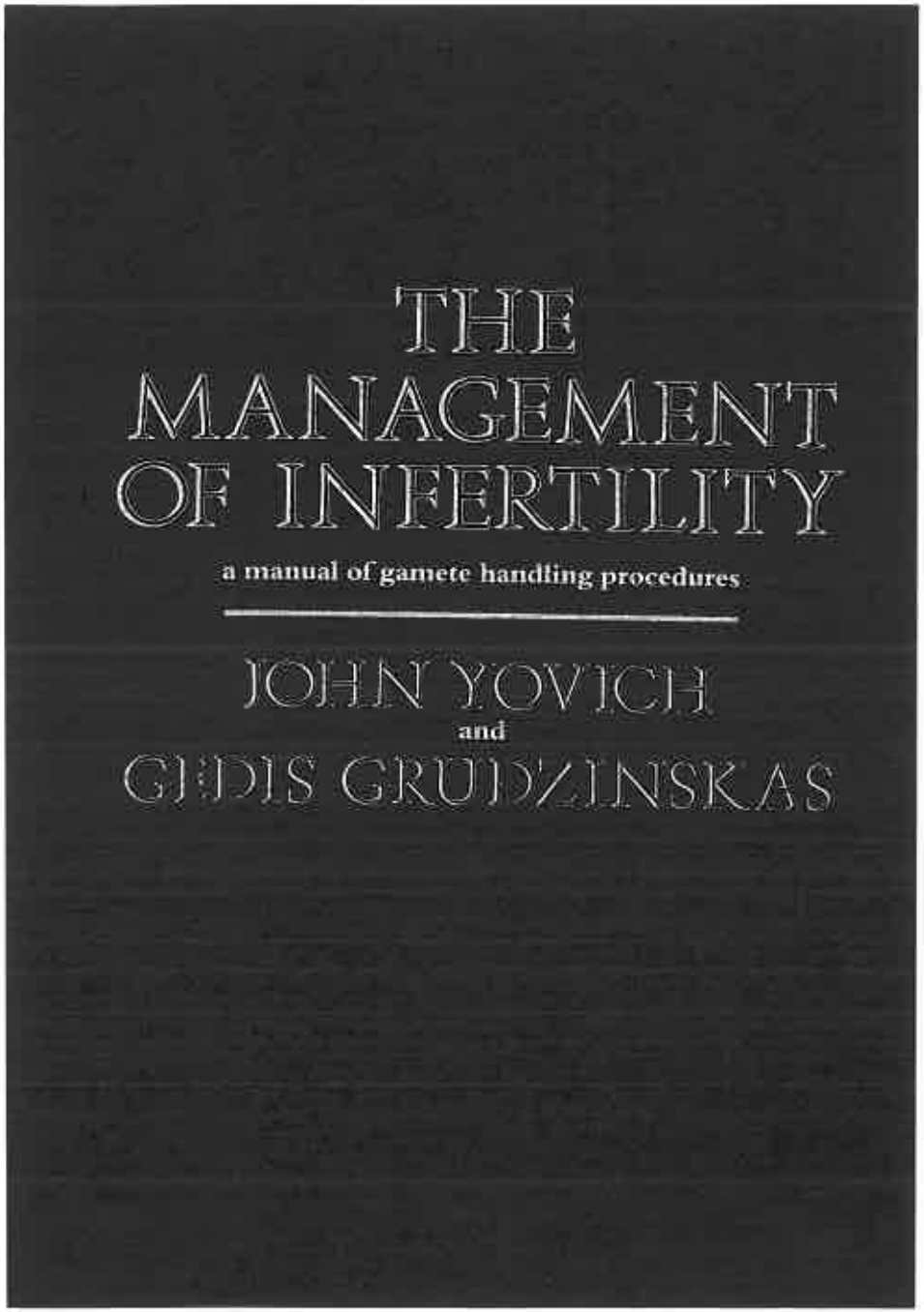

The Management of Infertility

A manual of gamete handling procedures

John Yovich, MBBS, MD, FRCOG, FACOG
Medical Director, PIVET Medical Centre, Perth, Western Australia;
Honorary Research Fellow, University of Western Australia, Perth

Gedis Grudzinskas, MD, FRCOG, FRACOG
Professor, Joint Academic Unit of Obstetrics, Gynaecology and Reproductive Physiology, The London Hospital Medical College and St Bartholomew's Hospital Medical College, London

Foreword by
Ricardo H. Asch

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Contents

Foreword

The introduction of assisted reproductive techniques such as IVF, GIFT and tubal pronuclear oocyte and embryo stage transfer in the last two decades has changed the entire pattern of management and teaching of infertility.

By the development of such technology a vast, increasing scientific knowledge has emerged in areas of biochemistry, embryology, molecular biology and genetics, with the immediate benefit of opening up treatment options for infertile couples, many of whom were previously considered untreatable.

However, we are still at a developmental stage, learning how to improve the fundamental technology based on very few appropriate animal models and facing the ethical problems raised by research and experimentation of various techniques in humans.

The main areas of current research involve new protocols of controlled ovarian stimulation, oocyte handling, gamete and embryo cryopreservation, sperm microinjection techniques and sperm retrieval from the epididymis and testes, as well as exploring the pathway of genetic diagnosis in preimplantation embryos. Developments in all these areas will certainly increase the number of patients able to be treated successfully in the future and will lead to a rewriting of the textbooks in the area of gamete physiology and human reproduction.

Looking with historical perspective we should not only concentrate on the great advances in the clinical and scientific issues but also focus our attention on the legal, ethical, moral and psychological issues to seek the optimum position of social acceptability of all current and future assisted reproductive techniques.

The authors of this book have succeeded in preparing a comprehensive state-of-the-art presentation for the application of modern assisted reproductive techniques. They have applied very clear descriptions and terminology that will greatly benefit the practising physician interested in human reproduction at any level.

This book provides an appraisal of the most advanced state of our knowledge in areas applied today for the treatment of infertile couples, from the induction of follicular development to the final gamete or embryo transfer, in a very practical fashion. The authors succeed in providing balanced advice in ethical and moral considerations, and treating patients rather than focusing on the disorders and techniques

alone. They are to be congratulated on these perspectives, and in my opinion, their work provides one of the definitive texts for current fertility management. They have drawn from a wealth of personal knowledge, much of which has been gained by trial and error during earlier years and which has currently been complemented by drawing on the experiences of other workers from around the world. The combined knowledge is leading to advancing sophistication of our understanding in the area of assisted reproduction and readers will be able to follow, learn and transfer the gained knowledge for the improved management of their own patients.

Ricardo H. Asch

Preface

The problem of infertility has gained prominence in the last decade for two main reasons. Firstly, because it is a health problem affecting an increasing proportion of couples (at least 15%) as they defer starting their families until a later stage in their reproductive lives due to the pressure of socioeconomic developments. Secondly, medical and technological developments involving ovarian stimulation and in vitro fertilization (IVF) techniques have led to a dramatic improvement in the diagnosis and prognosis for infertile couples. Peripheral developments such as the control of fertility by effective contraception have reduced the number of babies available for adoption in most countries, as well as reducing the number of children per household, so that several developed countries have now expressed serious concern regarding the diminishing birth rate which has led to static or falling populations, as well as a marked fall in the ratio of young to aged numbers in those populations. At the XIth World Congress of FIGO (International Federation of Gynecology and Obstetrics) in Berlin in 1985, the Mayor of the host city remarked on the importance of continuing work and research in the IVF-related area to assist those couples with a strong desire for children and family life in achieving their ambition. The benefits would be reflected in resolving the anguish of infertile couples as well as resolving community concerns.

We were pleased to be invited by Heinemann to prepare this book on infertility management as it combined overlapping interests in two major disciplines—the management of infertility and the concept of implantation. An effective understanding of the latter has only recently been evolving with improved knowledge regarding the identification of messages from the fetus, the trophoblast, the endometrium and the ovary. The identification of these messages has required developments in protein chemistry, purification techniques and assay procedures to enable the specific detection of local messages with the high degree of sensitivity required. Interpretation of those messages is a new science and the application of this knowledge at its threshold. Developments in this area are important because there is a high pregnancy wastage rate in those women who conceive after infertility treatments. This includes both the early and late pregnancy stages. Of particular concern is the high number of ectopic gestations which require early diagnosis for effective conservative management.

