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## Factors of Importance for Implantation

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# Implantation failure: clinical aspects

JOHN YOVICH
ADRIAN LOWER

Successful embryo implantation in the human involves a complex interaction between the hatched blastocyst and the luteal phase endometrium. The physiological aspects of this interaction have been covered in other chapters. The clinical perspectives concerning the factors of importance for implantation are brought into sharp focus in four areas:

1. Controlling fertility.
2. Treating infertility.
3. Managing threatened pregnancy wastage.
4. Managing the problem of recurrent pregnancy wastage.

This chapter will deal with the clinical implications of enhancing implantation and treating disorders of implantation in the last three areas of infertility and pregnancy wastage. In particular, it will examine clinical strategies, including specific treatments, to enhance and maintain successful embryo implantation.

## TREATING INFERTILITY

### Current developments

The last decade has witnessed a rapid evolution in the application of new knowledge and techniques in reproductive medicine to treat infertility. Specialized clinics adopting a team approach have emerged around the world for the specific treatment of infertility and early pregnancy disorders. This follows on the model introduced by the pioneers of in vitro fertilization and embryo transfer (IVF-ET) (Edwards et al, 1980), and which requires a very close working relationship between clinic and laboratory, particularly where any form of gamete handling is used. Today's teams generally comprise clinicians, nurse co-ordinators, scientists, specialized laboratory technologists and counsellors who provide information, emotional support and psychological services. Such organizations are in addition to conventional medical facilities and are generally supervised by institutional ethics committees and a central supervising body, e.g. the Interim Licensing Authority in the United Kingdom, the Society for Assisted Reproductive

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Technology in the United States of America, and the Reproductive Technology Accreditation Committee in Australia. In some countries there are statutory controls governing and limiting both service and research applications.

### Aetiological factors

The aetiological factors underlying infertility are numerous and the predominant causes vary with geographical location, socioeconomic factors and the changing face of health problems within different areas in given periods of time. For example, tuberculous disease was the prominent underlying condition in one location (Bahadori, 1986), whilst ovulatory disorders appeared most common in another (Cox, 1975). The broad categories of infertility recognized in industrialized societies are ovulatory dysfunction (25–45%), spermatozoal disorders (mostly unexplained; 20–35%), tubal disease (15–30%), pelvic endometriosis (10–15% as the attributed cause; up to 45% as an identified factor), poor sperm–mucus interaction (5–15%), antispermatozoal antibodies (ASABs; 5–15%) and completely unexplained (5–15%). Following the comprehensive investigation of both partners the infertility problem will often be identified as having a multifactorial basis, although one underlying condition usually predominates. Some uncommon causes of infertility include genital tract anomalies, such as congenital absence of vital structures (e.g. müllerian agenesis, androgen insensitivity syndromes, congenital absence of vasa deferentia), as well as those caused by surgical procedures, diethylstilboestrol (DES) exposure in utero, and uterine synechiae causing Asherman's syndrome. Sexual dysfunctions and ejaculatory disorders are occasional causes of infertility due to failure of sperm deposition in the vagina.

### Range of procedures

A wide range of techniques and procedures enables a much improved chance of achieving conception for infertile couples than was achievable in the early 1980s. These include tubal microsurgery, ovarian stimulation with close monitoring, a variety of techniques involving gamete handling or manipulation namely, donor insemination (DI), the intrauterine insemination of husband's washed spermatozoa (AIH or IUI), IVF-ET and related procedures such as gamete intrafallopian transfer (GIFT), pronuclear or zygote intrafallopian transfer (PROST or ZIFT), and tubal embryo stage transfer (TEST). Additional procedures which have been described but are not widely used include direct intraperitoneal insemination (DIPI), peritoneal ovum and sperm transfer (POST), and fallopian replacement of eggs with delayed insemination (FREDI). Ovum donation of supernumerary oocytes from GIFT programmes is widely practised but ovum donation with in vivo insemination and uterine lavage is not widely accepted for ethical reasons. Surrogacy arrangements may involve the surrogate woman allowing her own egg to be fertilized, which is the common form of commercial surrogacy practised in some American States. Alternatively, the surrogate woman may

carry the embryo resulting from the fertilization of the gametes of an infertile couple (IVF surrogacy). The latter is usual in altruistic compassionate surrogacy arrangements within families.

## INFLUENCING IMPLANTATION

In counselling and treating couples who present with infertility the clinician will be concerned with: (1) the available treatment options for the underlying diagnosis; (2) the relative chance of achieving pregnancy by any given treatment option; and (3) the chance of the resultant pregnancy ending in the birth of a healthy live infant. Extensive data is available covering these considerations but the prognosis for the individual case is uncertain because of the numerous variables involved in a treatment cycle, the wide range of results reported from clinics and the changes occurring in the technology, in addition to other factors within clinics, over time.

Treatment by IVF has provided an excellent model for studying the question of influencing the chance of implantation, as it maximizes the opportunity of measuring the various parameters, and there has been an enthusiastic response by infertile couples invited to participate in approved research studies. A number of developments in the procedure over the past 3 years have significantly improved the chance of pregnancy (Yovich et al, 1989d) and will be considered with respect to their respective impact on implantation. In general terms, the likelihood of successful implantation in IVF is dependent upon embryo factors, uterine receptivity and certain technical or mechanical considerations.

## EMBRYO FACTORS

The quality of embryos generated from IVF is dependent upon the quality of the oocyte fertilized, the normality of fertilization and the laboratory conditions for in vitro culture.

### Oocyte quality

In practice, oocyte quality is measured directly by morphological criteria and the ability to undergo fertilization. It is also measured indirectly by the morphological quality characteristics of the resultant embryos and their outcome following transfer. Reported oocyte grading systems are based on that originally proposed by Marrs and his colleagues (Marrs et al, 1984). Criteria considered include characteristics of the cumulus, the coronal layer, the presence of the first polar body and evidence of germinal vesicle breakdown (Figure 1).

Several influencing factors govern the quality of oocytes recovered from follicle aspirations. These are considered below.

*Stimulation regimen.* Until recently clomiphene citrate (CC) and human

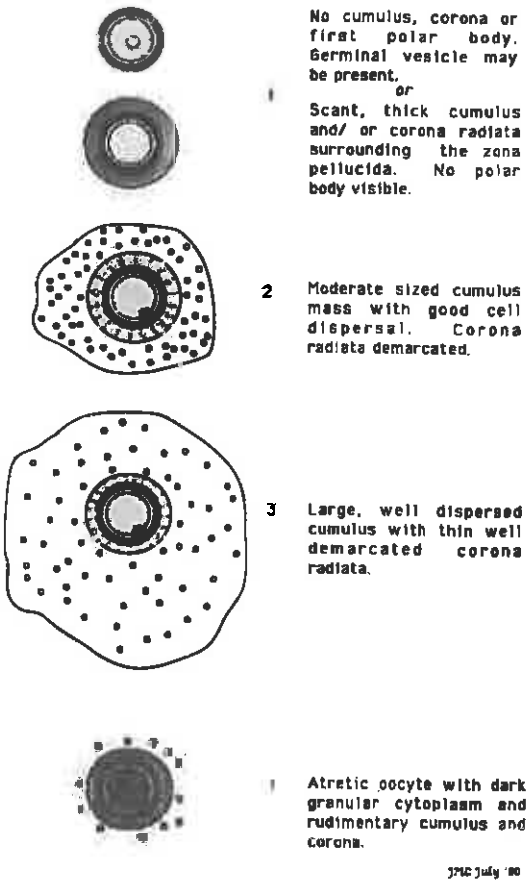


**Figure 1.** Schematic diagram indicating oocyte grading system in use at PIVET. After Marrs et al, 1984.

menopausal gonadotrophins (hMGs) have been widely used both separately or in combination to develop ovarian follicles to an 'appropriate' stage for oocyte recovery. Based on the ultrasonic determination of the size of follicles and/or the pattern of the oestradiol-17β ($E_2$) rise during the follicular phase, a clinical decision is made to trigger the final stage of follicle and oocyte maturation using human chorionic gonadotrophin (hCG) rather than await the spontaneous luteinizing hormone (LH) surge. Some programmes do monitor LH and progesterone, with a view to collecting oocytes on the basis of a spontaneous surge or an hCG-augmented surge. However, most prefer the hCG trigger mainly, for logistic reasons (e.g. for more orderly and convenient scheduling of retrieval procedures).

Marked improvements in IVF results have recently ensued from the diminishing use of CC and the increasing use of gonadotrophin releasing hormone agonist analogues (GnRHa) such as buserelin (Suprefact, Hoechst Laboratories) and leuprolide acetate (Lucrin, Abbott Laboratories). These

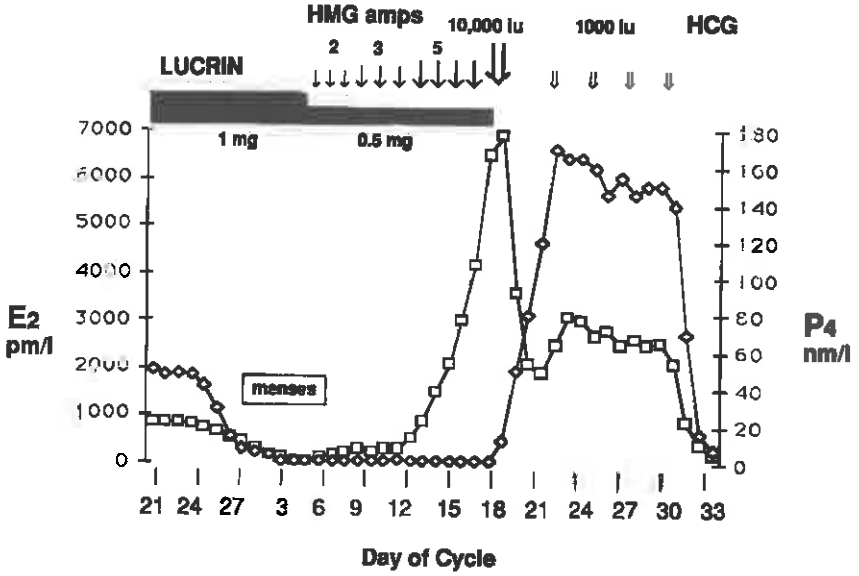


**Figure 2.** Preferred stimulation regimen for IVF and related procedures. hCG trigger is given on seventh day of $E_2$ rise. Courtesy of Blackwell Scientific Publications Ltd.

agents can be used in various regimens combined with hMG. Optimum results appear with a pituitary downregulation schedule. A successful regimen is shown in Figure 2 and involves commencing Lucrin 1 mg s.c. daily in the midluteal phase of the preceding cycle. Pituitary downregulation is usually achieved by day 3–5 of the ensuing cycle and is demonstrated by serum follicle stimulating hormone (FSH) and LH levels both $<5$ IU/litre and $E_2$ $<200$ pmol/litre. Thereafter 0.5 mg Lucrin daily will maintain suppression and hMG injections are given daily, with appropriate increases after 3 days of any given dosage, in order to increase $E_2$ by approximately 50% per day, and the hCG trigger is given on the 7th day of sustained $E_2$ rise. Spontaneous LH surges do not occur with this regimen. Ultrasound monitoring can provide additional useful information and may occasionally lead to delaying the LH trigger until a cohort of follicles have reached 1.6 cm or greater. However, follicle dimensions are not closely correlated with oocyte quality, as satisfactory fertilization, embryo development and pregnancy may be achieved across the range of 10–30 mm diameter follicles (Haines et al, 1989). One aims for $E_2$ levels around 6000 pmol/litre which equates with the recovery of 6–8 oocytes. It is unwise to stimulate higher $E_2$ levels as the generation of higher numbers of follicles increases the risk of the ovarian hyperstimulation syndrome (OHSS), which can be life threatening.

The use of GnRHa has been shown to have significant benefits in older patients ($>35$ years), those with underlying polycystic ovary (PCO) disease, those with raised androgens, those with raised basal LH or those with previous premature LH surges (Cummins et al, 1989). There are also advantages in using GnRHa in women who normally respond well to

conventional regimens, but this is mainly by preventing a premature LH surge, thereby reducing the cancellation rate. Those who have previously demonstrated poor ovarian responsiveness to a downregulation regimen may often respond to the *flare technique* which involves commencing both the GnRHa and hMG together at the beginning of the cycle, when the analogue will initiate pituitary release of gonadotrophins as a normal effect prior to downregulation, and so supplement the exogenous drug. An *ultra-short flare regimen* (Macnamee et al, 1989) has also been described and may prove equally useful and have certain cost benefits. However, the persistently poor responder group remains difficult to treat effectively and cancellation rates due to an inadequate response is of the order of 5–8%. Current research indicates the *combined use of growth hormone* may improve the response or at least reduce the amount of hMG required to effect successful stimulation (Homburg et al, 1988, 1990). However, the expense of such treatment is currently prohibitive for consideration in clinical service.

