

Preliminary Experiences with IVF & ET at Maharaj Nakorn Chiang Mai Hospital

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Abstract : *This is the first report of 35 patients who registered for IVF-ET at Maharaj Nakorn Chiang Mai Hospital from January 1 to September 30, 1991. The patients' ages ranged from 29-42 years, with a duration of infertility of 2-16 years. Gonadotropin releasing hormone agonist and human menopausal gonadotropins were prescribed for superovulation. Follicular growth was monitored by serial measurement of serum estradiol levels and pelvic ultrasound. Human chorionic gonadotropin was given intramuscularly when sonogram demonstrated two or more follicles ≥ 17 mm in diameter. GnRH-a was discontinued and oocyte retrieval was performed 34-36 hours later. Sperm was prepared by the washing and swim-up technique. Mature oocyte was inseminated with 100000 motile sperms and was observed for evidence of fertilization 12-16 hours later. Embryo transfer was performed 48-72 hours after oocyte was retrieval.*

Of the 336 harvested oocytes, 183 (55.3%) achieved fertilization. Embryo transfer was done in 37 cycles at 2- to 8-cell stages and resulted in 4 clinical pregnancies (10.8% of ET). Two pregnancies ended in spontaneous miscarriages at 11 weeks of gestation, the other two are well on-going. (Thai J Obstet Gynaecol 1992;4: 23-32.)

Key words : IVF & ET, preliminary experience

Stephoe and Edwards⁽¹⁾ reported the birth of the first baby after *in vitro* fertilization and embryo transfer (IVF-ET) in 1978. It opened a new era in the treatment of infertility. Today,

many clinics around the world are practicing human IVF with varying degrees of success. In this report, we describe our preliminary experience with this technique at Maharaj Nakorn

Chiang Mai Hospital.

Materials and Methods

Setting of the unit

Maharaj Nakorn Chiang Mai Hospital is the only University Hospital in Northern Thailand and a major tertiary care center. The team of assisted reproduction consists of four full-time reproductive endocrinologists and infertility specialists with two full-time scientists. All hormonal assays are done in the human reproduction laboratory, using commercial radioimmunoassay kits. Oocyte/embryo culture and medium preparation are done in another separate laboratory, which is located in a semi-sterile area inside the delivery suite. The culture laboratory is within a short walking distance from the operating room where oocyte pick-up is routinely performed. Media preparations, dilutions and transfers are done under a vertical laminar flow hood. Ham's F-10 medium is used in our system. The medium is prepared once a week by dissolving 0.981 g of F-10 nutrient mixture powder (GIBCO, New York, Cat. No. 430-1200 EB) in 100 ml of ultra-pure (HPLC-grade) water (SeroMed®, Berlin) in a sterile disposable tissue culture flask (Nunclon®, Denmark). To this are added the followings: calcium lactate 0.0308 g, sodium bicarbonate 0.2106 g, penicillin-G sodium 0.0075 g and streptomycin sulfate 0.0075 g (Sigma cell culture reagents®, USA). The culture medium is checked for osmolar-

ity, which invariably works out between 280 and 285 milliosmol/kg. The culture medium is then filtered through disposable syringe filter (Minisart® 0.2 µm, Sartorius) using a sterile 20-ml disposable syringe. Disposable 4-well multidish (Nunclon®, Denmark) is used for culture of gametes and embryos. Disposable 25 and 50 µl micropipettes (Clay Adams, NJ) are packed individually in aluminium foil and sterilized by heat in a hot air oven. These micropipettes are used to transfer harvested oocytes/embryos instead of Pasteur pipettes. Other glassware and equipment which come into contact with gametes are rinsed with distilled-deionised water and soaked in 1% hydrochloric acid solution for 60 minutes, then flushed through with distilled-deionised water, soaked in hot 1% solution of "7X" tissue culture detergent for 10 minutes, rinsed three times in distilled-deionised water and soaked overnight with ultra-pure water before sterilization by dry heat at 120° C for 6 hours.

Patient selection

Pretreatment screening involved a review of relevant medical history, a complete physical examination of the couples and basic infertility investigations which included at least two semen analyses; confirmation of ovulation and corpus luteum function with endometrial biopsy or mid-luteal serum progesterone assay; post-coital tests and laparoscopic tubal patency tests. A hysterosalpingogram was not

routinely required prior to treatment. Patients with tubal problems were treated surgically. Oligospermic males, defined as a sperm count of less than $20 \times 10^6/\text{ml}$, were treated with mesoterolone acetate for 6 months, with or without split-ejaculate and intrauterine insemination. If these conventional therapies were unsuccessful, they were considered for IVF-ET after a detailed discussion of the procedure.

