### Review

### IVF, IVM, natural cycle IVF, minimal stimulation IVF – time for a rethink



Professor Bob Edwards

Professor Bob Edwards succeeded with colleagues in fertilizing the human egg in vitro in 1969. Then he applied IVF clinically with Patrick Steptoe working from Cambridge University, later establishing Bourn Hall Clinic. He has been awarded numerous honorary DSc degrees and published many scientific papers and books, including A Matter of Life with Patrick Steptoe in 1980. Recent awards include the Lasker Clinical Medical Research Award (2001). Grand Hamdan Award for Clinical Science (2002), and the Pioneer in Stem Cells Award from Pittsburgh Development Center, USA (2004). In 2005, he won the highest award of the RCOG, the Eardley Holland Gold Medal. In 2006, he received the 30th Joseph Bolivar DeLee Humanitarian Award from Chicago Lying-in Hospital, as well as an Honorary Doctorate of Medicine from the Karolinska Institutet, Sweden. This year (2007) he was honoured twice in France, becoming a Chevalier dans l'Ordre National de la Legion d'Honneur, and winning the Jacques Salat-Baroux

Prize. He was also appointed Honorary Fellow of the Institute of Biology in the UK.

#### **RG Edwards**

Chief Editor, Reproductive BioMedicine Online, Duck End Farm, Dry Drayton, Cambridge CB23 8DB, UK Correspondence: e-mail: rge@rbmonline.com

#### Abstract

Considerable changes are afoot in the practice of assisted human conception. Doubts about its methods, especially over endocrinology, concern its complexity and its expense. IVF has spread worldwide since its beginnings in the UK, but its current practice, termed routine IVF, is being challenged by simpler routines. These include natural cycle IVF, which has been in the background for many years, minimal stimulation IVF, where doses of hormones are reduced, and the in-vitro maturation of human oocytes ready for fertilization in vitro (IVM). These three approaches are now practised in increasing numbers of IVF clinics, and may well replace routine IVF. The events leading to current interest in these methods will be discussed briefly in this review

Keywords: gonadotrophins, IVF, minimal stimulation, oocyte maturation in vitro, ovarian hyperstimulation

### Introduction

I am honoured to open this important meeting. It is very timely. It stresses that a new phase of assisted human conception is opening with the realization that methods currently in use to stimulate follicle growth and ovulation in infertile patients are too extreme and too expensive. Routine IVF, as we know it, is currently under close scrutiny as queries arise about the need for high doses of costly recombinant hormones, gonadotrophinreleasing hormone (GnRH) agonists and antagonists. The cost of these hormones and the complexities of their use in ovarian stimulation are the primary objections, although simpler laboratory techniques may also be introduced, based on IVF practice in animals. Alternative approaches are therefore being sought, including minimal stimulation IVF, natural cycle IVF and maturing human oocytes in vitro to prepare them for fertilization in vitro. Each of these approaches avoids the use of large doses of human menopausal gonadotrophins (HMG) and human chorionic gonadotrophin (HCG) that have become essential for routine IVF. The following text is based largely on contributions to these new approaches to IVF from my colleagues and me.

### Early studies on the endocrine induction of ovulation in animals

Routine IVF emerged from research on animal reproduction that was gained in the nineteenth and twentieth centuries when very little was known about human conception. Studies were pursued on follicle growth, ovulation, fertilization and early embryonic growth in mammals, as well illustrated when Heape (1880) introduced embryo transfers in rabbits and obtained full-term offspring. The tempo increased in the early twentieth century as shown, for example, by Lewis and Gregory (1929), who filmed cleaving rabbit eggs to blastocyst stages including hatching from the zona pellucida, and by Lewis and Hartman (1933), who studied cleaving eggs of Rhesus monkeys. Studies on 32 human embryos flushed from the oviduct after intercourse revealed anomalous forms, including chromosomal defects and abnormal forms that increased with advancing maternal age (Hertig et al., 1952). Analyses on the timing of ovum transport in the female reproductive tract revealed the various stages of embryogenesis that occurred in segments of the oviduct and in the uterus timed from the onset of the LH surge in mid-cycle (Croxatto *et al.*, 1978). Such insights were to prove invaluable for the introduction of human IVF.

Studies on the endocrinology of mammalian reproduction also accelerated in the early twentieth century. Research by Ascheim and Zondek (1927) led to a greater awareness of pituitary gonadotrophins and ovarian feedback systems, and too many studies on the role of the hypothalamus (see Lunenfeld, 1969). Endocrine aspects of the complex hypothalamic/pituitary/ ovarian axis, for example, clarified hypothalamic responses to sensory systems and secretions from the limbic system (MacLean, 1973). The decapeptide structure of LHRH and its role in controlling the release of FSH and LH were discovered by Schally in 1978, and its pulsatile release was clarified by Knobil (1974). Knowledge also accumulated on its daily, nycterine (sleep), hourly and sporadic rhythms (Kleitman, 1969; Rebar and Yen, 1979; Edwards et al., 1980a). The hypothalamic co-ordination of these systems was sealed by the discovery of the hypothalamic/pituitary portal vessels (Harris and Naftolin, 1970; Holmes and Ball, 1974), since this anatomical knowledge revealed how LHRH stimulated pituitary gonadotrophes to release FSH, LH and prolactin, and so stimulate gonadal differentiation (Everett, 1969). In turn, greater understanding of the role of gonadotrophins in regulating the growth of ovarian follicles and their oocytes led to studies on endogenous hormones that could be purified from pituitary glands, blood or urine. The availability of these hormones then resulted in their use as endocrine preparations for use in the stimulation of reproductive systems in animals and humans (Rebar and Yen, 1979; Edwards, 1980).

Epidemiologists had clarified various aspects of human reproduction. Birth rates, the epidemiology of conception and infertility, and the frequency of abortion were linked to studies on the chances of conception in women during natural mating (Leridon, 1979). Conception often required several menstrual cycles before pregnancy was established, with involuntary childlessness afflicting ~10% of UK couples and 28% of US couples aged 35 and older (Southam, 1960; Hirsch and Mosher, 1987). Problems with ovulatory disorders, primary and secondary amenorrhoea, tubal blockage, cervical hostility, oligozoospermia and azoospermia were well recognized among other factors leading to male and female infertility. Diagnoses of infertility included rising body temperature in women, tests on cervical mucus, disturbed hormonal rhythms, tubal repair, sperm assays and artificial insemination.

Treatments with available exogenous hormones were now designed to stimulate ovarian function in animals, and hormonal extracts from the pituitary gland, blood or placenta were utilized to induce ovulation and oestrus in various animals. A belief emerged that such stimulatory treatments were effective in immature animals but not in adults, e.g. in rabbits (Walton and Hammond, 1928; Fee and Parkes, 1929; Parkes, 1976). Similar beliefs applied to studies involving induced oocyte maturation and ovulation in immature female mice injected with pregnant mare's serum (PMS) as a source of FSH, and then with human chorionic gonadotrophin (HCG) as a source of LH (Runner and Palm, 1953). The immature mice entered oestrus, mated and ovulated soon after the HCG injection. Large numbers of eggs were recovered from their oviducts and uteri,

some of them being transferred into uteri of non-pregnant adult females to establish many pregnancies. This evidence showed how embryos established via injections of gonadotrophins into immature mice were fully capable of implantation and growth to full term (Gates and Beatty, 1954).

