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Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome

R.C.Chian[1], W.M.Buckett, T.Tulandi and S.L.Tan

McGill Reproductive Center, Department of Obstetrics and Gynecology, Royal Victoria Hospital, McGill University, Montreal, Quebec, Canada H3A 1A1

[1]To whom correspondence should be addressed

The present study examined whether the rates of oocyte maturation, fertilization and development, as well as pregnancy rate could be improved by human chorionic gonadotrophin (HCG) priming 36 h before immature oocyte retrieval in patients with polycystic ovarian syndrome (PCOS). Immature oocyte retrieval was performed on day 10–14 of the cycles and patients were randomly allocated either to be primed with 10 000 IU of HCG before the retrieval, or not primed. Immature oocytes were cultured for 24–48 h in TC-199 medium with 20% (v/v) inactivated fetal bovine serum (FBS) supplemented with 75 mIU/ml follicle stimulating hormone (FSH) and luteinizing hormone (LH). Intracytoplasmic sperm injection (ICSI) was performed in all mature oocytes and the resulting embryos were transferred on day 2 or 3 after ICSI. A total of 17 patients underwent 24 completed treatment cycles. Thirteen cycles were primed with HCG and 11 other cycles were not primed. The mean number of oocytes retrieved was comparable in the two groups (7.8 ± 3.9 versus 7.4 ± 5.2). The percentage of oocytes achieving maturation at 48 h was significantly higher ($P < 0.05$) in the HCG-primed group (84.3%, 86/102) than in the non-HCG-primed group (69.1%, 56/81). Oocyte maturation was hastened in the HCG-primed group. Following 24 h of culture, 78.2 ± 7.1% of oocytes were matured in the HCG-primed group compared with 4.9 ± 2.5% of oocytes in the non-HCG-primed group ($P < 0.001$). There were no significant differences in the rates of oocyte fertilization and cleavage in these two groups. There were five clinical pregnancies (38.5%) in the HCG-primed group, and three pregnancies (27.3%) in the non-HCG-primed group.

Key words: HCG/human/immature oocyte/in-vitro maturation/polycystic ovarian syndrome

Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common reproductive disorders in women of childbearing age. It has a heterogeneous presentation, which is clinically characterized by anovulation and hyperandrogenism, and on pelvic ultrasound examination shows numerous antral follicles within the ovaries (Adams *et al.*, 1986). Women with PCOS often present with anovulatory infertility, with a significant proportion being resistant to induction of ovulation by clomiphene citrate. Although ovulation can be induced successfully in 75% of non-responders with human menopausal gonadotrophin (HMG), gonadotrophin use requires intensive monitoring and involves a distinct risk of ovarian hyperstimulation syndrome (OHSS) (Healy *et al.*, 1980; Wang and Gemzell, 1980). In addition, many patients ovulate but do not achieve pregnancy even after their anovulation has been corrected. For these women, in-vitro fertilization (IVF) is the standard treatment, but there is a significantly higher risk of OHSS compared with treatment in women with normal ovaries (MacDougall *et al.*, 1993).

Recovery of immature oocytes followed by in-vitro maturation (IVM) of these immature oocytes is a potentially useful treatment for women with PCOS-related infertility since the immature oocytes from these women retain their maturational and developmental competence (Trounson *et al.*, 1994). In comparison with conventional IVF, the major benefits of IVM treatment include avoidance of the risk of OHSS, reduced cost, and less complicated treatment. However, the maturation rate of immature oocytes retrieved from women with PCOS is lower than that of those retrieved from women with normal menstrual cycles (Cha and Chian, 1998). There is a paucity of information about the maturational capacity of immature oocytes derived from women with PCOS.

