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Reviews

The history of assisted human conception with especial reference to endocrinology

R. G. Edwards

London Women's Clinic, London; Churchill College, Cambridge, UK

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Summary: This review lecture is primarily concerned with the study of assisted human conception and especially in-vitro fertilization (IVF). It also places in perspective the role of endocrinology in history of IVF. A knowledge of the hypothalamic-pituitary control of ovulation, and of ovarian follicle dynamics is assumed. A detailed consideration of these topics, together with extensive references, are available in a recent textbook (Edwards and Brody, 1995).

Many of the early pioneers studying animal reproduction combined reproductive physiology and endocrinology, especially Marshall, who analysed oestrous and menstrual cycles in many mammalian species. The clarification of the roles of pituitary gland and hypothalamus in the menstrual cycle and ovulation, and their regulation by steroidal feedbacks from the gonads gave an immense stimulus to studies on human reproduction (Smith and Engle, 1927; Lewis and Gregory, 1933; Harris, 1970). Three periods of research into assisted human conception are covered in this lecture including the initial work on the introduction of the endocrinology and embryology of human IVF, the rapid advances in technique as it expanded worldwide, and finally some of the recent remarkable advances in the field.

A. Early work on the endocrinology and embryology of assisted human conception

Introduction of controlled ovarian hyperstimulation in female mammals and in women

Initial concepts on the introduction of IVF into assisted human conception began in Edinburgh, during studies on experimental embryology in mice (Edwards, 1957). During these studies the occurrence of ovulation during the natural oestrous cycle was assessed from the examination of serial daily vaginal smears. Studies on embryology were made difficult by the need to use this cumbrous and inefficient method of obtaining gametes and achieving fertilization. It was also inadequate to yield sufficient freshly-ovulated oocytes and embryos for study on topics suc te experimental induction of gynogenesis, androgenesis and heteroploidy in mouse embryos (Beatty, 1957; Edwards, 1957). Essentially, some form of ovarian stimulation was needed to stimulate the controlled growth of several Graafian follicles and induce a timed ovulation.

The work of Ascheim, Zondek and many others in the 1920's and 1930's had led to the availability

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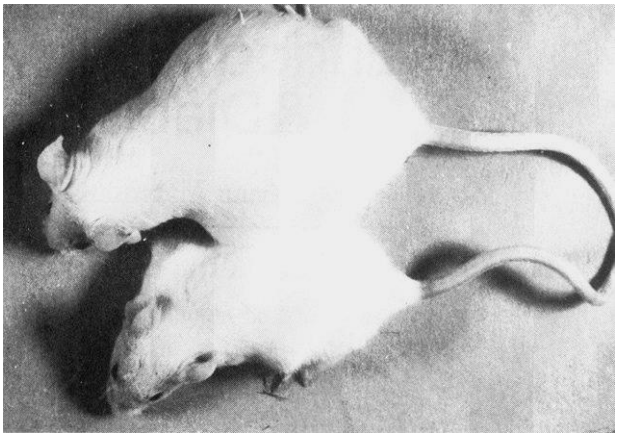


Fig. 1 The upper picture shows a pregnant mouse which was stimulated by Gestyl (pregnant mares' serum, Organon, Oss) and Pregnyl (human chorionic gonadotrophin, Organon, Oss); a non-pregnant female is shown in the lower part of the figure. Many multiple pregnancies resulted after this method of inducing controlled superovulation in adult mice, with up to 50 or more implants in some females (Fowler and Edwards, 1957).

of partially purified gonadotrophin preparations, prepared from extracts of various biological sources such as pregnant mare's serum (PMS) and human chorionic gonadotrophin (HCG). Several authors had used various methods of inducing superovulation and oestrus in immature animals, e.g. in mice (Evans and Simpson, 1940; Pincus, 1940; Parkes, 1942; Runner and Gates, 1954). The overall conclusion was that immature animals could be induced to superovulate and enter oestrus, but not mature females. Indeed, in many studies, oocytes or embryos were collected from immature females after ovarian stimulation, and transferred to adult female recipients to continue their growth to full term (Gates and Beatty, 1954).

The first successful application of any method of controlled ovarian hyperstimulation in adult mammals was carried out in mice. This resulted from the frustrations of using the natural oestrous cycle to obtain mouse oocytes and embryos, as outlined above. Controlled ovarian stimulation was incredibly successful (Fowler and Edwards, 1957). An injection of PMS to stimulate follicle growth, followed 40 hr later by HCG induced multiple follicular growth, oestrus in the great majority of females, superovulation, mating and fertilization. Many full-term multiple pregnancies and deliveries were obtained in the adult mice (Figure 1). These injected hormones imposed a strict uniformity on folliculogenesis, the time of oestrus, oocyte maturation and ovulation (Figure 2) (Edwards & Gates, 1959). All stages of oocyte maturation occurred at precise times with hardly any variability between females. Oestrus began at 8 hours approximately, and virtually all stimulated mouse females ovulated at 12 hours post-HCG. In some high multipregnancies, the combined weight of 40 or more fetuses exceeded the mother's body weight. These results led to the first concepts of inducing timed superovulation in women and animal species.

A year or so later, Gemzell began his work on superovulation in adult amenorrhoeic women using human pituitary extracts to stimulate follicle growth and HCG to invoke ovulation (Gemzell, 1967). Lunenfeld then introduced the clinical use of preparations of human menopausal gonadotrophins (HMG), prepared by Donini, as follicular stimulants for amenorrhoeic women (Lunenfeld, 1969), and an alternative approach utilizing clomiphene was introduced a few years later (Kistner, 1972). Similar results to those in mice were observed, with many high-order multiple pregnancies.

During those years, the methods of ovarian stimulation pioneered by Gemzell and his colleagues offered no chance of obtaining human oocytes for studies on maturation and fertilization. There was only limited information on the number of follicles that had been stimulated to grow, and none on the timing of human ovulation after the injection of HCG. Nor was it known if cyclic women would respond to these forms of ovarian stimulation. The necessary surgical methods to permit a simple approach to the ovary had not been pioneered, so that laparotomy would be needed for this purpose. These early advances were astonishing at the time, but each of the associated problems had to be solved before any attempt could be made to introduce human IVF.

Knowledge and oocytes and embryos in the 1960s

Many early pioneers had established a fundamental basis of animal embryology. In 1890, Heape was sufficiently familiar with the collection and short-term culture of rabbit ova and with their transplantation to adult recipients as to obtain full-term offspring from the transferred ova. Preimplantation embryos of rabbit and monkeys had been cultured, and had developed through cleavage and blastulation in vitro (Lewis and Gregory, 1933; Lewis and Hartman, 1933). Many investigators had studied human oocytes and embryos, as shown especially by the detailed manuscripts in the Carnegie Institute Contributions to Embryology and other journals (e.g. Hertig et al., 1954; Menkin and Rock, 1948; Shettles, 1955). Methods of tissue culture were being steadily improved, and many of the modern methods had been introduced by the 1950's. Defined media for the culture of mam-

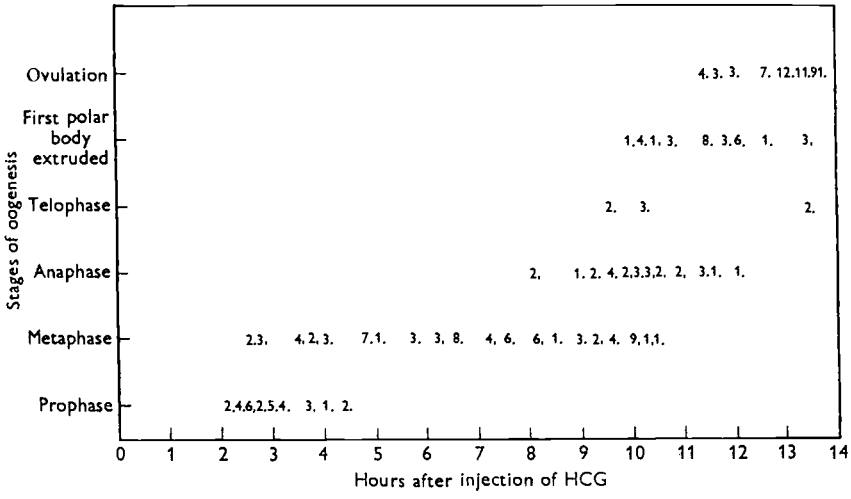


Fig. 2 Timing of the meiotic stages during oocyte maturation to metaphase 2 and ovulation in mice (Edwards and Gates, 1959)

Table 0 Human gonadotrophic preparations

Before, 1955	Human chorionic gonadotrophin available commercially
Gemzell, 1967	Anterior pituitary extracts to stimulate follicle growth
Lunenfeld, 1996	Urinary extracts to stimulate follicle growth
Various sources, 1990's	Purified FSH
Various sources, 1990's	Recombinant FSH/LH

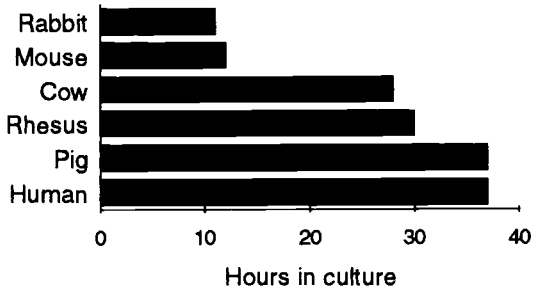


Fig. 3 Estimates of the time required for oocyte maturation from diakinesis to metaphase 2 in various species. The timing of maturation in vivo was subsequently shown to be similar in each species (Edwards 1965a,b).

malian embryos were steadily improved during those years (Hammond, 1949; Paul, 1961; Whitten and Biggers, 1968).

Initial work on the introduction of IVF was led by studies on the maturation of human oocytes released from their follicles into culture medium. If IVF was to be successful, mature human oocytes would be needed to achieve fertilization and normal embryonic growth. Pincus and his colleagues initiated the study of oocyte maturation in vitro in the 1930's. Rabbit oocytes required 11–12 hours to mature, and the maturation of human oocytes in vitro was thought to require 12 hours (Pincus and Saunders, 1939). As it happened, this figure was incorrect, and it probably led to later investigators adding spermatozoa to human oocytes which had been matured in vitro for 12 hours (Menkin and Rock, 1948; Shettles, 1955). This error, and possibly the general lack of understanding of the composition and handling of culture media, must have hampered these investigators.

Studies on oocyte maturation on several mammalian species were carried out in the late 1950's and early 1960's. They showed beyond doubt how each species had its own unique duration of oocyte maturation from the germinal vesicle stage until the formation of metaphase of the second meiotic division (metaphase 2) (Edwards, 1965a). Moreover, in all species examined, the interval required to complete oocyte maturation in vitro was exactly the same time as that needed for maturation in vivo. The interval between the LH surge or an injection of HCG and the onset of ovulation could thus be predicted exactly. Examples were 12 hours in mice and 37 hours in pigs.

