Hg. During OPU the bevel of the needle faced downward (9). If possible, all visible follicles (i.e., follicles > 3 mm) in both ovaries (between 5 and 20 per patient) were punctured.

Patients received the same hormonal substitution regimen as used in our donor oocyte IVF program, the aim being to get the endometrium in the phase as needed for transfer and implantation. From the day of oocyte retrieval estradiolvalerate, 4 mg, was given orally daily. Two days later this dose was reduced to 2 mg daily and combined with micronized progesterone (P), 600 mg daily, administered vaginally. ET was planned 6 days after OPU if the endometrial thickness was greater than 5 mm (i.e., measurement of the double layer using ultrasound) and no vaginal bleeding was present. If no ET was performed, an endometrial microbiopsy was taken on the same day. Dating was performed according to the criteria of Noyes *et al.* (18).

Blood samples were taken on the day of OPU and 6 days later on the day of ET or endometrial microbiopsy for measurements of follicle-stimulating hormone (FSH), estradiol (E_2), and P levels.

Laboratory Procedure

Immature oocytes were cultured in maturation medium for approximately 48 hr. The medium, basically consisting of a mixture of Earle's and Ham's F-10 medium, contained 8.8% pasteurized plasma protein solution (Central Laboratory of the Blood Transfusion Service, Amsterdam) as previously described (19,20) and was the same as used for routine IVF. For our maturation protocol 0.5 IU/ml human chorionic gonadotropin (Pregnyl; NV Organon, Oss, The Netherlands), 0.075 IU/ml human menopausal gonadotropin (Humegon; Organon), and 1 mg/l E_2 (E_2 4389; Sigma Chemical Co., St. Louis, MO) were added (21).

Two days after OPU, oocytes were mechanically denuded of their cumulus-corona-cell complex using a 160-mm glass pipette. Mature oocytes, i.e., oocytes showing the first polar body as a reflection of meiotic maturation (metaphase II [MII]), were transferred to routine medium without the additional hormones and subsequently inseminated. Inspection for fertilization took place the next morning.

In our hospital a maximum of two embryos is transferred on day 3, 4, or 5 after insemination. In this study it was decided to perform ET only on day 4 after insemination (22). Good-quality embryos would

Table I. Patient Characteristics

	Mean $\pm$ SD
Age	31.5 $\pm$ 2.7
BMI ^a	28.6 $\pm$ 6.7
Cycle history	28 days–7 months (20% oligomenorrhea)
LH	8.8 $\pm$ 3.2 IU/L
FAI ^b	6.8 $\pm$ 5.0
T	2.1 $\pm$ 1.2 nMol/L
DHEAS ^c	8.2 $\pm$ 4.4 μ Mol/L
Polycystic ovaries	Right: >10 follicles volume: 14.3 $\pm$ 5.2 stroma: 2.6 $\pm$ 0.5 Left: >10 follicles volume: 12.9 $\pm$ 5.2 stroma: 2.7 $\pm$ 0.5

^a Body mass index (weight/height²).

^b Free androgen index [(T/SHBG) $\times$ 100].

^c Dehydroxyepiandrosterone-sulfate.

Table II. Laboratory Results of Immature Oocyte Retrieval Followed by Maturation and Fertilization In Vitro in 10 PCOS Patients^a

Patient No.	Oocytes retrieved (n)	Findings after 48 hr of incubation				No. oocytes showing 2 pronuclei	Total No. oocytes showing polar body extrusion after 72 hr
		Deg.	GV	MI	MII		
1	7	1	—	2	4	1	6
2	5	1	—	—	4	1	4
3	7	1	1	2	3	1	4
4	1	—	—	—	1	—	1
5	4	—	1	2	1	—	4
6	2	1	—	1	—	—	1
7	6	1	—	—	5	—	5
8	7	4	1	—	2	—	3
9	2	2	—	—	—	—	—
10	13	—	2	1	10	—	13
Total	54	11	5	8	30	3	41

^a Deg., degenerated oocyte; GV, oocyte showing a germinal vesicle; MI, oocyte showing no polar body extrusion; MII, oocyte showing polar body extrusion.

be frozen if more than two embryos would be available. Furthermore cryopreservation would also take place whenever an ET could not be performed due to insufficient endometrial thickness or vaginal bleeding.

RESULTS

Of five patients who had previously undergone OPU, three found the study procedure to be less or similarly painful as routine OPU in a stimulated cycle, whereas two patients found it to be more painful. In general all women recovered quickly and could leave the hospital after 1–2 hr. In one case acute bleeding was observed into the pouch of Douglas at completion of OPU from the right ovary. The procedure was terminated and the patient was given an intravenous infusion. After 5 min the bleeding stopped spontaneously. No hemodynamic disturbances were observed, and the patient was discharged following overnight observation.

In all patients immature oocytes were obtained (median, 5.5; range, 1–13). In 8 of 10 cases, at least one oocyte reached the MII stage and insemination took place. In three patients pronuclei were observed but these embryos degenerated before any cleavage was observed, therefore ET was not performed in any of the patients. Oocyte maturation and fertilization results are shown in Table II. Eleven (20%) oocytes degenerated, whereas 41 (76%) of the oocytes finally reached the MII stage after 72 hr of incubation. Thirty oocytes reached the MII stage after 48 hr of incubation, but only three (10%) of these oocytes were normally fertilized.

Since no ETs were carried out, endometrial microbiopsies were performed in all patients 6 days after OPU. Table III shows data about the endometrium. As P was given for 4 days, the expectation is that the endometrium should correspond with approximately cycle day 17. It seems that using the classical criteria for histologic dating of the endometrium (18), eight patients had an endometrium in phase with the hormonal status.