Several authors have reported pregnancies following the transfer of embryos produced from immature oocytes derived from stimulated ovaries (Veeck *et al.*, 1983; Prins *et al.*, 1987; Nagy *et al.*, 1996; Edirisinghe *et al.*, 1997; Jaroudi *et al.*, 1997; Liu *et al.*, 1997; Tucker *et al.*, 1998). However, the pregnancy rate reported is correspondingly low. It has been demonstrated that morphological and molecular differences exist between immature oocytes retrieved from stimulated and unstimulated ovaries (Chian *et al.*, 1997). Although the time courses of germinal vesicle breakdown (GVBD) and oocyte maturation are different between immature oocytes retrieved from stimulated and unstimulated ovaries, the final rates of oocyte maturation are not different in these two groups (Cha and Chian, 1998). It appears that the oocytes retrieved from small follicles in women undergoing ovarian stimulation respond to human chorionic gonadotrophin (HCG) which promotes the initiation of oocyte maturation. However, it remains unclear whether priming with HCG before immature oocyte retrieval in unstimulated ovaries could improve the rates and time course of oocyte maturation and fertilization, as well as the embryo quality.

The present study was designed to determine whether priming with HCG 36 h before immature oocyte retrieval

alters the rates of oocyte maturation, fertilization and early embryo development in patients with PCOS.

Materials and methods

A total of 17 patients with PCOS underwent 24 completed treatment cycles. All patients were under 41 years of age (mean 35.4 ± 4.9 years) and presented with irregular menstrual cycles, anovulation (determined by ultrasound; see below) and a minimum 2-year history of infertility. All patients had an ultrasound appearance of polycystic ovaries and elevated serum testosterone concentrations. An ultrasound diagnosis of PCOS was made when there were more than 10 small cysts/follicles (2–8 mm diameter) around a dense core of stroma. The serum testosterone concentration was >1.2 nmol/l on day 2 or 3 of the baseline ultrasound scan. Body mass index (BMI) was not calculated for these patients. The serum luteinizing hormone (LH) concentration was >10 IU/ml on day 2 of bleeding for all patients. All patients had failed to conceive after at least six cycles of ovulation induction with clomiphene citrate or gonadotrophins and intrauterine insemination. The study was approved by the Research Ethics Board of the Hospital.

The treatment cycle was initiated by the administration of intravaginal progesterone (Prometrium; Schering, Pointe-Claire, Quebec, Canada) in a dose of 300 mg daily for 10 days. The timing of the start of treatment was random, as all patients had irregular menstrual cycles. Withdrawal bleeding occurred within 3 days after the last dose. On day 2 or 3 following the onset of menstrual bleeding, the patients underwent a baseline ultrasound scan to ensure that no ovarian cysts were present. Transvaginal ultrasound scans were repeated on day 8 to exclude the development of a dominant follicle. The size of all follicles on ultrasound scan had to be <10 mm diameter on day 8 of the cycle.

Oocyte retrieval was performed on day 10 to 14 of the cycle. Using a computerized random table, the patients were assigned either to be primed with 10 000 IU HCG (Profasi; Serono, Oakville, Ontario, Canada) (group A), or not primed (group B). In group A the HCG was given s.c. to patients 36 h before oocyte retrieval. Transvaginal ultrasound-guided oocyte collection was performed using a specially designed 17G single-lumen aspiration needle (K-OPS-1235-Wood, Cook, Australia) with an aspiration pressure of 7.5 kPa. Aspiration of all small follicles was performed under spinal anaesthesia. Oocytes were collected in 10 ml culture tubes (Falcon, Franklin Lakes, NJ, USA) containing 2 ml warm 0.9% saline with 2 IU/ml heparin (Baxter, Toronto, Ontario, Canada).

