OUR EXPERIENCE OF *IN-VITRO* FERTILISATION AND EMBRYO TRANSFER

by

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SUMMARY

With limited resources and restricted facilities, the infrastructure of an I.V.F. clinic has been initiated in Calcutta since Sept., 1983.

One hundred and four infertile couples have been monitored and sixtyfive were accepted in the treatment programme.

Details of patient assessment, stimulation regime, monitoring of response to ovarian stimulation, culture and transfer technique have been outlined.

Results in two stages of our work (September, 1983 to December, 1984 and January, 1985 to August, 1985) have been reported. Though we have not been able to achieve an ongoing pregnancy so far, our results in terms of oocyte recovery, fertilisation and cleavage rates have significantly improved in the second phase (70%, 70% and 58.3% respectively) compared to those (52.2%, 43.5% and 40%) in the first phase of our experience.

Introduction

Infertility is a major area of interest in our clinic. In order to provide the service as comprehensive as possible, the essentiality of establishing an *in vitro* fertilisation programme was clearly indicated, specially for the treatment of patients with irreparable tube damage. An attempt to initiate such a programme has been made, bearing in mind the availability of skilled manpower, the limitation of resources and above all the restricted budget.

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Accepted for publicaton on 23-12-85.

Implementation and Stage-wise Upgrading of the Programme

We undertook the project since 1982. The initial phase of the work was aimed to achieve reasonable experience in monitoring of ovarian stimulation, recovery of preovulatory oocytes by laparoscopic aspiration and attempt to fertilise them *in-vitro*. But it took another year when we could standardise the culture technique to achieve the first biochemical pregnancy following transfer of *in-vitro* fertilised egg grown in culture (Reported to BOGS & FOGSI, September, 1983). The details of gradually developing technology and the results of our clinic were presented recently in III World Congress of *In-vitro* Fertilisation and Embryo Transfer held in Helsinki, Finland (Chakravarty and Ghosh Dastidar, 1984). The objective of this communication is to present the current work-up of our clinic with results we have been able to achieve so far in our environment.

Selection and Assessment of Couples for In-vitro Fertilisation Embryo Transfer

Indications

Irreversible tubal damage or bilateral tubal resection for ectopic pregnancies were the main indications for I.V.F. A check laparoscopy was performed as a routine to assess laparoscopic assessibility of the ovaries for subsequent oocyte recovery. Gross oligospermia with or without moderate grades of asthenospermia was the second indication. Few cases of unexplained infertility were also included in the I.V.F. programme.

Acceptability of the programme by the couple

Success rates in other centres and probable success rate in our centre are discused. Theoretical probability of congenital malformation of the baby is explained though a major defect has not yet been reported amongst several hundred babies born by I.V.F. and E.T. so far (Lopata *et al*, 1980).

Ability of the husband to donate semen on demand is ascertained. Once in the programme, the couple is informed that they may have to follow an irregular time schedule during monitoring of ovulation, timed laparoscopic oocyte recovery and transfer of cleaving embryo.

While explaining these points the psychological set up of the husband and wife are assessed and those who are hesitant are excluded from the programme.

Bacteriological study of husband's semen and wife's cervical mucous

Routine culture of semen and cervical mucus is carried out and when infection is detected, appropriate antibiotic therapy is instituted till the culture becomes sterile.

Sperm survival test

Apart from motility and total count, 24 hour sperm survival in the specific media (in identical conditions to be subsequently used for I.V.F.) is always carried out in the monitoring cycle.

Trial transfer and Endometrial dating

Trial transfer with the embryo transfer catheter is carried out in the monitoring cycle as 70-80 per cent of nulliparous women will have a cervical spur at the region of the internal os. If present, the cervical canal is dilated in the pretreatment cycle.

Strip biopsy in the luteal phase is obtained to exclude the existence of "Out of Phase" endometrium. Such endometrium is unsuitable for implantation and indicates administration of progesterone in the luteal phase of the treatment cycle.