Several years elapsed before the correct interval of 37 hours between the onset of culture and the formation of metaphase 2 and the first polar body in human oocytes was clarified (Edwards, 1965a,b). This discovery was made during studies on the few human ovarian oocytes available in London and Glasgow, and confirmed in larger-scale studies in Baltimore in collaboration with Georgeanna and Howard Jones in the Johns Hopkins Medical School, and it proved to be decisive for the introduction of human IVF (Figures 3, 4). Human oocytes could now be obtained from excised pieces of ovarian tissue and cultured to timed stages of maturation. They could be used to assess chromosomal and other aspects of maturation, eg a study of diakinesis and metaphase 1 using

Stages of meiosis	0–24	25–27	28–31	32–36	>36
Germinal vesicle	31	3	1	1	16
Diakinesis		6	1		
Metaphase 1		2	13	5	3
Telophase 1				1	
Metaphase 2	1			1	46

Hours in culture

Fig. 4 Timing of the meiotic stages during maturation of human oocytes in vitro (Edwards 1965b and unpublished).

oocytes matured for 24–30 hours. Oocytes matured completely for 37 hours in vitro to metaphase 2 were invaluable for studies on later meiotic stages and to begin detailed analyses on human fertilization in vitro (Edwards et al., 1969). The exact timing of human ovulation in vivo could be estimated at 37 hours after the onset of the LH surge or an injection of HCG, since it would be identical to the timing of complete oocyte maturation. This prediction was made entirely on estimates gained from oocyte maturation in vitro, and before the onset of ovulation has been examined in any woman. Oocytes that had been matured in vitro for 37 hours could also be used for the first attempts at human fertilization in vitro.

Many previous studies had identified a need for animal spermatozoa to undergo capacitation, then a poorly understood process, before they could fertilise eggs in vivo or in vitro (Austin, 1951; Chang, 1951). The earliest studies on the fertilization of animal eggs in vitro depended on the use of capacitated spermatozoa collected from the uterus or oviduct (Dauzier & Thibault, 1956). Only in one species, the golden hamster, could fertilization be achieved in vitro using spermatozoa taken from the epididymis (Yanagimachi and Chang, 1964).

Nevertheless, in our studies, human fertilization in vitro was achieved using ejaculated spermatozoa that had been washed free of most seminal plasma by gentle centrifugation (Edwards et al., 1969). Oocytes matured in vitro were inseminated with these washed spermatozoa, and incubated at 37.5 °C. All the early and late stages of human fertilization were later identified in vitro, and the incidence of fertilization was quite high. Studies on human embryos in vitro were now becoming possible. However, there were many problems to solve, because rabbit oocytes that had matured entirely in vitro were unable to develop into normal embryos after fertilization, since they degenerated before the blastocyst stage (Chang, 1955). Human oocytes that had been matured and fertilised in vitro also displayed some anomalies. This meant that

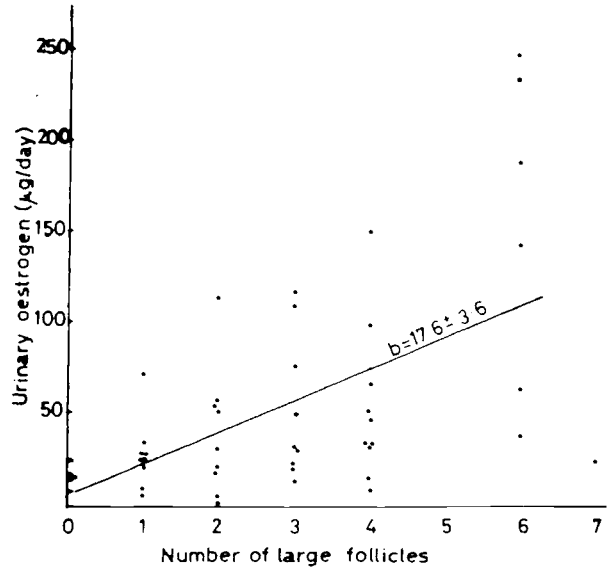


Fig. 5 Numbers of large Graafian follicles in relation to urinary oestrogens in women given HMG and HCG, with the correlation coefficient (Steptoe and Edwards, 1970).

successful fertilization and the culture of embryos in vitro, and their development to late stages of fetal life, would demand the aspiration of fully-mature oocytes from their ovarian follicles. It also meant that mild superovulation and oocyte mauration would have to be introduced for cyclic women, and their oocytes would have to be aspirated just before ovulation occurred.

At this time, I began a wonderful collaboration with Patrick Steptoe, perhaps the world's foremost laparoscopist at the time. Laparoscopy had come of age in the mid-twentieth century, led primarily by Palmer (1946), Fragenheim (1964) and Steptoe (1967). It now made a full contribution to the development of human IVF, by permitting a much simplified method for approaching the human ovary for the aspiration of oocytes from their follicles.

The routine of human IVF was established in the late 1960's. Infertile patients attending hospital for tubal repair were asked to take three injections of 225 iu of human menopausal gonadotrophins (HMG) on days 3, 5 and 7 of their menstrual cycle. This was followed by an ovulatory dose of 5000 IU HCG on day 10 or 11. The HCG injection was timed to pre-empt the chances of an endogenous LH surge and so avoid a spontaneous ovulation out of clinical control. Urinary oestrogens were measured once or twice during the stimulation cycle. Laparoscopy was used to inspect the ovary for follicles as they were being aspirated to collect their oocytes. Ultrasound was not available in those days!

Table 1 Number of Graafian follicles in the human ovary after priming patients with HMG and HCG (Edwards, 1980)

Dose of HMG (iu)	Mean number of follicles (± SE)
300–375	9.2
675	8.8 ± 0.8
900	10.0 ± 0.4

Table 2 Success in aspirating human oocytes from their follicles after ovarian stimulation with HMG and HCG (Edwards, 1973)

Follicle diameter (mm)	No of follicles aspirated	Oocytes collected	Preovulatory oocytes
<10	115	53 + 2? (46%)	7 + 4?
10–17.5	26	18 + 1? (69%)	4
>19	33	24 (73%)	9 + 2?

Oocytes classified from their appearance

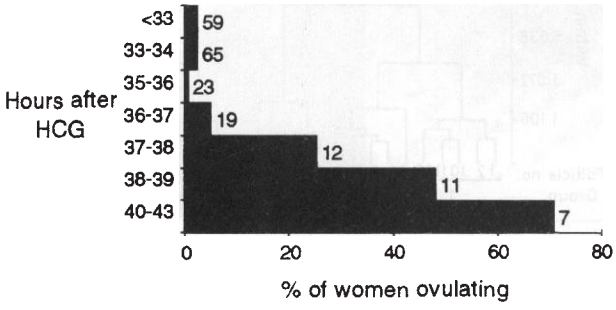


Figure 6 Timing of the onset of follicular rupture after an injection of HCG in women given controlled ovarian stimulation with HMG and HCG (Edwards, 1980).

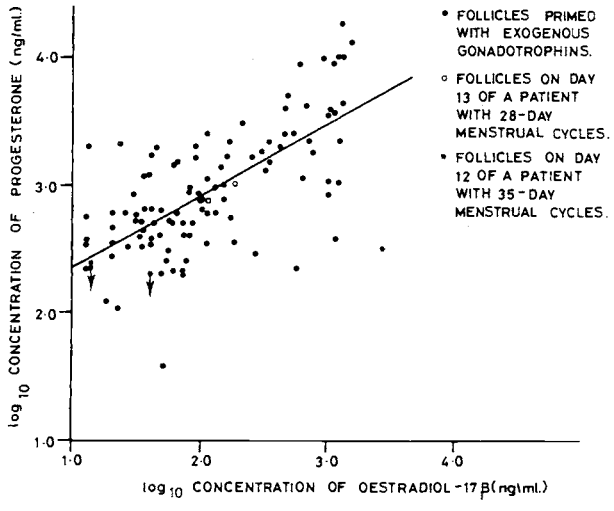
Many treated patients had four or more follicles when examined by laparoscopy at various intervals after the HCG injection (Figure 5; Table 1). Follicle rupture was observed to begin at 37 hours post-HCG (Steptoe and Edwards, 1970); this interval was exactly as predicted from the timing of oocyte maturation in vitro (Figure 6; Table 2). Laparoscopy for the aspiration of preovulatory follicles was accordingly timed at 36 hours post-HCG. Follicle numbers and diameters were recorded (Table 1) and Steptoe's laparoscopy delivered a steady number of preovulatory and other oocytes (Figure 7). The superovulation of cyclic women using HMG and HCG had begun, and marked another step along the road to human births after in vitro fertilization (IVF) (Steptoe and Edwards, 1970).

Preovulatory human oocytes were enclosed in a mass of viscous follicular fluid, and an average of two per patient recovered in the initial work rose to four or more later. Many other oocytes had only

Fig. 7 Steptoe was always filming his laparoscopic operations as shown in this picture taken in his operating theatre in the mid-1960's.

partly matured, as assessed by the state of their cumulus oophorus. Wide variations between oocytes collected from each patient were revealed by assays of up to nine steroids in their accompanying follicular fluids, using cluster analyses (Fowler et al., 1978a,b). Several cohorts of follicles had apparently been stimulated by the HMG, and this had seemingly led to the varying steroid ratios in the follicular fluids (Figure 8a). In contrast, only two classes of follicles were identified during the natural menstrual cycle, i.e. non-ovulatory and preovulatory respectively.