Following oocyte collection, the oocytes were evaluated for the presence or absence of a germinal vesicle (GV) in the cytoplasm of the oocyte (Figure 1), and the immature oocytes were then transferred into maturation medium for culture. To identify whether GV was present in the oocyte cytoplasm, the special technique of observation called 'sliding' was employed under the stereomicroscope. Briefly, the cumulus–oocyte complex (COC) was allowed to slide slowly down from one side to the other on the bottom of the tissue culture dish (60×15 mm; Falcon), the COC being observed under the microscope. During COC sliding, it was possible to observe clearly whether or not the oocyte cytoplasm contained a GV. If no GV was seen in an immature oocyte, the oocyte was defined as germinal vesicle breakdown (GVBD). The mature oocytes were determined by the presence of a first polar body extrusion. All oocyte handling procedures were conducted on warm stages and plates at 37°C. COC were washed in TC-199 medium (Sigma Chemical Co., St Louis, MO, USA) with 10% inactivated (56°C, 30 min) fetal bovine serum (FBS; Sigma Chemical Co.). The immature oocytes were then incubated in an organ tissue culture dish (60×15 mm; Falcon)

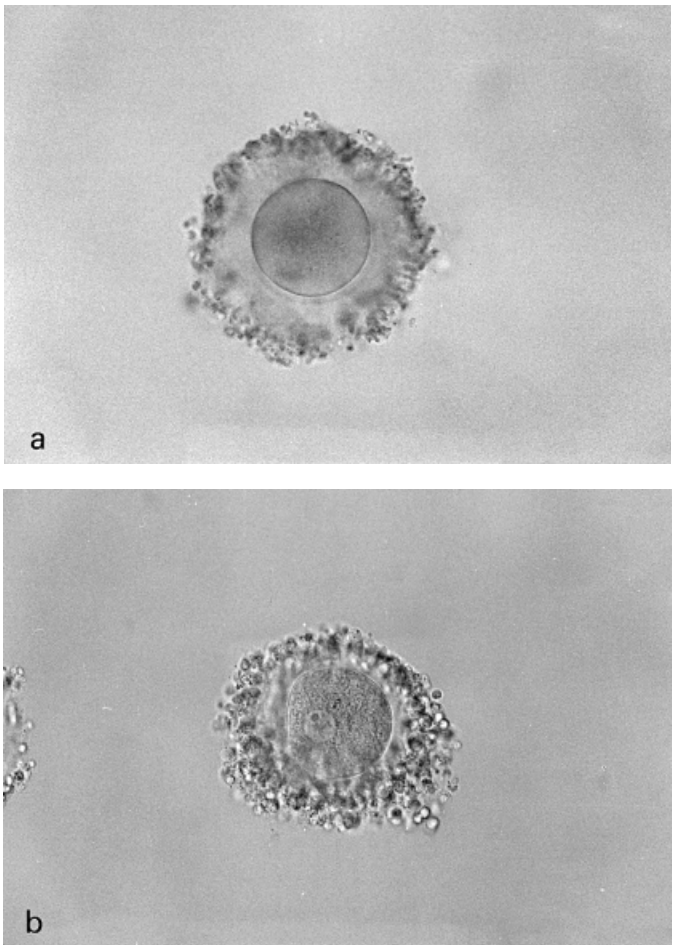

Figure 1. Immature human oocytes retrieved from (**a**) human chorionic gonadotrophin (HCG)-primed and (**b**) non-HCG-primed groups. Note that germinal vesicle breakdown has occurred in (**a**), while in (**b**) the intact germinal vesicle is clearly observed. (Original magnification ×400).

containing 1 ml of maturation medium, TC-199 medium supplemented with 20% FBS, 25 mol/l pyruvic acid (Sigma Chemical Co.), 75 mIU/ml follicle stimulating hormone (FSH) + LH (Humegon; Organon, Scarborough, Ontario, Canada) at 37°C in an atmosphere of 5% CO_2 and 95% air with high humidity. Following culture, the maturity of the oocytes was determined under the microscope at 12 h intervals for up to 48 h.

The oocytes which were mature at the time of checking were denuded of cumulus cells using finely drawn glass pipettes following 1 min exposure to 0.1% hyaluronidase solution (Medi-Cult, Hopkinton, MA, USA) ready for intracytoplasmic sperm injection (ICSI). Spermatozoa for ICSI were prepared by mini-Percoll separation (45% and 90% gradients) at 560 *g* for 20 min. Following Percoll separation, the sperm pellet was washed twice (200 *g*) with 2 ml of Medi-Cult IVF medium. A single spermatozoon was injected into each metaphase II oocyte. Following ICSI, each oocyte was transferred into 20 µl droplet of Medi-Cult IVF medium in a tissue culture dish (35×10 mm; Falcon) under mineral oil. Fertilization was assessed 18 h after ICSI for the appearance of two distinct pronuclei and two polar bodies.

