Treatment Variables in Relation to Oocyte Maturation: Lessons from a Clinical Micromanipulation-Assisted In Vitro Fertilization Program

ORI M. AVRECH,¹ GIL A. GOLDMAN,¹ ONIT RUFAS,¹ ANAT STEIN,¹ SHOSHANA AMIT,¹ ISRAEL YOLES,¹ HAIM PINKAS,¹ and BENJAMIN FISCH^{1,2}

Submitted: February 6, 1997

Accepted: March 17, 1997

Objective: In an effort to understand the mechanism underlying the improved pregnancy rate observed in IVF cycles when gonadotropin-releasing hormone analogues (GnRH-a) are applied, we investigated a possible relationship between treatment variables and oocyte-nuclear maturity.

Design: Nuclear maturity was retrospectively assessed in cumulus-free, denuded oocytes, obtained from women undergoing micromanipulation-assisted IVF treatment following controlled ovarian hyperstimulation with GnRH-a and menotropins.

Setting: The setting was the infertility and IVF unit of a tertiary academic medical center.

Participants: Two hundred twenty-one patients underwent 435 treatment cycles.

Main Outcome Measure: This was the proportion of germinal vesicle-intact immature (GVII) oocytes.

Results: One hundred fifty-four of the 3520 oocytes studied (4.4%) were in the GVII stage. These oocytes were found in 66 of the treatment cycles (15.2%) and in 54 of the patients (24.4%). Cycles in which GVII oocytes were detected did not differ from those in which all the aspirated oocytes were mature in the following respects: patient age, type and duration of infertility, controlled ovarian hyperstimulation protocol and time of ovum pickup. However, the GVII group was characterized by a significantly higher peak estradiol level, as well as a higher number of mature follicles visualized sonographically (diameter, >14 mm) and oocytes retrieved.

Conclusions: Comparing the present findings with previously published data, it appears that the inclusion of

GnRH-a in the stimulation regimen is associated with a lower proportion of immature oocytes. A higher occurrence of oocyte-nuclear immaturity is apparently associated with a significantly better ovarian response to stimulation. The high incidence of immature oocytes observed in patients with normospermic partners and low fertilization rates in previous cycles may suggest that the fertilization failure in some of these cases is due to oocyte, rather than sperm, dysfunction.

KEY WORDS: in vitro fertilization; gonadotropin releasing hormone analogues; oocyte maturation.

INTRODUCTION

Preovulatory mammalian oocytes are maintained in meiotic arrest by an inhibitory follicular environment (1). The luteinizing hormone (LH) surge overcomes this inhibition and stimulates oocyte maturation to the first polar body (PB) stage, in which the oocyte becomes capable of fertilization. Oocyte maturation may be induced by functional uncoupling of the oocyte-corona-cumulus complex (OCCC) while removing the oocyte from the follicular environment (2). Alternatively, this process may be enhanced by agents that elevate cAMP levels in the cumulus cells, inhibit protein kinase-A activity, or activate maturation-promoting factors. Despite the employment of sophisticated superovulation protocols, complete maturation synchrony of oocytes aspirated for in vitro fertilization (IVF) is rarely achieved. Even if they undergo nuclear maturation in vitro, the immature oocytes that still possess an intact germinal vesicle (GV) are unlikely to yield a normal pregnancy (3,4). Concomitantly with the resumption of oocyte maturation, the OCCC undergoes a morphological transformation from a compact cell mass into an expanded, mucified structure (5,6). Although the indirect method

¹ Infertility and In Vitro Fertilization Unit, Department of Obstetrics and Gynecology; Beilinson Medical Center, Petah Tiqva, Sackler Faculty of Medicine, Tel Aviv University, Israel.

² To whom correspondence should be addressed at Infertility and In Vitro Fertilization Unit, Department of Obstetrics and Gynecology, Beilinson Medical Center, Petah-Tiqva 49100, Israel.

of assessing oocyte maturity according to OCCC appearance is widely accepted (1), it has been reported to be in poor correlation with nuclear maturity (7).

