Humphrey K W (1969) *Journal of Endocrinology* **44**, 299–305
McLaren A (1973) In: Regulation of Mammalian Reproduction. Ed. S J Segal, R Crozier, P A Corfman & P G Condliffe. Thomas, Springfield, Illinois; pp 321–328
Martin L & Finn C A
(1968) *Journal of Endocrinology* **41**, 363–371
(1971) In: Basic Action of Sex Steroids on Target Organs. Ed. P O Hubinant *et al.* Karger, Basel; pp 172–188
Mehrotra S & Finn C A
(1974) *Journal of Reproduction and Fertility* **37**, 405–409
Neill J D, Johansson E D B & Knobil E
(1967) *Endocrinology* **81**, 1161–1164
Nilsson O (1966) *Zeitschrift für Anatomie und Entwicklungsgeschichte* **125**, 152–159
Pollard R M, Bredl J C S & Finn C A
(1973) *Journal of Reproduction and Fertility* **33**, 343–345
Pollard R M & Finn C A
(1972) *Journal of Endocrinology* **55**, 293–298
Potts M
(1966) *American Journal of Obstetrics and Gynecology* **96**, 1122–1128
(1969) In: Advances in Reproductive Physiology. Ed. A McLaren. Logos, London; pp 241–267
Psychoyos A
(1967) *Advances in Reproductive Physiology* **2**, 257–277
Psychoyos A & Bitton-Casmiri V (1969) *Comptes rendus hebdomadaires des séances de l'Académie des sciences (Paris)* **268**, 188–190
Somerville B W (1971) *American Journal of Obstetrics and Gynecology* **111**, 419–426
Weitlauf H M (1973) *Journal of Experimental Zoology* **183**, 303–308

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Control of Human Ovulation, Fertilization and Implantation

We have been studying the earliest stages of human development, including aspects of follicular growth, ovulation, fertilization, cleavage of the embryo and implantation. The primary clinical intention of the work is to attempt the cure of certain forms of infertility, especially those due to the occlusion of the fallopian tubes, oligospermia, and some other conditions. For this purpose, a great deal of information is needed about human physiology, for example the timing of ovulation or the requirements for culturing ova, since the cure of these conditions demands the reimplantation of cleaving embryos into the mother. All the work carried out on reimplantation of embryos has involved the use of the oocytes and spermatozoa from a wife and her husband. Our further intention is to gather as much knowledge as possible about these early stages in human growth because their detailed analysis would help in understanding or developing certain methods of contraception, and the origin of certain genetic anomalies during early development.

Several major difficulties had to be overcome before these clinical approaches could be established. Major difficulties concern the collection of oocytes from the ovary by an acceptable surgical method, and the exact timing of the preovulatory changes in follicles so that oocytes could be collected just before ovulation. If ovulation occurred in patients with occluded oviducts, the oocyte would have been lost in the abdominal cavity; if the collection was made too soon, the incompletely matured oocytes may not have been capable of implantation, a phenomenon well known in animals. Laparoscopy is used for oocyte collection (Steptoe 1969) and is timed just before ovulation is expected. The timing of ovulation was judged by studies on the maturation of oocytes *in vitro* and could be predicted to occur some 36–40 hours following the treatments of patients with gonadotrophins (Table 1) (Edwards 1973). The use of gonadotrophins for the cure of amenorrhœa and oligomenorrhœa has been amply described by Gemzell, Lunenfeld, Crooke and their collaborators, and the methods applied in our work to patients with a normal menstrual cycle were essentially similar to theirs. Our initial studies thus involved the use of human menopausal gonadotrophin (HMG: Pergonal, G D Searle & Co) as a means of stimulating follicular growth, followed by the use of human chorionic gonadotrophin (HCG: Pregnyl, Organon) to stimulate ovulation.

Response of Patients to Gonadotrophins

We will describe the response of patients to this form of treatment. The amount of HMG did not exceed 1125 iu in any patient during one course of treatment and a single injection of HCG, usually 5000 iu, was given to induce ovulation. In some patients, clomiphene was used as an alternative to stimulate follicular growth, followed by an injection of HCG to induce ovulation. The excretion of urinary œstrogens was used to follow follicular changes in the ovary, based on the methods described by Brown *et al.* (1969), and assays of urinary pregnanediol were used to determine the activity of the corpus luteum. Estimates of plasma levels would have provided more valuable data than urinary assays, but could have imposed repeated trauma on our patients.