To achieve fertilization in vitro, ejaculated spermatozoa washed in culture medium and used to inseminate the oocytes one hour after collection. More than 60% of the oocytes were fertilised in vitro (Figure 9), and many of the resulting human embryos cleaved in vitro to morulae and blastocysts (Figure 10; Table 3). The embryos had approximately a diploid chromosome number. No triploid embryos typical of dispermy or digyny were found. Cleavage rate, blastomere structure and nuclear number, and the hatching of the blastocysts from their zonae pellucidae in vitro were each assessed. Some hatched embryos grew for several days in vitro, and one developed to day 9 post-fertilization before being fixed for electrom microscopy (Figure 11). Its embryonic disc was prominent, and presumably contained many primary stem cells for various organ rudiments. Attempts to obtain these stem cells (ES cells, as had been achieved in rabbits during the years in Glasgow (Cole et al., 1965, 1966) by disaggregating blastocysts or by culturing monolayers of cell outgrowths from human blastocysts) did not succeed,



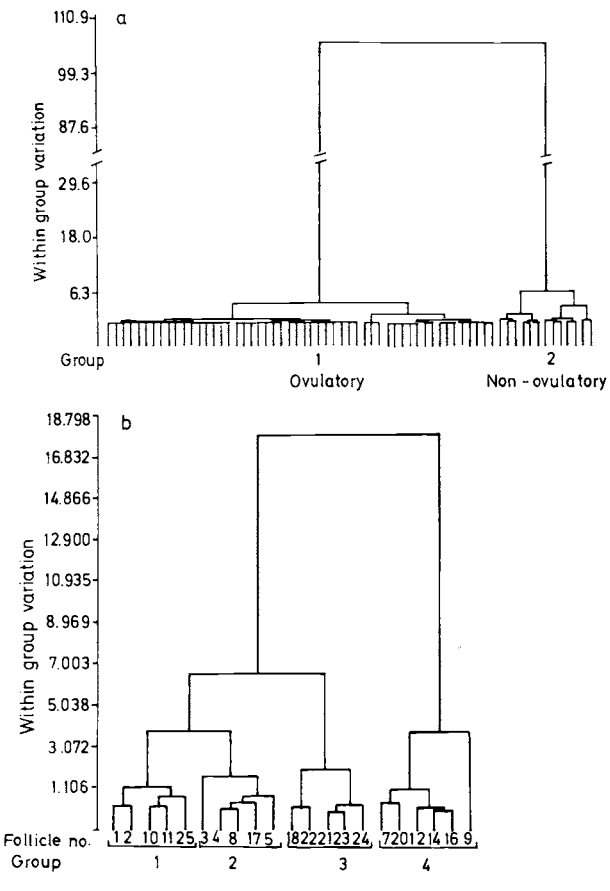


Fig. 8a Correlation between the log concentrations of oestradiol-17b and progesterone in fluids taken from human prevovulatory follicles.
b Dendograms showing the clustering and classification of the stage of growth of individual follicles in women when laparoscopy for oocyte aspiration was performed just before the time of ovulation. The height of the dendogram indicates increasing variability between follicles. Upper (a). Natural cycles, showing how the individual follicles are easily combined into two groups, ovulatory and non-ovulatory, with a huge variance between the two classifications. Lower (b). HMG/HCG cycles, showing the considerable variation between individual numbered follicles; they have fallen into four statistical groups with various levels of difference between the groups. Groups 1, 2 and 3 could be grouped with modest increase in variance, but not group 4. (Fowler et al., 1978 a,b).

and have not succeeded even to the present day. By 1971, studies on human embryology had completely surpassed animal studies, since the complete growth of oocytes from before fertilization to blastocysts had not been achieved in any mammalian species, and the embryos of most species did not develope at all in vitro.

The replacement of human embryos into their mothers with the intention of establishing pregnancies began in 1971 (Edwards, 1973). The IVF routine had became established: patients were given HMG and HCG, and laparoscopy for oocyte aspiration was performed just before ovulation was expected. Fertilization and cleavage in vitro had been achieved, and embryos were replaced in their mothers at various times between 2.5 and 5 days after aspiration.

The endocrine difficulties in establishing IVF pregnancies

Endocrine problems in the luteal phase soon emerged. Stimulation with HMG and HCG shortened the luteal phase to as little as 7–8 days in some patients, and its duration was directly proportional to their output of urinary oestrogens during the follicular phase of their cycle. This early menstruation severely restricted the chances of implantation (Figure 12) (Edwards and Brody, 1995), and luteal support was essential to overcome it. It was possible that progesterone and oestradiol supplements would impair the activity of the corpus luteum even more. Moreover, these supplemental steroids would have to be given daily until week 9 or 10 when the placenta became fully active endocrinologically (Csapo et al., 1974). This form of long-term treatment using daily injections of progesterone in oil would cause scabbing and pain to the patients.

An ethical decision to avoid scabbing was therefore taken to give the patients depot Primulot (17-hydroxyprogesterone acetate) at 5-day intervals since it was believed to save threatened miscarriages later in pregnancy. In fact, Primulot was luteolytic since progesterone levels fell disastrously within a day of its administration. Moreover, although we did know it until ten years later, our

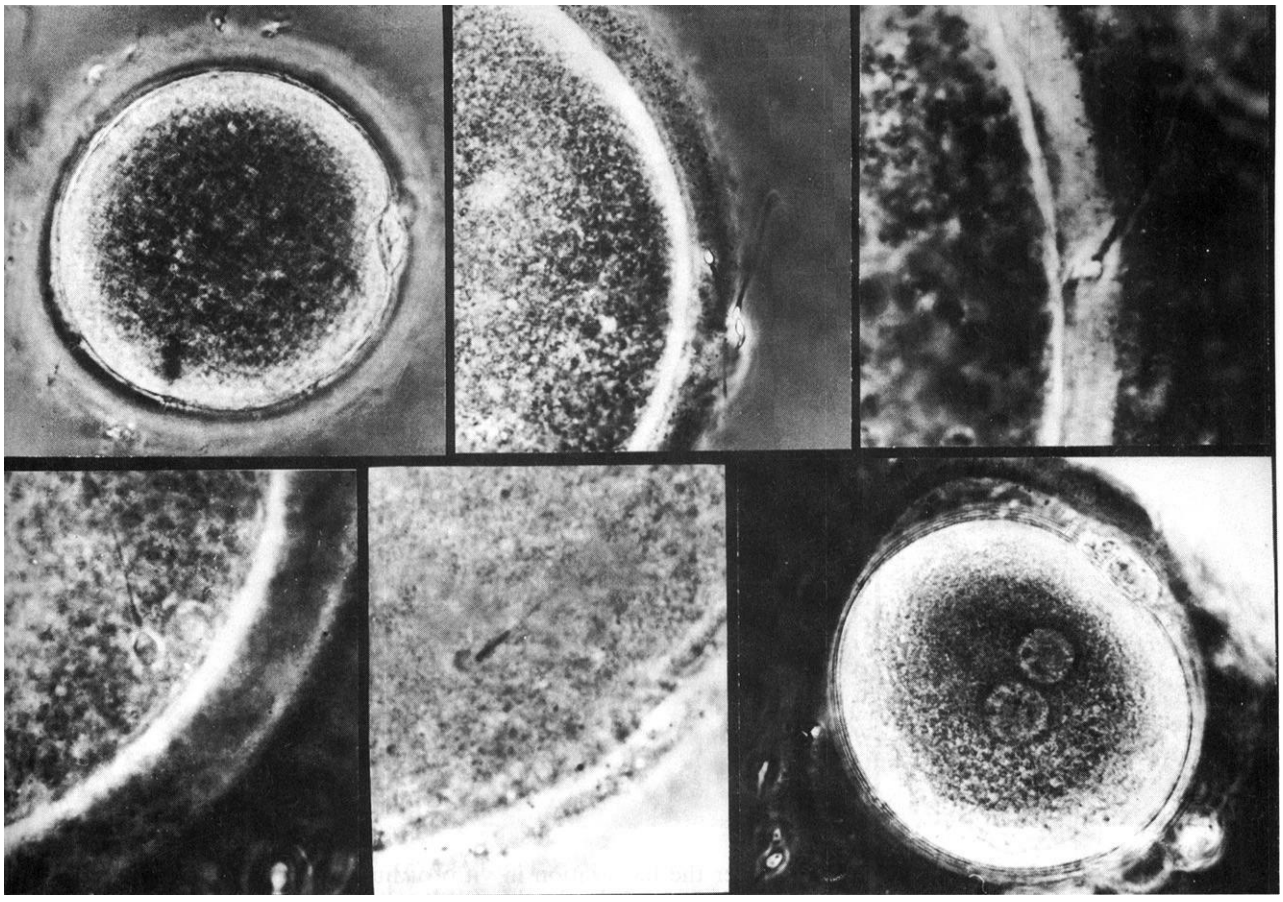


Fig. 9 Stages of fertilization in vitro, using preovulatory oocytes aspirated from the ovary. The successive stages show (upper) sperm attachment to the zona pellucida, their migration through it to the oolemma, and (lower) the spermatozoon inside the ooplasm, the expanding male pronucleus and a fertilised egg with two pronuclei and polar bodies. (Edwards, 1980).

belief that Primulot sustained the endometrium was totally mistaken for it actually offered nothing to the luteal endometrium. This ethical decision led to three years of frustration after we began to transfer embryos to their mothers. There were brief rises in HCGβ levels after embryo transfer in some patients indicating that short-lived pregnancies had been established. Several years later, we discovered that Primulot caused the early abortion of implantated human embryos between days 18–25 after fertilization!

Meanwhile, after thoroughly and repeatedly testing all the systems involved in IVF, suspicions about the cause of the failure of implantation fell upon Primulot. It was phased out, and at last pregnancies were established. The first clinical pregnancy arose as more natural steroids were used for luteal phase support. A blastocyst implanted after being replaced in its mother after 5 days in culture, and the living fetus was diagnosed on ultrasound. It later proved to be a tubal ectopic pregnancy, and had to be removed at 11–12 weeks of gestation (Figure 13). Another brief pregnancy identified by rising levels of HCGβ indicated that the endocrinology of early pregnancy was being brought under control and that full-term births were near (Edwards et al., 1980a).

Several new endocrine approaches were accordingly tested (Table 4). Some oocytes and embryos were cryopreserved with the intention of thawing them during a later natural cycle for replacement in the mother. Many were in good condition after thawing (Edwards et al., 1980a; Edwards and Steptoe, 1980; Steptoe and Edwards, 1976; Steptoe et al., 1980). Bromocryptine was introduced into ovarian stimulation to reduce high prolactin levels. Clomiphene and HMG were combined for ovarian stimulation to overcome the short luteal phase with HMG alone (Table 4). Many oocytes were harvested and embryo transfers were achieved with all these approaches.

IVF was also tested during the natural cycle. Measurements were made of urinary LH, oestrogens and pregnanediol in samples collected eight times daily, at 11 pm, 3, 7, 9, 11 am and at 2, 5 and 8 pm. LH was measured using Higonavis,