In a tentative move to the use of gonadotrophins in adult females, Fowler and Edwards (1957) initiated the use of PMS and HCG to induce multiovulation and pregnancy in adult female mice. Given small doses of hormones, virtually every adult female simultaneously entered oestrus from 6 h post-HCG, ovulated numerous oocytes at 12 h post-HCG, mated with a male and produced large numbers of fertilized eggs. Endocrine systems such as diurnal rhythms or the stage of the oestrus cycle when PMS was given had few or no effects on responses to exogenous gonadotrophins. Oocytes in each of the stimulated mice matured and ovulated at exactly the same rate (Figure 1). Diakinesis was observed 2–3 h post-HCG (Figure 2), with full maturation to metaphase 2, the extrusion of the first polar body and the onset of ovulation occurring at ~11.5 h. Fertilization and cleavage of the embryos also progressed in synchrony in virtually all of the treated mice. Close timing was found to be typically species-specific in each of the mammalian species that were examined. Numbers of ovulated oocytes and embryos varied between 10 to 50 or more, and embryos implanted in considerable numbers in their mothers' uteri, far more than in natural cycles. Accordingly, multiple pregnancies were established, averaging 30 implanted embryos or thereabouts (Edwards and Fowler, 1959).

#### Oocyte maturation in vitro

An alternative approach now emerged to the use of endocrine stimulation to obtain pre-ovulatory oocytes. It involved aspirating oocytes in germinal vesicle stages from their Graafian follicles and then placing them in culture media. This initiated their maturation *in vitro* to metaphase II and the extrusion of their first polar body, showing they were ready for fertilization *in vitro*. These studies began in the 1930s when Pincus and Enzmann (1935) and Pincus and Saunders (1939) reported that immature rabbit oocytes matured spontaneously *in vitro* over 12 h after liberation from their follicles into culture media. Their investigations also concluded that human oocytes likewise matured in 12 h, but this was an unfortunate error.

Seeking to introduce human IVF into clinical care for infertility, and wishing to stimulate oocyte growth from the germinal vesicle stages to metaphase II and an extruded polar body, Edwards (1965) rediscovered the work by Pincus and Enzmann (1935) and Pincus and Saunders (1939). These authors reported that immature mouse, rat and rabbit oocytes matured in 12 h or so, and that human oocytes required the same amount of time. Edwards (1965) concurred with the timings as being  $\sim 12$ h in mice, rats and rabbits, whereas oocytes from cattle, sheep, rhesus monkey and humans required longer intervals in vitro, e.g. ~31 h in cows and rhesus monkeys, ~37 h in humans and ca 40 h in pigs (Figures 1 and 2). Maturing oocytes in vitro was simple, required no hormones and opened pathways to studies on oocyte maturation and fertilization in vitro. Moreover, the timings for full maturation in vivo to metaphase II after an injection of HCG in various species were found to be identical with the timing of oocyte maturation in vitro (Edwards, 1965).





**Figure 1.** Timing the stages of meiosis I after an injection of HCG, showing the onset of prophase I, the timings of metaphase I, anaphase I and telophase I until the extrusion of the first polar body and the onset of metaphase II and ovulation. Numbers represent individual oocytes, for example the female autopsied at 8 h provided six oocytes in metaphase I and two in anaphase I. Note how the first ovulation began at 11 h, and the great majority had ovulated by 13 h.



Figure 2. Examples of diakinesis, a late stage in the first meiotic division, in maturing oocytes of (a) mice and (b) humans. Mouse oocytes required 4 h to reach this stage of meiosis and 11.5 h to complete maturation, whereas human oocytes (right) needed  $\sim$ 23 h to reach diakinesis and 37 h to complete their maturation (Edwards, 1965).



Pincus and Saunders (1939) had sadly misled later workers (Chian *et al.*, 2004) contemplating the introduction of in-vitro maturation (IVM) because of a lack of awareness of the structure of meiotic chromosomes, especially those in diakanesis, which were excellent markers of the meiotic resumption after a long delay in diplotene (**Figure 2**).

Since human oocytes required 37 h to mature *in vitro*, it was a simple matter to predict that women would ovulate 37 h after an injection of HCG. This was confirmed some years later when laparoscopy enabled follicles in human ovaries to be visualized very clearly as they ruptured during ovulation at 37 h post-HCG (Steptoe and Edwards, 1970). This information proved to be invaluable for the development of human IVF.

## Endocrine stimulation of ovarian and oocyte maturation

Attention in the later twentieth century was increasingly paid to the endocrine stimulation of follicle growth and oocyte maturation in humans and in adults of various animal species. Gemzell et al. (1975) induced ovulation in amenorrhoeic women by initially administering FSH preparations extracted from human pituitary glands followed a few days later by an injection of HCG to induce ovulation. Pregnancies were established, but many were multiple including octuplets, since numbers of ovulated eggs could not be controlled as shown previously in mice (Fowler and Edwards, 1957). Samples of human urinary menopausal gonadotrophins (HMG) were now given to oligomenorrhoeic women to stimulate the growth of several ovarian follicles, followed by HCG to induce ovulation (Klopper et al., 1974). Methods improved further when Donini and Lunenfeld (1974) prepared highly purified, FSH-rich preparations of HMG that could be obtained commercially (named Pergonal), and used it with HCG to stimulate ovulation in oligomenorrhoeic and amenorrhoeic women.

Two distinct means of obtaining human eggs at metaphase II and with an extruded first polar body were now available. They could be aspirated from growing Graafian follicles and matured in vitro for 37 h as just described, or gained by administering modest doses of HMG and HCG to patients in order to stimulate several follicles to mature and then aspirating their pre-ovulatory oocytes at 36 h post-HCG, just before the follicles were about to ovulate. Ripe oocytes had to be aspirated an hour or so before the follicles ruptured for ovulation, otherwise they could have been lost in the peritoneal cavity. While pondering on whether to choose hormonal stimulation or maturation in vitro as the best approach to human IVF, Chang (1955) reported that he had matured rabbit oocytes in vitro and then transferred them to oviducts of mated female recipients to see if the embryos developed normally. Some were found to be abnormal. This discovery militated against choosing to mature human oocytes in vitro for IVF, especially since there had been no reports of anomalies among children born to amenorrhoeic mothers after stimulation by HMG/HCG. The decision was therefore taken to use the endocrine method for human IVF.

My interest in alleviating human infertility via human IVF had now extended to using mild doses of HMG and HCG to induce oocyte maturation in the patients and then to aspirate ripe oocytes from their follicles, ready for fertilization. The practical application of this work would be based on earlier work in stimulating ovulation in adult cyclic mice, on studies by Gemzell and others that would involve testing various hormonal treatments in patients, and by the availability of Pergonal (**Figures 3** and **4**). It would be essential to work in rhythm with the menstrual cycle to avoid any hormonal complications. Human IVF would thus involve controlling follicle maturation by means of HMG and HCG, and a clinically acceptable method of approaching follicles to aspirate their oocytes. Low doses of hormones were selected which should produce an average of five or six oocytes per patient. A good rate of fertilization *in vitro* would be expected to provide sufficient embryos for replacement to the mother's uterus.