Monitoring of ovulation and endocrine profile

This is done in two consecutive pretreatment cycles—one unstimulated and the other stimulated cycle. Stimulation in the pretreatment cycle is given with clomiphene citrate 150 mgm daily from day 3 to day 7. Monitoring of ovulation and endocrine profile in the monitored cycle is carried out by BBT, cervical mucus, radioimmunoassay and ultrasonography. The objective is to determine the preovulatory LH surge in order to ascertain the exact time of ovulation (estimated time being 21-27 hours after spontaneous LH surge and 34-36 hours after stimulation with exogenous MCG). Levels of plasma oestradiol in the late follicular phase and plasma progesterone and prolactin in the mid luteal phase are evaluated by radioimmunoassay. The findings are corroborated by serial ultrasonographic measurement of growth and size of follicular diameter and follicular fluid volume from day 10 to day 14. In our clinic we have evolved a new cervical scoring system—slightly different from what has been described by Insler. This has been designed by one of us (S.G.D.) and has been named as "Hanging Drop Cervical Score". With few exceptions, this hanging drop score has become a valuable and quick clinical guide for pinpointing the oestradiol peak which means that LH surge is imminent. From this the exact time of exogenous HCG stimulation in the treatment cycle can be ascertained. Timing of HCG injection is important as premature injection will result in recovery of immature eggs and consequently of bad quality embryo (Wood and Kovacs, 1983). Our prediction by cervical score for optimum follicular maturation, oestradiol peak and hence anticipated LH surge has been corroborated by ultrasonography and laparoscopic mature oocyte recovery in 90 per cent of cases. The details of hanging drop score, its interpretation and correlation with Insler's score are given in Figs. 1 and 2.

Stimulation for Multiple Follicular Maturation in the Treatment Cycle

Clomiphene citrate (Fertotab, Biddle—Sawyer 100 mgm daily) is given from day 3 to day 7 combined with Human menopausal gonadotrophin (Pergonal-Serono) 2 amps each on day 6, 8 and 10. Though superovaluation may bring about many endocrinal disasters tending to antagonise subsequent implantation of the embryo and adequate luteal function (Lopata *et al*, 1980; Williams *et al*, 1979; Karsch *et al*, 1973), the regime at present followed in most of the clinics is either clomiphene/HCG, Clomiphene/HMG/HCG or HMG/HCG; pharmacological dose schedule is adjusted to guard against hyperstimulation (Michelmann *et al*, 1984; Katainen and Nikkanen, 1984; Vankooji *et al*, 1984). Jones et al, 1984, prefer pure FSH (Metrodin-Serono) rather than HMG.

The ovarian response is monitored by daily real time ultrasound scan (Fig. 3), cervical mucus study and estimation of plasma oestradiol level. Ultrasonic measurement of 18 mm or more of transverse diameter of the dominent follicle, a value of 1000 pg/ml or more of plasma oestradiol 17 ß (each mature follicle elaborating 400 pg/ml of oestradiol) and grade III cervical mucus (hanging drop cervical score) are considered to be dependable optimum parameters for induction of ovulation with exogenous HCG (Profasi-5000 I.U.—Serono) injection. Laparoscopy is scheduled approximately 36 hour following HCG administration.

Follicular Aspiration and Oocyte Recovery

The bulging ovarian follicles measuring 1.5 cm. or more with occasional formation of a stigma are identified through laparoscope. The ovarian ligament is grasped with Palmar's forceps as close to the ovary as possible. The follicles are punctured sharply in such a way that the opening is not big enough to allow escape of follicular fluid into the peritoneal cavity. The aspiration is carried out by a teflon coated two way metallic needle (Craft's—Rocket of London) attached to an electronically monitored foot controlled suction pump. The foot control maintains the prefixed aspiration pressure of 100 to 120 mm of Hg. The aspirate is collected in 10 ml. sterile, siliconised glass test tubes containing 1 ml. heparinised (1 I.U./ml) culture media (Figs. 4 & 5). Gentle rinsing of the follicle with culture media and repeat aspiration is sometimes necessary if oocyte has not been recovered by primary attempt. Aspiration of oocytes in the straw coloured follicular fluid aspirate is immediately confirmed under low power stereo dissecting microscope in the laboratory situated just by the side of the operating theatre (Fig. 6).