Following fertilization check, oocytes with two pronuclei (maximum five) were transferred into 1.0 ml of Medi-Cult IVF medium in the organ tissue culture dish (60×15 mm; Falcon) for further culture. Embryos were transferred on day 2 or 3 after ICSI. Since the oocytes were not matured and inseminated at the same time following maturation in culture, the developmental stages of embryos

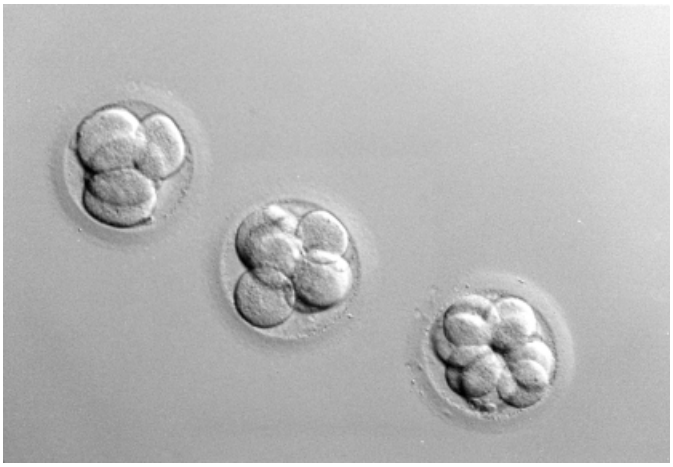

Figure 2. The stages of embryo development cultured for 2 or 3 days following in-vitro maturation and intracytoplasmic sperm injection from a woman with polycystic ovarian syndrome primed by human chorionic gonadotrophin before immature oocyte retrieval. Note that the embryos were at different stages (from 4-cell to 16-cell) of development. Original magnification, ×400.

were variable both within and between patients. Before transfer, all embryos for each patient were pooled and selected for transfer. Therefore, the developmental stages of each embryo transferred may have been different in individual women (Figure 2). However, the range of variation for embryo growth in the two groups of patients should have been the same.

For the preparation of the endometrium, the patients were given oestradiol (Estrace; Roberts Pharmaceutical, Mississauga, Canada) depending on the endometrial thickness on the day of oocyte retrieval in divided doses, starting on the day of oocyte retrieval. If the endometrial thickness on the day of oocyte retrieval was <4 mm, a 10 mg dose was administered; if it was >6 mm, a 6 mg dose was given. More precise measurements of endometrial thickness were not possible with the equipment available at this stage of the treatment. Luteal support was provided by 400 mg intravaginal progesterone (Prometrium) twice daily for 16 days starting from the day of ICSI. On the day of embryo transfer, the endometrial thickness was measured again by transvaginal ultrasound scan. If the endometrial thickness was <7 mm, the couples were offered embryo cryopreservation and transfer in a subsequent cycle. Clinical pregnancy was defined as an intrauterine gestation with a fetal heartbeat seen by transvaginal ultrasound scan.

The statistical significance of the differences between the means and percentages of the groups was determined and compared by Student–Newman–Keuls' test (Steel and Torrie, 1980). A P-value < 0.05 was considered statistically significant.