Giving patients three doses of HMG in their follicular stage, each dose composed of three ampoules, should be sufficient to stimulate the maturation of several follicles containing mature oocytes (Figure 3a). Daily measurements of urinary oestrogens and pregnanediol would help to assess follicular responses in each patient. HCG would be injected around day 10-11 when oestrogen concentrations should have risen to confirm that several follicles had responded to HCG. Pre-ovulatory follicles were aspirated 36 h later, just before the onset of ovulation. This course of action become possible as Jean Purdy (nurse) and Patrick Steptoe (gynaecologist and world expert in laparoscopy) joined the team. Proposals designed to introduce human IVF therefore began in the late 1960s, and it had to be arranged in the Oldham and District General Hospital where Patrick had his patients. Oldham was almost 200 miles from Cambridge so I was destined to travel this journey there and back for 10 years in order to maintain my duties with PhD students in Cambridge University.

#### Fertilization in vitro

The major problem in introducing human IVF now lay in the successful fertilization of human oocytes in vitro. Sufficient mature oocytes would be available, but an apparently difficult problem lay in the need to capacitate the spermatozoa. Research on several animals had revealed this process to be seemingly essential for sperm attachment to oocytes and later stages of fertilization, and it was often achieved by collecting uterine spermatozoa for use in insemination (Austin, 1952, 1975). Only a single study had succeeded using oocytes in vitro, namely Yanagimachi and Chang (1964) working with gametes from golden hamsters. In 1969, human oocytes aspirated from their follicles after hormonal stimulation were successfully fertilized in vitro without any need for capacitation unless it had occurred spontaneously (Edwards et al., 1969). All stages of fertilization to late pronuclear stages were identified. The way was now open to mature and fertilize human oocytes in vitro, and replace the resulting embryos into the uterus of infertile couples. Unexpected problems now arose in relation to hormonal stimulation.

# Ovarian stimulation and its problems

Low doses of administered gonadotrophins provided sufficient follicles to sustain IVF. Mean numbers of follicles averaged 9.2 with 300–377 IU of HMG, rising to 11.4 with 975–1125





Figure 3. (a) Treatments with HMG and HCG in Oldham during early 1970s. Usually three ampoules were given per injection (Edwards, 1965). (b) Treatment with clomiphene/HMG/HCG.



Figure 4. Cluster analysis of follicles in natural or induced cycles based on concentrations of progesterone,  $17\alpha$ -hydroxyprogesterone, oestradiol-17β, oestrone, androstenedione, testosterone, pregnanediol and 17αhydroprogesterone in follicular fluid (Fowler et al., 1978); (a) natural cycle, (b) induced cycle. Follicles could be classed as non-ovulatory or ovulatory from their appearance during laparoscopy when their follicular fluids were aspirated. Notice the clear separation into ovulatory and non-ovulatory follicles in natural cycles. In contrast, numerous classes of variant follicles followed treatment with HMG/HCG and it was difficult to decide which of four major groups of follicles were ovulatory or non-ovulatory. These immense variations implied that enormous differences arose between follicles after ovarian stimulation.

110

IU (Edwards and Steptoe, 1975; Edwards *et al.*, 1980a). Large and presumably ripe follicles increased from a mean of 2.2–5.6 within the same dose limits. Oocytes were aspirated from 46% of follicles sized <1 cm, rising to 73% from those sized >1.75 cm (Steptoe and Edwards, 1970). Aspirating oocytes did not harm the formation of the corpus luteum, which synthesized progesterone in luteal phase of the cycle. The types of steroids in aspirated follicular fluids reflected the stage of the menstrual cycle at the moment of aspiration. Oestrogens and some androgens typified follicular stages, and progesterone dominated follicles approaching ovulation.

Cluster analyses on concentrations of several steroids in follicular fluids aspirated in mid-cycle revealed two major follicular classes, separated by a large within group variance (**Figure 4a**). One class was ovulatory and the other non-ovulatory, as would be expected in a normal menstrual cycle. In stimulated patients cluster analyses differed, since differences between follicles were far less and there seemed to a continuum from one class to the other. In contrast, concentrations of various steroids in fluids aspirated from follicles of patients stimulated with HMG and HCG displayed immense variations (**Figure 4b**) (Fowler *et al.*, 1978). Such varying degrees of maturity might have led to variations in embryo quality, although this matter has not yet been solved even today (Wang *et al.*, 2006). It was nevertheless very clear that injecting HMG had stimulated some follicles to grow that would have otherwise decayed.

Second, several variations in follicular activity concerning diurnal and other rhythms in LH activity in some IVF patients was noted. For example, gonadotrophins are released into peripheral circulation in pulses every 90-100 min coincident with rapid eye movement (REM) sleep (Kleitmen, 1969). No attempts were made to utilize pulsatile injections of HMG when patients enrolled for IVF. A daily rhythm was discovered in the onset of the LH surge in UK patients, with concentrations beginning to rise at 3 a.m. in synchrony with rising cortical concentrations. In contrast, newly arrived US patients attending Bourn Hall for IVF initiated their daily LH rhythm at 7 a.m. after the onset of their treatment (Figure 5a). Those staying in the UK until their second attempt at IVF shifted their diurnal rhythm to 3 a.m., indicating they were adapting to UK time (Edwards, 1980). Variable cortisol and LH rhythms hence characterized patients coming from North America as they waited for their second cycle, although once again it was unclear if such variations influenced success with IVF. The strength of seasonal rhythms also modified the onset of patients' LH surge (Figure 5b), another endocrinological factor that may have affected success with IVF, since this rhythm was well marked in winter but weakened in summer. Once again, numbers were too few to study the implications of their varying menstrual rhythms on the efficacy of treatment with HMG/ HCG (Edwards, 1980).

A complex endocrine situation emerged in patients stimulated with HMG/HCG after a decision had been taken to transfer a single blastocyst to them on the assumption that humans would be as fertile as mice, rabbits and other animal species. Unexpectedly, many of them displayed a shortened luteal phase. This was associated with a lack of IVF pregnancies, since the duration of their luteal phases varied between 6 and 14 days, which was insufficient to sustain embryo implantation in many of them (**Figure 6**). Its causes remained unknown, although it was positively related to the total output of urinary oestrogens during their luteal phase. Treatments with HMG/HCG had apparently led to varying concentrations of oestrogens, which had harmed the corpus luteum. Feichtinger *et al.* (1982) attributed luteal phase deficiency as being at least partially due to aspirating many follicular cells just as ovulation was imminent. Daily progesterone support was clearly needed to correct this and any other cause of luteal phase defect in order to establish pregnancies. Patients would have to be given this treatment until week 8 of pregnancy, when the placenta begins to produce progesterone.

These short luteal phases in our patients led to near-disaster in our IVF programme. It was clear that patients required daily injections of progesterone in oil over several weeks until the placenta began to produce progesterone. This approach could cause scabbing in our patients, so instead Primulot depot was given three or four times during the luteal phase, since it was sold by Schering as a progestagen. It should have sustained the single blastocyst that had been transferred to their mother but, unfortunately, Primulot depot was also an abortifacient. It actually destroyed embryos soon after they had implanted, a fact revealed some time later when patients' blood samples over the expected period of implantation after embryo transfer were found to contain short-term increases in HCG, indicative of a lost early pregnancy. Several short-term pregnancies had obviously been established but then destroyed and overlooked in our patients. We named these brief implantations 'biochemical pregnancies'.