Controlled ovarian hyperstimulation

In our clinic, both long and short ovarian stimulation protocols, as described by others⁽²⁾ are in use. The allocation of patients to these two treatments was made at the discretion of the attending physicians, and not randomly. In brief, buserelin acetate (Suprefact®, Hoechst) was administered intranasally at a dose of 100 µg six times per day, starting in the mid-luteal phase of the preceding cycle in the long protocol, or on the first day of the current cycle in the short protocol. In both protocols, human menopausal gonadotropins (hMG 75 IU, Pergonal®, Serono) were given 2-4 ampoules intramuscularly, according to patients' age and previous responses, from cycle day 3 onward. Pelvic sonogram was done on the first day of the cycle as baseline, and then on a daily basis from day 8 of the cycle to monitor follicular growth. Daily measurement of serum estradiol by radioimmunoassay was done from day 6 of the cycle. Human chorionic gonadotropin (hCG, Profasi®, Serono)

10000 IU was given intramuscularly when ultrasound demonstrated two or more ovarian follicles exceeding 17 mm in diameter and estradiol level reached ≥ 300 pg/ml per dominant follicle. Buserelin was stopped on the day of hCG injection and oocyte retrieval was scheduled 34-36 hours later.

Oocyte recovery

Transvaginal oocyte retrieval under ultrasonic guidance was performed on an out-patient basis. The procedure was done in the morning after at least 6 hours of NPO, under intravenous pethidine (75mg) and diazepam (10mg) sedation. The analgesic and sedative were given just prior to the operation. The patient was placed in the lithotomy position and draped as in vaginal operation. The vagina was then thoroughly cleansed with normal saline solution. The 5-MHz vaginal ultrasonic transducer was covered with a sterile rubber glove. To improve the ultrasound image, contact jelly was placed inside between the transducer and the glove. A specially designed needle guide was then attached to the transducer and the transducer was connected to an Aloka SSD-650 ultrasound scanner with a viewing monitor. In the puncture mode, a dotted line representing the needle path was displayed on the monitor. A disposable double-lumen 16-gauge, 30 cm long needle with echogenic tip (Cook, Australia) was used for follicular aspiration. The

needle was rinsed with heparinized phosphate buffer saline (PBS) before being connected to the suction system.

The vaginal probe, with the needle guide in place, was introduced into the vagina. The pelvis was scanned and the number and size of follicles noted. Usually, we aspirated the most proximal follicles that were located in line with the needle path first. After determining and selecting the angle of puncture, the needle was placed in the needle guide and advanced into the follicle with a sharp jabbing action. Negative pressure of 100 mmHg was applied to collapse the follicle under vision on the ultrasound monitor. During aspiration, the needle was gently rotated and moved in all directions around the inner circumference of the follicle to give a curettage effect which increased the chance of oocyte collection. We routinely flushed each follicle twice with heparinized PBS to ensure recovery of oocyte. The flush volume used was approximately three-fourths of the original follicle volume. The operator then moved onto the next follicles and the same procedure was repeated until all follicles larger than 1 cm had been aspirated. The patient was observed for 2 hours and then discharged home on doxycycline 200mg/day for 5 days as prophylactic antibiotic.

Fluid from follicular puncture was examined carefully to recover oocytes under a dissecting microscope. Each harvested oocyte was placed in 1 ml of Ham's F-10 supplemented with 10% patients's serum in a 4-well

plate, and kept in an incubator under 5% CO₂ in air at 37° C.

Sperm preparation

Male partners were requested to produce semen samples after oocyte retrieval. Semen was allowed to liquefy for 30 minutes at room temperature before analysis, and the results were recorded on a standard form. Semen was then gently mixed with Ham's F-10 at a ratio of 1:2 (vol/vol) and centrifuged for 10 minutes at 300xg. The supernatant was discarded and the sperm pellet resuspended in 1 ml of medium. After a second wash, 0.5 ml of medium was layered onto the loosened pellet and motile sperm were allowed to swim up for 45 minutes in a 5% CO₂ incubator at 37° C. Sperm concentration in the supernatant was reassessed and adjusted to 100000/25 µl.

Oocyte insemination

After 4 hours of preincubation, mature oocytes were inseminated with 100000 actively motile spermatozoa and left undisturbed at 37° C in a humidified atmosphere of 5% CO₂ in air. After 12-16 hours, the ova were observed for evidence of fertilization and were transferred into fresh medium. Unfertilized oocytes were reinseminated in the same way.