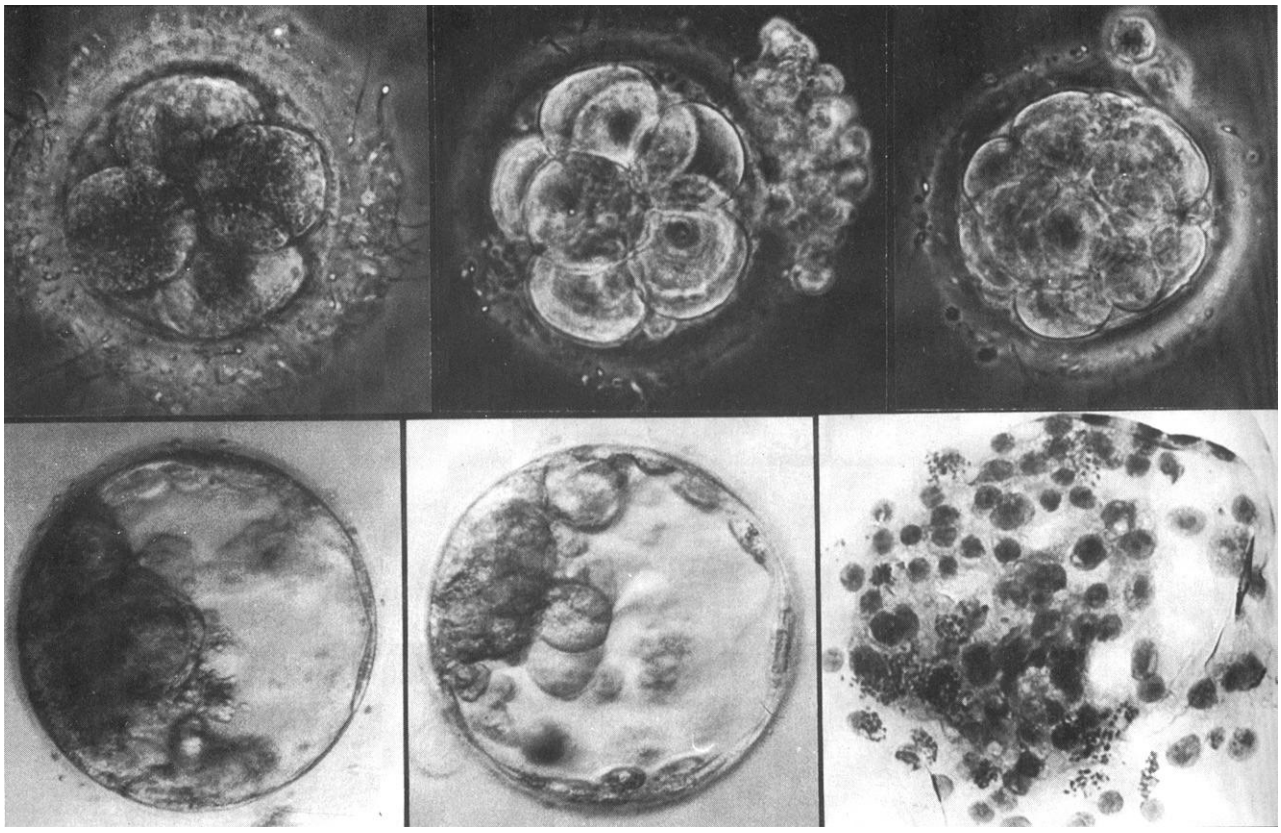


Fig. 10 Stages of embryonic growth in vitro after the fertilization in vitro of human oocytes aspirated from their follicles (Edwards et al., 1980). The stages shown include along the top row 4 cell, 8 cell, a compacting morula, and on the bottom row two living blastocysts and a flattened stained blastocyst with more than 120 cells and many mitoses (Edwards, 1980).

Table 3 Interval in hours between the insemination of human oocytes in vitro and development to various embryonic stages (Edwards, 1980)

Stage of development	Observations on interval (hr) between insemination and embryo cleavage	Estimate of (mid-)point of each cleavage stage (hr)
2-cell	26	34.9 ± 1.9
4-cell	38	51.2 ± 1.9
8-cell	46	67.9 ± 2.5
16-cell	68	84.6 ± 3.4
Morula	100	100.2 ± 3.0
Blastocyst	120	112.7 ± 3,8

From Edwards RG. Conception in the Human Female. London, Academic Press, 1980

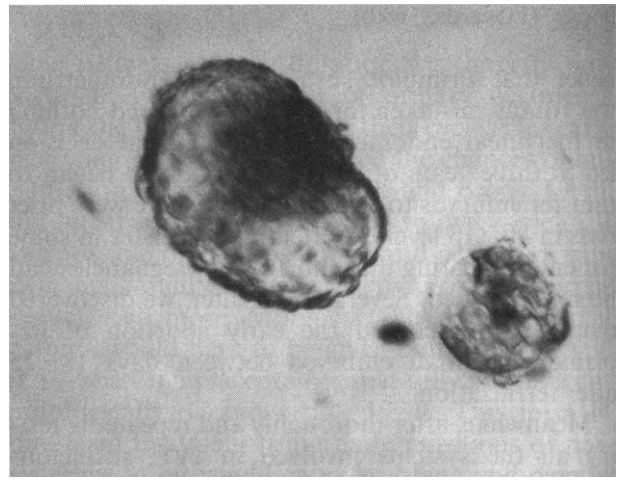


Fig. 11 A hatched human blastocyst grown in vitro until day 9 post-fertilization. Notice its greater diameter than that of the discarded zona pellucida. Notice also the prominent embryonic disc.

which was actually a urinary 'pregnancy test' for HCG with a 2-hour turn-round time (Figure 14a) (Edwards et al., 1980a,b). LH surges appeared in urine 4 hours after their onset in plasma, most of them beginning at 7 am, so the plasma LH surges must have begun at 3–7 am. The success of oocyte recovery during the natural menstrual cycle, and the changing steroidogenic activity of preovulatory follicles as they approched rupture, were assessed. The optimal time for follicular aspiration was identified.

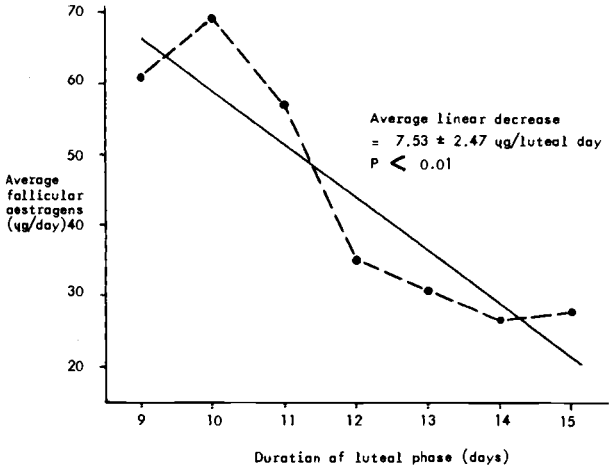


Fig. 12 Regression coefficient of the relationship between oestrogen excretion and the duration of the luteal phase in patients given HMG and HCG (Edwards et al., 1980). All urine samples were collected during the follicular phase from the patients and used to estimate total daily output of oestrogens.

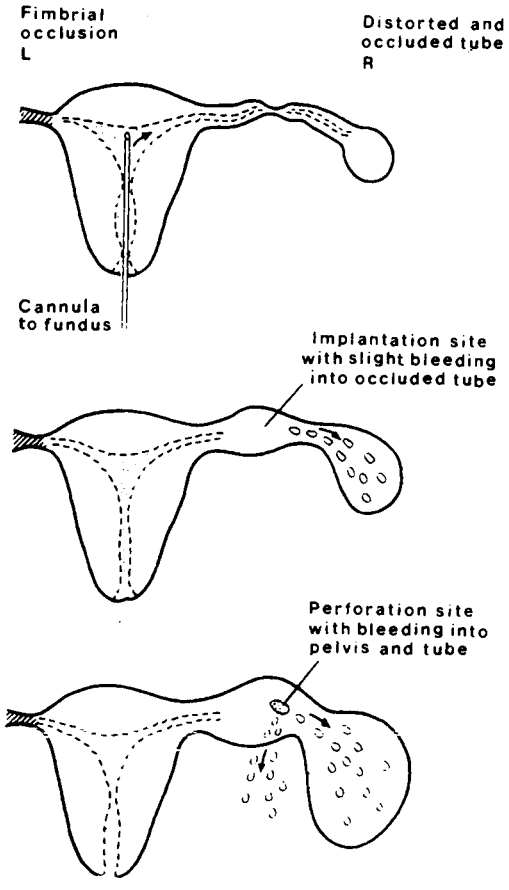


Fig. 13 Steptoe's drawing of the ectopic pregnancy showing the state of the uterus at the time of implantation (upper), the probable state of the oviduct at 8 weeks after embryo transfer (middle), and conditions in the right oviduct and pelvis during laparoscopy 10 weeks after transfer (lower) (Steptoe and Edwards, 1976).

This was also the first time such a diurnal rhythm in the onset of the LH surge had been reported. It showed that most women ovulated at 4 o'clock in the afternoon, i.e. 37 hours after the onset of the plasma LH surge (Figure 14b). This finding led us then and later to question previous work on the timing and control of the endocrinology of the human menstrual cycle, and also of the primate menstrual cycle; the diurnal rhythm of the LH surge had obviously been overlooked by several investigators who reported LH surges in the middle of the day. It has also led us to question Knobil's hypothesis that the medial basal hypothalamus (MBH) regulated ovulation in primates, since he did not know of this diurnal rhythm which could have been controlled by centres outside the MBH such as the suprachiasmatic nucleus.

The second patient treated during her natural cycle was Lesley Brown. Her ripening oocyte was aspirated at 11 am, i.e. 26–28 hr after the onset of the urinary LH surge, immediately inseminated, fertilised and replaced approximately 58 hours later as an 8 cell embryo (Steptoe et al., 1980). Three more pregnancies were established out of 32 replacement cycles. One triploid fetus aborted (Figure 15), a normal boy was delivered, and a second normal boy died 3 days after a premature delivery at 19 weeks of gestation, at one week after an amniocentesis (Steptoe et al., 1980). Three normal children were therefore delivered in this first series of IVF pregnancies, but one of them had died after a most unfortunate premature birth.

Scientific, clinical and ethical advances in human reproduction during this period

Many other attempts to advance knowledge of human reproduction were made during these years (Tables 5, 6). These included the first attempts at GIFT, in an attempt to simplify IVF (Figure 16), and the first studies on preimplantation diagnosis using whole-mount analyses or the excision of small pieces of trophectoderm from rabbit blastocysts. The presence of sex chromatin in the trophectodermal nuclei was used to identify female embryos, and its absence indicated male embryos. Initially, phase-contrast and fluorescent microscopy was applied to intact blastocysts pressed gently between a microscopic slide and a cover slip (Edwards & Gardner, 1967), but diagnosis and embryo survival were jeopardised by this approach. The excision of small pieces of trophectoderm using micromanipulation, followed by embryo

Table 4 Details of the incidence of pregnancy in patients given various treatments in Oldham (Edwards et al., 1980a)

Stimulation	Luteal phase treatment	Pregnancies
HMG/HCG	None	0/15
HMG/HCG	HCG and progesterone	0/7
HMG/HCG	Clomiphene	0/8
HMG/HCG	Hydroxyprogesterone hexanoate and HCG	2/16
HMG/HCG	Hydroxyprogesterone hexanoate, progesterone and HCG	1/10
HMG/HCG	Bromocryptine	0/12
Clomiphene/ HMG/HCG	None	0/2
Natural cycle	None	4/32
Nat. cycle/HCG	HCG and progesterone	0/7

transfer of the sexed blastocysts, led to the successful determination of sex in all 18 offspring (Gardner and Edwards, 1968). Similar attempts to identify sex chromatin and the Y body in human blastocysts were unsuccessful. Micromanipulation was also used to place a single donor cell in mouse blastocysts in order to produce chimaeric offspring (Figure 17a) (Gardner, 1968). This work was the forerunner of studies on transgenic mice, using a genetically-engineered donor cell for injection into a blastocyst (Figure 17b).

The first papers on the ethics of human IVF were also published in the 1970's (Edwards and Sharpe, 1970; Edwards, 1974). These were to set the scene for many far-reaching debates in the 1970's and 1980's, a debate that continues today. It is worth noting that these debates were initiated by a scientist in the field, accompanied by a lawyer (Edwards and Sharpe, 1970).

