# Pregnancies Resulting from In Vitro Matured Oocytes Collected from Women with Regular Menstrual Cycle

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***Purpose:*** *To demonstrate that human immature oocytes retrieved from women with regular menstrual cycles can undergo maturation and fertilization, and that the resulting embryos can establish pregnancies.*

***Methods:*** *Immature oocytes (n = 568) were retrieved from women with regular menstrual cycle. The intact immature oocytes (n = 506) were allowed to mature in YS medium supplemented with 70% human follicular fluid (hFF); the matured oocytes were fertilized with husband sperm. Two pronuclei oocytes were cocultured with cumulus cells in YS medium supplemented with 10% hFF until 2 or 3 days after insemination. The cleaved embryos were transferred in uteri.*

***Results:*** *Follicles were aspirated on Day 9.2 ± 5.3 of 63 natural cycles from 51 patients (mean age = 34.8 ± 4.0 years). The average number of retrieved immature oocytes was 9.0. The maturation rate was 74.3% (376/506). The two PN and cleavage rates were 72.6% (273/376) and 89.0% (243/273), respectively. Embryo transfer was achieved in 51 cycles and clinical pregnancy rate was 17.6% (9/51).*

***Conclusions:*** *The results suggest that in vitro matured oocytes can undergo fertilization and the resulting embryos may successfully lead to pregnancies. However, further research is needed to improve IVM technique to achieve success rate comparable to gonadotrophin stimulated cycles.*

**KEY WORDS:** Coculture; cumulus cell; immature oocytes; IVM, YS medium.

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## INTRODUCTION

Controlled ovarian stimulation (COH) is used to achieve multifollicular recruitment, enabling an increased number of embryos to be transferred. However, there are disadvantages associated with COH. COH increases the cost of the cycle. The major side effects of superovulation are ovarian hyperstimulation and deep vein thrombosis (1,2). Other possible long-term side effects of fertility drugs include cancers (3). Thus, production of competent oocytes by IVM and fertilization in natural cycles would be an attractive option to eliminate several problems of the COH used for conventional in vitro fertilization (IVF).

Recently, knowledge regarding IVM of immature human oocytes and its clinical application has accumulated. IVM, fertilization, embryonic development, and term pregnancies have been reported employing immature oocytes from stimulated cycles (4–6), ovariectomies (7), PCO patients (8,9), or natural cycles (10).

However, the efficiency of current IVM techniques is suboptimal in terms of obtaining the number of mature oocytes by natural cycle compared with that by COH cycles (9,11). Also, the quality of maturation appears to be suboptimal showing frequently retarded cleavage and blockage of development of in vitro matured oocytes (12,13), which may be related to the poor pregnancy outcomes. Although the poor pregnancy rate has been seen in human IVM, it is important to continue to find ways to improve the developmental competence of human immature oocytes.

This report is to demonstrate that human immature oocytes retrieved from women with regular menstrual cycles can undergo maturation and fertilization, and that the resulting embryos can establish pregnancies.

## MATERIALS AND METHODS

### Patients

Before the study, approval was obtained from the Institutional Review Board of the Maria Infertility Clinic. This study was conducted from October 1999 through March 2000. The patient group consisted of 51 women with regular menstrual cycles who were scheduled for ICSI or IVF treatment. The patients had male factor infertility or tubal disease. None of the women had polycystic ovaries or anovulation.

### Oocyte Recovery

Follicle development on the ovaries was monitored by transvaginal ultrasonography (Aloka, Tokyo, Japan) beginning on Cycle Day 3, and immature oocytes were aspirated between Cycle Days 7 and 13 after the leading follicle had reached 10 mm in diameter. A transvaginal ultrasound machine with 19-gauge aspiration needle (Cook, Eight Mile Plains, Queensland, Australia) was used to aspirate follicles that were between 5 and 14 mm in diameter. A portable aspiration pump was used with a pressure between 80 and 200 mmHg. The aspirates were collected in tubes containing prewarmed heparinized Ham's F-10 medium that contained bicarbonate and HEPES buffers supplemented with 0.3% BSA. Follicular aspirates were filtered (70-$\mu$m mesh size, Falcon 1060; Life Technologies) and washed with addition of much medium to filtrate. The filtrate was further washed with medium by vigorous pipetting using 10 ml of serological pipette (Becton Dickinson & company, NJ, USA) to remove erythrocytes and small cellular debris. The retained cells were then resuspended in the medium. The oocytes were isolated under a stereomicroscope and washed twice in the same medium.