While laparoscopic oocyte recovery is still practised in most of the centres there are others who are practising ultrasonically guided follicle aspiration under local anaesthesia (Feichtinger and Kemeter, 1984; Goswammy *et al*, 1984). This method is more suitable in cases where laparoscopically ovaries are inaccessible due to pelvic adhesions.

Identification and Incubation of Aspirated Oocytes

Correct grade of tissue culture petri dishes (Sterilin, U.K.) are extremely helpful for speedy identification and grading of morphological maturity of the oocyte. The grading of maturity of the oocyte is determined by the density of the cumulus cells surrounding the egg. Immature eggs are clustered by dense, while the mature ones have thin layers of cumulus cells surrounding the zona pellucida (Fig. 7). The eggs after identification are transferred by glass pipettes to clear follicular fluid containing 5 I.U. HCG, Pencillin (100 I.U./ml) and incubated at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% Nitrogen, relative humidity (RH) being 98 per cent (Fig. 8). All subsequent steps of experiments are carried out strictly in the similar environment.

Oocytes classified as mature (Grade I) and nearly mature (Grade II) are incubated for 8 and 12 hours respectively prior to insemination. Immature oocytes (Grade III) are subjected to preinsemination incubation for about 24 hours.

Preparation of Sperm Suspension, Insemination and Fertilisation

Two hours before the scheduled time for insemination, husband's semen is collected in a sterile glass container and is allowed to liquify in room temperature. 0.5 ml of liquified semen is then diluted with 2.5 ml of Ham's F-10 media containing 10 per cent heat inactivated maternal serum. The whole mixture is centrifuged twice at 300 G for 10 minutes each time, the supernatent fluid being decanted off and the sperm pallet at the bottom is resuspended with 2 ml culture media. After 2nd centrifugation the sperm pallet mixed with culture media is incubated at 37°C for 45 minutes. After incubation the most vigorously motile spermatozoa float at the top of the suspension in the tube. 95-100% of these floating sperms are collected and a fresh

suspension consisting of 1 to 5 x 1^5 sperm/ml is prepared using Ham's F-10 media and 10% inactivated maternal serum. The counting of sperm is done in Makler's chamber (Fig. 9).

The oocytes are taken out of incubation and washed quickly in 2-3 changes of Ham's F-10 media at 37°C in a petridish using the disecting microscope and then transferred to culture tubes (Falcon Plastics or Nunc Well, Denmark). The tubes are then incubated in identical condition for about 14 hours. Culture under cover of paraffin (as practised by Bourne Hall Clinic) is not done in our laboratory —for possible toxic factors which may exist in the quality of paraffin which we get in India.

Preparation of the Media

Culture media is freshly prepared by dissolving Ham's F-10 powder (GIBCO Laboratories 430-1200 USA) in 3 times distilled water. Approximately 2.1 gms of Sodium Biocarbonate is added per litre of culture media. Prepared media is filtered through 'Milex' luer adaptable micro filter (Milipore USA) and stored in refrigerator. The pH of media is always equilibriated in the gas phase 5% CO_2, 5% O_2 and 90% N_2 for at least 12 hours before actual use. Osmolarity of the medium is adjusted to 280 m.osm. Ham's F-10 is a complex media containing more than 180 ingredients. A controlled trial to find the best and simple media is on the way (Wood and Kovacs, 1983).

Embryo Culture

After the incubation period, the media containing sperms and oocytes are quickly inspected under the disecting microscope for evidence of fertilisation. Presence of pronucleus or polar bodies in the perivitelline space confirms evidence of fertilisation (Fig. 10). They are quickly transferred to the growth media containing 15% maternal serum in freshly prepared Ham's F-10 media mixed up with 75 I.U.% FSH and LH (Pergonal-Serono) and incubated in the same environment.

24 hours after incubation in growth media the eggs are inspected under inverted microscope using phase optics to record cleaving blastomeres. The embryo is judged to be developing normally in culture if the cells divide uniformly every 10-12 hours (Steptoe and Edwards, 1979) and the dividing cells are approximately equal in size, uniformly regular in shape homogenous in appearance and occupy most of the space within the zona pellucida (Fig. 11).

