BIOLOGICAL INFLUENCES ON THE CYTOGENETICS OF EARLY HUMAN DEVELOPMENT: AN IVF STUDY

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Thesis submitted for the degree of Doctor of Philosophy
to the University of Glasgow, Faculty of Medicine
September, 1992

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For my parents

I certify that this thesis does not contain material previously published or written by any other person, except where referred to in the text. The results presented in this thesis have not been submitted for any other degree or diploma.

Mary Elizabeth Jamieson.

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ACKNOWLEDGEMENTS

I am indebted to Dr JRT Coutts for his expert supervision and generous commitment of time. His advice kept this project on the straight and narrow when it showed signs of straying and was invaluable during preparation of this manuscript.

I would like to thank Professor JM Connor for his supervision and for critical reading of this manuscript. His assistance in cytogenetic aspects of this work was much appreciated. My thanks are also due to Professor MA Ferguson-Smith who initiated the project.

Dr R Fleming has acted as sounding board for most of my theories. I am very grateful for his help in distinguishing between the good and the fanciful.

This work would not have been possible without the assistance of Miss K Ross. Her expertise in the IVF laboratory allowed me the freedom to complete this study and I am very indebted.

I am grateful to Drs RWS Yates, MJ Haxton, RAL Low, MRP Hamilton, ME Carter, D McQueen and S Kader for their co-operation in these studies and to Dr J Gudmundsson for co-ordinating LORD study 1. The assistance of nursing staff of the ACS units at Glasgow Royal Infirmary and the Glasgow Nuffield Hospital was greatly appreciated.

My thanks are due to all staff of the Department of Obstetrics and Gynaecology at Glasgow Royal Infirmary for hormone assays, computing assistance, information, discussion, encouragement and many cups of tea.

I would like to thank Mr J Colgan for access to the cytogenetic laboratories and dark-room at the Duncan

Guthrie Institute of Medical Genetics and for his advice on staining techniques. Dr DA Aitken and Dr J Crossley provided information on the AFP screening programme.

I am most grateful to Dr RR Angell for invaluable discussion on the origin of meiotic abnormalities.

The assistance of the Department of Medical Illustration at Glasgow Royal Infirmary was much appreciated. I am particularly grateful to Mr T Parker for advice on photo-microscopy and for processing of photographs.

The support and patience of my family through these trying times will always be appreciated.

ABBREVIATIONS

cht	Chromatid
CC	Clomiphene citrate.
E2	Estradiol.
EDR	Embryo development rating.
ER	Endoplasmic reticulum.
ET	Embryo transfer.
FD	Follicular diameter.
FF	Follicle fluid.
FSH	Follicle stimulating hormone.
GIFT	Gamete intrafallopian transfer.
GnRH-a	Gonadotrophin-releasing hormone analogue.
GV	Germinal vesicle.
GVBD	Germinal vesicle breakdown.
hCG	Human chorionic gonadotrophin.
hMG	Human menopausal gonadotrophin.
IVF	In vitro fertilisation.
LORD	Luteinising stimulus to oocyte retrieval delay.
NOR	Nucleolus organiser region.
OR	Oocyte retrieval.
P	Progesterone.
PCC	Premature chromosome condensation.
PB	Polar body.
PB1	First polar body.
PB2	Second polar body.
PM	Post-mature.
PN	Pronucleus (nuclei).
SD	Standard deviation.
u/s	Ultrasound.

SUMMARY

This study investigated the cytogenetics of human oocytes and early embryos and related the findings to maternal factors and aspects of IVF methodology. Chromosome analyses supplemented embryological assessments in evaluation of the effects of luteinising hormone (LH) during follicular growth, the interval from administration of human chorionic gonadotrophin (hCG) to ovulation, the relative effects of *in vivo* and *in vitro* oocyte maturation and correlations between follicular and oocyte maturity.

Embryo quality assessed by morphology (score 1-10) and growth rate (embryo development rating; EDR, 'ideal' = 100) was predictive of implantation potential but not diagnostic. The mean score of embryos which implanted (8.84) was significantly higher ($p<0.001$) than those which failed to implant (8.07) and the minimum score associated with pregnancy was seven. Implanting embryos had a significantly higher ($p<0.002$) mean EDR (101.34) than those which failed to establish pregnancy (96.64). Assessment on day 3 after insemination allowed observation of a larger spectrum of development rate but observations on day 2 were highly predictive ($p<0.001$) of both assessment parameters on day 3 and no advantage of delayed embryo transfer (ET) was seen in pregnancy rates (day 2, 16.9%; day 3, 16.4%).

Suppression of endogenous pituitary function by adjuvant treatment with a gonadotrophin-releasing hormone analog (GnRH-a) during ovarian stimulation with human menopausal gonadotrophin (hMG) eliminated LH fluctuations and surges and all initiated cycles (n=32) proceeded to oocyte retrieval (OR) without complication. The cancellation rate (24/75; 32.0%) was significantly higher ($p<0.001$) in hMG alone cycles. Pregnancy rates after ET in the two groups (GnRH-a/hMG, 19.3%; hMG, 18.1%) confirmed that embryo

quality was similar after both stimulation protocols. Post-mature (PM) oocytes were recovered from 10 hMG alone cycles and were absent ($p<0.01$) in analog treated cycles. Significantly fewer ($p<0.01$) oocytes cleaved in PM associated (37.7%) than unaffected (60.4%) cycles although not all oocytes were affected. Prior to hCG administration, all PM cycles showed an attenuated LH surge which was undetected by monitoring of serum progesterone concentrations.

A preliminary study investigated the interval between administration of hCG and ovulation in 73 women undergoing laparoscopic OR after ovarian stimulation with hMG and adjuvant GnRH-a. The earliest ovulation was observed at 39.5 hours and oocyte quality, assessed by cumulus expansion and fertilisation rates, improved with extended *in vivo* maturation (mature oocytes, <36h, 70.8% ; ≥36h, 85.7%: fertilisation rate, <36h, 61.9%; ≥36h, 75.8%). These data indicated that extension of the luteinisation to oocyte retrieval delay (LORD) could be practiced with benefits at the level of the oocyte.

It is standard IVF practice to preincubate oocytes for 4-6h before insemination to minimise the incidence of polyspermic fertilisation and maximise embryo quality. The above study was extended to compare the relative effects of *in vivo* and *in vitro* maturation in 60 women randomly allocated to 34 or 39h LORDs. Each patient's oocytes were alternated between immediate insemination and 5h preincubation. The incidence of polyspermic fertilisation was highest in oocytes inseminated immediately after a short LORD (17/100) and was significantly reduced ($p<0.05$) by preincubation and/or an extended LORD. Fertilisation rates were significantly higher ($p<0.05$) after a 39h (84.2%) than a 34h LORD (76.8%) and the incidence of delayed fertilisation was reduced by extension of the *in vivo* maturation time (34h, 12.9%; 39h, 3.9%; $p<0.001$).

The relative importance of follicle size and cumulus expansion as indicators of oocyte maturity was investigated by retrospective analysis of IVF data on 6922 oocytes. Cleavage rates and embryo quality were significantly better ($p<0.001$) if the oocyte was retrieved from a large follicle ($\geq$2.5ml of follicle fluid; 63.4% cleaved, mean score 7.69) than a small follicle (<2.5ml; 51.3% cleaved, mean score 7.22). Significantly higher ($p<0.001$) cleavage rates were observed if the associated cumulus was fully expanded (79.2%) than if the oocyte was scored as immature (73.8%); embryo quality was unaffected. The two parameters of assessment were not independent but combined data revealed that follicle size was a better indicator of fertilisation potential and subsequent embryo quality than cumulus expansion.

The nuclear status of 466 (71.4% of 653) oocytes could be determined after cytogenetic preparation; the majority (399, 85.6%) were at meiotic metaphase II and had a haploid chromosome complement. The first polar body had not been extruded in 6% (28) of oocytes which were classified as diploid metaphase II. Prematurely condensed chromatin of sperm chromosomes, indicating cytoplasmic immaturity, were observed in 9.3% (37/399) of metaphase II cells and 32.1% (9/280) of diploid metaphase II oocytes. The same phenomenon was present in 11/17 oocytes remaining uncleaved after a short LORD and immediate insemination. Approximately 5% (22) of uncleaved oocytes had mitotic chromosomes, three had been parthenogenetically activated. Chromosome fragmentation was significantly higher ($p<0.001$) in women over 39 (9/20, 45.0%) than younger groups (<30, 3.7%; 30-34, 6.3%; 35-39, 7.6%).

Karyotypes were prepared from 178 metaphase II and eight diploid metaphase II cells. Abnormalities were detected in 43 (24.2%) haploid oocytes comprising 32 cells with whole chromosome aneuploidy, 11 with individual chromatids of

one homologue, and two structural anomalies. Two cells had more than one abnormality and all diploid cells had a 46,XX karyotype. Aneuploidy of acrocentric chromosomes (D and G group) was most frequent (10/20 hyperploid chromosomes) and 6/10 of cases with apparent pre-division of chromatids involved chromosome 16. The incidence of hyperploidy was significantly ($p<0.05$) higher in women ≥ 35 (7/28; 25.0%) than those <35 (11/113; 9.7%). No association between abnormality and any aspect of IVF methodology was determined. The incidence of abnormality was similar in oocytes inseminated with normal (31/130; 23.8%) and poor (8/24; 25.0%) quality sperm, suggesting that no selection against fertilisation of abnormal oocytes occurred.

Karyotype analysis of 178 diploid embryos revealed abnormalities in 40 (22.5%) cases. Single chromosome aneuploidy was detected in 19.1% of embryos, five cases (2.8%) involved two chromosomes and one embryo (0.6%) had both hyperploidy and hypoploidy involving three chromosomes. E group was most frequently involved in aneuploid karyotypes (10/23 hyperploid embryos) and trisomy 16 was detected in 2.2% of all embryos. Two structural anomalies were recorded and four embryos were mosaic. The incidence of aneuploidy increased with maternal age but did not reach statistical significance (<30, 7/52 [13.5%]; 30-34, 18/91 [19.8%]; 35-39, 6/26 [23.1%]; ≥ 40, 1/2 [50.0%]). Embryo morphology and growth rate, assessed by EDR, did not distinguish between normal (mean score, 7.9; mean EDR, 96.1) and aneuploid (mean score 8.1; mean EDR, 92.1) embryos.

Numbers of hyperploid (n=17) and hypoploid (n=11) embryos (non-mosaic cases involving single chromosomes) were not statistically different. Only one case of sex chromosome monosomy was detected. An excess of female karyotypes was detected in abnormal cases (sex ratio 0.48); the 74:64

XY:XX ratio in normal cases was significantly ($p<0.05$) different. Small numbers of karyotyped triploid embryos revealed equal proportions of XXX, XXY and XYY embryos (3:2:3).

In conclusion, this study detected a high incidence of chromosome abnormalities in human oocytes and preimplantation embryos. *In vitro* fertilisation methodology influenced oocyte maturity and IVF success rates but did not affect the frequency of aneuploidy. Factors influencing maternal meiotic nondisjunction require further investigation but this fundamental aspect of human reproduction is likely to be a limiting factor in the success of assisted conception treatments.

SECTION 1
OPTIMISING BIOLOGICAL CONDITIONS FOR IVF

CHAPTER 1
INTRODUCTION

1.1 Background

Natural fertility in the human female has evolved to produce approximately 10 children during the reproductive years between menarche and menopause (Edwards, 1980). However, the probability of a viable pregnancy occurring in any one month during which frequent intercourse occurred is only 15-28% (Short, 1979). Considerable evidence from cytogenetic study of spontaneously aborted pregnancies (Boue *et al.*, 1985) has shown that approximately 50% of these are chromosomally abnormal. This led to the conclusion that "a considerable part of embryonic death is unavoidable and should be regarded as a normal way of eliminating unfit genotypes in each generation" (Bishop, 1964).

Development of techniques for the recovery of human preovulatory oocytes from the ovary (Steptoe & Edwards, 1970), their fertilisation *in vitro* and subsequent cleavage in culture (Edwards *et al.*, 1970) led to the birth of the first *in vitro* fertilisation (IVF) babies (Edwards *et al.*, 1980; Steptoe *et al.*, 1980). Widespread use of such methods for the treatment of infertility has allowed direct observation of oocyte maturation, fertilisation, early embryonic development and cytogenetic examination of oocytes (Zenses *et al.*, 1985) and embryos (Angell *et al.*, 1983).

Pregnancy rates after IVF and embryo transfer (ET) remain low 14 years after the birth of the first IVF baby. Eleven percent of cycles initiated in the United Kingdom during 1989 resulted in the birth of a live child (ILA, 1991) although individual clinics reported higher 'take home baby' rates of up to 28%. Initial IVF successes were obtained after aspiration of single oocytes from natural cycles (Edwards *et al.*, 1980) but improved pregnancy rates after transfer of multiple embryos (Jones *et al.*, 1982;

Edwards et al., 1984) led to increased use of methods for induction of multiple follicular growth. Ideal ovarian stimulation will achieve optimal follicle growth, provide the correct oocyte microenvironment and allow oocyte retrieval (OR) to be timed when oocyte maturity is reached. Synchrony with cyclical endometrial changes and adequate luteal function must also be attained.

It can be argued that the viability of an embryo is largely determined by the quality of the female gamete. Protein synthesis is dependent on maternal transcripts until the four to eight cell stage when the embryonic genome is activated (Braude et al., 1988) and all ribosomes responsible for protein synthesis during the first three cell divisions are present at ovulation (Tesarik et al., 1986a). An endogenous supply of transcripts for the growth factors platelet-derived growth factor-A and transforming growth factor-alpha has been identified in unfertilised mouse oocytes (Rappolee et al., 1988) and may be essential for early development and implantation.

Preimplantation diagnosis of genetic disease has become a reality (Handyside et al., 1989) but the applicability of the complex and expensive techniques will be minimal while IVF pregnancy rates remain low. Accurate information on the incidence of chromosome anomalies in early embryos, including the extent of mosaicism, is also essential for future development of the methodology.

1.2 Human gametogenesis and embryogenesis

1.2.1 Meiosis

Meiosis is the modified cell division which reduces the diploid chromosome complement to the haploid number present in male and female gametes. The significance lies in genetic variation induced by recombination after crossing-over between paired homologous chromosomes and the subsequent random segregation of each pair.

Female

Meiosis is observed in the human foetal ovary from nine weeks gestation (Hulten et al., 1985) and the last oogonia enter meiosis at seven months (Peters, 1976). A single cycle of DNA synthesis occurs in early or preleptotene after which homologous pairs align and form bivalents by synapsis (zygotene). Chiasma formation occurs in pachytene which is first seen at week 11 and is predominant at 18-22 weeks. This stage lasts for approximately two weeks when meiosis becomes arrested in dictyotene. Oocytes reach this modification of diplotene, in which homologous pairs repel each other until chiasmata form the only attachment, from week 13 and all have done so by birth. This nucleated stage remains until meiosis is reinitiated by the luteinising hormone (LH) surge preceding ovulation (Jagiello et al., 1975).

Approximately 2,000,000 oocytes in dictyotene are present at the time of birth but numbers continually decline by atresia and additionally, after puberty, by growth and ovulation (Edwards, 1980).

At the start of the LH surge the nucleus is visible in the cell periphery as a germinal vesicle (GV) with a large nucleolus (Bomsel-Helmreich et al. 1987). The first sign of resumption of meiosis is germinal vesicle breakdown

(GVBD); the nucleolus pales, condensation of chromosomes continues and invaginations of the nuclear membrane signal its disappearance. Oocytes reach metaphase of the first division when bivalents assemble on the centre of a spindle. The first division is completed by centromeres of each bivalent moving towards opposite poles. Half of the chromosomes are absricted with a small amount of cytoplasm to form the first polar body (PB1) in the perivitelline space. These do not form a nucleus but remain associated with residual microtubules of the first meiotic spindle (Lopata et al., 1980). After normal meiosis, 23 chromosomes, each composed of two chromatids form themselves on the equatorial plate of the second metaphase. The process is shown diagrammatically in Figure 1. The spindle is radially oriented, with one pole close to the vitelline membrane and the other pointing towards the centre of the oocyte (Szollosi et al., 1986). Oocytes remain in this condition during ovulation until induced to resume meiosis by the stimulus of sperm penetration.

The most extensive data on the timing of meiosis in vivo comes from stimulated cycles where OR was performed at intervals after human chorionic gonadotrophin (hCG) administration (Bomsel-Helmreich et al., 1987). In mature follicles ($\geq$16 mm) the first signs of GVBD were seen 14 hours after initiation of luteinisation, metaphase I was observed between 20 and 26 hours and nuclear maturation was completed by extrusion of PB1 at 35 hours. Lopata and Leung (1988) studied oocytes removed from follicles <10mm in diameter in unstimulated cycles and suggested that the process was more rapid. Both estimates for progression to metaphase II are less than early observations by Edwards (1965a).

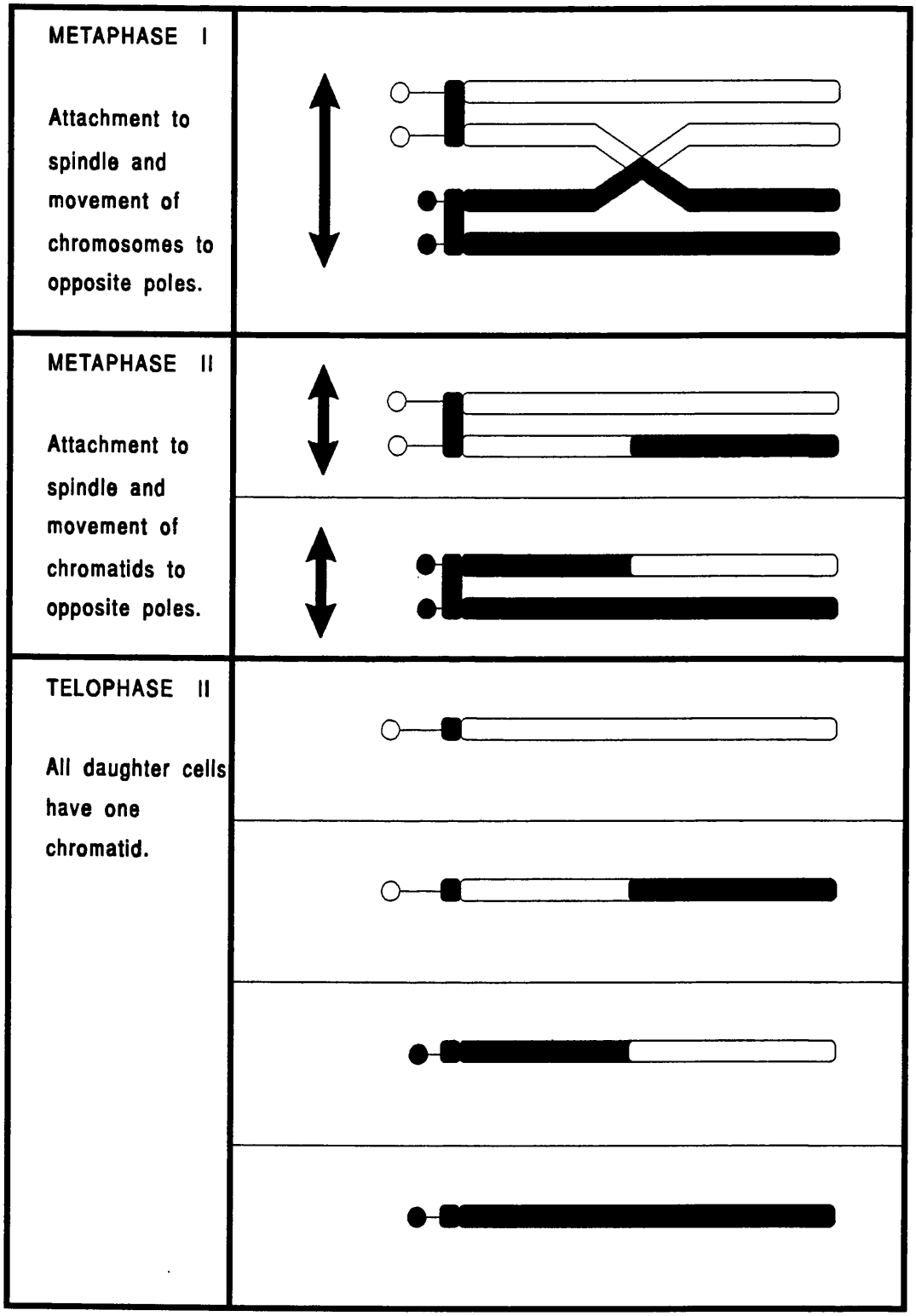


Figure 1

Diagrammatic representation of normal meiotic segregation. A chromosome of the D group with polymorphic satellites is illustrated.

Male

Primordial germ cells in the testis differentiate to form spermatogonia at the time of puberty. These in turn undergo differentiation to primary spermatocytes (Clermont & Leblond, 1959) which undergo meiosis I to form secondary spermatocytes. The second meiotic division follows immediately to produce two spermatids. These cells undergo considerable morphological changes including acrosome formation, nuclear condensation and formation of head, middle piece and tail formation to form spermatozoa (Leblond & Clermont, 1952). Spermatozoa are produced continually after puberty with a total time from spermatogonium to maturity of 61 days (Heller & Clermont, 1963).

1.2.2 Fertilisation

In vivo fertilisation occurs in the ampulla when a capacitated and acrosome reacted spermatozoa penetrates the cumulus oophorus, corona radiata and zona pellucida and fuses with the plasma membrane. In vivo capacitation occurs in the oviduct, appears to involve changes in the spermatozoal membrane (Friend et al., 1977) and is a pre-requisite for the acrosome reaction. The process is not fully understood but can be achieved in vitro by exposure of sperm to defined culture media (Edwards, 1980). The acrosome reaction occurs in the vicinity of the oocyte and involves fusion of the plasma membrane and the outer acrosomal membrane, releasing enzymes necessary for oocyte penetration. Hyaluronidase is implicated in breakdown of the cumulus matrix, which begins after two to three hours in vitro exposure to sperm (Edwards et al., 1970), and is present in the oviduct (Edwards, 1980). Acrosin bound to the inner spermhead and outer acrosomal membrane (Bhattacharyyta & Zaneveld, 1978) is thought to be responsible for zona lysis.

Fusion of gametes induces cortical granule release (Sathananthan & Trounson, 1982a) which forms the basis of the block to polyspermic fertilisation, probably at the level of the zona pellucida (Soupart & Strong, 1975). Meiosis is reinitiated and abstriction of the second polar body (PB2) in late telophase II is observed three hours after insemination *in vitro* (Lopata *et al.*, 1980). The second PB forms a nucleus after extrusion.

After sperm penetration, female pronucleus (PN) formation is similar to reconstruction of an interphase nucleus after mitosis but male PN development from the highly condensed sperm head is more complex (Longo, 1973). The process can be divided into four stages (Tesarik & Kopecny, 1989). One and two involve rapid chromatin decondensation and can occur within an hour while the timing of three and four is variable and dependent on cytoplasmic factors for reformation of the nuclear envelope, reorganisation of chromatin and assembly of nucleolar precursors. Fully formed male PN are observed after 12 hours (Tesarik & Kopecny, 1989) and, unlike some rodents, cannot be distinguished from the female equivalent (Wiker *et al.*, 1990).

1.2.3 Follicular development during the natural ovulatory cycle

Follicles available for gonadotrophin recruitment in the late luteal/early follicular phase are 2-5 mm in size, contain a fluid filled antrum, approximately one million granulosa cells and a single oocyte (McNatty, 1982). This stage develops after approximately ten weeks of growth from the primordial follicle, characterised by a single layer of granulosa cells around the oocyte (Gougeon, 1982).

An intercycle rise in pituitary follicle stimulating hormone (FSH), concurrent with corpus luteum regression (Baird et al., 1975), is implicated in initial recruitment of 'precursor' follicles, acting via receptors in the avascular granulosa layer. Early follicular phase secretion of FSH stimulates proliferation and differentiation of granulosa cells (Hillier, 1991), activation of aromatase enzymes (Dorrington et al., 1975) and development of LH receptors (Zeleznik et al., 1974).

Relatively low concentrations of LH in the initial stages of the cycle maintain cell function (Zeleznik, 1981), including androgen biosynthesis (Tsang et al., 1979), in the vascularised thecal cells. Development of granulosa cell aromatase activity results in utilisation of androgens to synthesise oestrogens (Baird, 1977). It is thought that these oestrogens act directly on granulosa cells to enhance FSH sensitivity, increasing LH receptor and aromatase production (Hillier et al., 1985a), and have an interactive role with FSH to increase granulosa cell proliferation (McNatty & Baird., 1978).

Preovulatory follicles, which acquire functional LH receptors, can respond to both FSH and LH (Zeleznik et al., 1977) and subsequent development can be supported by either, or both, hormones. The follicle which first acquires this reduced requirement for circulating FSH is 'selected' from the precursor cohort in the latter stages of the follicular phase when pituitary secretion of FSH diminishes (Zeleznik & Hillier, 1984) under the influence of increased levels of circulating oestrogen (Marshall et al., 1983). The remaining follicles, devoid of FSH, undergo atresia.

After further growth, over four to five days, the preovulatory follicle is approximately 20 mm in diameter (Hackeloer et al., 1979), contains around 50 million

granulosa cells (McNatty, 1982) and is responsible for >90% of the secreted estradiol (E_2; Baird, 1983). The circulating E_2 is believed to trigger the mid-cycle gonadotrophin surge (Liu & Yen., 1983) but this may not be the sole mechanism since supraphysiological levels produced by ovulation induction therapies do not always induce the response or, when produced, the response may be attenuated (Messinis et al., 1986).

Functions of the mid cycle LH surge include initiation of the processes leading to follicle rupture (Strickland & Beers, 1979), stimulation of luteal phase progesterone (P) secretion (Katz, 1981), reinitiation of meiosis in the oocyte (Jagiello et al., 1975) and induction of cumulus expansion with subsequent detachment from the follicle wall (Dekel et al., 1979).

1.3 Assisted conception methodology

1.3.1 Induction of multiple follicular growth

Multiple follicular growth can be effected by increasing gonadotrophin concentrations to allow development of all follicles acquiring sensitivity and thereby negating the 'selection' process. Endogenous and exogenous gonadotrophins, and combinations of the two, have been utilised. Clomiphene citrate (CC) has anti-oestrogenic effects which stimulate LH and FSH secretion through poorly understood effects on the hypothalamo-pituitary axis (Adashi, 1984). Initial sources of exogenous gonadotrophins were derived from human pituitaries and contained LH and FSH in varying ratios (Bertrand et al., 1972); a 1:1 ratio was considered most effective. Such preparations carry the risk of transmitting Jakob-Creutzfeldt disease (Baird, 1991) and are generally unavailable for clinical use. Commercially available human menopausal gonadotrophin (hMG) is obtained by purification from the urine of postmenopausal women and has bioactive

LH and FSH present in equal amounts (Schwartz & Jewelewicz, 1981). Purified FSH is now available (Shaw et al., 1985) and a recombinant DNA derived product (Germond et al., 1992) has been developed.

Most women undergoing ovarian stimulation in IVF programmes have normal ovulatory cycles and their functional pituitaries may respond to elevated E_2 levels by producing positive feedback LH rises. Where standard criteria for retrieval have been reached and the start of the surge accurately timed, it is possible to time OR in response to such endogenous LH rises with (Dreosch et al., 1988) or without (Eibschitz et al., 1986) hCG augmentation. This approach requires intensive monitoring and places heavy demands on staff and facilities. Spontaneous luteinisation before attainment of mature follicles results in cancellation of 20-30% of initiated cycles with both combined CC/hMG (Lejeune et al., 1986; Harada et al., 1991) and hMG (Vargyas & Marrs, 1987) stimulation regimens.

It is well accepted that cycles affected by an inappropriately timed LH surge, before development of mature follicles, should be cancelled (Lejeune et al., 1986). However, such rises are frequently attenuated (Messinis et al., 1986) and may not be detected. Oocytes lack gonadotrophin receptors (Tsafiri et al., 1982) and reported detrimental effects of abnormal gonadotrophin environments (Moor & Trounson, 1977) may be mediated by follicular fluid steroids.

The paradoxical anti-gonadotrophic properties of agonistic gonadotrophin-releasing hormone analogs (GnRH-a) have been used to develop stimulation methods with improved clinical control for induction of multiple follicular growth (Fleming et al., 1982). Following initiation of the GnRH-a therapy there is an initial rise in LH and FSH; the flare

effect. Thereafter the pituitary becomes desensitised and exogenous gonadotrophins can be administered without risk of complication from endogenous LH rises or surges. The original protocol initiated GnRH-a in the luteal phase of the cycle preceding stimulation and has become known as long course combined therapy. Luteinising hormone concentrations during subsequent hMG induced follicular recruitment and growth are at, or close to, the limits of assay sensitivity (Fleming & Coutts, 1990). The relatively short half life (Diczfalusy & Harlin, 1988) of the hormone suggests that the 50% component of LH in hMG contributes minimally, if at all, to follicular stimulation.

Long course combined therapy is characterised by an initial delay in ovarian response (Coutts et al., 1989) and the cost implication of increased hMG requirements has resulted in development of adapted analog therapies using the flare effect for stimulation by initiation in the follicular phase (Frydman et al., 1988; MacNamee et al., 1989).

1.3.2 Oocyte maturity

Morphological assessment

During IVF treatment, all oocytes are inseminated and selection of embryos for ET, based on morphological criteria, is made from those which fertilise. Oocyte selection for gamete intrafallopian transfer (GIFT) requires identification of those with the highest potential for normal fertilisation and cleavage. The requirement for identification of 'good' oocytes is, as with IVF, to maximise conception rates while minimising high risk multiple pregnancies. Rapid assessment is required since selection is made at OR and immediately prior to replacement of gametes in the fallopian tubes, while the patient remains under anaesthetic. Information

from the oocyte/cumulus complex and the follicle of origin can be utilised.

The most common method of assessment of oocyte maturity is indirect, using cumulus expansion (Marrs et al., 1984). Accurate assessment of nuclear maturity can be achieved by spreading the cumulus mass on a flat surface (Veeck, 1988) but the technique has not been universally adopted because of the perceived dangers of excessive handling of oocytes (Fischer et al., 1988; Pickering et al., 1990). A dissecting microscope does not generally have the necessary resolution and depth of field to visualise the PB and the method has been reported as invasive and impractical, especially when large numbers of eggs are involved (Khan et al., 1989).

Follicle size

The ultrasound (u/s) study of natural cycles which established the correlation between follicular diameter (FD) and circulating concentrations of E_2 also showed that the dominant follicle achieved a mean diameter of 20 mm (range 17-28 mm) on the day of the ovulatory LH peak (Hackeloer et al., 1979). Further measurements during spontaneous conception cycles showed ovulation of follicles reaching a maximum FD of $\geq$20 mm during the periovulatory period (Fleming et al., 1984).

In vitro fertilisation programmes differ in the u/s criteria considered minimal for administration of hCG (day 0) or salvage of cycles following a spontaneous LH surge. Reported day 0 criteria range from two follicles of >15 mm (Edelstein et al., 1990) to one with a FD >19 mm (Lejeune et al., 1986) while other programmes require at least three structures in the 'mature' range (Gudmundsson et al., 1990) or a specific number of additional medium sized follicles (Lopata & Hay, 1989).

Asynchronous development of the cohort due to recruitment as follicles reach gonadotrophin sensitivity throughout the stimulation period (Hillier et al., 1985a) leads to a range of FDs at the time of aspiration. Oocytes can be recovered from all sizes with varying efficiency. A recovery rate of 82% from follicles in the 18-20 mm range has been reported while only 39% of aspirated follicles less than 12 mm in diameter yielded an oocyte (Scott et al., 1989).

1.3.3 Interval from luteinising stimulus to ovulation

The luteinisation stimulus to oocyte retrieval delay (LORD) employed in most IVF programmes has been 32 to 36 hours (Hill et al., 1987; Khan et al., 1989; Simonetti et al., 1985). This represents a compromise between maximum in vivo maturation of oocytes and retrieval before there is significant risk of ovulation and was based on studies in patients in whom follicular growth was induced using CC (Testart & Frydman, 1982) and/or hMG (Edwards & Steptoe, 1975; Trounson et al., 1982a). Ovulation was observed in 50% of patients in the period 37-39 hours after initiation of luteinisation (Edwards & Steptoe, 1975; Testart & Frydman, 1982).

The influence of endogenous LH fluctuations in such regimes may have contributed to the large variability in the reported time of observed ovulation. Precise timing of initiation of luteinisation processes can now be achieved by induction of multiple follicular growth in a low and stable LH environment, using combined GnRH-a/hMG therapy but LORDs have not generally been altered (Chetkowski et al., 1989; Dandekar et al., 1990; Garcia et al., 1990, Tanbo et al., 1990).

1.3.4 Interval from oocyte retrieval to insemination

Adoption of conservative LORDs may have influenced other aspects of IVF methodology. It is a common practice to include, before insemination, an *in vitro* oocyte preincubation (usually four to six hours) to minimise the incidence of polyspermic fertilisation and increase the proportion of oocytes which fertilise and develop into "normal embryos" (Trounson *et al.*, 1982b).

The requirement for *in vitro* maturation before insemination was established by Trounson *et al.*, (1982b). Results of this study are shown in Table 1.3.1. Oocyte retrieval was performed 34-37 hours after hCG administration or 26-29 hours after detection of urinary LH levels >2 standard deviations (SD) above baseline. Patients were either stimulated using CC or were monitored during a natural cycle. No difference was found between the groups and results were combined. Immediate insemination was associated with low fertilisation and cleavage rates of 'activated oocytes' (definition not given) and a high (30%) incidence of polyspermic fertilisation. Preincubation for four to six and a half hours maximised the yield of normal embryos.

1.3.5 Assessment of embryo quality

It is well established that the number of transferred embryos is a major factor affecting IVF pregnancy rates (Jones *et al.*, 1982; Edwards *et al.*, 1984; ILA, 1990). However, a conflict exists between achieving acceptable success and minimising the incidence of multiple pregnancies which has been identified as the main detrimental factor for the health of babies born after assisted conception (MRC, 1990).

Table 1.3.1
Effect of oocyte preincubation on fertilisation and cleavage; from Trounson et al., 1982(b)

Preinc Time (h)	'Activated' Oocytes (n)	Pro-nuclear (n)	Multi Pro-nuclear (n)	Normal Cleavage (n)
0-0.5	42	20	6 (30%)a	11 (26%)b
4-4.5	4	3	0 (0%)	2 (50%)
5-5.5	37	34	0 (0%)	33 (89%)
6-6.5	36	28	2 (7%)	25 (69%)

a Percent is expressed as a proportion of pronucleate zygotes.
b Percent is expressed as a proportion of activated oocytes.

Factors affecting implantation after IVF and successful ET can be attributed to either embryo quality or uterine receptivity. These parameters may be related in some cases but an adequate uterine environment can be assumed if at least one embryo implants. Analysis of these pregnancies confirms that the potential of individual embryos for optimal development is variable. In 3,058 transfers of three embryos in UK clinics in 1988 (ILA, 1990) the overall implantation rate per embryo was 11.3% and in cycles with $\geq$1 gestation sac was 45%. A further study following 81 multiple pregnancies, in which at least one conception reached term, showed that the number of viable foetuses can reduce during gestation (Acosta et al., 1988).

The aim of embryo assessment is to identify those with the highest potential for full development. As well as the selection criteria for ET, the information is a useful adjunct to fertilisation and pregnancy rates for comparison of treatment regimes. Methods should be non-

invasive, objective, able to be performed rapidly and amenable to statistical analysis. The two parameters used on a day to day basis for embryo assessment selection are gross morphology and growth rate (Cummins et al., 1986; Claman et al., 1987, Puissant et al., 1987).

Morphology assessments are usually composites of blastomere regularity, number and size of cytoplasmic fragments and quality of cytoplasm, as assessed by granularity and vacuolisation. Scoring systems frequently use a range of one to four and assume that adverse parameters are correlated (Puissant et al., 1987; Bolton et al., 1989; Cummins et al., 1986).

Estimation of growth rate presents difficulties since cells spend 90% of their cycle in interphase. If observed on a single occasion it is not possible to differentiate between an embryo which has just cleaved and one about to undergo cleavage. Several approaches have been developed. Fishel et al. (1985) observed individual embryos at regular intervals to obtain a calibration curve for cell-doubling time. Growth variables of subsequent embryos were assessed by multiple observations and their growth curve compared with the observed mean rate. Standardisation of assessment has also been attempted by observation at a fixed time after insemination (Claman et al., 1987, O'Neill et al., 1988). Both of these methods present practical problems for routine laboratories.

Cummins et al. (1986) attempted to develop a method which would allow embryos to be assessed, irrespective of the time of observation, and which was also amenable to statistical analysis. Embryo development rating (EDR) was based on the ratio of the time at which the embryo was observed at a particular stage and the time it would be expected to reach that stage. The 'expected' time was calculated from a growth rate graph constructed by linear

regression analysis of a sample of embryos from their IVF programme. This ideal growth rate is a baseline for comparison and is not intended as an absolute measure but should represent an individual laboratory's conditions. Using this method a normally growing embryo would receive an EDR of 100.

Correlation of embryo quality and implantation is complicated by multiple embryo transfer. Precise information can only be obtained when all replaced embryos implant but some parameters can be deduced when similar embryos are transferred. Another approach is to give equal weighting to each embryo in cycles resulting in pregnancy.

1.3.6 Timing of embryo transfer after IVF

Embryo transfer has been performed at all stages of development from pronucleate zygotes (Quinn *et al.*, 1990) to blastocyst (Steptoe & Edwards, 1976). Replacement at two to four cells, two days after OR is most commonly practiced (Lejeune *et al.*, 1990; Lowe *et al.*, 1988; Tam *et al.*, 1990).

The observation that a high proportion of *in vitro* cultured embryos cease development before blastocyst formation (Bolton *et al.*, 1989, Fishel, 1986) prompted clinical trials of delayed embryo transfer to eliminate this population (Dawson *et al.*, 1988; Bolton *et al.*, 1991). Results did not compare well with conventional ET or transfer of donated blastocysts fertilised *in vivo* and recovered by uterine flushing (Buster *et al.*, 1985), suggesting an adverse effect of prolonged culture. This interpretation is supported by encouraging results from transfer of blastocysts co-cultured with monkey kidney cells (Boyer *et al.*, 1990). It has been shown that the main stage of arrest is at four to eight cells (Bolton *et*

al., 1989), possibly related to the switch from maternal to embryonic gene expression (Braude et al., 1988). Replacement on day three after OR/insemination has been shown to be feasible (Belaisch-Allart et al., 1991; Van Os et al., 1989) and may represent a compromise between improved selection and detrimental culture effects.

1.4 Aims

The aims of the work presented in section one of this thesis were:

1. To assess EDR as a means of quantifying embryo growth rate. To investigate the relative merits of embryo morphology and growth rate as predictors of implantation potential and to identify the optimum time for embryo selection for ET.

2. To compare ovarian responses, oocyte/embryo quality and the outcome of treatment in patients in whom multiple follicular growth was induced using either hMG without endogenous gonadotrophin suppression or using combined GnRH-a/hMG where endogenous LH was suppressed.

3. To determine the time of ovulation after hCG administration in patients treated with combined therapy.

4. To investigate whether a prolonged LORD obviates the requirement for preincubation.

5. To compare the effects of *in vivo* and *in vitro* oocyte maturation upon development of the block to polyspermy, the fertilisation rate and development of ensuing embryos.

6. To determine the relationship between cumulus expansion and follicular size, determined by FF volume, and investigate their relative importance as indicators of oocyte maturity.

CHAPTER 2
MATERIALS AND METHODS

2.1 Chemicals

Chemicals used for the preparation of culture media and solutions used in cytogenetic methods and the abbreviations used for them in the text are listed in Table 2.1.1.

Table 2.1.1
Chemical components

Chemical	Abbreviation	Supplier
Barium hydroxide	BaOH	Sigma
Calcium chloride dihydrate	$CaCl.2H_2O$	Sigma
Ethanol		BDH
Glucose		Sigma
Hydrochloric acid	HCl	BDH
Magnesium chloride	$MgCl_2.6H_2O$	Sigma
Methanol		BDH
N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid	HEPES	Sigma
Penicillin G		Sigma
Phenol red		Sigma
Potassium chloride	KCl	Sigma
Sodium bicarbonate	$NaHCO_3$	Sigma
Sodium chloride	NaCl	Sigma
tri-Sodium citrate	Sodium citrate	Sigma
di-Sodium hydrogen orthophosphate dodecahydrate	$Na_2HPO_4.12H_2O$	BDH
Sodium hydroxide	NaOH	BDH
Sodium pyruvate	Na pyruvate	Sigma
Sodium lactate	Na lactate	Sigma
Streptomycin sulphate	Streptomycin SO_4	Sigma

2.2 Induction of multiple follicular growth

2.2.1 Pituitary down regulation

Gonadotrophin-releasing hormone analog (Buserelin, Hoechst UK Ltd) was administered intranasally (5x100 µg daily, during the waking hours) or subcutaneously (12.5 µg every three hours) by pulsatile pump. Treatment was initiated in the mid-luteal phase (day 21) of the previous cycle and continued until administration of the ovulatory dose of hCG. Menstruation occurred 5-10 days after Buserelin initiation and the hypogonadal state was confirmed by the absence of follicular growth as assessed by ovarian u/s and basal plasma estradiol concentrations.

2.2.2 Stimulation of follicular growth

Human menopausal gonadotrophin (Pergonal, Serono UK Ltd), provided in ampoules containing 75 IU LH and 75 IU FSH, was used throughout the study period. Preliminary studies utilised a starting dose of four ampoules/day (im) but a lower dosage regime of three ampoules/day was later introduced. Doses were titrated depending on responses assessed by ovarian u/s and E_2 concentrations. Each cycle was reviewed before further treatment and the initial dose increased or decreased as appropriate.

Exogenous gonadotrophin therapy was usually initiated on day three or four following menstruation but could be delayed to suit programme management in patients down regulated with GnRH-a.

2.2.3 Monitoring

Ovarian responses to gonadotrophin treatment were monitored by rapid plasma E_2 radioimmunoassays (Fleming et al., 1985). Estradiol concentrations were used to indicate

when serial u/s scans were required and the latter were used to time administration of hCG. Progesterone concentrations were estimated daily or twice daily (Fleming & Coutts, 1982a) in patients not down regulated by GnRH-a.

