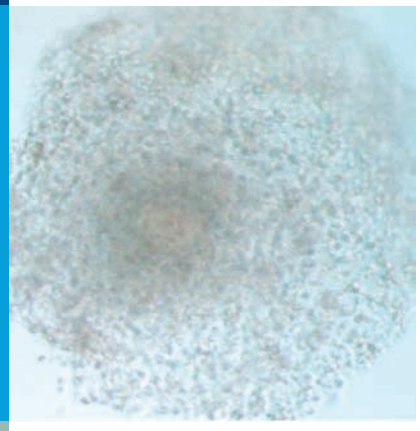
Handbook on In Vitro Maturation of Immature Human Oocytes



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SAGE®

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Preface

Although the initial live birth resulting from human in vitro fertilization (IVF) was produced by natural-cycle IVF, this procedure was gradually replaced by ovarian stimulation IVF because it was believed that the number of oocytes retrieved related to the embryos available for transfer which, in turn, directly affected successful pregnancy. However, repeated ovarian stimulation by gonadotropins has produced a number of serious long-term side effects in patients, including a greater possibility of ovarian hyperstimulation syndrome (OHSS) and the concern by some people about a possible increased risk of ovarian, endometrial and breast cancers. Therefore, more and more patients are becoming interested in natural-cycle IVF and in vitro maturation (IVM) treatment. The aim of this handbook is to share our experience and protocols with the assisted reproductive technology (ART) fraternity.

Recovery of immature oocytes followed by IVM of these oocytes is a potentially useful treatment for women with infertility. Recently, it was demonstrated that this method is particularly effective for women with polycystic ovaries (PCO) or polycystic ovarian syndrome (PCOS)-related infertility because there are numerous antral follicles within the ovaries of this group of patients. Today, given the efficiency of IVF and improvements in the culture system, natural-cycle IVF is more suitable for women undergoing infertility treatment. One very attractive possibility for enhancing the successful outcome of natural-cycle IVF treatment is combining it with immature egg retrieval and IVM. The use of IVM technology can thus be broadened to treat women suffering from all causes of infertility.

I would like to thank CooperSurgical and SAGE In Vitro Fertilization for inviting me to prepare this handbook on IVM treatment. I sincerely hope that the information it contains will be of value to embryologists and clinicians in the field of assisted human reproduction.

Acknowledgments

The contents of this handbook represent the culmination of many years of experience with IVM in different IVF centers. It would have been impossible for me to prepare this manual without the support and assistance of my colleagues at the McGill Reproductive Center, Royal Victoria Hospital, and the Department of Obstetrics and Gynecology of McGill University. I am grateful to Ms. Valerie Cameron for her excellent secretarial skills in helping me to prepare this handbook. I also thank all my other clinical colleagues, embryologists, nursing staff, ultrasound technicians and secretaries of our ART team for their cooperation and assistance.

Finally, I am grateful to my wife, Dr. Shan-Jin Cui, and two daughters, Jie-Jie and Julie, for their encouragement, support and patience during the difficult time of the initial IVM trial.

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Culture Media Involved in IVM Treatment

There are three major culture media involved in the protocol of in vitro maturation (IVM) treatment. The first is Oocyte Washing Medium; the second, Oocyte Maturation Medium; and the third, Embryo Maintenance Medium.

1 Oocyte Washing Medium

This medium is used for washing immature oocytes, cumulus-oocyte complexes (COCs), collected from the follicles before maturation in culture. This medium is buffered with HEPES; therefore, medium pH is not markedly changed at room temperature and atmosphere. This medium is ready for use following pre-incubation for at least 60 minutes at 37ºC.

2 Oocyte Maturation Medium

This medium is used for maturation in culture of immature oocytes in an incubator at 37ºC under an atmosphere of 5% CO_2 in air and high humidity. An incubator with a triple gas mixture (90% N_2, 5% CO_2, and 5% O_2) and 100% humidity is also suitable for maturation in culture of immature oocytes.

3 Embryo Maintenance Medium

This medium is used for fertilization and embryonic culture following insemination by intracytoplasmic sperm injection (ICSI). Although it is known that the fertilization rate of in vitro matured oocytes is not affected by in vitro fertilization (IVF), it is preferable to perform ICSI for in vitro matured oocytes because it can be guaranteed that more than 70% of oocytes will be fertilized if the sperm used have a normal morphology.

Figure 1 *Package of IVM media produced by SAGE, a product line of CooperSurgical, Inc.*

Product Description

IVM Kit, ART-1600, for the in vitro maturation of human oocytes

- 1 X 50 mL Oocyte Washing Medium
- 1 X 20 mL Oocyte Maturation Medium
- 1 X 5 mL Embryo Maintenance Medium

Introduction

PROGRESS IN IVM TREATMENT

To date, assisted reproductive technology (ART) has helped thousands and thousands of women to overcome infertility problems. Although initial attempts at human IVM and IVF were undertaken as far back as the 1940s (Rock and Menkin, 1946; Menkin and Rock, 1948), it was not until the 1960s that landmark work was done on IVM of immature human oocytes (Edwards, 1965a,b; Edwards et al., 1969). The laparoscope was introduced in the late 1960s to collect human oocytes from the Graafian follicle, resulting in the first live birth resulting from human IVF produced from an in vivo matured oocyte (Steptoe and Edwards, 1978). This natural cycle IVF treatment was gradually replaced by ovarian stimulation IVF because it was been believed that the number of oocytes retrieved related to the embryos available for transfer which, in turn, directly affected the achievement of a successful pregnancy. At the beginning, relatively inexpensive medications, such as clomiphene citrate, were used to stimulate ovaries to produce multiple follicles. However, current ovarian stimulation protocols call for the use of expensive gonadotropin-releasing hormone (GnRH) agonists or antagonists in combination with gonadotropins to generate multiple follicles in the ovaries. Some women are extremely sensitive to stimulation with exogenous gonadotropins and are at an increased risk of developing ovarian hyperstimulation syndrome (OHSS), which is a life-threatening condition (Beerendonk et al., 1998). In addition, there is concern that the long-term side effects of repeated ovarian stimulation may increase the risk of ovarian, endometrial and breast cancers (Tarlatzis et al., 1995; Duckitt and Templeton, 1998).