### In Vitro Maturation

The retrieved oocytes were classified as follows: multilayered cumulus, sparse cumulus, nude, or atretic. The IVM medium consisted of YS medium (Table I) supplemented with 70% human follicular fluid (hFF). The hFF was prepared using the method reported by Chi *et al.* (14). The oocytes were cultured in IVM medium at 37C in 5% $CO_2$ and humidified air. After 24 h in culture, the oocytes were denuded of cumulus cells with hyaluronidase (IVF Science, Gothenburg, Sweden) and mechanical pipetting. Oocyte nuclear maturation was assessed from the presence of the first polar body under the dis-

**Table I.** The Chemical Composition of YS Medium

| Chemicals | FW | mM | G/L |
|---|---|---|---|
| EDTA.2H$_2$O | 372.20 | 0.05 | 0.0186 |
| CaCl$_2$.2H$_2$O | 147.00 | 1.80 | 0.2646 |
| NaCl | 58.44 | 110.00 | 6.4284 |
| KCl | 74.55 | 5.00 | 0.3728 |
| MgSO$_4$.7H$_2$O | 246.50 | 0.80 | 0.1972 |
| Taurine | 125.10 | 0.20 | 0.0520 |
| Na-Pyruvate | 110.00 | 0.40 | 0.0440 |
| KHCO$_3$ | 100.10 | 5.00 | 0.5005 |
| NaHCO$_3$ | 84.01 | 20.00 | 1.6802 |
| L-Glutamine | 146.10 | 1.20 | 0.1753 |
| Na-Lactate(D,L) | 112.10 | 3.00 | 0.5 (ml) |
| MEM non-essential AA | 10 (ml) | 10 (ml) | 10 (ml) |
| RPMI 1640 AA | 10 (ml) | 10 (ml) | 10 (ml) |
| MEM Vitamin | 5 (ml) | 5 (ml) | 5 (ml) |
| Antibiotics | P/S | P/S | P/S |

secting microscope. Following examination, immature oocytes that remained at GV or MI stage were cocultured with cumulus cells in the same medium and reexamined for the meiotic status at 48 h and finally at 56 h of culture.

### Preparation of Cumulus Cells for Coculture

Cumulus cell pools were prepared from cumulus-oocyte complexes (COC) of matured oocytes. COCs were triple washed with medium immediately after oocyte collection. The portions of the cell mass, which have blood clots and cell debris outside the corona radiata of cumulus-oocyte complexes, were removed using two 30-gauge needles. And then, only corona radiator masses from the cleaned cumulus-oocyte complexes were excised using two 30-gauge needles. The masses were treated with a 100–150 $\mu$l YS medium containing 10% hFF and 0.003% hyaluronidase, and then the gap junctions were lysed by vigorous pipetting with pasteur pipette, resulting in change of the corona radiator masses to a pool containing many single cells. The cumulus cell layers used for coculture were made under 10 $\mu$l droplets by seeding cumulus cells (1 $\times$ 10$^4$/ml) obtained from the pool using a pipette. After 4 hours of incubation, YS medium containing 0.003% hyaluronidase was exchanged with a preequilibrated culture medium, and then the drop dishes containing cumulus layers were incubated overnight. The drops were used for in vitro maturation or in vitro development.