This book covers the modern management of infertility, bringing together the latest concepts in ovarian stimulation, combined with the precise description of gamete handling procedures required for the full range of treatments in infertility. It is written from a practical standpoint and includes all those techniques currently applied at the PIVET Medical Centre in Perth, Western Australia. We are particularly grateful to all those staff at PIVET who have contributed to this book in one way or another and helped us establish the described protocols which cover both clinical and laboratory areas. The book is intended to provide a comprehensive manual for all workers involved with infertility management.

The book concentrates on specific clinical protocols and associated laboratory techniques. It also includes a historical perspective on the evolution of the procedural methods which centre around IVF technology and the latest developments, such as tubal transfer procedures, cryopreservation, ovum donation and surrogacy. There is also a glimpse into future developments and it is anticipated that subsequent editions of this book will incorporate detailed descriptions of those techniques and their clinical applications when they have proceeded beyond the research stage of development.

John Yovich and Gedis Grudzinskas

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We gratefully acknowledge the direct contributions of embryologists Dr Jim Stanger, Dr Phillip Matson, Dr Linda Mohr, Dr Rohini Edirisinghe and Dr Jim Cummins who have worked at PIVET, and the large number of clinicians who have contributed in various ways. The on-going contributions of Drs Simon Turner and Rogan Draper are particularly acknowledged, together with the scientific staff who have assisted in the compilation of various chapters, particularly Jeanne Yovich, Paul Shenton, Jason Spittle, Ceinwen Gearon, Cheryl Hilliard and Barbara Bootsma. Advice regarding ultrasound was provided by Dr Peter Breidahl and his associates. Patient co-ordination and the maintenance of various registers was performed by Sue Devine, Fiona O'Halloran and Ann Tuvik. We are also grateful to the staff of PIVET Medical Centre who assisted with data compilation and the preparation of illustrations. The manuscript was compiled by Sharon Keyt.

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Abbreviations

A4	Androstenedione
Ab2	Second antibody reagent
AFS	American Fertility Society
AIDS	Acquired immune deficiency syndrome
AIH	Artificial insemination by husband
ARIC	Acrosome reaction following ionophore challenge
ASAB	Antispermatozoal antibody
B_0	Zero binding
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CPM	Cryoprotective medium
CT	Computerized tomography
CV	Coefficient of variation or chorionic villus
CVS	Chorionic villus sampling
DA	Daltons
DES	Diethylstilboestrol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DI	Donor insemination
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
E2	17β-oestradiol
ED	Estimated dose
EHT	Electron height threshold
ELISA	Enzyme-linked immunosorbent assay
EPF	Early Pregnancy Factor
ET	Embryo transfer
FFM	Follicle flushing medium
FROST	Frozen oocyte/embryo salpingo transfer
FSA	Fertility Society of Australia
FSH	Follicle stimulating hormone
GIFT	Gamete intrafallopian transfer
GnRH	Gonadotrophin releasing hormone
hCG	Human chorionic gonadotrophin
HIV	Human immune deficiency virus
hMG	Human menopausal gonadotrophin
hPG	Human pituitary gonadotrophin
HS	Human serum
HTFM	Human tubal fluid medium
HTLVIII	Human T-cell lymphotrohpic virus type III
ILA	Interim Licensing Authority
IRMA	Immunoradiometric assay

IS	International Standard
IU	International units
IVF	In vitro fertilization
IVF-ET	In vitro fertilization and embryo transfer
LAL	Limulus amoebocyte lysate
LH	Luteinizing hormone
LMP	Last menstrual period
MPA	Medroxyprogesterone acetate
N_2	Nitrogen (liquid)
NH&MRC	National Health and Medical Research Council
NPSU	National Perinatal Statistics Unit
NSB	Non-specific binding
OHSS	Ovarian hyperstimulation syndrome
OMI	Oocyte maturation inhibitor
PAF	Platelet Activating Factor
P4	Progesterone
PAPP-A	Pregnancy-associated placental protein-A
PB1	Phosphate-buffered medium 1
PBS	Dulbecco's phosphate buffered saline
PCO	Polycystic ovary
PCT	Post-coital test
PF	Pentoxifylline
PMSG	Pregnant mare's serum gonadotrophin
PN	Pronuclear stage
POD	Pouch of Douglas
PP12	Placental Protein 12
PP14	Placental protein 14
PRL	Prolactin
PROH	Propanediol
PROST	Pronuclear stage tubal transfer
PVC	Polyvinylchloride
QC	Quality control
RIA	Radioimmunoassay
RO	Reverse osmosis
RTAC	Reproductive Technology Accreditation Committee
SCMC	Sperm–cervical mucus contact
SHBG	Sex hormone binding globulin
SP1	Schwangerschaftsprotein 1
T	Testosterone
T6	Whittingham's modified Tyrode's solution
T9	Modified Tyrode's solution
TEST	Tubal embryo stage transfer
TSH	Thyroid stimulating hormone
VLA	Voluntary Licensing Authority
WHO	World Health Organization

Chapter 1

The spectrum of infertility

INTRODUCTION

Involuntary infertility is a worldwide problem which causes a sense of personal failure as well as carrying a social stigma in many cultures. Infertile couples describe a sense of anguish, and often desperation, when their desire to reproduce is unfulfilled. Without contraception, 75% of couples would achieve pregnancy within 12 months. Thereafter, the proportion conceiving is relatively low, and overall up to 15% of couples within the reproductive age range present for medical assessment, generally following two years of failed effort.

UNDERLYING CAUSES

The causes underlying infertility vary with geographical location, socioeconomic factors and the changing face of health problems within different areas and periods of time. A World Health Organization (WHO) report in 1975 documented the major causes of infertility on a global scale, and particularly in African countries south of the Sahara, as being largely due to four disorders:

1 Tuberculosis causing chronic ill health and genital tract infection in both males and females;
2 Venereal disease, especially gonorrhoea, causing tubal occlusion and obstructive azoospermia;
3 Post-abortal and post-partum infections;
4 Malnutrition, mainly causing increasing pregnancy wastage.

As recently as 1986, Bahadori reported a 25% incidence of tuberculosis in 291 infertile women in Iran, half of whom showed characteristic histological appearances on endometrial biopsy. In fact, he suspected the true incidence of tuberculosis among Iranian infertile patients to be greater than 40%.

However, in industrialized communities, the pattern of underlying causes differs quite markedly. Over the past decade, infertility clinics in the USA, UK, Europe and Australia have noted the disorder to be more common in the higher socioeconomic classes and those of

advanced reproductive age. Some of this perception relates to group selection based on those more likely to have information about new developments in infertility management, and individual self-selection based on the high cost of modern treatments, a large proportion of which is borne by the patient.

In the USA, national surveys of family growth show the prevalence of involuntary infertility to be 14% (2.5 million couples in the USA in 1982). The figures reveal a disproportionate number of black couples, couples with less than a high school education and where the woman had not previously used contraception (either oral contraception or intrauterine devices). There was a clear association of age with infertility, revealing that 25% of women aged between 35 and 39 years were infertile and there was a sharp rise after this age (Soules, 1988). However, the impression of an 'epidemic' in infertility over recent years is more apparent than real and relates to increased public awareness combined with changing socioeconomic trends, which have created the desire by most couples to delay the age of having children. Improved contraceptive techniques—especially oral contraception—have provided the means to achieve this.

The apparent reduction in fecundity of women in industrialized societies is due to the following broad aetiological categories (Cox, 1975): ovulatory dysfunction (25–45%); spermatozoal disorders (mostly unexplained; 20–35%); tubal disease (15–30%); pelvic endometriosis (10–50%); poor sperm–mucus interaction (5–15%); antispermatozoal antibodies (ASABs; 5–15%), and totally unexplained (5–10%). In less than a quarter of cases is a single factor identified as the sole cause of infertility. Most cases reveal a multifactorial basis although often a single dominant condition appears to have importance over other identified factors. This leads to some difficulty in evaluating the relevance of some disorders of ovulation, moderate range oligospermia and lower grades of pelvic endometriosis.