Since Cha et al. (1991) reported the first pregnancy from in vitro matured oocytes derived from a caesarean section donor, fertilization, embryo development and pregnancy by immature human oocytes matured in vitro have been successfully achieved in women with polycystic ovary syndrome (PCOS) (Trounson et al., 1994; Cha and Chian, 1998). Recent improvements in culture condition and transfer techniques have demonstrated that immature oocyte retrieval followed by IVM is an effective treatment for women with polycystic ovaries (PCO) or polycystic ovarian syndrome (PCOS)-related infertility because there are numerous antral follicles within the ovaries in this group of patients. In general, clinical pregnancy and implantation rates for infertile women with PCO or PCOS have reached approximately 30-35% and 10-15%, respectively, (Chian et al., 2004a, b).

WHY IVM?

Recovery of immature oocytes followed by IVM of these oocytes is a potentially useful treatment for women with infertility. Compared to women with normal ovaries, women with PCO or PCOS have a significantly higher risk of developing OHSS when stimulated with exogenous gonadotropins. Although immature oocyte retrieval followed by IVM might be useful for approximately 35% of women undergoing IVF treatment who have polycystic-like ovaries seen on ultrasound scan, it is important to mention here that IVM technology is an attractive alternative for women with all types of infertility.

ADVANTAGES OF IVM

In comparison with conventional IVF, the major advantages of IVM treatment include: (1) avoidance of the side effects resulting from gonadotropin stimulation including the risk of OHSS, (2) reduced cost, and (3) simplified treatment.

WHO ARE CANDIDATES FOR IVM?

Theoretically, all infertile women are candidates for IVM treatment. However, pregnancy rates are directly related to the number of oocytes retrieved and embryos available for transfer. Therefore, the best candidates for IVM treatment are women under 35 years of age who have polycystic-like ovaries seen on ultrasound scan because younger women have a greater number of follicles that continue to grow through to the preovulatory stage of development during each menstrual cycle.

DEVELOPMENT OF IVM

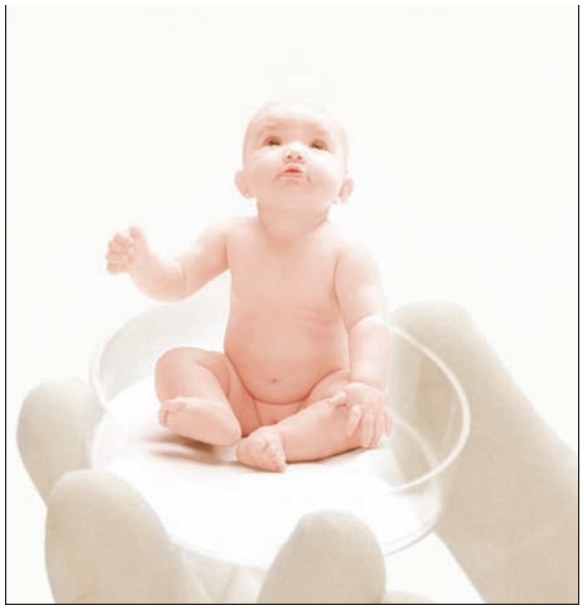
There is an increasing interest in natural cycle IVF among patients because it produces fewer side effects and less discomfort. Although natural cycle IVF may result in low success rates per oocyte collection due to a lack of oocytes and embryos available for transfer, by using life table analysis to calculate cumulative success rates of stimulated cycle IVF over the same time span, it becomes evident that the cumulative probability of achieving pregnancy is 46% after four natural cycle IVF treatments (Nargund et al., 2001). It is therefore possible to offer a treatment that combines natural cycle IVF with immature oocyte retrieval followed by IVM to women with all types of infertility, thereby eliminating the need to resort to ovarian stimulation and providing reasonable pregnancy and implantation rates. However, it is important to individualize natural cycle IVF treatment combined with IVM treatment for each patient in order to optimize the success rates. Further research must be done to address the mechanism of oocyte maturation in order to refine culture conditions and improve the implantation rate of in vitro matured oocytes.

Follicular Development

Although only a single follicle usually grows to the preovulatory stage and releases its oocyte for potential fertilization, many small follicles also develop during the same follicular phase of the menstrual cycle. It seems that approximately 20 antral follicles are selected and continue to grow through to the preovulatory stages of development during each menstrual cycle (Hillier,1994). Recently, two or three waves of ovarian follicular development have been documented in women during the menstrual cycle based on daily transvaginal ultrasonography, challenging the traditional theory that only a single cohort of antral follicles grows during the follicular phase of the menstrual cycle (Baerwald et al., 2003a,b). Interestingly, it seems that atresia does not occur in non-dominant follicles even after selection of the dominant follicle during folliculogenesis because immature oocytes retrieved from non-dominant follicles have been successfully matured in vitro and fertilized, resulting in several pregnancies and healthy live births (Paulson et al., 1994; Thornton et al., 1998). Animal model studies also support these findings that oocyte quality and early embryonic developmental competence of immature oocytes following maturation in vitro are not adversely affected by the presence of the dominant follicle in the ovaries (Smith et al., 1996; Chian et al., 2002).