In one study, comprising patients whose clinical characteristics combined with their previous IVF experience identified them as having a poor prognosis, the use of GnRHa treatment lifted their performance into line with that seen in 'good' prognosis groups (Cummins et al, 1989). In evaluating the advantage of one stimulation regimen over another, it is not always clear whether the difference relates to an improvement in the overall quality of oocytes recovered, or, if only a fixed proportion of oocytes within the ovary have the full potential for normal pregnancy, that simply more oocytes are recovered, leading to a greater chance that at least one oocyte will have the optimal characteristics. Furthermore, the drugs used in ovarian stimulation may influence luteal phase endometrial characteristics, thereby inhibiting the implantation of all embryos, regardless of their intrinsic potential for implantation.

*Natural (unstimulated) cycles*. Because there remain uncertainties regarding the effects of ovarian stimulation upon oocyte quality, some clinics have explored IVF in natural cycles. Indeed, the very first successful implantations which proceeded to livebirths were in IVF-ET treatment cycles involving the retrieval of an oocyte from the single, naturally developed follicle by monitoring the oestrogen output and spontaneous LH surge of natural cycles. It is salutary to recall that Louise Brown (born July 1978) was one of two livebirths after four such natural cycle pregnancies were achieved when embryos had been transferred to 32 women (i.e. pregnancy rate 12.5% per transfer in 1978–1979). However, the overall methodology was inefficient as 78 cycles were monitored in that series, leading to 68 attempts to collect the oocyte at laparoscopy, of which an oocyte was recovered from 45 women (i.e. pregnancy rate 5.9% per collection attempt). Other groups exploring IVF at the time experienced even less efficiency with natural cycle IVF; hence, it was not pursued very extensively, even by the original workers. However, given the methodological improvements in cycle monitoring, other technical developments for oocyte recovery (see below) and the possible benefits of oocyte preincubation (Garcia, 1989), natural cycle treatments are being explored once again. A recent study from Paris (Foulot et al, 1989) reported

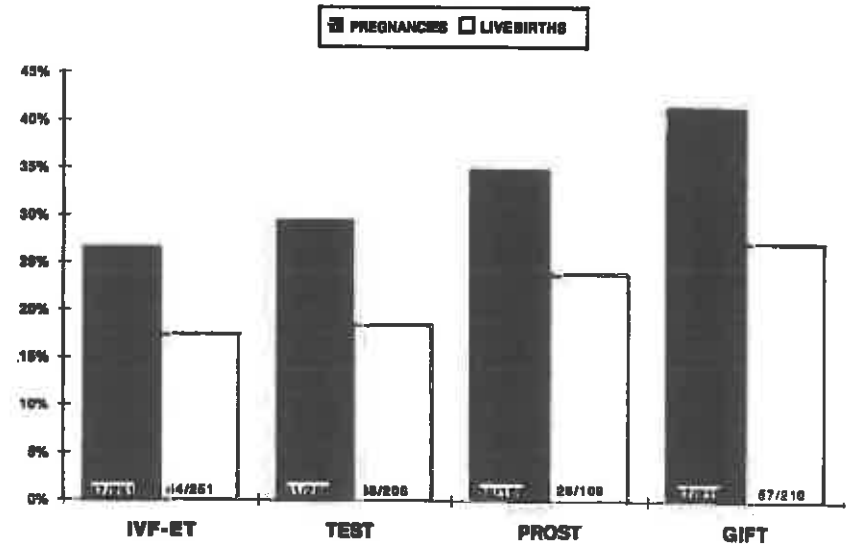


**Figure 3.** Data from PIVET for the 2-year period 1988–1989, showing pregnancy rates and livebirth rates (one or more infants) per transfer procedure for different IVF related techniques.

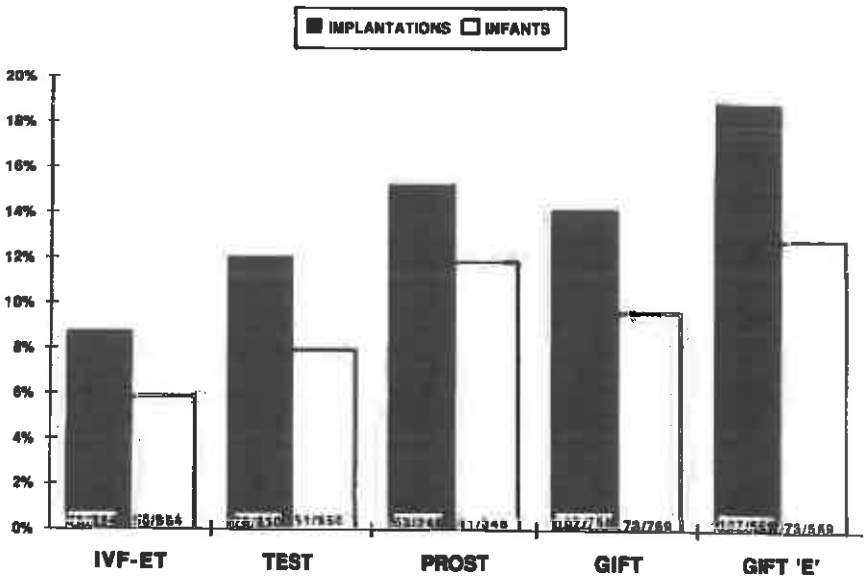


**Figure 4.** Gestational sacs diagnosed (implantation rate) and liveborn infants delivered per oocyte or embryo transferred for various IVF related procedures. GIFT 'E' refers to the rates obtained per estimated 'embryo' transferred if one assumes that 75% of oocytes will fertilize.

14 pregnancies advanced beyond 20 weeks from 80 unstimulated IVF cycles where the main infertility category was tubal disease and male factor cases were excluded (i.e. ongoing pregnancy rate 17.5% per cycle). Oocytes were recovered in 63 of 68 attempts (92.6%) and 53 oocytes fertilized (77.9%), with an apparently higher rate for those oocytes collected after an hCG trigger injection (37/41, 90.2% versus 16/22, 72.7%; not significant).

The best results reported from comprehensive annualized data in large series of stimulated cycles (e.g. Figures 3 and 4) indicate that around 10–15% of selected embryos have the capacity for implantation and complete development to a livebirth infant. The three embryos transferred are selected from a mean of 8–10 recovered oocytes. The supernumerary oocytes are known to have a reduced potential for fertilization (around 50%) (Yovich et al, 1989a), and probably for further development as well; hence, it appears that less than 10% of oocytes recovered from stimulated IVF cycles are of optimal quality. The data on unstimulated cycles should be considered in this context (i.e. a higher ongoing implantation rate per oocyte recovered) and, if the rates reveal a persisting difference, a possible adverse effect of stimulation on oocyte quality must be examined, along with the consideration that stimulation is 'capturing' a number of oocytes otherwise destined for the process of natural atresia.

*Technical aspects of oocyte collection.* Traditionally, oocyte recovery developed as a laparoscopic procedure but has increasingly become replaced by ultrasound-directed techniques, particularly the transvaginal approach. The optimization of oocyte recovery has been shown to depend upon three main aspects (Yovich et al, 1989c):

1. Timing the recovery following LH surge or hCG induction and inducing the surge at the appropriate stage of follicle maturation.
2. The instrumentation and techniques applied for aspiration of the oocytes from follicles.
3. Accessibility of the ovaries for aspiration.

With respect to timing, the LH surge or hCG trigger should occur on day 6 or 7 of the $E_2$ rise for CC/hMG cycles, and the optimal trigger is day 7 of the $E_2$ rise for cycles downregulated with GnRHa. Thereafter, follicles are aspirated $36 \pm 2$ h after initiation of the LH surge or hCG trigger. Oocytes aspirated earlier than 34 h may benefit by compensatory in vitro culture prior to insemination but embryo quality is poor and pregnancy rates are low if oocytes are recovered 4 or more hours earlier than optimal. Oocytes collected up to 4 h after the optimal time remain equally suitable but the risk of spontaneous oocyte release increases, although this appears to be $< 10\%$ up to 42 h in GnRHa cycles.

The first reports using ultrasound guidance for follicle aspirations were from Scandinavia (Lenz et al, 1981) and described a transcutaneous transvesical method. Subsequently, a transurethral method was explored briefly, and finally the transvaginal method has found popular acceptance (Wikland et al, 1989). The optimization of transvaginal ultrasound-directed aspiration is achieved by minimal anaesthesia (premedication is usually sufficient), an

abdominal pressure band to stabilize the ovaries, sharp disposable double-lumen needles which enable combined aspiration and follicle flushing (PIVET–Cook needles from William Cook, Australia, are ideal), controlled aspiration pressures which take into account the needle diameter and its length, a high resolution ultrasound image (e.g. General Electric electronic phased array sector scanner with 5.0 MHz vaginal probe is popular), and avoidance of oocyte-toxic sterilizing fluids in the vagina (10 ml culture medium provides a suitable cleansing and coupling medium) (Yovich and Grudzinskas, 1990).

*Chromosome abnormalities in gametes*

*Oocyte karyotypes*. The major causes of preimplantation embryo loss, implantation failure and spontaneous early pregnancy wastage are known to be chromosomal abnormalities and abnormal embryonic development. A reasonably large multicentre (IVF) study examined the chromosomal status of unfertilized oocytes, errors at fertilization and the chromosomal complement of cleaved embryos (Plachot et al, 1988). With respect to oocytes the study revealed:

1. Of 316 mature unfertilized oocytes, 234 were of the normal 23,X haploid status (74.0%). The abnormal findings were mostly oocytes with aneuploidy (76) and a few with diploidy (6). The rate of abnormal chromosomes was not related to the underlying infertility category, the stimulation regimen nor the amount of hMG used in the stimulation regimen. There was, however, a significant increase in the rate of aneuploidy for women >35 y (38%).
2. Of 1393 supernumerary oocytes examined at around 18 h postinsemination for pronuclear (PN) status, 92.4% showed a normal 2 PN complement, 6.4% showed multiple PN suggesting polyploidy, and 1.6% showed a single PN implying parthenogenetic activation. The fertilization abnormalities bore no relationship to maternal age or total dosage of gonadotrophins (in contrast to observations of pregnant mare's serum gonadotrophins in mice (Maudlin and Fraser, 1977)). Some minor differences in triploidy rates in relationship to stimulation schedules probably reflect total oocyte numbers collected, in that higher retrieval numbers are more likely to comprise some part-atretic or dysmature oocytes. With respect to the category of infertility, unexplained cases had a significantly higher rate of parthenogenetic zygotes (4.2%). Such causes might reflect oocyte abnormalities which are sensitive to activation of the oocyte under conventional IVF laboratory conditions. On the improvement side, there were fewer triple PN oocytes in male factor cases and this probably reflects a generally reduced fertilization (oocyte penetration) potential of such cases. This infers that multi-PN oocytes are due to polyspermic fertilization. There was also less triploidy when the preincubation of oocytes prior to insemination was short (2 h), rather than the usual longer (2–6 h) period.

*Spermatozoal karyotypes*. It is relevant in this discussion also to consider chromosomal abnormalities in spermatozoa. The technique of sperm

karyotyping is more complex than that for other cells and relies on a technique involving sperm preparation in a manner similar to that used for IVF, followed by insemination of zona-denuded hamster oocytes (Rudak et al, 1978). After 12–12.5 h incubation, a Colcemid solution is added to the oocytes, which are then incubated for a further period prior to fixation, staining with quinacrine dihydrochloride and reading under fluorescence microscopy. If one considers the combined results from three groups, involving the examination of more than 6000 spermatozoa from 78 donors (Martin, 1988), the following conclusions can be drawn:

1. Total karyotypic abnormalities can be identified in around 10% of spermatozoa from men with normal semen parameters (range 8.5–13.9%).
2. Aneuploidies occur in around 2% and these can be subdivided into hyperhaploids (range from 0.5% in Japan to 2.4% in North America) and hypohaploids (range from 0.5% in Japan to 3.4% in North America). All chromosome groups appear to have the same frequency of non-dysjunction, with the exception of group G, in which there is a significant excess of hyperhaploidy (X 2.4). These observations should be considered against the finding that human newborns display abnormalities, especially trisomies, of chromosomes 13, 18, 21 and X or Y far more often than any of the others and some, e.g. trisomy 1, are extremely rare.
3. Structural chromosome abnormalities occur in around 8% of spermatozoa (range from 3.3% in North America to 13.0% in Japan).
4. From studies in mice, it is apparent that chromosome constitution does not influence the ability of sperm to effect fertilization. The hamster studies on human sperm confirm that chromosomally abnormal sperm are not at a disadvantage in fertilizing zona-free hamster oocytes.
5. Sperm preparation techniques used for IVF, such as the overlay or swim-up method, do not show a difference in the frequency of sperm chromosomal abnormalities compared with sperm unselected for motility. Furthermore, other in vitro conditions, including cryopreservation, do not alter the sex ratio or the frequency of either numerical or structural abnormalities.
6. There was no relationship demonstrated between age of the male and the frequency of numerical chromosome abnormalities in sperm, although there may be some change in the ratio of the various complements (i.e. more hyperhaploids with advancing age). Furthermore, three independent studies find a negative association between non-dysjunction and paternal age. It is concluded that, unlike the situation with oocytes, there is no increased risk of trisomy with paternal age.
7. Structural spermatozoal chromosome abnormalities do show an increase with age, raising the question of a possible effect from mutagens. The technique of sperm karyotyping is therefore currently being widely explored with a view to developing a possible mutagen 'screening' system.