Embryo transfer

The embryos at 2- to 8-cell

stages were transferred into the uterine cavity using Tom Cat catheter 48-72 hours after oocyte retrieval. In brief, the patients were placed in the lithotomy position. The cervix was exposed with a bivalve speculum and the ectocervix was gently cleansed with normal saline solution. The embryos were loaded into the catheter with 30 μ l of culture medium supplemented with 50% patient's serum. The catheter was passed through the cervical canal into the uterine cavity until it reached the fundus. The catheter was then slightly withdrawn and the embryos were expelled. The catheter was gently withdrawn and promptly checked under a microscope to ensure that the embryos had not been retained. The patients were placed in Trendelenberg's position for 30 minutes and then discharged home.

Luteal phase support

All patients received hCG (Pregnyl®, Organon) 1500 IU intramuscularly on the day of embryo transfer and on days 3, 6 and 9 after the transfer.

Diagnosis of pregnancy

Pregnancy was diagnosed on day 14 post-embryo transfer when the level of serum β -hCG was greater than 25 mIU/ml, followed by a higher level in a subsequent assay 2 days later (biochemical pregnancy). Clinical pregnancy was diagnosed when a gestational sac was visualized

under vaginal ultrasound, which should be clearly seen from day 35 post-embryo transfer.

Results

Thirty-eight couples were enrolled for 46 cycles of IVF-ET. There were eight patients who underwent the IVF procedure twice. Of the 46 cycles, three cycles in three patients were cancelled before oocyte retrieval, two because of poor ovarian response to hMG and one because of decreasing estradiol level before oocyte pick-up.

The average age of the remaining thirty five patients was 31 years (ranged 29-42 years). Twenty eight of them had primary and seven had secondary infertility, for an average duration of 6.8 years (ranged 2-16 years). Their infertility diagnoses are shown in Table 1. One couple had both tubal obstruction and male factor infertility. Long ovulation induction protocols were used in 26 cycles and short protocols in 17 cycles. On the average, 29.7 ampoules of hMG were needed in the long protocol versus only 21.2 ampoules in the short protocol. Serum estradiol (E2) levels before hCG administration and the number of oocytes harvested were significantly higher in the long than in the short protocols (Table 2). Prewashed sperm motility increased from 51.9% to 81% after washing.

Three hundred and thirty one oocytes were harvested, but only 183 achieved fertilization, giving an overall

fertilization rate of 55.3%. Low fertilization rates were observed in older couples and in couples with male factor infertility. Unfortunately, we did not keep detailed records of oocytes quality to allow meaningful correlation with fertilization rates. In our series slightly less than 10% of reinseminated oocytes were fertilized. Embryo transfer was done 48-72 hours after oocyte pick-up, when the embryos reached the 2- to 8-cell stages (Table 3).

The average number of embryos replaced was 4.9 (ranged 2-10) and resulted in 4 clinical pregnancies (10.8% of ET). All pregnancies occurred in patients with tubal obstruction and none in the group with male factor infertility. The characteristics of pregnant cycles are shown in Table 4.

Table 1 Infertility diagnosis

Diagnosis	No.
Tubal obstruction	32
Male factor	4

Table 3 Stages of embryo development at the time of embryo transfer

Stages of embryo	Number
Pronuclear	3
2-cell	20
3-cell	8
4-cell	132
5-cell	2
6-cell	5
8-cell	13
Total	183

Two patients with singleton pregnancies had spontaneous miscarriages at 11 weeks 4 days and 11 weeks 2 days gestation. Chromosome study of the first abortus was normal 46 XX and the other was trisomy-21. The remaining two pregnancies are well on-going. One is a singleton pregnancy and the other is a triplets, at 22 and 10 weeks gestation respectively.

Discussion

Although indications for IVF-

Table 2 Outcome of IVF-ET

	Long protocol	Short protocol	P*
Number of cycles	26	17	-
Number of hMG used (ampoules)	29.7 ± 12.8	21.2 ± 10.7	< 0.02
Serum E2 level (pg/ml)	2173.1 ± 880.3	1391.2 ± 543.5	< 0.002
Number of oocytes harvested	9.7 ± 6.1	5.5 ± 3.4	< 0.01
Pregnancy	3	1	-

* Student's t-test was used for analysis

Table 4 Characteristics of pregnant cycles

Women's Age	Men's Age	E2# at OPU	hMG* (amp)	Oocytes retrieved	Oocytes fertilized	Embryos transferred	Stages			
							2-cell	3-cell	4-cell	8-cell
35@	36	1550	24	8	6	6	1	2	3	-
33\$	36	1300	24	7	5	5	1	-	4	-
30^	32	2500	16	14	12	10	1	-	5	4
35	37	2000	16	4	4	4	1	-	2	1

E2 in pg/ml, OPU = oocyte pick-up

* One ampoule of hMG contains 75 IU FSH

@ Triplet pregnancy

\$ Abortion with Down's syndrome

^ Spontaneous abortion at 11 weeks gestation

ET in our series of patients are limited only to tubal obstruction and male factor infertility, others^(3,4) have extended their indications to treat patients with other causes of infertility as a therapy of final resort in conditions such as hostile cervical mucus, immunologic infertility, ovulatory disorder, endometriosis, unexplained infertility and in ovum donation.

