Results of the *in vitro* fertilization programme at Tygerberg Hospital, phases II and III

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Summary

Phases II and III of the human *in vitro* fertilization programme at Tygerberg Hospital are presented. In phase II, 42 laparoscopies were performed and oocytes were obtained from 76% of the follicles aspirated, but with a fertilization rate of only 37%. The viable pregnancy rate per embryo transfer was 4%. Important changes took place in the programme, which led to a fertilization rate of 77% in phase III. Of the 78 patients subjected to laparoscopy, 65 (83%) reached the embryo transfer stage, resulting in a clinical pregnancy rate per embryo transfer of 23%, and with a 19% pregnancy rate per laparoscopy. The changes, methods and results of phases II and III are discussed.

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The methods used in the *In Vitro* Fertilization Unit at Tygerberg Hospital and the results of the first 24 laparoscopies and 11 embryo transfers, which resulted in 3 pregnancies, were recently published.[1]

In phase II of the programme (1 January - 30 April 1984) almost the same laboratory methods were used, except for a few small changes. The ovulation-induction drug programme was changed to a combination of clomiphene and human menopausal gonadotrophin (HMG), as outlined by Kerin *et al.*[2] The results were not satisfactory.

A reason for the poor results was sought and changes were instituted as from 1 May 1984. Phase III covered the period 1 May - 31 August 1984. These changes are discussed, as well as the results in phases II and III.

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Patients and methods

Patients accepted into the programme had tubal damage and their male partners had to be fertile, as outlined in our previous article.[1,3]

The ovulation induction programme in phase II was changed to a combination of clomiphene 100 mg and HMG 2 ampoules on alternate days, starting on the second day of clomiphene therapy. The HMG therapy was given 3 times, as discussed in a recent paper by Kerin *et al.*[2] We used 50 mg less clomiphene per day than Kerin's group. Ultrasound examinations were performed on day 1 of the menstrual cycle and the cycle was cancelled if a cyst was detected. Thereafter ultrasound examinations were performed daily after the oestradiol (E_2) values reached a level of 500 pmol/l. When a diameter of 18 mm was reached by one of the follicles with at least one other follicle more than 16 mm in diameter (measured in two planes with a Phillips ST 7000), the HCG 10 000 U was given intramuscularly. The laparoscopy was performed 36 hours later. This was a change from our previous method of giving low doses of HMG or clomiphene 100 mg as the only method of ovulation induction.

The laparoscopic technique also changed. The double-barrel Bourn Hall needle was replaced by the Monash needle, which is 14 cm in length, 17 mm in diameter and lined with Teflon.

Flushing of the follicle took place only if the oocyte was not discovered in the follicular fluid or needle rinse. No heparin was used in phase III but the blood-stained fluid was transferred immediately to the laboratory. The needle was rinsed with 4-times distilled water after the procedure and every needle was relined after 4 aspirations. Dry heat was used to sterilize the needles.

A 3033 Falcon tissue culture tube was used to obtain the follicular fluid and these bottles were prewarmed in an incubator to 37°C in phase III of the programme. This did not take place in phase II.

The follicular fluid was screened in the laboratory in 3003 Falcon Petri dishes. Care was taken to prewarm these dishes before use. The ovum was transported to a 3037 Falcon Petri dish as soon as possible after it was rinsed in T6 medium with no serum added. The rest of the methods followed were outlined in a previous article.[1]

Laboratory methods in phase II were as in phase I. Filtered T6 medium was used, made up once a week (filter 0,22 Gelman). The pH was 7,4 after 24 hours in a 5% carbon dioxide in air incubator (Forma Scientific 3157). The semen specimen was prepared by standard methods and described in a previous article;[1] 100 000 motile spermatozoa were used per ml of insemination medium. Transfer to the growth medium took place after 20 hours. Oocytes were dissected and pronuclei documented (Table I).

In phase III the medium was changed to Ham F10 because the poor fertilization rate in phase II could possibly have been caused by deterioration of the purivate quality in the medium. Fresh purivate could not be obtained immediately. The osmolarity was 280 mOsm/kg and the pH after 24 hours 7,4. The serum in the growth medium was 20%. No filters were used because wetting-agent free filters could not be obtained immediately. Because very satisfactory results were achieved, with no infection to date, reversion to the use of filters did not occur.