# Establishing the first full-term pregnancies after IVF

Steptoe's final days with the UK National Health Service were ending so we accelerated our Oldham IVF programme. A series of novel approaches were therefore tested in order to achieve an IVF pregnancy. One new approach stemmed from the discovery by Greenblatt et al. (1961) that clomiphene citrate induced ovarian stimulation and a full luteal phase in infertile patients (Kistner, 1975). Combinations of clomiphene and HMG (clomiphene/HMG) were tested to establish a normal luteal phase (Figure 3b). Bromocryptine/HMG was also assessed, since it reduced high prolactin concentrations in our patients. Injecting spermatozoa and a single oocyte into the Fallopian tubes was tested, a method later named gamete intra-Fallopian transfer, or GIFT. Primulot was discarded and the first clinical IVF pregnancy anywhere in the world was established by means of clomiphene/HMG (Steptoe and Edwards, 1976). Sadly for our patients, Steptoe diagnosed it as being ectopic at 10 weeks 'gestation and it had to be removed. Yet we now knew that two great problem had been solved, namely that my culture methods and ovarian stimulation with clomiphene/HMG had sustained a human embryo capable of developing for 10 weeks in vivo. Secondly the moderate use of HMG had indicated that minimal dose IVF had established and sustained this embryo. Human IVF was on the way!

Obsessed by attempts to avert short luteal phases after ovarian stimulation, we abandoned this in favour of natural cycle IVF. This demanded measuring urinary LH concentrations to time the onset of the LH surge preparatory to ovulation. Urinary concentrations run behind plasma concentrations, so







Figure 5. Two examples of endocrine rhythms in patients being treated for IVF (Edwards *et al.*, 1980b). (a) Diurnal rhythms in a US patient attending Bourn Hall for IVF treatment. Urinary LH concentrations rose at 7 a.m. during their first treatment cycle, and at 3 a.m. during their second treatment cycle as they corrected to UK time. (b) The onset of LH surges is shown in patients awaiting IVF treatment. Notice the distinct diurnal rhythm in winter and the weakest rhythm in summer.



**Figure 6.** The duration of shortened luteal phases in patients treated with HMG/HCG was related to the concentrations of urinary oestrogens in their follicular phase.

it was estimated that ovulation would occur ca. 32 h after the urinary LH surge. A sedimentation assay named Higonavis (Mochida Pharmaceuticals, Brocades), adapted to measure urinary LH, was a rapid assay fully capable of measuring LH concentrations in urine samples collected and assayed every 3 h (**Figure 7**). In practice, it was found to provide sufficient timely information on the LH surge to determine when laparoscopy would be needed to aspirate the maturing oocyte from its pre-ovulatory follicle. This form of natural cycle IVF accorded exactly with fundamental events of the menstrual cycle and avoided any need for exogenous gonadotrophins.

The second patient on natural cycle IVF was Lesley Brown. Her LH surge was detected, her pre-ovulatory oocyte aspirated and inseminated within an hour, and her 8-cell embryo replaced late on day 2 of her luteal phase. Transplanting 8-cell embryos instead of blastocysts shortened the period of embryonic growth *in vitro* and placed the embryo in its natural setting in the uterine cavity much sooner than offered by blastocyst transfers at day 5. Later cases of natural cycle IVF resulted in the first three more IVF babies, the first and second proceeding normally to term in 1978 and 1979. The third fetus was fully normal, as it was aborted at mid-term and lived for only 3 days, as a consequence of its parents taking a walking holiday in the Yorkshire hills. The fourth pregnancy was discovered to have a triploid fetus, which was removed from its mother's uterus.

These results ended the Oldham phase of IVF. Bourn Hall was opened 2 years after the birth of Louise, this delay being due to the need to convert it into the world's first IVF clinic. Numerous pregnancies were immediately established using natural cycle IVF (**Table 1**) or ovarian stimulation with clomiphene/HMG/HCG. Numbers rose to ~100 in a year or so and to 1000 in 6–7 years (Edwards *et al.*, 1984). By today, routine IVF has produced well over 3 million babies. Dissatisfaction with some of its aspects rose to the point where many clinics desired to modify the current routine of IVF and introduce simpler measures. Different IVF clinics decided their own approaches to new forms of IVF, a situation recalling the earliest days of IVF and the need to select either ovarian stimulation with mild doses of HMG and HCG or mature human oocytes *in vitro* and avoid any need for hormonal stimulation.

Clinics could now select routine IVF, minimal stimulation IVF, natural cycle IVF or IVM. Routine IVF still appealed to most clinicians and scientists as IVF clinics opened worldwide and established numerous pregnancies. Natural cycle IVF was practised spasmodically, and Lopata (1980) established a full-term pregnancy with it before switching to clomiphene/HMG and obtaining several more. Minimal stimulation IVF was achieved in some clinics, and IVM was pursued by rare clinics without establishing any pregnancies. Trounson *et al.* (1981) stimulated patients with clomiphene citrate or clomiphene/HMG, transferred two embryos and obtained several pregnancies, some of them twins. Other forms of treatment were introduced as experience with routine IVF spread widely (e.g. Testart *et al.*, 1983). These were the years when ultrasonic control over the aspirating needle began to replace the laparoscope (Wikland *et al.*, 1983).

## Hormonal stimulation becomes more complex

Methods for ovarian hyperstimulation now became much more

complex (Table 2). Contraceptives were used by some clinics to stabilize the menstrual cycle before Pergonal was injected. Endocrinologists introduced GnRH agonists and antagonists to provide a better control over FSH and LH concentrations during follicular and luteal phases of stimulated cycles including the correction of premature LH surges (Porter et al., 1984; Engel et al., 2005). Recombinant FSH, LH were introduced to avoid the use of urinary products which were claimed to be less specific in their actions and difficult to obtain in amounts sufficient to satisfy demand (Loumaye, 1990; Bergh et al., 1997). Occasional cases of hyperstimulation and polycystic ovaries arose during this treatment. Recombinant HCG was introduced later and was very effective in providing luteal phase support in hyperstimulated cycles and to induce ovulation in treatment cycles (Trenchard-Lugan et al., 2002). Pregnancy rates nevertheless remained very low at ca. 15-20%, whatever form of stimulation was employed.

Low pregnancy rates led to three and more embryos being replaced in some clinics, resulting in numerous multiple pregnancies, many of them twins, triplets and often more. It still remains uncertain if the use of recombinant gonadotrophins has offered better pregnancy rates than those gained with urinary preparations, despite their very high cost. Insulin was now recognized as an important factor in hyperstimulation, and metformin is being used to reduce aromatase activity and steroidogenesis and so reduce the risks of polycystic ovarian syndrome. It might also serve to replace FSH, when given at 8-h intervals from before the onset of stimulation, continuing until the first  $\beta$ -HCG assay for pregnancy. Claims have been made for its effectiveness and various investigators remain unconvinced about its value in controlling polycystic ovaries (la Marca et al., 2003; Genazzani et al., 2004; Khattab et al., 2006). Metformin may have other advantages in lowering concentrations of endothelial and coagulation markers, including soluble adhesion molecule and soluble intercellular adhesion molecule.

Pregnancy rates improved and multiple pregnancies declined as new methods identified the best-quality embryos for transfer. This was achieved by timing the first and second cleavage divisions *in vitro* and transferring one or two of the fastestgrowing embryos. This led to pregnancy rates of 12.9% when slow-growing embryos were transferred versus 42.3% with fast-growing embryos (Edwards *et al.*, 1984). High-quality embryos could also identified by scoring them for fragmenting blastomeres or multiple nuclei within blastomeres. New culture media also improved embryonic growth to blastocysts, and microarrays are currently entering clinical practice embryos to enable selection for desirable genes.