In all patients the serum FSH level was lower after 6 days of hormonal supplementation, probably due to rising E₂ levels (Figs. 1 and 3). Patient 4 had an LH surge on the day of OPU (Fig. 2), and in this particular woman only one oocyte, which was less immature compared to the other oocytes, was retrieved. The E₂ level varied from 140 to 1337 pMol/L on the day of OPU and from 352 to 3234 pMol/L after 6 days of hormonal suppletion. The P levels varied from 13.7

Table III. Endometrial Parameters 4 Days After Retrieval of Immature Oocytes and Steroid Hormone Substitution in 10 Unstimulated PCOS Patients

Patient No.	Thickness (mm) ^a	Histology ^b corresponding with cycle day
1	12.7	17
2	11.7	16
3	11.0	24
4	5.8	17
5	12.9	17
6	11.0	16
7	7.9	16
8	10.4	16
9	10.0	16
10	14.0	No dating

^a Assessed by transvaginal ultrasound (double layer).

^b Assessed according to criteria of Noyes *et al.* (18).

Fig. 1. Individual values of follicle-stimulating hormone (FSH) in 10 unstimulated patients with PCOS on the day of ovum pick-up (OPU) and 6 days later (OPU + 6).

Fig. 2. Individual values of luteinizing hormone (LH) in 10 unstimulated patients with PCOS on the day of ovum pick-up (OPU) and 6 days later (OPU + 6).

Fig. 3. Individual values of estradiol (E_2) in 10 unstimulated patients with PCOS on the day of ovum pick-up (OPU) and 6 days later (OPU + 6).

to 105.3 nMol/L after hormonal supplementation. Patient 3 had a postovulatory P level on the day of egg collection (Fig. 4). However, still seven immature oocytes were retrieved.

DISCUSSION

Until 1994 research on in vitro maturation and fertilization of human oocytes had two major goals. The first was to obtain a source for donor oocytes from patients undergoing pelvic surgery. The second was to develop a method to use immature oocytes found during routine IVF procedures (11,12). Gómez *et al.* used immature oocytes derived from unstimulated ovarian tissue from patients undergoing pelvic surgery (11). Retrieval of immature oocytes from unstimulated patients with PCOS became possible in 1994 (9). Our results indicate that OPU in unstimulated patients with PCOS can be performed without general anesthesia.

Published reports about maturation results vary. Gómez *et al.* (11) reported that approximately 16% of immature oocytes retrieved from unstimulated patients reached the MII cell stage after 48 hr of culture, whereas Trounson *et al.* (9) reported 60% and Cha *et al.* (12) 56%. Gómez *et al.* (11) reported an increased maturation rate after addition of epidermal growth factor and insulin-like growth factor I to the culture medium. In our laboratory 30 (56%) of 54 of the immature oocytes reached the MII stage within 48 hr. The same culture medium was used as by Trounson *et al.* (9). Although our maturation rate was similar, the fertilization rate was much lower: Trounson *et al.* (9) found normal fertilization in 23% of all inseminated

Fig. 4. Individual values of progesterone (P) in 10 unstimulated patients with PCOS on the day of ovum pick-up (OPU) and 6 days later (OPU + 6).

oocytes (i.e., oocytes showing two pronuclei), and 50% of these embryos developed to the eight-cell stage or further. Although in the present study the two PN-cell stage was found in only 10% of inseminated oocytes, the 95% confidence interval (2–26%) shows that these fertilization rates were in the same range as reported before (9). Low fertilization rates can result from different factors. Oocyte quality and subsequent fertilization rates might be impaired by arrested follicular development and hormonal influences (elevated androgenic environment) in patients with PCOS (15). The effect of T on immature murine oocytes during maturation reduces their ability to mature and undergo normal embryonic development (23). In addition, culture conditions might be suboptimal for complete maturation in vitro. Oocyte immaturity can explain some of the “fertilization failures” after IVF. In these cases sperm penetration is possible but oocytes remain arrested at MII (24). These findings are also observed in intracytoplasmic sperm injection failures (unpublished data) and probably will also be made in in vitro matured oocytes. Impaired fertilization rates might also be the result of a zona hardening process occurring during in vitro maturation (25,26). Improved fertilization rates can be reached by intracytoplasmic sperm injection of the in-vitro matured oocytes (27,28). Intracytoplasmic sperm injection also eliminates any male-factor bias. None of the two-PN oocytes showed any cleavage within an additional 24 hr of culture.

Endometrial quality seemed to be adequate in 8 of 10 patients as assessed by classical morphology. Although the same hormonal supplement dose regimen was given to each patient, large differences were observed in the E₂ and P serum levels. All luteal E₂ and P levels were in the same range as in normal responding IVF patients described by Pellicer (29). The differences in hormonal profiles were concluded to be irrelevant as similar histologic appearances were observed on endometrial biopsy. Furthermore, the majority of patients had an endometrium thickness that would be sufficient to perform ET according to the criteria stated in the study by Trounson *et al.* (9). Therefore, it appears to be possible to perform transfers of fresh embryos, which might give better pregnancy rates as compared to cryopreserved embryos.

In conclusion, the present study shows that it is possible to retrieve immature oocytes from unstimulated women with PCOS without general anesthetics and to mature these oocytes in vitro. However, fertilization rates were poor, and therefore, no ET could be performed. Although this method could be an alternative treatment in a group of patients at high risk for

OHSS, further studies are required to improve fertilization rates and embryonic development. Further studies should also be performed regarding the developmental competence of the in vitro matured oocytes. Because fertilization rates might be partly impaired by zona hardening, the application of intracytoplasmic sperm injection may be considered.

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