B. Forms of ovarian stimulation as IVF expanded worldwide after 1980

The years between 1980 and 1990 included rapid advances in many aspects of IVF (Table 7). These will be described only briefly, since many of them are thoroughly familiar to this audience.

Introduction of successive forms of ovarian stimulation

We had no laboratory or clinical facilities to continue our work for two years after Louise Brown was delivered, as a rapid expansion in IVF around the world began two years after her birth. Major attention was now directed at the optimal means of achieving follicular stimulation. The use of clomiphene and/or HMG during stimulated cycles was reintroduced (Trounson et al., 1981; Jones et al., 1982). The use of clomiphene alone or of HMG used with or without clomiphene now became essential features of follicular stimulation for IVF. Less HMG was needed when it was combined with clomiphene. This treatment overcame the short luteal phase typical of HMG alone (Edwards et al., 1980a), and it may have involved some form of compensation by the HMG for an FSH deficiency in the mid-follicular phase (Fleming and Coutts, 1990). Some investigators believed that stimulation with clomiphene and HMG impaired the endometrium, just as with clomiphene alone. Models for stimulation were developed for poor responders, e.g. giving HMG in pulses, but disadvantages with the use of HMG alone included the occurrence of elevated tonic levels of LH, premature or attenuated LH surges in 15% of cycles, and weak responses in many patients. These unwanted LH surges were often associated with rising progesterone levels and low pregnancy rates after embryo transfer (Howles et al., 1986). A transient hyperprolactinemia during oocyte collection had little effect on the success of the treatment.

The LHRH agonists became available commercially, followed more recently by the LHRH antagonists. These were combined with HMG and HCG for stimulation (Fleming and Coutts, 1990). Pulsatile treatment with the agonists stimulated follicular growth in men and women suffering from severe hypothalamic deficiencies, and was effective for idiopathic hypogonadotrophic hypogonadism (Leyendecker et al., 1990). Almost 90% of patients ovulated, and pregnancy occurred in >25% of cycles.

There was much ingenuity in the combination of the LHRH agonists, contraceptive steroids and HMG or FSH in IVF programmes. The agonists were given as a single depot injection, in several injections or as nasal sprays. LH pulses remained suppressed with long-term use whereas FSH levels rose gradually to early follicular levels. LH surges were abolished in many women if agonists were given in the preceding luteal phase or on the first day of menstrual bleeding, and many patients produced large numbers of oocytes and achieved pregnancies after IVF.

The LHRH agonists were sometimes given as an ultra-short flare-up protocol, i.e. for 3–4 days at the beginning of the cycle, followed by HMG (Figure 18). This protocol was originally introduced for poor responders since the agonists initially stimulated a short-term FSH release (Howles et al.,

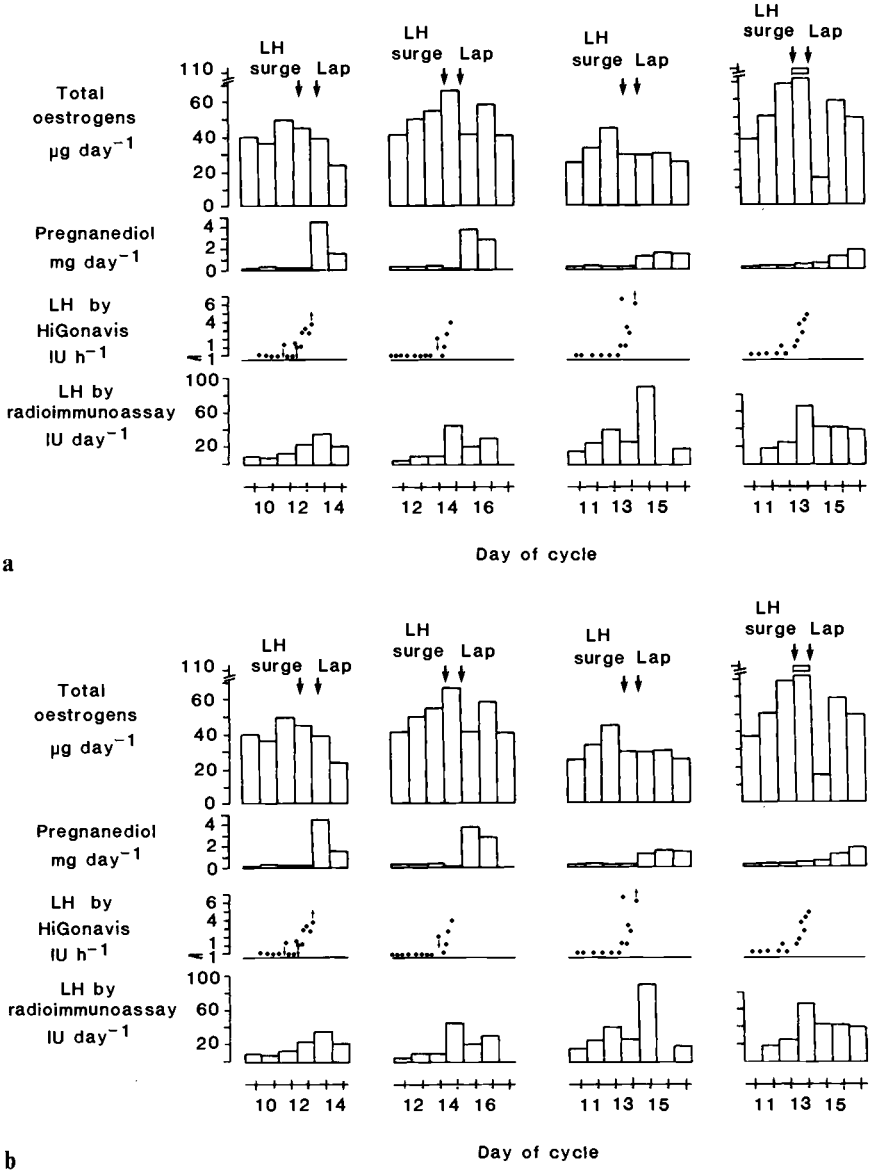


Fig. 14a Assays of urinary oestrogens, pregnanediol and LH in the follicular phase of the first patients being treated for natural cycle IVF. Eight samples of urine were collected at regular intervals throughout the day (Edwards et al., 1980a).

b Diurnal rhythm in the onset of the human LH surge in plasma (histograms), adjusted for its excretion in urine, and in the timing of human ovulation (line). Notice the stronger LH surge rhythm in spring than in autumn. The expected timing of ovulation was calculated by adding 37 hours to the onset of the LH surge. An interval of 37 hours is needed for oocyte maturation and follicle rupture in women, as shown earlier in this article. Approximately 75% of women ovulate before 16.00 hr in spring and 60% in autumn.

1986; Matthews et al., 1991). This form of stimulation is still suspected even today of providing insufficient flare-up release of FSH, and failing to down-regulate the pituitary gland sufficiently. Consequently, poor responses to HMG and endogenous LH surges were reported, jeopardizing the treatment cycle. Its protagonists stress the effectiveness of the protocol, and its low embryotoxicity and low teratogenicity since the agonist was withdrawn several days before ovulation.

A "short protocol" consisted of LHRHa administration from day 2 or from the mid-luteal phase of the preceeding cycle. HMG was given from day 3–4, with the LHRH agonist continuing until HCG was given to induce follicle maturation. Treatments beginning during the late luteal phase were introduced to avoid activating corpora lutea, but this protocol required more HMG than the ultra-short protocol. "Long protocols" involved LHRHa alone over many days, to completely down-regulate the pituitary gland. Treatment began during the previous cycle, from the follicular or luteal phase, and down-regulation could be continued for several weeks ("blocking protocols"). These treatments enabled the physician to choose the day when ovarian stimulation began, and so plan the timing of oocyte maturation well in advance. Oocyte aspiration could be planned to occur on a weekday ("Never on Sunday"!). Contraceptive steroids were also used for blocking and programming, and offered an equivalent and much less expensive treatment.

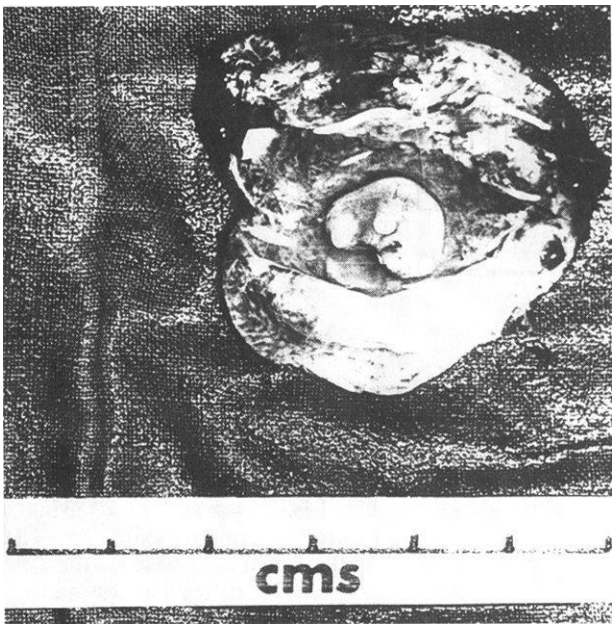


Fig. 15 Illustration of a triploid fetus which was the third clinical pregnancy established by IVF. It aborted late in the first trimester after embryo transfer (Steptoe et al., 1980).

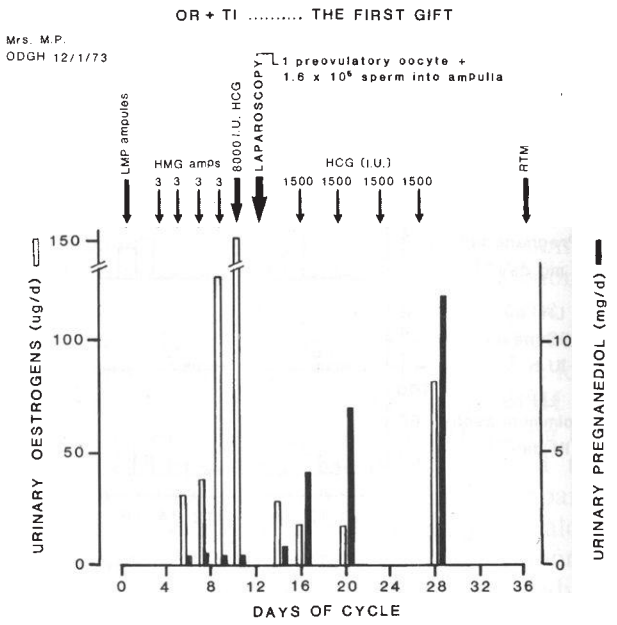


Fig. 16 The first GIFT, then called OR + TI (oocyte recovery with tubal insemination); a single oocyte was replaced with spermatozoa in most patients; occasionally two oocytes were replaced (Edwards and Steptoe, 1980).