2.2.4 Criteria for hCG administration

The ovulatory injection of hCG (5000 IU; Profasi, Serono UK Ltd) was given when at least three mature follicles with a diameter of $\geq$17 mm were seen on u/s with appropriate concomitant E_2 concentrations (>1.0 ng/ml and <10.0 ng/ml). The definition of follicle maturity derived from the minimum follicular diameter observed on the day of the LH peak in normal (Hackeloer et al., 1979) and conception (Fleming et al., 1984) cycles.

2.2.5 Luteal support

A second injection of hCG (2500 IU) was given on day 4/5 after the ovulatory dose and ovarian u/s on day 6/7 determined the requirement for continued support; a further 2500 IU was given if ovarian diameters were <5.0cm. Supplementary hCG was replaced by progesterone (Cyclogest-200; vaginal pessaries 200 mg bd; Hoechst UK Ltd) in patients considered to be at risk of severe ovarian hyperstimulation syndrome.

2.2.6 Assays

Rapid assays for monitoring were performed on fresh plasma and samples were subsequently stored at -20^{o}C until completion of the cycle. Selected samples were assayed for E_2, P, and LH using conventional radioimmunoassays (Coutts et al., 1981).

2.2.7 Luteinisation

Premature (pre-hCG) luteinisation was diagnosed when plasma P concentrations of >1.5 ng/ml were assayed in two consecutive samples before attainment of criteria for hCG administration. This derived from normal cycle data and represented the mean value for day +1 after the LH surge (Fleming & Coutts, 1986).

2.3 Oocyte retrieval

The majority of retrievals during the study period were performed by laparoscopy using a carbon dioxide (CO_2) pneumoperitoneum. Follicle fluid aspiration was effected by direct attachment of non-lubricated 10 ml syringes (Injekt; B. Braun Melsungen AG) to the aspiration needle. Follicles were routinely washed with an appropriate volume of flushing medium (2.4.3). Ultrasound guided OR by the vaginal route, introduced in the later stages of the study, utilised a controlled suction pump (100 mm Hg; Rocket of London Ltd) and double lumen needle (Casmed Ltd) for FF aspiration.

2.4 Culture methods

2.4.1 Disposable plastics

All vessels for media preparation and storage, sperm preparation, oocyte and embryo handling and culture were Falcon sterile disposable labware supplied by Becton Dickinson UK Ltd. Non-lubricated syringes (Injekt; B Braun Melsungen AG) of appropriate volume (2,5,10 or 20 ml) were used for media filtration and dispensing.

2.4.2 Glassware

Pasteur pipettes (230 mm; Volac) were soaked in detergent overnight (2% Decon 90, Decon Laboratories Ltd) then

rinsed with tap water in an automatic pipette washer for a minimum of five hours. Pipettes were transferred through two washes (minimum 15 hours) of distilled/deionised water, dried in a hot air oven (45°C) and plugged with non-absorbent cotton wool (BDH). Sterilised pipettes (dry oven; 160°C for two hours) were used for all manipulations of oocytes, sperm and embryos.

2.4.3 Medium

Whittingham's T6 modification of Tyrode's solution (Whittingham, 1971), prepared from basic ingredients as described by Hillier *et al.* (1985b), was used for embryo culture throughout the study period. Three variants were used; culture medium was bicarbonate buffered for use in a 5% CO_2 environment and HEPES buffer was used in flushing and handling media (Table 2.4.1).

Table 2.4.1
Composition of Whittingham's T6 modification of Tyrode's solution: culture, flushing and handling media

Component	Culture medium g/L	(mM)	Flushing medium g/L	(mM)
NaCl	5.719	(98.02)	6.084	(104.10)
KCl	0.106	(1.42)	0.106	(1.42)
$MgCl_2.6H_2O$	0.096	(0.47)	0.096	(0.47)
$Na_2HPO_4.12H_2O$	0.129	(0.36)	0.129	(0.36)
$CaCl.2H_2O$	0.262	(1.78)	0.262	(1.78)
$NaHCO_3$	1.681	(21.25)	0.420	(5.00)
Na pyruvate	0.052	(0.47)	0.052	(0.47)
Na lactate	2.791	(24.90)	2.791	(24.90)
Glucose	1.000	(5.56)	1.000	(5.56)
Penicillin G	0.060		0.060	
Streptomycin SO_4	0.050		0.050	
Phenol red	0.010		0.010	
HEPES	-		2.383	(10.00)

Handling medium: flushing medium + 4.0 g/L HSA.

Chemical components were tissue culture grade supplied by Sigma Chemical Company with the exception of NaOH and $Na_2HPO_4.12H_2O$ which were BDH 'Analar' grade (BDH Chemicals Ltd). Crystallised and lyophilized human serum albumin (HSA) prepared from Cohn fraction V was supplied by Sigma. Water for injections BP (Boots Company plc) was used for preparation of all stock solutions and culture media.

Stock solutions were freshly prepared for each batch of medium. Stock B3 was adjusted to pH 7.3 with 0.2N NaOH (Table 2.4.2).

Table 2.4.2
Stock solutions used for preparation of culture medium and flushing medium

Stock Solution	Component	g/100 ml	g/10 ml
A (x10)	NaCl	5.719	-
	KCl	0.106	-
	MgCl2.6H2O	0.096	-
	Na2HPO4.12H2O	0.126	-
	Na lactate	4.651a	-
	Glucose	1.000	-
	Penicillin G	0.060	-
	Streptomycin SO4	0.050	-
B1 (x10)	NaHCO3	2.101	-
	Phenol red	0.010	-
B2 (x10)	NaCl	1.461	-
	Phenol red	0.010	-
B3 (x10)	HEPES	5.958	-
	Phenol red	0.010	-
C (x100)	Na pyruvate	-	0.052
D (x100)	CaCl2.2H2O	-	0.262

a 60% syrup

Medium was prepared as required but at least once every two weeks. Volumes of stock solutions required per 100 ml are shown in Table 2.4.3. After assembly of stock

solutions and water the osmolarity of both media was adjusted to 285±2 mOsmol/kg using stock B2 or water. Culture medium was sterilised by Millex-GS filtration (Millipore SA) and aliquoted (18 ml) directly into 50 ml flasks. Flushing and handling media were passed through Sterivex-GS (Millipore SA) filters into 50 ml flasks and 16 ml tubes respectively.

Table 2.4.3
Preparation of culture and handling medium from stock solutions. Volume of stock solutions required for 100 ml of complete medium

Stock solution	Culture medium	Flushing medium
A	10.0 (ml)	10.0 (ml)
B1	8.5	2.0
B2	0-1.5[a]	2.5-4.0a
B3	-	4.0
C	1.0	1.0
D	1.0	1.0
H_2O	to 100.0	to 100.0

a variable volume to permit fine adjustment of osmolarity.

Each patient's own serum was used for culture medium supplementation. Venous blood (20 ml) was obtained onr to five days prior to OR (before or after hCG administration) and allowed to clot in a plain glass tube (Z10; LIP [Equipment and Services] Ltd.). Serum was removed after centrifugation at 1000 g for 20 minutes and spun for a further 10 minutes. Supernatant was transferred to a fresh tube and heat inactivated in a waterbath at 56°C for 30 minutes. After cooling to room temperature, serum was filtered through a 0.22 um membrane (Millex-GS; Millipore SA) into a Falcon storage tube. Samples were stored at 4°C until required. Cloudy or lysed sera were discarded.

Medium for each patient was prepared by adding 2 ml of prepared serum to a flask containing 18 ml of culture medium. This was used for sperm preparation, insemination tubes and oocyte and embryo culture. Culture vessels were individually gassed with humidified O_2:CO_2:N_2 (5:5:90; v:v:v; BOC Ltd) after each manipulation. Incubation at 37.0^{o}C was in a non-gassed, non-humidified chamber (Heraeus GmbH; model B5060).

2.4.4 Sperm preparation

A preliminary sample from the male partner was analysed within the laboratory to determine suitability for treatment. After measurement of volume, a wet film was prepared for assessment of motility and progressive motility (non, poor, normal, and good progression). Sperm density was determined by dilution of whole semen (1:9 in buffered formalin) and counting in an Improved Neubauer chamber. Poor and borderline samples were prepared using the standard IVF method (see below) to determine if sufficient motile sperm for assisted conception could be obtained.

Semen samples were produced before OR and processed in the laboratory within three hours. A standard analysis was performed to determine the preparation method or the requirement for a further sample.

Standard preparation: a 1 ml aliquot was centrifuged at 200g for five minutes, the pellet resuspended in 1 ml of culture medium and recentrifuged. After repeating the washing process, tubes were left at room temperature for up to one hour to allow motile sperm to 'swim-up'. The supernatant was removed without disturbing the pellet containing cells, debris and non-motile sperm and the concentration of motile sperm was estimated (Improved Nauebauer counting chamber). Samples were immediately

diluted to two million motile/ml for GIFT and 100,000 motile/ml for IVF after the number of oocytes obtained was known. Viscous samples were diluted (1:3) with culture medium before initial centrifugation and the pellet removed from under the supernatant and transferred to a fresh tube. Cryopreserved samples were thawed quickly and reconstituted by dropwise addition of medium to avoid osmotic shock.

Duplicate swim-ups were used in samples with low counts and/or poor motility. In extreme cases where the swim-up fraction did not contain sufficient motile sperm for the number of oocytes obtained, the pellet was resuspended and diluted. This method was not used for GIFT. In cases with severe agglutination, culture medium was layered over whole semen and left at room temperature for one hour. The supernatant was centrifuged once and vigorously resuspended in 1 ml of medium. Overall semen quality was recorded as poor if insufficient motile sperm were recovered from a single swim-up.

2.4.5 Oocyte recovery

An embryology work station was positioned in theatre or an adjacent room. Syringes, flushing medium and culture tubes were stored in an incubator (37^{o}C) until required. Handling medium was maintained at 37^{o}C on a heating block during the procedure. The volume of single FFs was recorded and the aspirate immediately transferred to a 60mm petri dish for microscopic examination (VMZ stereo microscope with transmitted light source; 10-40 magnification; Olympus Optical Co Ltd). Each identified oocyte/cumulus mass was assessed for maturity, washed in handling medium and transferred to a pre-equilibrated culture tube containing 1 ml of culture medium.

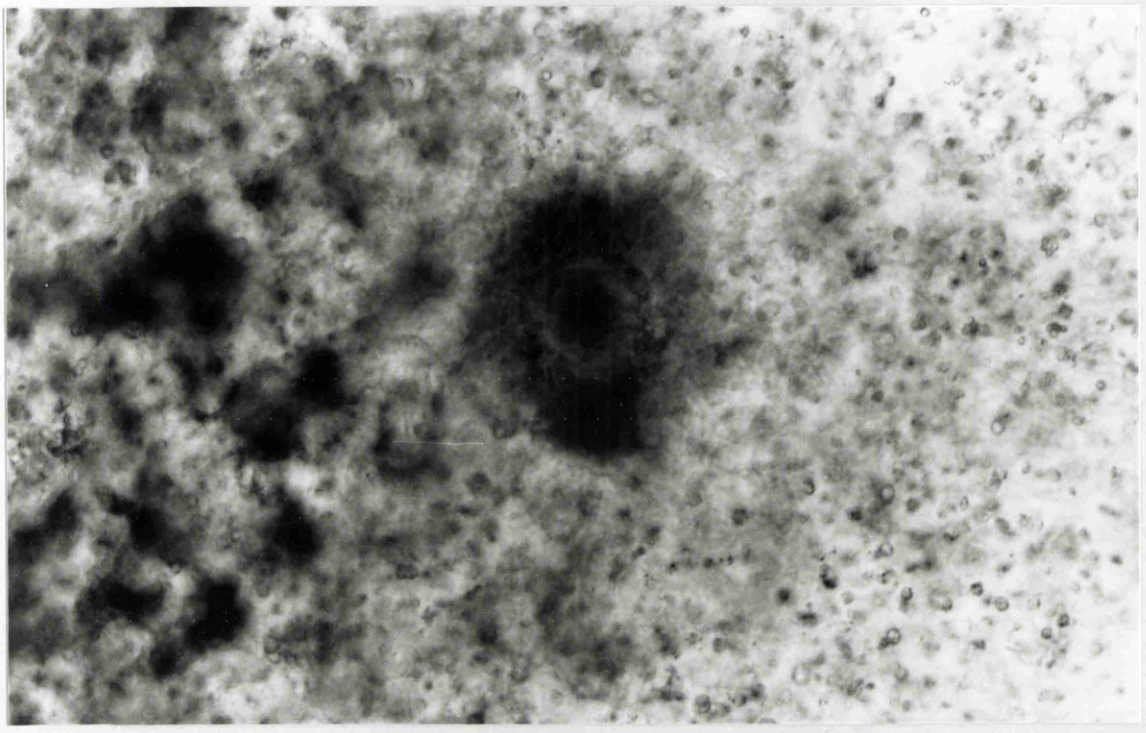
An initial assessment of oocyte maturity was made on the basis of cumulus expansion (Figure 2). Compact and partly expanded cell masses were scored as one and two respectively; these were considered immature. A score of three was given to fully expanded, mature cumuli and post-mature (PM) oocytes with very dispersed, friable cumuli containing aggregations of dark cells were recorded as four. All assessments during the study period were made by the same embryologist (MEJ).

2.4.6 Insemination

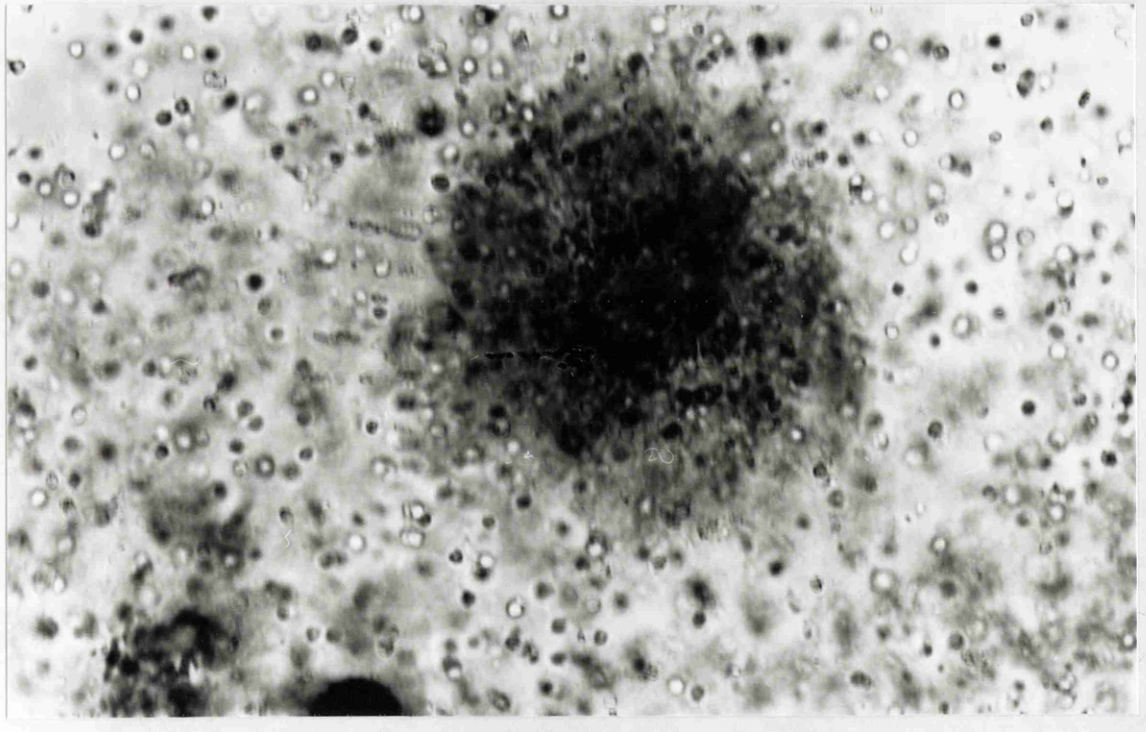
A sperm tube containing 100,000 motile sperm was prepared for each oocyte and heated to 37^{o}C before insemination; generally four to six hours after OR. Exceptions to this procedure are described in the text. Oocytes were transferred to the insemination tube to further reduce any FF and/or blood contamination.

2.4.7 Assessment of fertilisation

Examination for PN was performed 15-21 hours after insemination. The fragment of the cumulus mass containing the oocyte was identified in the culture tube using a stereo microscope (SV8 stereo microscope with transmitted light source; 10-80 magnification; Carl Zeiss (Oberkochen) Ltd) and transferred to a culture dish containing 4 ml of handling medium at 37^{o}C. Remaining cumulus and corona cells were manually removed by repeated aspirations into a finely drawn Pasteur pipette controlled by mouth suction. The ooplasm was examined for PN and the PB status recorded before the oocyte/zygote was transferred to a fresh culture tube. This process was performed prior to ET in cases not checked for the presence on PN. Figure 3 illustrates an unfertilised oocyte with 1PB, a normally fertilised zygote with 2PN and an apparently polyspermic zygote with 3PN.



a



b

Figure 2

Assessment of oocyte maturity based on cumulus expansion: a) immature, b) mature and c) post-mature (overleaf).

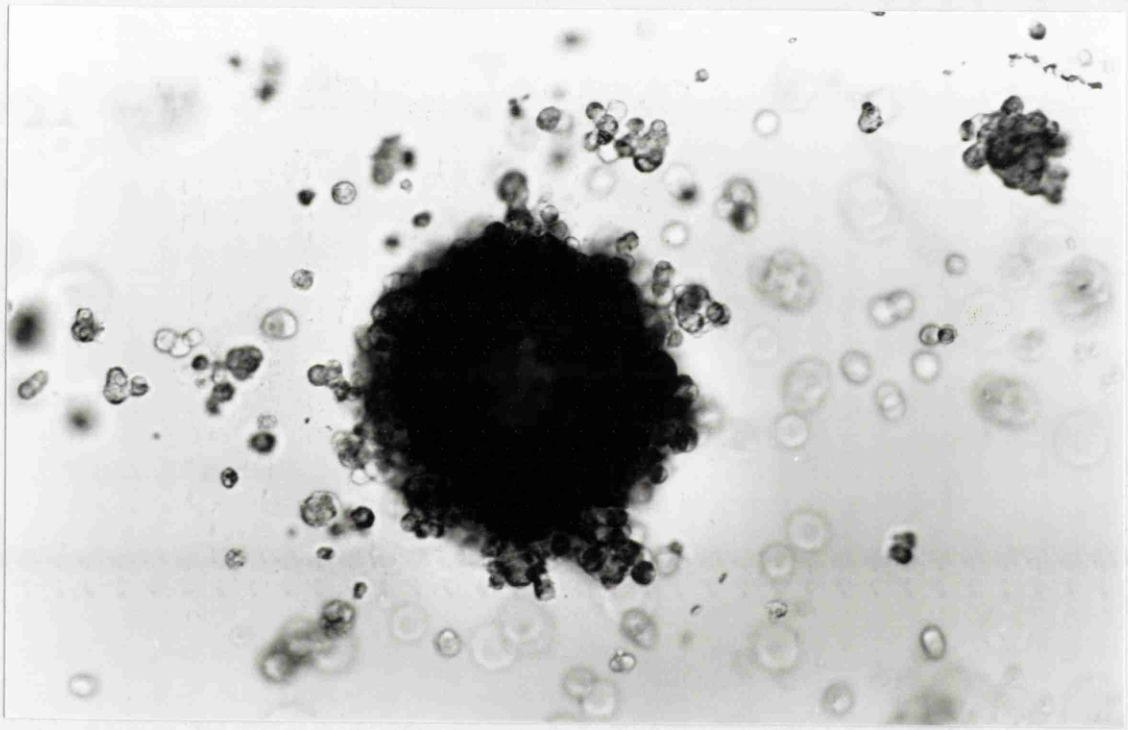
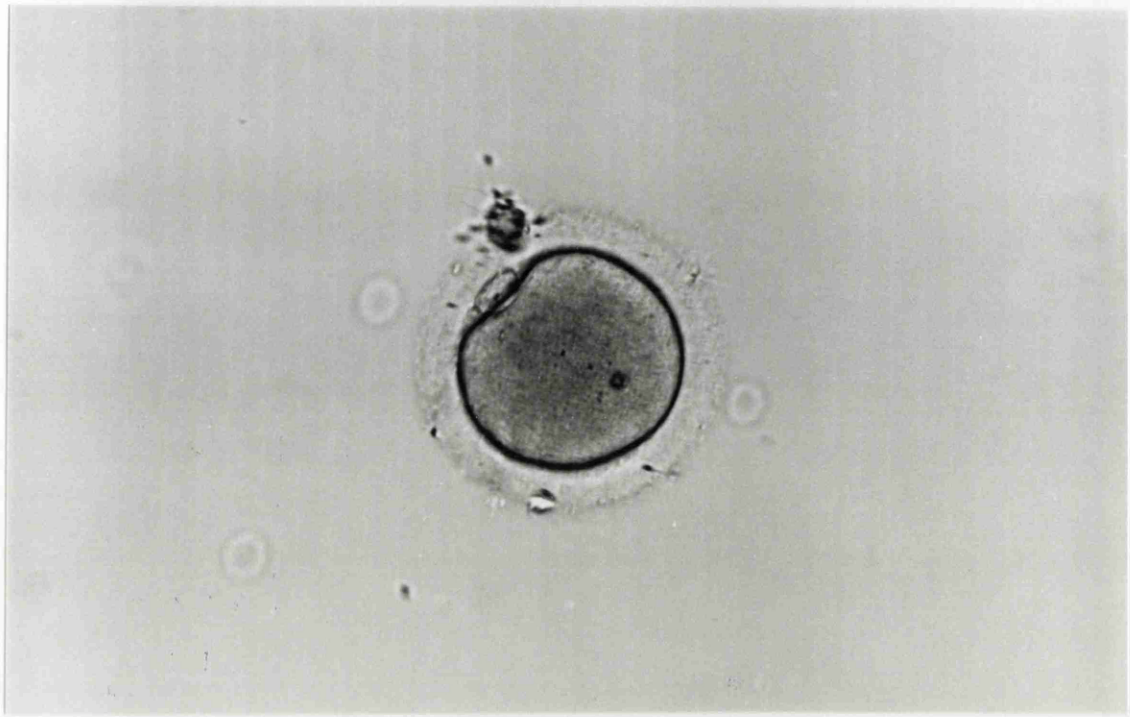
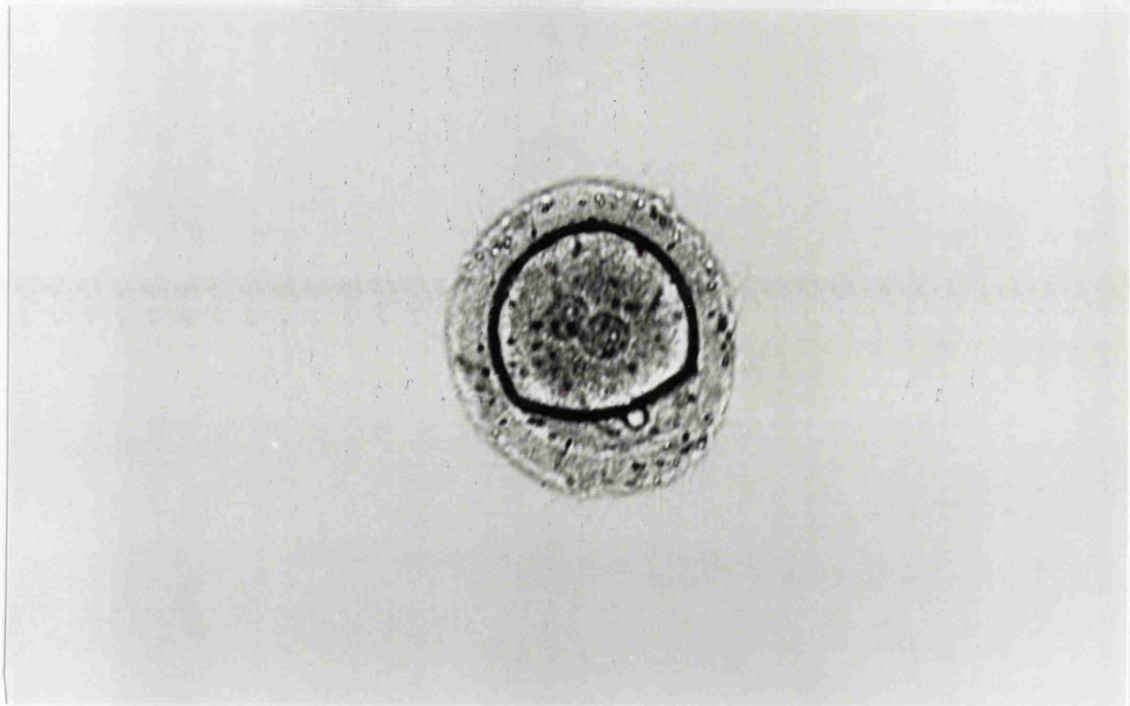


Figure 2c



a



b

Figure 3

Appearance of oocytes/zygotes 15-21 hours after insemination: a) an unfertilised oocyte, b) normal fertilisation and c) three pronuclei (overleaf).

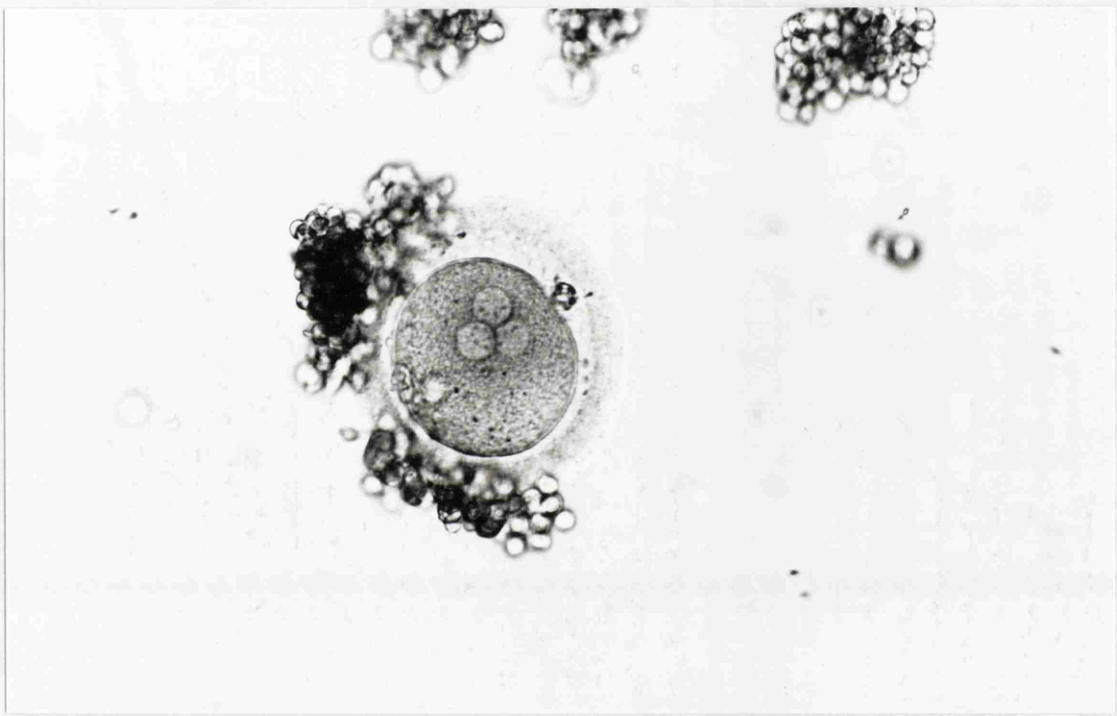


Figure 3c

2.4.8 Assessment of embryo morphology

Embryos were assessed prior to ET and given a score (range 1-10; Figure 4) based on a composite of two parameters; the regularity of cell size (1-5) and degree of fragmentation (1-5). Totally fragmented embryos were given a nominal score of one and cases with a single blastomere+fragments were recorded as two. All assessments during the study period were made by the same embryologist (MEJ).

2.4.9 Assessment of embryo growth rate

The number of blastomeres and the examination time (hours from insemination) were used to calculate an EDR based on the method described by Cummins et al (1986; 1.3.5).

Calculation of a growth rate graph to reflect laboratory standards was based on single observations made between 36h and 68h on 250 consecutive embryos. Two cell embryos were excluded from analysis since the earliest observations were made after the expected time of the first cell division (Cummins et al., 1986). Embryos were given a logarithmic score (S) of 2 $\log_2$ of the number of cells (Table 2.4.4) and a regression analysis of S in relation to hours from insemination was performed. Results of this analysis are shown in Table 2.4.5 and in Figure 5. In Table 2.4.5 the results are compared with those of the original authors. The estimated cell cycle length of 11.6 hours was comparable with that of other programmes (Cummins et al., 1986; Trounson et al., 1982b) and was adopted as the 'ideal' for subsequent analyses.

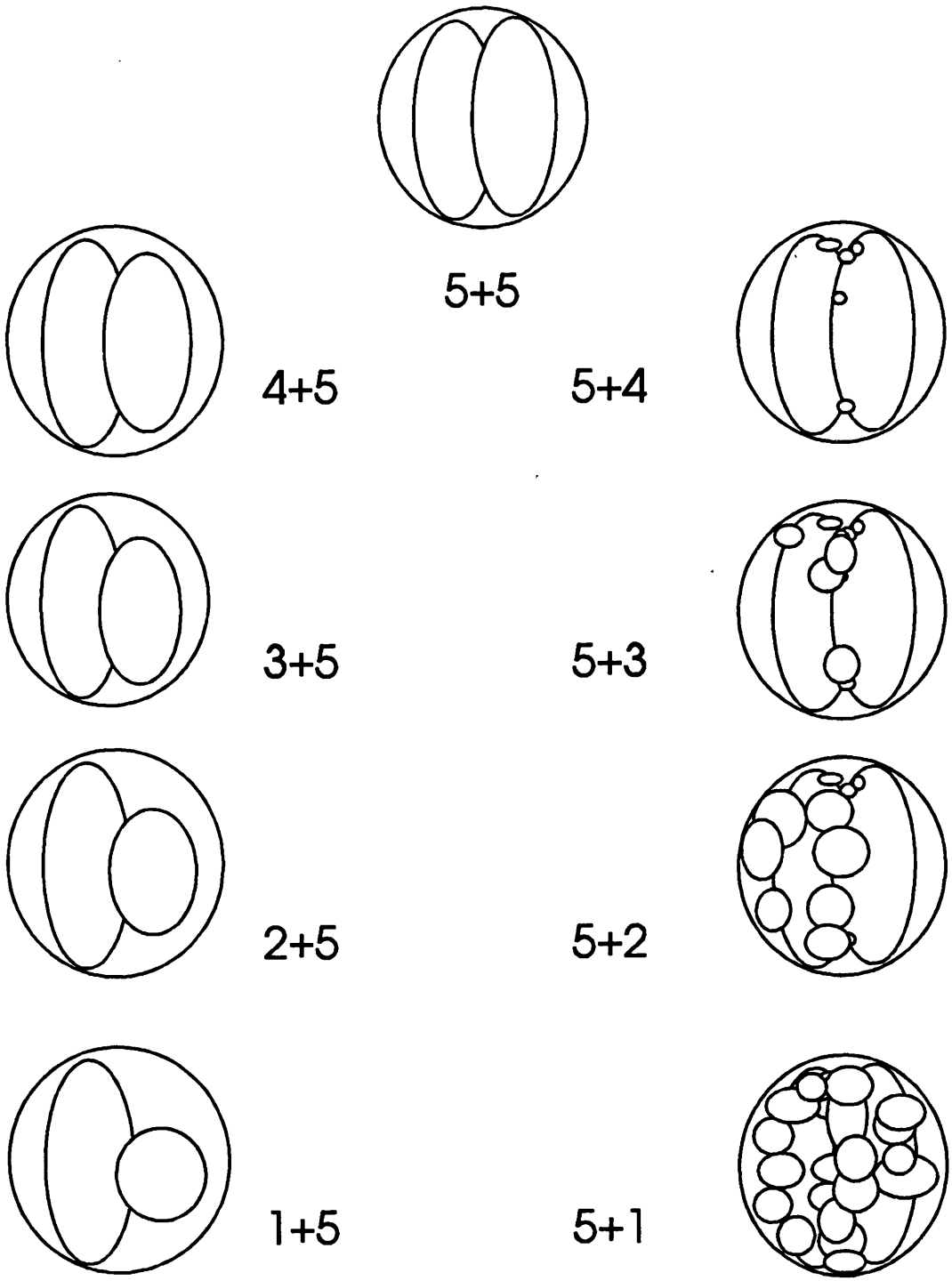


Figure 4

Examples of embryo morphology scores.

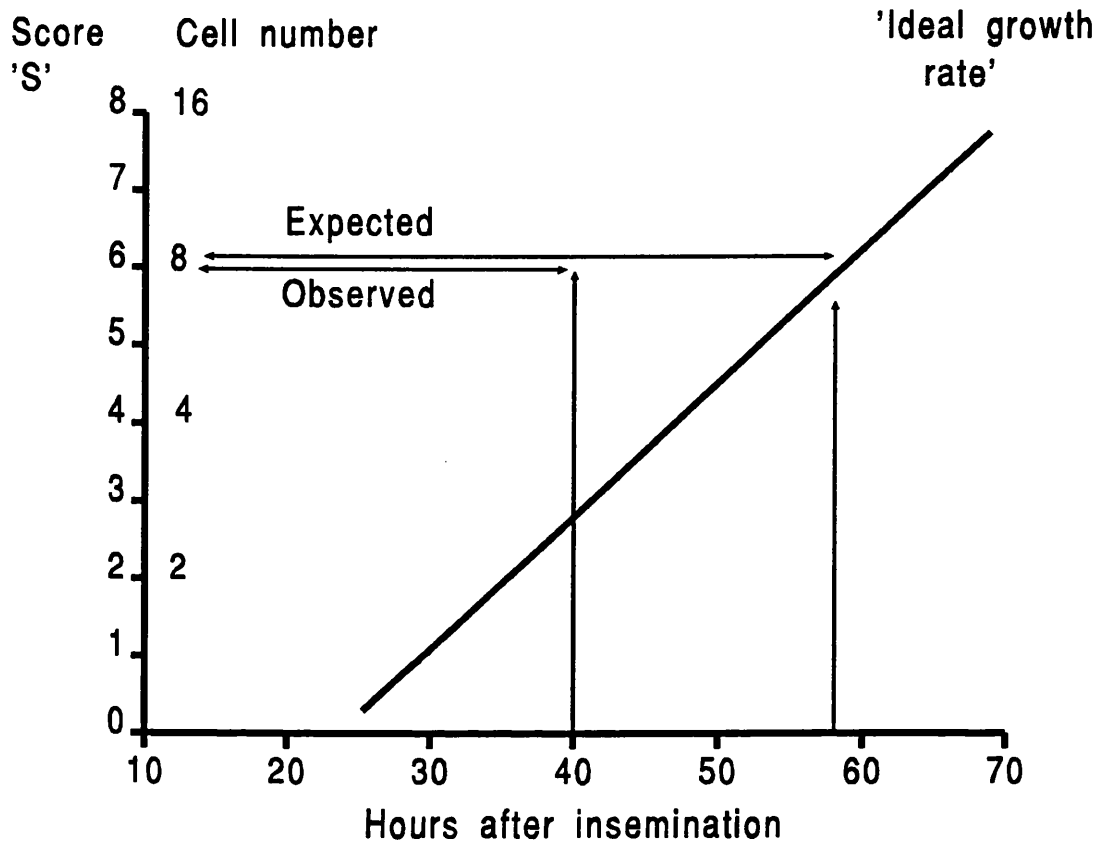


Figure 5

Ideal growth rate and calculation method for EDR.

The 'ideal growth rate' represents the mean growth rate of embryos in the programme. The cell cycle length was estimated at 11.6 hours.

Subsequent EDR calculations compared the observed time with the 'expected' time for that cell number.
The EDR for the example shown was calculated using the formula:

$$\frac{\text{Time expected (hours)}}{\text{Time observed (hours)}} \times 100$$

$$\frac{58.5}{40.0} \times 100 = 146.25$$

Table 2.4.4
Logarithmic conversion of number of blastomeres

Cell Number	S	Cell Number	S
2	2	7	5.5
3	3	8	6
4	4	8+	8
5	4.5	Morula	10
6	5		

Table 2.4.5
Expected time for cell stages (midpoint) calculated by regression analysis and comparison with Cummins *et al*. (1986)

Cell Number	Time Expected	Cummins *et al*.
2	35.4	33.6
4	46.9	45.5
8	58.5	56.4
16	70.0	68.3

The EDR for each embryo was calculated by comparing the 'observed' time with the 'expected' for that number of cells.

$$\text{ie} \quad \frac{\text{Time expected (h)}}{\text{Time observed (h)}} \times 100$$

In the example shown in Figure 5, an embryo observed at 40 hours had eight cells. The 'expected' time for this stage was 58.5 hours; EDR was calculated as 58.5/40 x 100 = 146.25 (rounded to 146). Appendix 1 lists EDRs subsequently assigned to all embryos.

2.4.10 Embryo transfer

Embryos for transfer were selected on the basis of morphology and growth rate immediately prior to transfer (day 2 or day 3 after OR). Transfer medium (25% culture medium: 75% prepared patient's serum) was prepared at least two hours before ET and incubated at 37°C. When the patient was prepared, embryos were transferred into the medium and drawn in a volume of 10-20 µl into a heated catheter (Edwards-Wallace replacement catheter; HG Wallace Ltd) using a 1 ml syringe (Plastipak; Beckton Dickinson). After ET the catheter was checked for the presence of blood and mucus and flushed through with medium to confirm that all embryos had been ejected. A maximum of four embryos was replaced until 1/4/88 after which the number was reduced to three.

2.4.11 GIFT

Oocytes selected for GIFT during or immediately after OR were transferred to an organ culture dish containing 2 ml of prepared sperm (2.2.4). Oocytes (1-4) were drawn with 50 ul of sperm into a catheter (GIFT catheter; Rocket of London Ltd) primed with culture medium. After the total contents of the catheter were ejected into the fallopian tube, the catheter was flushed through with medium and examined microscopically to check that all oocytes were transferred.

Supernumerary oocytes were inseminated to assess oocyte quality, the fertilising ability of sperm and compatibility of gametes.

2.5 Study design

2.5.1 Assessment of embryo quality

All embryos generated by IVF during the study period were included. Embryos from 'spare' GIFT oocytes were excluded.

Comparison of successful and non-implanted embryos was made using two methods.

1. Identification of individual embryos which implanted

Both morphology score and EDR of implanted embryos could be identified with certainty when all transferred embryos gave rise to a gestation sac. Deduction of one or both parameters could be made in cycles where similar embryos were replaced. In the example shown, both successful embryos had a score of 10 and one implantation from an embryo with an EDR of 109 could be identified.

Example: Twin pregnancy

	Score	EDR
Embryo 1	10	124
Embryo 2	10	109
Embryo 3	10	109

2. Identification of pregnancy associated embryos

All embryos transferred in pregnancy cycles were recorded as successful and comparison was made with those in non-pregnant cycles.

Comparison of embryo morphology and growth rate on day 2 and 3

Gross morphology and EDR were assessed on both day 2 and day 3 in 37 IVF cases designated for day 3 replacement. All embryos (234; range 1-10) were included in the prospective study. Comparison was made using the paired t-test.

2.5.2 Luteinising hormone: effects upon oocytes during the periovulatory period

One hundred and seven women undergoing induction of multiple follicular growth for IVF were included in the study. Patients were allocated to hMG alone (n=75) or long course combined GnRH-a/hMG therapy (n=32) with the analog administered by pulsatile pump. The initial dose of hMG in both groups was 4 amps/day and all ORs were performed by laparoscopy.

Oocytes were not checked for fertilisation at the PN stage during this study and results are expressed as cleavage rate (cleaved embryos as a percentage of viable oocytes).

Blood samples taken twice daily were assayed for P and E_2; treatment was discontinued if pre-hCG luteinisation (2.2.7) was diagnosed.

Endocrinology data on all combined therapy patients and the first 40/51 of hMG cycles reaching OR were determined by conventional radioimmunoassay.

2.5.3 Interval from luteinisation stimulus to ovulation in women treated with combined therapy: implications for assisted conception methodology

Study 1: Timing of the observation of ovulation and the effects of extended LORD on oocyte quality and fertilisation rates in patients treated with combined therapy

Seventy-three patients undergoing laparoscopic OR for IVF (n=50) or GIFT (n=23) were treated with long course combined GnRH-a/hMG therapy. All had normal menstrual rhythm (39 tubal and 34 unexplained infertility). Human menopausal gonadotrophin was initiated at a dose of three amps/day; monitoring and criteria for hCG administration were those used throughout these studies.

Luteinisation stimulus to oocyte retrieval delay was defined as the time from administration of hCG until aspiration of the first follicle at OR. Patients were randomly allocated to one of five groups, each with a two hour time span (Table 2.5.1). The shortest LORD was 33 hours and the longest 41 hours 25 minutes.

Table 2.5.1
Study 1: Distribution and range of LORD groups

Group Range (h:min)	Actual LORDs (h:min)	IVF (n)	GIFT (n)	TOTAL (n)
32:00-33:59	33:00-33:55	8	1	9
34:00-35:59	34:10-35:55	10	4	14
36:00-37:59	36:00-37:55	13	6	19
38:00-39:59	38:10-39:50	13	9	22
40:00-41:59	40:00-41:25	6	3	9

Ovulation

Ovaries were examined for stigmata of ovulation and fluid was aspirated from the Pouch of Douglas (POD) before the initial ovarian puncture. Ovulation was judged to have taken place if follicle collapse was observed or an oocyte was identified in the sample of POD fluid.

All inseminations were performed at the same time relative to OR (6+/-1 h). Oocytes were not checked for fertilisation at the pronuclear stage during this study and results are expressed as cleavage rates (cleaved embryos as a percentage of viable oocytes).

Statistical comparison was made between individual groups and between conventional (<36 h) and extended (≥36 h) LORDs.

Study 2: In vivo and in vitro fertilisation of human oocytes: effects upon embryo development and polyspermic fertilisation

Sixty women with tubal (n=50) or unexplained (n=10) infertility undergoing ovarian stimulation for IVF were included in the study. Male problems were excluded. Multiple follicular growth was induced by long course combined GnRH-a therapy with hMG initiated at three ampoules/day.