Early studies on IVF cycles, performed before the era of gonadotropin-releasing hormone analogues (GnRH-a), revealed that only 39 to 55% of the aspirated oocytes achieved a PB, and up to 50% still possessed an intact GV at the time of retrieval (3,8). Lower cancellation rates and greater yields of oocytes are two of the important causes for the higher pregnancy rate which has recently been reported in IVF cycles combining GnRH-a in the stimulation protocol (9,10).

In the present study we aimed at assessing oocyte nuclear maturation by examining the nuclear maturity of oocytes retrieved from women treated with a GnRH-a as part of their controlled ovarian hyperstimulation (COH) regimen. Since it is possible directly to visualize the ooplasm and perivitelline space after oocyte aspiration for IVF only when the oocytes are cumulus-free, we used denuded oocytes designated for micromanipulation-assisted IVF to investigate a possible relationship between treatment variables and a high incidence of immature oocytes. Maturity was classified on the basis of nuclear status: presence of the first PB or possession of an intact GV.

MATERIALS AND METHODS

Data on 221 normoovulatory patients who underwent 435 consecutive micromanipulation-assisted IVF treatment cycles which reached the stage of oocyte retrieval were retrospectively analyzed. They were all treated at the Infertility and In Vitro Fertilization Unit, Department of Obstetrics and Gynecology, Beilinson Medical Center, during the years 1990–1994. Micromanipulation technology was applied to assist fertilization when semen parameters were deemed unsuitable for standard IVF. The average age of the patients was 33.1 ± 4.5 years (range, 21–42 years); the mean duration of infertility was 7.7 ± 4.2 years (range, 1–22 years). Primary infertility was diagnosed in 70.6% (307) of the cycles, whereas secondary infertility was the cause of treatment in the remaining 128 cycles (29.4%).

The COH protocol used was a combination of GnRH-a (short protocol) and menotropins. Buserelin acetate (Suprefact; Hoechst A. G., Frankfurt am Main, Germany), 300 µg t.i.d., was administered transnasally from cycle day 2 until the injection of human chorionic gonadotropin (hCG) (Chorigon; Teva Pharmaceutical Industries, Petah Tiqva, Israel). A fixed dose of human

menopausal gonadotropin (hMG; Pergonal; Teva Pharmaceutical Industries) was given on cycle days 4 to 7 and adjusted according to the individual response from day 8 onward. Human chorionic gonadotropin (10,000 IU) was injected 16–40 hr after sonographic scanning showed two or more leading follicles with a minimal diameter of 16 mm. Transvaginal sonographically guided follicular aspiration was performed 33–34 hr later. Cycle monitoring, oocyte retrieval, sperm preparation, and insemination were performed as described previously (11). Before commencing micromanipulation procedures, all the aspirated oocytes were denuded of their cumulus cells by incubation (37°C, 15 min) in sperm preparation medium containing 80 IU/ml hyaluronidase and 5 mg/ml HSA (Medi-Cult, Copenhagen, Denmark), followed by mechanical dispersal using a glass pipette. The morphology of the cumulus-free oocytes was evaluated using an inverted microscope (Model IMT-2, Olympus Company, New Hyde Park, NY). Metaphase II oocytes were used for micromanipulation.

Statistical Analysis

Data analysis was performed with the assistance of the Department of Statistics of Tel Aviv University, applying BMDP software (1992). Descriptive statistics (mean $\pm$ SE) were performed on all parameters. Paired Student's *t* test, chi-square analysis, and multiple-parameter stepwise regression analysis were used to assess differences in ovarian response. Statistical significance was defined as $P < 0.05$.