All glassware was prepared as described by Whittingham.[4] The Pasteur pipettes were rinsed 3 times before use and handling of oocytes.

In phase III, dissection of the oocyte was seldom performed after 20 hours but cleavage was observed 40 hours after insemination.

In summary, the following changes took place: (*i*) T6 to Ham F10; (*ii*) osmolarity 285 mOsm/kg to 280 mOsm/kg; (*iii*) tubes and Petri

TABLE I. LABORATORY METHODS — COMPARISON OF PHASES II AND III

	Phase II	Phase III
Insemination medium		
Type	**T6 (filtered)**	**Ham F10 (not filtered)**
pH	**7,4**	**7,4**
Osmolarity	**285 mOsm/kg**	**280 mOsm/kg**
Gas phase	**5% CO_2 in air**	**5% CO_2 in air**
Serum	**10% patient's serum**	**10% patient's serum**
Tube/Petri dish	**Falcon 3037 organ culture dish**	**Falcon 3037 organ culture dish — prewarmed to 37° C**
Flushing medium	**Dulbecco's phosphate buffer + T6**	**Ham F10. Prewarmed Falcon 2025 bottle for oocyte retrieval**
Incubation of oocyte	**5-6 hours**	**5-6 hours**
Spermatozoa		
Time obtained	**2-3 hours before insemination**	**2-3 hours before insemination**
Wash	**1 cc semen 2 washes T6 200 g**	**1 cc semen 2 washes Ham F10 200 g**
Incubation in incubator	**30-45 minutes**	**30-45 minutes**
Number of spermatozoa/ml	**100 000 motile**	**100 000 motile**
Time of transfer to growth medium	**18-20 hours after insemination**	**18-20 hours after insemination**
Growth medium	**10% patient's serum + T6**	**20% patient's serum + Ham F10**
Gas phase	**5% CO_2 in air**	**5% CO_2 in air**
Dissection of oocytes: instrument used	**Yes; 18 hours after insemination; No. 30 needle**	**No; 41-43 hours after insemination to observe cleavage**
Observed development	**Pronuclei/cleavage**	**Cleavage occasionally pronuclei**

dishes prewarmed; (*iv*) Pasteur pipettes rinsed 3 times before use; (*v*) 20% human serum in growth medium; (*vi*) no dissection after 20 hours; and (*vii*) a second incubator was bought and has been in use since May 1984.

The embryo transfer technique also changed from that used in phase I in certain aspects. A preliminary examination was performed routinely early in the cycle without premedication. In phase II the dorsal lithotomy position was used if the uterus was in retroversion and the knee-chest position if the uterus was in anteversion. In phase III all embryos were transferred in the lithotomy position and the patient turned to the appropriate position, e.g. onto her back if the uterus was in retroversion.

The Norfolk catheter was used in phase II, but because we ran out of stock was changed to the Tomcat catheter (Monoject, St Louis, Missouri, USA).[2] A special stainless steel catheter was developed by the unit for use in conjunction with the Tomcat catheter in cases where embryo transfer was difficult and the uterus in acute anteversion.

The transfer medium was 10% serum + 90% T6 in phase II and 75% serum + 25% Ham F10 in phase III.[5] The volume was reduced from 90 μl in phase II to 30 - 60 μl in phase III.[6]

The air-bubble technique was used and the catheter introduced into the uterus up to the 6 cm level. In phase III after the fluid was injected, the Tomcat was turned through 180° and removed after 30 seconds.

The cell stage, number of embryos and hours after insemination with regard to the embryo transfer stayed the same in phase III. The most important change was that the transfer took place in the theatre about 30 m from the laboratory in phase II but in phase III this was done in the laboratory right next to the incubators. No medication was given after embryo transfer.

In summary, the following points were regarded as important in phase III: (*i*) only the dorsal lithotomy position was used; (*ii*) Tomcat catheter used as transfer catheter; (*iii*) 30 - 60 μl fluid injected; (*iv*) 75% human serum used; and (*v*) change to the laboratory as a transfer area not only made it more convenient but reduced the time involved dramatically (Table II).