### Laboratory conditions

Laboratory techniques have not changed significantly from the original model (Purdy, 1982), except where specialized sperm preparation, sperm

enhancement and micromanipulation procedures are used to enhance the chance of fertilization. Some studies assessing co-culture of embryos with endometrial cells or endosalpingeal cells suggest a benefit, but this has not yet been clearly demonstrated. The methodology of IVF is very simple but does require rigid control over:

1. *Temperature*, particularly for oocyte handling, sperm–oocyte incubation and the PN check at 16–20 h postinsemination.
2. *pH*, which should be appropriately set and buffered for follicle flushing, sperm preparation, insemination and postfertilization media (involves high carbon dioxide gassing for bicarbonate buffered solutions).
3. *Osmolality*, which for all media should be held constant at the appropriate level in the range of 280–295, depending on the solutions used. Generally high humidification is required in incubators and during handling procedures which must, therefore, be carried out deftly and rapidly.

Probably the most important laboratory influence over the chromosomal integrity of oocytes occurs during the metaphase stages of meiosis and fertilization. During this period the meiotic spindles are most prone to depolymerization followed by disruption and disorganization as a consequence of relatively minor reductions in temperature. For example, a reduction to room temperature for as little as 10 min caused a high proportion of changes, including chromosome dispersal from the metaphase plate (Pickering et al, 1990). The effects have been known for some time but many IVF units do not rigidly control for reductions in temperature during the crucial oocyte recovery, gamete handling and early insemination stages, possibly in the mistaken belief that meiotic spindles will repolymerize and reorganize normally when the temperature is raised to 37°C. Indeed, this does occur in the mouse (Pickering and Johnson, 1987) but the damage to human oocytes is usually irreversible, the difference being due to the manner in which the pericentriolar material is located in the human. It appears likely, therefore, that poor temperature control during IVF procedures may affect the rate of aneuploidies in the resultant embryos.

## Embryo quality

Following fertilization the resultant embryos are selected on morphological criteria prior to transfer. Other methods of assessment, including karyotypic analysis, biochemical microassays and genetic analysis by deoxyribonucleic acid (DNA) probes, have to date been conducted as research studies. However, the pace of development in both embryology (particularly with embryo biopsy) and molecular biology (especially the development of polymerase chain reaction (PCR) techniques) means that direct clinical applications are now feasible.

### *Morphological aspects*

Embryos can usefully be graded according to four main criteria (Yovich, 1985; Cummins et al, 1986):

1. Clarity of blastomeres.
2. Regularity of blastomeres.
3. Degree of cytoplasmic fragmentation.
4. Time frame of cleavage stages.

At PIVET a point scoring system using these criteria is in current use (Yovich and Grudzinskas, 1990). High quality embryos can achieve a maximum of 4 points with ½ or 1 point deducted in accordance with the degree of deviation from the optimum for each parameter. The findings with respect to implantation rates (i.e. pregnancy sacs detected by ultrasound around the seventh week of pregnancy) are similar to those of other reports (Puissant et al, 1987; Grillo et al, 1991) and can be summarized as follows:

1. Grossly abnormal embryos with irregular, dark, granular and highly fragmented blastomeres do not have the capacity for successful implantation and should be discarded.
2. The implantation rate is significantly reduced when two or more blastomeres of a 4-cell embryo are irregular in size and shape when compared with the other two.
3. The implantation rate is significantly reduced if the degree of fragmentation is greater than 20% of total blastomere volumes.
4. If all blastomeres display dark, granular cytoplasm, indicating vacuolation, this indicates the likelihood of some toxic influence within the culture medium or infection and such embryos rarely implant.
5. Mild degrees of single criteria are insignificant but if total scores of embryos fall below 2/4, there will be significant reductions in implantation rates.

There remains a question of one embryo influencing the chance that another will implant, a concept which has been proposed and debated but so far remains unresolved (see discussion arising at second Bourn Hall meeting following an assessment of two mathematical models for embryo implantation (Walters, 1985)). There is some supportive data for the concept, particularly when one compares single embryo transfers (success clearly dependent upon grade of embryo) with the transfer of several embryos of mixed quality (when the individual embryo implantation rate may be higher than expected). From the later discussion on luteal support therapy, a suggested mechanism may be via an embryo–endometrial signal enhancing uterine receptivity.

### *Chromosome abnormalities in preimplantation embryos*

In the aforementioned multicentre study by Plachot and her colleagues, 252 embryos were examined with the following findings:

1. Ninety-two per cent of the embryos were diploid, with 1.6% haploid, presumed to be parthenogenetically activated oocytes (all had an X chromosome), and 6.4% showed triploidy, presumed to be a consequence of polyspermy.
2. Of the diploid embryos which appeared morphologically normal,

21.4% displayed chromosome abnormalities as described for the PN oocytes.

3. There was a higher proportion of chromosome abnormalities (32.6%) in diploid embryos which were graded as morphologically abnormal.

From these and other karyotype studies on human gametes and embryos, some of which have been specifically generated for research (Angell et al, 1986; Veiga et al, 1987), a model on the natural selection process against chromosome abnormalities has been proposed and is summarized in Figure 5. Regardless of the severity of chromosomal defects, it is apparent that early cleavage stages usually proceed normally. However, once embryonic gene expression is manifested, the anomalies undoubtedly cause implantation failure or early pregnancy failure.

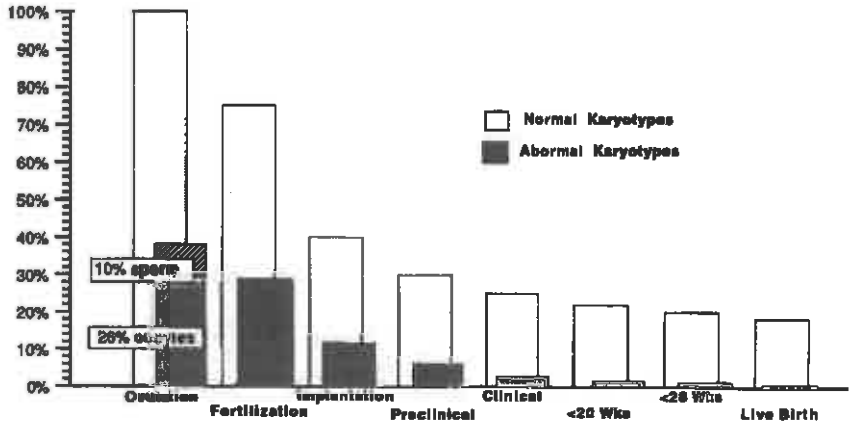


**Figure 5.** Bar chart displaying the inefficiency of normal human reproduction (19 infants per 100 ovulations) but a selective trend against generating infants with chromosomal abnormalities (e.g. 30–40% abnormal embryos but only 0.6% abnormal infants).

### *Other assessment of embryos*

Over the past three years, research has focused on improvements in culturing blastomeres obtained from embryo biopsy as well as by advances in diagnostic methods. The stage has now been reached whereby genetic diagnoses can be made on a small number of cells, often a single cell, and this can obviate the need for propagating blastomere cell lines or having to cryopreserve biopsied embryos pending the outcome of such culture. Diagnostic methods have proceeded along three lines: biochemical microassays, chromosome analysis and probing DNA sequences.

*Biochemical microassays.* For example, a model has been developed with the potential for a four-enzyme study from a single assay of a single cell, i.e. HPRT (hypoxanthine phosphoribosyl transferase; a deficiency of which causes Lesch–Nyhan syndrome), ADA (adenosine deaminase; a deficiency

of which causes severe combined immunodeficiency), PNP (purine nucleotide phosphorylase; a deficiency of which causes combined immunological and neurological disease) and APRT (adenine phosphoribosyl transferase; a deficiency of which causes another disorder of purine metabolism with nephrolithiasis and renal failure) (Monk, 1989).

*Chromosome analysis*. Obtaining unequivocal chromosome data from the few cells available in a human preimplantation embryo presented significant technical difficulties to the cytogeneticists undertaking the previously described studies on research embryos. Improved methods of fixation of human and mouse preimplantation embryos have been described to facilitate G banding and karyotypic analysis, enhancing the possibility of diagnosis on a single biopsied blastomere (Roberts and O'Neill, 1988), but this has not yet reached the stage of clinical feasibility. For sexing human embryos, Y-chromosome-specific DNA probes are now preferred. Successful probes have been described using in situ hybridization methods on entire 2–8-cell embryos (West et al, 1988), and recently PCR amplification (see below) has been applied to the single biopsied blastomere from 30 human embryos at the 6–10-cell stage (Handyside et al, 1989). The results of the latter technique matched the former (applied to the whole embryos) accurately. This development has now been applied successfully in clinical practice with two women, both carriers of X-linked disorders, implanting twin female pregnancies after preimplantation biopsy of a single cell at the 6–8-cell stage, and sexing by DNA amplification of a Y-chromosome-specific repeat sequence (Handyside et al, 1990). Subsequent chorionic villus sampling (CVS) and delivered outcomes have confirmed that the diagnoses have been accurate.

*Probing DNA sequences*. A rapidly increasing number of genetic diseases have had their specific gene sequence encoded, the most recent exciting report being the identification of the cystic fibrosis gene (Rommens et al, 1989), including the cloning and characterization of its cDNA (Riordan et al, 1989) and its complete genetic analysis (Kerem et al, 1989). Applying DNA probes for preimplantation embryo diagnosis using restriction fragment length polymorphisms required a large number of cells, initially many thousands. Subsequent DNA amplification methods reduced the numbers required to around a hundred but the development of the polymerase chain reaction (PCR), which can amplify DNA sequences by $10^7$–$10^{10}$, enables the detection of a single copy gene from a single cell by amplification of the specific base sequence of that gene (Saiki et al, 1985, 1988). The feasibility of diagnosing cystic fibrosis and muscular dystrophy from a single biopsied cell has been demonstrated by applying the PCR technique to the DNA from a single human oocyte and detecting markers closely linked to those genes within a few hours of cell isolation (Coutelle et al, 1989). Now that the actual gene for cystic fibrosis has been cloned, greater diagnostic specificity is achievable. Similarly the feasibility of diagnosing β-thalassaemia has been demonstrated in single blastomeres from mouse preimplantation embryos using this approach (Holding and Monk, 1989), which also relied on the

ingenious selection of nested primer DNA sequences to improve the sensitivity and specificity of amplification.

It is now entirely feasible that all the DNA markers which have been successfully applied to chorionic villus biopsy specimens, including linked DNA polymorphisms such as applied for the predictive testing of Huntington's disease (Brock et al, 1989), will be applicable to preimplantation embryo diagnosis. However, whilst PCR is clearly a very powerful tool in diagnostic genetics, a number of identified problems, particularly its ability to amplify any contaminant DNA, require caution about its use at this stage. Detection of gene sequences in individual sperm cells is now also possible using a PCR approach (Li et al, 1988), and this will probably also be used ultimately along with karyotyping approaches in prefertilization genetic screening.