RESULTS

Treatment variables concerning the 435 cycles studied are presented in Table I. In all, 3520 oocytes were

Table I. Average Values and Range of Follicular-Phase Variables

Variable	Value ^a	Range
Total FSH dose (IU) ^b	1387 ± 75	375–6000
COH duration (days)	12.7 ± 0.1	10–19
E ₂ on hCG day (pmol/L)	5979 ± 141	1554–15,170
Progesterone on hCG day (nM)	2.6 ± 0.1	0.3–12.2
Diameter of leading follicle (mm)	17.5 ± 0.1	15–24
Number of leading follicles (>14 mm)	5.4 ± 0.2	1–22
Oocytes retrieved	8.1 ± 0.2	1–20

^a Means $\pm$ SE. Conversion factors to SI units: E₂, 3.671; P, 3.180.

^b Calculated from the total number of hMG ampoules used per patient (75 IU/ampoule).

Table II. Treatment Variables by Oocyte Maturity^a

Parameter	Cycles with		P value ^b
	GVII oocyte	Mature oocytes	
Cycles	66(15.2%)	369 (84.8%)	
Age (years)	32.3 ± 0.5	33.3 ± 0.2	NS
Infertility duration (years)	7.5 ± 0.5	7.7 ± 0.2	NS
COH duration (days)	12.7 ± 0.3	12.7 ± 0.1	NS
Total FSH dose (IU) ^c	1372.5 ± 70.0	1387.5 ± 77.5	NS
E ₂ on hCG day (pM)	6449.0 ± 327	5894 ± 155	0.03
Progesterone on hCG day (nM)	2.7 ± 0.2	2.5 ± 0.1	NS
Leading follicle diameter (mm)	17.8 ± 0.3	17.5 ± 0.1	NS
No. of leading follicles (>14 mm)	6.3 ± 0.5	5.2 ± 0.2	0.02
No. of oocytes	9.2 ± 0.6	7.9 ± 0.2	0.02
E ₂ /progesterone	3285 ± 310	3290 ± 179	NS
E ₂ per oocyte	848 ± 62	888 ± 26	NS

^a Values are means ± SE.^b $P < 0.05$ was considered to be significantly different.^c Calculated from the total number of hMG ampoules used per patient (75 IU/ampoule).

retrieved and assessed for nuclear maturity as described. In 369 cycles (84.8%) all the aspirated oocytes were classified as mature, whereas in 66 cycles (15.2%) at least one oocyte was found to be in the germinal vesicle intact immature (GVII) status (Table II). GVII oocytes constituted 4.4% of all the oocytes retrieved. However, in those cycles where immature oocytes were detected, their average number was 2.3 ± 0.34 per patient ($25.6 \pm 2.2\%$ of the oocytes in these cycles). Of these 66 cycles, in 32 (48.5%) a single immature oocyte was noted. The cycles with immature oocytes did not differ significantly from the rest of the treatment cycles in the following parameters: patient age, duration of infertility, duration of COH treatment, hMG dose needed for stimulation, progesterone level at the time of hCG administration, and mean diameter of the leading follicle (Table II). However, these cycles were characterized by a significantly higher peak serum estradiol (E₂) level ($P < 0.03$), as well as a higher number of mature follicles visualized (diameter, >15 mm; $P < 0.02$) and oocytes aspirated ($P < 0.002$), than in cycles in which all the retrieved oocytes were mature.

The calculated ratio of serum E₂/progesterone level on the day of hCG administration or the calculated ratio of the serum E₂ level on that day to the number of retrieved oocytes yielded no difference between the two groups of cycles.

Using stepwise logistic regression analysis, we found that the determinant most strongly associated with the occurrence of GVII oocytes was the total number of oocytes retrieved per treatment cycle ($P < 0.023$). Other factors, in descending order of significance, were serum E₂ level on the day of hCG adminis-

tration ($P < 0.04$) and its proportion to the number of retrieved oocytes ($P < 0.035$). When the number of oocytes is not taken into consideration, the type of infertility has the greatest influence ($P < 0.04$) on the occurrence of GVII oocytes; The highest incidence of GVII oocytes was noted in cases of previously failed fertilization after insemination with normal sperm.