Table 1

Time of follicular rupture following an injection of HCG

Interval after HCG (hours)	*No. of patients*	*No. with evidence of ovulation*
32–36	51	3?
37–38½	5	3
40–42	3	2

Most of the work has been carried out using HMG and HCG, and the response of patients to this treatment has been carefully monitored. The interval between the initial treatment with HMG, usually given on Day 2 or 3 of the menstrual cycle, and the injection of HCG appeared to have little effect on the follicular response of the patients. This interval has been extended gradually from six to nine days, the shorter interval being used initially in view of the possibility of spontaneous ovulation during the course of treatment. Since the patients being treated had a normal menstrual cycle, and hence an intact hypothalamic-pituitary-ovarian system, the production of large amounts of œstrogen during treatment with HMG might have caused an endogenous surge of LH and spontaneous ovulation before the HCG was given. We would therefore have lost control of ovulation and of the preovulatory oocytes. Experience has taught us that spontaneous ovulation need not be troublesome, because very few, if any, patients have ovulated before the HCG was given, except in recent work where the interval between the HMG and the HCG was extended to nine days. We have also found that the level of urinary œstrogens should well exceed 50 μg per day when HCG is given, because lower levels often indicate insufficient follicular stimulation, leading to a dearth of preovulatory follicles. The careful monitoring of urinary œstrogens avoids needless laparoscopies and the patients can be treated again with slightly higher doses of HMG.

The number of follicles found in the ovaries in response to the treatments with HMG are shown in Table 2; many follicles of different sizes are found, some up to 3 cm in diameter and in general the largest follicles are preovulatory, as described later. No significant increase occurs in the total number of follicles with larger doses of HMG, although the number of large follicles is slightly greater. Estimates of the number of large follicles from the levels of œstrogens in urine are far from exact, and in some patients small follicles can evidently produce sufficient œstrogen to confuse these estimates. Nevertheless, provided the levels of œstrogen in urine are monitored closely, there has been hardly any occasion when no preovulatory oocytes were collected from the patient; usually one or more are found.

Table 2

Follicular response to different amounts of HMG

Amount of HMG (iu)	*Average number of follicles per patient*	*Average number of large follicles > (1·5 cm) per patient*
675	10.1	2.6
900	10.6	3.6
1125	11.6	5.1

Further data is given in Edwards & Steptoe (1974)

Follicular fluids aspirated from graafian follicles may serve as a guide to the stage of growth of the follicle. Studies in animals have shown that the amount of œstrogen in follicular fluid increases as the follicle develops, the highest concentrations being found just after the follicle begins its preovulatory changes. The quantities then decline precipitously until virtually none is synthesized just before ovulation. In fluids taken from human follicles, a considerable variation was found in the concentrations of œstradiol 17β and progesterone, and this could be due to the presence of follicles of different developmental stages in the ovary in response to the HMG and HCG (Edwards *et al.* 1972). Low values would be associated with follicles in early stages of growth or approaching ovulation. Clearly, there is a considerable heterogeneity in the stage of development of graafian follicles for some 32–34 hours after HCG is injected.

The Preovulatory Oocyte, Fertilization and Cleavage

Oocytes taken from preovulatory follicles have a highly typical appearance. They are surrounded by a dense mass of cumulus cells embedded in thick viscous fluid and the size of the whole mass is such that it can be recognized immediately by eye and so transferred rapidly into the media used for culture. The appearance of this viscous follicular fluid, in addition to the free follicular fluid in the antrum at large, accords with earlier descriptions of the development of follicles in animals towards ovulation (Zachariae 1957), and is so characteristic that follicles can be typed retrospectively as preovulatory or nonovulatory according to the appearance of the oocyte. Oocytes from nonovulatory follicles are enclosed in a few layers of cumulus cells but are not associated with either follicular fluid or the loose accumulation of cumulus cells. The vascularity of the stigma and surface of the preovulatory follicle is also fairly typical, with distinct blood vessels traversing the surface of the graafian follicle; such an appearance coincides closely with observations on preovulatory follicles in some nonhuman primates (Dukelow 1974). A further guide to the preovulatory nature of the follicle may be gained from assays of prostaglandins in the follicular fluid, since the levels of the prostaglandins E and F have been reported to rise considerably in rabbit follicles just before ovulation was expected (Le Maire *et al.* 1973). Initial assays on levels of prostaglandins in the fluids of human preovulatory follicles have indicated that a similar situation might also exist (Edwards 1973).

Preovulatory oocytes are the most suitable material for studies on fertilization and cleavage.