*Functional tests of embryo integrity*

The above diagnostic procedures all carry relevance to implantation in the specific instance of known genetic carrier states and certain high-risk situations, e.g. for Down's syndrome. However, it is only the chromosome assessment of preimplantation embryos which is likely to be rewarding in preselecting embryos for transfer on a routine basis with the view to improving the chance of successful implantation. Even then, at best one might expect one-third of embryos to be discarded on the basis of chromosomal defects, yet, in the best hands, less than one-third of embryos can be successfully cultured through to morphologically normal blastocysts and less than one-fifth can implant with normal outcomes. Therefore, it appears likely that a proportion of embryos (perhaps 25–30%) may lack the functional capacity for implantation and full development. Some degree of laboratory limitation can be conceded, although this appears minor for IVF and embryo culture to the 4- and 8-cell stages, given tight adherence to the aforementioned principles. It is more likely that certain degrees of oocyte immaturity or dysmaturity may be reflected by deficient metabolic or other functional processes, and perhaps this might be measured in vitro for embryo preselection. A number of tests have been explored in research models: e.g. seeking the presence of β-hCG, platelet aggregating factor (PAF) (O'Neill and Spinks, 1988) or other early pregnancy factor (EPF) (Morton, 1984) within supernatants of the culture environment of the developing embryo; and metabolic uptake studies using agents such as fluoroscein diacetate, radiolabelled glucose (Wales et al, 1987) and both glucose and pyruvate in non-isotopic studies using a non-invasive technique (Hardy et al, 1989). Other studies have examined the follicle fluid of the relevant oocytes, e.g. for steroid hormones, pregnancy associated placental protein A (PAPP-A) (Stanger et al, 1985) and insulin-like growth factor (IGF-1)-binding protein. It suffices to summarize that the limitations of toxicity, development of appropriate microassay techniques and the relevance of any given marker or measurement for human embryos are inadequate at this stage to consider introducing such a test into clinical practice. For example, the PAF studies, which proved to be significantly relevant for mouse embryos, have so far not

demonstrated the same relevance for human embryos. This might reflect assay limitations, and the development of a commercial radioimmunoassay that is sensitive in the range of 10–1000 pg (Baldo et al, 1990) is welcome.

## UTERINE RECEPTIVITY

Given optimal embryo quality, the chance of implantation is dependent upon two further factors: uterine receptivity and mechanical factors (concerning embryo placement and transport within the genital tract). The question of uterine receptivity focuses upon the endometrium: its functional integrity and physiological considerations concerning the implantation window which influences the timing of embryo transfer procedures. These are extensively discussed in other sections of this book, hence the clinical considerations presented here will address the application of current

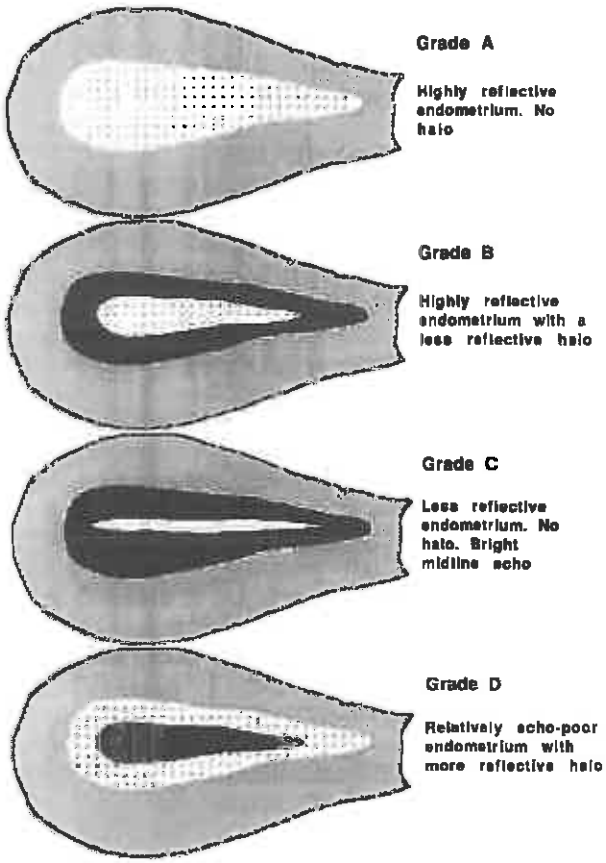


**Figure 6.** Schematic diagram showing the four endometrial patterns recognized on real-time transvaginal ultrasound scans.

knowledge. This covers two areas: determining the optimum biophysical characteristics for successful implantation, and treatment to enhance endometrial receptiveness.

### Seeking optimal characteristics

At PIVET a prospective study examining endometrial characteristics has been conducted using real-time transvaginal ultrasound. Over a 6-month period, scans were routinely performed in women attending for treatment of infertility during the follicular phase, and also in the luteal phase on days 10, 13 and 16 after hCG trigger or LH surge in the case of unstimulated, monitored cycles. Endometrial grades, based on earlier descriptions of the pattern of reflectivity seen at transvesical ultrasound scans (Smith et al, 1984), are depicted in Figure 6. The thickness of the inner and outer endometrial layers were recorded in each case.

Preliminary analysis of the results of 57 scans performed within 24 h of the hCG trigger in 39 women, and 54 scans performed in 31 women between days 12 and 16 after trigger, are shown in Figure 7. The data were analysed separately for scans performed around the time of trigger or during the luteal phase. There was no difference between the endometrial grade ascribed in scans in those women who failed to conceive and those who conceived either ongoing pregnancies or pregnancies which resulted in early pregnancy loss. We also failed to confirm earlier reports that endometrial thickness (Figure 8) is of predictive value in identifying those cycles in which conception is likely to occur (Gonen et al, 1989).

Measurement of the level of the placental protein PP14, the major secretory protein of the human endometrium, has been suggested as a marker of endometrial maturity (Joshi et al, 1986; Bell and Drife, 1989). Initial optimism has been confounded for a number of reasons: in particular, the wide variation among individuals and the close relation to β-hCG and progesterone levels. We measured PP14 levels in blood samples taken at the time of ultrasound scan in the above patients. Again, no significant correlation was demonstrated between PP14 level and endometrial thickness, and although PP14 is known to rise more rapidly in the late luteal phase of conception cycles, the wide individual variability precluded its use in a predictive capacity (Figure 8).

Other attempts to assess luteal phase characteristics include the measurement of $E_2$ and progesterone:$E_2$ ratios, with excessive $E_2$ indicating a diminished chance of implantation (Gidley-Baird et al, 1986; Forman et al, 1988). However, these measurements can reflect vagaries in stimulation regimens and appear to have no influence over outcomes when luteal support therapy is used (see below). At this stage a reliable and useful marker of endometrial quality has yet to be identified.

### Luteal support therapy

Luteal support therapy, using progesterone, progestogenic compounds and/or hCG, has become a common component of infertility treatments

involving ovarian stimulation, particularly where this is combined with assisted reproduction. There appears to be a reasonable rationale for luteal support in anovulatory women treated by hMG, as non-conception treatment cycles often display a markedly shortened luteal phase (Brown et al, 1980). This has also been observed among cycling women treated by hMG for assisted reproduction, particularly those with a high oestrogen output during the follicular phase (Edwards et al, 1980; Yovich, 1988b), hence luteal support is likely to be beneficial in hMG stimulated cycles. This

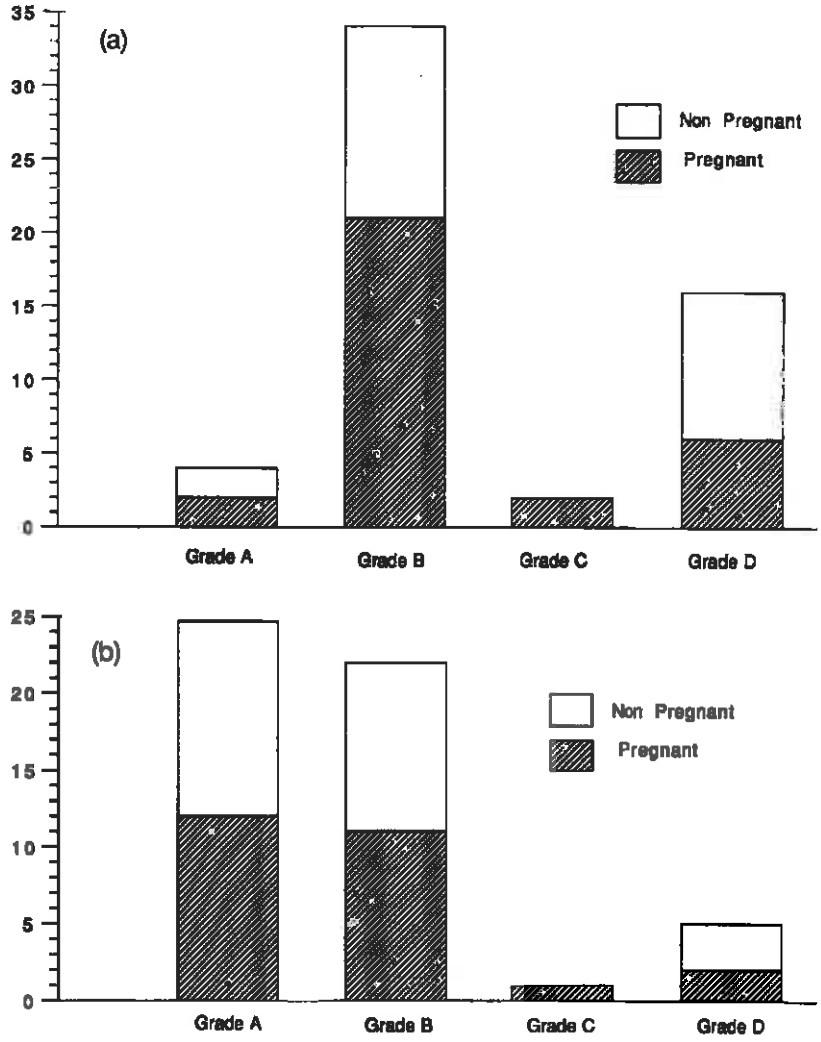


**Figure 7.** Distribution of ultrasound-graded endometrial patterns performed (a) within 24 h of the hCG trigger, and (b) during the luteal phase, categorized with respect to the achievement of pregnancy. (No significant relationship recognized.)

view is supported by a recently reported randomized matched study, which examined hormonal and pregnancy data in a small series (Hutchinson-Williams et al, 1990).

However, cycles stimulated by CC, with or without additional hMG, do not display shortened luteal phases (Yovich, 1988b). Furthermore, the early published reports of randomized controlled trials assessing luteal phase treatments using progesterone (Leeton et al, 1985; Yovich et al, 1985a; Trounson et al, 1986) or hCG support (Yovich et al, 1984; Mahadevan et al, 1985; Buvat et al, 1990) failed to show significant improvements in pregnancy rates, although there was usually a trend implying a benefit. However, despite the careful methodology in these studies, they have all been far too small to enable the significant detection of even a 10% variation in the pregnancy rate. Furthermore, they comprised a heterogeneous population of subjects within IVF programmes, which have usually been evolving through constant internal changes and which are generally reflected by inexplicable periods of poor pregnancy rates. One group attempting to study

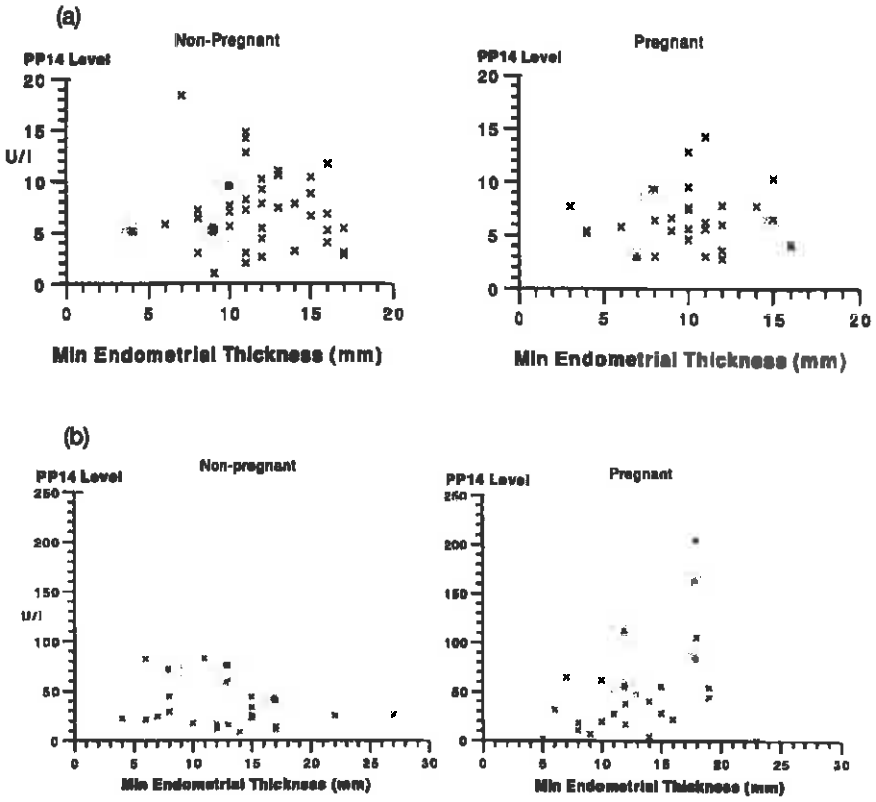


**Figure 8.** Scattergrams showing serum PP14 levels and endometrial thickness measured (a) within 24 h of the hCG trigger, and (b) during the luteal phase, categorized with respect to the achievement of pregnancy. (No significant relationship recognized.)

the effects of a progestagenic compound given during the luteal phase of IVF treatment cycles calculated that it would require two groups of 1500 subjects in each category to significantly detect a 5% improvement in the pregnancy rate, e.g. from 15% to 20% (Belaisch-Allart et al, 1987).