Further developments improved the practice of IVF and preimplantation genetic diagnosis during the 1990s. Intracytoplasmic sperm injection (ICSI) was introduced by Palermo *et al.* (1992) and spread worldwide. Offering a simple method of replacing normal fertilization by injecting a single spermatozoon into a mature oocyte, it replaced the need to inseminate oocytes with hundreds or more spermatozoa. Its benefits included treatments for severe male infertility when very few testicular spermatozoa were available. It also avoided the attachment of many spermatozoa to the zona pellucida after insemination *in vitro*, which raised problems for preimplantation genetic diagnosis.





**Figure 7.** Endocrinology of patients attending for natural cycle IVF using HiGonavis as a rapid sedimentation assay for LH. Total oestrogens and pregnanediol in urine measured the growth and differentiation of the follicles, and Higonavis enabled the onset of the LH surge to be identified in the majority of patients. Radioimmunoassays were used as a confirmation (Edwards *et al.*, 1980b)

Table 1. Experience with natural cycle IVF in Bourn Hall.

Results October 1981–January 1982		Fate of first 101 natural cycle and clomid pregnancies	
Cases	35	Patients delivered	57
With aspirated oocyte	29	Babies	59
Fertilized eggs	25	Third trimester	3
Replacements	24	Abortions	26 (30)
Clinical pregnancies (%)	6 (25)	Biochemical	15 (15)

Adapted from Edwards (1983).

**Table 2.** Successive stages in the history of ovarian stimulationfor human IVF between 1970 and 2000.

Time period	Stimulation agents	
1970s	HMG, HCG or clomiphene, HCG	
1980s	Clomiphene, HMG, HCG	
1990s	GnRH agonist, HMG, HCG	
1995	HMG, GnRH antagonist, HCG	
1998	GnRH agonist, HMG, HCG/GnRH/rLH	
2000	GnRH, rFSH, rLH	

Italics indicate agents used to induce ovulation.



Another novelty raised pregnancy rates to still higher levels. Involving the use of FISH (fluorescent in-situ hybridization), chromosomal complements in fertilized eggs could be identified and those that were heteroploid, polyploid, carried translocations or other cytogenetic anomalies could be discarded (Kuliev *et al.*, 2005; Munné, 2006). Chromosomal anomalies were found to arise in as many as ~50% of all human embryos growing *in vitro*, and pregnancy rates reached as high as 50% or more when one or two embryos diagnosed as normal were replaced. This considerable improvement above previous success rates occurred just as doubts began to emerge about the cost and efficacy of natural cycle IVF.

### Can new concepts offer simpler alternatives for ovarian stimulation?

Has the time now arrived to jettison routine IVF in favour of minimal stimulation IVF, natural cycle IVF or IVM? Can improvements be achieved in routine IVF, just as several of its problems were solved in Oldham in the 1970s? In fact, several possibilities of improving routine IVF that were known in the earliest days of routine persist today. For example, the fact that LHRH has pulsatile release during natural cycles, as shown by Knobil (1974) working with rhesus monkeys, has simply been overlooked together with any deleterious effects that may have been induced in IVF clinics administering large doses of GnRH agonists and antagonists, or using stimuli leading to high concentrations of oestrogens in their patients.

Three successive events occurring during natural menstrual cycles are controlled by timing the actions of different gonadotrophins, steroids and other regulatory factors (Le Nestour et al., 1993). The first step in follicular recruitment is decided by FSH dominance, and the last step in follicular maturation involves LH dominance. The former is difficult to detect, so its exact timing in stimulated cycles has been largely overlooked. Substituting the first day of menses as a satisfactory measure of the onset of a new cycle thus has a weak endocrinological basis. Le Nestour et al. (1993) also suggest that the exact timing of the LH surge leading to ovulation cannot be predicted prospectively during the natural cycle, although it was tediously achieved, for example, during Leslie Brown's menstrual cycle thatled to the birth of her daughter. Timing this phase is usually achieved by triggering ovulation through an injection of HCG, but this can be easily mistimed and may shorten the final part of the follicular phase. Likewise, the incorrect timing of GnRH antagonists may result in falling concentrations of oestrogens, which disturbs menstrual rhythms.

A better approach controlling the correct concentrations of FSH and LH is achieved by administering controlled concentrations of oestradiol  $\sim$ 3 days before menstruation is expected. Le Nestour *et al.* (1993) stress the significant role of gonadotrophin surge attenuating factor (GnSAF) in co-ordinating the menstrual cycle. It controls LH and its mid-cycle surge by antagonizing the surge-promoting activity of oestradiol, and further refinements of this hypothesis may improve results with both routine and minimal stimulation IVF (see de Ziegler *et al.* in this Symposium). Other major developments may yet be introduced to control menstrual rhythms and improve routine IVF.

Minimal stimulation IVF, and natural cycle IVF to a lesser degree, could replace routine IV inhibin, activin and follistatin F in many IVF clinics. In a sense, both represent steps in the development of routine IVF. Their techniques are virtually identical and highly sensitive methods are now available to detect the onset of the LH surge (Stenman et al., 1985). Novel techniques such as these have stimulated some clinics to abandon the use of large doses of urinary or recombinant gonadotrophins in normal or poor responders in favour of tests on minimal stimulation IVF and natural cycle IVF. Caution was long overdue as evidence accumulated on the delicacy of feedback systems such as the systemic roles of hypothalamic GnRH, the ovarian steroids oestradiol and progesterone, and the gonadal peptides inhibin, activin and follistatin on the secretions of pituitary gonadotrophins (Le Nestour et al., 1993). Oestradiol accordingly has a major role in determining concentrations of FSH at the luteal-follicular transition, and the need for care in stimulating patients became increasingly clear. The need for change was first mooted by an appeal to reduce doses of gonadotrophins and pay close attention to doses given to patients with specific defects in their menstrual cycles (Edwards et al., 1996). Minimal stimulation IVF is obviously very close to routine IVF, and differs mainly in its utilization of smaller doses of hormones. It has a major advantage of avoiding the production of high oestrogens that may damage maturing oocytes and even distort chromosomal segregation. Used in the earliest days of routine IVF, it could have been chosen by many clinics, although it fell out of favour as treatments involved increasing doses of urinary or recombinant gonadotrophins in the early 1990s (Howles, 2006). It may indeed have been chosen as routine practice in the 1970s, had we chosen a different progestagen than Primulot depot in attempts to avert short luteal phases.

Is IVM the preferred choice for human assisted conception today? Its clinical application has recently been described in detail by Tan et al. (2007). A major departure from routine IVF, the long period when many investigators failed to obtain births with this technique has been left well behind (Mikkelsen, 2005). By today, many clinics now achieve results with IVM that are equivalent to routine IVF (Table 3). Until recently, many patients attending for IVM had polycystic ovaries where numerous small follicles lie just below the ovarian surface, but similar treatments are now given to patients with normal ovaries. Methods initially successful when used for patients with polycystic ovaries have now been applied to those with normal cycles. Practitioners do not need high doses of HMG and use very small doses or none at all. A similar situation applies to HCG. Bleeding from aspirated small follicles has been reduced by modifying structure of aspirating needles. As many as 50 or more oocytes can be plucked from available follicles on some occasions, which challenge success rates attained with routine IVF. Improvements in oocyte cryopreservation imply that follicles can be aspirated at any stage of the menstrual cycle and their oocytes preserved until needed by their parents for later use (Boldt et al., 2006). With hindsight, should we have developed IVM rather than ovarian stimulation in 1969? Even then, its obvious advantages in avoiding high gonadotrophin doses and the availability of vast numbers of oocytes, especially from polycystic ovaries and the numerous small follicles growing in the follicular stage, was apparent. New methods of aspirating oocytes will enable them to be collected from Graafian follicles in early stages of growth and before they become sensitive



Table 3. Recent studies on maturing human oocytes *in vitro*.
(a) Data from Chian *et al.* (2004), on IVM in Seoul and in Montreal.
(b) Data of Mikkelsen *et al.* (2001) in Copenhagen.