Results

In phase II of the programme 42 laparoscopies were performed, 146 follicles aspirated, 112 oocytes obtained and 42 fertilized (37% fertilization rate per oocyte) (Tables III and IV). Embryos were transferred to 25 patients and 4 became pregnant. One had an ectopic pregnancy, two had biochemical pregnancies, and 1 patient became pregnant with twins after the transfer of 4 embryos. The babies were delivered on 17 September 1984 by caesarean section after 35 weeks' pregnancy. The first baby was a healthy female weighing 1 900 g with an Apgar score of 6 after 1 minute and 9 after 5 minutes. The second baby was a healthy male weighing 1 790 g with an Apgar score of 7 after 1 minute and 9 after 5 minutes. The postoperative course of both mother and babies was uncomplicated.

In phase III of the programme 78 laparoscopies were performed, 4 unsuccessfully; 341 follicles were aspirated and 288 oocytes obtained (84,5% oocytes per follicle) (Tables V and VI). The fertilization rate was 77,7% per oocyte and 83% of patients reached the embryo transfer stage. There were 17 pregnancies of which 15 were clinical. The clinical pregnancy rate per embryo transfer was 23% and per laparoscopy 19,2% (Tables V and VI).

Discussion

The fertilization rate was 37% per oocyte in phase II of the programme. Because the fertilization rate per oocyte in phase I was 62%,[1] we realized that something was radically wrong. Routine evaluation of the medium with two-cell mouse embryos did not detect any abnormality. The possibility existed that the quality of the purivate and its deterioration were the reason for the problem.[4] Although T6 medium contains lactate this substance cannot sustain the development of the mouse oocyte and fertilized ovum *in vitro*.[7] Fresh purivate was not

TABLE II. EMBRYO TRANSFER PROCEDURE — COMPARISON OF PHASES II AND III

	Phase II	Phase III
Measurement of uterine cavity before ET	Preliminary examination early in the cycle; Tomcat catheter used to determine whether uterus can be entered easily; valsellum used if necessary	As in phase II
Premedication	None	None
Position of patient	Dorsal lithotomy if uterus retroverted; knee-chest if in anteversion	Always dorsal lithotomy
Embryo transfer procedure	Norfolk Teflon catheter	Tomcat catheter
Medium/serum	10% human serum + 90% T6	75% human serum + 10% T6
Volume, intra-uterine method	Up to 60-90 μl 6-6,5 cm in uterus; air-bubble technique	30-60 μl 6-6,5 cm in uterus; air-bubble technique
Cell stage	2,4-8 cell stage	2,4-8 cell stage
Hours after insemination	42-46 hours	42-46 hours
No. of embryos	1-4 embryos	1-4 embryos; occasionally 5 embryos
Care after transfer	4 hours on stomach if uterus in anteversion or on back if retroverted. Discharged next day	As in phase II
Where done	In theatre $\pm$ 30 m from laboratory	In laboratory 2 m from incubator
Medication after transfer	None	None

ET = embryo transfer.

TABLE III. RESULTS OF PHASE II — 1 JANUARY - 30 APRIL 1984

	No.	%
Laparoscopies	42	
Successful laparoscopies	38	
No. of follicles aspirated	146	
Oocytes obtained	112	76,7
Oocytes/laparoscopy	2,6	
Oocytes fertilized (cleavage)	42	37,5
Transfer to patients	25	
Transfer/laparoscopy	25/42	59,5
No. of pregnancies	4	
Pregnancies/transfer	4/25	16
Pregnancies/laparoscopy	4/42	9,5
Viable pregnancies/transfer	1/25	4*

* —1 ectopic pregnancy; —2 menstrual abortions (biochemical pregnancies).

TABLE IV. PHASE II

No. of embryos transferred	No. of patients	Pregnant
1	17	2*
2	5	1†
3	2	—
4	1	1‡

* 1 ectopic; 1 menstrual abortion.
†Menstrual abortion.
‡ Twins.

TABLE V. RESULTS OF PHASE III — 1 MAY - 31 AUGUST 1984

	No.	%
Laparoscopies	78	
Successful laparoscopies	74	
No. of follicles	341	
Oocytes obtained	288	84,5
Oocytes/laparoscopy	3,6	
Oocytes fertilized	224	77,7
Transfer to patients	65	
Transfer/laparoscopy	65/78	83,3
No. of pregnancies	17	
Pregnancies/transfer	17/65	26,15*
Pregnancies/laparoscopy	17/78	21,79*
Clinical pregnancies/transfer	15/64	23,43
Clinical pregnancies/laparoscopy	15/78	19,23

* 2 menstrual abortions (biochemical pregnancies).