To minimize the inherent problems in luteal phase supplementation studies, a relatively large randomized controlled trial was performed at PIVET within a GIFT programme (Yovich et al, 1991), beginning around 9 months after it was established and shown to be characterized by stable pregnancy rates. Subjects were carefully screened to include a single infertility subcategory and exclude all known factors which might adversely affect the chance of pregnancy. Over a 12 month period (1986–1987), 280 couples were recruited after screening out all cases comprising any pre-existing factors deemed to be likely to influence the chance of pregnancy (e.g. age, ovulation disorders, presence of ASABs, endometriosis, male factors, previous IVF-related procedures) or any adverse factors in the treatment cycle (i.e. both poor and excessive responders excluded).

Couples fulfilling these criteria and providing informed consent were randomly selected by sequential allocation at the commencement of a treatment cycle into one of four groups:

1. Nil support.
2. hCG support: 1000 IU hCG (Profasi; Laboratoires Serono SA, Aubonne, Switzerland) given i.m.i. on days 4, 7, 10 and 13 of the luteal phase, where the day of oocyte retrieval is nominated as day 0.
3. Progesterone support: 50 mg progesterone in oil (Proluton; Schering AG, Berlin, Germany) given i.m.i. on days 0, 1, 2, 3 and 4 (i.e. 5 days), beginning in theatre immediately after oocyte retrieval. This regimen follows an implied benefit reported in a previous study (Yovich et al, 1985a).
4. Combined hCG/progesterone support: combines regimens (2) and (3), i.e. progesterone 50 mg i.m.i. on days 0–4 inclusive, followed by hCG 1000 IU i.m.i. on days 4, 7, 10 and 13 of the luteal phase.

The 280 cases selected into this trial had completely normal investigations, mild pelvic endometriosis only (American Fertility Society grades 1 or 2 only) or unexplained poor sperm–mucus interaction. They were therefore categorized as unexplained infertility. Such cases were usually treated by four cycles of ovarian stimulation therapy and, if postcoital tests were persistently negative, by additional intrauterine insemination of husband's washed, precapacitated spermatozoa (Yovich and Matson, 1988b) prior to inclusion in the GIFT programme. This was seen to be an ideal group for study as the patients had a good prognosis for pregnancy without luteal support therapy, hence any benefits of therapy which might be demonstrated would be likely to be of greater benefit in cases of repetitive unexplained failures.

The GIFT protocol applied a standard CC/hMG stimulation schedule to all cases, followed by laparoscopic oocyte recovery (the trial predated our introduction of the routine use of the transvaginal technique), followed by the selection of the highest graded oocytes for transfer. During the trial

period the routine was for two oocytes into each tube with occasional patients having up to six oocytes in total (current protocols set a maximum limit of three oocytes per patient, usually all into one tube). Only clinical pregnancies were recorded in the trial, i.e. those demonstrating a rising β-hCG after day 16 of the luteal phase and the subsequent demonstration of a pregnancy sac(s) on ultrasound performed routinely in the 7th week, or histologically if an ectopic occurred. Pregnancy losses were diagnosed as

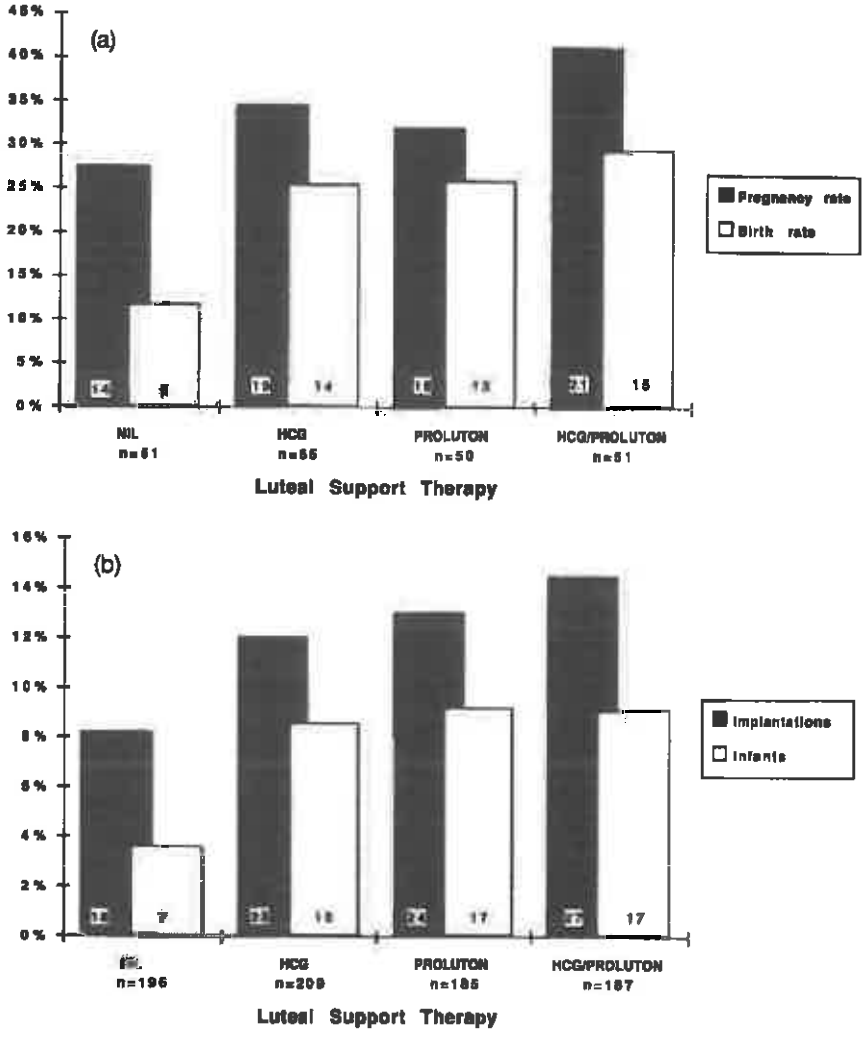


Figure 9. The effect of various luteal support therapy regimens on (a) total and ongoing pregnancy rates, and (b) total and ongoing implantation rates. Ongoing pregnancy rate ($P<0.05$) and ongoing implantation rate ($P<0.02$) significantly higher with luteal support. Courtesy of *Fertility and Sterility*.

*blighted ovum* if a viable fetus was not shown within the sac, or *spontaneous miscarriage* if a viable fetus was subsequently lost before the 20th week. All first trimester, pregnancy losses were classified as having single sacs. Those pregnancies progressing beyond 20 weeks were classified as ongoing or births.

The results are summarized in Figure 9 and indicate a significant benefit for luteal support therapy. The benefit is clearly shown with respect to

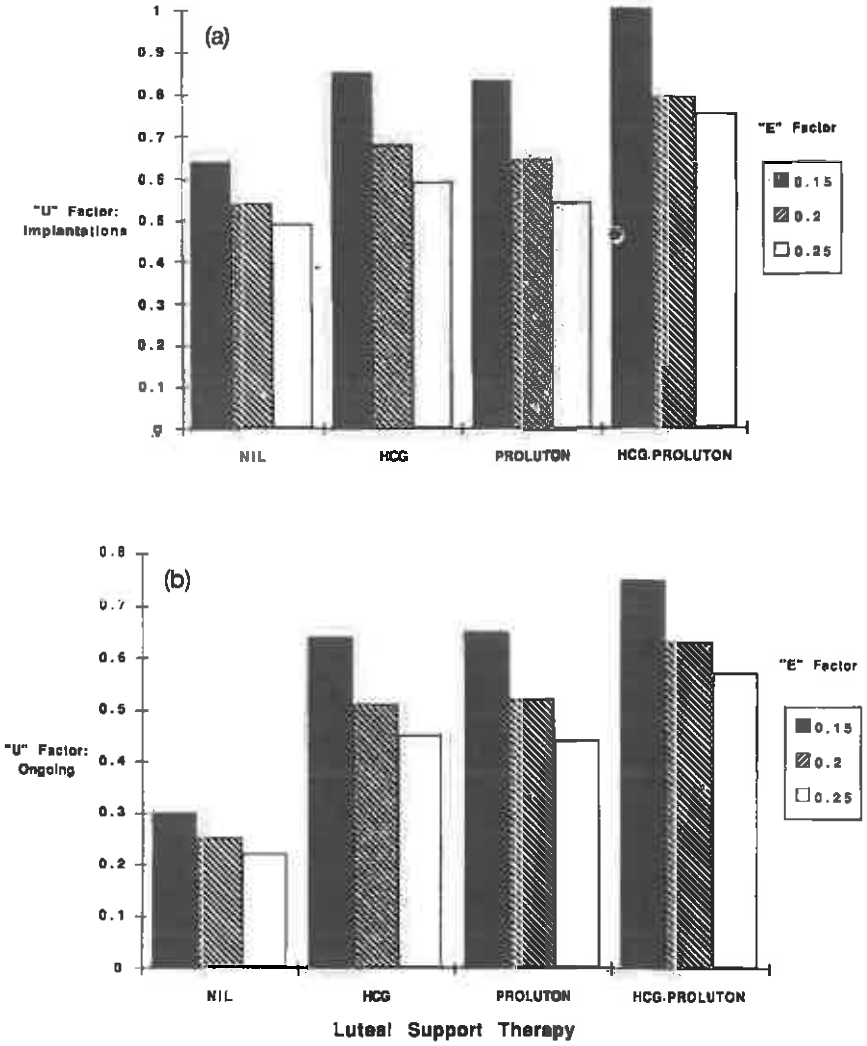


**Figure 10.** Statistical modelling of luteal support data showing that the uterine receptivity factor 'U' is improved most by luteal support therapy when embryo quality 'E' is lower. The improvement is apparent for all implantations (a) but highly significant for ongoing implantations (b). Courtesy of *Fertility and Sterility*.

ongoing pregnancies and is apparent for individual implantations, particularly those which proceed to births. By applying a modelling technique on the data (using iterative procedures based on the binomial formula to derive uterine receptivity ('U' factors) for both implantation and ongoing pregnancy sacs, given various likely values for the proportion of optimum quality embryos ('E' factor); Figure 10), two interesting extrapolations could be made from the observed data. Firstly, it demonstrates the relationship between embryo quality and uterine receptivity in a manner which enables the relevance of luteal support to be seen in perspective. That is, the poorer the quality of the embryo, the greater the benefit of improvements in uterine receptivity. Secondly, there appears to be an interdependence of uterine receptivity to embryo quality. The optimum 'U' and 'E' factors both tended to be high where the outcome was best, and both were low where the outcome was poorest. This latter observation assumes the mean embryo quality was similar in all groups prior to the embryos reaching the uterus. It was not possible to measure embryo quality in this trial but it was designed to exclude every known bias. The observations are therefore considered to be real and support the concept of preimplantation embryo–endometrial interactions, whereby quality factors in one can benefit the other, and vice versa.

The study does not enable conclusions to be drawn regarding the best regimen of luteal support but the hCG/Proluton regimen is now incorporated as a routine at PIVET and is believed to be a significant contributing factor to the higher pregnancy and livebirth rates recorded overall during 1988 and 1989, as shown in Figures 3 and 4. Of 212 GIFT transfers during that period, 88 pregnancies arose (41.5%) and 57 (26.9%) proceeded through to birth. It is also now applied routinely in all other IVF related treatments, and during the same period 166 pregnancies arose after 566 embryo transfer procedures (29.3%) with 108 (19.1%) proceeding to births. These results are a significant improvement on our own data from the years preceding this trial (Webb, 1988) and from those reported nationally in Australia (Lancaster, 1990). Although other beneficial factors have been identified (Yovich et al, 1989d), luteal support therapy is believed to be a major contributor to the improvement.

## MECHANICAL, TECHNICAL AND OTHER FACTORS

This section will consider the technical aspect of embryo transfer in so far as it relates to the chance and location of embryo implantation; the consideration of tubal as opposed to uterine transfers, as well as the relevance of transferring at different stages of oocyte to embryo development; and the relevance of certain underlying infertility disorders which appear to influence the chance of implantation.

### Embryo transfer technique

A variety of techniques are applied with the aim of depositing embryos in the upper uterine cavity with the least degree of disturbance to the cervix, the

uterine body and the endometrium. At PIVET a double cannulation technique is used. The external catheter is a soft polyurethane material which negotiates the cervical canal to a distance of 4 cm from the cervical os. Thereafter, an embryo-laden Teflon catheter is introduced through the outer sheath to a distance of 55–70 mm (as determined by prior evaluation by sounding and hysteroscopy during the investigatory work-up), to deposit the embryos a few millimetres short of the fundus. Prior to the process of embryo deposition, the outer catheter is withdrawn from the cervix and a settling period observed. Ultrasound control can be useful but is not usually required and the process should be totally atraumatic, i.e. instrumentation to grasp the cervix or dilate the canal should be avoided and both catheters should be blood-free on withdrawal. Best results are achieved by placing the patient in the Trendelenburg position with around 10° head-down tilt (knee–chest position is not required, regardless of the uterine position) and, after the transfer, the patient should maintain head-down tilt in bed for a minimum period of 4 h. A maximum of three embryos are transferred in 15–20 μl of culture medium containing 50% deactivated maternal serum.