Results

As shown in Table I, there was no difference in the number of immature oocytes retrieved between the HCG-primed (13 cycles) and the non-primed groups (11 cycles) (7.8 ± 3.9 versus 7.4 ± 5.2). However, the maturation rate of oocytes at 48 h of culture was significantly different following culture, with HCG priming increasing the maturation rate of immature oocytes (84.3% versus 69.1%; $P < 0.05$). The fertilization and cleavage rates and the embryo quality were comparable between the HCG-primed group and the non-primed group. There were no cycles cancelled because of thin endometrium, and all patients had an endometrial thickness of 8 to 13 mm. Following embryo transfer, there were five clinical pregnancies (38.5%) among 13 treatment cycles in the HCG-primed group, and three clinical pregnancies (27.3%) among 11 treatment cycles in the non-HCG-primed group. The details of the pregnancies are shown in Table II.

The time course of GVBD is shown in Figure 3. At the time of oocyte retrieval in the HCG-primed group there were 46.2 ± 5.2% GVBD oocytes; in contrast, in the non-HCG-primed group, no GVBD had occurred at the time of oocyte collection. After 48 h of culture, there was no significant difference in GVBD rates between the oocytes retrieved from the HCG-primed and non-primed groups (93.8 ± 6.8% and 87.5 ± 4.5% respectively). The time course of oocyte maturation was hastened in the HCG-primed group (Figure 4), with 8.8 ± 3.6% of oocytes being matured to metaphase II at 12 h after oocyte retrieval. At 24 h following IVM, there were 78.2 ± 7.1% of mature oocytes in the HCG-primed group compared with 4.9 ± 2.5% in the non-HCG-primed group ($P < 0.001$). At 48 h of culture, there were 85.2 ± 4.1% of mature oocytes in the HCG-primed group compared with 68.0 ± 2.7% of mature oocytes in the non-HCG-primed group ($P < 0.05$).

Discussion

The results of the present study indicate that the maturation rate of immature oocytes retrieved from women with PCOS was improved by HCG priming. The first report of pregnancy in a woman with anovulatory infertility following IVM of immature oocytes and IVF was made in 1994 (Trounson *et al.*, 1994), while the following year, a pregnancy was reported in a group of patients with PCOS treated with IVM, combined with ICSI and assisted hatching (Barnes *et al.*, 1995). Unfortunately, only about 60% of immature oocytes retrieved from women with PCOS mature *in vitro*, and the pregnancy rates reported have been correspondingly low, at approximately 22% (Cha and Chian, 1998). It has been reported that although immature oocytes recovered from untreated ovaries can be matured, fertilized and developed *in vitro*, the implantation rate of these cleaved embryos is disappointingly low, indicating that the oocyte culture system may have specific requirements for successful human oocyte maturation (Barnes *et al.*, 1996; Trounson *et al.*, 1998). The results of the current study demonstrate that the rate of oocyte maturation is significantly higher with HCG priming before immature oocyte retrieval, which may potentially increase the pregnancy rate, although this study was too small to detect any possible significant difference.

The cyclic pattern of FSH and LH secretion in women with PCOS is typically absent, and there is often a disproportionately high secretion of LH with a relatively constant low rate of FSH secretion (Yen, 1980). The exact mechanism by which anovulation occurs in these women is unknown. There is a consensus that the common feature in women with PCOS is arrested follicular development at the stage when selection of the dominant follicle should normally occur (Erickson and Yen, 1984). The initial steps of folliculogenesis, follicle recruit-

Table I. Effects of human chorionic gonadotrophin (HCG) priming before immature oocyte retrieval on rates of oocyte retrieval, maturation, fertilization, cleavage and pregnancy in women with polycystic ovarian syndrome (PCOS)[a]

With (+) or without (–) HCG priming	No. of cycles	No. of oocytes retrieved (Mean ± SE)	No. of oocytes matured at 48 h (%)	No. of oocytes fertilized (%)	No. of oocytes cleaved (%)	No. of embryos transferred (Mean ± SE)	No. of clinical pregnancies (%)
+	13	102 (7.8 ± 3.9)	86 (84.3)[b]	78 (90.7)	74 (94.9)	36 (2.8 ± 0.9)	5 (38.5)
–	11	81 (7.4 ± 5.2)	56 (69.1)[c]	47 (83.9)	45 (95.7)	27 (2.5 ± 1.1)	3 (27.3)

[a]Mean age of women in HCG-primed group 35.3 ± 4.8 years; mean age in non-HCG-primed group 34.5 ± 5.5 years.
[b,c]$P < 0.05$.