TABLE VI. PHASE III

No. of embryos transferred	No. of patients	Pregnant	Percentage
1	13	1	7,6
2	24	5	20,8
3	14	4	28,5
4	11	5	45,45
5	3	0	—

available immediately and a trial was run with Ham F10 medium, which resulted in an immediate improvement and excellent fertilization rates. The fertilization rate in phase III in which Ham F10 medium was used was 77,7% and compared well with rates quoted in the literature; these range from 60% to 90%.[8-10]

Although different media compare well in terms of fertilization and pregnancy rates,[11] one point in favour of the Ham F10 medium is its simplicity of preparation. Trounson *et al.*[12] have stated that a simple culture medium with relatively few chemical components has the advantage of ensuring quality control, since there is less chance of introducing unsuitable

chemicals at the time of preparation. Since the preparation of Ham F10 medium is simpler than Whittingham's T6, this is an advantage.

Filtering of the medium was discontinued; up to now no infection has occurred. The filters tested at that stage were not wetting-agent free[13] and after obtaining good results we decided not to use them. The medium is routinely prepared in a laminar airflow cabinet.

Another factor that could have played a role in improving the results was the acquisition of an extra incubator which created a more stable atmosphere for culturing the embryos. Opening and closing an incubator door could lead to an unstable temperature and change in osmolarity. A constant osmolarity is of the utmost importance in culturing embryos.[14]

As mentioned previously,[1] one of our aims was to improve the oocyte recovery per follicle aspirated. In phase I of the programme the percentage of oocytes recovered per follicle was 58%, in phase II it was 76,7% and in phase III 84% (Tables III and V). The technique for collecting mature oocytes has been outlined by Steptoe and Edwards.[15] Lopata *et al.*[16] reported a 45% recovery rate per follicle in 1974. Feichtinger *et al.*[17] obtained 58% rate of oocytes recovered and pointed out that a needle with a diameter of 1,4 mm was superior to one of 2 mm or 0,9 mm. Improvement of the aspiration system and concentration on improved surgical technique led to an 83% success rate in stimulated cycles and 92% in natural cycles at Monash University.[18] We think the improvement in our technique and equipment was also the most important factor leading to the 84% oocyte recovery rate per follicle in phase III.

The transfer rate of 83% per laparoscopy in phase III is satisfactory (Table V). The aim of an IVF programme is to get as many patients to the embryo transfer stage as possible. In a large series the Norfolk group[19] quotes a figure of 74% of laparoscopies reaching the transfer stage; of 176 patients who had a laparoscopy in Adelaide, 77% had an embryo transfer.[2] These figures reflect a good ovulation induction programme, an excellent oocyte recovery rate and a laboratory with a fertilization rate per oocyte that is constant and not fluctuating.

The clinical pregnancy rate per embryo transfer in phase III of this series was 23%. One of the most important factors in the higher pregnancy rate is the transfer of two or more embryos (Table VI). From the literature, this view is held generally.[2,8]

It is very necessary to define what is considered a pregnancy in an IVF programme. We follow the definitions laid down by Jones *et al.*;[20] these are important because a falsely high pregnancy rate can result if they are not taken into consideration. A chemical pregnancy or HCG pregnancy refers to a positive pregnancy test before expected menstruation but with no delay in the menses. The term menstrual abortion can also be used. The term preclinical abortion refers to the termination of the pregnancy by the onset of spontaneous menstruation no later than 28 days after oocyte aspiration. A clinical abortion refers to spontaneous termination of pregnancy more than 4 weeks after oocyte aspiration and before viability. Use of these definitions will ensure international standardization.

The clinical pregnancy rate per transfer of 23% and per laparoscopy of 19% achieved in this programme compares well with figures from internationally famous units (Table V); for example: a clinical pregnancy rate between 16,5% and 29% has been quoted lately at Bourn Hall,[21] 17% per laparoscopy and 23% per embryo transfer in the Norfolk series 1 - 8[19] and 16% per laparoscopy and 21% per embryo transfer at Queen Elizabeth Hospital, Adelaide, since 1982.[2] The group at Texas Medical School started their programme in July 1981 and has a clinical pregnancy rate per embryo transfer of 16,2% and 11,6% per laparoscopy.[22]

We realize that there are numerous variables involved in the human IVF work, but with a dedicated team and precision as the keyword, success can be achieved.

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