The principles of the embryo transfer technique are simplicity of method, minimal disturbance to the mental and physical senses of the patient, atraumatic technique of catheter placement, slow entry and withdrawal of catheters, small transfer volumes, and minimum exposure of the embryos to the external environment between incubator and uterine cavity. Failure to adhere to any one of these principles reduces the chance of successful implantation. For example, rapid withdrawal of the transfer catheter can lead to a suction effect and up to 20% of embryo transfers may fail due to embryos exiting the uterine cavity via the cervix.

Once embryos have been deposited in the uterine cavity, it is clear that even with the best technique and confirmation of satisfactory uterine placement (Kovacs et al, 1987), embryos find their way into the fallopian tubes. This leads to ectopic pregnancies occurring in around 5% of IVF pregnancies as a universal experience. However, it is almost invariably tubal factor patients who are at risk from ectopics and the rate can be significantly raised if embryos are deliberately transferred to the fallopian tubes (e.g. by TEST (Yovich, 1990)) or inadvertently transferred by faulty techniques (Yovich et al, 1985c). In the latter situation, direct cannulation of the fallopian tube or rapid forced ejection of embryos in excessive fluid volumes may cause embryos to be pushed into the fallopian tube in cases with narrowed interstitial segments. Such embryos may be unable to return because of the narrowing or additional disorders of the distal intratubal lumen, as combined proximal/distal fallopian tube disorders often coexist.

### Tubal versus uterine transfer

Since January 1988, the policy at PIVET for IVF related procedures has been to treat all tubal factor cases by IVF-ET; cases caused by endometriosis, unexplained infertility, poor sperm–mucus interaction or failed donor sperm therapy, by GIFT; and male factor cases, those with ASABs, those for donor oocytes or postcryopreservation and GIFT failures, by either

PROST or TEST. The last two techniques are still under comparative evaluation and include a laparoscopic or transcervical approach (TC-TEST) (Yovich et al, 1990). The results of oocyte and embryo transfers from these procedures over the 2-year period 1988–1989 are shown in Figures 3 and 4.

There were 253 pregnancies diagnosed following 776 transfer procedures (32.6%) and 165 pregnancies proceeded to livebirth deliveries (21.3% of transferred cases). The pregnancy rates were significantly higher following GIFT (3 d.f., $P<0.01$), although pregnancy wastage was higher. None the less, the GIFT procedure still had a significantly higher chance of livebirth delivery per transfer procedure (3 d.f., $P<0.05$). Conversely, the pregnancy rate and 'take-home-baby' rate for IVFET was significantly lower than the combined tubal transfer groups (1 d.f., $P<0.02$ and $P<0.005$ respectively).

With respect to individual implantations, 313 pregnancy sacs were diagnosed in the 7–8th week following the transfer of 2621 oocytes or embryos (failing pregnancies were diagnosed as single sacs). This indicated the overall implantation rate was 11.9%, and the rates ranged from a low of 8.7% for IVFET embryos to a high of 14.1% of GIFT oocytes or 18.8% of estimated GIFT 'embryos' (if one assumes that 75% of transferred oocytes will fertilize in vivo). The findings were highly significant (3 d.f., $P< 0.001$), mainly due to the lower implantation rate of embryos transferred to the uterus compared with the combined groups of oocyte and embryo transfers to the fallopian tubes (1 d.f., $P<0.001$). Of further interest is the comparison of implantation rates for GIFT, PROST and TEST. It appears that in vivo generated embryos may (if the fertilization rate assumption holds) have an improved chance of implantation as GIFT 'E' rates are higher than combined PROST–TEST (1 d.f., $P<0.005$).

Overall, 215 live infants were born providing an 'efficiency' level of 8.2% of oocytes or embryos transferred. The 'take-home-baby' rate ranged from 5.8% of IVF-ET embryos to 11.8% of PN oocytes transferred in the PROST procedure. The differences were highly significant (3 d.f., $P<0.002$), virtually entirely due to the relatively low rate for uterine transfers compared with all tubal transfers (1 d.f., $P<0.002$). Applying the aforementioned assumption to estimate GIFT 'embryos', an efficiency rate of 12.8% ongoing implantations was significantly higher than other tubal transfer procedures (1 d.f., $P<0.05$). Again, the data indicates that tubal transfers are significantly better than uterine transfers and the improvement is greater the earlier the stage of transfer.

The above data confirms previous reports from PIVET (Yovich et al, 1988a, 1989b) but the precise reason for the benefit of tubal transfer remains uncertain. It is likely that oocytes and embryos transferred to the fallopian tube are more securely housed than with uterine placement, but the main reason is more likely to relate to the relatively unfavourable milieu within the uterine cavity in the early postovulatory phase, and this may be more marked after CC has been used for stimulation (Nelson et al, 1990). It is also possible that the fallopian tube contains factors of special benefit to the developing embryo, but one would expect greater differences if some crucial factor was not present in the uterus. The variation in implantation rates of tubal procedures with respect to the day of transfer implies that in vitro

culture techniques are still not ideal and that further research is warranted in this area.

## Underlying infertility disorder

It appears that factors in the female, associated with the underlying infertility disorder, may influence the likelihood of implantation. However, the differentiation between embryo factor and uterine receptivity is not always clear and specific studies in this area are required. The following conditions show variations from the general results in IVF.

### *Polycystic ovary disease and associated syndromes*

The polycystic ovary (PCO) syndrome remains a contentious subject, with wide variations in its definition and management. In a decade of experience with subfertile couples treated by a broad range of methods, including IVF related procedures and GIFT, such patients have generally performed poorly when the stimulation regimen involved CC ± hMG. The characteristics of the group included women with clinical PCO disease (i.e. infertility, oligomenorrhoea, obesity, hirsutism); those with histologically diagnosed polycystic ovaries; those with multifollicular ovaries diagnosed on ultrasound scans performed early in a non-treatment menstrual cycle; those with high basal LH:FSH ratio; and those women with raised serum androgens. Both reduced fertilization of oocytes when LH levels are raised (Stanger and Yovich, 1985) and reduced implantation of embryos when serum androgens are raised (Yovich, 1988b) have been described.

During 1988–1989, an alternative stimulation regimen was applied to the above group; this involved pituitary downregulation with GnRHa (Lucrin) followed by hMG stimulation as described earlier. The outcome was significantly improved in all the subcategories of the PCO syndrome. For example, 14 women with clinical PCO disease had 20 IVF related treatment cycles, resulting in the retrieval of 305 oocytes (15/patient). Following the transfer of up to four oocytes in GIFT or three embryos in IVF, nine pregnancies ensued with eight livebirths (57% of women). However, OHSS occurred in five treatment cycles (25%), and one patient required paracentesis. Three of the pregnancies were twin gestations (38%), and each had a successful outcome.

It appears the prognosis of women with PCO disease and PCO syndrome is markedly improved in assisted reproduction by avoiding CC and creating pituitary downregulation prior to stimulation; however, this group appears particularly predisposed to OHSS and multiple pregnancies.

### *Endometriosis*

A wide range of abnormalities have been described in attempting to explain the cause of infertility in women with endometriosis, including hormonal anomalies, disordered ovarian function, peritoneal factors and immunological disturbances. A study from PIVET compared the outcome of 30

women with grade IV (i.e. most severe) pelvic endometriosis with a similar group of non-endometriotic tubal factor causes treated by IVF-ET. No differences were found in the stimulated hormone profiles but the endometriosis cases had a significantly reduced pregnancy rate (Yovich et al, 1988b). Significantly fewer oocytes were obtained from the endometriosis cases but the fertilization rate was normal, unlike the findings in another study (Wardle et al, 1985). The PIVET study also indicated no morphological differences in embryo quality but the implantation rate was significantly reduced, implying the presence of an implantation inhibitory factor. However, the subsequent experience of treating endometriosis patients by tubal transfer procedures, particularly GIFT, showed no limitations in the chance of pregnancy or implantation (Figure 11), regardless of the grade (Figure 12) or whether the women had been treated previously by either hormonal or surgical therapy (Yovich et al, unpublished data). There are some apparent differences related to the stimulation regimen, with the best results obtained after GnRHa downregulation. This further data appears not to support the presence of an implantation inhibitory factor but implies that embryos generated from women with endometriosis may be more susceptible to damage in utero, possibly when this follows CC stimulation. It is current policy at PIVET to downregulate women with grades III and IV (i.e. moderate and severe) pelvic endometriosis for 6 weeks prior to hMG stimulation for IVF related procedures, GIFT being preferred where applicable.

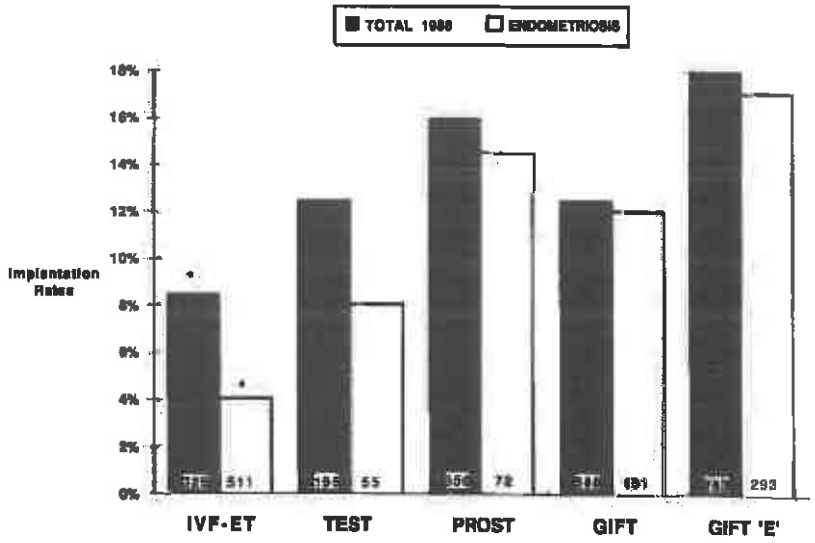


**Figure 11.** A comparison of implantation rates of embryos and oocytes for cases with endometriosis (1986–1988) compared with total cases treated from mixed causes during 1988 by various IVF related procedures. *IVF-ET had significantly lower implantation rates generally than tubal transfer procedures (combined data $P<0.001$) and endometriosis cases were further reduced from other IVF-ET cases ($P<0.05$). GIFT 'E' refers to estimated embryos (as Figure 4).

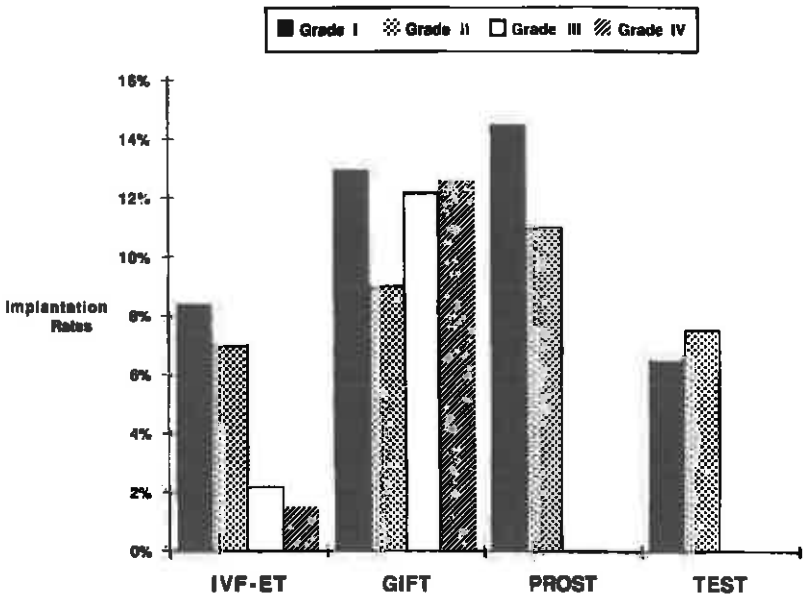


**Figure 12.** The implantation rates for oocytes and embryos transferred in various IVF related procedures according to the grade of endometriosis of the cases in Figure 11. (Total transferred = 1039; $*\chi^2$ 3 d.f., $P<0.001$.)