Patients were randomly allocated to a short (34 hours, n=30) or long (39 hours, n=30) LORD. Each patient's oocytes were alternated between immediate (0) insemination and a 5 hour (5) preincubation (Figure 6). The total time between hCG and insemination (total maturation time) therefore varied from 34 hours (short/0) to 44 hours (long/5). The two intermediate groups (short/5 and long/0)

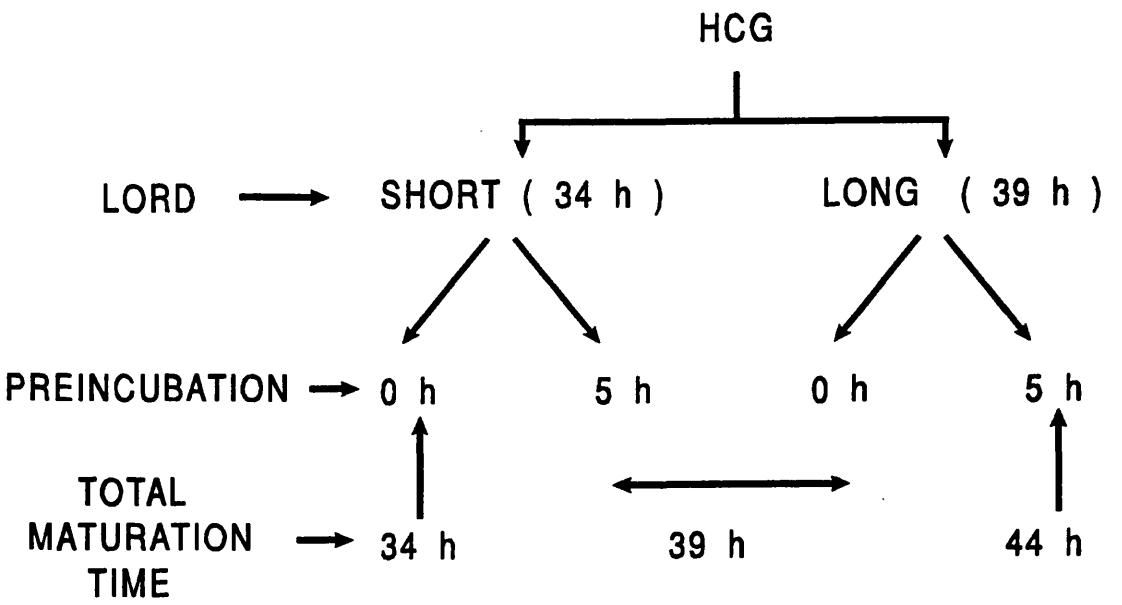


Figure 6

LORD study 2: diagrammatic representation of experimental design.

each had 39 hours, with differing proportions *in vivo* and *in vitro*.

Examination for the presence of pronuclei was performed 15-21 hours after insemination; checking was performed at the same time relative to insemination in both short (mean = 18.1 h, range 16-21 hours) and long (mean = 18.1, range 15-20 hours) LORD groups. Oocytes with a single PN were considered to be parthenogenetically activated and were not included in calculations of fertilisation rates or embryo evaluations. Embryo quality was assessed by the standard scoring system (2.4.8) prior to replacement on day 2 or day 3 after OR. The preincubation time of oocytes was not considered at embryo selection for ET.

Because of possible detrimental effects of immediate insemination, only patients with seven oocytes or more were included in this study and at least four oocytes were given standard treatment (5 hours preincubation). Patients in whom <7 oocytes were obtained or whose partner's semen sample was poor on the day of OR were excluded from the study and replaced by the next patient fulfilling all criteria. This requirement necessitated initial recruitment of >60 patients. The cancellation rate was minimised by excluding women with poor ovarian responses to stimulation and those whose partners had previous semen problems.

2.5.4 The relationship between follicle size and cumulus expansion and their relative importance as indicators of oocyte maturity

Estimation of follicle size

All oocytes recovered in IVF cycles were included in the study. Follicular size was estimated by volume of follicular fluid aspirated at OR. Follicles were considered to be spherical and the formula 4/3 pi r^3 used

to calculate diameter (2 x radius). Only data from discrete follicles was included in analyses.

Follicles were grouped according to diameter and appropriate cut off points of FF volume selected (Table 2.5.2).

Table 2.5.2
Limits of follicle diameter and volume used in analyses

Follicle Size	Diameter (mm) Minimum	Maximum	Volume (ml) Minimum	Maximum
Small	----	12.9	---	1.0
Medium	13.0	16.9	1.1	2.4
Large	17.0	24.9	2.5	7.9
Very Large	25.0	----	8.0	---

Where individual groups were too small for statistical comparison, data has been combined ie. <2.5 ml; small and ≥2.5 ml; large.

A proportion of oocytes were not checked for fertilisation during the study and results are expressed as cleavage rate (cleaved embryos as a percentage of viable oocytes).

2.6 Data storage and analysis

Data for all studies was recorded on Dbase III files (Ashton-Tate) and analysed using Kwikstat (Mission Technologies) statistics package.

CHAPTER 3
RESULTS

3.1 Assessment of embryo quality

3.1.1 Assessment of EDR as a means of quantifying growth rate

Embryos were observed prior to embryo transfer on either day 2 (range 35-51h) or day 3 (range 59-70h). Embryos from the day 2/3 comparison were included in this study but only the assessments on the day of ET were used. The mean EDR (+/-SD) for each one hour interval is shown in Table 3.1.1 and Figure 7. Values ranged from 106 at 38h to a minimum of 66 in the group observed at 70h. The mean EDR of all embryos was 94.5.

Table 3.1.1
Mean EDR values at one hour intervals from insemination

Day 2				Day 3			
Time (h)	EDR (mean)	SD	n	Time (h)	EDR (Mean)	SD	n
35	93.0		3	59	92.7		3
36	104.1	20.2	29	60	91.9	16.6	23
37	98.6	12.2	53	61	94.8	16.5	40
38	106.4	18.0	150	62	88.0	14.5	143
39	98.8	14.4	179	63	88.5	15.3	131
40	101.0	14.9	340	64	82.3	17.6	136
41	99.6	15.3	330	65	82.1	14.5	63
42	98.2	15.4	302	66	89.1	16.7	103
43	96.7	15.9	416	67	80.4	15.9	84
44	96.2	15.8	199	68	80.6	14.7	68
45	93.8	14.5	131	69	77.9	15.7	62
46	89.5	16.2	78	70	66.0		6
47	93.1	15.5	87				
48	90.6	13.3	61				
49	89.8	13.9	18				
50	94.7	9.7	10				
51	92.0		3				

Mean EDR values of pregnancy associated embryos were compared with those from non-pregnant cycles observed at the same interval from insemination (Table 3.1.2). Embryos replaced in pregnant cycles had a significantly higher (t-

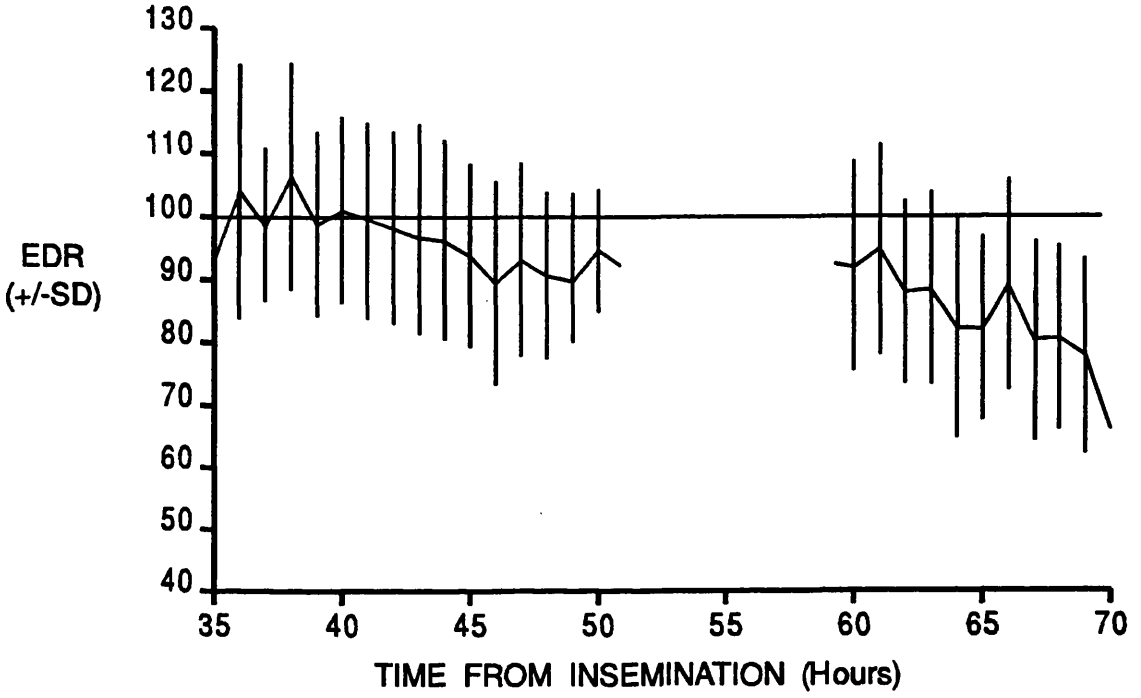


Figure 7

Distribution of EDR values (+/-SD) for one hour intervals after insemination.

test, $p<0.001$) EDR than the non-pregnant group. This difference was apparent whether embryos were observed on day 2 or day 3.

Table 3.1.2
Mean EDR of embryos transferred in pregnant and non-pregnant cycles

Time (h)	Pregnant EDR	(n)	Non Preg EDR	(n)	p (t-test)
38-39	111	36	104	125	0.031
40-41	104	0	101	267	0.034
42-43	100	59	99	294	0.440
44-45	98	39	98	125	0.895
46-47	90	25	91	140	0.781
48-49	93	4	90	75	0.665
50-61	Insufficient data				
62-63	100	40	89	101	0.001
64-65	85	53	81	146	0.201
66-67	93	46	82	141	0.001
68-69	83	27	78	103	0.131
Day 2	99	209	96	791	0.011
Day 3	89	234	83	665	0.001
All embryos	98	205	93	795	0.001

3.1.2 Investigation of embryo morphology and growth rate as predictors of implantation potential

The EDR values of embryos known to have implanted are shown in Table 3.1.3. The mean value representing growth rate was significantly higher (t-test, $p<0.002$) in the implanted embryos than the unsuccessful group. No implantation from an EDR less than 70 could be deduced and the fastest growing embryos (EDR ≥ 110) were most frequent in the successful group.

Table 3.1.3
EDR of individual embryos known to have implanted and those which failed

EDR	Implanted n (%)	Not implanted n (%)
<50	0	0
50-59	0	26 (2.0)
60-69	0	21 (1.6)
70-79	1 (0.9)	99 (7.7)
80-89	25 (22.7)	302 (23.6)
90-99	25 (22.7)	277 (21.6)
100-109	28 (25.4)	288 (22.5)
≥110	31 (28.2)	268 (20.9)
Total	110	1281
Mean EDR	101.34a	96.64

a t-test, p<0.002

The morphology scores of implanted and non-implanted embryos are detailed in Table 3.1.4. The minimum score of an implanted embryo was seven and 38.3% of all successful embryos had a 'perfect' score of 10 compared with 19.7% of those which did not implant. The mean morphology score of successful embryos was significantly higher (t-test, p<0.001) than that of embryos replaced in unsuccessful cycles.

Eighty-two embryo transfers were performed where all embryos scored less than seven. One pregnancy (1.2%) resulted. The pregnancy rate after transfer of embryos scoring seven or more was significantly (chi-sq., p<0.001) higher (165/694; 23.8%).

Table 3.1.4

Morphology score of individual embryos known to have implanted and those which failed

Score	Implanted n	(%)	Not implanted n	(%)
1	0		0	
2	0		2	(0.2)
3	0		2	(0.2)
4	0		15	(1.2)
5	0		43	(3.4)
6	0		98	(7.6)
7	6	(8.8)	195	(15.2)
8	18	(26.5)	374	(29.2)
9	18	(26.5)	300	(23.4)
10	26	(38.3)	252	(19.7)
Total	68		1281	
Mean score	8.84a		8.07	

a t-test, $p<0.001$

The relative contribution of embryo growth rate and morphology to pregnancy rate was investigated by placing embryos in an array based on both parameters. Table 3.1.5 shows the numbers of embryos in each group, the number transferred and those replaced in pregnancy cycles. Table 3.1.6 shows the same data expressed as percentages. The number of pregnancy associated embryos in each group was compared with all other transferred embryos using the chi-squared test. The probability of differences between groups and the whole population is shown in Table 3.1.5.

Table 3.1.5

Comparison of EDR and embryo morphology as predictors of implantation

Embryo quality	Embryo Development Rating				
	<80	81-90	91-100	>100	Total
1-4					
n	36	32	19	53	140
ET	1	8	2	10	21
Pr	0	2	0	0	2
p	0.599	0.820	0.457	0.096	0.174
5+6					
n	91	106	98	210	505
ET	29	38	33	65	165
Pr	1	7	2	10	20
p	0.007	0.621	0.028	0.208	0.002
7+8					
n	177	377	312	569	1435
ET	62	163	180	324	729
Pr	7	29	48	76	160
p	0.043	0.203	0.087	0.391	0.824
9+10					
n	132	305	211	410	1058
ET	71	189	150	316	726
Pr	6	47	26	95	174
p	0.006	0.261	0.175	<0.001	0.047
Total					
n	436	820	640	1242	3138
ET	163	398	365	715	1641
Pr	14	85	76	181	356
p	<0.001	0.851	0.647	0.002	

n = Number of embryos.
ET = Number of embryos transferred.
Pr = Number of embryos associated with pregnancy.
p = Significance of difference of pregnancy associated embryos from general (ET) population (tested using chi-sq).

Table 3.1.6

Comparison of EDR and embryo morphology as predictors of implantation; data expressed as percentages

Embryo quality	Embryo Development Rating				
	<80	81-90	91-100	>100	Total
1-4					
% n	1.1	1.0	0.6	1.7	4.5
% ET	2.8	25.0	10.5	18.9	15.0
% Pr	0	25.0	0	0	9.5
5+6					
% n	2.9	3.4	3.1	6.7	16.1
% ET	31.9	35.8	33.7	30.9	32.7
% Pr	3.4	18.4	6.1	15.4	12.1
7+8					
% n	5.6	12.0	9.9	18.1	45.7
% ET	35.0	43.2	57.7	56.9	50.8
% Pr	11.3	17.8	26.7	23.5	21.9
9+10					
% n	4.2	9.7	6.7	13.1	33.7
% ET	53.8	62.0	71.1	77.1	68.6
% Pr	8.5	24.9	17.3	30.1	24.0
Total					
% n	13.9	26.1	20.4	39.6	
% ET	37.4	48.5	57.0	57.6	52.3
% Pr	8.6	21.4	20.8	25.3	27.7

% n = % of total number of embryos.
% ET = % of embryos transferred.
% Pr = % of transferred embryos associated with pregnancy.

Embryos with EDR values <80 or morphology scores of five and six were infrequently associated with pregnancy cycles. The fastest growing embryos (EDR >100) and those with the highest morphology scores were significantly

(chi-sq., p=0.002; p=0.047) associated with pregnancy. Results from individual groups suggested that both parameters contributed to the probability of implantation. Only 13.1% of embryos were in the fastest growing group with morphology scores of nine or ten. Seventy-seven percent of these were selected for ET and the proportion associated with pregnancy (30.1%) was significantly higher (chi sq., p<0.001) than the general population.

3.1.3 Investigation of the optimum time for selection of embryos for transfer

Both embryo morphology and EDR were strongly correlated (paired t-test, p<0.001) when examined on successive days (Table 3.1.7).

Table 3.1.7
Comparison of embryos quality assessments on day 2 and day 3 following OR

	Day 2	Day 3
Time of observations:		
Range (h)	40-53	61-72
Mean (h)	45.1	66.5
Embryo assessment:		
Mean embryo score	8.0	7.6a
Mean EDR	100.0	87.2a

a paired t-test, p<0.001

Comparison was made between all IVF replacements performed on day 2 and those delayed until day 3. It should be noted that the day of ET was decided only by logistical considerations. Pregnancy rates (Table 3.1.8) and implantation rates (Table 3.1.9) were similar on day 2 and day 3, irrespective of whether the number of embryos available for ET exceeded the number replaced (selected

replacements) or all available embryos were transferred (non-selected replacements). Pregnancy rates were not significantly different if selection on day 2 was made before 42 h (87/371; 23.4%) or between 42 and 52h (19/97; 24.4%).

Table 3.1.8
Comparison of pregnancy rates when ET was performed on day 2 or day 3 after OR

	Day 2			Day 3		
	ET (n)	Preg (n)	(%)	ET (n)	Preg (n)	(%)
Non-selected replacements	305	27	8.8	143	11	7.7
Selected replacements	386	90	23.3	161	39	24.2
All cycles	691	117	16.9	304	50	16.4

Table 3.1.9
Comparison of implantation rates when ET was performed on day 2 or day 3 after OR

	Day 2			Day 3		
	ET (n)	Implant (n)	(%)	ET (n)	Implant (n)	(%)
Non-selected replacements	645	37	5.7	297	12	4.0
Selected replacements	1212	128	10.6	500	59	11.8
All cycles	1857	165	8.9	797	71	8.9

3.1.4 Summary

Embryo development rating was an effective method of assessing growth rate if groups under comparison were examined at the same time, relative to insemination. Embryo quality assessed by morphology and EDR was predictive of implantation potential but not diagnostic. The mean score of embryos which implanted (8.84) was significantly higher ($p<0.001$) than those which failed to implant (8.07) and the minimum score associated with pregnancy was seven. Implanting embryos had a significantly higher ($p<0.002$) mean EDR (101.34) than those which failed to establish pregnancy (96.64). Assessment on day 3 after insemination allowed observation of a larger spectrum of development rate but observations on day 2 were highly predictive ($p<0.001$) of both assessment parameters on day 3 and no advantage of delayed ET was seen in pregnancy rates (day 2, 16.9%; day 3, 16.4%).

3.2 Luteinising hormone: effects upon oocytes during the periovulatory period

3.2.1 Patient responses

Twenty-four (32.0%) of cycles on hMG alone were cancelled as a result of premature luteinisation (Table 3.2.1). All patients on combined therapy attained minimum criteria for hCG administration without any evidence of luteinisation.

The mean number of oocytes recovered per cycle and the proportion which subsequently cleaved were similar in the two groups (Table 3.2.2). Post-mature (PM) oocytes were recovered from 10 patients on hMG alone and were absent in combined therapy cycles; this difference was statistically significant (chi-sq., $p<0.01$). Embryo quality assessed by morphology and growth rate was not affected by the stimulation regime.

The pregnancy rate per ET was similar in the two groups but success per cycle was higher after combined therapy since all cycles proceeded to OR (Table 3.2.1).

Profiles of estradiol, LH and P during the eight days prior to day 0 are shown in Figure 8 and Table 3.2.3 lists the plasma hormone concentrations on the day of hCG administration. These results omit those of cycles cancelled prior to OR.

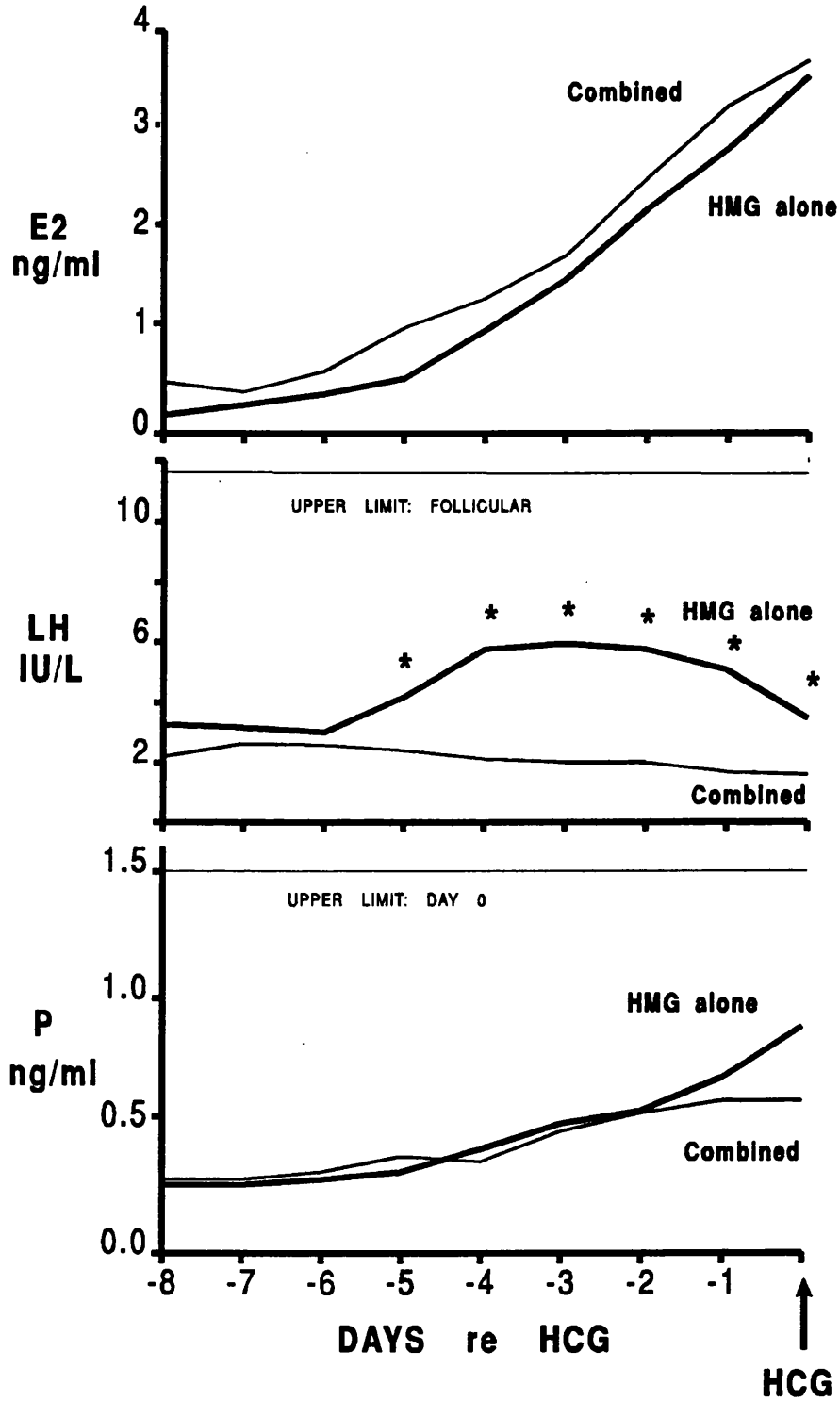


Figure 8

Mean plasma concentrations of E2, LH and P during the eight days prior to hCG administration in women treated with hMG alone or combined therapy.

* t-test, $p<0.01$.

Table 3.2.3
Steroid and gonadotrophin concentrations at hCG administration in cycles treated with hMG alone or in combination with GnRH-a

	hMG	GnRH-a/hMG
Day 0 estradiol (+/- SD) [ng/ml]	2.63(1.77)	3.16(2.01)
Day 0 P (+/- SD) [ng/ml]	0.74(0.32)	0.64(0.34)
Day -1 LH (+/- SD) [IU/L]	4.8 (6.46)	1.45(1.02)a

a t-test, $p<0.01$

Concentrations of estradiol on day 0 were similar in the two groups, reflecting the degree of stimulation required for hCG administration. Luteinising hormone concentrations in the hMG group were significantly higher throughout the final five days of stimulation but did not exceed normal follicular phase levels at any point. Elevations were not reflected in P concentrations which were similar in the two treatment regimes and were within normal laboratory limits.

3.2.2 Characteristics of cycles yielding post-mature oocytes

Cycles yielding PM oocytes were characterised by significantly reduced (chi-sq., $p<0.01$) cleavage rates, absence of pregnancies after ET (Table 3.2.4) and P concentrations $\geq$1ng/ml at the time of hCG administration (Table 3.2.5). Mean endocrine profiles of PM cycles (Figure 9) disguise an attenuated LH surge, with subsequent P rise, occurring in each patient. An

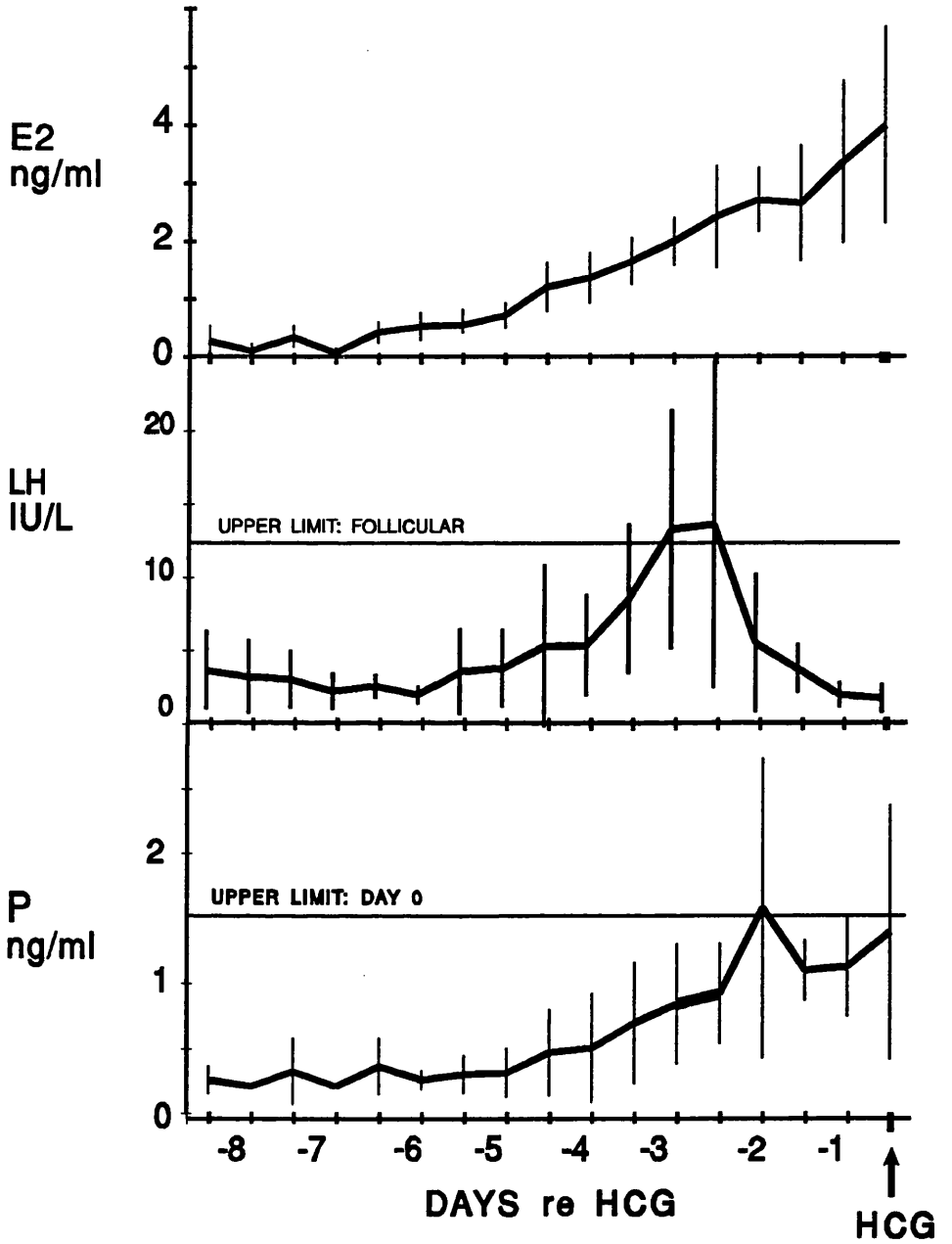


Figure 9

Mean (+/- SD) plasma concentrations of E2, LH and P prior to hCG administation in cycles which yielded post-mature oocytes.

individual profile in shown in Figure 10. Progesterone levels exceeded the 1.5 ng/ml cut-off in one sample only and the luteinisation escaped detection.

Table 3.2.4
Comparison of cleavage rates and pregnancies in cycles where PM oocytes were or were not recovered

	Cycles (n)	% Oocytes cleaved	Pregs (n)
PM Absent	73	60.4	14
PM Present	10	37.7	0
Chi sq.		p<0.01	NS

Rises in circulating LH levels occurred during the final five days of stimulation with the mean LH peak 57 hours before hCG was given. Maximum P concentrations were detected 27 hours later (Table 3.2.6).

Table 3.2.5
Progesterone concentrations and the occurrence of PM cycles

Progesterone Day -1 (ng/ml)	Cycles (n)	≥1 PM oocyte recovered
<1.0	59	0
≥1.0	24	10
	chi-sq., p<0.001	

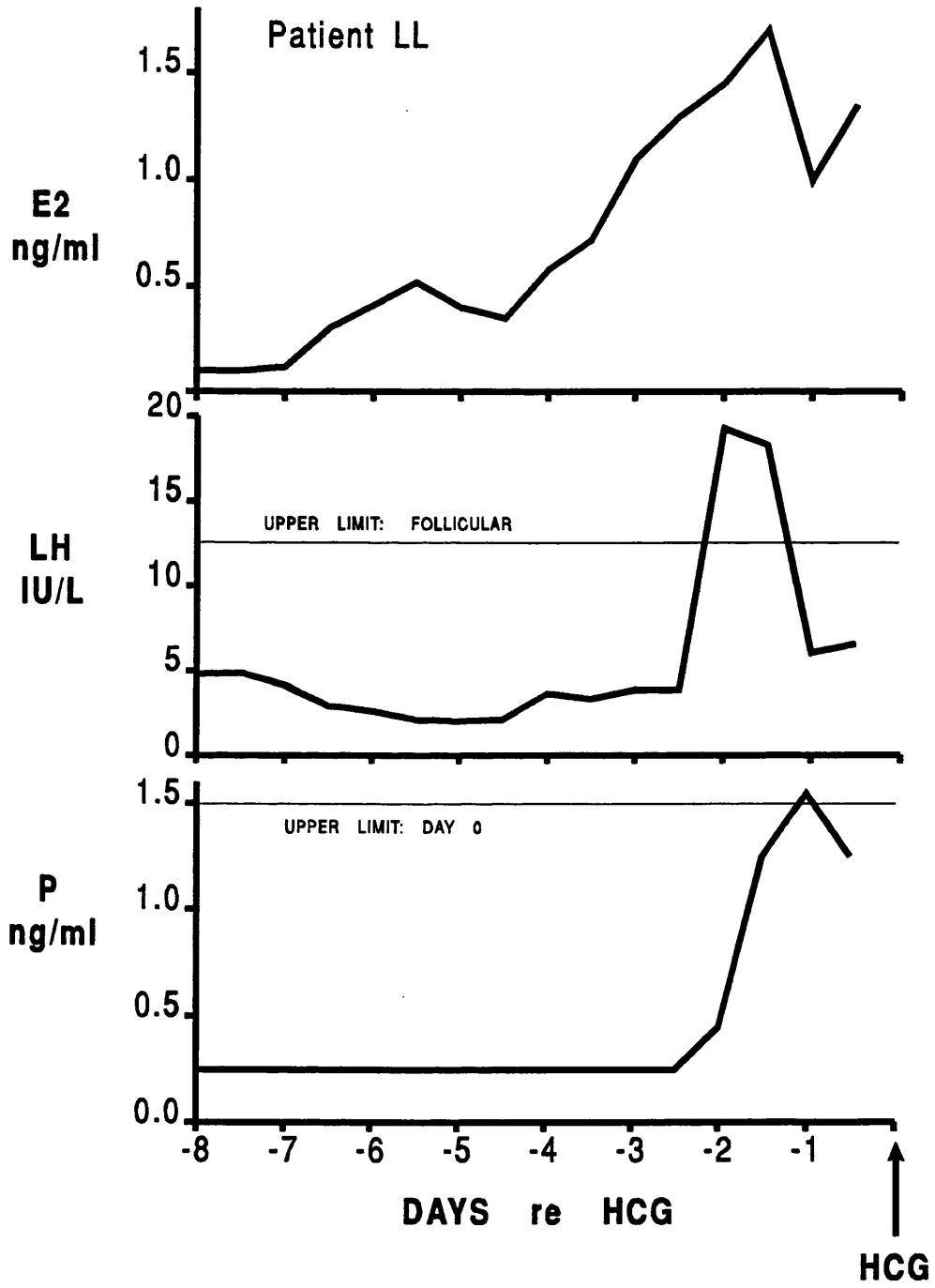


Figure 10

Plasma hormone concentrations prior to hCG administration in a cycle which yielded post-mature oocytes.

Table 3.2.6
Timing of LH and P peaks in cycles yielding PM oocytes

	LH Peak (h pre-hCG)	P Peak (h pre-hCG)	Delay (h)
Mean	57	32	27
SEM	8.5	8.6	4.4

In cycles affected by pre-hCG LH rises, only 16/68 recovered oocytes were scored as PM. In only one case were all oocytes (2/2) PM.

3.2.3 Characteristics of post-maturity

During the total study period 75 PM oocytes were recovered. These were associated with larger FF volumes (mean 6.1ml) than those scored as immature or mature (n=8322; mean 4.1ml) and a reduced cleavage rate (27.3% v 61.8%) was observed. Embryo quality was similar in the two groups (PM; mean score 7.1, mean EDR 92.3: non-PM; mean score 7.5, mean EDR 94.0).

The majority of PM oocytes were recovered during a period when PN checking was not routinely performed and information on fertilisation is limited. However, 7/48 PM oocytes which had not cleaved on day 2/3 had multiple PN, one had 3PN, twelve had 1PB, three had no PB and a further four showed signs of degeneration. Remaining oocytes were not informative. Apparently normal oocytes recovered from PM cycles did not display fertilisation anomalies but 4/18 which remained unfertilised had failed to reach metaphase II.

3.2.4 Summary

Suppression of endogenous pituitary function by adjuvant treatment with GnRH-a during ovarian stimulation with hMG eliminated LH fluctuations and surges and all initiated cycles (n=32) proceeded to OR without complication. The cancellation rate (24/75; 32.0%) was significantly higher ($p<0.001$) in hMG alone cycles. Pregnancy rates after ET in the two groups (GnRH-a/hMG, 19.3%; hMG, 18.1%) confirmed observations that embryo quality was similar after both stimulation protocols. Post-mature oocytes were recovered from 10 hMG alone cycles and were absent ($p<0.01$) in analog treated patients. Significantly fewer ($p<0.01$) oocytes cleaved in PM associated (37.7%) than unaffected (60.4%) cycles although not all oocytes were affected. Prior to hCG administration, all PM cycles showed an attenuated LH surge which was undetected by monitoring of serum progesterone concentrations.

3.3 Interval from luteinisation stimulus to ovulation in women treated with combined therapy: implications for assisted conception methodology

3.3.1 Study 1: Timing of ovulation

The mean number of oocytes obtained was 9.5 and there was no difference between any of the five study groups (Table 3.3.1). Two patients in each of the 39h and 41h groups had at least one collapsed follicle at initiation of OR. The earliest observation of ovulation was at 39h 30min. Oocytes were recovered from unruptured follicles in all cases and from a number of collapsed follicles, flushed with culture medium, in patients with evidence of ovulation.

Table 3.3.1
Study 1: Yield of oocytes and incidence of ovulation in LORD groups

Group (Midpoint) (h)	Oocytes (n) Range	Mean	Ovulation (patients)
33	5-10	8.00	0
35	6-18	9.79	0
37	4-17	10.37	0
39	2-23	9.41	2
41	2-14	8.89	2

3.3.2 Study 1: cumulus expansion

The proportion of oocytes with an associated mature cumulus increased with extension of the LORD (Table 3.3.2) and this increase was significant (chi-sq., $p<0.001$) when conventional and extended groups were compared. No post-mature oocytes were recovered in any group.

Table 3.3.2
Study 1: Cumulus expansion in LORD groups

Group (Midpoint) (h)	Immature (n) (%)	Mature (n) (%)	Total (n)
33	25 (34.2)	48 (65.8)	73
35	32 (26.2)	90 (73.8)	122
37	25 (13.1)	166 (86.9)	191
39	31 (16.2)	160 (83.8)	191
41	9 (12.3)	64 (87.7)	73
Combined groups			
<36	57 (29.2)	138a (70.8)	195
≥36	65 (14.3)	390a (85.7)	455

a chi-sq., $p<0.001$

3.3.3 Study 1: Cleavage rate

Table 3.3.3 shows the proportion of viable oocytes which cleaved in individual and combined LORD groups. GIFT cycles and any case with an apparent male factor were excluded. Cleavage rate increased with extension of the LORD with the exception of the small 41 h group. Significantly (chi-sq., $p<0.02$) more oocytes cleaved after extended *in vivo* maturation.

3.3.4 Study 1: Pregnancy rate

The pregnancy rate per OR was 22.0% (11/50) for IVF and 30.4% (7/23) for GIFT (Table 3.3.4). Significantly (chi-sq., $p<0.05$) more IVF pregnancies were achieved in the extended LORD groups (10/32; 31.2%) than those treated conventionally (1/18; 5.6%). Pregnancy rates after GIFT were not different in the different LORD groups.

Table 3.3.3

Study 1: Cleavage rate in LORD groups. Results from IVF cases excluding those with male factors

Group (Midpoint) (h)	Cleaved (n) (%)	Not cleaved (n) (%)	Total (n)
33	28 (52.8)	25 (47.2)	53
35	42 (70.0)	18 (30.0)	60
37	63 (75.0)	21 (25.0)	84
39	61 (80.3)	15 (19.7)	76
41	23 (67.6)	11 (32.4)	34
Combined groups			
<36	70 (61.9)a	43 (38.1)	113
≥36	147 (75.8)a	47 (24.2)	194

a chi-sq., $p<0.02$

Table 3.3.4

Study 1: Pregnancies in LORD groups

Group (Midpoint) (h)	IVF OR (n)	IVF ET (n)	IVF Pregs (n)	GIFT OR (n)	GIFT Pregs (n)
33	8	6	0	1	0
35	10	10	1	4	1
37	13	12	6	6	1
39	13	11	2	9	4
41	6	5	2	3	1
Combined groups					
<36	18	16	1 a	5	1
≥36	32	28	10 a	18	6

a $p < 0.05$

3.3.5 Study 2: Patient details

Table 3.3.5 details LORD times and the numbers of oocytes recovered.

Table 3.3.5
Study 2: Details of LORD groups

	SHORT	LONG
Number of cycles	30	30
LORD (h:min): mean	34:20	39:20
LORD (h:min): range	33:20-35:00	38:10-40:00
Oocytes (n): total	321	328
Oocytes (n): mean/cycle	10.7	10.9
Oocytes (n): range	7-20	7-23

3.3.6 Study 2: Cumulus Expansion

The proportion of oocytes scored as mature on the basis of cumulus expansion was higher in the 39 h group than in the 34h group (83.1% v 77.6%). The difference was not statistically significant (Table 3.3.6).

Table 3.3.6
Study 2: Cumulus expansion in LORD groups

	Immature n (%)	Mature n (%)
Short LORD	70 (22.4)	243 (77.6)
Long LORD	55 (16.9)	270 (83.1)

3.3.7 Study 2: Fertilisation Rate

The proportion of oocytes which fertilised was significantly (chi-sq., $p<0.05$) higher in those oocytes with a long hCG/OR delay (long/0 + long/5) than in the combined short groups (Table 3.3.7). The same trend was seen in individual groups but the differences were not statistically significant (Table 3.3.8). Preincubation time did not affect the fertilisation rates (Table 3.3.9). Oocytes with a single PN observed between 15 and 21 hours (n=7) were considered to be parthenogenetically activated and were classified as unfertilised for the purposes of this study.

Table 3.3.7
Study 2: Details of fertilisation and pregnancies in groups with short or long LORD

	LORD	
	SHORT (n)	LONG (n)
Viable oocytes	302	303
Fertilised (%)	232 (76.8) **a**	255 (84.2) **a**
Fertilisation not observed (%)	30 (12.9) **b**	10 (3.9) **b**
Pregnancies (%/OR)	6 (20.0)	8 (26.7)

a chi-sq., $p<0.05$
b chi-sq., $p<0.001$

Table 3.3.8
Study 2: Details of fertilisation and implantation in groups with short or long LORDs, with or without additional in vitro incubation

	SHORT/0	SHORT/5	LONG/0	LONG/5
Viable oocytes (n)	152	150	155	148
Fertilised (n)	116	116	129	126
Fertilised (%)	76.3	77.3	83.2	85.1
2 PN (n)	83	94	118	111
3/4 PN (n)	17 a	8	7	9
3/4 PN (%)	17.0	7.8	5.6	7.5
Fertilisation not observed (n)	16	14	4	6
Fertilisation not observed (%)	13.8	12.0	3.1	4.8
Embryo morphology (mean score)	7.7	7.5	7.3	7.4
Implanted embryos (n)	3	2	6	1

a chi-sq., $p<0.05$

3.3.8 Study 2: Polyspermic Fertilisation

Table 3.3.8 shows the incidence of polyspermic fertilisation in those oocytes where PN were visualised at the first observation between 15 and 21 hours. Forty-one out of 447 oocytes had >2PN. The majority of abnormal fertilisations had 3 PN but two showed four. These were combined for analysis. The incidence of polypronuclei was highest in the short/0 group and was significantly lower (chi-sq., $p<0.05$) in all other groups.

Table 3.3.9
Study 2: Details of fertilisation in oocytes inseminated immediately or after preincubation

	Preincubation Time	
	0 (n)	5 (n)
Viable oocytes	307	298
Fertilised (%)	245 (79.8)	242 (81.2)
Fertilisation not observed (%)	20 (8.2)	20 (8.3)

3.3.9 Study 2: Delayed Fertilisation

Forty oocytes which did not have recognisable PN at first examination subsequently showed evidence of fertilisation or had cleaved on day 2 (Table 3.3.8). Significantly fewer (chi-sq., $p < 0.001$) oocytes in the 2 long groups (3.9%) showed late fertilisation and/or cleavage compared with those in the short groups (12.9%; Table 3.3.7). Preincubation time did not affect the incidence of delayed fertilisation (Table 3.3.9).