The factors that were found to be most strongly associated with the occurrence of more than one GVII oocyte per cycle were (in descending order of statistical significance) the total number of oocytes retrieved ($P < 0.0005$) and the calculated ratio of E₂ on the day of hCG administration to the number of oocytes ($P < 0.001$). When the number of oocytes was not taken into consideration, the occurrence of multiple GVII oocytes was found to correlate with unexplained infertility and with the serum E₂ level ($P < 0.06$).

DISCUSSION

In the mammalian oocyte, meiosis is initiated during fetal life but oocyte maturation is arrested at the diplotene stage of the first prophase. At birth, the oocyte presents a nuclear structure known as the GV (1,12). Meiotic arrest persists until sexual maturity, when one or more oocytes (the number depends on the species) reinitiate their reductive division at each cycle. The preovulatory mammalian oocytes are maintained in meiotic arrest by an inhibitory follicular environment. The series of events initiated by the breakdown of the GV and completed with the formation of the first PB leads to the production of a mature, fertilizable oocyte (13). Thus, maturation of the oocyte is an essential

prelude to fertilization. Normally, meiosis is reinitiated by the preovulatory LH surge, but when meiotically arrested oocytes are removed from the antral follicles, they resume meiosis spontaneously in vitro. Removal of the oocyte from the follicular environment results in a functional uncoupling of the OCCC, a fall in the oocyte concentration of cAMP, inhibition of protein kinase-A activity, and activation of the maturation-promoting factor, all of which lead to the onset of meiosis and oocyte maturation (14). It is possible that epidermal growth factor (EGF) produced by the cumulus cells has a direct, positive effect (through EGF receptors which are probably primed by gonadotropins) on the final nuclear and cytoplasmic oocyte maturation in vivo (15). Investigations of the intrafollicular hormonal milieu (16) have not demonstrated any differences in follicular fluid estrone or E_2 levels between follicles containing mature and those containing immature oocytes, whereas higher follicular fluid progesterone levels and progesterone/ E_2 and androstenedione/ E_2 ratios were found in follicles from which mature oocytes were obtained. Itskovitz et al. (17) demonstrated that progesterone, E_2 and prorenin levels were lower in follicular fluid surrounding immature oocytes associated with a compact or expanded cumulus than in those containing mature oocytes. Interestingly, oocytes retrieved from binovular follicles (18), though exposed to the same intrafollicular milieu, may show a difference in nucleus maturity and cytoplasm characteristics in both oocytes.

Previous studies have demonstrated that immature oocytes exhibit a reduced fertilizing capacity. In rhesus monkeys, a positive correlation exists between the degree of nuclear maturation of oocytes at collection and their potential for fertilization in vitro (19). Assessment of the degree and normality of the nuclear maturation with the fluorochrome Hoechst 33342 demonstrated that oocyte nuclear immaturity was the major cause of fertilization failure, while other human oocytes from the same retrieved cohort were successfully fertilized (4). It appears that, apart from nuclear immaturity, there may be other features of the immature oocyte that reduce its fertilizing capacity, such as the characteristics of the zona pellucida (ZP). The ZP of mature eggs differed in its sensitivity to proteolysis and sperm binding from the ZP of GVII oocytes (20). It has been shown that the chromatin of the spermatozoa does not undergo decondensation within GVII oocytes (21) and that oocytes penetrated by uncondensated sperm retain their cortical granules, whereas these granules are absent in fertilized, mature oocytes (21). Flood et al. (22) suggested that cytoplasmic factors

essential for continued developmental competence are lacking in oocytes containing a GV at harvest. Thus, even when these oocytes undergo nuclear maturation, fertilization, and cleavage in vitro, transfer of such embryos rarely results in pregnancy reaching delivery. It is possible that even if GVII oocytes undergo nuclear morphological alterations that are accepted as evidence of oocyte maturation, these changes may not necessarily indicate normal fertilization capability in these oocytes.