(a)	Seoul	Montreal
Cycles	419	254
Patient age	32.2	32.4
Oocytes	6860	3079
Matured	5021 (75)	2426 (79)
Fertilized	3967 (78)	1679 (69)
Cleaved ova	n/a	1509
Transferred	1816 {4.3}	865 {3.4}
Pregnancies/cycle	137 (30.3)	61 (24.1)
Implants/trans	211 (11.6)	96 (11.1)

Values in brackets represent percentages, those in curly brackets represent means. n/a = not available.

(b)	Medium with maternal serum	Medium with serum albumin	
M2 oocytes Pregnancies Children	47/74 5/18 7 (2 twin sets)	26/73 0/23	

Patients: mostly PCO/PCOS, some cyclic. Treatment: FSH day 5–6, aspiration day 8–9.

Medium: 199, pyruvate, oestradiol, rFSH, HCG, maternal serum/serum albumin, granulosa cells.

Luteal support: oestradiol and progesterone.

to FSH. At any one time, the numbers of such follicles are considerable and vary from several thousands in younger ages to more than 1000 at the age of 50 years (**Table 4**) (Faddy and Gosden, 1995). Perhaps we were over-influenced by the advantages of induced ovulation, for example the safety offered to pre-ovulatory oocytes as they pass through their maturation phases within their follicles. Their immediate insemination *in vitro* very soon after their aspiration is a further advantage.

Nevertheless, current evidence indicates that IVM has come to stay. It will switch attention away from studies on embryos, which have dominated with routine IVF, in favour of studies on oocytes in ovulatory and non-ovulatory follicles. Attention will be given to the genetics and biochemistry of follicles and oocytes as they grow and mature for 37 h after aspiration (e.g. Harris and Picton, 2007). The nature of the regulatory genes involved in crucial stages of oocyte formation and maturation will be clarified by microassays, and genes regulating maternal influences on early development should enhance knowledge on quantitative inheritance and the roles of particular genes that are active between gamete formation and implantation. Research on oocytes as they are placed in culture media to initiate their maturation may help to identify and correct those meiotic factors leading to disorders in chromosomal segregation. Averting chromosomal disorders and their induction by high concentrations of ovarian oestrogens in follicular fluid must be another potential advantage of IVM. It would help every clinic practising IVM and other forms of IVF.

What are the embryonic risks of IVM? One concerns the period of 37 h needed for maturation *in vitro*, since inadequate media, poor temperature control or incorrect gas phases might enhance damage to oocytes as they mature. Another risk may be associated with the formation of anomalous embryos as described by Chang (1955). So far, reports on chromosomal anomalies in embryos derived using IVM are lacking and are urgently awaited. Does the fact the oocytes were never exposed to events occurring during a normal menstrual cycle imply that they may not be fully normal? Yet, all in all, the advantages of IVM seem to far outweigh its disadvantages.

To conclude this brief review, mention must be made of the immense amounts of information already gained on genetics and biochemistry of the oocyte gained from IVM (Table 5). These topics have been discussed in detail in the recent publication of In-Vitro Maturation of Human Oocytes, edited by Tan et al. (2007). Excellent chapters on oocyte growth and developmental competence are discussed by Thomas and Vanderhyden (2006), follicular metabolism by Harris and Picton (2007) and Picton et al. (2007), gene expression in oocytes during growth and maturation by Eichenlaub-Ritter (2007) and Swain and Smith (2007), and epigenetic changes during oocyte growth by Fortier and Trasler (2007) (Table 5). The development in vitro of small ovarian follicles is covered by Abir et al. (2007) and Hreinsson et al. (2007). Together with the other chapters on clinical aspects of IVM, this wealth of information is essential to those intent on practising IVM. These papers point to an astonishing future **Table 4.** Maternal age and the numbers of human ovarian follicles egressing from the follicular pool. Data on age given in two sections, the upper one covering daily egress from stage III and the lower one showing the numbers of follicles remaining in the ovaries at ages 19 and 50. Modified from Faddy and Gosden (1995).

Age (years)	Follicle numbers <sup>a</sup>
29-30	31
39–40	9
49–50	1
	<i>Follicles<sup>b</sup></i>
19	300,000
50	1500

<sup>a</sup>Age and daily egress from stage III. 2+ granulosa layers. <sup>b</sup>Remaining follicles with age. High death rates of small follicles.

Table 5. Factors affecting meiotic arrest and its resumption chromosomal segregation and maternal effects in early development.

Cytogenetic and molecular aspects of oocyte growth and maturation

Event	Determinants
Arrest in early growth	Cumulus cAMP; oocyte protein kinase A
Meitoic resumption	Cumulus protein kinase C (PKC), oocyte MPF, CDK1/cyclin B
Cohesion	Physical association of chromosome until anaphase
Microtubular polymerization	GSk3 controls
Cohesion relieved	Microtubular forces overcome
Chromosomal separation	Separatase
Crossovers disjoin	Perhaps via PP1/PP2A
Disordered segregation	To be found
Meiotic re-arrest	Metaphase two cytosol, MPF, Mos
Polar body extrusion	MAPK, actin microfilaments
Oocyte transcripts functioning at meiotic resumption	
Factor	Determinants
Nucleoplasmin	Heat-shock factor 1
Brahma-related gene	Formin 2
Zygotic arrest	Mater
Dnmtlo (transferase)	Stella
Epigenetic factors	
Epigenetic factor	Mediator
Histones 3 and 3	DNA methylation
Transcriptional regression	Polycomb group
Translocational activation	Trithorax group
TrxG enzymes	Enhanced by histone acetylation

Based on data from Eichenlaub-Ritter (2006); Swain and Smith (2006); Fortier and Trasler (2007).

and provide back-up for what may be the largest surprise of all, namely that new follicles may develop from bone marrow in children and adults (Johnson *et al.*, 2004, 2005). If they descend from haemangioblasts, then follicles and other tissues might be prepared from a drop of blood in the same manner as discussed in relation to the use of cord blood stem cells (Edwards and Hollands, 2007). In other words, we would carry stem cells in our blood including those that may be able to form oocytes? Time will tell.

#### References

- Abir R, Nitke S, Ben-Haroush A, Fisch B 2007 In-vitro development of small ovarian follicles. In: Tan SL, Chian R-C, Buckett WM (eds) In Vitro Maturation of Human Oocytes, Basic Science to Clinical Application. Informa Healthcare, Toronto, Canada, pp. 123–136.
- Ascheim S, Zondek B, 1927, Das hormon der
- hypophysenvorderlapens: Testobjeckt zum nachweis des hormons. Klinische Wochenschrift **6**, 248–252.
- Austin CR 1975 Membrane fusion events in fertilization. Journal of Reproduction and Fertility 44, 155–166.
- Austin CR 1952 The 'capacitation' of mammalian sperm. *Nature* **170**, 526.