It has been customary for most IVF programs not to offer treatment of patients above the age of 40⁽⁵⁾. We began with the same rule in respect of age. However, this rule was occasionally violated to accommodate patients who persistently request IVF as a therapy of final resort and could afford the cost of treatment. In our series, there were two such patients who were willing to try IVF despite the known fact of a diminished success for this age group. One of them had poor response to hMG and the cycle was cancelled prior to oocyte retrieval, the other ended up in embryo

transfer but did not become pregnant. In view of the fact that pregnancies from IVF have been reported in patients at age 40 and above⁽⁵⁾, we believe it is justified to consider IVF in a few selected patients around the age of 40 years.

In most instances, there is no effective therapy for infertility⁽⁵⁾. With the discovery that relatively few spermatozoa were required for IVF, it was proposed that subnormal semen samples could be used to fertilize in vitro if sufficient numbers of spermatozoa could be obtained⁽⁶⁾. In our series, couples with male factor experienced very low fertilization rate and no pregnancy occurred in this group. However, others have reported success with IVF for the treatment of male infertility⁽⁷⁾. With the promise of newer techniques, such as zona drilling, sperm microinjection and sperm stimulation still on the horizon, IVF may be of major use in the future treatment of male infertility⁽⁸⁾.

In our study, significantly more ampoules of hMG were needed in the long versus the short protocol, probably reflecting the absence of endogenous pituitary contribution when hMG stimulation was begun after down regulation has been accomplished⁽²⁾. Moreover, the number of oocytes harvested was also significantly higher in the long than in the short protocol. Our data seem to support Mordel et al⁽⁹⁾, who reported that only the long protocol achieved significantly better result over hMG-only stimulated cycles in the number of aspirated oocytes. However, we would like to point out that the observed differences in our results could have been influenced by other confounding factors because patient allocation to either the short or long protocol was not made at random. In a study by Frydman et al⁽¹⁰⁾, they could not demonstrate any advantage of long versus short protocol regarding folliculogenesis, oocytes recovered and pregnancy rates. Until the advantage of either protocol is established, we feel that the choice should be tailored according to the patients' convenience, cost and side effects.

Observations from many clinics have been that the incidence of pregnancy increases with the number of embryos replaced⁽¹¹⁾. However, because of the concern about multiple pregnancies of higher orders ($\geq$ triplets), many fertility centers restrict the number of embryos transferred to 3 or 4^(11,12). In so doing, difficulty arises in the selection of quality embryos. To

date a precise method to determine the quality of human conceptus is not available. So far, cleavage rates and morphological criteria have been only rough indicators of viability.⁽¹³⁾ Pregnancy has been established after transfer at 2-, 4-, 6-, 8-, 16-cell morula or blastocyst stage of concepti; homogeneous and regular blastomeres implant, as well as those with blebs and granular aspects⁽¹³⁾. To by-pass this technical problem and to avoid the ethical dilemma of discarding surplus human embryos, we decided to replace all fertilized ova unless the couples specify otherwise such as donation to other couples. We agree that in places where cryofacilities are available, it is advisable to replace fewer embryos for each cycle, with consideration given to cryopreservation of excess embryos for transfer in subsequent cycles.

Embryo transfer is an important step which requires skill and patience. Any delay or difficulty in entering or passing through the cervical canal can result in changes in the composition of the culture medium. This, in turn, may jeopardize the embryos and reduce the chance of implantation⁽¹⁴⁾. Steptoe et al⁽¹⁴⁾ suggested that the use of a single cannula instead of the outer sleeve and inner cannula made the placement of embryo easier, which is in agreement with our experience using Tom Cat catheter. In our series, we encountered difficulty in passing the catheter into the uterine cavity in 5 patients and had to dilate the cervix using the

smallest Hegar's dilator.

In this report, the pregnancy rate is only 10.8% per embryo transfer, which is relatively low compared to other well-established centers⁽⁴⁾. This may be partly due to our lack of expertise in this relatively new field of assisted reproduction. However, we hope to achieve a better result in the future.

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Addendum :

Since the first submission of this manuscript, we have achieved 4 more IVF pregnancies (as of February 7, 1992), giving the current pregnancy rates of $8/67 = 11.9\%$ per cycle or $8/59 = 13.5\%$ per embryo transfer. The first IVF baby in Northern Thailand was born by caesarean section at Maharaj Nakorn Chiang Mai Hospital on January 7, 1992. It was a boy, weighing 3680 g. The triplets in this report is now at 28 weeks of gestation.

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