3.3.10 Study 2: Embryo Quality

No difference in embryo quality related to the hCG/oocyte retrieval interval or preincubation time was observed (Table 3.3.8). Examination times relative to insemination were not equivalent in the two LORD groups and EDR could not be utilised to evaluate embryo growth rate (3.1.1).

3.3.11 Study 2: Pregnancy rate

The pregnancy rates in the short and long groups was not different (Table 3.3.7). Twenty-one embryos implanted; the preinsemination treatment of 12 could be deduced. Nine followed immediate insemination (Table 3.3.8).

3.3.12 Summary

A preliminary study investigated the interval between administration of hCG and ovulation in 73 women undergoing laparoscopic OR after ovarian stimulation with adjuvant GnRH-a. The earliest ovulation was observed at 39.5 hours and oocyte quality, assessed by cumulus expansion and fertilisation rates, improved with extended *in vivo* maturation (mature oocytes, <36h, 70.8% ; ≥36h, 85.7%: fertilisation rate, <36h, 61.9%; ≥36h, 75.8%). These data indicated that extension of the LORD could be practiced with benefits at the level of the oocyte.

It is standard IVF practice to preincubate oocytes for 4-6h before insemination to minimise the incidence of polyspermic fertilisation and maximise embryo quality. The above study was extended to compare the relative effects of *in vivo* and *in vitro* maturation in 60 women randomly allocated to 34 or 39 hour LORDs. Each patient's oocytes were alternated between immediate insemination and five hours preincubation. The incidence of polyspermic fertilisation was highest in oocytes inseminated immediately after a short LORD (17/100) and was significantly reduced ($p<0.05$) by preincubation and/or an extended LORD. Fertilisation rates were significantly higher ($p<0.05$) higher after a 39 hour (84.2%) than a 34 hour LORD (76.8%) and the incidence of delayed fertilisation was reduced by extension of the *in vivo* maturation time (34h, 12.9%; 39h, 3.9%; $p<0.001$).

3.4 The relationship between follicle size and cumulus expansion and their relative importance as indicators of oocyte maturity

3.4.1 Follicle size

The cleavage rate increased with follicle size and was significantly different between all individual groups using the chi-squared test (small/medium; $p<0.05$: all others; $p<0.001$) with the exception of large/very large (Table 3.4.1). Combined data showed a significantly (chi-sq., $p<0.001$) higher cleavage rate of oocytes from large follicles than those from small volumes (63.4% v 51.3%).

Table 3.4.1
Proportion of oocytes which cleaved related to follicle size

Follicle size	Total oocytes (n)	Oocytes cleaved (n)	Percentage cleaved (n)
Small	412	194 **a**	47.1
Medium	718	386 **a**	53.8
Large	2080	1304 **a**	62.7
V. large	412	276 **a**	67.0
Combined data:			
Small	1130	580 **b**	51.3
Large	2492	1580 **b**	63.4
Total	3622	1462	59.6

a Cleavage rates of all individual groups with the exception of large/very large were significantly different.
b $p<0.001$

3.4.2 Cumulus expansion

Table 3.4.2 details the cleavage rate of oocytes related to cumulus expansion. The proportion of oocytes which cleaved increased with apparent cumulus maturity. All individual and combined groups were significantly different using the chi-squared test (1/3 & 2/3; $p<0.001$: 1/2; $p<0.01$: immature/mature; $p<0.001$).

Table 3.4.2
Cleavage rate related to cumulus expansion

Cumulus score	Total oocytes (n)	Cleaved embryos (n)	Percentage cleaved (%)
1	38	12 **a**	1.6
2	1433	802 **a**	56.0
3	5451	3356 **a**	61.6
Combined data:			
Immature	1471	814 **b**	55.3
Mature	5451	3356 **b**	61.6
Total	6922	4170	60.2

a Cleavage rates of all individual groups were significantly different (chi-sq.).
b chi-sq., $p<0.001$

3.4.3 Combined data

The relationship between follicle size and the degree of cumulus expansion of the associated oocyte is shown in Table 3.4.3. The proportion of oocytes with a fully expanded cumulus increased with FF volume. The difference was significant (chi-sq., $p<0.001$) when combined groups for follicle size were compared.

Table 3.4.3
Relationship between follicle size and expansion of the associated cumulus.

Follicle size	Cumulus expansion	
	Immature	Mature
	n (%)	n (%)
Small	121 (27.9)	313 (72.1)
Medium	185 (25.3)	547 (74.7)
Large	451 (21.0)	1698 (79.0)
V large	83 (19.7)	339 (80.3)
Combined data:		
Small	306 (26.2)a	860 (73.8)
Large	534 (20.8)a	2037 (79.2)

a chi-sq., $p<0.001$

Embryo morphology, as assessed by the mean score of all cleaved embryos, was significantly (t-test, $p<0.001$) better if the follicle of origin was large than if the oocyte was recovered from a small volume (Table 3.4.4). The proportion of embryos with a score of ≥ 7 (minimum score associated with pregnancy; 3.1.2) was also significantly (chi-sq., $p<0.001$) higher in the large FF volume group (78.7% v 67.6%). Embryo morphology was not affected by the degree of cumulus expansion as assessed at OR (Table 3.4.4).

No correlation was found between embryo growth rate and either follicle size or cumulus maturity.

Figure 11 shows the cleavage rate and mean embryo score when oocytes are grouped according to both follicle size and cumulus expansion. The fertilisation/cleavage rates of oocytes from large follicles were significantly (chi-sq., $p<0.001$) higher than those from small volumes, regardless of the degree of cumulus expansion. Mature oocytes had a

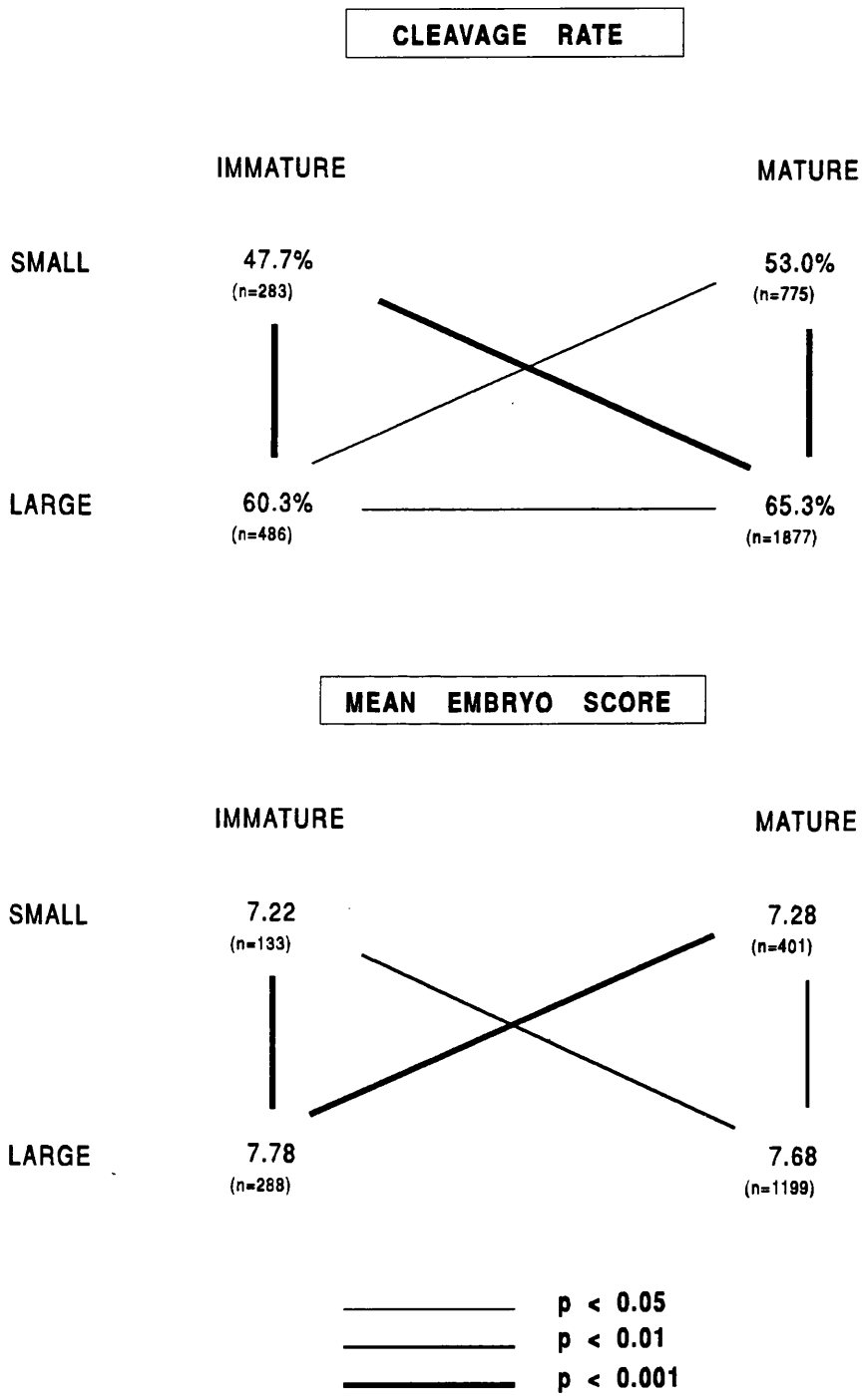


Figure 11

Relationship of cleavage rate and embryo morphology with follicle size and cumulus expansion: combined data.

higher fertilisation rate than those with non-expanded cumuli but the difference only reached statistical significance when the follicle of origin was large. Embryo quality was better if oocytes originated from large follicles, regardless of apparent maturity at OR.

Table 3.4.4
Effects of follicle size and cumulus maturity upon embryo quality; combined groups

	Embryo quality	
	Morphology mean score (n)	EDR mean (n)
Follicle size:		
Small	7.22a (558)	95.1 (575)
Large	7.69a(1517)	94.5 (1559)
Cumulus expansion:		
Immature	7.54 (808)	96.6 (810)
Mature	7.55 (3294)	95.2 (3308)

a t-test, $p<0.001$

3.4.4 Summary

The relative importance of follicle size and cumulus expansion as indicators of oocyte maturity was investigated by retrospective analysis of IVF data on 6922 oocytes. Cleavage rates and embryo quality were significantly better ($p<0.001$) if the oocyte was retrieved from a large follicle ($\geq$2.5ml of follicle fluid; 63.4% cleaved, mean score 7.69) than a small follicle (<2.5ml; 51.3% cleaved, mean score 7.22). Significantly higher ($p<0.001$) cleavage rates were observed if the associated cumulus was fully expanded (79.2%) than if the oocyte was

scored as immature (73.8%); embryo quality was unaffected. The two parameters of assessment were not independent but combined data revealed that follicle size was a better indicator of fertilisation potential and subsequent embryo quality than cumulus expansion. These data suggested that follicle size should be the primary criterion when selecting oocytes for GIFT.

CHAPTER 4
DISCUSSION

4.1 Assessment of embryo quality

4.1.1 Predictive value of morphology and EDR for pregnancy potential of embryos

Mean values for EDR assessed at different times relative to insemination varied within, and more markedly, between days 2 and 3. Two factors appeared to contribute. The earlier observations on day 2 identified the modal time of transition from two to four cells. The majority of embryos had reached four cells when examined during the latter part of day 2 and received successively lower EDRs (Appendix 1). The greater spread of cell stages observed at 62-63 h (Figure 12) suggests the reason for lower mean EDR values on day 3.

Cummins et al., (1986) concluded that the objective of 'normalising' growth rate data was only partially met by the EDR method but that it's advantage lay in the ability to compare groups of embryos at disparate stages. This study suggests that this is only true when groups have been assessed at the same time relative to insemination.

Both embryo morphology and growth rate were predictive of implantation potential but neither were specific. Lower values were more indicative of lack of viability than high scores were of success. For example, only one pregnancy could be attributed to an embryo scoring less than seven but more than 75% of replacements where all embryos were ≥ 7 did not result in pregnancy.

The distribution of embryos in the analysis which combined both assessment parameters (Tables 3.1.5 and 3.1.6) suggested growth rate and morphology were not correlated. The finding that rapid cleavage could not compensate for poor morphology and vice versa suggests that a combined assessment might be feasible. A scoring system combining a morphology score and an addition for embryos reaching four

cells 48h after 'fertilisation' has been reported (Puissant et al., 1987). The present study suggests that the choice of cut-off used by these authors is inappropriate.

Mean values of EDR were maximal when embryos were assessed before 42h. This was also the period during day 2 when the EDR of pregnancy associated embryos was significantly higher than those in non-pregnant cycles (Table 3.1.2). Figure 12 illustrates the total embryo population and those selected for ET at 38-39 h, 44-45 h and on day 3 at 62-63h. In the first group only 30% of embryos had reached four cells and were preferentially selected for ET. Six hours later, 50% were at four cells and comprised the majority of replaced embryos. This data suggests that EDR is predictive of implantation potential if selection takes place at a time when the fastest growing population can be identified. In the 62-63h group 30% had four cells or fewer, 71% of transferred embryos were at eight or more cells and EDR was highly predictive ($p<0.001$) of pregnancy potential. The methodology used could not determine if those embryos with fewer than eight cells were arrested, growing slowly or were growing normally after delayed fertilisation.

4.1.2 Timing of embryo replacement

Despite apparent separation of slow and rapidly growing embryos on day 3 (Figure 12), improved selection was not apparent in pregnancy (Table 3.1.8) or implantation (Table 3.1.9) rates. It would appear that the correlation between both selection criteria on days 2 and 3 (Table 3.1.7) allowed prediction on day 2 of those most likely to continue normal growth. This data also excluded an influence of embryo/endometrium synchrony.

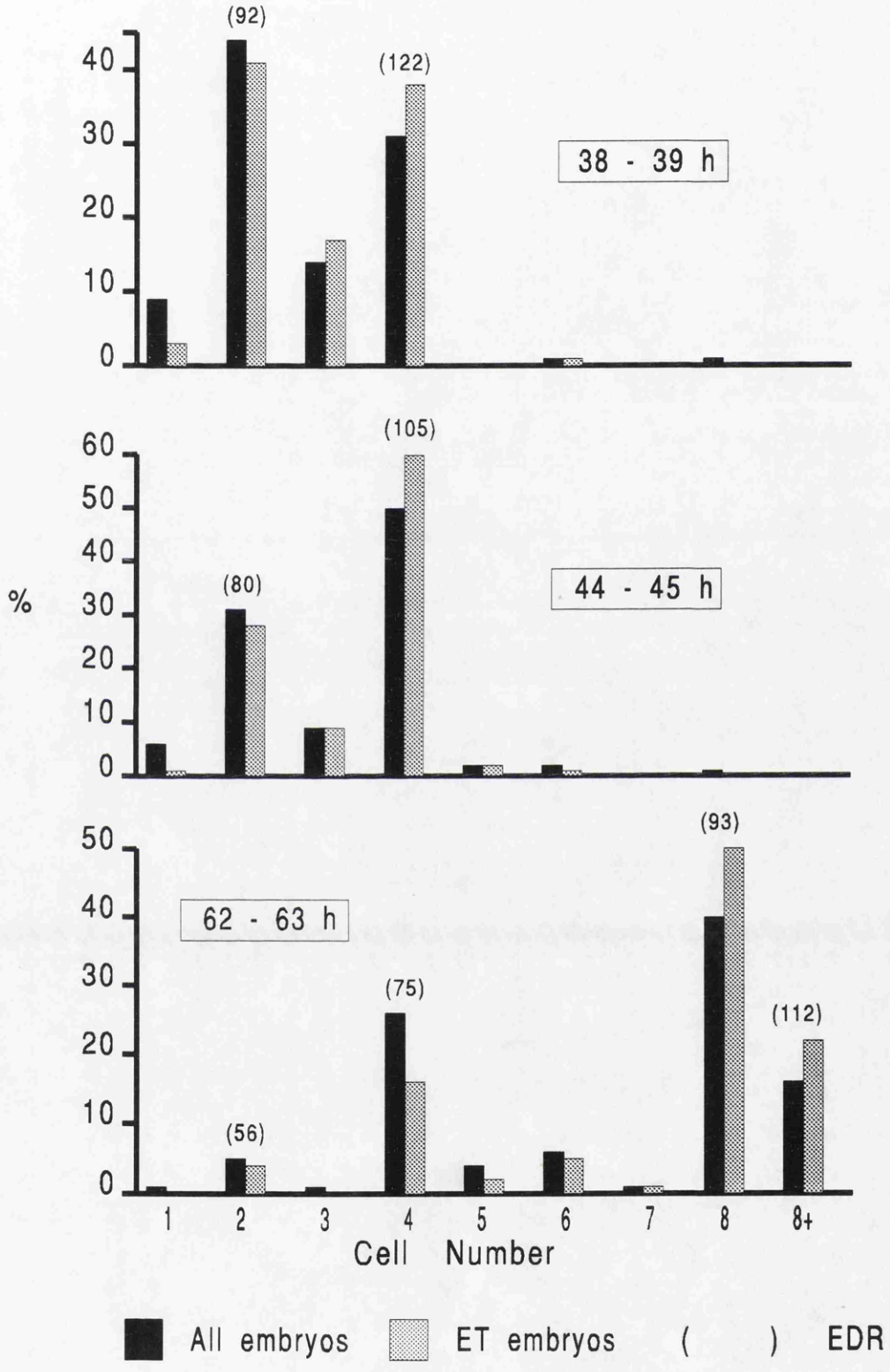


Figure 12

Developmental stage of embryos at 38-39, 44-45 and 62-63 hours after insemination.

4.1.3 Other methods of assessing embryo quality

The conclusion that gross assessment of preimplantation embryos is an insensitive predictor of implantation potential is supported by several studies investigating aspects of embryo metabolism, production of maternal pregnancy recognition factors and culture methods.

Sensitive assays capable of monitoring the uptake of energy substrates from droplets of embryo culture medium have been developed (Hardy et al., 1989). This non-invasive technique showed that the main nutritional source changed from pyruvate in the early cleavage stages to glucose from days 2.5 to 4.5 post-fertilisation. Pyruvate uptake was 20% higher in embryos which developed to blastocyst than those which arrested in culture. This difference was detectable before any apparent change in growth rate. Although embryo morphology was better in the group which reached blastocyst, 70% of the group which arrested were equally good.

Embryo-derived platelet-activating factor (PAF) is secreted by human (O'Neill & Saunders, 1984) and mouse (O'Neill, 1985) preimplantation embryos and has been identified as possibly having a function in maternal recognition of pregnancy. The biologically active phospholipid causes platelet aggregation and sequestration leading to thrombocytopoenia when injected into splenectomised mice. This formed the method for the original bioassay in which PAF containing solutions were injected to mice (O'Neill, 1985). Analysis of culture medium showed that embryos which resulted in pregnancy secreted significantly more PAF than those which failed to implant (O'Neill et al., 1987). An improved, in vitro, assay using purified phospholipid extracted from the test solution showed higher secretion of PAF from preimplantation embryos with good morphology and growth

rates, but identified a macroscopically normal population with deficient production (O'Neill et al., 1988).

The potential effects of light and room temperature on pre-implantation rabbit embryos were assessed by thymidine incorporation, electron microscopy and gross morphology (Fischer et al., 1988). Adverse effects of simultaneous exposure were apparent first as a reduction in thymidine incorporation indicating decreased cell proliferation. After longer exposure, signs of degeneration such as fatty vacuolisation, lamellar bodies, clustering of organelles and dead blastomeres within the perivitelline space were detectable by EM. Gross morphology proved to be too inaccurate to evaluate embryo damage.

Individual cells within the embryo may have differing potential. Low levels of extranuclear RNA synthesis associated with absent nucleolar activity were observed in a proportion of blastomeres in morphologically normal embryos (Tesarik et al., 1986b). Although destruction of up to 50% of blastomeres after cryopreservation is not incompatible with implantation (Troup et al., 1990), it is not a good prognostic factor.

Current methods for determination of energy substrate uptake (Hardy et al., 1989) and PAF production (O'Neill et al., 1988) are impractical for routine embryo assessment but may provide a basis for future developments. Another method is suggested by a study using videocinematography which allowed detailed study of 11 morphological parameters of fresh and cryopreserved embryos (Cohen et al., 1989) without time constraints imposed by protecting the embryo. The system was found to be easily learned by inexperienced embryologists and was considered relatively objective. Initial results for the predictive value of interleukin-1-alpha and immunosuppressive factors secreted into culture fluid (Sheth et al., 1991) are encouraging

but require verification using media from individual embryos.

4.1.4 Conclusions

In conclusion, embryo morphology and growth rate are both indicators of embryo viability but are not specific. Both show correlations with direct measurements of metabolism, ability to continue *in vitro* development and to transmit a maternal recognition message. They presently offer the best method for rapid selection of embryos for ET and it can be assumed that with fertilisation and pregnancy rates they offer a method of comparing and contrasting the relative success of treatment regimes. Embryo morphology and EDR can predict implantation potential and prevention of multiple pregnancy will involve analysis of these and assessment of other factors such as maternal age and previous pregnancy (Dawson *et al.*, 1991; Waterstone *et al.*, 1991).

4.2 Luteinising hormone: effects upon oocytes during the periovulatory period

4.2.1 Patient responses during cycles stimulated using hMG or combined GnRH-a/hMG therapy

All cycles treated with long course analog therapy during this study attained standard criteria for hCG administration without complication from endogenous pituitary activity, confirming previous reports that the use of adjuvant GnRH-a therapy significantly reduces the necessity for cycle cancellation due to inappropriate LH surges (Fleming & Coutts, 1986; Rutherford et al., 1988).

The number of oocytes recovered in the two groups was similar, as were cleavage rates and indices of embryo quality. Comparable pregnancy rates per ET confirmed that embryos had similar potentials for implantation. In analog treated patients this rate (19.3%) was similar to that per OR (18.7%), and perhaps of most importance to patients, per cycle started (18.7%). Cancellation of 32% of hMG cycles resulted in a disparity between the rate per ET (18.1%) and that per initiated treatment (10.7%).

4.2.2 Advantages and disadvantages of adjuvant GnRH-a therapy

Published studies comparing adjuvant GnRH-a therapy with exogenous gonadotrophins (Berg van de-Helder et al., 1990; Thanki & Schmidt, 1990) or CC/hMG (Rutherford et al., 1988; Abdalla et al., 1990; Lejeune et al., 1990; Kubik et al., 1990) agree that elimination of spontaneous LH surges results in increased success, when related to initiated stimulation cycles. Five studies reported higher oocyte yields but, with the exception of Lejeune et al. (1990), fertilisation rates were similar. An increased conception rate/ET and a high (33%) incidence of multiple pregnancy

suggested improved embryo quality in one programme (Rutherford et al., 1988) but this was the exception. No other group reported significantly increased success/ET and Lejeune et al. (1990) found that assessments of embryo quality and subsequent implantation rates were similar.

Interpretation of individual studies is complicated by variations in methodology between treatment groups. Different criteria for hCG administration were applied to the treatment regimens compared in two studies (Rutherford et al., 1988; Thanki & Schmidt, 1990) and Abdalla et al. (1990) utilised the flexibility afforded by analog therapy to avoid weekend recoveries. Since follicle size has an influence on the recovery rate of oocytes (Scott et al., 1989) and subsequent fertilisation, cleavage and embryo quality (3.4) some apparent advantages of pituitary desensitisation may be attributable to extended follicular growth.

A further investigation suggested improved uterine receptivity as the factor responsible for higher pregnancy rates/ET in analog cycles compared with those following CC/hMG treatment (Testart et al., 1989). A detrimental influence of CC may be implicated (Rogers et al., 1986) but improved synchronisation between the embryo and endometrium by elimination of pre-hCG luteinisation might also be important.

4.2.3 Oocyte post maturity

It is of interest that embryology results in the present study were comparable despite inclusion of a sub-population, adversely affected by pre-hCG luteinisation, in the group treated with hMG alone.

Post-maturity was exclusively associated with cycles in which an LH surge (often attenuated) was retrospectively

detected and affected fewer than 25% of the oocyte population. Typically, PM oocytes were recovered from large acellular cysts, cumulus cells were aggregated within a friable matrix and the oocyte was obscured by a darkened, contracted corona radiata. The aspiration process or gentle pipetting was often sufficient to detach cumulus and corona cells. Fertilisation anomalies and meiotic failure were frequent.

A relationship between these phenomena and subnormal luteinisation is supported by experimental evidence from the rabbit showing that functions of the mid-cycle LH surge can become dissociated if the signal is insufficient (Bomsel-Helmreich et al., 1989). Superovulated does were given hCG doses ranging from five to 100 IU. Although 5-10 IU could reinitiate meiosis, progressively higher doses were required for completion of each meiotic stage and >15 IU induced asynchronous nuclear development among oocytes in preovulatory follicles of the same ovary. Some oocytes attained metaphase II after 15-20 IU of hCG but the associated cumulus was only partially dissociated. Thirty IU were required for luteinisation of granulosa cells but a proportion of follicles showed cystic transformation and condensation of cumulus cells around the oocyte. Ovulation was the function requiring the largest stimulus with the number of ruptured follicles being dose proportional.

4.2.4 Classification of sub-optimal oocytes

A retrospective study of 219 patients undergoing OR after CC or CC/hMG ovarian stimulation grouped cycles according to the pre-hCG concentration of LH (Munabi et al., 1990). Luteinising hormone levels >50% above baseline were associated with a significant reduction in pregnancy rate and higher concentrations (>2 x baseline) resulted in elevated P levels. These results suggest that cycles were affected by attenuated LH surges but abnormal oocytes

recovered from affected cycles were considered 'atretic'. This highlights the fact that, although there is broad agreement on classification of 'normal' oocytes, considerable confusion exists over the terms atresia, post-maturity, over-maturity and dysmaturity. Clarification is important since different factors may be involved in their origin.

Trounson et al., (1983) reported that if hCG was given when E_2 showed a marked fall the follicles were 'atretic' and recovered oocytes had no potential for fertilisation or development. A similar report found that if hCG was withheld until E_2 plateaued or declined following CC or CC/hMG stimulation (Kerin et al., 1984), 'postmature' oocytes were recovered and the pregnancy rate declined. These cycles did not have a detected LH surge but were associated with rising concentrations of P and low levels of FF androstenedione. Mechanisms involved may be luteinisation, reducing FSH levels in CC cycles failing to maintain follicular growth (Fleming & Coutts, 1982b) or a mixture of both.

A high (35%) incidence of follicular 'atresia' and recovery of morphologically abnormal oocytes has been reported in a programme where patients are 'coasted' for 2-3 days between final administration of gonadotrophin and hCG (Jones, 1984). This anomaly may also be due to cessation of developmental stimuli. Oocytes recovered from leading follicles of >23mm, which were associated with increased polyspermy, decreased recovery, fertilisation and cleavage rates (Ben-Rafael et al., 1986a) may represent another phenomenon. Lobo et al. (1985) described 'dysmature' oocytes from CC/hMG cycles and compared FF steroid concentrations with those of classically atretic follicles recovered from unstimulated patients. The abnormal oocytes were aspirated from large (>18mm) follicles, had no cumulus, corona or first PB but had

undergone GVBD. They were associated with higher levels of P and E_2 and lower androgen concentrations than atretic FF.

Some aspects of follicular luteinisation and atresia are similar (Bomsel-Helmreich & Huyen, 1982) and this may be reflected in oocyte morphology. However, if recovery of abnormal oocytes is considered relevant, publication of full descriptions and photographs might lead to a consensus on nomenclature.

4.2.5 Abnormal luteinising hormone profiles in human reproduction

Elevated follicular phase concentrations of LH have been implicated in several aspects of impaired human reproduction. Stanger and Yovich (1985) found reduced *in vitro* fertilisation, embryo quality and pregnancy rate in patients with basal LH levels >1SD above the population mean. Similarly reduced implantation and an increased abortion rate occurred when high tonic levels of urinary LH were measured during CC/hMG stimulation for IVF (Howles *et al.*, 1986). Cycles affected by LH surges were eliminated from both studies. Thomas *et al.* (1989) questioned the relevance of these results since parametric statistical methods were applied to non-Gaussian distributions and investigated the hypothesis by comparing follicular phase profiles of 596 consecutive patients with LH ranges from similarly stimulated conception cycles. No relationship between LH and IVF outcome was found.

However, *in vivo* studies have added further evidence for an adverse effect of LH elevations on fecundity. Homburg *et al.* (1988) investigated patients with polycysic ovarian syndrome undergoing ovulation induction with pulsatile luteinising hormone releasing hormone. Basal LH levels were significantly lower in patients who conceived than

those whose treatment failed. Elevated concentrations during the latter stages of follicular development were correlated with non-ovulation and early pregnancy loss. A similar relationship with conception and abortion rates was reported in a population of normally cycling women followed over an 18 month period during which they were attempting to conceive (Regan, 1991).

In the absence of direct experimental evidence, the mechanism for these effects upon oocyte development can only be a matter of speculation. Investigations using isolated mouse follicles have shown that oocyte health can be adversely affected by changes in the FF hormonal milieu caused by altered patterns of steroid synthesis (Tyler & Collins, 1980). Contrary evidence is provided by an investigation of the predictive value of pre-hCG P concentrations in patients stimulated with hMG and/or FSH after suppression with the GnRH-a leuprolide acetate (Edelstein *et al.*, 1990). No significant effect on the number or classification of oocytes recovered, fertilisation rates or resulting pregnancy was found. A slight but significant correlation was found between P and E_2. Another possible effect at the follicular level is suggested by *in vitro* studies showing that LH reduces granulosa cell DNA synthesis (Hillier, 1991).

The mid-cycle LH surge initiates resumption of meiosis, mediated by a complex array of inhibitory and stimulatory factors (Plachot & Mandelbaum, 1990). Premature initiation of these processes might result in ovulation of 'aged' oocytes still capable of fertilisation. Evidence from IVF programmes has shown reduced implantation rates after late fertilisation (Oehninger *et al.*, 1989; Trounson & Webb, 1984; Pampiglione *et al.*, 1990), possibly reflecting an increased incidence of genetic anomalies (Ben Rafael *et al.*, 1986b; Plachot *et al.*, 1988a). An observed association between increasing miscarriage rate and

extension of the delay from ovulation to intercourse would support a similar effect *in vivo* (Guerrero & Lanctot, 1970; Guerrero & Rojas, 1975).

Meiosis in the rabbit can be reinitiated by relatively low doses of hCG (Bomsel-Helmreich *et al.*, 1989) but completion of the process may be dependent on other LH controlled functions. One feature of cumulus expansion is a reduction in coupling of the oocyte with the corona cells (Eppig, 1982). The cytoplasmic processes through the zona pellucida which form gap junctions with the oolemma permit low molecular weight substances to pass between the cells and may permit one cell type to influence another (Lawrence *et al.*, 1978). These processes are completely retracted at the time of ovulation (Dekel *et al.*, 1978) under the influence of the oviductal environment. Both Eppig (1982) and Moor *et al.* (1981) suggest that meiotic resumption is not a function of reduction in intercellular coupling but that the gap junctions are required for transmission of the signal.

4.2.6 Conclusions

A great variety of methods for induction of multiple follicular growth for IVF and GIFT are currently utilised, reflecting a lack of consensus on which regime provides the most acceptable balance of efficacy and costs. Adjuvant GnRH-a therapy has considerable advantages for clinical management and allows oocyte development without interference from LH fluctuations. The effects of detectable LH surges are reflected in oocyte morphology and developmental potential but the full role of LH in human fecundity remains unclear.

4.3 Interval from luteinisation stimulus to ovulation in women treated with combined therapy: implications for assisted conception methodology

4.3.1 Oocyte maturation *in vivo* and *in vitro*

The hours following the luteinising stimulus are a period of intense nuclear and cytoplasmic activity in human oocytes. The resumption of meiosis from the dictyotene stage to metaphase II when the process is again arrested, awaiting fertilisation, is accompanied by changes in the zona pellucida and ooplasm. These allow recognition and penetration by a spermatozoon, formation of pronuclei, further reduction of the maternal genome, early cleavage stages and transition from maternal to embryonic gene expression.

Pincus and Enzmann (1935) first showed that mammalian oocytes, removed from healthy antral follicles, resumed meiosis when cultured *in vitro*. This has been shown to be true for all mammalian species studied, including humans (Edwards, 1965b), but the capacity of such oocytes for normal fertilisation and development is minimal without an inductive *in vivo* period followed by further *in vitro* maturation (Moor & Trounson, 1977; Niwa & Chang, 1975). Zona pellucida maturation (Plachot & Mandelbaum, 1978) and development of factors involved in male pronucleus formation (Thibault & Gerard, 1973) have been implicated in this phenomenon.

4.3.2 Timing of ovulation after hCG administration

Study 1 (3.3) showed that, in patients whose endogenous LH was suppressed, a 39 h post-hCG *in vivo* maturation time could be practiced without risk of pre-operative ovulation (Table 3.3.1). Cumulus expansion increased with extended LORD (Table 3.3.2) and cleavage rates were significantly

higher ($p< 0.02$) in oocytes retrieved ≥36 hours after the luteinising stimulus than in those from ORs <36 hours after hCG. All oocytes were preincubated for the same period (6 +/-1 h) in this preliminary study.

4.3.3 Ovulation processes

It is of interest that oocytes were successfully recovered from a proportion of 'ovulated' follicles by introducing the aspiration needle through the point of rupture and flushing with medium. Evidence suggests that ovulation is not an explosive event. *In vivo* experiments on the baboon (Beck & Blair, 1977) using a method to distinguish between ovarian contractions and movement of surrounding ligaments and mesenteries could not confirm previous reports of spontaneous ovarian contractions (Virutamasen *et al.*, 1973). No significant change in intrafollicular pressure could be measured in the periovulatory period in the rabbit (Espey & Lipner, 1963) and direct observation in the human described a gradual flow of fluid (Edwards & Steptoe, 1975) without evidence of muscle contractions.

4.3.4 *In vitro* oocyte maturation before insemination

The study which indicated a requirement for an *in vitro* incubation before insemination in human IVF programmes was conducted on patients treated using a variety of stimulation regimes (Trounson *et al.*, 1982b; Table 1.3.1). Oocyte retrieval was timed from either spontaneous or induced luteinisation and although minimum criteria for hCG administration included u/s visualisation of a follicle ≥17mm in diameter, some decisions to proceed with OR were based on changes in cervical mucus in the absence of detectable follicle growth. Subsequent observations have suggested that a more individual approach to each oocyte could be beneficial. A preincubation period for preovulatory oocytes from leading follicles (≥18mm)

appeared to be unnecessary for CC/hMG stimulated oocytes recovered 36 h post-hCG but the fertilisability of secondary follicles was enhanced by *in vitro* maturation (Lopata, 1983). Optimal timing of OR after an endogenous LH surge also obviated the requirement for an incubation period before insemination (Edwards *et al.*, 1984).

Variation of preincubation time from 6-24h, according to apparent oocyte maturity based on the degree of cumulus expansion achieved comparable fertilisation rates among three categories of oocyte maturity (Laufer *et al.*, 1983). This approach was extended by examining nuclear maturity and delaying insemination until extrusion of the first polar body was observed (Veeck, 1985). More than 80% of immature oocytes were successfully matured *in vitro* and 82.3% subsequently fertilised. However, fertilisation rates were lowest where *in vitro* maturation was necessary from the GV stage. No pregnancies resulted from 27 transfers of embryos derived solely from this group but embryos from oocytes which had undergone GVBD but had not extruded PB1 had implantation rates similar to those from mature oocytes (Veeck, 1988).

Transfusion of ooplasm from metaphase II to GV oocytes has been shown to overcome this developmental block (Flood *et al.*, 1990). Ribonuclease A was shown to interfere with the process, implicating a messenger RNA as the cytoplasmic factor necessary for oocyte competence.

4.3.5 Influence of *in vivo* maturation time upon fertilisation

In study 2, the highest incidence of fertilisation was again observed in oocytes removed from the ovary after an extended (39 hour) hCG/OR delay (Table 3.3.7). Normal fertilisation occurs by penetration of the oocyte by a single spermatozoon. Subsequent penetration is prevented

by modification of the zona pellucida by release of cortical granule contents into the perivitelline space. Cortical granules are produced in the prematurational period by the Golgi complex and migrate to the cortex during maturation (Sathananthan & Trounson, 1982b; Bomsel-Helmreich *et al.*, 1987). Close association of cortical granules with the vitelline membrane is considered essential for cortical granule release and the zona reaction (Longo, 1973).

The high incidence of polyspermy observed in the short/0 group was significantly reduced ($p<0.05$) by either *in vitro* preincubation and/or extension of the *in vivo* maturation, suggesting that migration of cortical granules and development of the block to polyspermy are phenomena dependent on the time from initiation of the luteinising stimulus.

4.3.6 Animal models

The outcome of oocytes inseminated after premature removal from the follicular environment has been investigated in two animal species. Oocytes were removed from preovulatory follicles of sheep at various intervals from hCG administration and transferred to the oviducts of simultaneously inseminated recipients (Trounson *et al.*, 1982a). Embryos were recovered seven days after transplantation and classified as single-cell, abnormal or fully differentiated blastocysts. The number of unfertilised oocytes decreased as duration of *in vivo* maturation increased and an initially high incidence of abnormality decreased with time, the lowest frequency being from ovulated oocytes. Normal embryos were found in significant numbers after a 12h follicular maturation and comprised 97% of those originating from ovulated oocytes.

Badenas *et al*. (1989) investigated the effects of immaturity (immediate insemination of oocytes removed from ovaries 2-3 and 3-4h before ovulation and overmaturity (3,6 and 12h *in vitro* preincubation) on fertilisation rate, polyploidy, polyspermy and digyny in the mouse. The control group comprised oocytes obtained from the oviducts immediately after ovulation. Fertilisation rates were lowest in immature oocytes. Only 16.6% of those harvested 3-4h before ovulation fertilised, despite 81% having attained nuclear maturity. Cytoplasmic immaturity was suggested by arrest at the pronuclear stage (16.7%) and failure of male pronucleus development (45.5%). Fertilisation was improved in oocytes obtained 2-3 before ovulation (65.6%) but was still significantly lower than controls (78.2%).

In vitro maturation of ovulated oocytes for 3 hours increased fertilisation but the effect decreased after 6 h and significantly fewer oocytes fertilised after 12h preincubation. Increased cumulus expansion has been implicated in the short term improvement by *in vitro* maturation (Yanagimachi, 1981) and zona hardening (Inoue & Wolf, 1974) in the subsequent reduction in sperm permeability. No such effects of overmaturity were observed in the present study, but the intervals may not be comparable.

Chromosome analysis revealed an increased incidence of polyploidy in embryos derived from immature oocytes and the 3 and 6h overmature groups. This was related to polyspermy in all groups and to fertilisation of diploid eggs, mainly caused by endoreduplication, in the immature group. This anomaly, a duplication of the haploid chromosome complement without cytokinesis, would be visualised as 2PN after fertilisation and is not therefore implicated in the high incidence of 3PN in the short/0 group of study 2.

4.3.7 Cytoplasmic maturation during the periovulatory period

A final maturational stage in pre-ovulatory oocytes matured *in vitro* has been reported (Sundstrom & Nilsson, 1987). In oocytes with a first polar body extruded, the distribution of mitochondria and the appearance of endoplasmic reticulum (ER) was influenced by the duration of hCG and *in vitro* culture time. Aggregates of ER surrounded by mitochondria appeared after 3-5h *in vitro* culture and were found in an oocyte recovered at mid-cycle in a natural cycle. It was suggested that asynchrony between nuclear and cytoplasmic maturity was corrected by preincubation and that this maturation was responsible for the increase in the proportion of normal embryos described by Trounson *et al.* (1982b).

4.3.8 Timing of *in vitro* fertilisation

In the 2 short groups (short/0 and short/5), 30 (12.9%) of the 232 fertilised oocytes did not have PN visualised between 15 and 21 hours after insemination. The incidence was significantly less (3.9%, $p< 0.001$) in the groups with 39 hours *in vivo* maturation. Failure to observe multiple PN will allow genetically abnormal embryos, which may be morphologically indistinguishable after cleavage, to be transferred. Embryos which develop after late fertilisation, either spontaneously (Oehninger *et al.*, 1989; Trounson & Webb, 1984) or following reinsemination, (Oehninger *et al.*, 1989, Trounson & Webb, 1984; Ben-Rafael *et al.*, 1986b; Pampiglione *et al.*, 1990) are associated with reduced implantation rates. This may be related to the observed increase in the incidence of polyspermy, chromosome anomalies and mosaicism (Ben-Rafael *et al.*, 1986b; Plachot *et al.*, 1988a).

Several factors may be implicated in the apparent delay in fertilisation. Post luteinisation changes include modification of the zona pellucida which decreases its resistance to sperm penetration (Tesarik et al., 1988) and development of factors which support pronucleus formation (Lopata & Leung, 1988). Complete failure of male PN formation can be visualised as prematurely condensed chromosomes (Schmiady et al., 1986). The inverse association of this phenomenon with elapsed time from hCG has been demonstrated experimentally in the mouse (Calafell et al., 1991). Lopata and Leung (1988) have shown that resumption of meiosis and extrusion of the second polar body occurs rapidly in spontaneous cycle oocytes which have been matured in vitro for 30 hours, but more slowly after a 24 hour incubation despite apparent nuclear maturity. Mouse oocytes recovered either 13 or 17 hours after hCG administration exhibited a similar pattern of oocyte response to fertilisation and showed an increased rate of PN development in the older oocytes (Fraser, 1979).

4.3.9 Conclusions

Embryo quality, as assessed by gross morphology, was unaffected by the hCG/OR interval or by immediate or post-incubation insemination. Previous investigation has reported that fragmentation and abnormal cleavage were common sequelae of the attempted fertilisation of immature bovine oocytes and those matured in vitro (Trounson et al., 1977). Incubation in vitro of oocytes matured in vivo for 39 hours had no observed influence on normal fertilisation, embryo development or implantation compared with 39 hour oocytes inseminated immediately.

Laboratory management can be simplified to allow insemination at a time suitable for PN checking the following day. Insemination at the time of OR would reduce

the number of manipulations required, minimising exposure to the potentially harmful effects of temperature change, light (Fischer et al., 1988) and pH fluctuations when incubators are opened.

In conclusion, extension of the in vivo maturation time of human oocytes increased fertilisation rates and obviated the requirement for preinsemination incubation, allowing simplification of laboratory procedures.