BASELINE ULTRASOUND SCANS

If the patient has irregular menstrual cycles or indeed no menstrual period, it is necessary to induce a menstrual bleed with progesterone (Prometrium 300 mg/day for 10 days or Provera 5 mg twice daily for 5 days). Once the medication is stopped, menstrual bleeding will generally occur within 3 days. When the patient has a withdrawal bleed, she should be examined by baseline ultrasound scan to determine the status of follicular development in both ovaries. If the patient has regular menstrual cycling, she does not need withdrawing menstrual bleeding and can be monitored naturally. In both cases, a transvaginal ultrasound examination is required during the first 2-3 days of the menstrual cycle. At this point, the number and size of the follicles should be recorded and measured.



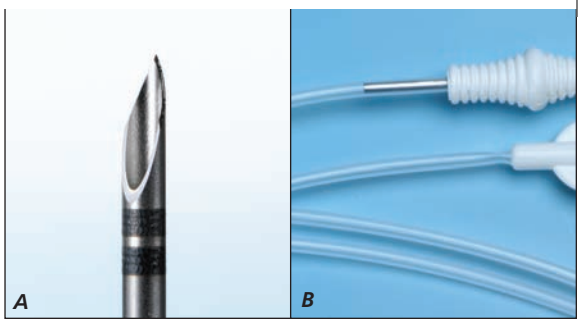
SECOND ULTRASOUND SCAN

Between day 6 and day 9 of the menstrual cycle, a repeat ultrasound should be performed to assess the lining of the uterus (the endometrium) and to confirm follicular development. If all is normal, the patient can be scheduled for oocyte retrieval. At this point, it is very important to mention that the thickness of the endometrium should not be less than 6 mm when the patient is scheduled for administration of human chorionic gonadotropin (hCG) 36 hours before oocyte collection.

HCG PRIMING 36 HOURS PRIOR TO EGG COLLECTION

The patient is given an injection of 10,000 IU hCG (Profasi) 36 hours before oocyte retrieval. Oocyte retrieval is usually preformed between days 10 and 14 of the menstrual cycle, depending upon the thickness of the lining and the size of the follicles. It is extremely important to prevent ovulation from the dominant follicle due to a natural LH surge. Our experience indicates that hCG can be administered when the maximum size of the dominant follicle reaches 12-14 mm in diameter. Most oocytes collected from the dominant follicle are at metaphase-II (M-II) stage.

Immature Oocyte Retrieval



SAGE Oocyte Retrieval Needles have ultra-precision distal tips that are sharpened, beveled and polished (A) with Polyethylene tubing that eliminates kinks and enhances flow (B).

OOCYTE RETRIEVAL NEEDLES

A specially designed aspiration needle is required to retrieve immature oocytes from the ovary and can be purchased from the following companies:

SAGE Oocyte Retrieval Needles
Single Lumen
19 Gauge

REF#	Description	Needle Length	Tubing Length
ORN-1930-7	SAGE Oocyte Retrieval Needle	30 cm	75 cm
ORN-1935-7	SAGE Oocyte Retrieval Needle	35 cm	75 cm
ORN-1930-9	SAGE Oocyte Retrieval Needle	30 cm	95 cm
ORN-1935-9	SAGE Oocyte Retrieval Needle	35 cm	95 cm

CooperSurgical Inc.
95 Corporate Drive
Trumbull, CT 06611
Phone: +1.203.601.5200
Fax: +800.262.0150
www.coopersurgical.com

IVM Ovum Aspiration Needle
Single Lumen
Ref. K-OPS-7035-RWH-ET
Needle 19 G
Length 35 cm
Total Aspiration Lumen Volume 0.71 ml

Cook Australia
12 Electronics Street
Eight Mile Plains
Queensland 4113 Australia
Phone: +61 7 3841 1188
Fax: +61 7 3841 1288
www.cookgroup.com

KITAZATO Aspiration Needle
Single Lumen
Code No. FS-NKS2030-K
Needle 20 G
Length 30 cm
Permission No. 20900BZZ00905000

KITAZATO Supply Co.
Shizuoka-Ken, Fuzimiya-Shi
418-0004
Phone: +81-0544-27-8831
Fax: +81-0544-27-6060

Figure 2
Aspiration pressure is at approximately 85-100 mmHg connected with a single lumen of aspiration needle.

ASPIRATION PRESSURE

Immature oocytes are retrieved from the ovaries under ultrasound guidance in the same way that oocyte retrieval is performed during a conventional IVF cycle. Aspiration pressure of approximately 7.5-8.0 kPa 85-100 mmHg (Figure 2) should be used to aspirate immature oocytes with the above designated single-lumen aspiration needle. All visible follicles are aspirated under local or general anesthesia.

FLUSHING MEDIUM

COCs are collected in 10 ml culture tubes (Falcon) containing approximately 2-3 ml of heparinized warm flushing medium (usually containing 2 IU/ml of heparin). Any commercially available flushing medium, such as modified human tubal fluid (mHTF) or Ham's F-10 medium, can be used. However, it is important to make sure that the medium used contains heparin to prevent clouding (solidification) of the aspirates. It is also possible to use 0.9% saline containing 2 IU/ml heparin (Baxter, Toronto, Ontario, Canada) as flushing medium (Figure 3).