Table 5 Scientific advances in assisted conception between 1968 and 1978

Ovarian follicular models
Successive follicle cohorts after ovarian stimulation in women
ES cells in vitro
Mouse chimaeras from single-cell injections into blastocysts
Preimplantation diagnosis in rabbits

Table 6 Clinical advances in assisted conception between 1968 and 1978

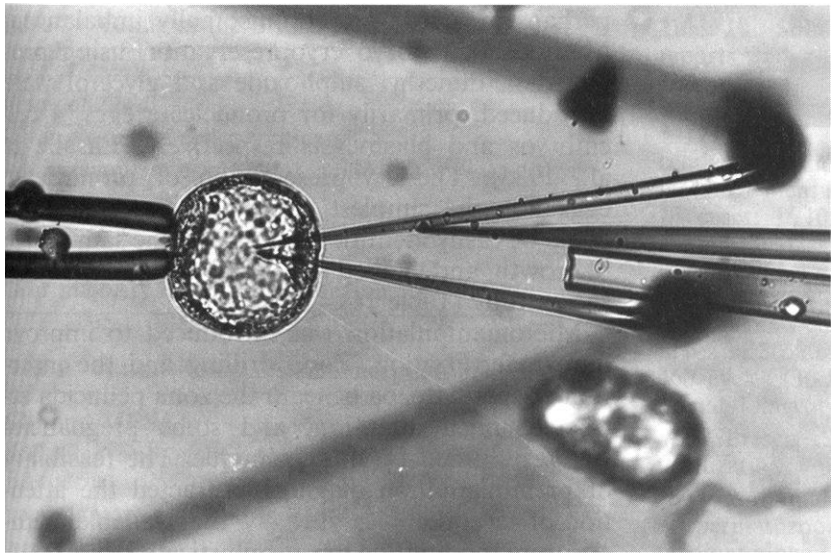
Human births
First ectopic pregnancy after IVF
5 forms of controlled ovarian stimulation
First GIFT cases done
Human oculation timed
Human oocytes/embryos cryopresvd
Ooyte donation
First ethical debates on IVF
Human preimplantation diagnosis begun

Knowledge gained from controlled human ovarian stimulation

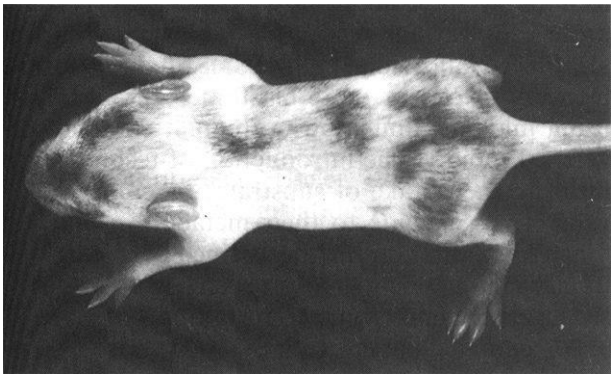
Much knowledge on human ovarian function has arisen from the use of controlled ovarian stimulation in women. Simple endocrine assays were introduced including dual-analyte enzyme immunoassays for measuring urinary oestrone-glucuronide and pregnanediol-glucuronide. Home monitors were introduced to measure urinary glucuronides, and home kits or dipsticks for urinary LH.

Most protocols of ovarian stimulation resulted in large numbers of successive follicle populations being recruited, in multifolliculation and in considerable developmental variations between neighbouring preovulatory follicles (Figure 8b). A dominant follicle produced 400 pg oestradiol several days after stimulation began. The sonography of follicular development using first transabdominal and then transvaginal probes became increasingly important for monitoring follicles, and replaced endocrine assays in many clinics. The time of HCG administration could be delayed for 1–2 days after follicles reached 18 mm, without any loss of pregnancy rates. HCG could be replaced by LHRH or LHRHa to induce ovulation, and progesterone supplements given in the late follicular phase could also induce an LH surge in stimulated patients. Luteal phase support with progesterone or intermittent doses of HCG were usually given after embryo transfer.

Several sequelae of ovarian stimulation became well recognised. The consequences of elevated tonic levels of FSH or LH, of high androgen levels or luteal phase deficiencies were recognised. The nature of treatments for patients with polycystic ovarian disease (PCOD) was clarified. They displayed inappropriate negative feedback effects,



a



b

Fig. 17a Micromanipulation of a mouse blastocyst to insert a single donor cell and so form a chimaeric embryo. The three needles open a hole in the trophectoderm, allowing the injection pipette to be inserted into the blastocoel. **b.** Chimaeric mouse born after the injection of a single stem cell into the blastocoelic cavity of a recipient blastocyst. The host blastocyst was albino, and the donated cell was pigmented; the areas of pigment shown the sites of colonisation of melanocytes derived from the donor cell. In many chimaeras, the testis contained spermatogonic cells derived from the donor cell which could achieve fertilization. Backcrosses involving his daughters and a chimaeric male led to the birth of offspring derived originally from the donated cell. (Courtesy of Richard Gardner).

high androgen levels, and changes in gonadotrophin secretion. They were at risk of hyperstimulation and premature luteinization, and were usually high responders. New forms of endocrine stimulation for them involved the use of low doses of FSH and perhaps HMG to avoid hyperstimulation, or the stepwise administration of FSH.

Ovarian hyperstimulation syndrome was the most serious complication of ovulation induction. It arose in 1.2% of all patients treated with LHRHa/HMG. Severe hyperstimulation arose after HMG treatment in 5% of pregnancy cycles and 1% of non-conception cycles. It was more frequent with large cohorts of medium-sized follicles than with two or three hyperfunctioning dominant follicles, high levels of oestradiol, and the occurrence of pregnany. Moderate and severe forms were a serious health risk to patients (Lunenfeld, 1969). Attempts to avoid it included continuing the administration of LHRHa until menstruation, replacing HCG with LHRHa, injecting albumin intravenously and freezing all embryos after IVF to avoid luteal phase treatments.

Advances in IVF in the 1980's

The major advance during these years included the introduction of ultrasound for the examination of follicles, oocyte aspiration and for studies on blood flow to the ovary and uterus (Table 7). It would be hard to imagine IVF today without these major inputs. Its diagnostic value is incalculable, for it provides rapid, non-invasive sightings of the number and size of follicles, warnings of PCOD and hyperstimulation, and may help to witness follicular rupture. Blood flow to the ovarian follicles and the uterus can be calculated. Echogenic patterns in the uterus up to mid-cycle are predictive of implantation. Ultrasound of the implantation sac at day 16 has become possible, and fetal heartbeat and other parameters of growth can be scored from day 20. Invasive methods relying on ultrasound have enabled the use of coelocentesis to collect samples for prenatal diagnosis from the coelomic cavity of human post-implantation embryos aged 28 days and older (Jurkovic et al., 1993).

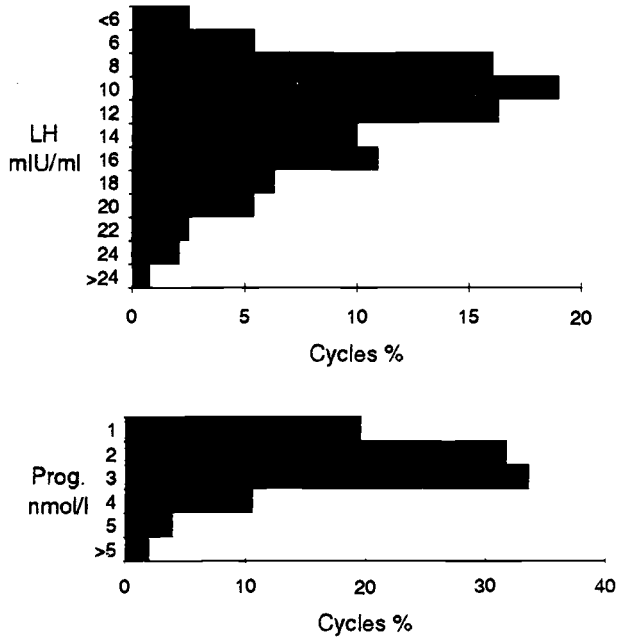


Fig. 18 Proportion of cycles showing flare-up responses in LH and progesterone on day 2 after leuprolide administration (Courtesy of CD Matthews. Matthews et al., 1991).

Table 7 Advances in assisted conception between 1980 and 1990

Approx. 2000 babies in Bourn Hall
Ultrasound of follicles and blood flow
Laws from UK and Spanish Parliament
Many new forms of ovarian stimulation
Embryo cryopreservation introduced
Many embryo studies
Sperm donation/HRT cycles
DNA probes for preimplantation diagnosis
Embryo implantation rates low

GIFT and ZIFT were also introduced as routine forms of treatment during these years. Their value in assisted human conception has become somewhat controversial. Pregnancy rates during GIFT could be artificially raised by the fertilization of those oocytes remaining in their follicles despite attempts at their aspiration. There are suspicions that ZIFT involves selection for patients with many embyros, which could raise the pregnancy rate by selecting the most fertile patients. These two techniques became the primary reasons for the retention of laparoscopy for the collection of ocytes, and the need for this operation is perhaps the greatest handicap with these methods.

Much research was carried out on preimplantation human embryos in culture. It became clear that many human embryos growing in vitro, and perhaps in vivo, were chromsomally imbalanced. Methods of embryo cryopreservation using propanediol, dimethyl sulphoxide and glycerol were introduced, primarily for pronucleate eggs, 4 cell embryos and blastocysts respectively (Lasalle et al., 1985). The cryopreservation of pronucleate eggs was the simplest approach, since the eggs could be easily identified, were all in the same stage of growth, and seemed to tolerate cryopreservation successfully (Table 16).

Micromanipulation was introduced to improve human fertilization. Zona drilling and the insertion of spermatozoa beneath the zona pellucida attracted much attention, and some pregnancies were estabished by this technique. The feasibility of preimplantation diagnosis attracted the attention of several clinics. Initially, the available methods were too limiting or unsophisticated, but DNA technology soon improved sufficiently to produce probes for various nucleotide sequences (Jones et al., 1987; Handyside et al., 1988). Methods for the recovery of one or more blastomeres for analysis, using micromanipulation on human embryos, developed rapidly. The introduction of techniques to measure the uptake of substrates by embryos was valuable as a guide to their metabolism, but not for preimplantation diagnosis since persisting maternal characteristics in the embryos evidently swamped the embryonic contribution.

An agonadal patient with the XO syndrome conceived after oocyte donation and hormone replacement therapy (HRT) and delivered a child (Lutjen et al., 1984). The method was applied to women with other forms of agonadism and worked equally effectively for them. This study would also transform attitudes to pregnancies in post-menopausal women in the 1990's.