4.4 The relationship between follicle size and cumulus expansion and their relative importance as indicators of oocyte maturity

4.4.1 Predictive value of follicle size and cumulus expansion for fertilisation and normal embryo development

The capacity of oocytes for fertilisation and cleavage was related to both follicle size and the degree of associated cumulus cell expansion. This study confirmed the relationship between the two parameters previously determined using both FF volume (Simonetti et al., 1985) and direct measurement by u/s at OR (Scott et al., 1989).

When the outcome of each viable oocyte was assessed in relation to both criteria, follicle size was a more reliable indicator of the probability of fertilisation/cleavage. Embryo quality was correlated with FF volume alone (Fig 11).

4.4.2 Limitations of assessment methods

Accurate estimates of follicle size are best obtained by u/s measurement at the time of aspiration but this method cannot be utilised at laparoscopy and is not suitable for routine use during u/s recoveries due to time restraints. Follicle fluid volume has been reported to correlate well with u/s measurements (O'Herlihy et al., 1980). Although measurement does not present the same logistic problems, apparent FF volume can be affected by leakage at the site of aspiration, accidental puncture of adjacent cysts and variation in fluid content of the dead space in collection needles and tubing.

In spontaneous cycles the degree of cumulus mucification and corona dispersal has been found to correlate well with the stage of meiotic maturation (Testart et al., 1983a)

but this relationship may be disrupted by ovarian stimulation regimes. The degree of cumulus mucification quantified by time required for enzymatic digestion showed no correlation with nuclear maturity of enclosed oocytes following hMG therapy (Laufer et al., 1984). Use of cumulus expansion as an assessment criterion may be further compromised by the optical limitations of dissecting microscopes. Stereoscopic observation was unable to differentiate between cell densities of 210 and 40 cells/mm^2 corresponding to the limits of cumulus expansion observed during the 18 hours preceding ovulation (Bomsel-Helmreich et al., 1987).

4.4.3 The relationship between follicle growth and oocyte maturation

Follicle size has been implicated in many aspects of oocyte and follicle function. A cytological examination of in vivo matured oocyte/cumulus complexes at various intervals after hCG suggested that nuclear synchrony could be assumed in follicles of >16mm, regardless of the degree of cumulus dissociation (Bomsel-Helmreich et al., 1987). In comparison, oocytes from 16mm follicles showed delayed resumption of meiosis and those from 8 - 15.5mm did not complete GVBD. Secretion of PAF, shown to be related to implantation potential, was found to be higher in embryos originating from 4 - 6ml follicles than from either larger or smaller (O'Neill et al., 1987). Spontaneous luteinisation of cultured granulosa cells, shown by secretion of P, is likewise dependent on follicle size (Suzuki et al., 1981).

The pregnancy potential of embryos related to their follicle of origin was investigated in a programme using CC/hMG ovarian stimulation (Nayudu et al., 1987). Volumes between 2.5 and 6.5ml were associated with normal pregnancy and followed six days of sustained E_2 rises.

Embryos derived from larger follicles led to either abnormal pregnancy (biochemical, blighted ovum, early abortion) or no conception. Those from small volumes did not cleave or if replaced, did not implant. These results differ from those reported by Simonetti et al. (1985) who used hMG stimulation and found that pregnancies could be obtained from preovulatory oocytes derived from very small follicles. However, the subsequent abortion rate (10/21; 47.6%) in the small size group was high while all conceptions derived from large (>2ml) follicles (n=16) continued to term. The report concluded that embryo quality was compromised despite apparent oocyte maturity.

It thus appears that the capacity to respond to the luteinisation stimulus by meiotic maturation is acquired earlier than full cytoplasmic and follicular maturity and that small follicles are immature in some respects despite their ability to yield oocytes which have reached metaphase of the second meiotic division. Oocytes from follicles in the mature size range should, therefore, be the primary choice for replacement during GIFT treatment.

4.4.4 Implications for timing of hCG administration

These results have implications for the criteria used to time administration of hCG prior to OR and choice of follicles for aspiration. Trounson et al., (1982b) reported that if hCG was given before follicles reached 17mm diameter, oocytes did not have typical preovulatory appearance and rarely fertilised. An IVF programme utilising CC stimulation found that the probability of a patient having embryos available for ET was related to the number of follicles $\geq$20mm in diameter (Quigley et al., 1982) and suggested that follicles <20mm should not be aspirated; preferring to retain the enclosed granulosa cells to support luteal function. Results from this study would not support such extreme action but suggest that the

size of follicles should be considered if aspiration involves any surgical risk such as proximity of vascular structures or bowel.

Conservative criteria for proceeding with OR may be related to the possibility of complications due to endogenous LH rises. The use of GnRH-a has eliminated this difficulty and improvement in fertilisation and pregnancy rates resulted when administration of hCG was delayed for at least 24h after attainment of standard criteria to avoid weekend ORs (Conaghan et al., 1989). Follicle size at the time of hCG was not reported but it can be assumed that an average increase of 2-4mm in follicle diameter would result from this delay (Bomsel-Helmreich, 1985). No patient in the delayed group suffered from severe ovarian hyperstimulation syndrome suggesting that a change in u/s criteria for hCG administration could be considered.

4.4.5 Conclusions

In conclusion, both the size of the follicle of origin and expansion of the associated cumulus were indicators of the ability of an oocyte to fertilise and cleave. Combined data suggested that FF volume should be the primary selection criterion for oocyte selection for GIFT .

SECTION 2
THE CYTOGENETICS OF EARLY HUMAN DEVELOPMENT

CHAPTER 5
INTRODUCTION

5.1 Cytogenetics

5.5.1 The human chromosome complement

The normal human diploid chromosome number is 46; comprising 22 homologous pairs of autosomes and two sex chromosomes. This number was determined in 1956 (Tjio & Levan, 1956) following some years of controversy. Twenty-three meiotic bivalents were counted in a primary spermatocyte (Ford & Hamerton, 1956) the same year. A nomenclature system based on relative chromosome size and centromere position (Denver Study Group, 1960) was agreed in 1960.

Group A (1-3) have approximately median centromeres and can frequently be distinguished from each other on the basis of size and centromere position.
Group B (4 and 5) have distal centromeres and are generally indistinguishable.
Group C (6-12) is the largest group and consists of medium sized chromosomes with sub-median centromeres. The X chromosome is similar to the largest components of this group.
Group D (13-15) contains three chromosomes with almost terminal centromeres (acrocentric). Variable (polymorphic) satellites representing a region of reduced coiling at the site of a nucleolus organiser region (NOR) may be present on the short arm.
Group E (16-18) have median or submedian centromeres and can be distinguished from each other.
Group F (19 and 20) consists of two small metacentric chromosomes.
Group G (21 and 22) contains the two smallest chromosomes which are acrocentric and polymorphic. The Y chromosome is similar.

5.1.2 Chromosome identification

Individual homologues can be differentiated by staining techniques reliant on variation in DNA and protein composition of chromosomes. Distinctive banding patterns on each chromosome were first visualised after staining with quinacrine mustard (Caspersson et al., 1970) which shows enhanced fluorescence in AT-rich DNA (Comings, 1978). This affinity also highlights the polymorphic short arms of the acrocentric D and G group chromosomes. The most widely used technique in clinical laboratories is G-banding which involves partial chromosome digestion with the proteolytic enzyme trypsin. The mechanism is poorly understood (Comings, 1978). C-banding stains heterochromatin at the centromere region and on the long arm of Y (Sumner, 1972) and is used to visualise the polymorphic regions of chromosomes 1, 9, 16 and Y.

Polymorphisms on 10/22 human autosomes can be readily detected using standard cytogenetic methods (Hassold, 1985). These techniques identify variation in inherited heterochromatic chromosome regions which, because of their proximity to the centromere are rarely involved in crossing-over (Mikkelsen et al., 1980). Visually assessed staining techniques are subject to limitations; scoring is somewhat subjective and is affected by technical factors. Use of restriction fragment length polymorphisms (RFLPs) and the Xg blood are more objective but, because many identify sites affected by genetic exchange, are only useful for identification of the parental origin, not the meiotic division.

Cells spend a relatively small proportion of their cycle in mitosis, when contracted chromosomes can be visualised (Zetterberg, 1970), and cytogenetic techniques frequently utilise mechanisms for maximising the proportion of cells in metaphase. Colchicine was the first mitotic arrestant

found and, with its synthetic derivative Colcemid, remains the most frequently used. The alkaloid, derived from Colchicum species, prevents tubulin polymerisation into microtubules without interference in cell growth. Cells entering mitosis are blocked at metaphase but chromosome condensation continues (Hsu & Satya-Prakash, 1985).

5.1.3 Terminology

Aneuploidy is a condition in which an organism (or cell) possesses fewer (hypo) or more (hyper) chromosomes than an exact multiple of the haploid number.

Nondisjunction is the term commonly used to describe any defect giving rise to aneuploidy. Used more specifically (Bridges, 1913), it refers to the failure of chromosomes to disjoin during division. Both homologues pass to one pole and hypoploid and hyperploid products result. Figure 13 illustrates non-disjunction at metaphase I; the bivalent passes to one cell during anaphase I and, after normal separation of chromosomes at the second division, two nullosomic and two disomic gametes are formed. Centromeric polymorphisms are heterozygous. This feature differentiates the phenomenon from non-disjunction at the second division (Figure 14) which results in homozygous polymorphisms in disomic daughter cells.

Nonconjunction (Sturtevant & Beadle, 1940) is lack of homologue pairing, whether due to initial failure of the process (asynapsis) or it's subsequent breakdown (desynapsis).

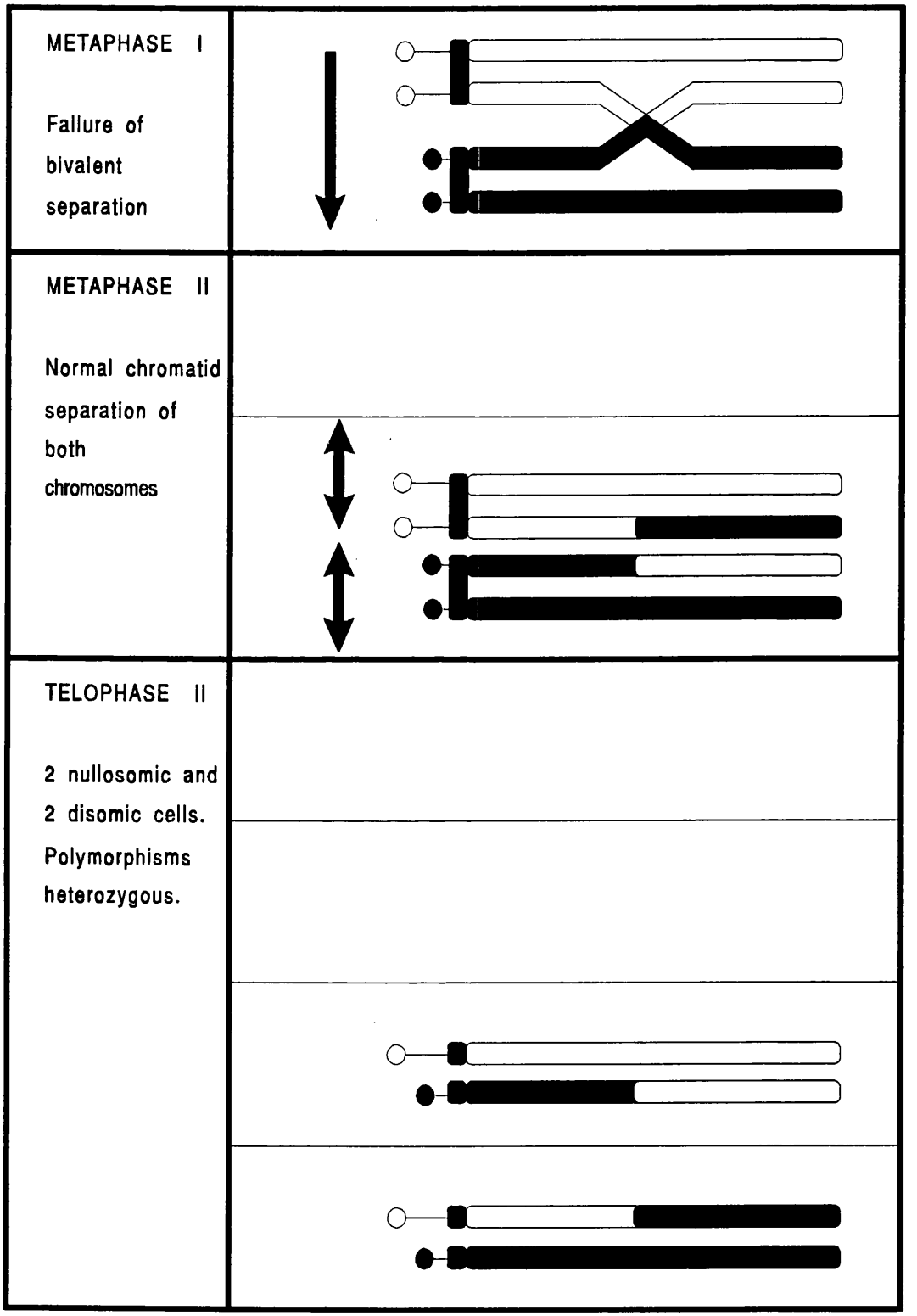


Figure 13

Diagrammatic representation of primary non-disjunction.

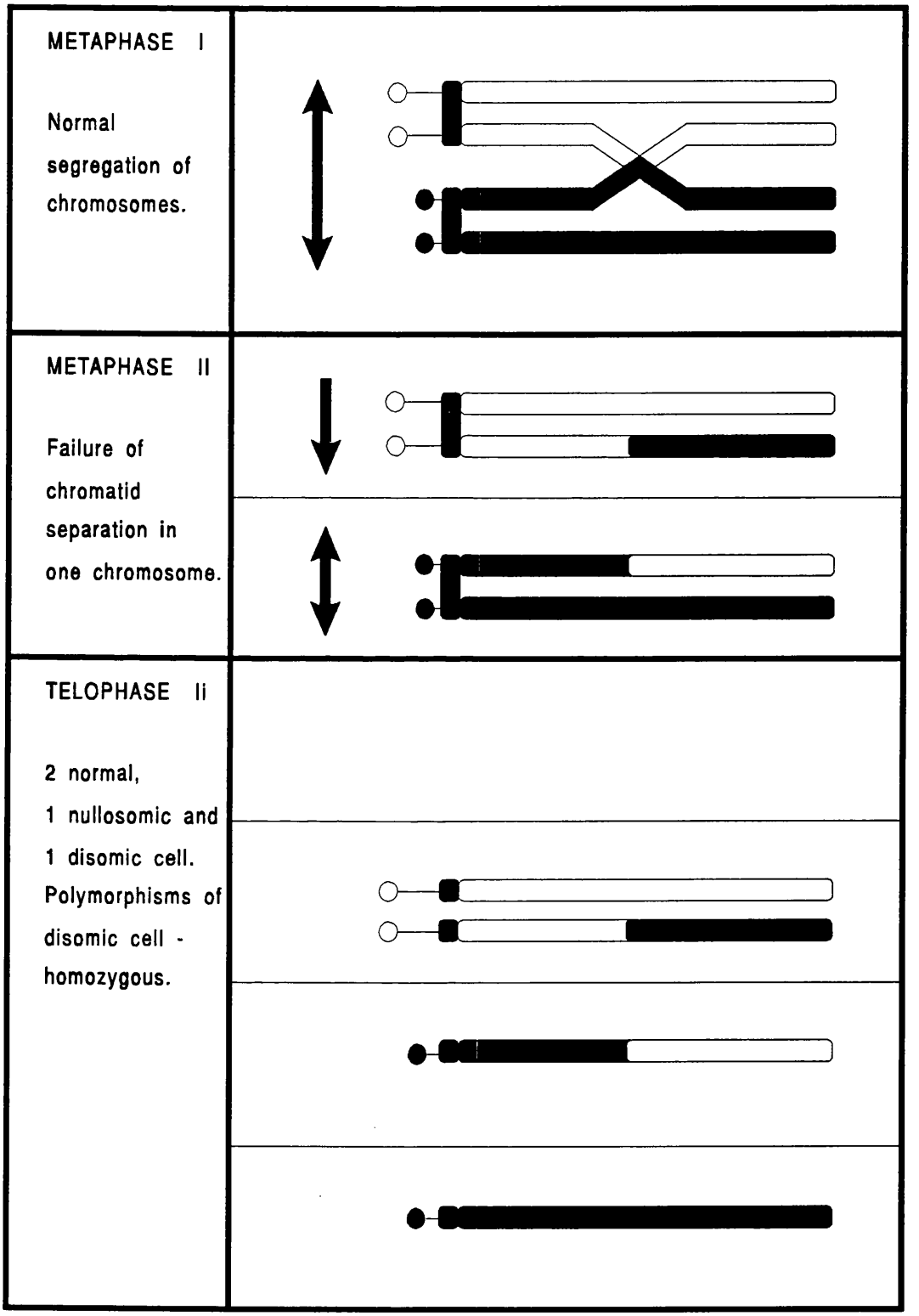


Figure 14

Diagrammatic representation of secondary non-disjunction.

5.2 Human fecundity

5.2.1 Definition and estimate of fecundity

Demographic studies of populations ranging from 18th century rural French villagers to modern day British women ceasing non-hormonal contraception have shown remarkably similar estimates of fertility (Short, 1979). Fecundability, defined as the probability of full term pregnancy per menstrual cycle during which intercourse took place, ranged from 21 to 28% between the ages of 20 and 30 with a suggestion of maximal rates at age 25; an incidence considerably lower than domestic mammals (Hanly, 1961).

5.2.2 Foetal mortality

Evidence that low fecundity is related to early foetal mortality has accumulated from several sources. A statistical model devised by Roberts and Lowe (1975) postulated that 2,437 million married women in England and Wales participated in 63,362 million acts of unprotected coitus of which 1/14 (2,263 million) would be within 48h of ovulation. They assumed a 50% fertilisation rate and concluded that 505,000 recorded deliveries represented only 22% of zygotes, the majority being lost before any delay in menstruation.

Evidence from investigations using B-hCG assessments, in normal women attempting to conceive, initially suggested postconceptual pregnancy losses of 62% (Edmonds et al., 1982) and 43% (Miller et al., 1980). Walker et al. (1988) criticised these studies on the basis of assay crossreaction with LH in cycles where ovulation was predicted from menstrual dates alone. Their own investigation used a specific but insensitive B-hCG assay and failed to find any evidence of 'biochemical' pregnancy in 75 cycles, which included 25 clinical pregnancies.

Investigation of 707 cycles with an equally specific, but more sensitive, assay detected biochemical evidence of pregnancy in 198 patients. One hundred and fifty-five were clinically confirmed, suggesting 22% subclinical pregnancy loss (Wilcox et al., 1988). Confirmation that early studies may have overestimated the phenomenon comes from the reported 15% incidence of transitory B-hCG rises after IVF-ET (Edwards, 1986).

Hertig et al. (1959) painstakingly examined the reproductive tracts of 107 women of known fertility undergoing therapeutic hysterectomy. The group comprised patients with demonstrated ovulation, coital dates within 24 hours of ovulation and absence of ovarian, tubal or uterine pathology. The success of ova recovery was related to the expected site, based on menstrual dates and endometrial morphology, at the time of hysterectomy. Recovery was poorest (1/9) when specimens were expected to be in the "relatively voluminous" fallopian tube. Uterine flushing was efficient for recovery of pre-implantation morulae and blastulae (7/15) but early implantation was difficult to observe (2/30). The authors considered that embryonic stages after day 12 (>day 25 of the menstrual cycle) "gave us the best opportunity to deduce the fertility rate in patients who are known to be fertile". Five of 18 implanted embryos observed at this stage were morphologically abnormal. All 34 recovered 'ova' had undergone cleavage and it was assumed that unfertilised oocytes had degenerated, although the actual number could not be deduced. Overall, 10/34 embryos were considered abnormal and the relative proportions of normal and abnormal conceptuses in pre (4:4) and post (20:6) implantation stages lead to the conclusion that "some bad eggs are worse than others" and that the degree of abnormality was reflected in implantation rates.

Limited information on the earliest stages of *in vivo* development was provided by 53 cycles of uterine lavage of women donating embryos for transfer to infertile recipients (Buster *et al.*, 1985). Artificial insemination was consistently performed on day 0 or +1 relative to the LH peak followed by lavage, initially attempted on day five, when 24 of 25 recovered ova were identified. Recovered ova suggested a trimodal distribution with peaks at one cell (4/25), 8-15 cells (7/25) and blastocyst (5/25). All intrauterine pregnancies (n=3) resulted from blastocyst transfer. This source of information ceased because of the problem of retained pregnancy in donors (Sauer *et al.*, 1989).

5.2.3 Involvement of chromosome abnormality in pregnancy loss

Two reports, in 1961, of triploid cell lines derived from abortus material (Delhanty *et al.*, 1961; Penrose & Delhanty, 1961) were the stimuli for systematic cytogenetic investigation of spontaneous and induced abortions. A review of initial studies revealed inadvertent selection of both patients and culture tissue (Carr, 1971) but implicated karyotypic anomaly as a causative factor in pregnancy loss.

The incidence of spontaneous pregnancy loss after delayed menses has been estimated at 15% on the basis on personal interviews (Warburton & Fraser, 1964) and life table analysis (Harlap *et al.*, 1980), with highest losses during the first trimester. Comparison of published studies with the distribution pattern of reported miscarriage has shown a not unexpected deficiency of karyotyped samples from the first weeks of pregnancy (Burgoyne *et al.*, 1991), attributable to the occurrence of abortions at home and difficulty in obtaining suitable tissue for culture from the earliest developmental stages (Kajii *et al.*, 1980).

However, it is clear that approximately 50% of abortions occurring between eight and eleven weeks are associated with a major chromosome abnormality, regardless of geographical location (Kajii et al., 1980; Hassold et al., 1980; Creasy et al., 1976; Warburton et al., 1980a). Given the limitations of these studies, perhaps the most reliable data on the incidence of numerical abnormalities in clinical pregnancies has come from surveys of early induced abortions (Burgoyne et al., 1991; Yamamoto & Watanabe, 1979; Zhou et al., 1989). These studies found similar rates of abnormality (4.6%, 6.1%, 5.1% at 7-8 weeks gestation) but represented an abnormal maternal age distribution. Burgoyne et al. (1991) corrected the pooled data for the general population and obtained a figure of 4.7% for a gestation equivalent to five weeks of foetal development; an incidence considerably higher than any other mammalian species investigated (Chandley, 1981).

Expulsion of chromosomally abnormal conceptions continues throughout pregnancy but they comprise a lower proportion of later abortions (Creasy et al., 1976; Warburton et al., 1980a) and are detected in only 5% of stillbirths (Angell et al., 1984). Natural processes are therefore responsible for reduction of the heavy genetic load apparent in early conceptions to 0.6% of newborns (Evans, 1977).

5.2.4 Types and origin of chromosome abnormalities detected in spontaneous abortion

Table 5.2.1 summarises the types and proportions of chromosome defects diagnosed in five surveys of unselected spontaneous abortions (Boue et al., 1975; Creasy et al., 1976; Kajii et al., 1980; Hassold et al., 1980; Warburton et al., 1980a). There was considerable agreement between studies and with the smaller numbers from induced abortion series. (Yamamoto & Watanabe, 1979).

5.2.5 Autosomal aneuploidy

The presence of an additional autosome was responsible for 50% of all abnormal karyotypes (Table 5.2.1). Composite data from studies which utilised banding techniques to differentiate individual chromosomes (Table 5.2.2) showed that all autosomes, with the exception of number 1, were represented in trisomies (Boue et al., 1985). The frequency of abnormality showed considerable variation among chromosomes.

Table 5.2.1
Types of chromosome abnormalities detected in 2196 spontaneous abortions and their relative frequency; from Boue et al., 1985

Abnormality	Number detected (n)	Proportion of total (%)
Numerical abnormalities		
Autosomal trisomy	1105	50.3
Autosomal monosomy	2	0.1
45,X	410	18.7
Triploidy	361	16.4
Tetraploidy	123	5.6
Mosaicism	48	2.2
Structural abnormalities	85	3.9
Other	62	2.8

Almost one third of cases involved number 16, a sub-metacentric member of the E group with a large heterochromatic region at the centromere. Acrocentric D and G group chromosomes collectively comprised more that 37% of karyotypes but individually did not approach the

frequency of trisomy 16. Trisomies 3, 5, 6, 11, 12 and 17 occurred rarely.

Table 5.2.2
Autosomal trisomies found in spontaneous abortions and their relative frequency; from Boue et al., 1985

Trisomy	Number	Percent	Trisomy	Number	Percent
1	0	0	12	9	1.0
2	53	5.1	13	63	6.1
3	8	1.0	14	51	5.0
4	26	2.5	15	75	7.2
5	1	1.0	16	326	31.4
6	3	1.0	17	7	1.0
7	46	4.4	18	51	4.9
8	37	3.6	19	1	1.0
9	33	3.2	20	28	2.7
10	22	2.1	21	89	8.6
11	2	1.0	22	107	10.3

The recognised association between maternal age and Down's Syndrome (Penrose, 1934) in livebirths has also been found for trisomies 13 and 18 detected at amniocentesis (Ferguson-Smith, 1983). Aneuploid abortions show a similar overall relationship (Kajii et al., 1980; Hassold et al., 1980) but individual chromosomes, again, are variable. Maternal ages were highest for trisomies involving the smallest chromosomes (13-22) with the exception of number 16 which, with groups A, B and C was only moderately associated with increasing maternal age (Hassold et al., 1984). Suggestions of a paternal age effect for trisomy 21 (Stene et al., 1977) were not confirmed by a larger series of cases detected at amniocentesis (Ferguson-Smith, 1983).

The polymorphic nature of the most common trisomic chromosomes has allowed investigation of the parental origin of the additional material in both livebirths and abortions. Seventy-six percent of 110 families with a

Down's syndrome child were informative using fluorescence heteromorphisms (Mikkelsen et al., 1980). The majority (80.5%) of errors occurred in the ovary with 60.9% of informative cases attributable to a defect of maternal meiosis 1 although DNA polymorphisms have suggested that this may be an underestimate (Antonarakis & Down Syndrome Collaborative Group, 1991). A similar distribution of parental origin has been detected in the larger variety of abnormalities found in abortus material (Hassold & Jacobs, 1984). Maternal first division errors were predominantly responsible for the small numbers of trisomies 3,4,9,14 and 15 which were informative. Nine of 17 trisomies 21 and 21/28 trisomies 22 were similarly assigned. It is of interest that the same error was responsible for 25/34 supernumerary 16s (Hassold & Jacobs, 1984), despite a limited association with maternal age.

Theoretically, non-disjunction gives rise to equal numbers of trisomic and monosomic gametes but autosomal monosomy occurs very rarely in human clinical pregnancies (Table 5.2.1). Only deficiency of chromosome 21 has been consistently noted (Ohama & Kajii, 1972).

A mouse model for production of trisomic and monosomic embryos by matings involving carriers of Robertsonian translocations has been developed to investigate the viability of aneuploid offspring. Initial studies demonstrated trisomies but no monosomies after 12-13 days of gestation (Epstein et al., 1977) and prompted investigation of preimplantation stages (Epstein & Travis, 1979). Equal numbers of trisomic and monosomic embryos were found in three day late morulae and early blastocysts but 24 hours later monosomy for chromosome 19, the smallest in the complement, had virtually disappeared. The phenomenon did not appear to involve expression of a lethal recessive allele. Differential survival of trisomies has also been demonstrated with only trisomy 19

compatible with viability beyond term (Gropp et al., 1974). A similar mechanism is probable in man since both the number and range of aberrations reduce during gestation and those detected in late abortions and perinatal deaths are, mainly, compatible with birth (Angell et al., 1984). A strong correlation between individual anomalies and developmental age, regardless of gestational age at expulsion, would also support this hypothesis (Boue et al., 1985).

5.2.6 Sex chromosome monosomy (45,X)

The most common abnormal karyotype detected in human abortions is 45,X; monosomy X (Table 5.2.1). Losses in early pregnancy occur at a developmental age of 6 weeks, frequently with embryonic maceration (Boue et al., 1985). Later abortions can be identified by oedema, cystic hygroma and horseshoe kidneys (Singh & Carr, 1966) and the phenotype of those who survive to term constitutes Turner's Syndrome (Turner, 1938).

In contrast to autosomal anomalies, the incidence shows an inverse relationship with maternal age (Warburton et al., 1980b; Kajii & Ohama, 1979) and maternal meiotic non-disjunction is not implicated. Restriction length fragment polymorphisms of the X chromosome have indicated the absence of a male sex chromosome in 6/10 45,X spontaneous abortions (Hassold et al., 1985); an incidence not inconsistent with 77% identified by Xg blood grouping in women with Turner's syndrome (Sanger et al., 1977). This incidence is incompatible with the demonstrated frequency of sex chromosome nullisomy in sperm (Martin & Rademaker, 1990) and the observed relationship between expected reciprocal karyotypes (XXX and XXY) and maternal age (Kajii & Ohama, 1979). A similar phenomenon in the mouse can largely be attributed to loss of the paternal

chromosome after fertilisation (Russell & Montgomery, 1974).

5.2.7 Triploidy

Using the above data for the incidences of miscarriage, chromosomally abnormal abortuses and types of abnormality (Table 5.2.1), it can be deduced that approximately 1% of recognised pregnancies are triploid; an incidence corroborated by data from induced abortions (9/1250, 0.7%; Yamamoto & Watanabe, 1979). Analyses using chromosome polymorphisms (Jacobs et al., 1978; Uchida & Freeman, 1985) and HLA typing (Couillin et al., 1987) indicate that the majority (69%) of triploids arise from diandry: an additional haploid set of paternal chromosomes. Thirty-eight percent could be attributed to dispermy; fertilisation of a haploid oocyte by two sperm, and this origin could not be excluded in cases attributed to a male meiotic error. The apparent rarity of diploid sperm would support this interpretation (Brandriff et al., 1984 & 1985; Martin et al., 1983).

A strong correlation between the origin of the additional haploid set and phenotype has been demonstrated (Jacobs et al., 1982). Partial moles, with hydatidiform changes in the placenta, are associated with paternally derived triploids and probably represent a weaker version of complete hydatidiform moles, which are paternally derived parthenogones (Kajii & Ohama, 1977). They are not, however, associated with trophoblast disease (Szulman & Surti, 1978). The expected ratio of XXX:XXY:XYY sex chromosome complements in triploids of dispermic origin is 1:2:1. The ratios of XXX to XXY (Jacobs et al., 1982) generally fit this hypothesis but XYY complements are under-represented (Kajii & Niikawa, 1977). The observation is generally explained by non-implantation or early expulsion of XYY triploids but poor growth in culture

prior to chromosome analysis may artificially affect data (Boue et al., 1985).

The anomaly rarely continues to term and no correlation with maternal age has been detected (Boue et al., 1975; Creasy et al., 1976; Kajii et al., 1980).

5.2.8 Tetraploidy

The sex chromosome constitutions of all analysed tetraploids have all been XXXX or XXYY, suggesting replication of a normal diploid complement without cytokinesis at an early stage of development (Kajii & Niikawa, 1977). Superimposed aneuploidy has also been duplicated in a 94,XXXX,+16,+16 abortus (Boue et al., 1985). Embryonic development is rarely observed (Boue et al., 1985) and development to term is uncommon (Golbus et al., 1976; Pitt et al., 1981). An observation of three paternal contributions was made in a complete mole investigated by Sheppard et al. (1982).

5.2.9 Mosaicism

The mean figure of 2.2% mosaicism given in Table 5.2.1 disguises considerable variation among studies (1.6-8.7%), probably reflecting differences in methodology such as tissue used for culture, number of cells and tissues analysed, length of culture and number of passages. The occurrence of 'true' mosaicism in amniotic cell cultures is similarly variable (Ferguson-Smith, 1983) but in some cases, abnormal lines are confined to extra-embryonic tissues and normal healthy infants have been born (Ferguson-Smith & Ferguson-Smith, 1983). This finding has been confirmed at earlier gestations investigated by chorionic villus sampling (Goldberg & Golbus, 1988). Mosaicism in abortuses rarely involves acrocentric

chromosomes (Stene & Warburton, 1981) and meiotically derived trisomy with subsequent loss of one homologue may be the origin of the normal cell line (Hassold, 1982).

5.2.10 Structural abnormalities

Surveys of liveborn children have shown a mean rate of 0.05% for unbalanced structural abnormalities and 0.19% for apparently balanced rearrangements (Evans, 1977). Warburton et al. (1980a) concluded that 85% of unbalanced karyotypes with implicit partial trisomy and/or monosomy were aborted. Both miscarriage and livebirth data show approximately equal numbers of Robertsonian and reciprocal translocations and a similar ratio of inherited and de novo anomalies. An excess of apparently balanced de novo translocations in surveys of individuals in mental institutions (Funderburk et al., 1977) was thought to reflect undetected deletions or duplications. No such surplus was detected in spontaneously aborted pregnancies (Warburton et al., 1980a).

5.2.11 Chromosome anomalies at conception

Estimates of the incidence of chromosome abnormality at conception have been based on extrapolation of data from spontaneous abortion and have assumed that abnormalities arise with equal frequency in each chromosome. Boue et al., (1975) postulated that the most frequent anomalies detected (trisomy 16 and 45,X) represented the true incidence for all chromosomes and that other anomalies perished before establishment of a clinical pregnancy. This gave an estimate of 50% abnormality at conception. Burgoyne et al., (1991) used another approach; using a calculation based on the proportion of anomalies attributable to male meiotic abnormality and their observed incidence in sperm, he suggested that 20% of embryos would be aneuploid. This figure is in good

agreement with the small numbers of observations from IVF embryos (Angell *et al.*, 1986a).

5.3 Aims

The aims of the work presented in section two of this thesis were:

1. To determine the nuclear condition of oocytes remaining uncleaved after insemination *in vitro* and relate cytogenetic findings to aspects of IVF methodology.

2. To determine the incidence of chromosome abnormalities in human oocytes and investigate the origin of anomalies.

3. To investigate maternal factors affecting the occurrence of chromosome abnormalities and determine whether selection against fertilisation of abnormal gametes operates.

4. To investigate the incidence and types of chromosome abnormality in early cleavage stages of human embryos and correlate cytogenetic and morphological assessments of embryos.

CHAPTER 6
MATERIALS AND METHODS

6.1 Cytogenetic techniques

6.1.1 Microscope slides

Glass slides (pre-washed; Berliner Glas KG) were soaked in 2% Decon 90 (Decon Laboratories Ltd) overnight, washed (minimum one hour) under running tap water and soaked overnight (x2) in distilled water. Slides were dried in a hot air oven (45^{o}C) and stored in sealed containers until required.

6.1.2 Oocyte and embryo fixation

Solutions

Colchicine (Sigma) stock solution: 80 ug/ml in Hanks Balanced Salt Solution (Gibco BRL); stored frozen.
Colchicine working solution (0.4 ug/ml) was prepared daily in culture medium supplemented with 1% patient's serum (2.4.3).
Hypotonic solution: 1% sodium citrate in distilled H_2O; freshly prepared.
Fixative 1: 100% methanol.
Fixative 2: 5:1; methanol:acetic acid; freshly prepared.
Fixative 3: 3:1; methanol:acetic acid; freshly prepared.
Leishman's stock solution was prepared by dissolving 1.5 g of Leishman's stain (BDH Chemicals Ltd) in 1 l methanol and was filtered before storage.
Leishman's stain was prepared daily from stock solution in Gurrs buffer; pH 6.8 (BDH). Dilutions are given in the text.

Methods

Oocytes for cytogenetic analysis were re-examined for PB and PN before fixation and were processed individually. After an initial 15 minute incubation in hypotonic solution at room temperature, each oocyte was transferred in a minimal droplet of solution to a microscope slide.

Three to five drops of each fixative (1, 2 and 3) were sequentially dropped onto the cell from a height of 1-2 cm under microscope observation (microscope as for 2.4.7). Premature collapse or movement of the oocyte was recorded and the final position of the cell noted. Slides were air dried, stained in 25% Leishman's stain, rinsed in tap water and air dried.

Embryos were reassessed before transfer to individual culture tubes containing colchicine solution; an overnight incubation (mean 19.3 hours) was generally used. Fixation and staining methodology was identical to that described for oocytes.

6.1.3 C-banding

Solutions

HCl: 50% (in distilled H_2O); used immediately.
BaOH: a saturated solution was prepared by dissolving 0.5g in 50 ml distilled H_2O using a magnetic stirrer. The solution was filtered and used immediately.
2 x saline sodium citrate (2 x SSC): 17.5 g NaCl + 8.8 g sodium citrate, 2 hydrate in 1 l distilled H_2O; adjusted to pH 7.0 using 1M NaOH.

Method

After initial analysis slides for C-banding were de-stained in methanol and air dried. Slides were treated for 30 minutes in 50% HCl, rinsed in distilled water and transferred to BaOH at 60°C. An initial exposure of 45 seconds was used on test slides and time in BaOH was adjusted as required. A further incubation in 2xSSC at 60°C for 90 minutes was performed, slides were rinsed, air dried and stained in 10% Leishman's stain.

6.1.4 Microscopy and photography

Analyses were performed using a BH-2 microscope (Olympus Optical Co Ltd) fitted with x10 (D Plan 10), x40 (D Plan 40) and x100 (S Plan 100 oil immersion) lenses and a x3.3 photographic eyepiece. Chromosome spreads were photographed using a PM-10AK automatic exposure photomicrographic system and C-35AD4 camera back (Olympus Optical Co Ltd).

Photographs were taken on Kodak Technical Pan film rated at 32 iso and processed film was printed on Ilford Multigrade or Ilfospeed paper. Karyotypes were prepared after cutting individual chromosomes from the photographs.

6.2 Study design

6.2.1 Cytogenetic analysis of uncleaved oocytes.

Patients

A total of 653 uncleaved oocytes from 166 patients undergoing 188 cycles of IVF/GIFT were fixed for cytogenetic analysis (Table 6.2.1). All had multiple follicular growth induced using long course GnRH-a/hMG therapy and comprised three groups.

1. Four hundred and sixty-one oocytes from cases where semen parameters appeared normal.
2. Cases with impaired semen quality as defined in 2.4.4 (113 oocytes).
3. Seventy nine oocytes from 27 cycles in the study investigating the effects of LORD and preinsemination incubation (3.3.7).

Table 6.2.1
Cytogenetic analysis of uncleaved oocytes: cycles, recovered oocytes and fertilisation rates

	Semen Quality	
	Normal	Poor
Cycles (n)	141	20
Oocytes recovered (mean)	9.7	10.5
Fertilisation rate (% of viable oocytes)	48.2	11.0
Oocytes fixed	461	113

Criteria for classification of ploidy

Preparations were classified as haploid if chromosome counts were 23+/-5 and diploid if 46+/-5. These criteria allowed classification of cells with poorly spread or slightly distorted chromosomes.

Detailed analysis

Karyotype analysis was only carried out if the chromosomes were in a discrete group and an accurate count could be performed. The quality of each karyotype was assessed at the time of preparation. A score of one was given for preparations of outstanding quality, with well spread chromosomes of good morphology. Slightly poorer cells with either overlapping chromosomes or less defined chromatin were scored as two. Assessments of three and four were given for cells where placing of individual chromosomes proved difficult but counting and grouping were possible. Photographed spreads which proved ambiguous when karyotyping was attempted were discarded and assessment was made on the basis of ploidy alone. Haploid cells with fewer than 21 chromosomes were considered incomplete and not karyotyped.

Chromosome, centromere and chromatid breaks and gaps were ignored if components remained aligned and were attributed to fixation artefacts.

6.2.2 Cytogenetic analysis of early embryos

A total of 816 cleaved embryos from 187 ORs (164 patients) were mitotically arrested with colchicine and fixed for cytogenetic analysis (Table 6.2.2). The majority of patients (147 cycles) were undergoing IVF. This number includes four cases where partner's semen was assessed as poor and 36 patients included in the study investigating

the effects of LORD and preinsemination incubation (2.6.3). The fertilisation rate in GIFT cycles (n=40) was based on spare oocytes, inseminated for investigation of gamete compatibility, after selection of 'best' oocytes for immediate transfer.

Table 6.2.2
Cytogenetic analysis of embryos: cycles, treatment, recovered oocytes and fertilisation rates

	Total (n)	IVF (n)	GIFT (n)
Patients (n)	187	147	40
Oocytes recovered (mean)	10.7	11.0	9.5
Fertilisation rate (% of viable oocytes)	63.6	69.5	38.7
Embryos fixed (n)	816	678	138

Criteria for classification of ploidy

All haploid embryos were classified on the basis of complete grouping or karyotyping of one or more cells. Classification as diploid was based on a single cell only if grouping or full karyotyping was possible. An approximate count (46+/-5) was considered sufficient if more than one cell could be estimated. Triploidy was similarly assessed but all greater degrees of polyploidy were based on approximate counts of single metaphase spreads.

Detailed analysis

Criteria for detailed analyses and assessment of karyotypes were those used for oocytes (2.6.5).