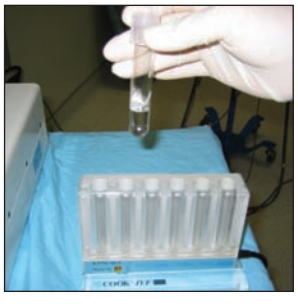


Figure 3
Follicular aspirates collected into tubes (10 ml, Falcon) containing approximately 2-3 ml of heparinized warm (37°C) flushing medium (0.9% saline containing 2 IU/ml heparin; Baxter, Toronto, Ontario, Canada).

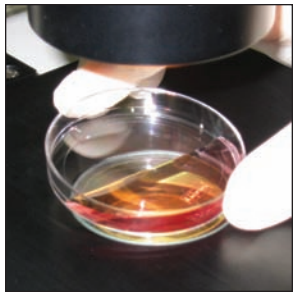


Figure 4
Sliding technique for observation of oocyte maturity. COCs are allowed to slide slowly from one side to the other on the bottom of the Petri dish, while being observed under the stereomicroscope.

IDENTIFICATION OF IMMATURE OOCYTES

There are two ways to look for and collect COCs from follicular aspirates: (1) Dish research: the follicular aspirates are poured directly into Petri dish (or Tissue Culture Dish) and examined for COCs under a stereomicroscope; (2) Cell strainer: the follicular aspirates are filtered through a cell strainer (Falcon, Cell Strainer 352350, 70 µm Nylon; www.bd.com/labware). After filtering, the collected aspirates can be rinsed with pre-warmed IVM Washing Medium and transferred to a Petri dish (Falcon, 60 X 15 mm) to search for COCs under a stereomicroscope. All handling procedures should be conducted on warm stages or plates at 37°C.

In order to determine whether the oocyte is mature or not, a special observation technique called 'sliding' can be employed. Briefly, the COC is allowed to slide slowly from one side to the other on the bottom of the Petri dish, while being observed under the stereomicroscope (Figure 4). During COC sliding, it is possible to observe clearly whether or not the oocyte cytoplasm contains a germinal vesicle (GV) or if the oocyte has extruded a first polar body (1PB) into perivitelline space (PVS). If neither GV is seen in the oocyte cytoplasm nor 1PB found in PVS, the oocyte is defined as germinal vesicle breakdown (GVBD) or metaphase-I stage (M-I) (Figure 5). If any mature oocytes are found, they should be inseminated by either IVF or ICSI within three hours after collection.

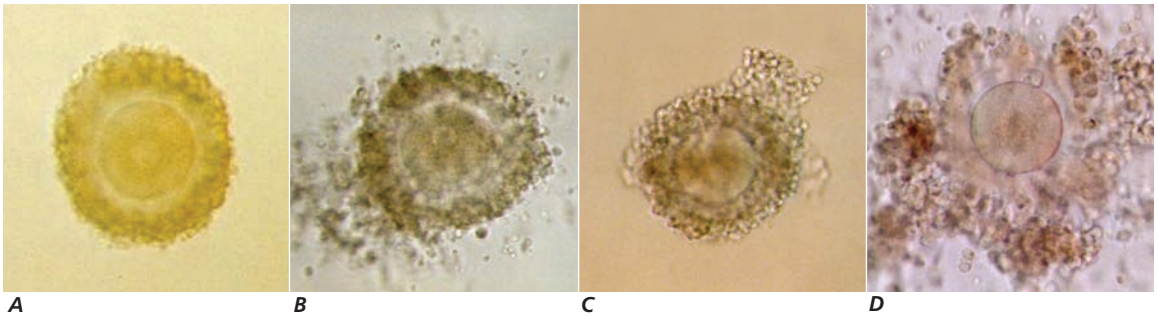


Figure 5

*During COC sliding, it is possible to observe clearly whether or not the oocyte cytoplasm contains a germinal vesicle (GV) (**A** and **B**) or the oocyte has extruded a first polar body (1PB) into perivitelline space (PVS) (**D**). If neither GV is seen in the oocyte cytoplasm nor 1PB found in PVS, then the oocyte is defined as germinal vesicle breakdown (GVBD) or metaphase-I stage (M-I) (**C**).*

PREPARATION OF OOCYTE WASHING MEDIUM

Oocyte Washing Medium must be prepared at least one hour before oocyte collection and kept at 37ºC. Briefly, three Petri dishes (Falcon, 35 X 10 mm), each containing approximately 2.0-2.5 ml of Oocyte Washing Medium under mineral oil, are prepared for every patient (Figure 6). If a cell strainer is to be used to collect COCs, a flask (Falcon, 50 ml) containing approximately 25-30 ml of Oocyte Washing Medium must also be prepared for each patient and kept in an incubator (with the flask cap tightened).

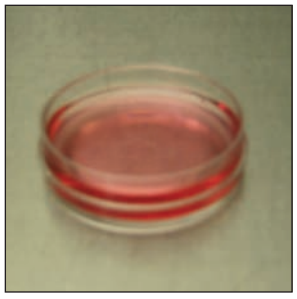


Figure 6

For each patient prepare three COC washing dishes (Falcon, 35 X 10 mm), each containing approximately 2.0-2.5 ml of Oocyte Washing Medium under mineral oil.