### In Vitro Fertilization (IVF), In Vitro Development (IVD), and Embryo Transfer

Conventional IVF or ICSI were used to fertilize the mature oocytes. The zygotes were cocultured

with cumulus cells in 10 $\mu$l YS medium supplemented with 10% human follicular fluid. Embryos were transferred on Day 2 or 3 after insemination by the transcervical route in standard fashion. After the embryo transfer (ET), surplus embryos were cultured until Day 6, and only the embryos that developed to the expanded blastocyst stage were cryopreserved by Ménézo's method (15).

### Endometrium Preparation

For the preparation of the endometrium, the patients were given 10,000 IU HCG 48 h after of oocyte retrieval. Progynova (6 mg) was administered daily since the day of immature oocyte retrieval. Progest (100 mg) was administered daily starting 2 days after oocyte retrieval. Both medications were continued until either a negative pregnancy urine test or a positive fetal heartbeat was confirmed in women with regular menstrual cycles. Embryos from patients with poor endometrial preparation (<7 mm endometrial thickness) at the time of ET were further cultured for 5 or 6 days, and resulting blastocysts were cryopreserved.

### RESULTS

Follicles were aspirated on Day 9.2 ± 5.3 of 63 natural cycles from 51 patients (mean age = 34.8 ± 4.0 years). A total of 568 oocytes were retrieved. The oocytes with multilayered cumulus, sparse cumulus, nude, and atretic were 59.5%, 17.8%, 11.8%, and 10.9%, respectively. Oocytes ($n = 506$) that were classified as having a multilayered, sparse, and nude cumulus were used for this experiment. The maximum size of the leading follicle on the day of oocyte aspiration was 10–14 mm, and that of subordinate follicles was 5–8 mm. After 24 h of maturation, 40.7% (206/506) of oocytes had a visible first polar body. By 48 h of culture, 362 (71.5%) of 506 oocytes were matured to metaphase II and by 56 h, a total of 376 oocytes (74.3%) reached meiotic maturity. The result of 376 in vitro matured oocytes obtained from IVM cycles in terms of maturation rate to metaphase II stage, fertilization and cleavage rates, and pregnancies are summarized in Table II. After insemination, 72.6% (273/376) of the oocytes were fertilized to two pronuclei, and the cleavage rate was 89% (243/273). The embryos were cultured for 2 or 3 days after insemination. Cycles in nine patients in IVM did not undergo ET because of poor endometrium (<7 mm

**Table II.** Maturation, Fertilization, Development, and Pregnancy Rate Using In Vitro Matured Human Oocytes

| | |
|---|---|
| Patients | 51 (34.8 ± 4.0) |
| No. of cycles | 63 |
| No. of oocytes collected | 568 |
| No. of oocytes cultured | 506 |
| No. of MII oocytes | 376 (74.3%) |
| No. of 2PN oocytes | 273 (72.6%) |
| No. of oocytes cleaved | 243 (89.0%) |
| No. of cycles with ET | 51 |
| No. of clinical pregnancies | 9 (17.6%) |

endometrial thickness) at the time of transfer. Cycles in three patients also did not undergo ET because of lack of maturation, fertilization failure, or poor embryo quality. Cycles in the 51 patients that underwent ET received a mean of 3.6 embryos. Cycles in nine patients developed into clinical pregnancy. The details of the pregnancies are shown in Table III. Cycles in three patients had spontaneous abortion at <12 weeks of gestation. The other six pregnancies are ongoing.

### DISCUSSION

This study demonstrates that human immature oocytes retrieved from women with regular menstrual cycles can undergo maturation and fertilization, and that the resulting embryos can establish pregnancies.