In our work, ejaculated spermatozoa are used for fertilization *in vitro*, most of the seminal plasma being removed by gentle centrifugation in medium used for fertilization. This medium is composed basically of Tyrode's solution and albumin; pyruvate and penicillin are added to it. After washing, the spermatozoa are incubated in this medium at 37°C for at least 1½ hours, because animal studies have shown that the changes termed 'capacitation' begin in culture and fertilization occurs rapidly when the oocytes are added. The concentration of spermatozoa is usually held at varying levels between 5 and 20×10^6/ml, and reduced in the insemination droplets to 10^6/ml depending on the quality of the sperm sample. The oocytes are pipetted through two or three changes of medium and into the insemination droplets within a few minutes of collection, the interval between their recovery from the ovary and insemination being a matter of a few minutes. The osmotic pressure and pH of the culture media must be strictly controlled; our experience has shown that a pH of 7.4, obtained by using sodium bicarbonate and a gas phase of 5% CO_2, 5% O_2 and 90% N_2, and an osmotic pressure of approximately 285 mosmol/kg, are excellent for fertilization.

All the criteria normally used for fertilization have been seen in human oocytes, including spermatozoa traversing the zona pellucida or in the perivitelline space, the remains of the midpiece and tail in the oocyte, the formation of pronuclei, and the first and second polar bodies. There has been no evidence of polyspermy with oocytes collected just before ovulation, as judged by the presence of two pronuclei in all of them so far examined. Clearly, the block to polyspermy is fully operative, which is an important factor if triploidy or more complex forms of aneuploidy are to be excluded in the embryos. The timing of pronuclear growth and syngamy has been timed, and the embryos are transferred – usually before the first cleavage – into Ham's medium F10 for further growth.

Pronucleate eggs are thus transferred at approximately 12–15 hours after insemination into medium F10 which has been supplemented by fetal calf serum or albumin, together with a small amount of serum from the patient in order to guard against infections in the culture. The pH gas phase and osmotic pressure are identical to those used for fertilization. Many embryos have cleaved normally in this medium, some to the blastocyst stage of development. Cleavage and the formation of the blastocyst has been timed (Table 3) and accord closely with the data obtained from the collection of embryos directly from the oviducts and uterus of women during hospital treatments (Croxatto *et al.* 1972).

Table 3

Timing of human preimplantation development *in vitro*

Stage of development	*Time after insemination when embryos have been observed (hours)* ●
2-cell	26– 46
4-cell	38– 58
8-cell	46– 70
16-cell	67– 97
Morula	111–135
Blastocyst	123–147

● Based on examinations at irregular intervals

Various aspects of cleavage have been studied, including examination of the morphology and chromosomal complement in the embryo, and initial studies on formation of the blastocelic cavity.

Reimplantation of Embryos into the Mother

The evidence available indicates that the human embryo enters the uterus between the 8- and the 16-cell stages of development (Croxatto *et al.* 1972), and this stage was chosen as the optimal time to reimplant embryos into their mothers. Passage through the cervix was adopted as the method giving least trouble to the mother; a fine catheter of 2.0 mm diameter was passed through the cervical canal in the uterine lumen, and a smaller canula containing the embryo was threaded through it, as described by Steptoe (1971). No anæsthetic is needed, the method takes little time and is painless. The total amount of solution injected does not exceed 0.05 ml.

Reimplantation via the cervix could result in a low rate of implantation, as shown by work on the cow, where surgical transfers of embryos directly into the uterus are much more successful (Rowson *et al.* 1969). Transfers via the cervix also have a poor reputation in laboratory animals, based on early reports of a low rate of success, although recent studies in mice show that the rate of implantation can be close to that achieved by surgical transfers (Marsk & Larsson 1974). Reimplantation via the cervical route should be tested fully in patients, to avoid the necessity of a second laparoscopy or laparotomy to gain access to the uterine cavity. Problems with the cervical route could include the formation of deciduomata, the stimulation of uterine contractions or the release of prostaglandins when the catheter is passed into the uterus, and injections of Ritodrine (Philips-Duphass) or indamethacin are being used as a precaution against uterine contractions. Most of the embryos were in the 8-cell stage when replaced in the mother, and by now at least 14 reimplantations have been attempted. None have implanted. A delay in the return to subsequent menstruation has been noted, but this may have been due to the use of various endocrine treatments designed to maintain the corpus luteum as

outlined below. Similar experience has been reported by another group (de Kretzer *et al.* 1973).