Uncommon causes of infertility include genital tract anomalies such as absence of reproductive structures (Rokitansky–Küster–Haüser syndrome, testicular feminization syndrome, congenital absence of vas deferens), as well as those caused by DES exposure in utero, intrauterine synechiae causing Asherman's syndrome, sexual dysfunction causing failure of sperm deposition in the vagina, and ejaculatory dysfunctions.

PROGNOSIS

Before 1960, no more than 20% of couples presenting with infertility were successfully treated. In fact, those conceptions which did occur following assessment were considered to be mostly unrelated to treatment (Jeffcoate, 1975).

The prognosis improved during the 1960s with the introduction of clomiphene citrate and pituitary gonadotrophin therapy for anovulation. Later in the decade, laparoscopy was introduced in the UK and has now rapidly established its role as a primary investigatory tool.

During the 1970s, infertility management improved with the introduction of sensitive and specific radioimmunoassays (RIA), the appreciation of the role of anaerobic organisms in the causation of pelvic inflammatory disorders, bromocriptine for the treatment of hyperprolactinaemia, the establishment of donor semen banks in frozen straws, the introduction of microsurgery (initially on the female and subsequently on the male genital tract), hysteroscopy and the detection of antibodies against spermatozoa and zona pellucida.

Developments during the 1980s included the establishment of artificial insemination using husband's washed spermatozoa (AIH) for intrauterine insemination, in vitro fertilization and embryo transfer (IVF-ET) and related technologies such as gamete intrafallopian transfer (GIFT), tubal transfer procedures for pronuclear stage (PROST) and cleaving embryo (TEST), embryo and ovum donation, the introduction of pulsed gonadotrophin-releasing hormone (GnRH) for certain ovulatory disorders followed later by GnRH analogues for pituitary down-regulation, the appreciation of *Chlamydia trachomatis* and *Ureaplasma hominis* as organisms underlying pelvic inflammatory processes, and improved knowledge regarding events leading to folliculogenesis, oocyte release and luteal function in the ovarian cycle.

The potential prognosis for successful treatment of couples presenting now for treatment has risen to at least 75%. However, in practice the prognosis is limited by cost considerations.

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Chapter 2

Infertility investigations and management

The assessment and treatment programmes which are implemented at a secondary and tertiary referral unit such as the PIVET Medical Centre, Australia have evolved in the past decade. The underlying philosophy regarding both diagnosis and management reflects an understanding that most infertility cases present with a complex and multifactorial basis. The effective identification of each of the underlying factors and selection of the appropriate treatment mode require an appropriate facility where this may be performed. Within the infertility management facility, there should be certain defined areas for specific functions:

1 *Consultation*: an area which relates to the specific consultation and examination of couples.
2 *Counselling*: an area for counselling for the exchange of information and for emotional support.
3 *Co-ordination*: a nurse co-ordination area where patient management is organized. In this area, all patient activities are co-ordinated. Management instructions can be translated into arrangements for specific tests, and the modification of management on the basis of results can be rapidly instituted.
4 *Laboratories*: on-site laboratory areas for embryology, andrology, cryopreservation and hormone assays. Ready access to general pathology areas is also required.
5 *Ultrasound*: ultrasound (for ovarian follicle monitoring and early pregnancy assessment) and radiology facilities (e.g. for hysterosalpingograms).
6 *Results*: central site attended by key personnel from each area at a fixed time, for example 3.00 p.m. each day. At the results session, the case file for each patient undergoing a treatment cycle in any programme is reviewed. A summary of clinical information combined with laboratory and ultrasound results is presented by the co-ordinating nurse for interpretation by the assessment group. Based on the deliberations of the team, the managing clinician will issue written instructions to modify that specific patient's management, e.g. drug dosage, schedule for ovarian stimulation, timing of insemi-

nation in AIH or donor insemination (DI) programmes, giving the human chorionic gonadotrophin (hCG) trigger, timed oocyte recovery, etc. Such instructions are then taken over by the co-ordination area on completion of the results session.

7 *Treatment*: apart from consultation and examination rooms, a number of treatment areas are required within the clinic to enable patients to undergo insemination treatments, have cervical scores assessed, post-coital test (PCT) evaluated, hormonal injections given, routine swabs undertaken during in vitro fertilization (IVF) cycles, and an area where blood specimens can be collected. The clinic may commence typically at 7.00 a.m., permitting all blood specimens for the day's assays to be collected by 9.00 a.m. in order to be included in the assay runs which commence around 9.30 a.m. In addition, an early start allows many working patients to avoid interrupting their schedules and having to explain their late arrival at work.

8 *Semen collection rooms*: these should be directly attached to the laboratory, and should be easily accessible from outside the main building on an appointment basis. The rooms should be comfortable, sound-protected, connected to the central air conditioning arrangement, and conveying a sense of privacy. Appropriate literature should be discreetly available, along with specimen collection jars and clear instructions regarding the labelling of individual specimens. Once the ejaculate is collected, the specimen jar is placed in a double hatchway area. The patient presses a buzzer prior to leaving the semen collection room, and usually proceeding to work. The laboratory will retrieve the specimen from its side of the double hatchway as soon as the buzzer is triggered.

9 *Operating theatre*: ready access to operating theatre facilities is mandatory for procedures such as oocyte recovery and transfer where anaesthesia is required. A dedicated IVF laboratory must be a component part of the theatre suite, preferably located immediately adjacent to the oocyte collection theatre.

INVESTIGATING INFERTILITY

Clinical examination

The clinical examination of both partners in a combined clinic is ideal. The examination should concentrate on the endocrine systems and genital tracts of both. However, regardless of obvious clinical conditions, e.g. polycystic ovary (PCO) disease, hirsutism, varicocele, etc., a standard investigation profile is essential as there is often a multifactorial basis for the infertility. Occasionally, one detects absolute

infertility conditions such as Turner's syndrome in the female and Klinefelter's syndrome in the male. In such cases, the depth of investigations in the other partner depends upon treatment plans.

Assessment and testing

For the woman, this includes a routine endocervical swab for aerobic and anaerobic cultures and a second swab for detection of *Chlamydia* antigen and a routine cervical smear. Semen analysis should be arranged for the male (after 4 days' abstinence) and at least two repeat samples should be obtained if results show volume <1 ml, total sperm density <20 million/ml, motile sperm density <12 million/ml, <60% sperm motility, <60% normal morphology or ⩾5 leucocytes/hpf (×400). Antispermatozoal antibodies should be checked in the serum of both partners as well as in semen and cervical mucus. Full antibody characterization such as the direct or indirect immunobead test (Clarke et al., 1985; Figs 2.1 and 2.2) is preferred over MAR (IgG) test.

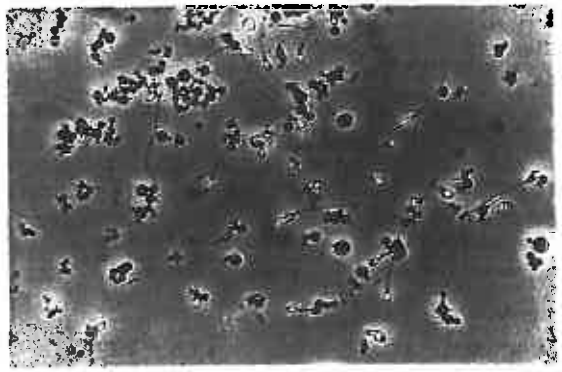

Fig. 2.1 Antigen-coated immunobeads bound to the head and tail regions of motile spermatozoa.

Serum prolactin (PRL), follicle stimulating hormone (FSH) and luteinizing hormone (LH) should be assessed in the early follicular phase for oligomenorrhoeic, amenorrhoeic and galactorrhoeic women. Routine hepatitis B antigen/antibody screening and human T-cell lymphotropic virus type III (HTLVIII) antibody screening of both partners and rubella titre of the female should be conducted.