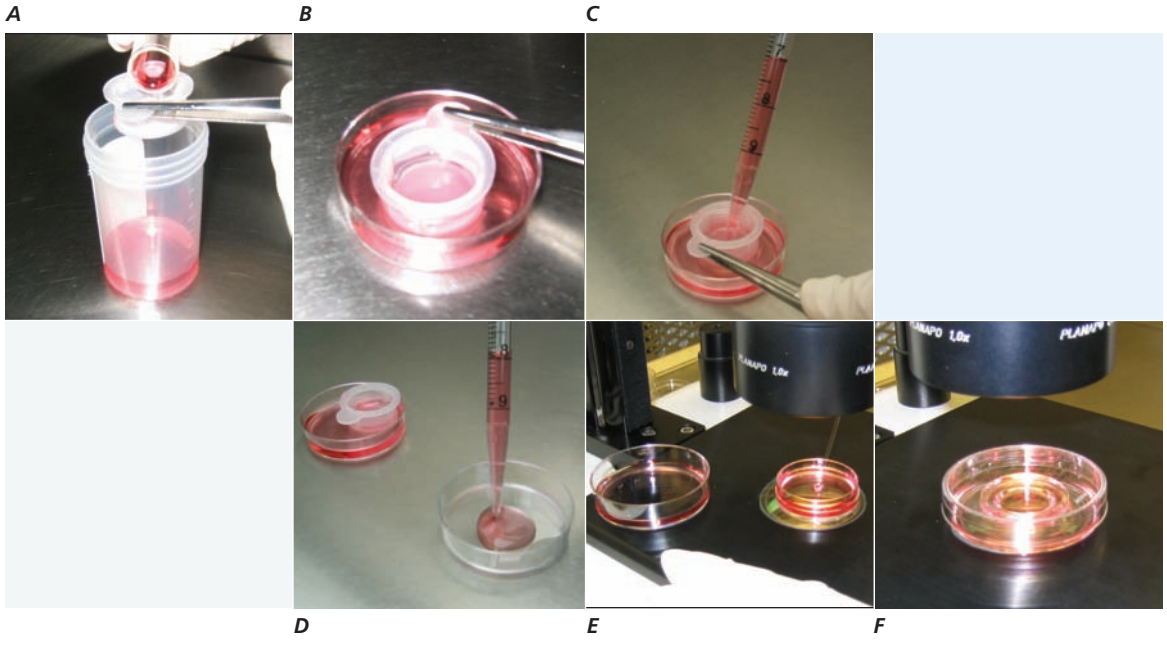


Figure 7

*Follicular aspirates are filtered with a Cell Strainer (**A**). The Cell Strainer is kept in a Petri dish (Falcon, 60 X 15 mm) containing Oocyte Washing Medium between tube collection and filtering (**B**). After filtering, the collected aspirates can be rinsed and transferred into another Petri dish in order to search for COCs under a stereomicroscope (**C** and **D**) after washing two or three times with pre-warmed Oocyte Washing Medium in a washing dish (Falcon, 30 X 10 mm) (**E**), COCs are transferred to Oocyte Maturation Medium for maturation in culture (**F**).*

To use a cell strainer, each tube of follicular aspirate is poured through the cell strainer immediately after collection. The cell strainer can be placed in a Petri dish (Falcon, 60 X 15 mm) containing 3-5 ml of Oocyte Washing Medium on a warm stage or plate in order to prevent the COCs from drying in the strainer between the time of follicular aspiration and tube collection. Once follicular aspiration has been completed, the COCs contained in the cell strainer are collected with a pipette, then immediately transferred into Petri dishes for scanning of the COCs under a stereomicroscope. To identify the status of oocyte maturity, the sliding technique of observation is then used as described previously. COCs are transferred to Oocyte Washing Medium to wash for three times, and then COCs are transferred to Oocyte Maturation Medium for maturation in culture (Figure 7).

WASHING IMMATURE OOCYTES

COCs are picked up with a sterile cotton-plugged Pasteur pipette fitted to a pipette pump and transferred into the pre-warmed Oocyte Washing Medium for washing several (at least three) times before maturation in culture.

LINING PREPARATION WITH ESTRADIOL

On the day of oocyte retrieval, the patient should begin taking estradiol valerate tablets (Estrace) in order to prepare the endometrium of her uterus to receive the embryos. Depending upon the endometrial thickness on the day of oocyte retrieval, the patient will be given a divided dosage. If endometrial thickness is ≥6.0 mm, then a dosage of 6 mg will be administered daily until the pregnancy test after embryo transfer.

In Vitro Maturation of Immature Oocytes

PREPARATION OF OOCYTE MATURATION MEDIUM

The immature COCs (maximum of 10) are incubated in an Organ Tissue Culture Dish (Falcon, 60 X 15 mm) containing 1 ml Oocyte Maturation Medium supplemented with a final concentration of 75 mIU/ml FSH and 75 mIU/ml LH at 37ºC in an incubator with an atmosphere of 5% CO_2 and 95% air with high humidity (or with triple gas mixture (90% N_2, 5% CO_2, and 5% O_2) and 100% humidity). Oocyte Maturation Medium should be prepared for equilibration at least two hours before immature oocyte retrieval (practically, it can be made one day before). A brief description of the procedures involved in the preparation of Oocyte Maturation Medium follows (Figure 8):

A Place 10.0 ml of Oocyte Maturation Medium into a test tube;

B Dissolve completely 1 ampoule of 75 IU FSH and 75 IU LH into (a);

C Place 9.90 ml of fresh Oocyte Maturation Medium into a test tube;

D Take 100.0 µl FSH and LH dissolved as per item (a) and transfer into (c);

E Prepare 3 Organ Tissue Culture Dishes for each patient. In each dish, the inner well contains 1 ml of (c) and the outer well 2 ml of (c);

F Cover the Organ Culture Dish with the dish cover and place it in the incubator.