Table II. Details of eight pregnancies from immature oocytes obtained in women with polycystic ovarian syndrome (PCOS)

With (+) or without (–) HCG priming	Age (years)	No. of oocytes retrieved	No. of embryos transferred	Results
+	38	4	3	Twins
+	32	10	3	Singleton
+	41	2	1	Singleton
+	39	10	4	Miscarriage[a]
+	41	4	3	Miscarriage[b]
–	35	7	4	Singleton
–	31	1	1	Singleton
–	35	14	3	Twins

[a]Miscarried at 6 weeks.
[b]Miscarried at 7 weeks.

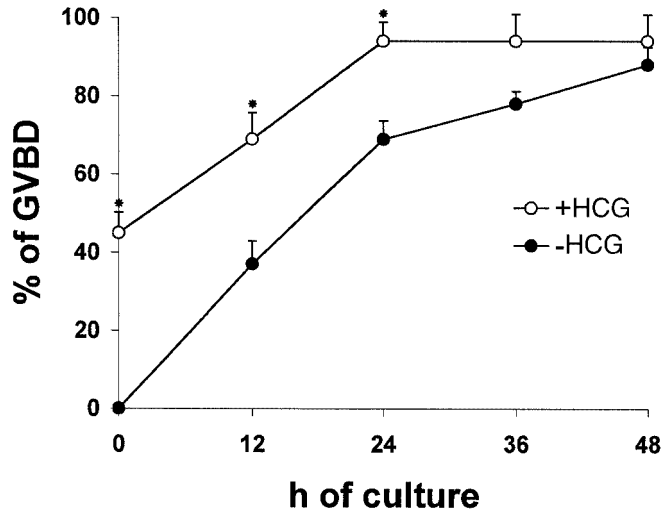

Figure 3. The time course of human oocyte germinal vesicle breakdown (GVBD) during culture *in vitro.* At the time of retrieval in the human chorionic gonadotrophin (HCG)-primed group, 46.2 ± 5.2% of oocytes showed GVBD. At 48 h culture, the percentage of GVBD was similar in oocytes retrieved from the HCG-primed (○) and non-HCG-primed (●) groups (total 183 oocytes). *Significant difference between two groups at the time point ($P \leq 0.05$).

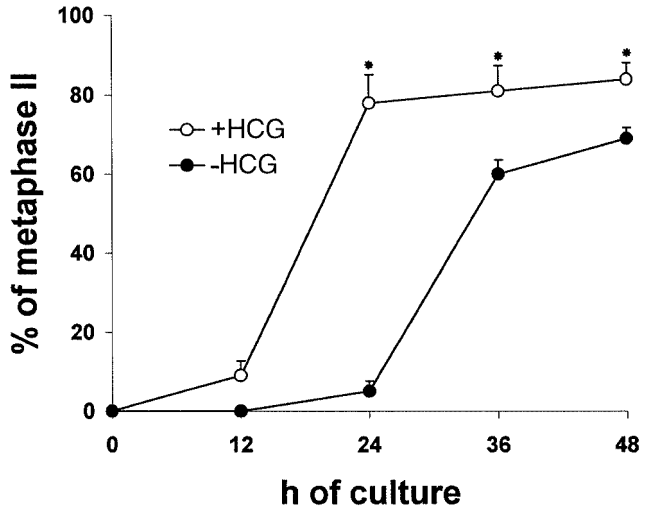

Figure 4. The time course of human oocyte maturation during culture *in vitro.* At 24 h after culture in the human chorionic gonadotrophin (HCG)-primed group, 78.2 ± 7.1% of oocytes were matured. Final percentages of oocyte maturation were significantly different between oocytes retrieved from the HCG-primed (○) and non-HCG-primed (●) groups (total 183 oocytes). *Significant difference between two groups at the time point ($P \leq 0.05$).