When the resumption of oocyte maturation occurs, the OCCC synchronously undergoes a morphological transformation from a compact cell mass into an expanded and mucified structure, which was thought to be well correlated with the stage of meiotic maturation (1). Cumulus maturity per se has not been shown to bear any importance in the fertilization process. However, because it is often impossible to visualize directly the nucleus, ooplasm, and perivitelline space of retrieved oocytes, the degree of OCCC dispersal has been extensively used for indirect assessment of oocyte maturity (5,6,13). Since the introduction of IVF for clinical purposes, a considerable body of evidence has accumulated to suggest that in stimulated cycles the type of COH protocol used may affect the rate and synchrony of the maturation of oocytes aspirated. In menotropin-only stimulated IVF cycles, resumption of meiosis and expansion of the OCCC have been noted to be asynchronous (1,3,13,23,24). As noted, (1,3,8), only 39 to 55% of oocytes had a PB at the time of retrieval and sometimes even half still possessed a GV at retrieval. Laufer et al. (3) observed a high fertilization rate of denuded oocytes which had a PB at the time of insemination, whereas oocytes remaining at the GV or germinal vesicle breakdown (GVBD) stage failed to fertilize. The difference in oocyte maturity could be a result of retrieval prior to adequate exposure to gonadotropins or a different OCCC ability to respond to gonadotropin stimulation. Flood et al. (22) reported that most of the oocytes collected undergo GVBD with extrusion of the first PB during culture before insemination, though 3.75% of them remain GVII.

Employment of more sophisticated superovulation protocols, which included GnRH-a, has yielded a higher maturation synchrony of oocytes aspirated for IVF (9). Combined GnRH-a/hMG stimulation protocols are associated with lower cycle cancellation rates and a higher yield of mature oocytes, probably through pituitary suppression of endogenous LH release (10). This may explain the observation that an improved pregnancy rate has been reported in IVF cycles when

GnRH-a was used in the stimulation protocol (9,10,25). It is important to assess carefully the nature of GnRH-a action on the oocyte. Although it is still arguable whether receptors for GnRH are present in the human ovary (26,27), the fact that the time course of GnRH-a-induced oocyte maturation parallels that of meiotic maturation suggests that GnRH-a may act directly on the ovary to trigger the meiotic maturation of oocytes within the follicles (28,29). GnRH-a-induced oocyte maturation is probably a protein kinase C-dependent reaction (28,30). Conversely, a deleterious effect of GnRH-a, an increased rate of oocyte degeneration, has been demonstrated in large follicles in monkeys (31).

Despite the widespread use of GnRH-a in COH protocols, there have been only a few reports on the effect of these compounds on follicular fluid composition (32) and oocyte maturation (25,32). Hammitt *et al.* (7) assessed oocyte meiotic maturity in 4961 oocytes aspirated following COH with GnRH-a. In comparison with rates previously reported for menotropin-only stimulation protocols, a higher percentage (67%) of oocytes possessing a PB at retrieval was found. A discrepancy in oocyte maturation estimation using OCCC morphology and direct nuclear maturity assessment was noted in 28% of the 2336 oocytes evaluated.

To understand better the causes for improved fertilization and pregnancy rates seen when GnRH-a is used for COH, we investigated a possible relationship between treatment variables and a high incidence of immature oocytes which still possess an intact GV. A total of 3520 oocytes was retrieved in 435 micromanipulation-assisted IVF treatment cycles from 221 women using a GnRH-a stimulation regimen. Nuclear maturity was directly assessed in cumulus-free oocytes which were denuded of their corona-cumulus complexes. The results indicated that the most effective determinant associated with the occurrence of GVII oocytes was the total number of oocytes retrieved per patient and the response of the granulosa cells to stimulation (E_2 serum levels on the day of hCG administration). The same tendency was demonstrated in cycles in which more than one GVII oocyte was aspirated. Comparing the present findings with previously published data, it appears that the inclusion of GnRH-a in the stimulation regimen is associated with a lower proportion of immature oocytes. A higher occurrence of oocyte-nuclear immaturity is apparently associated with a significantly higher ovarian response to stimulation. The high incidence of immature oocytes observed in patients with normospermic partners and low fertilization rates in previous cycles may suggest that the

fertilization failure in some of these cases is due to oocyte, rather than sperm, dysfunction.