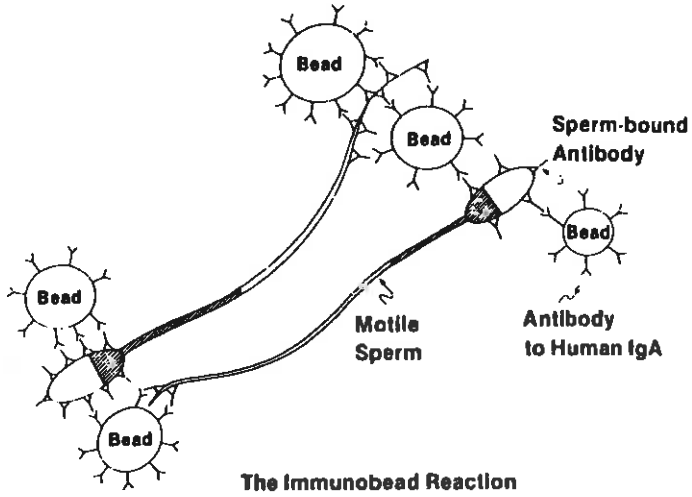

Fig. 2.2 A positive immunobead test result showing a typical appearance of greater than 20% binding to motile spermatozoa.

Endocrine profiles

Specific endocrine profiles should be undertaken according to clinical indications, e.g. androgen profile for PCO in hirsute women (serum testosterone, sex hormone binding globulin (SHBG), androstenedione (A4), dehydroepiandrosterone (DHEA) and its sulphate (DHEAS) as well as LH and FSH, thyroid, adrenal, pituitary and pancreatic function where relevant.

Ovulation profile

An ovulation profile (Yovich et al., 1987; Fig. 2.3)—should be composed, preferably comprising daily serum 17 β-oestradiol (E2), progesterone (P4) and LH in the preovulatory build-up to LH surge (generally days 11–14), and subsequent mid luteal (7–10 days post LH surge) serum E2 and P4. On the day before the LH surge, E2 should be $\geq$620 pmol/l. An effective LH surge is accompanied by raised P4 levels. Ultrasound monitoring of follicular development should be undertaken in the immediate pre-surge period and up to 3 days after if one wishes to observe follicle dispersal. Ultrasound is also useful in the early cycle to determine whether multifollicular ovaries are present and to assist in the identification of women with PCO (Adams et al., 1985).

The development of fertile cervical mucus is assessed in the imme-

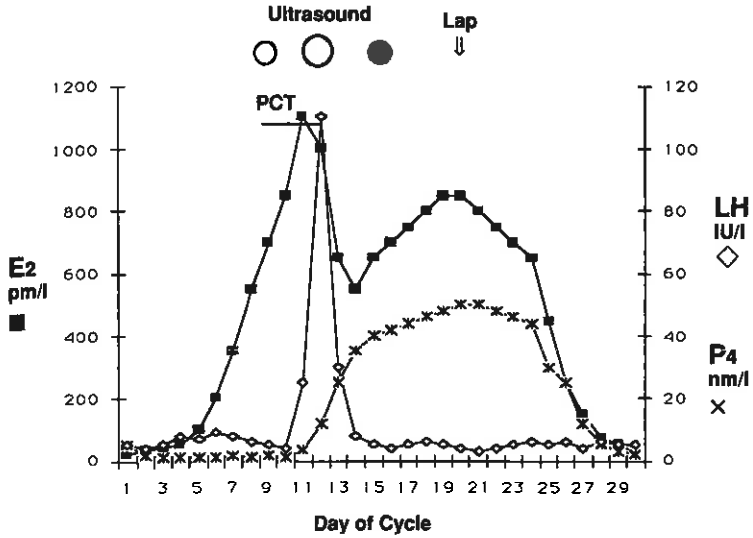

Fig. 2.3 Schematic diagram to depict hormonal parameters in an ideal ovulatory cycle and noting the preferred times for postcoital test (PCT) evaluation, periovulatory ultrasound and laparoscopy/endometrial biopsy (Lap).

diate preovulatory phase. Insler scoring notes four characteristics—dilatation of the cervical canal (Insler et al., 1972); quantity and clarity of the mucus; spinnbarkeit and complexity of the ferning pattern after the mucus is allowed to dry on a slide. These are given a score out of 3, giving a possible total of 12. Fertile mucus is that which achieves a score $\geq$6/12.

Sperm–mucus interaction

Sperm–mucus interaction is evaluated as a Sims–Huhner post-coital test performed in the presence of fertile mucus and preovulation hormonal parameters (before P4 rise), following 4 days of abstinence and assessed 6–12 hours post coitus. Cervical mucus must be obtained from at least 1.5 cm up to 3.0 cm into the cervical canal, using long forceps; it is immediately examined under the microscope. The result is classified as negative (no progressively motile spermatozoa/hpf); equivocal (up to 9 progressively motile spermatozoa/hpf) or positive ($\geq$10 motile spermatozoa/hpf). In vitro assessment of sperm–mucus interaction such as the sperm–cervical mucus contact test (SCMC) for a crossed hostility study may have a role in detecting whether

the fault resides in semen or mucus. In the past this was considered to be an indication for specific treatment, e.g. DI, but nowadays treatment options are broader.

Laparoscopy

Laparoscopic appraisal of the pelvis with dye pertubation to assess tubal patency is usually necessary. This is combined with dilatation of the cervix, sounding and exploring the uterine cavity, and endometrial biopsy. The latter investigation is retained more for the exclusion of endometritis (including *Mycobacterium tuberculosis*) than for histological dating. Luteal hormonal evaluation (i.e. P4) is considered more relevant in the assessment of the luteal phase.

Hysteroscopy and hysterosalpingography

These procedures are performed in women with conditions such as recurrent abortions, scant menses, suspected uterine anomalies and intrauterine irregularities detected during the laparoscopy appraisal or follow-up assessment of tubal patency.

Counselling

Counselling of all new couples is valuable at an early stage. Because of the complexity of management, patient education plays an integral role. In addition, couples need to be familiar with counsellors who may assist at times of frustration, failed treatment cycles and domestic tension. Their role may also be wider in attending to staff reactions arising from emotional sympathy with the frustrations of infertile couples.

Infertility diagnosis

A full investigative assessment of the infertile couple will generally reveal more than one factor associated with their infertility. Often a dominant factor such as severe oligospermia or grade IV pelvic endometriosis may be considered to be the cause. Occasionally, a sole factor is identified in the male or female partner, while other parameters are shown to be normal. However, the usual situation is depicted in Figure 2.4, which shows that the range of infertility factors indicate the majority of couples will display a multifactorial basis for their infertility. It is essential to identify these factors in the preliminary investigations before treatment begins. Management may centre around what is perceived to be the dominant condition, but patients must be aware of the additional factors, which may also require treatment should the initial approach not prove successful. All factors must be identified so that couples have a clear idea of the basis of their infertility and do not lose confidence in their therapists if unidirectional treatments do not achieve pregnancy. This may particularly bear consideration in the management of endometriosis and surgery for peritubal adhesions.

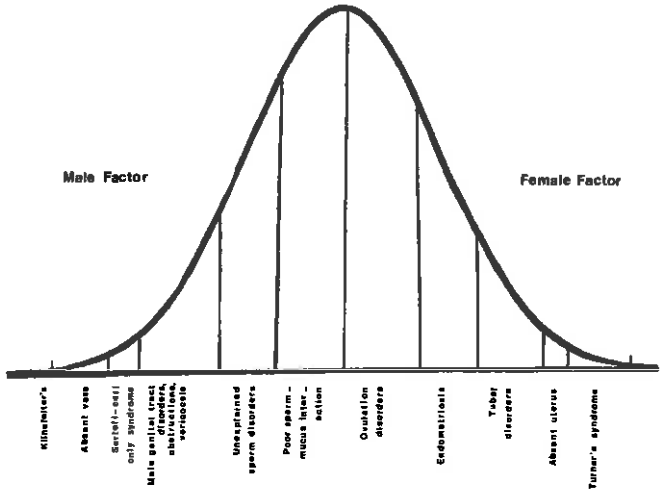

Fig. 2.4 Range of infertility factors which need to be considered in the assessment of all couples, showing the multifactorial basis of infertility seen in the majority of patients.