CHAPTER 7
RESULTS

7.1 Cytogenetic assessment of uncleaved oocytes: nuclear and cytoplasmic maturity

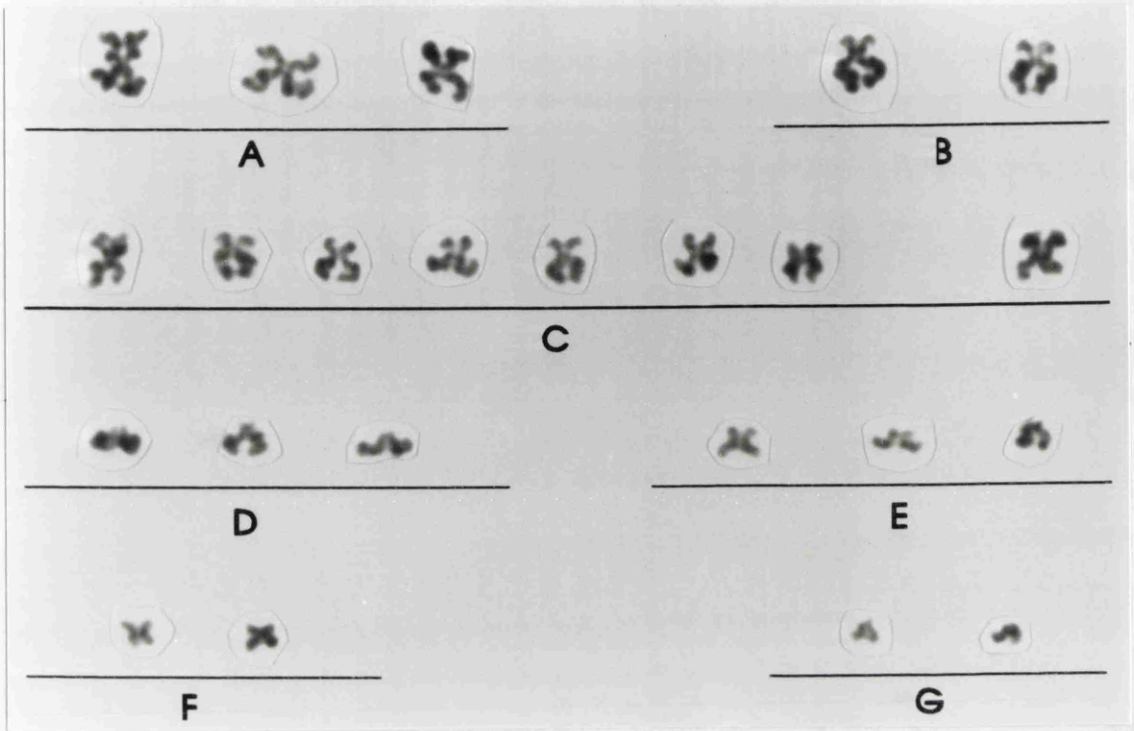
7.1.1 Assessment of nuclear status: total data

Analysis of uncleaved oocytes was possible in 71.4% (466/653) of attempted chromosome preparations (Table 7.1.1). The main reason for unsuccessful analysis (n=167) was the complete absence of chromosomes despite the visible outline of a zona pellucida. Estimation of ploidy was not possible in 20 cells due to insufficient spreading (n=10) or overdispersion of nuclear contents (n=10).

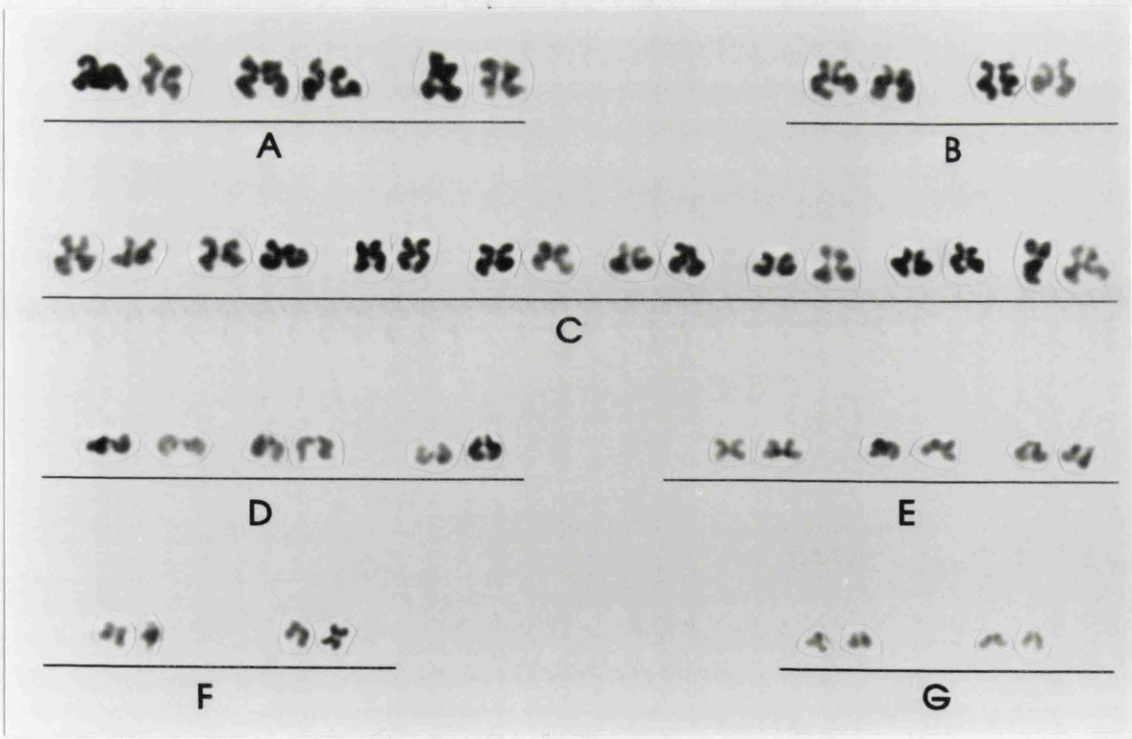
Human meiotic chromosomes were readily differentiated from those in mitotic metaphase by their curled chromatids, in close proximity only at the centromere. Figure 15a shows the haploid chromosome complement of a normal metaphase II oocyte which failed to fertilise after insemination *in vitro*.

Polar body chromosomes were fuzzy, indistinct and rarely analysable (Figure 16a). The group was spatially well defined and rarely interfered with observation of oocyte chromosomes. Elongated single stranded chromatids were identified as prematurely condensed chromosomes (PCC) of sperm, as originally described by Schmiady *et al*. (1986; Figure 16b).

The majority of analysed oocytes (399; 85.6%) had a grossly haploid number of meiotic chromosomes consistent with metaphase II. This second 'resting' phase of meiosis is normally maintained until fertilisation triggers completion of the reduction division but the presence of condensed sperm chromosomes in 37 (9.3%) cells indicated that sperm penetration had occurred in some without response.



a



b

Figure 15
Oocyte karyotypes: a) normal metaphase II (23,X) and
b) diploid metaphase II with a 46,XX chromosome constitution.

a

b

Figure 16

a) Polar body chromosomes and b) Premature condensation of sperm chromosomes in a metaphase II oocyte.

Table 7.1.1

Cytogenetic analysis of uncleaved oocytes: total data

Analysis	Number of oocytes
Oocytes fixed	653
Uninformative analyses	187
No chromosomes found	167
Chromosomes unanalysable	10
Chromosomes overspread	10
Informative analyses	466
Metaphase II	362
Metaphase II + PCC	37
Metaphase 1	2
Diploid metaphase II	19
Diploid metaphase II + PCC	9
PCC sperm chromosomes only	7
Mitotic chromosomes	22
Haploid	3
Diploid	7
Triploid	1
Tetraploid	5
Polyploid	4
Undetermined	2
Other	8
PN chromosomes	3
Endoreduplication	1
Meiotic tetraploid	1
Polyploidy + PCC	1
Interphase nucleus	1
Elongated degenerated chromosomes	1
Fragmented chromosomes	38
Metaphase II	32
Diploid metaphase II	4
Unanalysable	2
Karyotyped cells	192
Metaphase II	178
Diploid metaphase II	8
Mitotic	5
Metaphase 1	1

7.1.2 Nuclear status of oocytes failing to cleave after insemination with normal and poor semen

Cytogenetic outcome of cases with normal and poor semen is detailed in Table 7.1.2. The proportion of cells which yielded informative analyses (72.1% and 69.9%) was similar in the two groups and it can be assumed that results are representative of each population. More than 90% of oocytes which failed to fertilise in the presence of deficient sperm were at metaphase II. None showed evidence of sperm penetration. Significantly more (chi-sq, $p<0.01$; 20.9%) uncleaved oocytes in the group with normal semenology demonstrated fertilisation or oocyte activation.

Karyotype results from Tables 7.1.2 to 7.1.8 will be discussed in section 7.2.

Arrest at an earlier stage occurred in 28 cells which had undergone GVBD but retained the haploid set of PB chromosomes within the ooplasm (Figure 15b). These oocytes were classified as diploid metaphase II and a significantly higher (chi-sq, $p<0.001$) proportion (9/28; 32.1%) contained PCC of sperm than metaphase II oocytes (37/399; 9.3%). The meiotic stage of seven cells which retained only sperm chromosomes after fixation could not be determined but multiple penetration had occurred in 6/7. Only two oocytes, from the same cycle, remained paired in metaphase I. These were distinguished from endoreduplicated chromosomes by their meiotic morphology and observation of heterozygous polymorphisms of centric heterochromatin after C-banding.

Despite lack of cleavage, mitotic chromosomes were found in 22 oocytes. The largest single group (n=11) were diploid but three had been parthenogenetically activated and triploidy, tetraploidy and polyploidy were detected.

A number of individual anomalies which could not be included in the above categories are listed in Table 7.1.1. Three cells contained haploid sets of single chromatids consistent with PN complements prior to replication.

Thirty-eight cells contained severely fragmented chromosomes. The proportion of metaphase II to diploid metaphase II cells (32:4) was similar to that in the total series (399:28).

Table 7.1.2
Cytogenetic analysis of oocytes from cycles with normal or impaired semen parameters

Analysis	Semen Quality Normal (n)		Poor (n)	
Oocytes fixed	461		113	
Informative analyses (%)	335	(72.1)a	79	(69.9)
Metaphase II (%)	265	(79.1)b	73	(92.4)
Metaphase II + PCC	19		0	
Diploid metaphase II	14		2	
Diploid metaphase II + PCC	6		0	
PCC sperm chromosomes only	7		0	
Mitotic	17		3	
Other	7		1	
Karyotyped metaphase II cells	130		32	
23,X	99		24	
Aneuploid (%)	24	(18.5)c	6	(18.7)
Individual chromatids	7	(5.4)	2	(6.2)
Structural abnormality (%)	2	(1.5)	0	(0.0)
All abnormalities (%)	31	(23.8)	8	(25.0)

a Informative analyses expressed as a percentage of fixed oocytes.
b Metaphase II oocytes expressed as a percentage of informative analyses.
c Analyses expressed as a percentage of karyotyped metaphase II cells.

7.1.3 Nuclear status of uncleaved oocytes from short and long LORDs; with and without preincubation

Patient selection for LORD study 2 ensured a high fertilisation rate (3.3.7). Cytogenetic analyses of oocytes which remained uncleaved in the four study groups are detailed in Table 7.1.3. Nuclear maturity was determined as metaphase II in 17/20 (85.0%) of informative S/0 cells; 11 (64.7%) of these contained prematurely condensed sperm chromosomes. During the total period of cytogenetic study only two cells in diakinesis were analysed. Both were recovered after a short LORD; one with and one without preinsemination incubation.

Table 7.1.3
Cytogenetic analysis of uncleaved oocytes: short and long LORD with or without preincubation

Analysis	LORD/Insemination Time S/0 (n)	S/5 (n)	L/0 (n)	L/5 (n)
Oocytes unfertilised	36	34	26	22
Oocytes fixed	28	19	13	19
Informative analyses	20	15	8	9
Metaphase II	6	9	5	4
Metaphase II + PCC	11	3	2	2
Diploid metaphase II	1	1	0	1
Diploid metaphase II + PCC	1	0	0	2
Mitotic	0	1	1	0
Other	1	1	0	0
Karyotyped metaphase II cells	8	6	1	1
23,X	5	5	1	1
Aneuploid	2	0	0	0
Individual chromatids	1	1	0	0
Structural abnormality	0	0	0	0
All abnormalities	3	1	0	0

7.1.4 The effect of insemination to fixation interval upon successful preparation and result of cytogenetic analysis

The proportion of oocytes which yielded an analysis was not different in those fixed on day 2 (67.6%), day 3 (76.6%) or day 4 (69.7%: Table 7.1.4). Analytical failure rates due to lack of chromosomes were also similar (day 2, 27.8%; day 3, 21.8%; day 4, 28.8%).

Mitotic cells were found more frequently (chi-sq., $p<0.001$) in oocytes fixed after three days in culture (21.7%) than with those processed on either day 2 (3.3%) or day 3 (2.0%). Prolonged fixation delay was also associated with an increase in cells with only sperm chromosomes identified (day 4, 6.5% v day 2, 0.5% and day 3, 1.3%) but the difference did not reach statistical significance. The incidences of chromosome fragmentation, aneuploidy and structural anomalies were not associated with culture time.

7.1.5 Relationship between nuclear status and indication for treatment

The majority of cycles treated had a tubal occlusion or unexplained infertility (Table 7.1.5). Eight had moderate or severe endometriosis as the primary indication and four had andrological problems. Eighteen patients with ovulatory dysfunction who had failed six cycles of ovulation induction with long course combined therapy (2.2) were treated with IVF.

No differences in the proportions of informative preparations or metaphase II complements were found. It is of interest that 91% of oocytes from patients with ovulatory problems were apparently normal.

Table 7.1.4

Cytogenetic analysis of uncleaved oocytes related to interval between insemination and fixation

Analysis	Day 2 (n)	Day 3 (n)	Day 4 (n)
Oocytes fixed	309	197	66
Uninformative analyses	100	46	20
No chromosomes found	86	43	19
Chromosomes unanalysable	8	2	0
Chromosomes overspread	6	1	1
Informative analyses	209	151	46
Metaphase II	164	121	28
Metaphase II + PCC	23	9	3
Diploid metaphase II	4	12	0
Diploid metaphase II + PCC	7	1	0
Sperm chromosomes only	1	2	3
Mitotic	7	3	10
Other	3	3	2
Fragmented chromosomes	18	10	5
Karyotyped metaphase II cells	71	75	7
23,X	53	57	5
Aneuploid	15	11	2
Individual chromatids	4	6	0
Structural abnormality	1	1	0
All abnormalities	18	18	2

Table 7.1.5
Cytogenetic analysis of uncleaved oocytes related to indication for treatment (tubal, unexplained, endometriosis, androlological or ovulatory)

Analysis	Indication				
	Tubal (n)	Unexp. (n)	Endom. (n)	Andro. (n)	Ovul. (n)
Cycles	100	58	8	4	18
Oocytes fixed	299	250	20	12	72
Informative analyses	218	169	15	10	54
Met II	159	132	15	17	49
Met II + PCC	27	6	0	2	2
Dip met I	9	10	0	0	0
Dip met I + PCC	7	0	0	1	1
PCC chroms only	2	5	0	0	0
Mitotic	9	11	0	0	2
Other	5	5	0	0	0
Karyotyped metaphase II cells	86	57	8	5	22
23,X	64	44	5	4	18
Aneuploid	15	10	3	0	4
Individual c'tids	8	2	0	1	0
Struct abnorm	0	2	0	0	0
All abnorm	22	13	3	1	4

7.1.6 Relationship between nuclear status and degree of stimulation

The degree of stimulation was assessed by the number of oocytes recovered at OR (Table 7.1.6). Although 90% of oocytes from cycles with one to five recovered oocytes were at metaphase II, the proportion of oocytes at this stage of nuclear development was similar if an average number of oocytes were obtained (73.9%) or patients were highly stimulated (>10 oocytes; 79.7%).

Table 7.1.6

Cytogenetic analysis of uncleaved oocytes: relationship with number of oocytes retrieved in cases with normal semenology

Analysis	Oocytes retrieved		
	1-5	6-10	>10
Oocytes fixed	40	190	339
Informative analyses	25	142	241
Metaphase II	23	105	192
Metaphase II + PCC	1	9	23
Diploid metaphase II	1	6	8
Diploid metaphase II + PCC	0	3	5
All other	0	19	13
Karyotyped metaphase II cells	9	47	99
23,X	7	33	78
Aneuploid	1	11	18
Individual chromatids	1	4	2
Structural abnormalities	0	0	2
All abnormalities	2	14	21

7.1.7 Relationship between nuclear status, size of follicle of origin and cumulus expansion

The relationships among follicle size, cumulus expansion and fertilisation rates have been previously described (3.4). A higher incidence of developmental competence was associated with oocytes derived from large ($\geq$2.5ml) follicles and those with fully expanded cumuli. The cytogenetic analysis of uncleaved oocytes in each of these groups is shown in Tables 7.1.7 (follicle size) and 7.1.8 (cumulus expansion). Paradoxically, the highest incidences of oocytes in metaphase II (without evidence of sperm penetration) were found in those groups with lower rates

of fertilisation although differences did not reach statistical significance.

Table 7.1.7

Cytogenetic analysis of uncleaved oocytes: relationship with follicle size in cases with normal semenology

Analysis	Follicle size Small (n)		Large (n)	
Oocytes fixed	79		158	
Informative analyses	62	(78.5%)a	115	(72.8%)
Metaphase II	53	(85.5%)b	87	(75.6%)
Metaphase II + PCC	3		3	
Diploid metaphase II	2		4	
Diploid metaphase II + PCC	1		3	
All other	3		18	
Karyotyped metaphase II cells	23		41	
23,X	18		32	
Aneuploid	3		6	
Individual chromatids	2		2	
Structural abnormalities	0		2	
All abnormalities	5	(21.7%)c	9	(21.9%)

a Informative analyses expressed as a percentage of fixed oocytes.
b Metaphase II oocytes expressed as a proportion of informative analyses.
c Analyses expressed as a proportion of karyotyped metaphase II cells.

Table 7.1.8

Cytogenetic analysis of uncleaved oocytes: relationship with cumulus expansion in cases with normal semenology

Analysis	Cumulus expansion			
	Immature (n)		Mature (n)	
Oocytes fixed	102		336	
Informative analyses	75	(73.5%)a	241	(71.7%)
Metaphase II	63	(84.0%)b	183	(75.9%)
Metaphase II + PCC	2		17	
Diploid metaphase II	1		13	
Diploid metaphase II + PCC	1		5	
All other	8		23	
Karyotyped metaphase II cells	25		90	
23,X	16		70	
Aneuploid	6		16	
Individual chromatids	3		4	
Structural abnormalities	0		2	
All abnormalities	9	(36.0%)c	20	(22.2%)

a Informative analyses expressed as a percentage of fixed oocytes.
b Metaphase II oocytes expressed as a proportion of informative analyses.
c Analyses expressed as a proportion of karyotyped metaphase II cells.

7.1.8 Nuclear status of oocytes failing to cleave after evidence of fertilisation

A small number (n=31) of oocytes in the study had failed to cleave after PN had been visualised on day 1 following insemination. The proportion of informative analyses was rather low (13/31; 42.0%); details are given in Table 7.1.9. The only cell with a single PN, and 5/9 of apparently normally fertilised (2PN) oocytes remained at meiotic metaphase II with only one showing evidence of

sperm penetration in the form of PCC of sperm. A high proportion of cells in this group (5/13; 38.5%) had nuclei undergoing mitotic division without cytokinesis. It is of interest that one oocyte with 2PN had a triploid chromosome constitution. Both cases with >3 PN contained multiple sets of PCC.

Table 7.1.9
Cytogenetic analysis of oocytes which remained uncleaved after PN formation

Analysis	Number of PN			
	1 (n)	2 (n)	3 (n)	>3 (n)
Oocytes fixed	1	25	3	2
Informative analyses	1	9	1	2
Metaphase II	1	4	0	0
Diploid metaphase II + PCC	0	1	0	0
PCC sperm chromosomes only	0	0	0	2
Mitotic	0	4 **a**	1 **b**	0

a Diploid x 2, triploid x 1, tetraploid x 1
b Hexaploid

7.1.9 Relationship between morphological and cytological assessment of nuclear status

Polar body status of oocytes was reassessed at the time of fixation and the number present, related to nuclear status is shown in Table 7.1.10. Absence of any PB is normally interpreted as failure of the first meiotic division but 33/60 (55.0%) informative analyses in this category displayed only metaphase II chromosomes, including eight

with PCC. Nineteen oocytes (31.7%) did retain polar body chromosomes, two remained at metaphase I and one contained a tetraploid complement of meiotic chromosomes. Despite the presence of the first PB in the perivitelline space, a small proportion (8/331; 2.4%) of oocytes appeared to retain all meiotic chromosomes. The most common finding in this group was a metaphase II complement, as was the case in oocytes with two, three and fragmented PB.

Table 7.1.10
Cytogenetic analysis of uncleaved oocytes related to polar body status

Analysis	Number of PB			
	0 (n)	1 (n)	2 (n)	3/Frag (n)
Oocytes fixed	77	452	21	7
Informative analyses	60	331	15	7
Metaphase II	25	284	11	5
Metaphase II + PCC	8	23	2	2
Diploid metaphase II	13	6	0	0
Diploid met 2 + PCC	6	2	0	0
PCC sperm chrom only	1	5	0	0
Mitotic	1	10	1	0
Other	6	1	1	0

7.1.10 Summary

The nuclear status of 466 (71.4% of 653) oocytes could be determined after cytogenetic preparation; the majority (399, 85.6%) were at meiotic metaphase II and had a haploid chromosome complement. The first polar body had not been extruded in 6% (28) of oocytes which were classified as diploid metaphase II. Prematurely condensed chromatin of sperm chromosomes, indicating cytoplasmic immaturity were observed in 9.3% (37/399) of metaphase II cells and 32.1% (9/280) of diploid metaphase II oocytes.

The same phenomenon was present in 11/17 oocytes remaining uncleaved after a short LORD and immediate insemination. Approximately 5% (22) of uncleaved oocytes had mitotic chromosomes, three had been parthenogenetically activated. Chromosome fragmentation was significantly higher ($p<0.001$) in women over 39 (9/20, 45.0%) than younger groups (<30, 3.7%; 30-34, 6.3%; 35-39, 7.6%).

7.2 Cytogenetic assessment of uncleaved oocytes: detailed analysis

7.2.1 Incidence and types of chromosome abnormalities detected in uncleaved oocytes

Thirty-two of 178 (18.0%) karyotyped oocytes were aneuploid (Figures 17a and 17b), two (1.1%) had structural anomalies and 11 (6.2%) displayed individual chromatids of one homologue Figure 18. (Table 7.2.1). Overall, 43 abnormal karyotypes were found, giving an estimate of 24.2% abnormality in unfertilised oocytes. The overall incidence of abnormality was not different in cases with normal or deficient sperm (Table 7.1.2) or in cells with or without PCC of sperm chromosomes (Table 7.2.2). It should be noted that a small number of oocytes had both aneuploidy and structural defects and that total abnormal is not always the sum of individual abnormalities.

Chromosome analysis was successfully performed on eight diploid metaphase II oocytes, four of which contained PCC. All had a 46,XX karyotype (Figure 15b).

7.2.2 Karyotype quality of metaphase II oocytes

Detection rate of karyotypic anomaly did not differ in relation to preparation quality (Table 7.2.3). The highest incidence of abnormality occurred in grade 3 cells but the difference between good (1+2) and poor (3+4) preparations was not significant and the majority (129/177; 72.9%) of analyses were based on good preparations with minimal ambiguity.

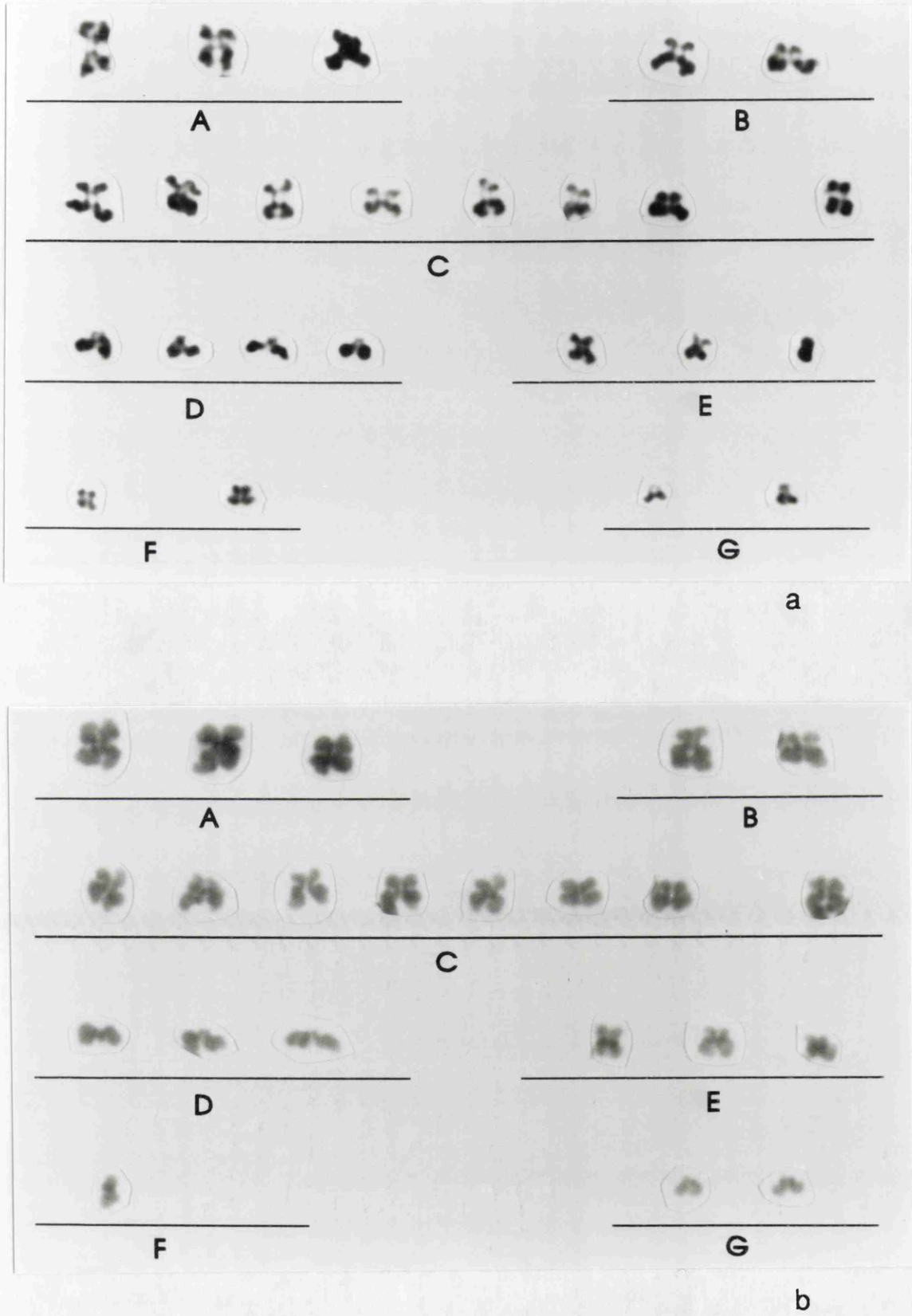


Figure 17

Oocyte karyotypes: metaphase II cells with a) an additional D group chromosome (24,X,+D) and b) nullosomy F (22,X,-F).

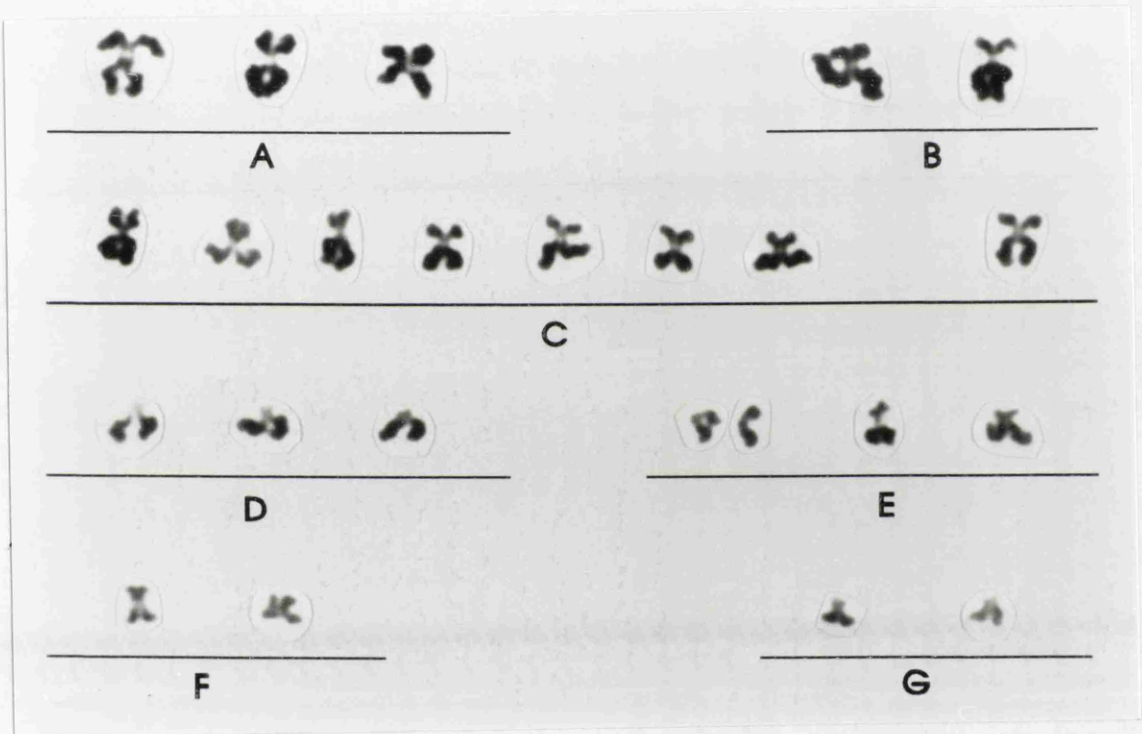


Figure 18
Oocyte karyotype: metaphase II cell with individual chromatids of chromosome 16 (22,X,-16,+cht(16),+cht(16)).

Table 7.2.1

Karyotype details of analysed oocytes

Analysis	n
Metaphase II	178
23,X	135
24,X,+C	1
24,X,+D	4
24,X,+D, cht sep(B)	1
24,X,+E	1
24,X,+18	2
24,X,+F	1
24,X,+G	4
24,X,+?	3
23,X,+D,-G	1
24,+?,+?,-C	1
22,-C	6
22,X,-D	1
22,X,-F	3
22,X,-G	1
21,X,-B,-C	1
23,-C,+ triradial	1
20,-C,-E,-G,+ cht(C)	1
22,X,-16,+ cht(16),+ cht(16)	6
22,X,-F,+ cht(F),+ cht(F)	2
22,X,-G,+ cht(G),+ cht(G)	2
Diploid metaphase II	8
46,XX	8

Table 7.2.2

Cytogenetic analysis of uncleaved oocytes: incidence of abnormality in metaphase II cells with or without PCC

Karyotype	Cell Type: Met 2 (n)	Met 2 + PCC (n)	Total (n)
Total cells	167	11	178
23,X	127	8	135
Aneuploid	30	2	32 (18.0%)a
Individual chromatids	10	1	11 (6.2%)
Structural abnormality	2	0	2 (1.1%)
All abnormalities	40 (23.9%)	3 (27.3%)	43 (24.2%)b

a Expressed as percentage of total cells
b Two cells had both aneuploidy and a structural anomaly.

Table 7.2.3

Cytogenetic analysis of uncleaved oocytes: influence of preparation quality on ascertainment of chromosome abnormalities

Preparation quality	Total cells	Number abnormal	Percentage abnormal
1	69	14	20.3
2	60	16	26.7
3	24	9	37.5
4	24	4	16.7

7.2.3 Chromosomes involved in abnormalities of metaphase II oocytes

Table 7.2.4 details involvement of chromosome groups in aneuploidy and structural defects. Groups D, E and G were most frequently responsible for hyperploidy and groups C, F and G for karyotypes deficient in whole chromosomes. Six of 11 karyotypes with individual chromatids involved chromosome 16. A composite picture is shown in Figure 19.

Table 7.2.4
Cytogenetic analysis of uncleaved oocytes: involvement of chromosome groups in abnormal karyotypes

Group	Aneuploid Hyper (n)	Aneuploid Hypo (n)	Individual chromatids (n)	Total Abnormalities (n)
A	0	0	0	0
B	0	1	0	1
C	1	8	1	10
D	6	1	0	7
E	3	1	6	10
F	1	3	2	6
G	4	3	2	9
Unknown	5	0	0	5
Total	20	17	11	48

7.2.4 Influence of maternal factors and IVF methodology on the incidence of chromosome abnormalities in metaphase II oocytes

The incidence of abnormality was not different in oocytes fixed on day 2, day 3 or day 4 after insemination (Table 7.1.4). Patients with tubal and unexplained infertility had similar proportions of normal and abnormal oocytes (Table 7.1.5). Only 18% of oocytes from patients who had failed ovulation induction were abnormal. Follicle size

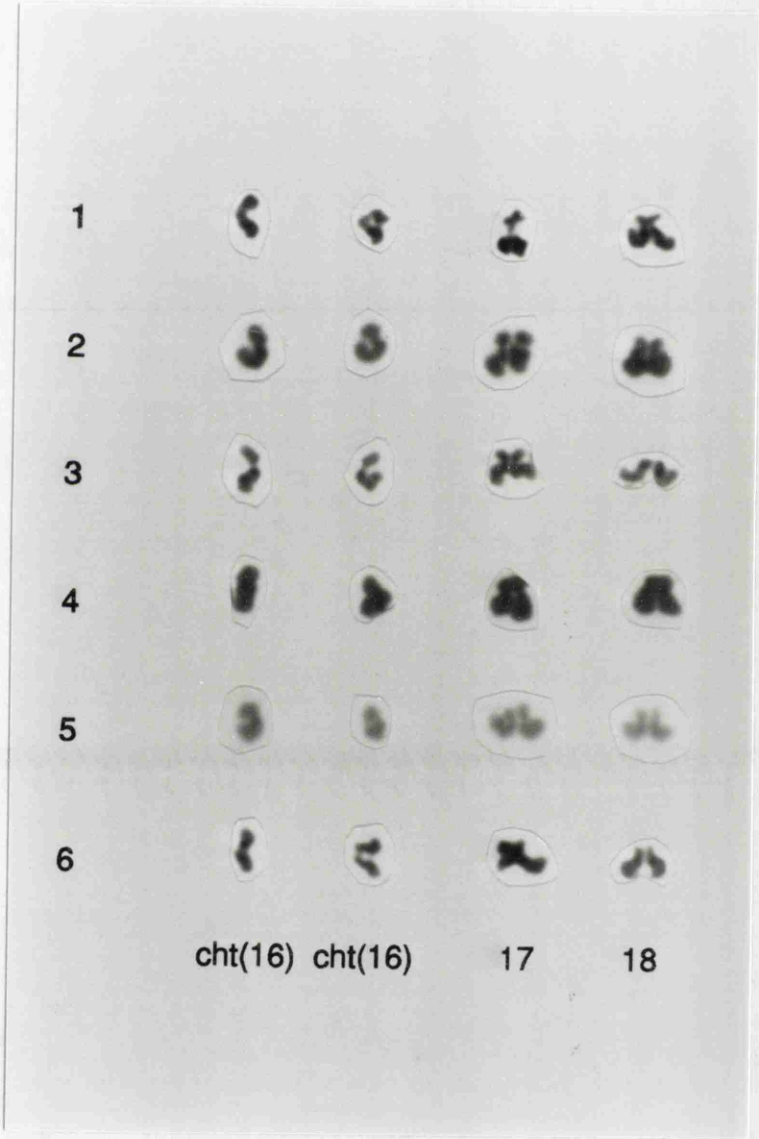


Figure 19
Composite photograph of the E group of six oocytes with individual chromatids of chromosome 16.

(Table 7.1.7) did not affect the incidence of aneuploidy or individual chromatids. Oocytes with an immature cumulus at the time of OR had a higher incidence of abnormality (36.0%) than those classified as mature (22.2%) but the difference was not statistically significant (Table 7.1.8).

7.2.5 The influence of maternal age on nuclear status, chromosome morphology and the incidence of chromosome abnormality

The fertilisation rate of oocytes decreased with maternal age (Table 7.2.5). The difference was statistically significant between ages <30 and 30-34 (chi-sq., $p<0.02$), 30-34 and 35-39 (chi-sq., $p<0.03$) and between those <35 and those ≥35 (chi-sq., $p<0.001$). The proportions of informative cells and of those determined as metaphase II were similar in all age groups (Table 7.2.5). Nine of 20 chromosome spreads from women ≥40 showed severe fragmentation. This incidence was significantly higher (chi-sq., $p<0.001$) than any other age group.

The proportion of metaphase II oocytes which could be karyotyped decreased with maternal age (Table 7.2.6). The difference was significant between ages <30 and 30-34 ($p<0.01$), 30-34 and 35-39 ($p<0.03$) and between those <35 and those ≥35 (chi-sq., $p<0.001$).

The overall incidence of abnormality was not different in the four age groups (Table 7.2.6) although the proportion which was aneuploid increased. The difference did not reach statistical significance but hyperhaploid cells were more frequent (chi-sq., $p<0.01$) in women ≥35 than the two younger groups.

Table 7.2.5

Cytogenetic analysis of uncleaved oocytes: relationship of chromosome constitution with maternal age and fertilisation rate.

Analysis	Maternal age <30 (n)	30-34 (n)	35-39 (n)	≥40 (n)
Oocytes fixed	139	340	119	25
No chromosomes (%)	28 (20.1)a	95 (27.9)	22 (18.5)	4 (16.0)
Informative analyses (%)	108 (77.7)a	237 (69.7)	92 (77.3)	20 (80.0)
Metaphase II (%)	87 (80.5)b	183 (77.2)	68 (73.9)	19 (95.0)
Metaphase II + PCC	8	14	13	0
Diploid metaphase II	4	12	2	1
Diploid metaphase II + PCC	1	7	1	0
PCC chromosomes only	0	2	5	0
Mitotic chromosomes	4	14	2	0
Other	4	5	1	0
Fragmented chromosomes (%)	4 (3.7)b	15 (6.3)	7 (7.6)	9 (45.0)
Oocyte retrievals	130	246	122	25
Oocytes inseminated	1101	1969	707	122
Oocytes fertilised (%)	787 (71.5)c	1322 (67.1)	442 (62.5)	75 (61.5)

Percentage of a) oocytes fixed b) informative analyses c) oocytes inseminated

Table 7.2.6

Cytogenetic analysis of uncleaved oocytes: relationship of chomosome constitution with maternal age.

Analysis	Maternal age <30 (n)	30-34 (n)	35-39 (n)	≥40 (n)
Total metaphase II cells	95	197	81	19
Karyotyped metaphase II cells (%)	59 (62.1)a	89 (45.2)	25 (30.8)	3 (15.8)
23X (%)	46 (78.0)b	67 (75.3)	19 (76.0)	1 (33.3)
Aneuploid (%)	9 (15.2)	16 (17.8)	5 (20.0)	2 (66.7)
Hyperploid	5 (8.5)	6 (6.7)	5 (20.0)	2 (66.7)
Hypoploid	4 (6.8)	10 (11.2)	0	0
Single chromatids (%)	4 (6.8)	6 (6.7)	1 (4.2)	0
Structural abnormalities (%)	0	2 (2.2)	0	0
All abnormalities (%)	13 (22.2)	22 (24.7)	6 (24.0)	2 (66.7)

a Karyotyped cells expressed as a percentage of metaphase II cells.
b Analyses expressed as a percentage of total cells karyotyped.

7.2.6 Summary

Karyotypes were prepared from 178 metaphase II and eight diploid metaphase II cells. Abnormalities were detected in 43 (24.2%) haploid oocytes comprising 32 cells with whole chromosome aneuploidy, 11 with individual chromatids of one homologue, and two structural anomalies. Two cells had more than one abnormality and all diploid cells had a 46,XX karyotype. Aneuploidy of acrocentric chromosomes (D and G group) was most frequent (10/20 hyperploid chromosomes) and 6/10 of cases with apparent pre-division of chromatids involved chromosome 16. The incidence of hyperploidy was significantly ($p<0.05$) higher in women ≥ 35 (7/28; 25.0%) than those <35 (11/113; 9.7%). No association between abnormality and any aspect of IVF methodology was determined and selection against fertilisation of abnormal oocytes was not observed.

7.3 Cytogenetic analysis of early embryos: assessment of ploidy

7.3.1 Ploidy assessment and reasons for failure of analysis

Analysis of ploidy was possible in 325 (39.8%) of embryo preparations and 215 (26.3%) were karyotyped (Table 7.3.1). The main reason for analytic failure (n=219) was lack of chromosomes despite the presence of interphase nuclei. Only 37 embryos had no identifiable cells after fixation.

One hundred and seventeen embryos had chromosome complements which could not be assigned ploidy. The majority appeared diploid but did not meet criteria for confirmation and were recorded as unanalysable. Since multiple chromosome sets were generally present, overspreading of individual metaphase plates interfered with analysis in a large number (109; 13.4%) of embryos. Severe fragmentation was recorded in 9 preparations.

7.3.2 Relationship between number of PN and ploidy

Table 7.3.2 details ploidy analysis in relation to number of PN visualised on the day following insemination. When the number available for fixation was corrected by subtraction of those transferred in IVF cycles from total numbers, it appeared that abnormally fertilised embryos (1, 3 and 4PN) had been preferentially fixed.

The highest proportion of successfully analysed embryos were those with one (9/18; 50.0%) or 2PN (167/393; 42.5%). Only 9/35 late fertilised embryos (0PN) were analysable and eight appeared diploid. Parthenogenesis was not the origin of all single pronucleate embryos. A haploid chromosome complement was found in three preparations but 5/9 were diploid and one was tetraploid. Activation of a

diploid oocyte could be excluded in 2/3 karyotyped preparations due to detection of 46,XY chromosome constitutions.

Table 7.3.1
Cytogenetic analysis of embryos: total data

Analysis	Number of embryos
Embryos fixed	816
Uninformative analyses	491
No cells	37
No chromosomes	219
Unanalysable	117
Overspread	109
Fragmented	9
Informative analyses	325
Haploid	7
Diploid	284
Triploid	22
Tetraploid	9
Polyploid	2
Other	1
Karyotyped cells	193
Haploid	5
Diploid	178
Triploid	11
Tetraploid	0
Polyploid	0
Other	1

Diploidy was confirmed in 155/167 (92.8%) of 2PN zygotes but one was haploid and four triploid. Three triploid embryos were fully karyotyped and two had sex chromosome complements compatible with fertilisation of an unreduced oocyte (XXX and XXY). However, the third was karyotyped as 69,XYY and could only be explained by abnormal fertilisation. The remaining embryos in this group were tetraploid (n=4) or octaploid (n=1).

A small number (n=12) of 3 PN zygotes were successfully analysed. The majority (n=9) were triploid but two were tetraploid and one hexaploid. No 4PN embryos were informative.