## EARLY PREGNANCY FAILURE

The clinician's interest in the phenomenon of implantation does not cease when pregnancy is diagnosed as there is a high proportion of wastage, mostly within the first 6 weeks after ovulation or embryo transfer. The pattern of wastage identified clinically at PIVET has been reported (Yovich and Matson, 1988a) and is shown in Figure 13. From a series of 1657 pregnancies diagnosed after infertility treatments to May 1989, 30.9% did not reach 20 weeks. The remaining 1145 pregnancies progressed to births, with the delivery of 1613 infants. These data are now incorporated into the routine counselling of patients seeking infertility treatments.

The clinical categories of pregnancy wastage were:

1. *Preclinical (biochemical) pregnancies* ($n=98$; 5.9%): where loss occurred before the ultrasound diagnosis around week 7.
2. *Blighted ovum (anembryonic) pregnancy* ($n=211$; 12.7%): early ultrasound examination revealed an empty intrauterine gestation sac associated with static or falling β-hCG levels.
3. *Spontaneous fetal death (spontaneous abortion or miscarriage)* ($n=98$; 5.9%): absent fetal heart activity and failure in growth of the gestation sac, when positive fetal heart activity had previously been demonstrated at ultrasound scan.
4. *Ectopic pregnancy* ($n=100$; 6.0%): an extrauterine implantation

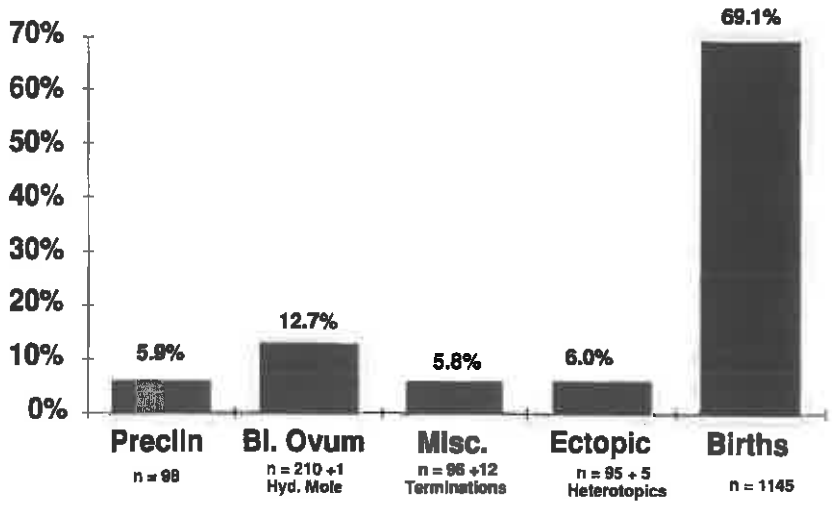


**Figure 13.** Outcome of 1657 monitored pregnancies conceived following infertility treatments at PIVET (July 1982–May 1989). Preclin., preclinical; Bl. Ovum, blighted ovum; Misc., miscarriage; Hyd. Mole, hydatidiform mole.

(usually tubal) with diagnosis made at laparoscopy if ultrasound examination failed to confirm the presence of an intrauterine gestation sac in association with raised serum β-hCG.

5. *Heterotopic pregnancy* ($n=5$): a multiple pregnancy with both intrauterine and extrauterine gestational sacs.
6. *Hydatidiform mole* ($n=1$): characteristic snowstorm appearance on ultrasound and hydropic chorionic villus vesicles at curettage.
7. *Therapeutic terminations* ($n=12$): mostly for genetic reasons following amniocentesis or CVS diagnosis.
8. *Late pregnancy outcomes* ($n=1145$): high incidence of multiple pregnancies (1.4 infants per birth) and preterm deliveries, even among singletons (13%). Fetal abnormalities ($n=33$; 3.0%) are not increased over that reported in the local population (Bower et al, 1989), nor were recurring abnormalities a feature. An early study on the followup of children indicated normal development (Yovich et al, 1986a).

### Patterns of early wastage

The patterns of early pregnancy wastage revealed some variations due to the underlying infertility disorder and the treatment method applied (Yovich and Matson, 1988a). For example, preclinical pregnancies were more common after GIFT and IVF-ET; blighted ovum pregnancies were more common after AIH or GIFT treatment for male factor infertility; and ectopic pregnancies were almost invariably found in women with known or suspected fallopian tube disease but were less common in male factor treatment groups, e.g. DI and PROST.

### Monitoring early pregnancies

In some studies at PIVET and elsewhere early pregnancies have been monitored by hormonal and certain pregnancy associated protein estimations each week until a definitive diagnosis could be made, usually by ultrasound scan in the 7th to 8th week (Yovich et al, 1985b, 1986b, 1986c; Yamashita et al, 1989). Recently, an analysis on 675 monitored treatment cycles in women conceiving a singleton pregnancy, either following spontaneous ovulation ($n=384$) or ovarian stimulation with CC/hMG ($n=291$) between 1985 and 1989, has been completed (Lower et al, unpublished data). The prospective serum collection has been analysed to establish the value of early postimplantation hormonal estimations in the prediction of early pregnancy loss (Lower et al, unpublished data).

As expected, in the early weeks of pregnancy the levels of $E_2$ and progesterone are considerably higher in pregnancies conceived after ovarian stimulation with CC/hMG when compared with unstimulated cases. However, somewhat surprisingly, β-hCG levels were found to be significantly higher up to week 7 in unstimulated conceptions than in those following stimulation ($P<0.005$). Frequency distribution curves plotting multiples of the median value (MoM, e.g. Figure 14) enabled comparisons of single and several hormone estimations for ongoing pregnancies and early pregnancy losses to be calculated as an odds ratio (i.e. true positives/false positives) (Wald and Cuckle, 1989). Significantly lower and falling β-hCG

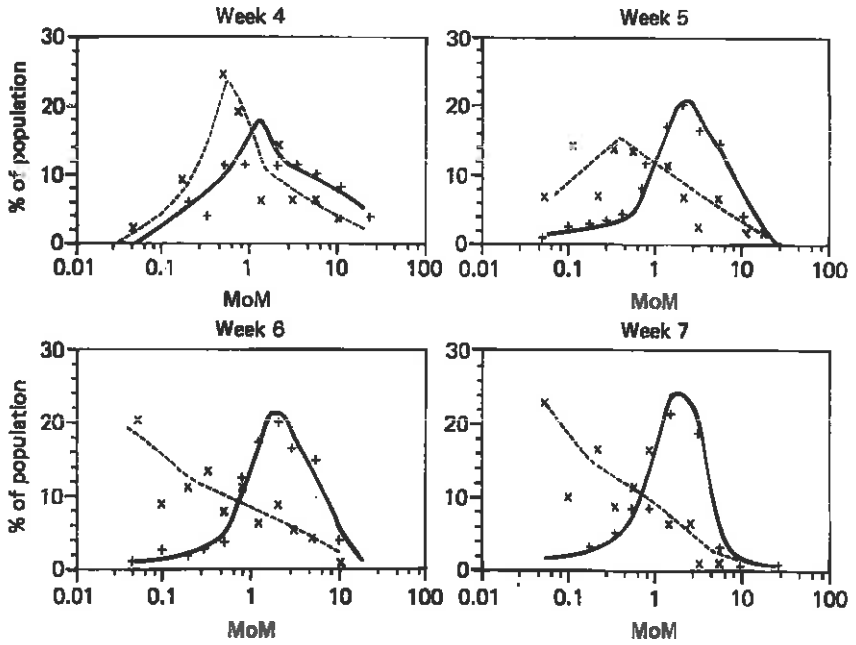


**Figure 14.** Multiples of the median value (MoM) curves for β-hCG in weeks 4–7 of ongoing (+——+) and failing (×---×) singleton pregnancies arising in unstimulated cycles.

levels discriminated the failing pregnancy group from ongoing pregnancies as early as the 5th week but the predictive value of any given result could often pose a clinical dilemma. Figure 15 considers the sensitivity and specificity of β-hCG estimations with receiver operating characteristic (ROC) (Vinatier and Monnier, 1988) curves for each week of gestation. For example, choosing a cut-off level of β-hCG below 0.5 MoM of the ongoing pregnancy range gives a sensitivity of 75% with an odds ratio of 8.61 at 7 weeks in unstimulated cycles. Similarly, a cut-off of 0.5 MoM for progesterone gives a sensitivity of only 41% and an odds ratio of 20. Such frequency

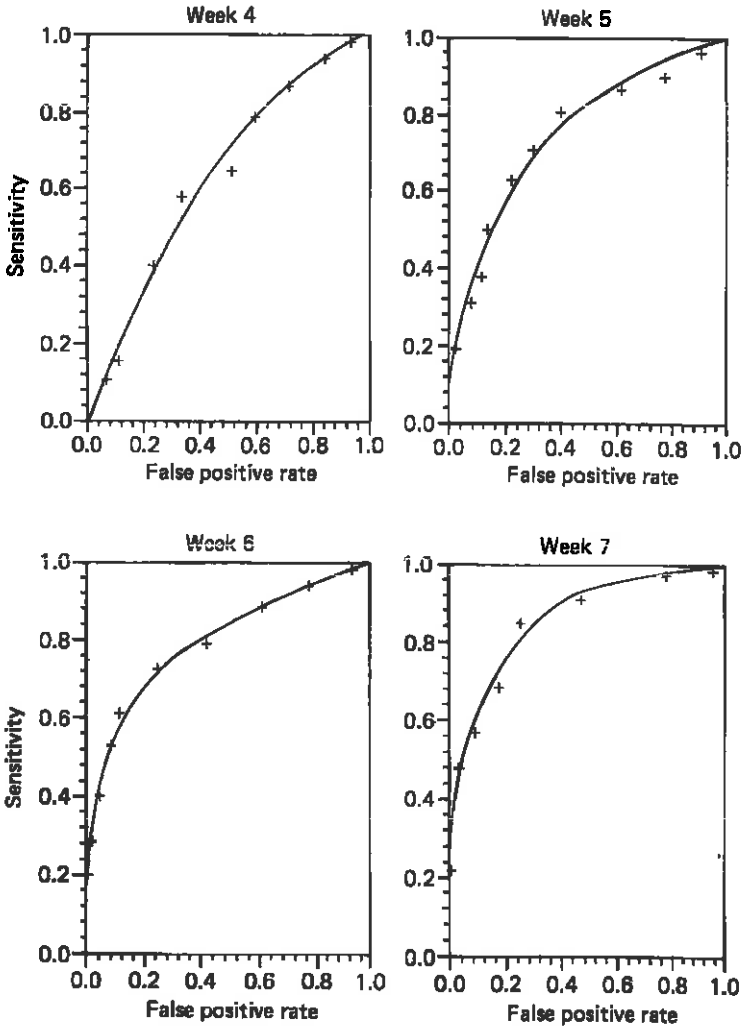


**Figure 15.** Receiver operating characteristic (ROC) curves for β-hCG estimations using a cut-off of 0.5 MoM for weeks 4–7 of singleton pregnancies.

distribution and ROC curves are currently being evaluated in prospective studies to determine their value in clinical practice.

## Therapeutic strategies for early pregnancy wastage

In considering a therapeutic approach for high-risk pregnancies and those threatening to abort, the nature and causes of pregnancy wastage bear consideration. These can be broadly considered in three areas: general maternal conditions, intrinsic embryo abnormalities, and local uterine-endometrial factors.

### *General maternal disorders*

Underlying maternal conditions may cause recurrent pregnancy losses, and comprehensive investigations with a view to specific counselling and treatments where applicable, should screen for the following.

*Genetic causes*. Detailed banded chromosome analysis is checked from both partners.

*Anatomical causes*. Detection of fibroids, cervical incompetence, active pelvic endometriosis, genital tract infections and uterine anomalies, including those from diethylstilboestrol exposure and intrauterine synaechiae, requires careful clinical evaluation, along with hysterography, laparoscopy and hysteroscopy.

*Infective causes*. Serological tests are performed for relevant antibodies in the detection of syphilis, brucellosis, toxoplasmosis and rubella immunological status. Endocervical swabs are cultured for aerobic and anaerobic bacteria and specific investigations are performed to exclude infection with cytomegalovirus, *Chlamydia trachomatis*, herpesvirus, *Listeria monocytogenes* and *Mycoplasma hominis*. *Mycobacterium tuberculosis* also requires consideration, depending upon clinical features and the patient's demographic background.

*Maternal diseases*. Tests are performed for systemic lupus erythematosis (Bresnihan et al, 1977), i.e. lupus anticoagulant and/or cardiolipin antibody (Exner, 1989), antinuclear factor and complement proteins C3 and C4, and fasting blood sugar for diabetes, as well as screening for cardiovascular, renal and thyroid diseases. Other considerations, such as exposure to anaesthetic gases, industrial chemicals and pesticides, the ingestion of antimetabolites and anticoagulants, and smoking/alcohol history, may be relevant in individual cases. The investigation of fertility parameters including ASABs (Junk et al, 1986; Haas, 1987) and luteal phase evaluation should be included.