Bergh C, Howles CM, Borg K *et al.* 1997 Recombinant human follicle stimulating hormone (r-hFSH; Gonal-F) versus highly purified urinary FSH (Metrodin HP): results of a randomized comparative study in women undergoing assisted reproductive techniques. *Human Reproduction* **12**, 2133–2139.

- Boldt J, Tidswell N, Sayer A et al. 2006 Human oocyte cryopreservation: 5-year experience with a sodium-depleted slow freezing method. *Reproductive BioMedicine Online* 13, 96–100.
- Chang MC 1955 The maturation of rabbit oocytes in culture and their maturation, activation, fertilization and subsequent development in the fallopian tubes. *Journal of Experimental Zoology* 128, 379–405.
- Chian RC, Buckett WM, Tan SL 2004 In-vitro maturation of human oocytes. *Reproductive BioMedicine Online* **8**, 148–166.

Croxatto HB, Ortiz ME, Diaz S *et al.* 1978 Studies on the duration of egg transport by the human oviduct. II. Ovum location at various intervals following the luteinising hormone peak. *American Journal of Obstetrics and Gynecology* **30**, 408–414.

Donini P, Lunenfeld B 1974 Clinical results of gonadotrophins. Acta Europa Fertilitatis 5, 305–316.

- Edwards RG 1980 Conception in the Human Female. Academic Press, London.
- Edwards RG 1965 Maturation in vitro of human ovarian oocytes. Lancet ii, 926–929.

Edwards RG, Fowler RE 1959 Fetal mortality in adult mice after superovulation with gonadotrophins. *Journal of Experimental Zoology* **41**, 299–322.

Edwards RG, Hollands P 2007 Will stem cells in cord blood, umbilical cord, bone marrow and peripheral blood soon be unnecessary in transplantation? *Reproductive BioMedicine Online* **14**, 396–401.

Edwards RG, Steptoe PC 1975 Induction of follicular growth, ovulation and luteinisation of the human ovary. *Journal of Reproduction and Fertility* **22** (Suppl.), 121–163.

Edwards RG, Lobo R, Bouchard P 1996 Time to revolutionise ovarian stimulation. *Human Reproduction* **11**, 917–918.

Edwards RG, Fishel SB, Cohen J *et al.* 1984 Factors influencing the success of in vitro fertilization for alleviating human infertility. *Journal of In Vitro Fertilization and Embryo Transfer* **1**, 3–23.

Edwards RG, Steptoe PC, Fowler RE, Baillie J 1980a Observations on preovulatory human ovarian follicles and their aspirates. *British Journal of Obstetrics and Gynaecology* **87**, 769–779.



fertilization in vitro of human oocytes matured in vitro. *Nature* **221**, 632–635.

Eichenlaub-Ritter U 2007 Gene expression in oocytes during growth and differentiation. In: Tan SL, Chian R-C, Buckett WM (eds) *In Vitro Maturation of Human Oocytes, Basic Science to Clinical Application.* Informa Healthcare, Toronto, Canada, pp. 49–82.

Engel JB, Schuktz-Mosgar A, Diedrich K 2005 Five years of clinical use of GnRH antagonist: evalution of safety and allergic potential. *Reproductive BioMedicine Online* **10** (Suppl. 3), 61–65.

Everett JW 1969 Neuroendocrine aspects of mammalian reproduction. Annual Review of Physiology **31**, 383–416.

Faddy MJ, Gosden RG 1995 A mathematical model of follicle dynamics in the human ovary. *Human Reproduction* 10, 770–775.

- Fee AR, Parkes AS 1929 Studies on ovulation: 1. The relation of the anterior pituitary gland to ovulation in the rabbit. *Journal of Physiology* 67, 383–388.
- Feichtinger W, Kemeter P, Szalay S, Janisch H 1982 Could aspiration of the Graafian follicle cause luteal phase deficiency? *Fertility and Sterility* 37, 205–208.

Fortier AL, Trasler JM 2007 In: Tan SL, Chian R-C, Buckett WM (eds) In Vitro Maturation of Human Oocytes, Basic Science to Clinical Application. Informa Healthcare, Toronto, Canada, pp. 103–122.

- Fowler RE, Edwards RG 1957 Induction of superovulation and pregnancy in mature mice by gonadotrophins. *Journal of Endocrinology* 15, 374–384.
- Fowler RE, Edwards RG, Walters DE *et al.* 1978 Steroidogenesis in preovulatory follicles of patients given menopausal and chorionic gonadotrophins as judged by the radioimmunoassay of steroids in follicular fluid. *Journal of Endocrinology* **77**, 161–169.
- Gates AH, Beatty RA 1954 Independence of delayed fertilization and spontaneous triploidy in mouse embryos. *Nature* **174**, 356–357.

Gemzell CA, Roos P, Loeffler FE 1975 Follicle stimulating hormone extracted from human pituitary. In: Behrman SJ, Kistner RW (eds) *Progress in Infertility*. Little, Brown and Company, Boston, pp. 479–493.

- Genazzani AD, Battaglia C, Malavasi B *et al.* 2004 Metformin administration modulates and restores luteinizing hormone spontaneous episodic secretions and ovarian function in nonobese patients with polycystic ovary syndrome. *Fertility and Sterility* 81, 114–119.
- Greenblatt RB, Barfield E, Jungck EC, Ray AW 1961 Induction of ovulation with MRL/41. Preliminary report. *Journal of the American Medical Association* **178**, 101–104
- Harris GW, Naftolin F 1970 The hypothalamus and control of ovulation. British Medical Bulletin 26, 3–9.

Harris SE, Picton HM 2007 Metabolism of follicles and oocytes during growth and maturation. In: Tan SL, Chian R-C, Buckett WM (eds) In Vitro Maturation of Human Oocytes, Basic Science to Clinical Application. Informa Healthcare, Toronto, Canada, pp. 15–36.

Heape W 1880 Preliminary note on the transplantation and growth of the mammalian ova within a foster mother. *Proceedings of the Royal Society* B, **48**, 457–458.

Hertig AT, Rock EC, Adams EC, Menkin EC 1952 Thirty-four fertilized human ova, good, bad and indifferent recovered from 201 women of known fertility. *Pediatrics* 23, 202–211.

Hirsch MB, Mosher WD 1987 Characteristics of infertile women in the United States and their use of infertility services. *Fertility and Sterility* **47**, 618–625.

- Holmes RL, Ball JN 1974 *The Pituitary Gland*. Cambridge University Press, Cambridge, UK.
- Howles C 2006 Recombinant gonadotrophins in reproductive medicine: the gold standard of today. *Reproductive BioMedicine Online* 12, 11–13.
- Hreinsson J, Carlsson IB, Hovatta O 2007 Maturation of primordial follicles – the next step. In: Tan SL, Chian R-C, Buckett WM (eds) *In Vitro Maturation of Human Oocytes, Basic Science to Clinical Application.* Informa Healthcare, Toronto, Canada, pp. 137–146.
- Johnson J, Bagley J, Skaznik-Wilkiel M 2005 Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow



and peripheral blood. Cell 122, 303-315.

Johnson J. Canning J, Keneke T *et al.* 2004 Germline stem cells and follicular renewal in the postnatal mammalian embryo. *Nature* 428, 145–150.

Khattab S, Fotouh IA, Mohesn IA *et al.* 2006 Use of metformin for prevention of ovarian hyperstimulation: a novel approach. *Reproductive BioMedicine Online* 13, 194–197.

Kistner RW 1975 Induction of ovulation with clomiphene citrate. In: Behrman SJ, Kistner RW (eds) *Progress in Infertility*. Little Brown and Company, Boston, p. 509.