The standard investigative profile described above should be completed in a single cycle and should be followed by a clinical consultation where the full spectrum of results and treatment options is discussed with the couple.

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Chapter 3

Infertility treatment: general considerations

The specific treatments offered to each couple will fall under the following five broad categories:

1 Management of specific conditions.
2 Ovarian stimulation.
3 Gamete manipulation.
4 Gamete substitution (donor).
5 Surrogacy.

The management of specific conditions such as pelvic endometriosis and fallopian tube obstruction will not be discussed in great detail here; such cases are best managed in a clinical environment which embraces total infertility management. This is not to say that a single clinician should be proficient in all areas of infertility treatment, but rather that the clinic in which the patient is being managed should have the facilities and the expertise within it to manage the broad range of infertility problems. It is not suitable for patients to be referred completely away from the clinic, e.g. for management of hyperprolactinaemia or for reconstructive tubal microsurgery, and then to be lost from continuing integrated management by the clinic. It is disappointing if such patients return years later to find that they have not conceived despite the seemingly successful management of their specific condition. This is often due to additional factors contributing to their infertility, or misperceptions, e.g. regarding the likelihood of success following reconstructive tubal surgery.

The following brief descriptions address specific conditions in the light of recent advances.

ENDOMETRIOSIS

Endometriosis is the proliferation of functioning endometrial-like tissue outside the uterine cavity. It commonly affects the ovaries, uterosacral ligaments, fallopian tubes, the broad ligament, the bladder and rectum. Sometimes it is a cause of a nodular obstruction in the fallo-

pian tube and it can also occur in other sites such as the appendix and wound scars outside the abdominal cavity. It may affect up to 25% of women in the reproductive age range; some degree of pelvic endometriosis can be identified in 40% or more of women in whom laparoscopy is undertaken for the investigation of infertility. Its severity can be graded as from I to IV according to the American Fertility Society classification (1985).

There is an increasing prevalence of endometriosis in developed countries associated with an increased total number of menstrual cycles, reduced number of pregnancies, short lactational periods and deferment of childbearing. It appears to be the main underlying cause of infertility in 10–15% of couples but the precise mechanism remains unknown.

Endometriosis can induce symptoms such as secondary dysmenorrhoea or pelvic dyspareunia and menstrual irregularities apart from infertility. However, the degree of symptoms is often unrelated to the extent of the condition. With grades III and IV pelvic endometriosis there may be interference with oocyte recovery and its transport in the fallopian tubes. Associated ovulation disorders have also been described, as well as reproductive endocrine anomalies such as a 'double peak' of LH and altered local secretion of prostaglandins. More recent data point to an implantation defect which may have an immunogenic basis (Yovich et al., 1988). Occasionally, very severe grades of pelvic endometriosis will be found in women as young as 20 years who generally have a preceding history of severe dysmenorrhoea.

Traditionally, treatment has been medical, surgical, or a combination of both. Increasingly it appears that gonadal steroid therapy should be the first treatment in all cases. In the past, progestagens or oestrogen–progestagen combinations were used but danazol, a mildly androgenic steroid, is now used as the preferred agent. When a progestagen is preferred, either medroxyprogesterone acetate (MPA) or dydrogesterone probably causes least side-effects.

Steroid treatment should be continuous, beginning with a dose regimen to create amenorrhoea (e.g. danazol 800 mg/day) and reducing to a maintenance level that continues to inhibit the ovarian cycle (e.g. danazol 400 mg/day). Danazol may cause side-effects such as nausea, weight increase, hoarseness, hirsutism, acne and a reduction in breast size. The latter may be seen as a benefit for those women who experience mastalgia. More recently, the use of GnRH analogues has been explored and promises to be successful. They cause pituitary down-regulation, which creates a hypogonadotrophic hypo-oestrogenic state and consequent involution of the endometriotic deposits.

Generally, a laparoscopic review is required following 6–12 months of medical treatment. Persisting endometriotic deposits and endometriomas may require conservative surgery. Currently, laser coagulation of small endometrial deposits is proving to be effective (Nezhat et al., 1988) possibly causing less post-operative adhesions and scarring than surgical or diathermy excisions. Constant neodymium (Nd:YAG) laser has greater depth of penetration and is preferred over the full-beam CO_2 laser. Major surgery is required to excise ovarian endometriomas and peritubal or periovarian adhesions. The principles of microsurgery should be applied. The Nd:YAG laser is also showing promise as a useful surgical tool for excision and adhesiolysis procedures.

IVF-related procedures, particularly GIFT, are applied where previous treatments have failed to generate pregnancies. Women with grades I and II pelvic endometriosis may be treated successfully without preliminary medical therapy but in grades III and IV disease, women should have preliminary medical therapy prior to undertaking IVF-related procedures in order to improve the chance of implantation (Yovich et al., 1988). Occasional patients will require radical surgery (hysterectomy and bilateral salpingo-oophorectomy) despite all efforts with conservative treatment and attempts to achieve pregnancy. However, it is the authors' experience that this is rarely necessary in women who are still trying to conceive—many pregnancies have been achieved in women who have had surgical treatment for grade IV pelvic endometriosis, including bowel and tubo-ovarian resections.

RECONSTRUCTIVE TUBAL SURGERY

Significant tubal disorders causing disturbances of the ovum dispersal mechanism or ovum transport may be present in up to 35% of infertility cases; almost half of these women will demonstrate proximal or distal tubal occlusion. Underlying causes such as pelvic endometriosis, tuberculosis and inflammatory disorders from a range of organisms must be controlled before undertaking corrective surgery. Where distal tubal disease is present, a major consideration is associated proximal disease which can lead to partial or complete occlusions which will limit the subsequent results achieved from salpingostomy and fimbrioplasty procedures. In the evaluation of such cases at the preliminary laparoscopy, palpation of the isthmic regions for nodularity and observance of these regions during dye pertubation confers important information. Salpingitis isthmica nodosum may be clearly palpable with Semm's grasping forceps. Other cases may be recognized by observing the isthmic segments 'standing up' during dye pertubation with impeded flow down to the distal fallopian tubes.

The results of infertility surgery have improved markedly in recent years since the principles of microsurgery have been adopted. These principles include the use of an atraumatic technique; careful attention to haemostasis; irrigation of tissues during procedures and careful

wash-out on completion to remove fibrinous clots; the complete excision of pathological tissue; fine needlepoint electrocautery or laser coagulation techniques; bipolar electrocoagulation of fine vessels within and near the fallopian tubes; non-abrasive packing; magnification; the use of delicate instruments and fine sutures; the precise alignment and apposition of tissue planes; serosal re-peritonealization using free grafts if necessary, and eversion of parietal peritoneum during closure. For re-anastomotic reversals of tubal sterilization, the patency rate is better than 90% with more than 60% of patients having a successful pregnancy (Winston, 1981). Results are better if the final tube length is greater than 5 cm and particularly if the ampullary or infundibular end is preserved. Ectopic pregnancies occur in 1–2% of women post-operatively.

Microsurgical tubal reconstructions performed for inflammatory disorders provide pregnancy rates of 20–40% for salpingostomy, 30% for fimbrioplasty, 40% for cornual implantation, 45% for salpingolysis and 60% for discrete resection/re-anastomoses (Winston, 1981). Comparative studies indicate that microsurgery offers improved results over conventional techniques (Winston, 1981), although this is not marked with salpingolysis or salpingostomy, the former being equally satisfactory and the latter being equally poor (indicating additional functional disturbance of the fallopian tubes). The ectopic pregnancy rate following surgery for inflammatory damage is variably reported as 5–20%.

Reconstructive tubal surgery should also be considered for the conversion of cases from IVF-ET to tubal transfer techniques such as GIFT or PROST if the procedure permits subsequent access to a patent and mobile fallopian tube. The tubal transfer techniques are associated with a significantly higher chance of pregnancy, although the advantages must be weighed against the increased risk of ectopic pregnancies (10% or more).