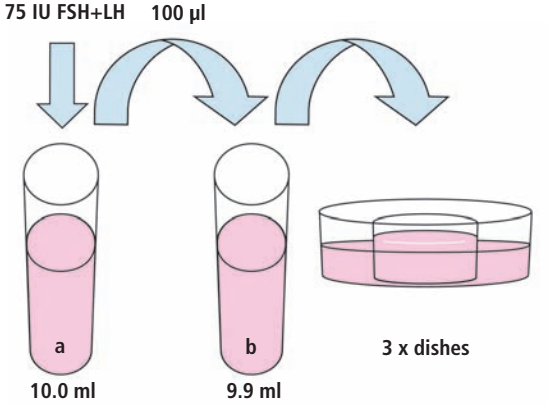
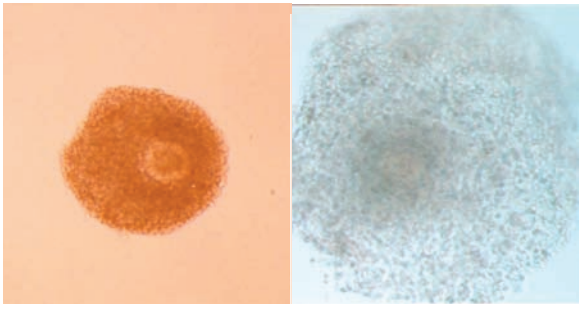


Figure 8
Preparation of Oocyte Maturation Medium when the IVM Oocyte Medium. Taking two tubes, the first tube (a) contains 10.0 ml of IVM Oocyte Medium, and the second tube (b) contains 9.9 ml of IVM Oocyte Medium, respectively. Using (a) tube's medium dissolves 75 IU of FSH and 75 IU of LH (gonadotropin stock). Placing 100 µl of the gonadotropin stock into the second tube (b). Preparing three Organ Tissue Culture Dishes (Falcon, 60 X 15 mm) with tube (b) for each patient, the inner well containing 1 ml of IVM Oocyte Medium and the outer well, 2 ml of IVM Oocyte Medium.



A B

Figure 9

*Morphological changes of immature COC before and after culture in the Oocyte Maturation Medium. (**A**) Immature COC immediately after retrieval from the ovary; (**B**) The same COC 24 hours after culture in the Oocyte Maturation Medium. Note that the cumulus mass has almost doubled in size compared with its mass before culture (two photos with the same magnification).*

SPERM PREPARATION

Semen can be collected and prepared for insemination on the day of oocyte retrieval if a mature oocyte has been retrieved from the dominant follicle. Otherwise, semen collection and preparation should be performed the day after oocyte retrieval. If possible, a fresh sperm sample should be obtained, which can then be prepared for the insemination. Since the procedure is identical to that used for sperm preparation for IVF or ICSI, it will not be repeated here.

STRIPPING OOCYTES 24 HOURS AFTER CULTURE

The The immature COCs are cultured in the Oocyte Maturation Medium in the incubator and allowed to begin the maturation process for 24 to 48 hours. Twenty-four hours after maturation in culture (Figure 9), all of the COCs are stripped for identification of oocyte maturity (Figure 10). COCs will be denuded using a finely drawn glass pipette following one minute of exposure to a commercially available hyaluronidase solution. The mature oocytes are then subjected to insemination by either IVF or ICSI after stripping. The remaining immature oocytes (GV and M-I) will continue to mature in culture for another 24 hours. At this point, it is not necessary to change the Oocyte Maturation Medium.

ENDOMETRIUM PREPARATION WITH PROGESTERONE

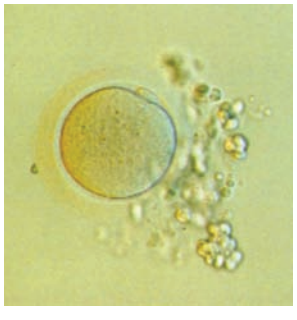
Preparation of the lining is also required using progesterone. Two hundred milligrams (200 mg) of intra-vaginal progesterone (Prometrium) is administered to the patient three times daily or progesterone injections are given subcutaneously with instruction starting from the day after egg collection and continuing until the pregnancy test.

IDENTIFICATION OF MATURE OOCYTES 48 HOURS AFTER CULTURE

Forty-eight hours after oocyte retrieval (or oocyte maturation in culture), the remaining stripped oocytes are re-examined and if any have matured (M-II) at this point, they will be inseminated immediately by either IVF or ICSI.

Figure 10

Mature oocyte following in-vitro culture 24 hours in Oocyte Maturation Medium after partially stripping the cumulus cells from the oocyte.



Insemination of Mature Oocytes

ICSI is recommended for the insemination of in vitro matured oocytes because we believe that this method offers a greater chance of successful fertilization than does IVF. Therefore, this manual describes only ICSI for insemination of in vitro matured oocytes (Figure 12).