The optimum age of embryo transfer is uncertain, as pregnancies have occurred following the transfer of 1-, 2-, 4-, 8- and 16- cell embryos (Steptoe *et al*, 1980; Lopata *et al*, 1980; and Wood *et al*, 1981c). Feichtinger and Kemeter (1984) have reported encouraging results following transfer of good quality pronucleate/syngamic eggs. We usually transfer between pronucleate to 8 cell stage (24 to 60 hours after insemination). Controlled trials will determine the optimum stage of embryo development for uterine transfer.

Technique of Embryo Transfer

Embryos are transferred through cervical canal and should preferably be placed near the fundus. For this the patient should be either in Trendelenberg Gynelithotomy or knee chest position. Intramuscular anaesthesia is not essential but Diazapam orally half an hour before transfer is desirable. Specially designed transfer bullet tipped or Crafts embryo transfer catheter (Fig. 12) may be used. Ext. os

is exposed by a bivalved speculum. Grasping of cervix is avoided in order to prevent uterine irritability. Embryo aspirated by a tuberculin syringe fitted with transfer catheter with 0.05 ml of culture media under microscope (Low power stereo) is negotiated through the outer canula into the uterine cavity and deposited near the fundus (Fig. 13). The cannula is withdrawn after one minute and inspected under the microscope to be sure that following transfer the embryo has not returned back to the catheter. The patient is kept in bed for 36 hours and then released.

In order to avoid discordance of luteal phase of the stimulated cycle, transfer of freeze thawed embryo in the subsequent unstimulated menstrual cycle is being speculated (Trounson *et al*, 1981b). Mukherjee *et al* (1978) however reported one success by such procedure.

Post Transfer Monitoring

Blood samples for progesterone are taken every week folowing transfer. In case of implantation !the progesterone level should show gradual rise following temporary fall between 25th to 28th day of cycle. HCG in blood is estimated every week (if positive) from 28th day onwards.

As a supporting measure progesterone (GESTONE, Lily USA) 12.5 mgm to 25 mgm is given every day in the luteal phase following the transfer.

Various factors are involved for success following *in-vitro* fertilisation and embryo transfer. The significant probables include collection of mature or near mature cocytes (Jones *et al*, 1984) speed and gentleness in egg manipulation, transfer of good quality embryos (Trounson *et al*, 1982) transfer of two or more embryos (Wood and Kovacs 1983) case of transfer (Diedrich *et al*, 1984) and physiological receptive uterine environment (Jones Jr. 1984) and a normal or regulated luteal phase (Lopata *et al*, 1980).

Result

Since September, 1983, 104 patients were monitored in the pretreatment cycle. Sixtyfive were selected for *in-vitro* fertilisation and embryo transfer. With increasing experience and expertise of our team the overall results are showing signs of promise. Therefore, we propose to record our results in two phases.

Ist Phase (September 1983 *to December,* 1984)

No. of Laparoscope	46
No. of follicles aspirated	88
No. of eggs recovered	46
No. of eggs fertilised	20
No. of eggs cleaved	8
EMBRYO TRANSFER	8
No. of Pregnancies	3
**Biochemical —2	
Clinical —1 (Abortion 10/52).	
Recovery rate per follicle	52.2%
Fertilisation rate per recovery	43.5%
Cleavage rate of fertilised eggs	40%
Pregnancy rate per Lap.	6.5%

2nd Phase (1st January, 1985 *to August,* 1985).

No. of laparoscope	19
No. of follicles aspirated	47
No. of eggs recovered	34
No. of eggs fertilised	24
No. of eggs cleaved	14
EMBRYO TRANSFER	12
No. of pregnancies	4
**Biochemical —3	
Clinical —1 (Abortion 8/52-	
Recovery rate per follicle	70%
Fertilisation rate per recovery	70%
Cleavage rate per fertilised gg	58.3%

Pregnancy rate per Lap. 21%

**Plasma HCG positive more than 50 mIu/ml but onset of normal period a few days following missed period.

Incidence of abortion after a missed period is very high following I.V.F. & E.T. Wood *et al* (1981b) reported the incidence as 50 per cent. This abortion rate may appear elevated as chemical pregnancies are not taken into consideration when determining the incidence of abortion after natural conception. As the quality of embryos improves, abortion rate can be expected to decline.

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See Figs. on Art Paper I, II, III, IV