Ethical arguments about IVF reached parliaments in various countries. Debates in Victoria were among the first and among the most vitriolic, and were dominated by a strict conservative line. Much more liberal attitudes prevailed in Spain and the UK (only after similar vitriolic debates in the UK parliament), with the passage of legislation that was sympathetic to research yet insisted on a close control being maintained on the clinical and research aspects of IVF. The majority in favour of IVF was 3:1 in the UK parliament, and a similar majority supported the creation of embryos for research, provided the studies were completed by 14 days (Table 8). Perhaps the efforts made by professionals in the field of assisted human conception to explain the ethics of IVF had convinced parliamentarians that research was essential and that it could be carried out within an acceptable ethical basis. A German law based acceptable forms of research on human preimplantation embryos on the

Table 8 Voting in the British Houses of Parliament on the ethics of IVF

House of Lords
3:1 in favour of embryo research
3:1 in favour of establishing embryos for research
House of Commons
>2:1 in favour of embryo research
Clear majority in favour of research embryos

Table 9 Advances in assisted conception after 1990

ICSI and MESA
Molecular approaches to ovary, fertilization, embryo and uterus
Preimplantation diagnosis introduced
Sperm sexing
Recombinant and pure FSH and LH
Pregnancies in women >50 years
Implantation rates still low!!
UK HFEA established

concept of totipotency (Beier, 1991). This form of deciding acceptable legislation is highly unreliable and subject to unexpected changes as scientific opinion on the nature of totipotency is modified as new studies are carried out on preimplantation embryos (Gardner, 1995).

C. Recent studies on IVF and especially on human implantation

Some modern studies on assisted human conception

The pace of research has accelerated even more during the 1990's, and many new advances during this time have led to fresh clinical opportunities and also ethical problems (Table 9). Pride of place among the advances in fertilization in vitro should perhaps be given to the introduction of ICSI (Table 10) (Palermo et al., 1993; Van Steirteghem et al., 1993). It is very successful, since 60% or more of the eggs contain two pronuclei, even using spermatozoa collected from the epididymis or testis. This technique now ensures that hardly any forms of male fertility remain untreatable, even including such severe conditions as the Sertoli cell only syndrome (Silber et al., 1995). Embryos are obtained today in couples who produce more eggs than spermatozoa! The introduction of spermatid injections is likely to increase even more the number of infertile men who can conceive.

The introduction of ICSI has shown how novel and simple techniques can lead to completely new forms of treatment, and how these treatments can be applied across the world in IVF clinics. ICSI is

Table 10 Some recent results with ICSI. Most of these reports were presented at the ESHRE Annual Meeting, 1994. (Edwards and Brody, 1995)

* 1275 ICSI cycles between October 1991 and December 1993 in one clinic included triple sperm defects in 49.6% men, and the use of testicular spermatozoa in 17 cases; 16109 cumulus eggs were injected, 82% in metaphase 2. Among 13407 injected metaphase 2 oocytes, 66,4% were fertilised and 11.4% damaged; three-quarters of the embryos were transferrable. Replacements were made in 91% of cycles, with 2 or 3 embryos in 80% of them; positive HGG tests occurred in 460 cycles (36.1%), clinical pregnancies in 28.3%, 268 prenatal karyotypes were made and 235 children born.
* ICSI rescues 1 day-old unfertilized eggs and gives 48% fertilizations in them.
* More human oocytes injected with medium plus a spermatozoon were activated (56%) than if given medium alone (14%).
* 25/27 GV oocytes and 6/23 metaphase 1 oocytes degenerated during ICSI, and many of the others had disordered polar body extrusion or pronucleus formation.
* Sperm morphology does not effect fertilization or implantation rates during ICSI.
* Epididymal and testicular spermatozoa have a lower potential to give full-term pregnancies with ICSI.
* Epididymal spermatozoa gave 42% fertilization in ICSI; if spermatozoa cannot be aspirated from the epididymis, a testis biopsy provides sufficient to give 42% fertilizations and many pregnancies.
* Pressing the tails of spermatozoa against the bottom of the dish during ICSI immobilises and damages the gametes, and PVP probably stabilises this damage.

overall a very safe method, although there are some unexpected genetic problems. There is apparently a higher risk of sex chromosome trisomies in embryos, and the gene causing congenital absence of the vas has proved to be a variant of cystic fibrosis. Patients must be carefully counselled and diagnosed, yet the overall frequency of anomalies in the children are virtually identical to those arising after IVF or natural conception (Bonduelle et al., 1995). The success of ICSI has also led to a rethinking of many fundamental aspects of fertilization, and has contributed to some fascinating studies on the inheritance of Y chromosome deletions in severely oligozoospermic men (Reijo et al., 1995).

Studies on embryos are also advancing quickly. The frequency of anomalies at fertilization has been well analysed, including a 5% frequency of tripronucleate eggs in vitro, despite the almost total absence of such eggs and of triploid embryos in Oldham. The biggest disappointment with modern embryological studies is that methods of embryo culture have not progressed far since the Oldham days. Many novel concepts have been applied to improve embryonic growth, including the use of

Table 11 Some recent data on preimplantation diagnosis for genetic diseases and other traits using the polymerase chain reaction. Most of these reports were presented at the ESHRE Annual Meeting, 1994. (Edwards and Brody, 1995)

* Deletion of exon 17 of DMD locus in single blastomere to identify non-afflicted male embryos.
* Single-copy X- and Y-linked amelogenin sequences (AMX and AMY) to sex cleaving embryos successful in most embryos.
* Rapid multiplexing of conserved amelogenin gene segments from both X and Y chromosomes plus Y-linked DYZ1 repetitive elements from biopsies and human blastocysts with <2% sexing errors and exclusion of aberrant samples.
* Multiplexing of microsatellite, amelogenin and CF primers gave simultaneous DNA fingerprinting, sexing and detection of CF status.
* Amplification of ZFX/ZFY followed by restriction enzyme digestion to sex single human blastomeres.
* Amplification of 1–2 cells from cleaving embryos for cystic fibrosis; affected and normal girl delivered after diagnosis.
* Amplification of 1- or 2-cell biopsies from 8 cell embryos of 7 couples at risk of cystic fibrosis, 2 for Lesch-Nyhan, and 25 for sex-linked diseases: 16 clinical pregnancies, 3 aborted, 4 ongoing and 9 normal babies.
* Diagnoses of W1282 and ΔF508 mutants of cystic fibrosis in single blastomeres identified homozygous normal, homozygous abnormal and heterotygotes.
* Correct diagnoses on 15 normal, 12 carriers and 7 homozygous cystic fibrosis patients (ΔF508) by a new method of PCR amplication.

feeder cell layers and more complex media, yet implantation rates per embryo remain stubbornly low. In contrast to this static situation, the introduction of preimplantation diagnosis of genetic disease is moving quickly (Table 11). Its molecular biology is exquisite, since several alleles can be amplified from a single cell using PCR (Li et al., 1989), whole genomic amplification (Zhang et al., 1992; Grifo et al., 1995; Handyside, 1996) and fluorescent PCR (Findlay et al., 1995). Perhaps 50 or more genes will soon be amplified from a single cell. FISH has also been perfected to give a rapid 2-hour method of identifying the X and Y chromosomes, chromosome 18 and chromosome 21 simultaneously. Some clinics are scoring all embryos in their patients aged >40, to check for trisomy or for monosomy for XY, 13, 18 and 21. Embryos with these conditions are not transferred to their mothers, to avert the birth of children carrying these chromosomal anomalies.

Other major advances in genetic diagnosis are imminent. Spermatozoa from several species, including human spermatozoa, can now be flow-sorted into two populations containing an overwhelming majority of X or Y spermatozoa respectively (Figure 19) (Johnson et al., 1993; Levinson et al., 1995). The success of X-sorting in 1993 was 70–80%, and better methods are certain to be introduced to improve this degree of sorting even more. Simple and reliable methods of sex choice, based on successful work with several animal species, are thus becoming available in clinical practice. These methods will probably be acceptable socially if they are used to avert the birth of children carrying sex-linked inherited disease. There will probably be many arguments if they are used for the choice of boy or girl for social reasons.

Fig. 19 Results of sorting rabbit sperm samples for X spermatozoa, using Johnson's methods. The upper histogram shows the proportion of X spermatozoa in samples sorted for Y spermatozoa (i.e., more than 80% of X spermatozoa have been removed). The lower histogram shows the results of sorting for X spermatozoa, and the middle histogram gives the data when sorted samples were recombined. The expected frequency is derived from the proportion of X spermatozoa in several samples, and the actual frequency gives the sex ratios of the offspring. Similar results were gained using human spermatozoa (Johnson et al., 1993).

Options for stimulation widened further as the LHRH antagonists entered clinical practice. LHRH antagonists such as Nal-Glu6 suppress immunoreactive and bioactive FSH and LH levels in women for 72 hrs or longer. FSH and oestradiol levels rise steadily, while LH levels remain low when Cetrorelix is administered from day 7 of ovarian stimulation with HMG/HCG (Table 12) (Diedrich et al., 1993). Purified and recombinant gonadotrophins also became available and were used successfully in IVF (Devroey et al., 1992). Doubts remain about the need for pure FSH in ovarian stimulation protocols, since it is expensive and the LH component of HMG may have little clinical significance. The contributions of many fine researchers in companies such as Organon, Serono and Schering in making these exciting new products available must be applauded. Many oocytes could be aspirated from the ovaries of

Table 12 Control of mean LH levels with daily injections of the LHRH antagonist Cetrorelix after stimulation with HMG/HCG (Diedrich et al., 1993)

Day of cycle	Mean levels of plasma hormone FSH mIU/ml	LH IU/ml	Oestradiol-17β pg/ml
5	7.0	4	50
7*	7.8	3.5	100
10	9.0	2.25	250
14+	11.75	2.0	950

* Cetrorelix administered; + Ovulatory HCG administered; progesterone levels remained consistently low throughout the follicular phase

Table 13 Oncogenes and oocyte maturation

cdc2+.	cdc2 34 kd kinase cyclins and cdc2 form MPF
cdc32.	phosphorylates MTOCs
c-kit.	cell signals, kinase receptor
c-fms.	Ligand is SL factor (kit)
c-myc.	nuclear DNA binding protein
c-fos.	regulates gene expression
c-abl.	protein kinase
c-mos.	mos protein in M 2 arrest

most patients, and very large numbers from a few, using stimulation protocols consisting of LHRH agonists, HMG or FSH and HCG. Yet, at this time, concern began to grow about the risks of ovarian epithelial cancer as a result of prolonged ovarian stimulation (Whittemore et al., 1992).

Molecular analyses became increasingly fundamental to many aspects of research into human conception. One example evolved from studies on *Xenopus* and other invertebrates and concerned the role of various oncogenes in oocyte maturation (Table 13). These genes, e.g. $pp39^{mos}$, are presumably active in human oocytes. The mos protein encoded by this gene has been detected in mature human oocytes, and as in other species it apparently arrests meiosis at metaphase 2 until the spermatozoon fuses with the oocyte membrane. The use of gene knock-out to eliminate $pp39^{mos}$ in mice has shown how the oocytes are unable to arrest at metaphase 2, and they immediately proceed to anaphase and beyond if this gene has been eliminated from their mother. Molecular studies are also being applied to human implantation, and I shall conclude this lecture with brief analyses of recent molecular and clinical studies on implantation and pregnancy after IVF.