PREPARATION OF EMBRYO MAINTENANCE MEDIUM

Embryo Maintenance Medium must be prepared at least one hour before ICSI and kept at 37ºC in an incubator with an atmosphere of 5% CO_2 and 95% air with high humidity or with triple gas mixture (90% N_2, 5% CO_2, and 5% O_2) and 100% humidity. Briefly, it is appropriate to prepare 20 µl of droplets under mineral oil in a Petri dish (Falcon, 35 X 10 mm) (Figure 11). The number of dishes used for each patient will depend upon the number of mature oocytes obtained after maturation in culture.

INSEMINATION BY ICSI

ICSI is a common procedure and for this reason, it is not described in this handbook (Figure 12). Although it is preferable to prepare sperm freshly before ICSI, it does not appear problematic to use sperm prepared on the day of egg collection or the day after for oocytes matured 48 hours after egg retrieval. Commercially available ICSI medium and PVP solution can be used to prepare the ICSI dish. It is also appropriate to use Oocyte Washing Medium for preparation of the ICSI droplets because the pH of the Oocyte Washing Medium is quite stable at room temperature and atmosphere. However, it is important to note that the ICSI dish should be prepared at least one hour before ICSI and kept at 37ºC in the incubator or on warm stage or plate for equilibration. After ICSI, the individual oocyte is transferred into a droplet (20 µl) of Embryo Maintenance Medium in a Petri dish for culture in the incubator.

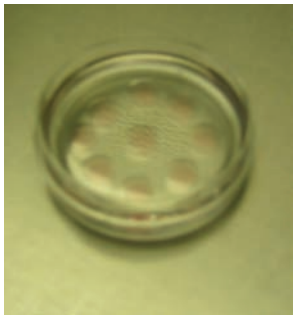


Figure 11
Preparation of fertilization and development dish with Embryo Maintenance Medium when insemination is performed by ICSI. Several 20 µl of droplets are covered by mineral oil in a Petri dish (Falcon, 35 X 10 mm).

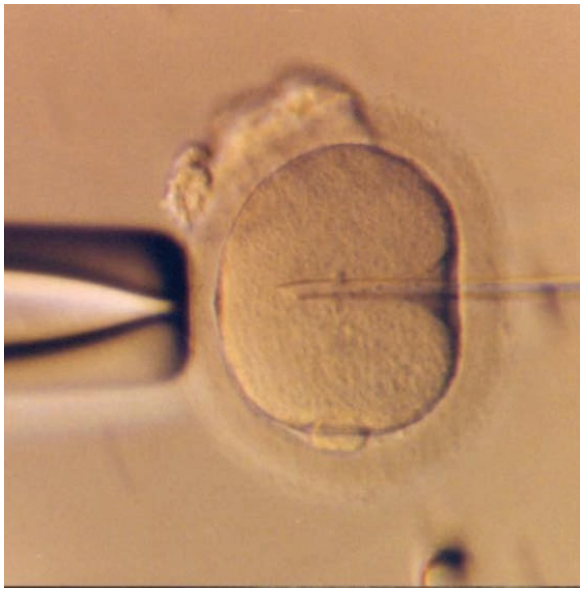


Figure 12
ICSI is recommended for the insemination of in vitro matured oocytes because we believe that this method offers a greater chance of successful fertilization than does IVF.

IDENTIFICATION OF FERTILIZATION

Sixteen to 18 hours after ICSI, fertilization of the oocytes is checked under a microscope for the appearance of two distinct pronuclei (2PN) and two polar bodies (sometimes, the polar bodies are fragmented). Pronuclear scoring can be carried out by rating embryos as "good" if they have aligned nucleoli, a cytoplasmic halo and abutting pronuclei or other systems. However, at this point, it is not necessary to transfer the fertilized oocytes (2PN embryos) into another medium (dish) for continued culture.

EMBRYO CULTURE

The fertilized oocytes are to be cultured in the droplets (20 µl) under mineral oil for one or two additional days, depending upon the number and quality of embryos achieved. If blastocyst culture is requested, the cleaved embryos should be transferred to new droplets (20 µl) in a Petri dish containing the same Embryo Maintenance Medium under mineral oil two days after ICSI. The cleaved embryos can be cultured until blastocyst stage in this Embryo Maintenance Medium.

Embryo Transfer

It seems that most embryo transfers (ET) in IVM treatment can be done on day 2 or day 3 after ICSI because no extra benefit is derived by culturing the embryos to blastocyst stage if the available number of embryos is small. In general, ET should be performed on day 2 after ICSI if the number of embryos obtained is ≤3; ET should be performed on day 3 after ICSI if the number of embryos obtained is ≥4. ET with blastocyst should only be considered if a total of more than four good quality 4-cell stage embryos are achieved on day 2 of embryo assessment after ICSI.

SCORING EMBRYOS

The scoring of cleavage-stage embryos for transfer is very crucial for pregnancy potential. Since the oocytes may not be matured and inseminated at the same time following maturation in culture, the developmental stages of embryos may be variable in the same patient. Therefore, before ET, all embryos for each patient should be pooled and selected for transfer (Figure 13). The final outcome of pregnancy may depend to a great extent on the experience of the embryologist. The cleavage speed of embryos and the morphological marker of each cleaved blastomere are usually used for scoring the embryo quality of in vitro matured oocytes. It is recommended that a maximum of three embryos be transferred into the uterus, based on the quality of embryos or if blastocysts are obtained the number of embryos for transfer should be only one or two. It is not true that transferring a greater number of poor-quality embryos increases pregnancy and implantation rates.