MALE FACTOR INFERTILITY

Until recently, the management of male factor infertility has been universally poor. A range of drug treatment approaches have been tried to improve spermatogenesis but none have shown convincing evidence of success, with the exception of gonadotrophin therapy for hypogonadotrophic hypogonadal males. Many male factor causes of infertility are not treatable, e.g. Klinefelter's syndrome, hypergonadotrophic hypogonadism, Sertoli cell-only syndrome and abnormal sperm morphology (microdefined, e.g. round-headed sperm and defined by electron microscopy, e.g. Kartagener's syndrome). However, in most cases of male factor infertility, undetermined causes of reduced sperm numbers (oligospermia), reduced sperm motility (asthenospermia) or

a reduced number of mature normal ovoid forms (teratospermia) are seen. A number of developments in the last decade have improved the prognosis for such cases of male factor infertility.

Surgical

The introduction of microsurgical techniques has led to an improved prognosis for discrete obstructions of the vas (including vasectomy), as well as epididymal obstructions (Silber 1981; Silber et al., 1977). The prognosis for vasovasostomy re-anastomoses is ≥80%. The prognosis for vasoepididymostomy procedures is dependent upon the level (caput, body or caudal region), with an overall prognosis of around 50%.

Furthermore, microsurgical treatment is now often combined hand-in-glove with IVF clinic treatment to improve the prognosis further. Male surgery performed in IVF theatres allows the pinpointing of the appropriate region of the epididymis where motile spermatozoa can be identified, enabling the surgeon to better select the site of anastomosis. Secondly, epididymal sperm can be retrieved prior to re-anastomosis and used in a combined procedure to fertilize the wife's oocytes on the same day that the husband is having a vasoepididymostomy procedure. The collection of spermatozoa at the very high caput region has led to fertilization and pregnancy has been reported (Silber et al., 1987).

In some cases, scrotal exploration reveals absent vasa deferentia which is usually identified clinically and shown by azoospermia combined with a low pH of the semen and absent seminal fructose, indicating the combined absence of vas and seminal vesicles. In such cases, scrotal exploration may still be of benefit to permit spermatozoa to be recovered for IVF of the wife's oocytes. Again, this is best performed using microsurgical principles in order to avoid trauma and post-operative adhesions, and allowing the consideration of further attempts if required.

Gamete handling

Sperm washing, precapacitation and sperm selection for IVF have led to the ability to retrieve and concentrate motile sperm preparations from men with varying degrees of oligospermia. In certain cases this has proven satisfactory for treatment by AIH, with pregnancy rates of approximately 15% per treatment cycle for moderate oligospermia (see Chapter 7). More severe cases can be treated by IVF and if necessary sperm penetration of the oocytes can be enhanced by either chemical agents, e.g. pentoxifylline (PF), or micromanipulation, e.g. zona splitting, zona drilling, or microinjection. These procedures are discussed

in Chapters 9 and 12. At this point it is relevant to state that gamete handling procedures have introduced treatment options for male factor infertility which are already promising to have an equivalent impact to female factor infertility.

Antispermatozoal antibodies

Male

ASABs can be identified in approximately 5–10% of male partners of infertile couples. However, despite considerable evidence to support a significant role, the importance of ASABs as a cause of male infertility has remained controversial. Recent studies have shown that the presence of both IgA and IgG in seminal plasma is associated with a decreased incidence of satisfactory PCT results and a reduced rate of fertilization of human oocytes in vitro (Junk et al., 1986). No significant differences were found for men with IgA and IgG alone when compared to men with no detectable ASABs (Matson et al., 1988). Such antibodies appear to exert their effect by causing spermatozoa to agglutinate one to the other, thereby reducing the number available for sperm–egg interaction. The treatment of such cases can involve the use of oral corticosteroids to suppress antibodies. This can be effective, but steroidal side-effects may be quite serious, e.g. psychotropic effects, hypertension and Cushingoid states. Alternative treatments have involved the use of washed spermatozoal preparations for AIH and IVF-related procedures. The success of sperm washing techniques is enhanced by collecting the ejaculate directly into culture medium and receiving the ejaculate to begin immediate washing prior to full liquefaction (see Chapters 7 and 9).

Female

Significant levels of circulating ASABs were identified in 7% of women seeking advice about infertility, using the indirect immunobead test. High levels of IgA correlate with poor sperm–mucus interaction and negative post-coital tests. At this stage, the association between circulating and mucus antibodies is unclear, as is the clinical relevance to the overall picture of infertility. However, it seems that the identification of circulating IgA is of major relevance, regardless of whether cervical mucus antibodies are identified; steroidal treatment can be considered for women but is not recommended. The preferred options are:

1 *AIH*. A 17% chance of pregnancy can be attained per treatment cycle (see Chapter 7). This suggests that the main functional defect is the failure of spermatozoa to pass through the cervical mucus. Although there is known to be a reduced fertilization rate in vitro, the failure of antibodies to totally block fertilization enables pregnancy to occur if spermatozoa can be rapidly flushed into the higher regions of the genital tract when an oocyte is present.

Table 3.1 Gamete handling techniques used in the treatment of infertility

Treatment mode	*Sperm preparation*	*Transfer*	*Site*	*Time*
DI	semen	sperm	cervix	Day 0 ± 1
AIH	washed	sperm	uterus	Day +1, +2
GIFT	washed	gametes	tube	Day 0
PROST	washed	2PN oocytes	tube	Day +1
IVF-ET	washed	embryos	uterus	Day +2
TEST	washed	embryos	tube	Day 2–4

2 *IVF*. The PROST method is the preferred, allowing IVF in the presence of antibody-free donor serum.

Poor sperm–mucus interaction

This category of infertility is identified by negative or equivocal PCT evaluation and can be further analysed by in vitro studies of sperm–mucus interaction, for example using the SCMC test. Negative PCTs are often found where there is an ovulation disorder limiting the development of suitable cervical mucus or a sperm disorder affecting sperm motility. However, poor sperm–mucus interaction can be identified in up to 10% of couples where ovulation and sperm function appear to be normal.

Genital tract infections and the presence of ASABs must be excluded before making a diagnosis of unexplained poor sperm–mucus interaction. Such cases have a good prognosis in AIH (16% per treatment cycle) and can also be treated by GIFT. Although some centres have suggested that negative PCTs are associated with poor IVF outcome, this has not been the authors' experience; we have found that the chance of pregnancy by GIFT is the same as for unexplained infertility, and is the same by IVF-ET as for tubal infertility (see Chapters 7, 10 and 11).

The following chapters will deal with ovarian stimulation and gamete manipulation techniques, but it has already been mentioned that these are generally applied in combination. Gamete handling procedures are summarized in Table 3.1 which introduces the acronyms applied, the general methodology of the technique and its timing in relation to the ovarian cycle.

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Chapter 4

Ovarian stimulation

Ovarian stimulation has an important role in infertility management and is considered in three situations—anovulation, disordered ovulation, and ovulatory women—either to enhance other therapies or to increase oocyte numbers for IVF-related procedures.

OVULATORY DISORDERS

Weight-related

Ovulation and ovulatory disorders may be weight-related, particularly in Caucasian women <45 kg or >75 kg, or may occur secondary to other endocrine disorders and underlying chronic diseases such as tuberculosis. Correction and control in these areas can improve ovulation. Plate 1 shows the normal body mass index (BMI) range for Caucasian individuals (derived from the formula weight (kg)/height^2 (m)). Most women have severe disturbances of ovulation if the BMI is <18 or >28. The adverse effects appear to extend to other areas of reproductive function also, such as implantation and pregnancy outcome.

Stress

Geographic relocation, emotional, physical or psychogenic factors and unresolved conflicts causing chronic stress can interfere with the ovulatory mechanism, in particular in younger women. Careful evaluation, counselling and other investigations to exclude subclinical disease in other systems should be considered. With respect to physical activity, high intensity training programmes (e.g. marathon running, high grade competitive sports and ballet training) would have to be modified if pregnancy is desired. In such women the BMI levels may need to be higher as the component of body mass contributed to by muscle rather than fat tissue is higher. With respect to employment, any decision regarding cessation of work should take into account where the woman feels most comfortable—most women enjoy their work and the social interaction within the work environment. It

should rarely be necessary for the woman to consider ceasing work in order to improve her fertility. However, this may be important to consider if there are unresolved conflicts, an excessive workload, or personality-related disturbances in the work environment.