Table 7.3.2
Cytogenetic analysis of embryos: PN and subsequent ploidy

	Number of PN				
	0	1	2	3	4
Total embryos (n)	142	33	1293	89	8
Available for fixation (n)	85	27	747	89	8
Embryos fixed (n)	35	18	393	54	6
Proportion of available (%)	41.2	66.7	52.6	60.7	75.0
Informative analyses (n)	9	9	167	12	0
Informative analyses (%)	25.7	50.0	42.5	22.2	0
Haploid	0	3	1	0	0
Diploid	8	5	155	0	0
Triploid	1	0	4	9	0
Tetraploid	0	1	5	2	0
Polyploid	0	0	1	1	0
Other	0	0	1	0	0

7.3.3 Relationship between ploidy and embryo quality

The relationship between cytogenetic analysis and embryo quality, as assessed by morphology and growth rate (EDR), is detailed in Table 7.3.3. Parameters of quality were not different in haploid, diploid and triploid embryos but those with $\geq$4n chromosome complements had significantly

poorer (t-test, $p<0.001$) morphology and lower (t-test, $p<0.01$) EDR. When embryos with abnormal ploidy were grouped, both assessment parameters were significantly poorer (t-test, morphology; $p<0.001$: EDR; $p<0.05$).

After fixation, slow growing embryos (EDR<81) had a significantly (chi-sq., $p<0.001$) lower proportion (152/254; 59.8%) of cells in metaphase than other EDR groups (81-90, 91-100, >100; Table 7.3.4). Other groups had similar proportions of mitotically active cells.

Table 7.3.3
Cytogenetic analysis of embryos: relationship between embryo quality and chromosome constitution

Analysis	(n)	Quality assessment: Morphology (Mean score)	Quality assessment: Growth Rate (Mean EDR)
Informative analyses			
Haploid	7	7.0	102.1
Diploid	276	7.8	95.5
Triploid	22	7.2	94.7
Polyploid	11	5.9 **a**	80.4 **b**
All non-diploid	41	6.8 **a**	90.1 **c**
Karyotyped diploid embryos			
46,XX or 46,XY	134	7.9	96.1
Non-mosaic aneuploid	33	8.1	92.1

Significance of difference when compared with diploid embryos (t-test): **a** $p<0.001$ **b** $p<0.01$ **c** $p<0.05$

Table 7.3.4

Cytogenetic analysis of embryos: relationship between mitotic index at time of fixation and growth rate

	EDR			
	0-80	81-90	91-100	>100
Embryos analysed (n)	77	144	98	201
Metaphase cells (n)	152 a	394	243	497
Interphase cells (n)	102	129	66	202
Total cells (n)	254	523	309	699
Proportion of cells in metaphase (%)	59.8	75.3	78.6	71.1

a Proportion of cells in metaphase significantly (chi-sq., $p<0.001$) lower than any other group.

7.3.4 Number of PN and ploidy analysis related to indication for treatment

The largest group of embryos (n=494) studied were obtained from patients with tubal infertility (Table 7.3.5) and a further 206 derived from treatment of unexplained infertility. The only other group with significant numbers comprised patients who had failed ovulation induction therapy for ovulatory disorders.

Eighteen of 520 (3.5%) embryos had a single PN when checked on day 1. The proportion was highest in ovulatory (7.3%) indications but the difference from all other indications was not statistically significant. Three PN were observed in 10.4% of embryos with a significantly higher (chi-sq., $p<0.01$) proportion (21.8%) in women with ovulatory dysfunction.

Table 7.3.5

Number of PN, ploidy and karyotype of embryos related to indication for treatment (tubal, unexplained, endometriosis, androlological, ovulatory)

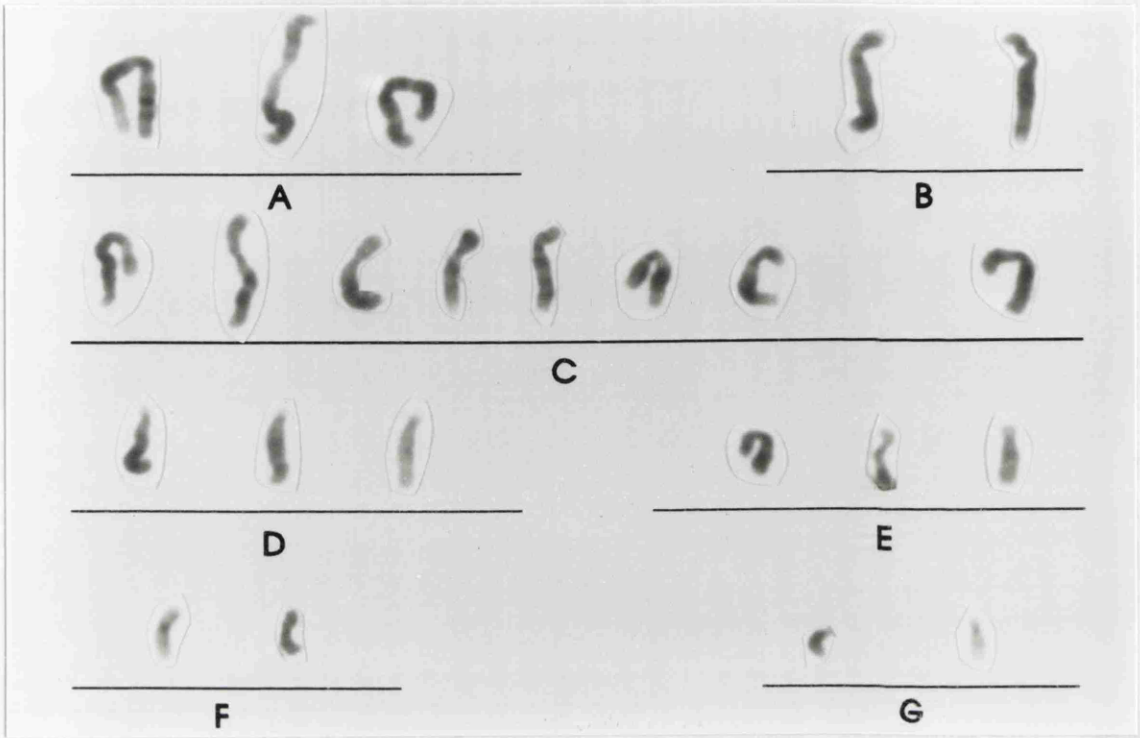
Analysis	Tubal (n)	Unexp. (n)	Endom. (n)	Andro. (n)	Ovul. (n)
Embryos fixed	494	206	22	4	90
Informative analyses	194	79	9	3	40
PN checked	322	135	4	4	55
1PN	7	7	0	0	4
2PN	244	106	3	4	36
3PN	35	6	1	0	12
≥4PN	6	0	0	0	0
Ploidy					
Haploid	4	2	1	0	0
Diploid	165	73	7	3	36
Triploid	18	1	0	0	3
Polyploid	7	2	1	0	1
Other	0	1	0	0	0
Karyotyped diploid embryos	104	43	4	1	20
46,XX or 46,XY	85	31	4	0	18
Non-mosaic aneuploid	19	12	0	1	2

7.4 Cytogenetic assessment of early embryos: detailed analysis

7.4.1 Total data

Karyotype details of all diploid embryos are given in Table 7.4.1 and the results are summarised in Table 7.4.2. Karyotypes of haploid, triploid and other embryos are given in Table 7.4.3.
Three of five karyotyped haploid embryos had a 23,X complement (Figure 20a) and one was hyperhaploid with an additional C chromosome. The fifth embryo had two 'normal' cells and one with only 22 chromosomes. Genuine mosaicism or a preparation artefact may have been responsible.

One hundred and thirty-eight diploid embryos had an apparently normal chromosome complement (Figure 20b) with a ratio of XY to XX karyotypes of 1.15. This sex ratio was significantly (chi-sq., $p<0.05$) different from the 0.48 ratio of aneuploid embryos. Thirty-four preparations showed consistent aneuploidy, four were mosaic and two exhibited structural anomalies; giving a total abnormality rate of 40/178 (22.5%). Involvement of chromosome groups in consistent hyperdiploidy and hypodiploidy is given in Table 7.4.4. Only chromosomes 16 (Figure 21a) and 18 could be identified individually in abnormal karyotypes and these chromosomes comprised 8/10 hyperploidies involving E group. Partial banding allowed the tentative diagnosis of 45,X in one embryo but an XX sex chromosome constitution with a missing C could not be excluded. It should be noted that unbanded cells with a 45,X karyotype would be included in the hypoploid group. Only three embryos with a 45,-C constitution were detected and two had a Y chromosome identified. Only one consistent case of chromosome rearrangement was identified in both cells of an embryo with a derived D group chromosome.

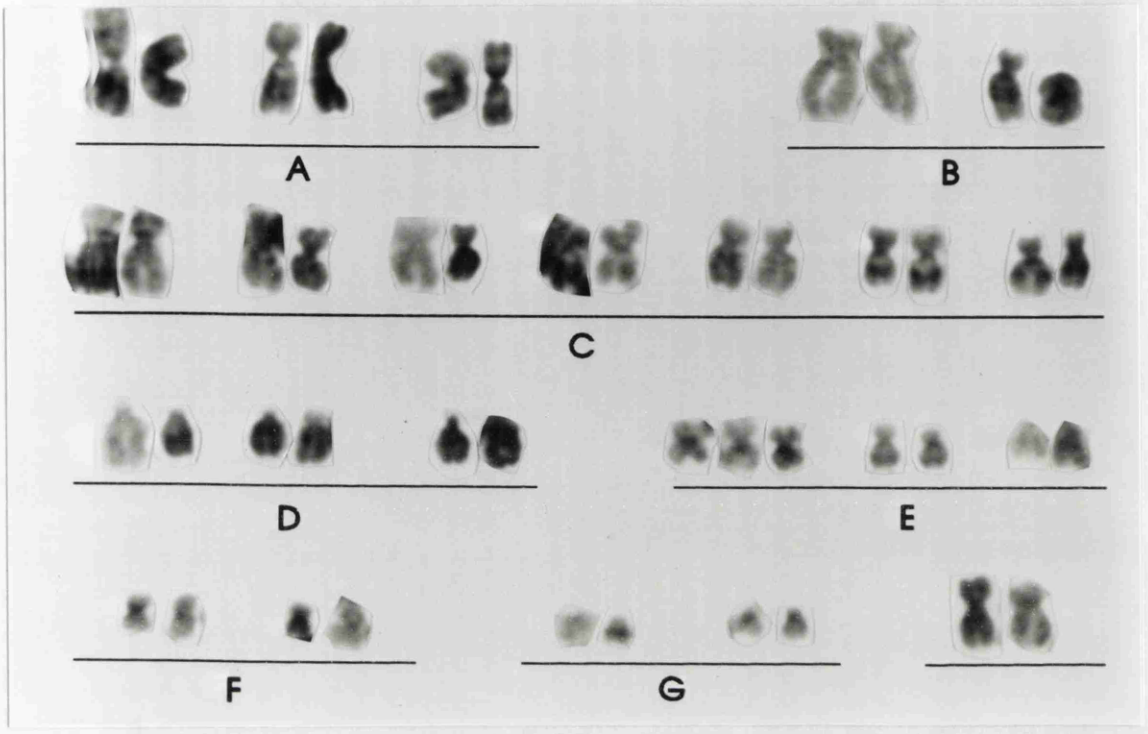


a

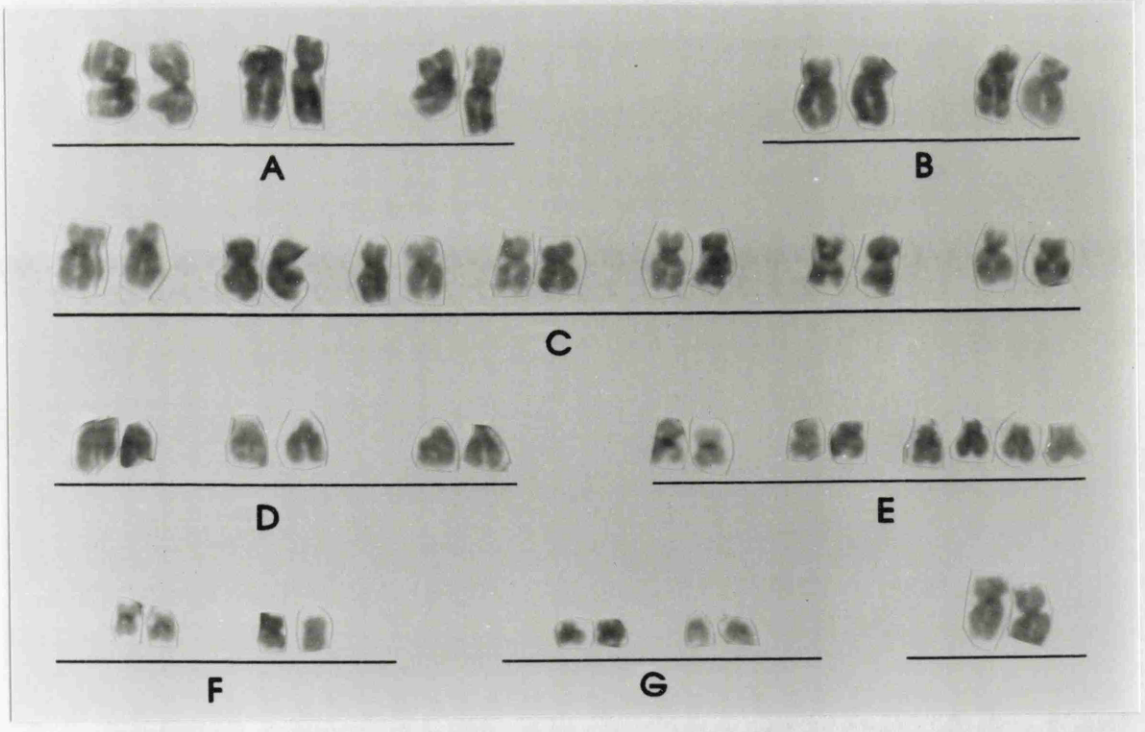
b

Figure 20

Embryo karyotypes: a) a parthenogenetic embryo (23,X) and b) a normal 46,XX chromosome constitution.



a



b

Figure 21
Embryo karyotypes: a) trisomy 16 (47,XX,+16) and b) tetrasomy 18 (48,XX,+18,+18).

Triploid karyotypes with large numbers of missing chromosomes may be artefacts but both hyperploidy (+16) and hypoploidy (-E) were superimposed on embryos with a 3n complement. A partially banded metaphase with 74 chromosomes appeared to be fundamentally triploid with five 'extra' chromosomes. The sex chromosome constitutions of nine triploids were equally distributed between XXX, XXY and XYY. No polyploid metaphase spreads could be fully analysed.

Three analysed metaphases from a four cell embryo had a 29/29/32 chromosome constitution with non-random loss. Two pronuclei were observed on the day following insemination and the resulting embryo had even blastomeres with minimal fragmentation.

Table 7.4.1

Karyotype details of diploid embryos

Analysis	n
Diploid	178
46,XX	64
46,XY	74
47,XX,+C	2
47,XY,+C	1
47,XX,+D	1
47,XY,+D	1
47,XX,+D or E	1
47,XX,+E	1
47,XX,+16	3
47,XX,+18	1
47,XY,+18	1
47,XX,+F	1
47,XX,+G	1
47,XY,+G	2
47,XX,+?	1
45,XX,-C	1
45,XY,-C	2
45,X (?)	1
45,XX,-D	2
45,XX,-D or E	1
45,XX,-E	1
45,XY,-E	1
45,XX,-F	1
45,XY,-F	1
48,XX,+18,+18	1
48,XX,+D(?),+E(?)	1
46,XY,+D,-C	1
44,XX,-3,-G	1
44,X,-F,-C or G/Y	1
45,+16,-?,-?	1
47,XX,+C / 46,XX	1
46,XX / 45,XX,-18	1
46,XX / 44,XX,-2,-21	1
46,XX / 44,XX,-C,-C	1
46,XX,-D,+FR (bisatellited)	1
46,XX / 45XX,-C,+FR,+FR	1

Table 7.4.2

Cytogenetic analysis of diploid embryos: karyotype details

Analysis	Number of embryos	
Total karyotyped	178	
Apparently normal	138	(77.5%) a
46,XX	64	
46,XY	74	
Aneuploid (non mosaic)	34	(19.1%)
Single chromosome	28	(15.7%)
Hyperploid	17	
Hypoploid	11	
Two chromosomes	5	(2.8%)
Hyper/hyper	2	
Hyper/hypo	1	
Hypo/hypo	2	
Three chromosomes	1	(0.6%)
Hyper/hypo	1	
Mosaic	4	(2.2%)
Structural anomalies	2	(1.1%)
Total abnormal	40	(22.5%)

a Expressed as percentage of total karyotyped.

Table 7.4.3
Karyotype details of non-diploid embryos

Analysis	n
Haploid	5
23,X	3
24,X,+C	1
23,X / 23,X / 22, -C	1
Triploid	
69,XXX	1
69,XXY	1
69,XYY	1
69	2
70,XXX,+16	1
68,XYY,-E	1
64,XYY,-1,-1,-2,-3,-6,-12,+18	1
64,XXY,-3,-3,-C,-C,-C	1
64,XXX,-18,-F,-G,-G,-G	1
74,+5 chromosomes	1
Other	
32 / 29 / 29	1

The proportion of embryos categorised as abnormal was not different in good (grades 1+2) quality preparations (29/138; 21.0%) and those considered poor (9/38; 23.7%, Table 7.4.5). The majority (138/176; 78.4%) of analyses were based on good quality metaphase spreads with minimal ambiguity.

Table 7.4.4

Cytogenetic analysis of embryos: involvement of chromosome groups in non-mosaic abnormal karyotypes

Group	Aneuploidy Hyper (n)	Hypo (n)	Total (n)
A	0	1	1
B	0	0	0
C	3	4	7
D	4	2	6
E	10	2	12
F	1	3	4
G	3	1	4
Sex chrom	0	1	1
Unknown	2	4	6
Total	23	18	41

Table 7.4.5

Cytogenetic analysis of embryos: influence of preparation quality on ascertainment of chromosome abnormalities

Preparation quality	Total cells	Number abnormal	Percentage abnormal
1	79	18	22.8
2	59	11	18.6
3	24	8	33.3
4	14	1	7.1

As another internal control, the incidence of consistent aneuploidy in embryos with >1 cell analysed was examined (Table 7.4.6). Nine of the forty two embryos (21.4%) in this category were aneuploid, a figure similar to the overall incidence. Forty-nine embryos had more than one cell karyotyped. Four (8.2%) were mosaic; all based on analysis of two cells.

Table 7.4.6
Cytogenetic analysis of embryos: number of cells on which individual analyses were based

Analysis	Number of cells analysed 1 (n)	2 (n)	3 (n)	4 (n)
All embryos	146	45	9	1
Diploid embryos	129	42	7	0
Apparently normal embryos	104	30	4	0
Aneuploid embryos	25	6	3	0
Mosaic embryos	0	4	0	0
Structural abnormalities	0	2	0	0

7.4.2 Factors influencing the incidence of aneuploidy in embryos

Embryo quality, as assessed by morphology and growth rate, was not different in apparently normal and aneuploid embryos (Table 7.3.3).

No significant influence of cause of infertility and aneuploidy was seen (Table 7.3.5) but, as in oocytes, the lowest incidence was observed in ovulatory indications.

Seventeen embryos derived from cycles with sperm problems were fixed and eight embryos were informative; all diploid. Of seven karyotyped embryos, five appeared normal and two were aneuploid.

7.4.3 Effect of maternal age on the incidence of aneuploidy

The incidence of non-mosaic aneuploidy increased with maternal age (Table 7.4.7). Unlike oocytes, the proportion of successful analyses was similar in all age groups, a slight reduction in patients >40 being statistically non-significant. The incidence of aneuploidy increased with maternal age but did not reach statistical significance. Hyperploidy and hypoploidy occurred with equal frequency in each age group and all four embryos with double and triple aneuploidy were found in the 30-34 group.

Table 7.4.7
Age distribution of successful analyses and non-mosaic aneuploidy in embryos.

Age	All n	Karyotyped n (%)	Embryos Aneuploid n (%)	Hyper n	Hypo n	>1 n
<30	223	52 (23.3)a	7 (13.5)b	4	3	0
30-34	396	91 (23.0)	18 (19.8)	8	6	4
35-39	109	26 (23.8)	6 (23.1)	3	3	0
≥40	12	2 (16.7)	1 (50.0)	1	0	0

a Successfully karyotyped embryos expressed as a percentage of the total number fixed.
b Aneuploid embryos expressed as a percentage of those karyotyped.

7.4.4 Summary

Karyotype analysis of 178 diploid embryos revealed abnormalities in 40 (22.5%) cases. Single chromosome aneuploidy was detected in 19.1% of embryos, five cases (2.8%) involved two chromosomes and one embryo (0.6%) had both hyperploidy and hypoploidy involving three chromosomes. E group was most frequently involved in aneuploid karyotypes (10/23 hyperploid embryos) and trisomy 16 was detected in 2.2% of embryos. Two structural anomalies were recorded and four embryos were mosaic. The incidence of aneuploidy increased with maternal age but did not reach statistical significance (<30, 7/52 [13.5%]; 30-34, 18/91 [19.8%]; 35-39, 6/26 [23.1%]; ≥40, 1/2 [50.0%]). Embryo morphology and growth rate, assessed by EDR, did not distinguish between normal (mean score, 7.9; mean EDR, 96.1) and aneuploid (mean score 8.1; mean EDR, 92.1) embryos.

Numbers of hyperploid (n=17) and hypoploid (n=11) embryos (non-mosaic cases involving single chromosomes) were not statistically different. Only one case of sex chromosome monosomy was detected. An excess of female karyotypes was detected in abnormal cases (sex ratio 0.48); this ratio was significantly ($p<0.05$) different from that observed in normal cases (74:64, XY:XX). Small numbers of karyotyped triploid embryos revealed equal proportions of XXX, XXY and XYY embryos (3:2:3).

CHAPTER 8
DISCUSSION

8.1 Cytogenetic assessment of uncleaved oocytes: study objectives and design

The cytogenetic study of oocytes remaining uncleaved after insemination *in vitro* had two aims. Firstly, to obtain corollary evidence to fertilisation rates and embryo quality for assessment of aspects of IVF methodology discussed previously. Secondly, metaphase of maternal meiosis II should reveal both the normal and abnormal products of the first meiotic division; the main source of chromosome anomaly in man (Hassold & Jacobs, 1984).

The initial division of data into three groups (6.2.1) was made to attempt to differentiate between these objectives. Oocytes remaining unfertilised in the presence of poor sperm should provide the most reliable information on the intrinsic abnormalities since they represent a largely unselected population. Oocytes remaining uncleaved after exposure to (apparently) good sperm may yield information on aspects of oocyte maturation which preclude normal fertilisation and/or cleavage. Differences between the two groups should suggest which, if any, chromosome abnormalities are incompatible with fertilisation. Patients treated in the LORD study (2.5.3) were highly selected and have been considered separately.

Chromosome preparations were considered at two levels, requiring different degrees of quality. Accurate karyotyping for assessment of aneuploidy necessitated containment of the metaphase within a defined area and, conversely, with sufficient spreading of the chromosomes to allow unambiguous counting, grouping and karyotyping. Classification as meiotic or mitotic, estimation of ploidy and fragmentation and identification of penetrated sperm were considered adequate for more general study of oocyte maturity and development.

8.2 Cytogenetic assessment of uncleaved oocytes: nuclear and cytoplasmic maturity

8.2.1 Absence of chromosomes

General analysis of 466 (71.4%) preparations was possible (Table 7.1.1). A proportion of the 167 preparations with apparent lack of chromosomes was certainly attributable to technical reasons but previous investigations suggest that some may reflect the true status of the oocyte.

A small proportion (4%) of whole oocytes stained with a fluorescent DNA specific stain showed lack of concentrated fluorescence within the ooplasm despite clear PB staining (Van Blerkom & Henry, 1988). Electron microscopy revealed chromosomes, each within a nuclear membrane, distributed throughout the oocyte. The phenomenon appeared to predominate in CC/hMG treated cycles and was not observed in a similar study (Van Wissen et al., 1991) of oocytes retrieved after hMG or GnRH-a/hMG stimulation. Small nuclei found in oocytes without identifiable chromosomes after conventional cytogenetic preparation (Wramsby & Fredga, 1987) may represent the same abnormality.

Complete lack of chromosomes after fixation has been reported at rates from 3.3 to 34% (Bongso et al., 1988; Ma et al., 1989; Pellestor & Sele, 1988; Selva et al., 1991; Tarin et al., 1991a; Wramsby & Fredga, 1987; De Sutter et al., 1991), with the majority similar to the 25.6% observed in the present study. A relationship with extended in vitro culture time was excluded (Table 7.1.4). The widely used technique of oocyte fixation developed by Tarkowski (1966) (with or without modification) has been criticised for causing chromosome scatter (Mikamo & Kamiguchi, 1983a) and is unlikely to yield reliable information on any real developmental defect.

8.2.2 Chromosome fragmentation

Total fragmentation of the chromosome complement was detected in 8.1% of informative analyses (Table 7.1.1). The inherent nature of material available for cytogenetic analysis has led to the suspicion that fragmentation represents an *in vitro* ageing process (Veiga *et al.*, 1987) but no relationship with insemination to fixation interval was detected (Table 7.1.4). These data support results from Tarin *et al.*, (1991a) who compared non-inseminated eggs fixed four hours after recovery with a group processed after 52 hours, following fertilisation failure.

Fragmentation was equally prevalent in age groups under 40 but significantly higher (45.0%; $p<0.001$) in oocytes recovered from women over 39, possibly contributing to the reduced fertilisation rate in older women (Table 7.2.5). The suggestion that oocytes may be directly affected by an age related increase in FSH (Tarin *et al.*, 1991a) should be treated with caution. Sub-optimal responses to ovarian stimulation in women with elevated FSH concentrations in the perimenstrual period (Toner *et al.*, 1991) have been reported and an influence of prolonged, high doses of exogenous gonadotrophins cannot be excluded.

8.2.3 Nuclear maturity

Meiosis had progressed to metaphase II in 85.6% of informative preparations (Table 7.1.1). Bivalent separation had occurred in 28 (6.0%) cells without evidence of migration of homologues to opposite poles. Classification of this stage has presented some difficulties since it is not a 'true' stage of meiosis; the term 'diploid metaphase II' has become accepted. The weight of evidence from experimental (Tarin *et al.*, 1991a) and cytological (Chandley, 1971; Plachot *et al.*, 1987) data suggests that these oocytes are arrested.

High ovarian response to stimulation facilitated by GnRH-a suppression was reported to produce significantly more immature oocytes, unable to proceed through nuclear maturation (Tarin & Pellicer, 1990). This led to the suggestion that criteria for hCG administration should be changed in analog treated cycles to increase the follicle size, and oocyte maturity, at OR. The present study failed to show any relationship between the frequency of diploid metaphase 2 oocytes and either number of oocytes recovered (Table 7.1.6), follicle size (Table 7.1.7) or maturation time before insemination (Table 7.1.3).

8.2.4 Cytoplasmic maturity

Schmiady et al. (1986) first described inappropriate condensation of G1 phase sperm chromosomes in apparently unfertilised metaphase II oocytes. The phenomenon has been explained by inability of the oocyte to respond to fertilisation and retention of chromosome condensing factors in the cytoplasm (Schmiady & Kentenich, 1989). Cytoplasmic capacity to decondense sperm heads develops with nuclear maturity (Lopata & Leung, 1988) as critical factors accumulate in the ooplasm after GVBD (Clarke & Masui, 1986). Normal PN formation is possible after metaphase I/II transition (Lopata & Leung, 1988; Iwamatsu & Chang, 1972).

A possible relationship between diploid metaphase II and cytoplasmic immaturity is suggested by the higher rate of PCC of sperm chromosomes in diploid meiotic cells (32.1%) than in those which had completed the first division (9.3%).

Relatively few oocytes remained uncleaved during LORD study 2, reflecting choice of patients for inclusion (2.5.3). The incidence of cytogenetic abnormality was high, with only 24/52 (46.1%) of informative oocytes in

metaphase II without evidence of fertilisation (Table 7.1.3). Eleven of 17 (64.7%) metaphase II cells recovered after 34 hours and inseminated immediately contained prematurely condensed sperm chromosomes. This compares with an incidence of 6.7% in non-LORD cases with normal semen (Table 7.1.2 and, although numbers were small, appeared higher than other LORD groups which had matured longer before insemination. These data suggest asynchrony between completion of meiosis I and development of cytoplasmic competence, correctable by additional in vivo or in vitro maturation.

The incidence of PCC of sperm chromosomes appeared to increase with the degree of ovarian response (Table 7.1.6). Development of more follicles, while applying the same day 0 criteria, would result in an increase in the number of medium and small follicles. Absence of an association between PCC and follicle volume (Table 7.1.7) did not support this hypothesis.

8.2.5 Zona/membrane permeability

Fertilisation rates were positively correlated with follicle size (Table 3.4.1) and cumulus expansion (Table 3.4.2). Despite their assumed immaturity, uncleaved oocytes from small follicles and those with an immature cumulus did not exhibit increased evidence of nuclear or cytoplasmic immaturity (Tables 7.1.7 and 7.1.8). Eighty-five percent of oocytes recovered from FF volumes <2.5ml and 84% lacking complete cumulus expansion were at metaphase II without evidence of sperm penetration. These data suggest that zona pellucida and/or vitelline membrane penetration may be dependent on the stage of follicular growth or optimal cumulus development; an association between these parameters has been demonstrated (Table 3.4.3).

A change in zona resistance to sperm penetration is concurrent with metaphase I/II transition Tesarik et al., (1988) and is associated with impregnation of the outer zona with cumulus cell-secreted proteoglycans (Tesarik & Kopecny, 1986). Changes are visible at the ultrastructural level (Familiari et al., 1988) and may be related to sperm-binding mechanisms.

It is of interest that total sperm penetration rates (fertilised oocytes + PCC , Tables 3.3.8 and 7.1.3) in LORD study 2 indicated that sperm penetration had occurred with equal frequency in each group. This suggests that a 34 hour interval from initiation of luteinisation to insemination is sufficient for final zona pellucida maturation, if development to that stage has been optimal.

8.2.6 Oocyte activation

Chromosome morphology was indicative of mitotic metaphase in 22 (4.7%) oocytes which had not cleaved. Only five of these oocytes had PN visualised prior to fixation. Colchicine had not been used during preparation and mitotic arrest rather than chance observation of the transient metaphase stage seems probable.

It is known that a proportion of oocytes fertilised in vitro subsequently fail to cleave (Fishel et al., 1988; Testart et al., 1983b) and this explanation must account for the 46,XY karyotype in the present study. Cytoplasmic incompetence due to immaturity, ageing or an effect of manipulation may be implicated.

Mitotic chromosomes were rarely identified in oocytes fixed on day 2 or 3 after insemination but represented 21.7% of informative cases fixed after 70 hours in culture (Table 7.1.4). In vitro ageing may eventually lead to an increase in zona permeability (Tesarik, 1989) with

concurrent ooplasm degeneration preventing cleavage but the ability of mouse embryos to react to parthenogenetic stimuli also increases with time (Kubiak, 1989). Extrapolation to the human may not be valid since the phenomenon appears species specific (Zeilmaker & Verhamme, 1974).

Three uncleaved oocytes with a haploid complement of mitotic chromosomes were parthenogenetic in origin (Table 7.1.1). This chromosome constitution is not incompatible with cleavage (Angell et al., 1986a, Table 7.3.1) but endoreduplication may be the origin of diploid (Angell et al., 1991) and tetraploid cases. Another origin was suggested by the data of Balakier and Casper (1991) who reported that 13% of oocytes fixed between 43 and 67 hours had undergone spontaneous activation while retaining PB2.

8.2.7 The relationship between cytologic and cytogenetic assessment of oocyte maturity

The presence of PB1 in the vitelline space is generally considered evidence of successful completion of meiosis 1 (Veeck, 1988). This observation appears to be subject to considerable error since only 22/60 oocytes with no PB visualised at the time of fixation (Table 7.1.10) had chromosome complements compatible with their PB status. Apparently normal metaphase II oocytes predominated. Degeneration (Balakier and Casper, 1991) or complete fragmentation may be responsible.

Metaphase II was correctly diagnosed by PB1 extrusion in the majority of cases (Table 7.1.10) but eight appeared to retain all meiotic chromosomes. Misdiagnosis was also made in 13/15 cases with 2PB and all seven with three PB or fragmented material remained at metaphase II. Electron microscopy has shown retention of coronal material in the vitelline space after retraction of cytoplasmic processes

(Sundstrom et al., 1985; Van Blerkom et al., 1987); this may be mistaken for extruded ooplasm. Initial extrusion of PB1 into two structures (Spielman et al., 1985) and spontaneous division of PB1 (Ortiz et al., 1983) have also been observed.

Thirty-one oocytes which failed to cleave after PN visualisation were fixed for analysis (Table 7.1.9). Five of 13 informative analyses were at metaphase II without evidence of sperm penetration. These errors may reflect the limitations of stereo microscopy for differentiating PN, restitution nuclei (Van Wissen et al., 1991), vacuoles (Van Blerkom et al., 1987) and mitochondria/vesicle complexes (Sundstrom et al., 1985).

Two oocytes with more than three PN were successfully analysed and showed only multiple sperm nuclei with PCC. Human oocytes penetrated by more than three sperm rarely undergo syngamy (Wiker et al., 1990), probably due to limited supply of cytoplasmic factors required for male PN formation (Tesarik & Kopecny, 1989). Penetration of mouse oocytes by more than six sperm caused decondensation or pycnosis of maternal chromosomes (Clarke & Masui, 1986). A similar process may explain the apparent lack of maternal chromosomes in oocytes with multiple PCC (Table 7.1.1).

8.3 Cytogenetic assessment of uncleaved oocytes: detailed analysis

Abnormalities were detected in 24.2% of oocytes in this study (Table 7.2.2) and were more diverse than previous published studies (Bongso et al., 1988; Ma et al., 1989; Wramsby & Fredga, 1987; Angell et al., 1991). The majority of abnormalities (32/43; 74.4%) were conventional aneuploids (Tables 7.2.1, 7.2.2) and, although hyperploidy and hypoploidy occurred with equal frequency, acrocentric were over-represented in aneuploid karyotypes. An apparent increase in the incidence of aneuploidy with maternal age (Table 7.2.6) was not statistically significant.

In addition, 10 chromosome complements contained 22 whole chromosomes with the missing homologue replaced by two individual chromatids; the majority (n=6) involved chromosome 16. A single chromatid represented a missing C chromosome in a karyotype with two other deficiencies. Two structural anomalies were recorded.

The overall incidence of abnormality was not different in cases with normal or deficient sperm parameters (Table 7.1.2) or between metaphase II cells with and without PCC of sperm chromosomes (Table 7.2.2). These observations imply that no selection against aneuploid oocytes had occurred at fertilisation.

Chromosome analysis of human female meiotic material presents considerable technical difficulties attributable to four factors; chromosome morphology, chromosome scatter, inapplicability of banding techniques and the presence of only one cell per case. An internal quality control (Table 7.2.3) suggested that results accurately represent the incidence of numerical abnormalities in the material studied.

8.4 Mechanisms leading to abnormality

8.4.1 Whole chromosome aneuploidy

Reciprocal hyperploidy and hypoploidy are implicit in classical non-disjunction, as described by Bridges (1913; 5.1.3) but other mechanisms lead primarily to chromosome loss (Ford & Lester, 1982; Eichenlaub-Ritter et al., 1988). The presence of only one cell per slide in oocyte cytogenetic preparations facilitates accurate diagnosis of hyperploidy but interpretation of chromosomally deficient cells is more problematical. Because it is impossible to differentiate between a chromosome lost artificially and genuine hypoploidy, some authors have ignored all complements of <23 (Angell et al., 1991) or included them for discussion but expressed the aneuploidy rate as 2 x hyperploidy (Tarin et al., 1991a). This problem would be obviated if the karyotype could be verified from other cells, as is considered mandatory for diagnostic cytogenetics (Ferguson-Smith & Ferguson-Smith, 1983).

Efforts were made to exclude over-spread cells and only metaphase spreads with >20 chromosomes were included in this study. This cut-off point was made retrospectively after analysis of oocytes and embryos showed a maximum of two additional chromosomes in any cell. Within these limitations, it can be concluded that hyperhaploidy and hypohaploidy of single chromosomes occur with equal frequency in metaphase II oocytes. The observation that hypoploidy was confined to younger (<35, Table 7.2.6) was not supported by analysis of embryos (Table 7.4.7).

The preponderance of trisomic acrocentric chromosomes in human spontaneous abortions may represent an excess of these anomalies in female gametes (Table 5.2.2). Fifty percent of disomic oocytes in this study had an extra D or G group present, 12/12 hyperhaploid oocytes reported by

Plachot et al. (1987) and 4/7 by Ma et al. (1989) involved groups D and G.

The phenomenon of satellite association between chromosomes carrying NORs was first observed in 1961 (Ferguson-Smith & Handmaker, 1961) in stimulated lymphocytes. During mitotic interphase the NOR regions of chromosomes 13, 14, 15, 21 and 22, containing the ribosomal genes, become fused into common nucleoli (Schwarzacher et al., 1978). The fibrillar centre of this structure contains an argyophilic protein which decreases during mitosis (Schwarzacher et al., 1978) but is still present in the bridge joining associated chromosomes at metaphase. Mirre et al. (1980) studied prophase I oocytes from 16-24 week foetuses and confirmed that the ribosomal genes of 4-12 chromatids (1-3 bivalents) are juxtaposed in the same silver-staining structure during meiotic prophase. The possibility that this phenomenon might lead to non-disjunction has been widely discussed (Henderson et al., 1973; Nakagome, 1973; Hansson, 1979; Jacobs & Mayer, 1981). Support for this theory comes from the observation that chromosome 21 is more commonly involved in satellite associations in parents of Down's syndrome children (Hansson & Mikkelsen, 1978) and an association between carriers of an acrocentric chromosome with a double NOR detected by silver staining and parents having children with Down's syndrome. The anomaly was found in the parent in which non-disjunction occurred but was not always on the trisomic chromosome (Jackson-Cook et al., 1984).

The applicability of mouse embryo cytogenetics to the human has been questioned since all mouse chromosomes are acrocentric. However, each mouse bivalent contains two independent nucleoli, without a common fibrillar centre. These important species differences suggest that attempts to find a model for human chromosome defects must consider more than simple chromosome morphology.

Eight diploid metaphase II cells were karyotyped; all had a 46,XX chromosome complement. These oocytes arise from retention of PB1 and thus contain all chromosomes present in the primary oocyte and would thus indicate the occurrence of pre-existing aneuploidy. This might arise from cryptic mosaicism in a parent or a de novo mitotic error in the ovary. This small number of analyses supports data from pachytene oocytes (Hulten et al., 1985) which had a very low incidence (1/459; 0.2%) of supernumerary chromosomes suggesting a minimal contribution of pre-existing aneuploidy to the genetic load in the female gamete.

8.4.2 Mechanisms leading to single chromatids in metaphase II oocytes

Angell (1991) was first to unambiguously describe single chromatids in unfertilised human oocytes although 6/411 metaphase II cells examined by Jagiello et al. (1976) "probably contained a separation of a small chromosome". Anomalies were detected in 5 of 44 completely analysed metaphase II cells. Four cells had 22 whole chromosomes plus 2 individual chromatids representing the missing homologue; groups D, E, F and G were involved. Only one cell had missing material; 22, +cht(C). It is of interest that these cases, and the present study, primarily detected cells with both chromatids present. Animal studies have almost exclusively reported missing or additional single chromatids (Hansmann & El-Nahass, 1979; Hummler et al., 1987; Polani & Jagiello, 1976; De Boer & van der Hoeven, 1980; Mikamo & Kamiguchi, 1983b), and different mechanisms might be involved.

These examples involve spatially separated chromatids of individual chromosomes and apparently differ from the more general phenomenon of chromatid separation observed in 20.2% of human oocytes (Tarin et al., 1991a). However,

artefact cannot be completely excluded (Polani & Jagiello, 1976), especially when both chromatids are present.

Two meiotic anomalies leading to the existence of individual chromatids in metaphase II oocytes have been postulated. The first results from the presence of univalents during the first reduction division. Darlington (1937) indicated that the behaviour of unpaired chromosomes is similar, irrespective of their method of origin, thus information from hybrids, organisms with numerical abnormalities and failure of bivalent formation are relevant. Univalents lie at random on the spindle at metaphase and their subsequent behaviour is dependent on their position relative to the poles and equator. Those close to a pole become incorporated in a daughter cell, either oocyte or polar body (post-division) or become lost in the cytoplasm (Figure 22). Unpaired chromosomes near the equator are able to orient themselves axially and divide into single chromatids (pre-division) which migrate to opposite poles. The movement of both chromosomes and chromatids may be delayed, relative to normal bivalents (Figures 23 and 24)

The consequences of univalent formation may be species dependent. Approximately 5% of Drosophila melanogaster X chromosomes do not undergo genetic exchange (Bond & Chandley, 1983), suggesting asynapsis. This incidence far exceeds that of aneuploid offspring and provides circumstantial evidence of regular segregation of unpaired bivalents. Cytogenetic examination of the univalent X chromosome of the mantid Humbertiella indica (Hughes-Schrader 1948) detected both lagging and segregation of chromatids to opposite poles. Michel and Burnham (1969) observed 13.1% equatorial division of univalents in maize and an additional chromatid was the most frequent abnormality detected in spermatocytes of trisomic newts (Guillemin, 1980).