During the course of this work, we noted that the amount of pregnanediol in urine during the luteal phase was rather low in some patients, and in others there appeared to be a delayed luteinization in that the levels of pregnanediol did not rise until two or three days after the expected time of ovulation. The embryos might therefore have been placed into a uterus insufficiently stimulated by progesterone, a situation resembling that created artificially in rabbits by the injection of œstrogens after mating, in which luteinization takes place some two or three days after the time expected (Beier 1974). This evidence of a delayed luteinization, or of weak luteinization, implied that progesterone support and stimulation of the corpus luteum were probably needed to reinforce potential endocrine deficiencies in the luteal phase of these induced cycles. Patients were therefore given injections of progesterone and/or 500–1000 units of human chorionic gonadotrophin every other day in order to support luteinization and the deficiency was corrected in some of them by this treatment; again, no evidence of implantation has been found.

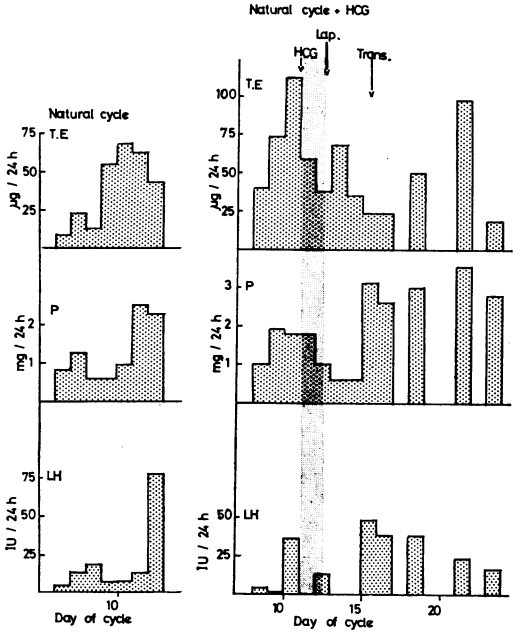

Fig 1 *Attempted control of ovulation, and reimplantation of embryos, following use of HCG during a normal menstrual cycle. Left, details from a preceeding menstrual cycle. Right, details of the cycle when HCG was given. In the cycle where ovulation was controlled by HCG, the shaded area indicates the interval between the injection of this hormone and the expected rupture of the follicle. The surge of LH had evidently just begun before HCG was injected; levels of urinary LH were surprisingly low on Day 13, the day following the injection of HCG. Notice the low levels of urinary pregnanediol for some days after the rupture of the follicle, presumably due to inadequate luteinization of the ovulatory follicle or to a succeeding ovulation. Levels of urinary œstrogen were also falling when the embryo was replaced in the mother.*
TE, *total urinary œstrogens.* P, *urinary pregnanediol.* LH, *urinary LH and HCG.* HCG, *time when HCG injected.* Lap., *time of laparoscopy for recovery of oocytes.* Trans., *time when embryo was replaced in mother's uterus*

Cycles induced by treatment with gonadotrophins may be relatively infertile. An example is shown by some of our own evidence on the use of clomiphene for the treatment of oligomenorrhœa: several cycles of treatment are needed on average to achieve one pregnancy (Edwards 1973). We do not have sufficient data on the use of HMG and HCG in patients with oligomenorrhœa, and so cannot determine the fertility of cycles induced by these gonadotrophins in our clinic. The emphasis of our work has recently been changed in attempts to utilize the natural fertility of the cycle and so increase the chances of a successful reimplantation. The fluctuating levels of urinary œstrogen, pregnanediol and LH during the natural menstrual cycle was followed in patients, in order to identify the day of the LH surge and the typical changes in steroidal excretion during the follicular and luteal phases. In a succeeding cycle, the œstrogen excretion was followed in the follicular phase but an injection of HCG was given just before the LH surge was expected (Fig 1), so that the activation of the preovulatory follicle could be induced without the risk of ovulation occurring naturally before laparoscopy was performed. The problems with this form of treatment are that presumably only single oocytes would be available for recovery (although since HCG has a longer half-life than LH it may stimulate more than a single follicle), and the need for sufficient surgical skill to recover the single oocyte from its follicle.

This treatment has now been given to several patients, and in most of them a single large follicle was present in the ovary at laparoscopy. The difference in size between the larger and smaller follicles was considerable, and far greater than that seen after treatment with gonadotrophins. The oocyte can be recovered from this large follicle, and resembles those preovulatory oocytes recovered after HMG-HCG treatment in being enclosed in a viscous mass of follicular fluid and cumulus cells. Moreover, the timing of ovulation appears to be similar during both treatments, for only in patients where a detectable surge of LH predated the injection of HCG has any indication of ovulation before laparoscopy been found. The single oocyte has been fertilized and grown *in vitro*, and replaced in the mother on

two occasions. Once again, however, there has been no evidence of implantation after this treatment. Evidence of delayed luteinization has been found in these natural cycles combined with HCG (Fig 1), just as found in cycles induced by HMG and HCG, and luteal support may be necessary here too.