Hyperprolactinaemia

Prolactin (PRL) levels should be assayed in serum samples early in the follicular phase around mid-day to early afternoon when surge levels are least likely. Hyperprolactinaemia (only 20% is associated with galactorrhoea) should be fully investigated. Computerized tomography (CT) scan sections of the pituitary gland provide important information prior to treatment. Bromocriptine is effective in controlling the hyperprolactinaemia, limiting microadenoma expansion and re-establishing normal ovarian function in most cases. Additional ovarian stimulation therapy may be required, as well as attention to other fertility factors.

Anovulation

Absolute anovulation, especially in amenorrhoeic women with hypo-oestrogenism and who are not hypergonadotrophic, has the best prognosis. Clomiphene citrate (50 mg up to 200 mg/day for 5 days) can be effective in those women who have a satisfactory withdrawal bleed following medroxyprogesterone acetate (MPA; 10 mg/day for 5 days). The effectiveness of therapy can be judged by basal body temperature changes but is better observed by more intensive monitoring of the ovarian cycle (see below).

Gonadotrophin therapy is indicated for those women who do not have a satisfactory MPA withdrawal bleed, fail to respond to clomiphene citrate, or fail to conceive despite 4–6 'satisfactory' treatment cycles on clomiphene. Hypergonadotrophic anovulation or amenorrhoea generally indicates ovarian failure in younger women or the menopausal state in women over 40 years. However, in some women in whom the diagnosis is not clear, GnRH analogues can be used to down-regulate pituitary activity thus permitting the successful use of high-dose human menopausal gonadotrophin (hMG) stimulation of the ovaries. For those hypergonadotrophic women who do respond to this regimen, the underlying ovarian condition may be that of ovarian resistance or incipient ovarian failure, which may be diagnosed by ovarian biopsy demonstrating the scarcity of primordial follicles. However, the diagnosis is esoteric, given that the diagnosis is possible by chemotherapeutic means and ovarian biopsy for this purpose is no longer recommended.

Disordered ovulation

Generally, ovarian stimulation is not recommended if evidence of ovulation has been observed. However, the more intensive assessment of ovulation indicates that many cycles have lesser disorders in the follicular and luteal phases while remaining reasonably regular in length. In such disorders the hormonal indices usually measured in the menstrual cycle can be outside the normal range for conception .(Fig. 4.1) and these can often be improved by ovarian stimulation

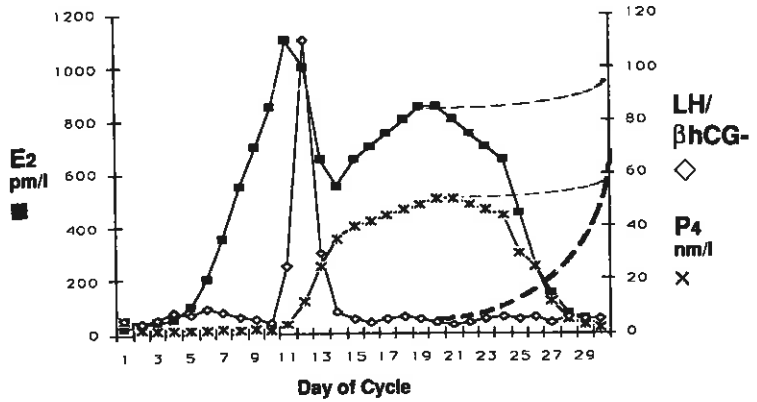

Fig. 4.1 Typical hormonal levels in an ovulatory cycle during which conception may occur.

therapy (Yovich et al., 1987). Some women will respond to clomiphene citrate used alone but the most effective approach appears to be the use of combined clomiphene (e.g. 100 mg/day, days 2–6 of the cycle) with hMG, triggering with hCG and also providing luteal phase support with hCG (Fig. 4.2; see below). Approximately 70% of women will respond appropriately, and half of these will conceive within three cycles.

OVARIAN STIMULATION REGIMEN

All disturbances of ovulation can be treated similarly using the following principles:

1 Correcting of any underlying disorders where possible;
2 Assessing response to clomiphene citrate in the first instance;

3 Enhancing the effect of clomiphene citrate using hCG to trigger ovulation;
4 Supporting the luteal phase using further hCG injections;
5 Introducing hMG combined with clomiphene citrate;
6 Removing clomiphene if adverse effects are recognized;
7 Introducing GnRH analogue if indicated.

Ovarian stimulation drugs have been widely used now for almost three decades and appear to be very safe. No serious long-term sequelae have been reported in women using the drugs, neither has any teratogenic effect been revealed.

Clomiphene citrate

Clomiphene citrate contains a mixture of cis- and trans-isomers, is well absorbed following oral injection, and has a half-life of 5 days. The main route of excretion is in the faeces following enterohepatic circulation. Although developmental abnormalities can be produced in animal studies, no teratogenic effect has been shown in humans. Women may experience primary side-effects such as hot flushes and visual disturbances such as scotomata which disappear when the drug is withdrawn. Some women may demonstrate marked elevations of androgens on clomiphene citrate treatment and others may demonstrate biliary stasis. There is no evidence that clomiphene citrate causes genital tract or breast cancer.

Clomiphene citrate is generally commenced at a dose of 50 mg/day, given on days 2–6 of the ovarian cycle, increasing up to 200 mg/day for 5 days. The majority of responders require 50 mg b.d., hence many clinicians choose this dose initially, avoiding the lower dose prior to progressing to the next stage. The effectiveness of therapy is judged in four ways:

1 Basal body temperature changes;
2 Hormonal assays;
3 Cervical mucus changes;
4 Ovarian follicle monitoring by ultrasound scan, described in detail below.

A triggering injection of hCG can then be given at the appropriate stage of folliculogenesis and luteal support may also lead to improved results. A triggering dose of hCG is 5000–10 000 IU and booster injections during the luteal phase should be 1000 IU given on days 4, 7, 10 and 13 after the initial triggering injection. Apparent ovulation occurs in 70% of cases but pregnancy ensues in only half of these, usually within three treatment cycles. The discrepancy is partly due

to mucus inhibition (22%) and probably failure of follicles to disperse in others.

The probable modes of action of clomiphene citrate include binding to E2 receptors in the hypothalamus and pituitary, a decreased negative feedback action by E2, sensitization of the pituitary gland to GnRH, and reduced ovarian steroidogenesis. Hyperstimulation is rare with clomiphene citrate, the multiple pregnancy rate is of the order of 5%, and the incidence of fetal abnormalities is not increased.

Human menopausal gonadotrophin

Gonadotrophin therapy is indicated for those who do not have a satisfactory MPA withdrawal bleed, fail to respond to clomiphene citrate, or fail to conceive despite 4–6 'satisfactory' treatment cycles. hMG is given preferably by daily injections. Human pituitary gonadotrophin (hPG) is no longer used in view of the potential risk of Creutzfeldt–Jakob disease, found in some patients given pituitary extracts for growth hormone. Most preparations contain an equivalent amount of FSH and LH (approximately 75 IU of each per ampoule). The hMG is usually given in association with clomiphene citrate (Fig. 4.2), commencing with 1–3 ampoules of intramuscular injection per day, beginning 2 days after clomiphene is started. The dose is increased by one ampoule every third day in response to daily monitoring of E2 levels.