*Immunological factors*. It has been suggested that the absence of an essential maternal immunoregulatory response to the genetically foreign fetus is the

cause of at least some cases of recurrent miscarriages (Taylor and Faulk, 1981). It was therefore speculated that immunizing such women with their husbands' prepared lymphocytes would stimulate the appropriate maternal response, and one controlled study indicated the approach was valid (Mowbray et al, 1985). Certainly, women who recurrently miscarry often have negative mixed lymphocyte reactivity (MLR), whereas most women who have successfully completed at least one pregnancy have a positive MLR. This test, combined with the aforementioned screening protocol, forms the basis of an international multicentre study of paternal lymphocyte immunotherapy, and in which PIVET is a member (Moloney et al, 1989). So far a clear benefit for immunotherapy has not been demonstrated.

*Embryo factors*

Workers in the field of human reproduction have long been concerned that their efforts should not cause congenital abnormalities. The birth of Louise Brown in July 1978 was the climax of many years of painstaking research. She was one of four pregnancies achieved in that period, two of which resulted in normal children and two which failed to proceed. One of the latter was a first trimester abortion and tests on the fetus demonstrated a karyotype of 69XXX (Steptoe et al, 1980). Interestingly, the other was a midtrimester loss several weeks after amniocentesis had revealed pleiomorphism of 15D and a large Y chromosome, paternally derived anomalies which were not thought to have contributed to the loss.

As previously discussed, studies of preimplantation embryos generated following IVF have demonstrated a high rate of chromosomal abnormalities (23–40%) (Plachot et al, 1987; Papadopoulos et al, 1989). This may be compared with the historic findings of Hertig and Rock (Hertig et al, 1952), who described that only four of the eight preimplantation embryos they identified were morphologically normal. Of those embryos which do implant, by far the most common identifiable defect in early pregnancy losses is chromosomal abnormality in the conceptus. Abnormalities were detected in 60% of spontaneous abortions when the products of conception were analysed after the diagnosis had been made at a relatively late stage on clinical grounds (Boué et al, 1975). One might speculate that a number of other aetiological factors peculiar to pregnancies conceived following IVF could have an adverse effect. However, in a study conducted by postal questionnaire of more than 200 European IVF centres, 21 out of 34 (62%) abortuses karyotyped demonstrated a chromosomal abnormality (Plachot, 1989), suggesting no increase in the rate.

Pregnancies monitored at PIVET, and which have been diagnosed as blighted ovum, fetal deaths or ectopics, have undergone CVS and subsequent culture for chromosome identification using GTG banding (Lower et al, unpublished data). Fifty pregnancies were examined in this fashion over a 2 year period. Four have been excluded because of failed culture. Of the remaining 46 pregnancies, there were 29 blighted ova, nine spontaneous fetal deaths and eight ectopic pregnancies. In ten of 29 blighted ovum pregnancies and one of nine spontaneous fetal death pregnancies, readily identifiable villi

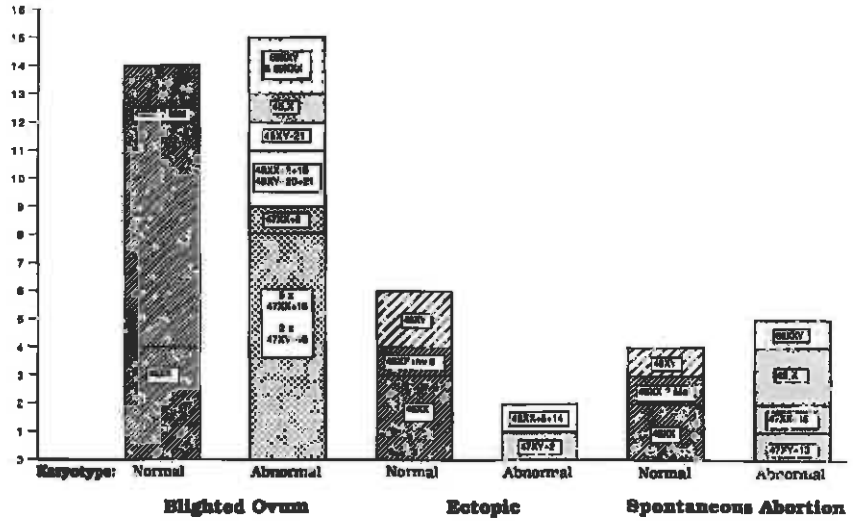


**Figure 16.** Distribution and type of chromosome abnormalities identified after chorionic villus sampling among 46 early failing pregnancies in a subfertile population.

could not be unequivocally separated and the culture specimen, consisting of membranous tissue, was classified as products of conception (POC). These 11 cases, mostly obtained early in the series when the initial assessment was less rigorous, all gave normal 46XX karyotypes. Whilst it is our belief that the majority of these karyotypes are of fetal origin, it was not possible to confirm conclusively that some were not of maternal origin.

Figure 16 summarizes the number and types of chromosomal abnormalities obtained in conceptions, both with and without gamete manipulation. The proportion of abnormalities in early pregnancy losses following gamete manipulation (14/26) was not significantly different from those following spontaneous conception (9/20). Anembryonic pregnancies and spontaneous abortions showed a higher proportion of chromosomal abnormalities than ectopic pregnancies. Maternal ages were not significantly different from those experiencing a loss showing a normal karyotype ($32.2 \pm 4.4$ years) from those with an abnormal karyotype ($32.8 \pm 4.7$ years).

These results from a subfertile population are in agreement with the findings of others in the general population. Data pooled from several series reveals an overall incidence of chromosomal abnormality in 46% of spontaneous abortions recognized in the first trimester (Simpson, 1989). The most common anomaly was autosomal trisomy, accounting for 50% of all abnormalities detected and 22% of all abortions for which karyotypes were available.

The imbalance between sexes in our data is interesting. Only 11 of 46 conceptuses karyotyped bore a Y chromosome, and of the 23 conceptuses with normal karyotypes only three were male. Even if the 11 pregnancies in which definite villi were not identified are excluded, only 25% of the karyotypically normal early pregnancy losses were male. This may represent

a sampling error, although it appears that female conceptuses are more likely to abort in early pregnancy than males, as the finding has been reported in several series of spontaneous abortions (Boué et al, 1975; Creasy et al, 1976; Hassold et al, 1980). Of interest, the overall sex ratio in singleton births following IVF procedures was 1.03 (M:F) in Australia and New Zealand in 1988 (Lancaster, 1990).

Chromosomal abnormalities were seen in only two of our eight cases of ectopic pregnancy. A third case was identified with inversion of chromosome 6 of maternal origin, which was not considered to have contributed to the pregnancy loss. The ratio of male to female karyotypes was 0.6 for ectopic gestation. Clearly, larger numbers are required before any firm conclusions can be drawn from this data, however it is interesting to speculate that chromosomal anomalies do not appear to predispose to ectopic gestation, nor does there appear to be any gross imbalance between the sexes.

There is a clear and well-documented correlation between maternal age and aneuploidy, in particular trisomy 21 (Penrose, 1933). However, in our data there was no significant difference in maternal age between women losing normal and abnormal conceptuses.

Two of the three cases of triploidy are of particular interest. These occurred in consecutive pregnancies in the same patient following spontaneous conception. Whether these lesions were the result of polyspermic fertilization or caused by a defect of cell division is unknown. Controlled fertilization in vitro is proposed as a diagnostic test for this couple. If polyspermic fertilization is demonstrated at the pronuclear stage then the condition may be amenable to specific treatment by microinjection of a single spermatozoon. The importance of careful examination of the fertilized oocytes at the pronuclear stage has been stressed (Yovich and Grudzinskas, 1990). In particular this will enable oocytes with odd numbers of pronuclei to be discarded. There were no cases of polyploidy amongst those pregnancies conceived following fertilization in vitro in the present series.

Early diagnosis of pregnancy failure has therefore enabled accurate karyotyping of the abortus in a high proportion of the early pregnancy losses arising after infertility treatment. It indicates that around half the losses related to chromosomal abnormalities, similar to that observed after spontaneous abortions diagnosed on clinical grounds at a later stage of gestation in the general community. The study is currently being extended to determine the impact, if any, on the pattern of chromosome related wastage to luteal and obstetric support therapy.

### *Endometrial factors*

The concept of potentially treatable hormonal deficiencies causing an inadequate endometrial bed for successful nidation remains an appealing but unproven hypothesis. Studies reported during the 1960s indicated that hormone support therapy was probably not effective (Shearman and Garrett, 1963; Goldzieher, 1964; Klopper and MacNaughton, 1965).

Furthermore, as shown in the preceding section, 50–60% of implantations and miscarriages demonstrate chromosomal abnormalities. However, in subfertile cases, pregnancy wastage appears to be more common than in the fertile population, and the demonstrated benefits of luteal support therapy in assisting implantation, as well as the finding that pregnancies will establish and be maintained in women without ovaries by simply providing exogenous oestradiol valerate and progesterone during the conception cycle and through the first trimester, lend support to the concept that implantation may be supported by hormonal therapy. The following regimens are undergoing trials at PIVET.

*Threatened abortion*. Women who present with vaginal bleeding in the first trimester are offered oral medroxyprogesterone acetate (MPA; Provera, Upjohn Pharmaceuticals). Early data from this study suggests a beneficial effect without causing the retention of abnormal fetuses or creating fetal abnormalities (Yovich, 1988a). The regimen commences with 4-hourly oral tablets to 120 mg/day, reducing to 6-hourly (80 mg/day) when bleeding ceases. Cases are monitored by hormonal and ultrasound investigations each week (see earlier) until the diagnosis is clear. If a viable fetus is demonstrated, hormonal support is continued until the 16th week, when it is ceased following a weaning regimen.

*Recurrent abortion*. In the belief that such cases may have a corpus luteal defect, hCG is used to maintain luteal activity and this is supported by MPA, which is thought to contribute further by maintaining uterine quiescence (i.e. dampening contractility). The regimen commences at the time of pregnancy diagnosis as follows:

hCG: 5000 IU i.m. twice each week to 10 weeks.
MPA: 80 mg/day by four divided doses to 16 weeks, thereafter weaning over the ensuing fortnight.

In selecting MPA as the support progestagen we were influenced by previous observations that the drug did not cause significant congenital abnormalities (Burstein and Wassermann, 1964). MPA is a substituted progesterone and therefore differs from the derivatives of the 19-nortestosterone group, which have been used in the past and have been clearly shown to have appreciable androgenic effects on the developing female fetus (Wilkins, 1960). The oral preparation of MPA appears satisfactory as it is well absorbed and stable plasma concentrations around 27 nmol/litre are established and maintained on the described regimens (Yovich et al, 1985d). That study also examined the profile of steroid metabolites in the maternal urine during the first trimester of pregnancy and showed no abnormal peaks on gas–liquid chromatography and mass spectrometry in a matched series.

## CONCLUSIONS

A large number of factors are known or suspected to influence the chance of

successful embryo implantation and these have been considered with a view to enhancing the process, where possible and appropriate. In clinical practice this falls into three broad areas: assisted reproduction for infertility disorders; enhancing the luteal phase and early weeks of pregnancy to minimize embryo wastage; and exploring management protocols to identify and treat pregnancies which threaten to abort or where women are at high risk for recurrent abortion.

Although the various techniques involved in assisted reproduction have developed rapidly over the past 15 years, the main limitation to their success relates to poor embryo quality. The numerous factors bearing on this have been considered and current research is being directed to improving the quality of embryos developed in vitro, and the identification of those embryos which have the full developmental potential to implant and proceed through to become healthy infants. Currently only around 10% of embryos have this potential. Certainly, a large part of the problem relates to underlying chromosomal abnormalities (up to 40% of preimplantation embryos and around 50% of both implanted gestational sacs and early developing fetuses). The rapidly evolving potential for preimplantation embryo diagnosis makes routine embryo selection seem a highly feasible proposition for the not-too-distant future, and one which can already be considered where there are known chromosome related disorders. However, other assessments of embryo quality are also important in order to improve the efficiency of assisted reproduction procedures for the management of both infertility and genetic disorders.

Treatments to enhance uterine receptivity appear to improve the chance of implantation and do significantly improve the chance of implantations proceeding to successful outcomes. This is most noticeable when embryos are of poorer quality. Luteal phase and early pregnancy treatments are currently given on an empirical basis and require further evaluation in relationship to the specific circumstances where benefits might be obtained. Although the treatments described here do not appear to cause maternal or fetal complications, caution must be expressed, in that numbers treated are still relatively small. Furthermore, the results obtained in studies on subfertile populations may not necessarily be relevant for fertile women.

## Acknowledgements

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