ment, and follicle growth to the small antral stage, are functional in women with PCOS, but the terminal step—the selection of a dominant follicle that is capable of ovulation—does not occur regularly (Jakimiuk *et al.*, 1997). Normally, the mechanism of LH action on the ovary starts with the binding to specific LH receptors on the theca and granulosa cells, followed by a rapid stimulation of adenylate cyclase activity. Stimulation of adenylate cyclase results in elevated concentrations of cyclic adenosine monophosphate (cAMP) which then interacts with the regulatory subunits of protein kinase, resulting in production or activation of the appropriate enzymes (Marsh, 1976). The action of oocyte maturation inhibitor (OMI) is itself inhibited by increased cAMP concentrations associated with the mid-cycle LH peak (Tsafriri and Pomerantz, 1986). Excessive LH secretion is frequently encountered in women with PCOS (Waldstreicher *et al.*, 1988), and it has been shown that excessive LH secretion is associated with anovulation, infertility and miscarriage (Regan *et al.*, 1990; Balen *et al.*, 1993; Shoham *et al.*, 1993).

It is postulated that acquisition of optimal competence for maturation of immature oocytes in women with PCOS may require the presence of a mid-cycle LH surge for priming. It is known that GVBD is initiated by the pre-ovulatory surge of LH, suggesting that LH induces the loss of communication between the oocyte–cumulus cell complex, thus terminating the flow of OMI (Fagbohun and Downs, 1991). The results of this study show that there were 46.2 ± 5.2% of GVBD oocytes at the time of oocyte retrieval 36 h after HCG administration, which may suggest that the small follicles in polycystic ovaries possess some LH receptors, even though there is no direct

evidence that LH receptors are present in the granulosa cells of these small follicles. This is an area that clearly requires further study.

In women with normal ovaries, the time courses of GVBD and oocyte maturation are different between immature oocytes retrieved from stimulated and unstimulated ovaries; however, the final rates of oocyte maturation are similar (Cha and Chian, 1998). The results of the present study indicate that the rate of oocyte maturation during the first 48 h of culture is increased in women with PCOS following HCG priming before immature oocyte retrieval. Three of the six women who achieved pregnancy and did not miscarry had failed to conceive following three cycles of conventional IVF treatment during the preceding 2 years. These results suggest that immature oocyte retrieval followed by IVM might be useful in 20% (Franks, 1989) to 37% (Buckett *et al*., 1999) of women undergoing IVF who have polycystic ovaries seen on ultrasound scan.

Extremely low fertilization rates are usually obtained after standard insemination of in-vitro-matured oocytes, suggesting that ICSI is the best option, even when the sperm parameters are not impaired (Nagy *et al.*, 1996). Qualitative changes, including zona hardening, occur in the zona pellucida during oocyte maturation *in vitro* that may reduce the fertilization rates using conventional IVF (DeFelici and Siracusa, 1982; Downs *et al.*, 1986; Choi *et al.*, 1987). There were no differences in the rates of fertilization and cleavage between the HCG-primed and non-HCG-primed groups. This suggests that although the time course and rate of oocyte maturation are hastened by HCG priming before immature oocyte retrieval, the rates of oocyte fertilization and development are not different in the two groups.

It has been suggested that the endometrial priming commencing at the mid-follicular phase may be optimal when embryos resulting from IVM are transferred in the same cycle in which the immature oocytes are retrieved (Russell *et al.*, 1997). However, the results of this study indicate that endometrial priming can be started from the late follicular phase. In this regard, endometrial priming from the late follicular phase seems effective for embryo transfer in the same cycle of immature oocyte retrieval.

In conclusion, the time course of oocyte maturation *in vitro*, i.e. the maturation rate, has been shown to be hastened by priming with HCG before retrieval of immature oocytes. It is possible that pregnancy rate may potentially be improved by this means in women with PCOS. However, the numbers of patients in this study were too small to detect any such difference.

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