One of the important factors regulating the number and quality of oocytes maturing in vitro is the culture conditions used for IVM. The composition of most media used for human IVM is based on experiences with other mammalian species. The common approach used by previous investigators had been that of supplementing various concentrations of hormones or serum to culture media (7,8,16). Follicular fluid is expected to contain high hormone levels and growth factors in addition to other putative

**Table III.** Details of Nine Pregnancies from Immature Oocytes Obtained in Women with Tubal or Male Factor Infertility

| Patient | Age (years) | Infertility factor | Results |
|---------|-------------|--------------------|---------|
| KHS | 30 | Tubal | Twin |
| KYL | 39 | Tubal | Miscarriage[a] |
| CHT | 37 | Tubal | Miscarriage[b] |
| KJS | 34 | Tubal | Singleton |
| KMY | 28 | Tubal | Singleton |
| SKY | 26 | Male | Singleton |
| JEJ | 31 | Male | Triple |
| LKM | 30 | Male | Singleton |
| OHJ | 34 | Male | Miscarriage[a] |

[a]Miscarried at 6 weeks.
[b]Miscarried at 8 weeks.

intrafollicular factors. Cha *et al.* (7) reported 55.8% of matured oocytes using 50% hFF. We observed that the maturation rate of human immature oocytes in YS medium supplemented with 70% hFF (74.3%) was higher than that with 50% hFF (58%). Thus, we selected YS medium supplemented with 70% hFF to culture the human immature oocytes. The percentage of matured oocytes (74.3%) is similar to those that are supplemented with hormones as reported elsewhere (8,11,12). Therefore, follicular fluid is a useful supplement enhancing the maturity of immature oocyte obtained from women with regular menstrual cycles.

We used YS medium for IVM of immature oocytes and cumulus cell coculture of human embryo. YS medium contains vitamins, amino acids, and taurine-omitting glucose (Table I). Gardner *et al.* (17) reported that concentration of glucose in fluid collected from the in situ human oviduct was 3.11 mM in the follicular phase but 0.5 mM at midcycles. Mean glucose concentration in hFF is in the range of 3.29–5.5 mM (18). We used YS medium supplemented with 70% hFF as a protein source for IVM. Therefore, immature oocytes were exposed to 2.30–3.85 mM glucose. Several studies showed that glucose was not required for early preimplantation embryos (19,20). We routinely used YS medium for embryo coculture with cumulus cell (21). Since YS medium was supplemented with 10% hFF, embryo culture medium contains approximately 0.44 mM glucose.

We examined the time course of in vitro meiotic maturation to define the optimum time for fertilization in our IVM system. When cumulus cells of oocytes were denuded at 24 h after culture, 40.7% of cultured oocytes matured into metaphase II. Indeed, 55.7% of oocytes reached metaphase II within 24 h from the onset of culture. Smith and his colleagues (22) observed that 24% of oocytes matured at 22–23 h after the onset of maturation. Chian *et al.* (23) reported that only 4.9% of oocytes were matured at 24 h following IVM. The low percentage of oocytes at Metaphase II, at 24 h, might be due to the evaluation of maturity without denuding cumulus cells. Another possible explanation could be the different culture conditions. In certain cases oocytes inseminated 48 or 56 h after maturation (8,11). Thus, most of the oocytes inseminated were arrested in metaphase II for 20–30 h. Developmental competence of the oocytes arrested for long time might become worse. However, critical evaluation of the optimal interval between in vitro oocyte maturation to Metaphase II and insemination remains to be further investigated.

Although the number of embryos transferred (mean = 3.6) in uteri was comparable to regular IVF/ICSI cycles, clinical pregnancy rate was only 17.6% in this study. The Implantation and pregnancy rates were lower than those obtained with conventional IVF/ICSI in our clinic (21). Other investigators also reported lower pregnancy and implantation rates (8,11,24). This low success rate might be attributed to asynchrony between the cytoplasmic and nuclear maturation of the oocyte. Cytoplasmic maturity might not be complete in spite of matured nucleus. Another possible explanation for the low success rate could be the asynchrony between endometrium and cleaving embryo. Although the number of IVM cycles was small, recent studies have shown improved pregnancy rates per embryo transfer (23,25).

In conclusion, immature oocytes retrieved from women with regular menstrual cycle can undergo maturation in vitro with hFF. The oocytes can fertilize and cleave, and the resulting embryos can also establish pregnancies. To make the use of immature oocytes more efficient, further work is needed to define the best conditions for both clinical and laboratory procedure.

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