Conclusions

The problems of follicular stimulation, the collection of oocytes from the follicles, fertilization and cleavage *in vitro* appear to have been solved, with a high rate of success, and this part of our study now appears to be a formality. The failure of implantation could arise for several reasons. Uterine contractions may occur on reimplantation, and more suitable methods for controlling the myometrium may be needed. The delayed luteinization in some patients might imply that the follicles were not ripe when HCG was given, in the sense that luteinization did not occur even though a preovulatory oocyte was collected; there may be a great deal to learn about the dynamics and growth of follicles in the ovary during mid-cycle. Another possibility is that the preovulatory oocytes are insufficiently matured when they are collected, for in animals embryos will fail to implant if oocytes had received insufficient stimulation in the follicle before fertilization. This possibility should be remote, since the oocytes are collected only 3–4 hours before ovulation, but collections even closer to the expected time of follicular rupture may be needed. Another problem may arise because embryos grown *in vitro* may not implant; in some animal species, rates of implantation decline as the embryos are cultured for longer periods *in vitro*. Nevertheless, the human embryos are cultured for only about 48 hours before being returned to the mother's uterus. There may be other forms of imbalance in the embryo, and trisomy has not been excluded. Once again, however, this possibility should be remote, for trisomic embryos should implant, as shown by observations on many aborted human fetuses; a delayed return to menstruation, possibly with some synthesis of HCG, would at least have been noted in some of the patients. The only genuine test of the embryos could be to use a volunteer recipient, and place pronucleate eggs or cleaving embryos in her oviduct or uterus to ensure that the embryos are capable of sustained growth from fertilization onwards. This method has not been tested, nor do we propose to adopt it, for an extra person unrelated to the embryo would become involved.

An alternative method still to be tested involves the placement of the embryo by surgery into the uterine cavity using laparoscopy or laparotomy. This routine might be acceptable to many patients, especially if high rates of implantation were attained, because the reimplantation of twin embryos would recompense for the two laparoscopies needed within a short time for oocyte recovery and reimplantation.

In conclusion, mention should be made of some of the other advantages gained from the present work. Information about human reproduction should provide a clearer understanding of the mode of action of established methods of contraception. Awareness of the time of ovulation after HCG is given, and of the growth of the blastocyst and its capacity to synthesize hormones or other compounds, should help to improve the rhythm method or with the development of effective IUDs. Increased knowledge of follicular growth in the human ovary after various endocrine treatments should clarify the nature of the ovarian response to various clinical methods designed to cure infertility. Finally, the availability of cleaving embryos *in vitro* should lead to detailed analyses of the chromosomal complement or expression of other genetic disorders in early human embryos. Such information could help to establish whether meiotic errors or delayed fertilization are the cause of human trisomy, and lead to new methods for averting the birth of children with congenital defects.

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REFERENCES

Beier H M
(1974) *Journal of Reproduction and Fertility* **37**, 221–237
Brown J B, Evans T H, Adey F D, Taft H P & Townsend D
(1969) *Journal of Obstetrics and Gynæcology of the British Commonwealth* **76**, 289–307
Croxatto H B, Diaz S, Fuentealba B, Croxatto H D, Carrillo D & Fabres C (1972) *Fertility and Sterility* **23**, 447
de Kretzer D, Dennis P, Hudson B, Leeton J, Lopata A, Outch K, Talbot J & Woods C (1973) *Lancet* ii, 728–729
Dukelow R (1974) *Journal of Reproduction and Fertility* (in press)
Edwards R G (1973) *American Journal of Obstetrics and Gynecology* **117**, 587–601
Edwards R G & Steptoe P C (1974) In: Progress in Infertility. Ed. S J Behrman & R W Kistner.
Edwards R G, Steptoe P C, Abraham G E, Walter E, Purdy J M & Fotherby K (1972) *Lancet* ii, 611–615
Le Maire W F, Yang N S T, Behrman H H & Marsh J M
(1973) *Prostaglandins* **3**, 367
Marsk L & Larsson K S
(1974) *Journal of Reproduction and Fertility* **37**, 393–398
Rowson L E A, Moor R M & Lawson R A S
(1969) *Journal of Reproduction and Fertility* **18**, 517
Steptoe P C
(1969) Laparoscopy in Gynæcology. Livingstone, Edinburgh & London
(1971) In: Proceedings of the Eighth Conference on Fertility and Sterility, Tokyo (in press)
Zachariae F (1957) *Acta endocrinologica* **26**, 215–223