It is tempting to speculate that the relatively high incidence of oocytes with a PB at retrieval noted in our study is associated with the use of a GnRH-a stimulation regimen. This protocol permits postponement of hCG administration without the risk of a premature LH surge, thus allowing the oocyte to achieve full maturity before aspiration. Further study is warranted in order to evaluate the net effect of GnRH-a on the occurrence of GVII oocytes in IVF. Such a study should compare the incidence of GVII oocytes in two groups of patients randomly allocated to COH with or without a GnRH-a.

REFERENCES

1. Veeck LL: Extracorporeal maturation: Norfolk, 1985. *Ann NY Acad Sci* 1985;42:357-367
2. Dekel N, Sherizly I, Phillips DM, Nimrod A, Zilberstein M, Naor Z: Characterization of maturational changes induced by a GnRH analogue in the rat ovarian follicle. *J Reprod Fertil* 1985;75:461-466
3. Laufer N, Tarlatzis BC, DeCherney AH, Masters JT, Haseltine FP, MacLusky N, Naftolin F: Asynchrony between human cumulus-corona cell complex and oocyte maturation after human menopausal gonadotropin treatment for in vitro fertilization. *Fertil Steril* 1984;42:366-372
4. Gwatkin RB, Conover JC, Collins RL, Quigley MM: Failed fertilization in human in vitro fertilization analyzed with the deoxyribonucleic acid-specific fluorochrome hoechst 33342. *Am J Obstet Gynecol.* 1989;160:31-35
5. Veeck LL: Morphological estimation of mature oocytes and their preparation for insemination. In *In Vitro Fertilization*, Norfolk. HW Jones Jr, GS Jones, GD Hodgen, Z Rosenwaks (eds). Baltimore: Williams and Wilkins, 1986; pp 81-93
6. Veeck LL: Human oocytes at the time of follicular harvest. In *Atlas of the Human Oocytes and Early Conceptus*, HW Jones Jr, GS Jones, GD Hodgen, Z Rosenwaks (eds). Baltimore, Waverly Press, 1986, p 5
7. Hammitt DG, Syrop CH, Van Voorhis BJ, Walker DL, Miller TM, Barud KM: Maturational asynchrony between oocyte cumulus coronal morphology and nuclear maturity in gonadotropin releasing hormone agonist stimulations. *Fertil Steril* 1993;59:375-381
8. Mahadevan MM, Fleetham J: Relationship of a human oocyte scoring system to oocyte maturity and fertilizing capacity. *Int J Fertil* 1990;35:240-244
9. Antoine JM, Salat-Baroux J, Alvarez S, Cornet D, Tibi Ch, Mandelbaum J, Plachot M: Ovarian stimulation using human menopausal gonadotrophins with or without LHRH analogues in a long protocol for in-vitro fertilization: a prospective randomized comparison. *Hum Reprod* 1990;5:565-569
10. Pieters MHEC, Dumoulin JCM, Engelhart CM, Bras M, Evers JLH, Geraedts JPM: Immaturity and aneuploidy in human oocytes after different stimulation protocols. *Fertil Steril* 1991;56:306-310