Kleitman RW 1969 Basic rest activity cycle in relation to sleep and wakefulness. In: Kales A (ed.) *Sleep Physiology and Pathology*. Lippincott, Philadelphia, pp. 33–38.

Klopper A, Aiman J, Besser M 1974 Ovarian steroidogenesis resulting from treatment with menopausal gonadotrophin. *European Journal* of Obstetrics, Gynaecology and Reproductive Biology **4**, 25.

Knobil E 1974 On the control of gonadotrophin secretion in the rhesus monkey. *Recent Progress in Hormone Research* 30, 1–46.

Kuliev A, Rechitsky S, Verlinsky O *et al.* 2005 Preimplantation diagnosis and HLA typing for haemoglobin disorders. *Reproductive BioMedicine Online* **11**, 362–371.

La Marca A, Morgante G, Palumbo M *et al.* 2002 Insulin-lowering treatment reduced aromatase activity in response to follicle-stimulating hormone in women with polycystic ovary syndrome. *Fertility and Sterility* **78**, 1234–1239.

Le Nestour E, Marraoui J, Lahlou N *et al.* 1993 Role of oestradiol in the rise in follicle-stimulating hormone levels during the lutealfollicular transition. *Journal of Clinical Endocrinology and Metabolism* **77**, 439–442.

Leridon H 1979 Patterns of fertility at later ages of reproduction. Journal of Biosocial Science **6** (Suppl.), 59–74.

Lewis WH, Gregory PW 1929 Cinematographs of living, developing rabbit eggs. *Science* 69, 226–229.

Lewis WH, Hartmann CG 1933 Early cleavage stages of the egg of the monkey (*Macacus rhesus*). Contributions to Carnegie Institute, Washington, 24, 187–201.

Lopata A 1980 Successes and failures in human in-vitro fertilization. *Nature* 288, 643.

Loumaye E 1990 The control of endogenous secretion of LH by gonadotrophin-releasing hormone agonists during ovarian hyperstimulation for IVF and embryo transfer. *Human Reproduction* **5**, 367–376.

Lunenfeld B 1969 The use of human menopausal gonadotrophins in gonadal pathology. In: Inguilla W, Greenblatt RG (eds) *The Ovary*. CC Thomas, Springfield, IL.

MacLean PD 1973 A triune concept of the brain and behaviour. In: Boag TJ, Campbell D (eds) *The Clarence M. Hincks Memorial Lectures 1969*, University of Toronto Press, Toronto.

Mikkelsen A 2005 Strategies in human in-vitro maturation and their clinical outcome. *Reproductive BioMedicine Online* 10, 593–599.

Mikkelsen AL, Host E, Blaabjerg J, Lindenberg S 2001 Maternal serum supplementation in culture medium benefits maturation of immature human oocytes. *Reproductive BioMedicine Online* 3, 112–116.

Munné S 2006 Chromosome abnormalities and their relationship to morphology and development of human embryos. *Reproductive BioMedicine Online* 12, 234–253.

Palermo G, Joris H, Devroey P, Van Steirteghem AC 1992 Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340, 17–18.

Parkes AS 1976 Patterns of Sexuality and Reproduction. Oxford University Press, London.

Picton HM, Muravi W, Jin P 2007 Interaction of oocytes and somatic cells. In: Tan SL, Chian R-C, Buckett WM (eds) In Vitro Maturation of Human Oocytes, Basic Science to Clinical Application. Informa Healthcare, Toronto, Canada, pp. 37–48.

Pincus G, Enzmann EV 1935 The comparative behaviours of mammalian eggs in vitro and in vivo. II The activation of tubal eggs in the rabbit. *Journal of Experimental Medicine* 62, 665–675.

Pincus G, Saunders B 1939 The comparative behaviour of mammalian eggs in vitro and in vivo. VI. The maturation of human ovarian

ova. Anatomical Record 75, 537.

- Porter RN, Smith W, Craft IL *et al.* 1984 Induction of ovulation for in vitro fertilization using buserilin and gonadotrophins. *Lancet* 2, 1284–1285.
- Rebar RW, Yen SSC 1979 Endocrine rhythms in gonadotrophins and ovarian steroids with reference to reproductive processes. In: Krieger DT (ed.) *Endocrine Rhythms*. Raven Press, New York, pp. 259–298.

Runner MN, Palm J 1953 Transplantation and survival of unfertilized ova of the mouse in relation to postovulatory age. *Journal of Experimental Zoology* **124**, 303–316.

Schally AV 1978 Aspects of hypothalamic regulation of the pituitary gland. *Science* **202**, 18–28.

Southam A 1960 What to do with the normal infertile couple? *Fertility* and Sterility **11**, 543–549.

Stenman U-H, Alfthan H, Koskimies A et al.1985 Monitoring the LH surge by ultrarapid and highly sensitive imunofluorometric assay. In: Seppala M, Edwards RG (eds) In Vitro Fertilization and Embryo Transfer. New York Academy of Sciences 442, 544–550.

Steptoe PC, Edwards RG 1976 Replacement of a human embryo with subsequent tubal pregnancy. *Lancet* I, 880–882.

Steptoe PC, Edwards RG 1970 Laparoscopic recovery of pre-ovulatory human oocytes after priming ovaries with gonadotrophins. *Lancet* I, 683–689.

Swain JE, Smith GD 2007 Mechanism of oocyte maturation. In: Tan SL, Chian R-C, Buckett WM (eds) In Vitro Maturation of Human Oocytes, Basic Science to Clinical Application. Informa Healthcare, Toronto, Canada, pp. 83–102.

Tan SL, Chian R-C, Buckett WM (eds) 2007 In-vitro Maturation of Human Oocytes: Basic Science to Clinical Application. Informa Healthcare, Toronto, Canada.

Testart J, Lasalle B, Frydman R, Belaisch JC 1983 A study of factors affecting the success of human fertilization in vitro. *Biology of Reproduction* **28**, 425–432.

Thomas FH, Vanderhyden BC 2006 Oocyte growth and developmental competence. In: Tan SL (ed.) *In-vitro Maturation of Human Oocytes*. Informa, UK, pp. 1–14.

Trenchard-Lugan I, Khan A, Porchet HC, Minafo A 2002 Pharmacokinetics and pharmacodynamics of recombinant human chorionic gonadotrophin in healthy male and female volunteers. *Reproductive BioMedicine Online* **4**, 106–115.

Trounson AO, Leeton H, Wood C et al. 1981 Pregnancies in humans by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. Science 212, 681–682.

Walton A, Hammond J 1928 Observations on ovulation in the rabbit. Journal of Experimental Biology 6, 190–204.

Wang Y, Ock S-A, Chian R-C 2006 Effect of gonadotrophin stimulation on mouse oocyte quality and subsequent embryonic development in vitro. *Reproductive BioMedicine Online* 12, 304–314.

Wikland M, Nilsson L, Hansson R et al. 1983 Collection of human oocytes by the use of sonoography. *Fertility and Sterility* 39, 603–608.

Yanagimachi R, Chang MC 1964 In vitro fertilization of golden hamster ova. Journal of Experimental Zoology 156, 361–376.

Paper based on a contribution presented at the First World Congress on 'Natural Cycle/Minimal Stimulation IVF' in London, UK, December 15–16, 2006.

Received 14 March 2007; refereed 3 April 2007; accepted 2 May 2007.