Molecular basis of human implantation

Preimplantation embryos apparently send various types of signals to the mother. Hatched blastocysts may signal the mother via embryo-derived platelet activating factor (EDPAF) within 48 hours of fertilization and by early pregnancy factor (EPF). Embryos also synthesise oestrogens, e.g. oestradiol is secreted by human blastocysts at 5–8 days post-fertilization (Edgar et al., 1993; Edgar, 1994). Trophoblast of the implanting embryo expresses many receptors (Table 14), so that the embryo increasingly resembles a complex tissue where cytokines, integrins, growth factors and their receptors are expressed.

Table 14 Trophoblast expression: some of the receptors and other properties displayed by trophoblast of human and animal embryos as implantation approaches

Integrins: fibronectin receptors (a_4b_1]
lamin receptors
vitronectin receptors (a_vb_3)
UPA receptors
IL-1 R activated by LIF
IGF-R, EGF-R, LIF receptor
CSF
Fibronectin

Modifications in the uterus involve the formation of decidua which is essential to sustain the implanting embryo. Another uterine activity involves the expression of progesterone-dependent pinopods on uterine epithelium between days 19 and 21. Their function is seemingly to extract fluid from the uterine lumen, so that the embryo and the epithelium are drawn into a very close physical contact. This tight apposition results in an extremely close association between embryo and epithelium. The association is so close that indentations forced into the trophoblast resemble the outlines of epithelial pinopodes and microvilli. It may also enable reactions to occur between complementary binding proteins and other factors on embryos and uterine epithelium during this first stage of implantation (Psychoyos, 1993; Nikas et al., 1995).

Embryo adhesion to the uterine epithelium via polar trophoblast marks the beginning of the implantation process. As trophoblast and epithelial cells are closely apposed, cytoplasmic projections from the implanting embryo extend long trophoblastic protusions between epithelial cells which penetrate to reach the basement membrane. Fibronectin and other substrates may then guide the implanting embryo until it is downgraded by trophoblast. Trophoblast also adheres to laminin, which is distributed around human decidual cells, and it may also secrete this substrate to help with mutual recognition with the uterus (Loke et al., 1989).

Many intermediary factors are expressed in the uterus and embryo during implantation, e.g. EGF,

which may stimulate epithelial cells and gland proliferation. Uterine cytokines include interleukins (IL-1 to IL-13), interferons (IFN-α, β, γ), colony-stimulating factors (M-CSF, C-CSF, GM-CSF), tumour necrosis factors (TNF-α, β), activin, inhibin, and nerve- and platelet-derived growth factors (Tabibzadeh et al., 1994) They regulate the uterine microenvironment via intercellular signals, and many of them are influenced by steroid hormones or locally-released interferons (Bischoff et al., 1995). The four classes of interferons are IFN-alpha, beta, gamma and omega. T cell interferons regulate cytokine activity and local cell-cell interactions, perhaps via the mediation of PGE_2 and HLA-DR. IFN-α, IFN-γ, and TNFα may mediate luteal regression. The cytokine cascade is one of the many complex interactions in the uterus involving cytokines, and interferons.

Various matrices, basement membranes and blood vessels, including gelatin, collagen, fibronectin and laminin, confront the implanting embryo. They may guide and anchor the embryo through the uterine epithelium and stroma. Adhesion molecules, e.g. integrins, immunoglobulin superfamily, selectins and cadherins, are expressed on cell surfaces and interact with matrix membranal substrates. Integrins mediate cell binding to fibronectin, which is cross-linked with collagens (especially collagen I), fibrin, heparin and DNA. Laminin is expressed, together with collagen IV, in blood vessel and gland basement membranes during the mid-secretory phase and in stroma and decidua later. Laminin, fibronectin and collagen IV in extracellular matrices are probably regulated by interferons.

The three integrin families, B_1, B_2 and B_3, are also present in the extracellular matrix, and ligands of B_1 include fibronectin, laminin and collagen IV. There is an immense variability of integrin expression, enabling them to bind with laminin, collagen, fibronectin, vitronectin and von Willebrand's factor. Glandular epithelium expresses mainly a_2, a_3 and a_6 (e.g. collagen/laminin a_6b_1 receptors) and a_1 during the secretory phase (Lessey et al., 1994). Stroma predominantly expresses a_5b_1 (the fibronectin receptor). A defective expression of a_vb_3 may cause infertility, and an absence of b_3 (called type I infertility) results in an out-of-phase endometrium which is correctable by progesterone. Growth factors may regulate a_vb_3 and a_4b_1 expression on endometrial microvilli as they do with specific receptors on trophoblast. a_vb_3 (the vitronectin receptor) is expressed cyclically and b_3 normally appears on day 20. The expression of a_1, a_4 and b_3 on glandular epithelium at days 20–24 of the menstrual cycle may define the implantation window in the human uterus (Table 15).

Table 15 Uterine integrins in epithelium and stroma, and during the implantation window (Lessey et al., 1994)

Eoithelium (glandular + luminal)	
Constitutive	a_2, a_3, a_4, b_1, b_4
Cyclic	a_1 & a_4 luteal only
Stroma	
Constitutive	a_5, b_1
Cyclic	a_1, a_vb_3 (prolif)
Implantation window days 20–24	
Coexpression of a_1, a_4, b_3	

Invasive trophoblast successively expresses different integrin classes. These various classes probably mediate the initial attachment of the embryo, invasion of extracellular matrix 10–15 hours after hatching, the activation of decidual metalloproteases and movement of extravillous trophoblast to the spiral arteries. The expressed integrins include receptors for fibronectin, laminin and vitronectin.

Cytotrophoblast secretes the matrix metalloproteinases (MMPs), i.e. collagenases, gelatinases and stromelysins. Collagenases digest collagen types I, II, III, VII and X, gelatinase digests collagen type IV and gelatin, and stromelysins digest fibronectin, laminin, collagens IV, V and VII (Matrisian, 1990). Type IV collagenases and serine protease uPA degrade matrices and remodel extracellular matrix during implantation. They are regulated by steroid hormones and growth factors, e.g. EGF, IL-1, TGF-β which balance the activities of proMMp-1 and TIMP-1. Invasive trophoblast also expresses cytokeratin, high affinity uPA receptors, HLA class I framework antigens, receptors for integrins and LIF, and the integrins a_3, a_5, b_1 and a_vb_3/b_5 (the vitronectin receptor) which may regulate the penetration of extravillous trophoblast to the spiral arteries. Extravillous trophoblast expresses the non-polymorphic histocompatibility antigen HLA-G (Bischoff et al., 1995), and migrates to the uterine blood vessels, probably via the expression of M-CSF and the c-*fms* and c-*kit* genes which encode for specific ligands. Targetted mutations of the LIF gene impair implantation in mice, whereas *op/op* mice (with a congenital absence of CSF) are infertile.

It is obviously essential not to oversimplify the paracrinology of the uterus during implantation. Many complicated interactions occur, each involved with specific uterine components, such as the limitation of trophoblast invasion, recognition of the embryonic HLA antigens and the regulation of blood flow to uterus and placenta. The close relationship between the duration of the implantation window, the expression of uterine integrins and the existence of the pinopods suggests that they are controlled by the same regulatory factors.

Table 16 Implantation rates per embryo in acyclic women given HRT therapy and in cyclic women using cryopreserved embryos (Edwards et al., 1991)

	No of cycles	Implantation rates All patients	Pregnant only
Cyclic	542	12.1	38.1
Acyclic	78	19.4	41.7

Table 17 Pregnancies in women aged between 45 and 63, using data collected from various clinics

	Details of pregnancies			
Clinic	1	2	3	4
Pregnancies	31	50	66	44
Clinical pregnancy rate	28.1		28.5	31.4
Implantation rate	14.4	17.4	11.9	12.6
Abortions	4	8		7

New clinical concepts on human implantation

Clinical studies attempting to raise pregnancy rates after assisted human conception provide much information on implantation. One remarkable finding concerns the high fertility of agonadal and postmenopausal recipients given donated oocytes plus HRT therapy (Edwards et al., 1991). These patients include women with ovarian dysgenesis, premature menopause, X-chromosome abnormalities, and those having an early menopause after surgery. They remain highly fertile up to the age of 60 and beyond, and age-for-age are more fertile than cyclic women of equivalent ages (Edwards and Brody, 1995). Agonadal women aged <45 are exceptionally fertile, with implantation rates per embryo >20% and even higher as compared with 11% in normal IVF patients. Similar results are gained for women in mid-late 40's, some of them post-menopausal (Serhal and Craft, 1989). Women aged 40–43, 44–47 and >47 established 31% clinical pregnancies per transfer, with no variation due to maternal age (Table 17). Couples aged 50–59 had 38% pregnancies per attempt and a 19% implantation rate, and pregnancies have been established in women up to age 63. These exceptional results are not due to differences in donor age.

Some investigators reason that their high rate of pregnancy after oocyte donation and HRT is due to a period of amenorrhoea (Marcus and Edwards, 1994; Borini et al., 1995). This conclusion is based on the high pregnancy rates (>40%) obtained when cyclic women treated for endometriosis are down-regulated with LHRH agonists for 4 months or more, to become highly fertile after IVF. Their pregnancy rates reach 40% and more, while controls remain at 10%. Similar values are obtained when perimenopausal cyclic women of the same ages are given down-regulation for several cycles (Borini et al., 1995).

Perhaps amenorrhoea enables uterine steroid-sensitive systems to recover from the effects of constant menstrual cycles. The pinopodes are highly progesterone-sensitive (Nikas et al., 1995). Their malfunction would distort the evacuation of uterine fluids, so that embryos may have difficulty in adhering to the uterine epithelium. New approaches to implantation could involve the use of progesterone antagonists such as RU 486 to reduce any overexposure of uterine organelles to progesterone (Beier et al., 1994).

Other approaches to raising pregnancy rates after IVF

Finally, two other approaches to raising pregnancy rates after IVF must be considered. One involves the use of aspirin to improve blood perfusion in women with an impaired blood flow (Wada et al., 1994). The second approach involves the immunology of reproduction. The treatment of women with anti-phospholipid antibodies who are undergoing IVF with heparin and aspirin raises their pregnancy rates from 10–20% to almost 50% (Sher et al., 1994). The positive effects of the treatment may involve some form of protection to the early syncitiotrophoblast, before or after implantation. Similar results are being gained using anti-gamma globulin injections in women undergoing IVF (Coulam et al., 1994).

Clearly, these leads into the improvement of implantation rates are of immense importance for IVF and its derivatives. The low rate of implantation per embryo (12–15%) has been a problem since Louise Brown was conceived; perhaps this period is finally coming to an end.

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Correspondence address: Prof. Dr. R. G. Edwards, Churchill College, Cambridge CB3 0DS, 113–115 Harley Street, London, United Kingdom