11. Fisch B, Kaplan-Kraicer R, Amit S, Ovadia J, Tadir Y: The effect of preinsemination interval upon fertilization of human oocytes in vitro. *Hum Reprod* 1989;4:954-956
12. Veeck LL: Oocyte assessment and biological performance. *Ann NY Acad Sci* 1988;41:259-262
13. Plachot M, Mandelbaum J: Oocyte maturation, fertilization and embryonic growth in vitro. *Br Med Bull* 1990;46:675-694
14. Dekel N: Regulation of oocyte maturation; The role of cAMP. *Ann NY Acad Sci* 1988;541:211-216
15. Das K, Stout LE, Hensleigh HC, Tagatz GE, Phipps WR, Leung BS: Direct positive effect of epidermal growth factor on the cytoplasmic maturation of mouse and human oocytes. *Fertil Steril* 1991;55:1000-1004
16. Seibel MM, Smith D, Dlugi AM, Levesque L: Periovulatory follicular fluid hormone levels in spontaneous human cycles. *J Clin Endocrinol Metab* 1989;68:1073-1077
17. Itskovitz J, Rubattu S, Rosenwaks Z, Liu HC, Sealey JE: Relationship of follicular fluid prorenin to oocyte maturation, steroid levels, and outcome of in vitro fertilization. *J Clin Endocrinol Metab* 1991;72:165-171
18. Ron-El R, Nachum H, Golan A, Herman A, Yigal S, Caspi E: Binovular human ovarian follicles associated with in vitro fertilization: Incidence and outcome. *Fertil Steril* 1990;54:869-872
19. Lanzendorf SE, Zelinski-Wooten MB, Stouffer RL, Wolf DP: Maturity at collection and the developmental potential of rhesus monkey oocytes. *Biol Reprod* 1990;42:703-711
20. Rufas O, Shalgi R: Maturation-associated changes in the rat zona pellucida. *Mol Reprod Dev* 1990;26:324-330
21. Lopata A, Leung PC: The fertilizability of human oocytes at different stages of meiotic maturation. *Ann NY Acad Sci* 1988;541:324-336
22. Flood JT, Chillik CF, van-Uem JF, Iritani A, Hodgen GD: Ooplasmic transfusion: Prophase germinal vesicle oocytes made developmentally competent by microinjection of metaphase II egg cytoplasm. *Fertil Steril* 1990;53:1049-1054
23. Testart J, Frydman R, De Mouzon J, Lassalle B, Belaisch JC: A study of factors affecting the success of human fertilization in vitro. I. Influence of ovarian stimulation upon the number and condition of oocytes collected. *Biol Reprod* 1983;28:415-424
24. Sundstron P, Nilsson BO: Meiotic and cytoplasmic maturation of oocytes collected in stimulated cycles in asynchronous. *Hum Reprod* 1988;3:613-619
25. Thanki KH, Schmidt CL: Follicular development and oocyte maturation after stimulation with gonadotropins versus leuprolide acetate/gonadotropins during in vitro fertilization. *Fertil Steril* 1990;54:656-660
26. Clayton RN, Huhtaniemi IT: Absence of gonadotropin-releasing hormone receptors in human gonadal tissue. *Nature* 1982;299:56-59
27. Bramley TA, Menzies GS, Baird DT: Specific binding of gonadotropin-releasing hormone and an agonist of human corpus luteum homogenates: Characterization, properties, and luteal phase levels. *J Clin Endocrinol Metab* 1985;61:834-841
28. Yoshimura Y, Nakamura Y, Yamada H, Nanno T, Ubukata Y, Ando M, Zuzuki M: Gonadotropin-releasing hormone agonists induce meiotic maturation and degeneration of oocytes in the in vitro perfused rabbit ovary. *Fertil Steril* 1991;55:177-183
29. Yoshimura Y, Nakamura Y, Ando M, Shiokawa S, Koyama N, Nanno T: Direct effect of gonadotropin-releasing hormone agonists on the rabbit ovarian follicle. *Fertil Steril* 1992;57:1091-1097
30. Lefevre B, Gougeon A, Testart J: Les primates dans l'etude de la maturation ovocytaire. *Pathol Biol Paris* 1990;38:166-169
31. Lefevre B, Gougeon A, Nome F, Testart J: Effect of a gonadotropin releasing hormone agonist and gonadotropins on ovarian follicles in cynomolgus monkey: A model for human ovarian hyperstimulation. *Fertil Steril* 1991;56:119-125
32. Brzyski RG, Hofmann GE, Scott RT, Jones HW Jr: Effects of leuprolide acetate on follicular fluid hormone composition at oocyte retrieval for in vitro fertilization. *Fertil Steril* 1990;54:842-847