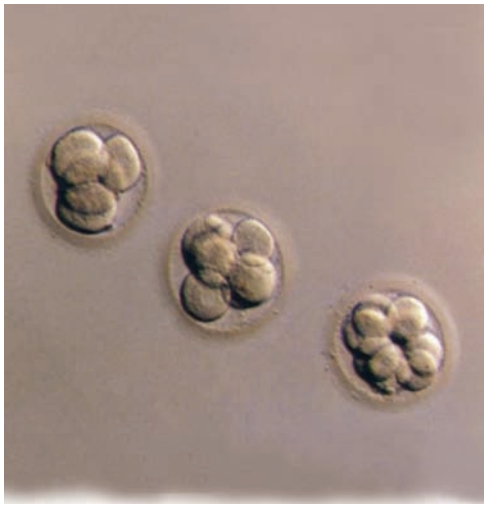


Figure 13
Embryos are pooled together 2 or 3 days after ICSI. Note that the embryos are at different stages of development.

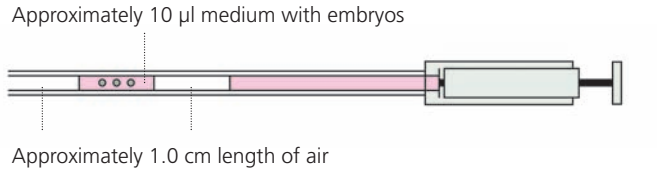


Figure 14
A diagram of embryo loading in ET catheter. Embryo with transfer medium loading into the ET catheter should be approximately 10 µl.

THICKNESS OF ENDOMETRIUM

On the day of ET, endometrial thickness should be measured by transvaginal ultrasound scan. At this point, the endometrial thickness should be at least ≥7.0 mm. If the endometrial thickness is <7.0 mm, the embryo should be cryopreserved and transferred in a subsequent cycle.

EMBRYO LOADING

It seems that selecting a good ET catheter is important for establishing successful pregnancy. The ideal ET catheter should be very soft and its tip should be rounded to prevent any trauma to the endometrial lining. In addition, it should be easy to aspirate embryos through the tip under the microscope without air bubbles. Furthermore, the ET catheter should be smooth enough to prevent the embryos from getting stuck to the inner wall of the catheter. Rinsing the inner wall of catheter with a syringe is very important prior to ET. At this point, the Oocyte Washing Medium (2-3 ml) contained in a test tube can be used for rinsing the inner wall of the catheter with a syringe. Embryos with transfer medium loading into the ET catheter should not be more than 10 µl (Figure 14).

EMBRYO TRANSFER

One of the final key contributory factors to a successful pregnancy is embryo transfer. Careful attention must be paid to both the scientific and clinical aspects of this event. A trial or mock transfer prior to the actual transfer provides very useful information to ensure a curved cervical canal, ascertain the position of the uterus and to any avert foreseeable problems during the actual transfer. It is important that as much mucus as possible is removed from the cervix with a sterile cotton ball before ET. An abdominal ultrasound guided ET may be recommended in selected cases in order to confirm that the embryos with the fluid contents of the catheter are in the uterus.

LUTEAL SUPPORT

To encourage the potential pregnancy, luteal support is required for the patient in the form of progesterone and/or hCG administration. Normally, luteal support for IVM treatment is provided by 200 mg intra-vaginal progesterone (Prometrium) three times daily, or progesterone injection intramuscularly subcutaneously with instruction starting from the day after egg collection and continuing until the pregnancy test.

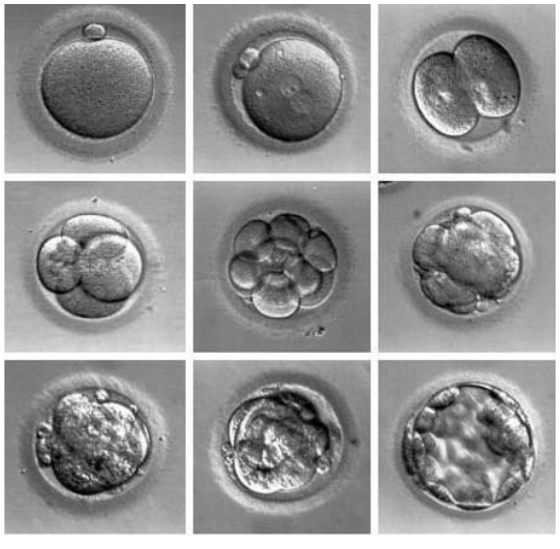
PREGNANCY TEST

A pregnancy test should be performed 14 days after ET. The level of hCG in serum or plasma should be raised to a value of ≥25.0 mIU/ml as a positive pregnancy. Also, if necessary, a second test can be arranged two days later to ensure a positive pregnancy. Normally, the values of hCG should be doubled by the second pregnancy test. At this point, the patient should continue taking progesterone and estradiol. Thereafter, hCG positive patients are scheduled to return for a trans-vaginal ultrasound scan two weeks later to ensure the presence of a fetal sac and heartbeat. It is recommended that the patient continue taking progesterone and estradiol until 10 weeks after ET, if a fetal sac and heartbeat are noted by trans-vaginal ultrasound scan at 4 or 5 weeks after ET. The rest of pregnancy management should be the same as a spontaneous pregnancy.

Pregnancy Outcome

In general, clinical pregnancy and implantation rates of IVM treatment have reached approximately 30-35% and 10-15%, respectively, in infertile women with PCO or PCOS. Based on recent survey, more than 1,000 healthy infants have been born following immature oocyte retrieval and IVM.

Observation From In Vitro Matured Oocyte To Embryo (Blastocyst Stage)



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