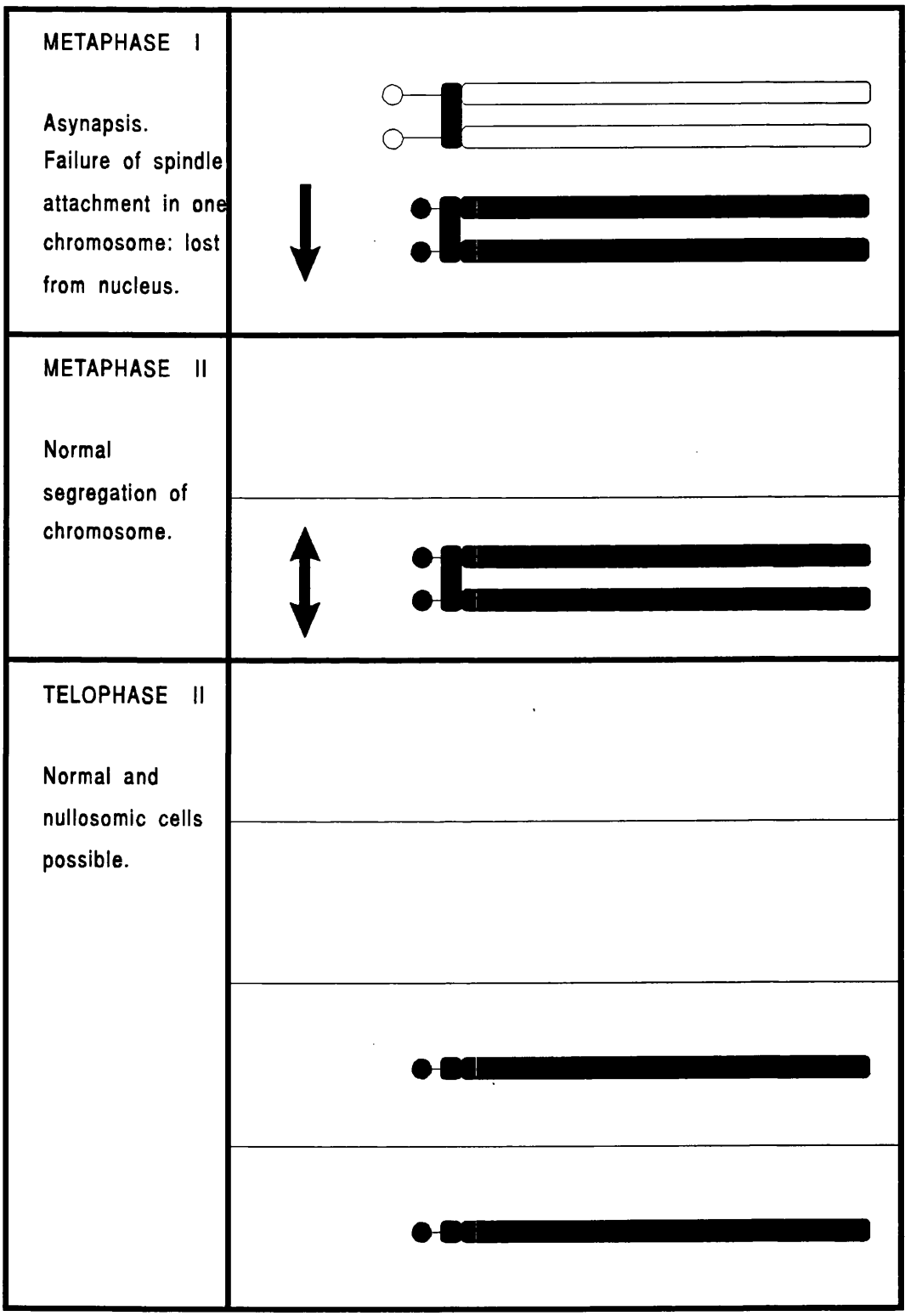


Figure 22

Illustration of random segregation of asynaptic univalents at metaphase I.

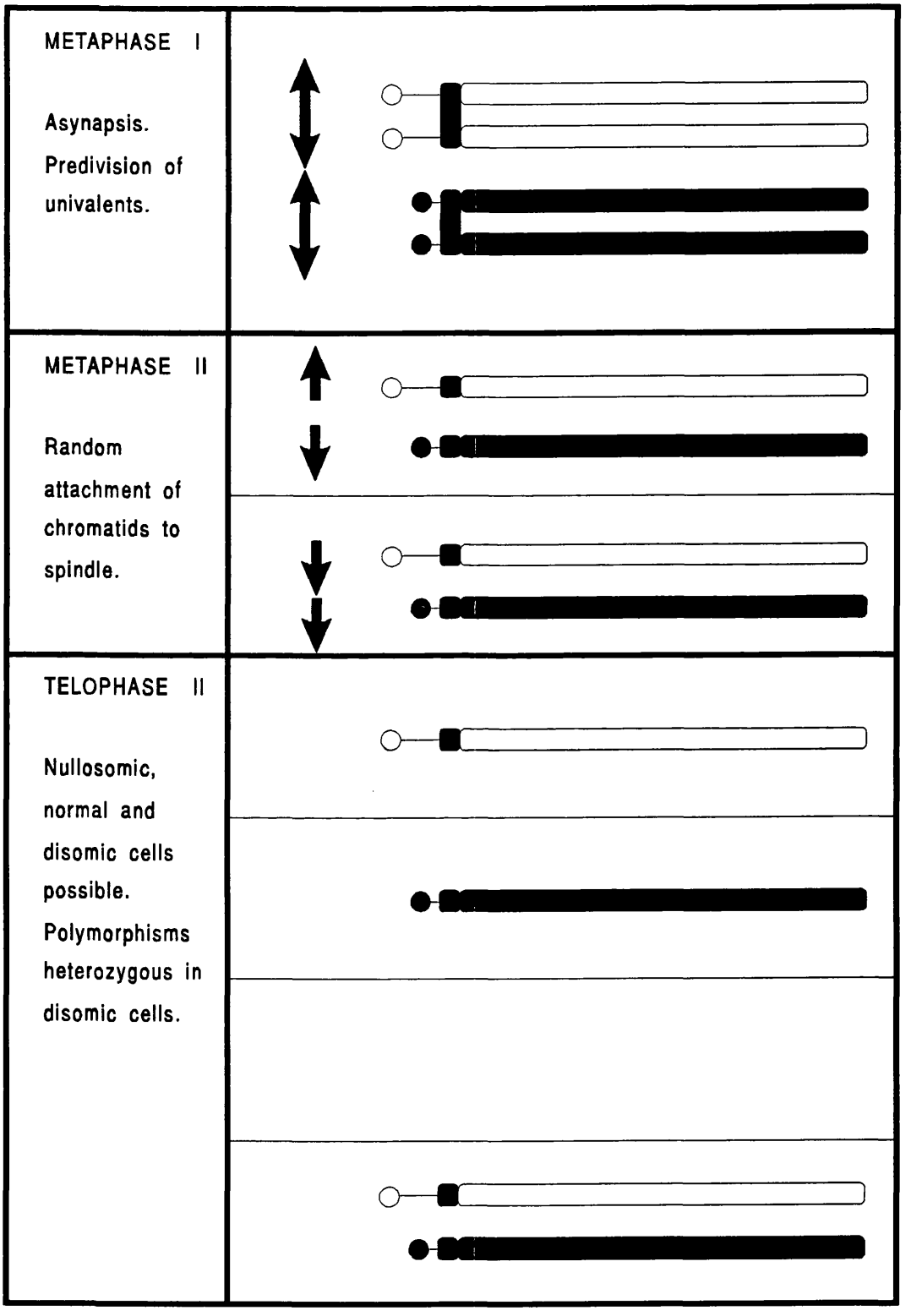


Figure 23

Diagrammatic representation of predivision of asynaptic univalents during meiosis.

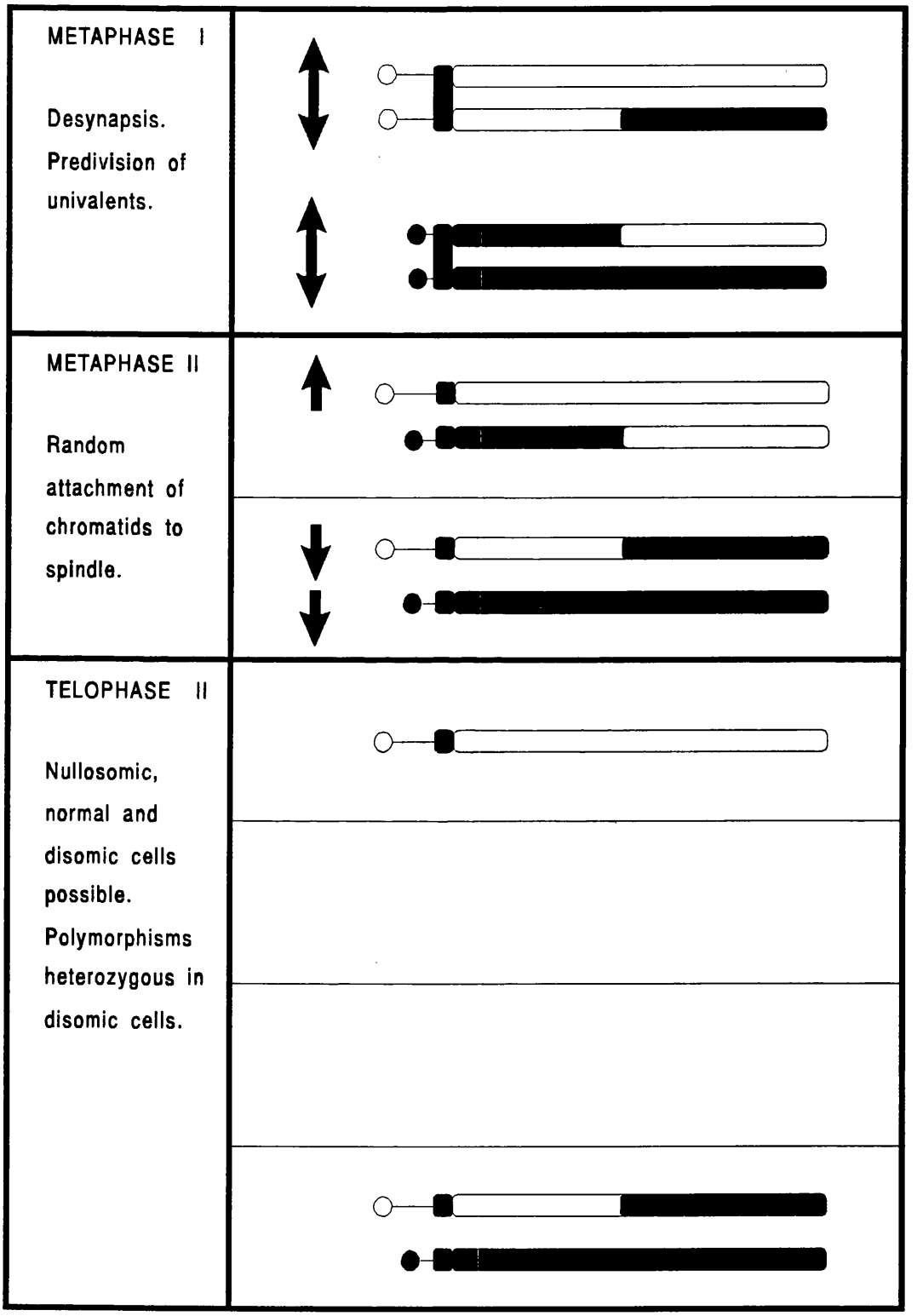


Figure 24

Diagrammatic representation of predivision of desynaptic univalents during meiosis.

Interpretation of the present results and those of Angell (1991) as chromosome pre-division is dependent on the occurrence of univalents during the prolonged human prophase due to either failure or breakdown of synapsis. Wallace and Hulten (1985) observed univalents in 1/122 pachytene preparations using light microscopy, an incidence similar to that detected by Speed (1985; 2/154). However, EM suggested a much higher rate of 10.5% (17/162; Speed, 1985); ten times the incidence of pairing anomalies detected in mouse by the same investigator (Speed & Chandley, 1983).

Combined data from Angell (1991) and the present study suggest that the majority of individual chromatids represent the smallest chromosome groups. Fourteen of 16 cases involved chromosomes identified as D, E, F or Gs, which represent only 45% of the autosome complement. The smaller components of both mouse and Chinese hamster are most frequently involved in univalents (Luthardt et al., 1973a; Speed, 1977; Sugawara & Mikamo, 1983), possibly related to lower chiasma frequency (Henderson & Edwards, 1968).

The relationship between univalents and aneuploidy at metaphase II has been questioned (Polani & Jagiello, 1976; Speed, 1977). The most convincing evidence of lack of relationship comes from investigation of the Chinese hamster (Sugawara & Mikamo, 1983), selected because of it's small number of chromosomes and four distinct morphological groups. Univalents were most frequent in the smallest class but hyperploidy occurred with equal frequency in all categories. A circumstantial relationship between univalent X and Y chromosomes during male meiosis in the human (Laurie & Hulten, 1985) and sex chromosome hyperploidy in sperm (Martin & Rademaker, 1990) does exist however.

The second meiotic abnormality leading to individual chromatids was first described in Neurospora crassa by Threlkeld and Stoltz (1970). They postulated that one homologue of a bivalent could undergo precocious division during meiosis I. One daughter cell would inherit a complete chromosome and a chromatid, the other a single chromatid. This mechanism is compatible with observations in mouse (Hansmann & El-Nahass, 1979; Polani & Jagiello, 1976) but the observed defects in human oocytes would require precocious division of both homologues.

Any relationship between individual chromatids present at metaphase II and subsequent trisomy would require retention of both chromatids within the oocyte nucleus after fertilisation and normal replication before syngamy. Little evidence exists but Ostergren (1947) reported equatorial division of univalents in Anthoxanthum resulting in single chromatids which were unable to divide at metaphase II and were lost from the spindle.

One consequence of this mechanism would be that centromeric markers would be heterozygous and, therefore, indistinguishable from the products of classic non-disjunction in the embryo (Figures 13, 23 and 24).

Chromosome 16

The involvement of chromosome 16 in 6/11 cases with individual chromatids in this study (Table 7.2.1) is of particular interest given the high incidence of trisomy 16 in human abortions (Table 5.2.2). Circumstantial evidence has suggested that the number of detected cases cannot be wholly attributed to differential survival of equally frequent abnormalities since trisomies 13,18 and 21 all occur less frequently but appear to be subject to reduced selection; surviving to later gestations or to term.

Analysis of chromosome 21 (Sherman et al., 1991) in trisomic individuals had shown a reduced frequency of genetic recombination. This would be consistent with a defect of synapsis which might predispose to univalent formation. The same pattern was not found in a similar investigation of 26 cases of trisomy 16 (Hassold et al., 1991). However, univalent formation can result from both non-conjunction and desynapsis and different mechanisms might operate in the two chromosomes as suggested by the different distributions of maternal age (Hassold & Jacobs, 1984). The presence of a large heterochromatic block at the centromere of number 16 may be relevant (Angell, 1991). The only chromosome showing an increased frequency of disomy in human sperm is number 9 (Martin & Rademaker, 1990), which also has a large C-banding centromeric region. Reduced pairing of regions thought to correspond to centric heterochromatin in chromosomes 1, 9 and 16 has been observed at pachytene (Wallace & Hulten, 1985). No relationship between heterochromatic variants and reproductive failure has been found (Bobrow, 1985) although assessments have been largely subjective.

8.4.3 Structural abnormalities

Only two structural anomalies, of doubtful significance, were recorded during the study. This may under represent the incidence of chromosome breakage and/or rearrangement in unfertilised oocytes. The decision to ignore centromere, chromatid and chromosome gaps and breaks without major displacement of components was imposed by the material under analysis. The recognised phenomenon of chromatid separation (Tarin et al., 1991a; Rodman, 1971) in mammalian metaphase II material and the propensity for fragmentation (Papadopoulos et al., 1989; Selva et al., 1991) encouraged conservative interpretation of anomalies which might be attributable to preparation artefact. This approach was supported by the low incidence of

rearrangements detected in embryos (Table 7.4.1). Absence of banding also precluded identification of deletions, translocations and inversions which did not dramatically alter chromosome proportions.

Excluding chromatid separation, extensive fragmentation and PCC, combined data from nine studies (Angell et al., 1991; Bongso et al., 1988; Djali et al., 1988; Martin et al., 1986; Plachot et al., 1987; Tarin et al., 1991a;b; Veiga et al., 1987; Wramsby et al., 1987) which specified the presence or absence of this category of abnormality reported 4/853 (0.5%) structural abnormalities. Eighteen percent (24/132) of oocytes examined by Selva et al., (1991) had 1-5 chromosome breaks. It is possible that, as in this study, authors have interpreted these anomalies as preparation artefacts.

8.4.4 Factors affecting the incidence of abnormality

The lack of association between the incidence of abnormality and any aspect of IVF methodology (7.2.4) is of considerable interest. It implies that IVF does not increase the genetic load during oocyte development and that the results of this study can be extrapolated to in vivo reproduction.

The overall incidence of abnormalities in oocytes was similar in all groups under the age of 40. However, 2/3 cells in the over 40 group were aneuploid and the incidence of hyperploidy, the more reliable numerical abnormality because of the fixation method, was significantly higher ($p<0.01$) in women $\geq$ 35 (Table 7.2.6). The marked reduction in preparation success with maternal age (Table 7.2.6) could not be fully explained by the concurrent increase in fragmentation (Table 7.2.5) but might be related to an age dependent effect on chromosome morphology, as found in the mouse (Speed, 1977).

The detected contribution of maternal age to the incidence of aneuploidy in human oocytes has varied in published reports. Both the multi-centre study reported by Plachot et al. (1988a) and smaller numbers from Ma et al. (1989) detected an increase with age but Pellestor and Sele, (1988), Selva et al. (1991) and Djalali et al. (1988) failed to find an association. The last study reported a considerable excess of hypoploid cells and this may highlight a problem of interpretation in all studies.

The most extensive data on maternal age and trisomy has accumulated for Down's syndrome and two components to the rate of change have been described (Hook, 1985), consistent with an age dependent and an age independent contribution to the overall incidence, estimated at 35-50% and 50-65% respectively. Trisomy 18 shows a parallel pattern but the apparent age dependent component for trisomy 13 is less marked. The age related increase for trisomy 16 in spontaneous abortions is much less pronounced. In the light of evidence that more than one mechanism, affecting different chromosomes, induces abnormality in human oocytes, it is of considerable importance to obtain reliable information on all age groups. Less destructive techniques might yield better information in older women but the nature of the population undergoing IVF generally precludes study of those under 25.

The theory of pre-division of chromosomes is dependent on an increased frequency of univalents in older women. This relationship has been established in the mouse (Polani & Jagiello, 1976; Henderson & Edwards, 1968; Luthard et al., 1973) and Chinese hamster (Sugawara & Mikamo, 1983) but the mechanism underlying the increase remains problematical. Henderson and Edwards (1968) postulated the production-line theory which supposed that those oocytes which enter meiosis late in foetal life fail to form

bivalents and that these are the last oocytes to ovulate. No relationship between foetal age and pairing defects was detected in two strains of mouse (Speed & Chandley, 1983) and an effect in the maternal gonad at the time of final maturation has been favoured (Speed & Chandley, 1983; Sugawara & Mikamo, 1983). However, evidence of reduced crossing-over in chromosome 21 in Down's suffers (Sherman et al., 1991) may redirect interest to foetal development.

8.5 Cytogenetic assessment of early embryos

8.5.1 Incidence and types of abnormality in diploid embryos

The incidence of abnormality detected in diploid embryos (40/178, 22.5%) was similar to that found in oocytes (24.2%). This is of interest given the almost exclusively maternal origin of the extra chromosome in trisomy 21, based on RFLP analysis (Antonarakis, 1991; Sherman et al., 1991). The data also implies a minimal contribution from metaphase II anomalies, when any temperature induced anomalies caused by IVF methodology would be manifest (Pickering et al., 1990).

This overall incidence is similar to that reported by Angell et al. (1986a; 23%, 7/30) but the present study is the first with sufficient numbers to address the question of relative frequencies of abnormalities. All previous estimates of the types and incidence of abnormalities at conception have relied on extrapolation of data from early abortions, both spontaneous (Boue et al., 1975) and induced (Burgoyne et al., 1991).

As in oocytes (Table 7.2.4), abnormalities were not detected with equal frequency in chromosome groups. Ten of 23 trisomic chromosomes were from the E group with trisomy 16 the most frequent single abnormality (Table 7.4.3), comprising 2.2% of diploid embryos. Whole chromosome aneuploidy for chromosome 16 was not seen in oocytes and the frequency of the anomaly in embryos provides corroborative evidence that pre-division, visualised as single chromatids at metaphase II, represents the origin of trisomy 16. Two embryos had one additional number 18 and tetrasomy 18 was found in three analysable spreads of a four cell embryo (Figure 21b). This last example can be explained by either a first and second meiotic error in the oocyte (or sperm) or the same anomaly arising in both

gametes. To the author's knowledge no similar tetrasomy has been identified in material from clinical pregnancies.

Hyperploidy of an A group was not found in either oocytes (Table 7.2.4) or embryos (Table 7.4.3). Trisomy 1 has not been detected in any clinical pregnancy but was found in a preimplantation embryo (Watt et al., 1987) and attributed to the presence of two male contributions. Disomy 1 has also been identified in 4/6821 sperm chromosome complements (Martin & Rademaker, 1990); an incidence similar to other aneuploidies.

Two structural abnormalities were detected (Table 7.4.1) but only one was consistent. A two cell embryo with even blastomeres and minimal fragmentation had a missing D group chromosome replaced by an apparently bisatellited fragment in both cells. Unfortunately parental karyotypes could not be obtained but this embryo may represent de novo origin of a marker chromosome which has caused considerable problems during diagnostic chromosome analysis, especially prenatal diagnosis (Buckton et al., 1985). The other anomaly involved apparent breakage of a C chromosome in 1/2 cells. Artefact cannot be excluded but Angell et al., (1986a) has shown evidence of a break being transmitted through at least two divisions.

The incidences of hyperploidy (n=17) and hypoploidy (n=11) for single chromosomes were not statistically significant. In the absence of chromosome banding, the hypoploid group would include 45,X karyotypes which represent almost 20% of abnormalities in spontaneous abortions (Table 5.2.1). One embryo was tentatively assigned this diagnosis (Table 7.4.1) but both sex chromosomes could be identified in all other embryos with a missing C group chromosome. This substantiates evidence that sex chromosome monosomy arises after fertilisation.

Single chromosome aneuploidy was detected in 19.1% (34/178) of diploid embryos, five (2.8%) embryos had double aneuploidy and one (0.6%) embryo has both hyperploidy and hypoploidy involving three chromosomes (Table 7.4.2). These incidences suggest that non-disjunction of individual chromosomes occurred independently with double and triple aneuploidy occurring at frequencies predicted by the basic incidence. A similar relationship has been observed in spontaneous abortion material (Boue et al., 1985).

An excess of male karyotypes was found in normal embryos (7.4.2) and the sex ratio of 1.15 was significantly ($p<0.05$) different from that of those with abnormalities (0.48). Male karyotypes were also found in excess in normal spontaneous abortions (Boue et al., 1985) but individual anomalies showed different patterns. The lowest sex ratio of 0.29 was found in trisomy 9, trisomy 16 has almost equal numbers of males and females and trisomy 21 has a ratio of 1.67. These results have been attributed to differential survival of embryos. Additional data from embryos will be required to confirm this unexpected finding.

The presence of more than one analysable cell per embryo allowed corroboration of the analysis in 49 diploid and six other embryos (Table 7.4.6). The proportion of aneuploidy in these cases (26.5%) was not different to that detected in single cells (19.4%). Three of four diploid mosaic cases had one normal and one hypoploid cell, making artefactual loss the most likely explanation. An embryo with one apparently normal and one hypoploid cell may be of more interest given the suggested origin of mosaic cell lines in abortus material (Hassold, 1982). Accurate estimates of the extent of mosaicism are required to validate methods of preimplantation diagnosis. One

unusual embryo with an unstable cell line (32/29/29) highlights this problem.

8.5.2 Incidence and types of abnormality in non-diploid embryos

Three of five successfully karyotyped haploid embryos had a 23,X complement and one was disomic for a C group chromosome; the fifth was mosaic, probably due to artefactual chromosome loss (Table 67). All reported haploids have had an X chromosome (Angell et al., 1983, Plachot et al., 1987), consistent with maternal origin and an incidence of aneuploidy similar to that detected in oocytes (26%; Plachot et al., 1989).

Although only a small number of triploid embryos were fully karyotyped, it is worth comment that 3/8 cases where the sex chromosome constitution was established had an XYY complement. This substantiated observations by Plachot et al. (1987), Ma et al. (1990) and Macas et al. (1988) and confirms suggestions that under-representation of these cases in clinical pregnancies is due to very early pregnancy loss or ascertainment difficulties. An additional chromosome 16 was present in one embryo with 70 chromosomes and four number 18s were identified in a triploid cell with several missing chromosomes.

The general availability of abnormally fertilised material has allowed extensive investigation revealing that most tripronuclear oocytes do not develop into triploid embryos (Kola et al., 1987; Angell et al., 1986b; Macas et al., 1988). Three patterns of cytokinesis/chromosome behaviour have been observed (Kola et al., 1987). The most frequent (62%; 18/29) event involved cleavage directly into three cells resulting in severely abnormal, but not triploid, cells. Correction to a diploid complement occurred in 2/29 cells after exclusion of one haploid complement; carrying

the theoretical risk of hydatidiform mole formation (Pattillo et al., 1981). Only 24% of 3PN embryos formed triploid embryos.

8.5.3 Success rate of preparations

The proportion of successfully karyotyped embryos (193/816; 23.6%) was similar to that reported by Angell et al., (1986a: 22/69; 31.9%) in a study of embryos generated for research purposes. This poor preparation efficiency has implications for preimplantation diagnosis which would be performed on those embryos intended for ET, rather than supernumerary examples such as the present study. Wilton & Kola (1990) have reported considerable success in analysing all cells of eight cell mouse embryos to the extent of identifying a specific translocation but it remains to be seen if preparations of sufficient quality for complete analysis can be obtained from human material with any reliability.

This series had disappointing results from 3PN and 4PN embryos. This may have been related to the development of the technique primarily for diploid embryos. Tarkowski (1966) commented that alterations in hypotonic exposure and concentration are necessary for different stages of embryo development.

8.5.4 The relationship between PN status and ploidy

Approximately 2% of oocytes in the study has a single PN visualised, an incidence similar to that reported by Plachot et al. (1988b) and indicative of parthenogenetic activation in the mouse (Kaufman, 1982). However, this diagnosis was only confirmed in three of nine cases; five embryos were diploid and the presence of a Y chromosome suggested normal fertilisation was probable in two cases. Although a simple error cannot be excluded, the transition

of 2PN into a single structure has been observed (Angell et al., 1986a). Asynchrony of male and female PN might also occur and would explain the opposite situation where an additional PN appeared during culture (Staessen & Van Steirteghem, 1992).

Observation of 2PN within the normal examination period was associated with a diploid chromosome complement in the majority (155/167; 92.8%) of cases. A further five embryos were tetraploid. Duplication of a normal complement would be consistent with the proposed origin in spontaneous abortions (Kajii & Niikawa, 1977) and provides evidence that the anomaly could occur during the earliest stages of development. The origin of the additional set of chromosomes could not be determined in the 3/4 triploid embryos which had 2PN but the sex chromosome constitution of 2/3 karyotyped cases was compatible with fertilisation of a diploid oocyte. The XYY karyotype of the fourth embryo suggests abnormal fertilisation and failure to observe one PN since diploid sperm have not been determined.

8.5.5 Maternal age

The incidence of aneuploidy increased with maternal age but the difference did not reach statistical significance (Table 7.4.7). No other study has included sufficient embryos to draw conclusions on the influence of age on the rate of aneuploidy but Bongso et al. (1991) found no difference between the mean age of women with normal embryos (35.5) and those with any abnormality (36.5); all embryos were normally fertilised and the abnormal category included aneuploidy, structural defects, mosaicism, PCC and chromosome fragmentation.

A relationship between maternal age and the incidence of trisomy 21, in spontaneous abortions (Hassold & Jacobs,

1984), prenatally diagnosed cases (Ferguson-Smith, 1983), and births (Penrose, 1934) is undisputed but the situation at conception has only been deduced. The theory of relaxed selection (Ayme & Lippman-Hand, 1982) is largely discredited (Hook, 1983) and an oocyte donation programme has implicated the age of the recipient (endometrial age) as the factor affecting implantation and that of the donor (oocyte age) as influencing subsequent abortion rates (Levran et al., 1991). These data imply an increased incidence of anomaly at the time of conception in older women and are supported by the present study.

8.5.6 Chromosome constitution and embryo quality

Although embryo morphology and growth rate were shown to predict implantation potential (3.1.2), these parameters did not distinguish between normal and aneuploid embryos (Table 7.3.3). It has been suggested that poor quality embryos might be aneuploid (Martin et al., 1986) and that embryos not selected for ET on morphological grounds would give an artificially high estimate of the true incidence. The present data contradict this suggestion and imply that transferred embryos carry a similar genetic load.

Similarly, haploid, diploid and triploid embryos could not be distinguished by morphological parameters (Table 7.3.3.). This confirms observations by Mettler and Michelman (1985) and corroborates the pattern of energy metabolism seen in abnormally fertilised embryos which did not differ from normal until after 3.5 days in culture (Hardy et al., 1989). The EDR of triploid embryos may be artificially high since a proportion divide directly into three cells (Kola et al., 1987).

Quality was significantly ($p<0.01$) impaired in polyploid cases, casting some doubt on the potential of the most abnormal group and suggesting that abnormal nuclear

behaviour may reflect incipient degeneration. Support for this hypothesis comes from the high rate of post fertilisation polyploidy, detected by measurement of the DNA content of the nuclei, in normally fertilised embryos which did not yield mitoses for cytogenetic analysis (Angell et al., 1987).

No attempt was made to compensate for slow growth rates by extension of colchicine exposure during embryo processing and mitotic index was used as an objective assessment (Table 7.3.4) of the rate of cell division. The proportion of cells entering metaphase during the time in colchicine was compared with their EDR. The number of metaphase plates was similar in all groups with an EDR greater that 80 but significantly ($p<0.001$) lower in the 'slowest' category. The mean colchicine time of 19.3 hours was 7.7 hours more than the calculated cell cycle length for the population and mitotic index would therefore be expected to indicate only severely retarded growth. Slight differences in cell cycle length in the three higher EDR groups might not have been detected.

8.6 Implications for in vivo reproduction

Although there appears to be no increase in the incidence of chromosomally abnormal conceptions in spontaneous abortions (Roesler et al., 1989; Plachot, 1989) after IVF or in births after assisted conception (M.R.C., 1990), extrapolation of IVF cytogenetic data to in vivo fertilisation should consider several factors which might influence results.

Firstly, infertile patients might be predisposed towards cytogenetic abnormality. No significant relationship between infertility diagnosis and aneuploidy could be detected in oocytes (Table 7.1.5) or embryos (Table 7.3.5) although patients with ovulatory disorders appeared to

have an increased incidence of one and 3PN at fertilisation and a low aneuploidy rate. Direct comparison of fertile and infertile patients are sparse. Tarin et al., (1991b) reported a lower overall frequency of anomalies in oocytes from women who underwent ovarian stimulation prior to sterilisation than non-inseminated oocytes from an IVF programme (Tarin et al., 1991a) but no hyperploidy was detected in either group. The difference was attributable to diploid oocytes and chromosome fragmentation.

Time required to diagnose infertility and waiting time for treatment results in a higher mean age in women undergoing IVF than the general pregnant population. Figure 25 shows the distribution of maternal age at OR during the present study compared with that of pregnant women undergoing alphafetoprotein (AFP) screening for neural tube defects in the West of Scotland.

Protocols for induction of multiple follicular growth have been reported to increase the proportion of chromosome abnormalities in spontaneous abortions (Boue and Boue, 1973). In the human, the cohort of follicles which grows during the follicular phase develops from follicles with a diameter of 4-6mm of which 50% show some evidence of atresia (Chikazawa et al., 1986). It has been suggested that mouse follicles in the early stages of atresia can be 'rescued' by pregnant mare's serum gonadotrophin (PMSG) administration (Peters et al., 1975) and that sheep oocytes from secondary and tertiary stages of atresia retain developmental competence (Moor & Trounson, 1977). Evidence that 50% human of oocytes recovered from follicles in advanced stages of atresia had nuclear abnormalities (Sanyal, 1979) has led to the suggestion that follicles re-recruited by exogenous gonadotrophins or GnRH-a (Banka & Erickson, 1985) might contribute significantly to anomalies detected in oocytes and embryos

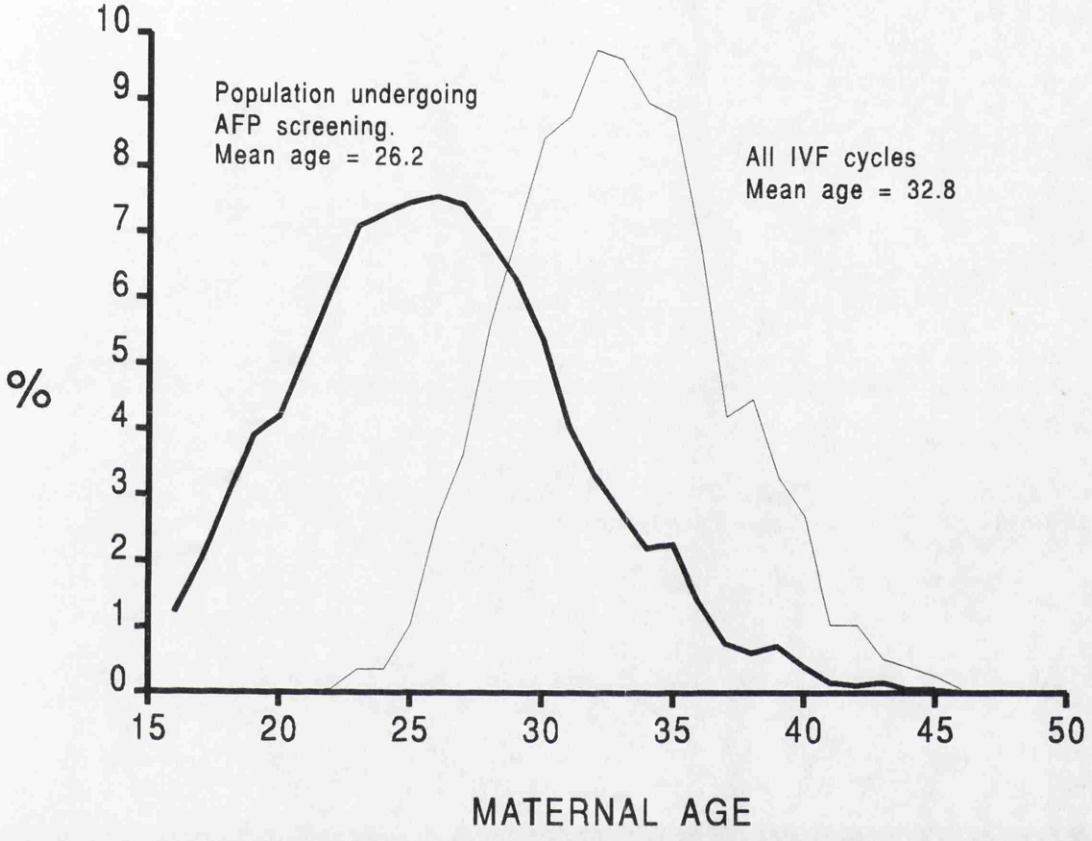


Figure 25

Age distribution of women undergiong AFP screening for neural tube defects and IVF for infertility.

from stimulated cycles (Pellicer et al., 1988). However, histology of human ovarian tissue surgically removed after hMG stimulation (Gougeon & Testart, 1990) suggested that all follicles were healthy at the time of recruitment/initiation of exogenous gonadotrophin.

Collaborative data reported by Plachot et al. (1988b) showed no difference in the proportion of chromosome abnormalities in oocytes after stimulation with CC/hMG, hMG, FSH or GnRH-a/hMG. Dosage of gonadotrophin did not affect the rate of anomaly in oocytes or proportion of 1, 2 and 3PN after insemination. Pre-hCG luteinisation, observed in 24% of cases, did not increase the incidence of non-disjunction in unfertilised, mature oocytes (Plachot et al., 1988a). Cytogenetic comparison of oocytes stimulated using CC/hMG had a significantly higher incidence of anomalies associated with immaturity than those from GnRH-a/hMG cycles but the incidence of aneuploidy was unaffected.

The incidence of sister chromatid exchange in considered a more sensitive marker of mutagenicity than visible damage (Perry & Evans, 1975). Exchanges were four times more frequent in embryos from superovulated mice than after spontaneous ovulation (Elbling & Colet, 1985). Increased breakage would dispose towards chromosome rearrangements.

Although haploid embryos have not been detected in any abortus material it is likely that the parthenogenetic nature of the 'embryo' would limit embryonic development (Whittingham, 1980). Some aspects of IVF methodology may dispose towards oocyte activation. Use of nitrous oxide for the pneumoperitoneum was implicated in the high incidence of haploid embryos in early studies by Angell et al (1986a). This substance is known to induce parthenogenesis in mice (Kaufman, 1983).

Triploid karyotypes are found in 1-3% of clinical pregnancies conceived *in vivo* and the majority are caused by polyspermy (Jacobs *et al.*, 1978). This figure does not include 3PN zygotes which did not develop to triploids (Kola *et al.*, 1987) and a proportion of XYY embryos thought to be lost in early pregnancies. The incidence of 3 PN after *in vitro* fertilisation has been shown to be influenced by differences in methodology among IVF programmes. Excessive growth of the leading follicle (Ben-Rafael *et al.*, 1986a), oocyte maturation time (Table 3.3.8), preincubation of oocytes before insemination (Trounson *et al.*, 1982b), method of follicle aspiration (Lowe *et al.*, 1988), the concentration of sperm for insemination (Wolf *et al.*, 1984) and biochemical markers of luteinisation (Ben-Rafael *et al.*, 1987; Webster *et al.*, 1985) have all been implicated.

8.7 Conclusions

Aneuploidy for different chromosomes occurs with variable frequency in human preimplantation embryos, probably due to different causative mechanisms during meiosis. Differential survival during development certainly operates and does eliminate the majority of hypoploid conceptions observed in preimplantation embryos. This mechanism is not totally responsible for the frequencies of abnormalities in spontaneous abortions.

8.8 Future work

Strains of mouse which show a strong maternal age effect (Gosden, 1973) have, like the human (Gosden, 1984), a short reproductive life compared with longevity. The nature of this chronological effect has been investigated using unilateral ovariectomy (uni-ovx) of CBA mice (Baker et al., 1980) which results in compensatory ovulation from the contralateral ovary and depletion of primordial follicles at an earlier age. This phenomenon was utilised to compare aneuploidy rates due to physiological and chronological age. An increase in aneuploidy was seen earlier in the uni-ovx group suggesting that investigation of factors affecting rates should be directed away from ageing per se. This might provide evidence for the production line theory but it also supports the hypothesis that age related changes in hormones could be responsible (Lyon & Hawker, 1973; Crowley et al., 1979).

Two other groups subject to fluctuations in hormonal imbalance have an high incidence of aneuploidy. The proportion of pregnancies affected by chromosome imbalance may be higher in peri-pubertal girls (Erikson, 1978) than after established menstrual rhythm although reports are not consistent. A small number of women with Turner's syndrome have sufficient ovarian tissue to allow intermittent ovulation (Ferguson-Smith, 1965) and these pregnancies have a high incidence of pregnancy wastage and Down's offspring (Reyes et al., 1976, King et al., 1978). This theory would also find support from the abnormality rate detected in breakthrough pregnancies on contraception (Alberman, 1978, Harlap et al., 1979) and could account for the exponential nature of the age related rise in trisomy (Hassold & Jacobs, 1984)

This theory might be usefully investigated by using a marker of the end of reproductive life such as inter-cycle

FSH which indicates potential lack of response to exogenous gonadotrophins and may be more important than actual age (Toner *et al.*, 1991). It is proposed to utilise ovarian tissue from women undergoing oophorectomy for gynaecological conditions not involving ovarian pathology to investigate the relationship between maternal age and aneuploidy. Preliminary data suggests that oocytes from unstimulated cycles can be successfully matured and fertilised *in vitro* (Cha *et al.*, 1991). Hormone profiles will be obtained prior to surgery and attention will be particularly directed to the mechanisms leading to malsegregation of chromosomes at meiosis 1. *In vitro* maturation will also facilitate manipulation of the hormonal milieu.

CHAPTER 9
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Appendix 1

EDR values of embryos assessed between 30 and 76h.

		Cell number								
Time	PN	1	2	3	4	5	6	7	8	8+
30	90	100	118	137	156	167	176	185	195	233
31	87	97	114	133	151	161	170	179	189	226
32	84	94	111	129	147	156	165	174	183	219
33	82	91	107	125	142	152	160	168	177	212
34	79	88	104	121	138	147	155	164	172	206
35	77	86	101	118	134	143	151	159	167	200
36	75	83	98	115	130	139	147	154	162	194
37	73	81	96	112	127	135	143	150	158	189
38	71	79	93	109	124	132	139	146	153	184
39	69	77	91	106	120	128	136	142	150	179
40	67	75	89	104	117	125	132	139	146	175
41	66	73	87	101	115	122	129	135	143	171
42	64	71	85	99	112	119	126	132	139	167
43	63	70	83	97	109	116	123	129	136	163
44	61	68	81	94	107	114	120	126	133	159
45	60	67	79	92	104	111	118	123	130	156
46	59	65	77	90	102	109	115	121	127	152
47	57	64	75	88	100	106	123	118	124	149
48	56	62	74	86	98	104	110	116	122	146
49	55	61	72	85	96	102	108	113	119	143
50	54	60	71	83	94	100	106	111	117	140
51	53	59	70	81	92	98	104	109	115	137
52	52	58	68	80	90	96	102	107	112	135
53	51	57	67	78	89	94	100	105	110	132
54	50	56	66	77	87	93	98	103	108	130
55	49	55	65	75	85	91	96	101	106	127
56	48	54	63	74	84	89	95	99	104	125
57	47	53	62	73	82	88	93	97	103	123
58	46	52	61	72	81	86	91	96	101	120
59	46	51	60	70	80	85	90	94	99	118
60	45	50	59	69	78	83	88	92	97	117
61	44	49	58	68	77	82	87	91	96	115
62	44	48	57	67	76	81	85	90	94	113
63	43	48	56	66	75	79	84	88	93	111
64	42	47	55	65	73	78	83	87	91	109
65	42	46	55	64	72	77	82	85	90	108
66	41	45	54	63	71	76	80	84	89	106
67	40	45	53	62	70	75	79	83	87	105
68	40	44	52	61	69	73	78	82	86	103
69	39	43	51	60	69	72	76	81	85	101
70	39	43	51	59	67	71	75	79	84	100
71	38	42	50	58	66	70	74	78	82	99
72	37	42	49	57	65	69	73	77	81	97
73	37	41	48	57	64	68	72	76	80	96
74	36	41	48	56	63	68	71	75	79	95
75	36	40	47	55	63	67	70	74	78	93
76	35	39	47	54	62	66	69	73	77	92

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