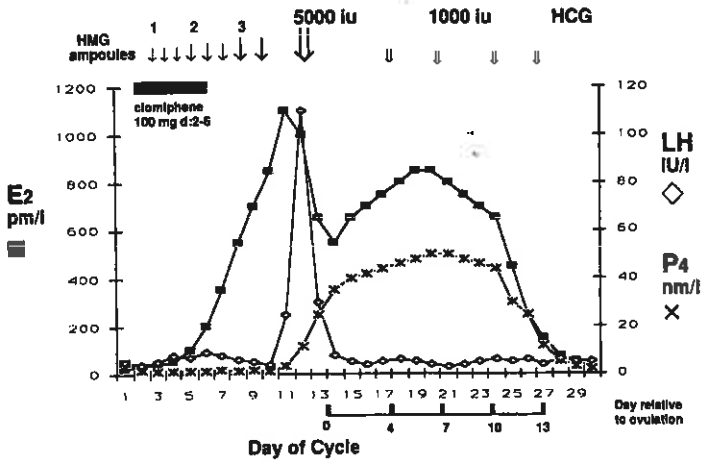

Fig. 4.2 Treatment protocol for clomiphene citrate and human menopausal gonadotrophin in the treatment of disordered ovulatory cycles.

The initiating dose is chosen with regard to the patient's age, weight and history. Slim young women with polycystic ovaries may be very sensitive to hMG, whereas obese women over 35 years are often resistant even if they suffer from PCO disease. When there is a significant E2 rise (≥150% of the baseline), the dosage usually remains the same to achieve satisfactory folliculogenesis. After a variable interval, a triggering injection of hCG is given when the E2 level is appropriate for the number of follicles generated (1000 pmol/l per follicle ≥1.5 cm) and this is generally on the sixth day of sustained E2 rise. hCG 5000–10 000 IU is required to trigger ovulation; luteal support with hCG is invariably required to sustain luteal function. This is particularly necessary if clomiphene citrate is not used in the regimen and the hMG has been applied alone as luteal phase lengths will be markedly foreshortened (see below). Clomiphene should be avoided if there is continuing inhibition of cervical mucus (11% in CC/hMG cycles) or there is an elevation of either serum LH or androgens.

hMG is highly effective in women with absolute anovulation, especially in amenorrhoeic women with hypogonadotrophic hypogonadism (success rate of 85% ovulation and 70% pregnancy). However, the multiple pregnancy rate is high (25–30%) but can be reduced by ceasing ovarian stimulation in those women with E2 rises too steep or too high, or who have developed excessive follicle numbers noted on ultrasound scanning. There is also an increased chance of heterotopic pregnancies (Yovich et al., 1985). Virtually all responsive cases develop cervical mucus and up to 3% may develop severe ovarian hyperstimulation syndrome (see Chapter 17).

Apart from the effects of ovarian stimulation, hMGs have not been associated with any maternal or teratogenic effects. hPG was withdrawn from use when cases of Creutzfeldt–Jakob disease were identified in the USA 15–20 years after children were treated with hPG extracts. This was thought to be a persisting slow virus disorder. No cases of Creutzfeldt–Jakob disease have been reported following treatment with hPG and it is considered to be unlikely with hMG as the method of preparation should prevent the transmission of any viral disorder.

Pure FSH

Purified FSH (e.g. Metrodin) is currently under evaluation for those women who have elevated basal serum levels of LH. Preliminary observations have not shown any apparent advantages over the use of hMG.

GnRH and analogues

Pulsatile injections of GnRH and GnRH analogues can effectively stimulate pituitary gonadotrophin release to achieve ovulation in anovulatory amenorrhoeic women (Leyendecker et al., 1980). There may be a reduced likelihood of multiple pregnancies (although these have been reported) and hyperstimulation syndrome. Continuous GnRH has been shown to produce a down-regulation effect in hypergonadotrophic women. Down-regulation can be achieved more effectively with GnRH analogues such as intranasal buserelin acetate (Suprefact) or subcutaneous leuprolide acetate (Lucrin). After an initial stimulatory period, pituitary down-regulation is achieved within 10–15 days and allows superimposed stimulation with hMG. This combination permits much more control over the ovarian cycle, keeping basal serum LH levels suppressed and effectively blocking the spontaneous LH surge which must therefore be induced artificially. It has shown particular benefit in women with the PCO disorder, particularly in IVF programmes (Fig. 4.3).

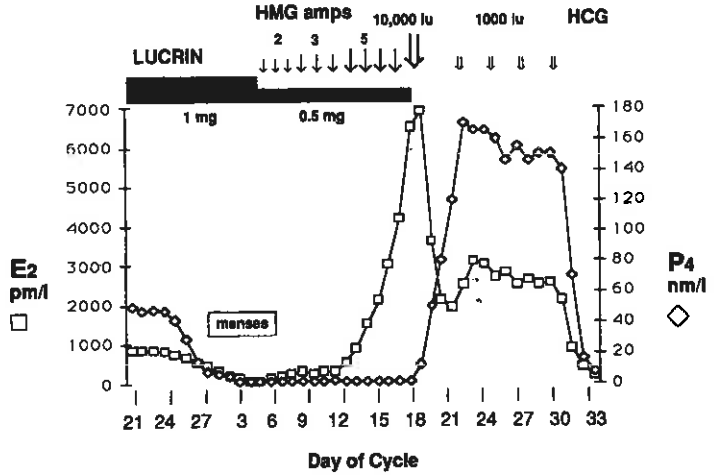

Fig. 4.3 Pituitary down-regulation regimen for Lucrin followed by hMG stimulation. This is of particular benefit in IVF programmes.

Over an 8-year period, the PIVET infertility programme has identified six groups of couples with female factors giving a poor prognosis in IVF-related treatments: poor responders, those of advanced years (>35 years); premature LH surge (before 6 days of E2 rise); raised androgens; PCO disease (raised LH:FSH ratio and/or multifollicular ovaries on day 2 ultrasound), and severe pelvic endometriosis. Following the early favourable trials with continuous GnRH therapy to create pituitary down-regulation, Lucrin was subsequently used when this became

available in Australia. It was applied in a matched study for the above cases (n = 118; non-Lucrin pregnancy rate 8%) using 1 mg/day in a long regimen beginning in the mid luteal phase of the previous cycle. hMG stimulation was commenced with E2 was <200 pmol/l and LH <10 IU/l, usually day 3–6 of the subsequent cycle. The hCG trigger was given on the seventh day of sustained E2 rise and patients were treated in one of the IVF-related programmes. Lucrin treatment was associated with the following observations:

1 There was a significant increase in the requirement for hMG;
2 There were significantly higher E2 levels prior to hCG trigger;
3 The trigger was delayed by a mean of 2 days;
4 Oocyte recovery was significantly elevated (from mean 5.4 to 10.2 oocytes per collection);
5 Fertilization rates were not different;
6 Pregnancy rates were significantly improved reaching an overall rate of 33.3% ($p < 0.01$)— the rate was elevated for each of the individual groups and the difference was statistically significant in all cases except poor responders.

These findings have implications for all patients undergoing ovarian stimulation, whether for IVF or otherwise. Subcategories of patients selected benefit significantly from the Lucrin/hMG protocol and generate high numbers of oocytes which are synchronously mature. In IVF this allows fertilized oocytes or cleaving embryos to be selected from a larger pool. There are also further advantages, namely, a reduced rate of cancellation, removal of the spontaneous LH surge, and androgen suppression. The current protocol applied at PIVET utilizes 1 mg/day Lucrin given subcutaneously beginning around day 21 of the previous cycle. The blood sample on day 3 of the treatment cycle usually shows an E2 level <200 pmol/l signalling the commencement of hMG treatment, at this stage the Lucrin dose is reduced to 0.5 mg/day and ovulation is triggered on the seventh day of E2 rise, usually between days 11 and 13 of the cycle.

MONITORING THE STIMULATED OVARIAN CYCLE

Fig. 2.3 on page 9 shows the desired monitoring profile in women undergoing ovarian stimulation. A number of parameters can be monitored as follows:

Basal body temperature

A basal body temperature (BBT) chart is useful for providing information on previous cycles and determining the likely day for LH surge,

which corresponds with the day of temperature elevation. This appears to be relatively fixed for most women by a 'hypothalamic clock', so that any stimulation regimen should aim to achieve the appropriate level of stimulation on a predicted day of LH surge—usually between days 11 and 13.

Hormonal monitoring (daily E2, P4 and LH)

Monitoring can commence on day 8 of the cycle but is more revealing if baseline levels are undertaken around day 2, followed by daily monitoring from day 6. Ideally, at the results review, decisions are made to maintain or increase the hMG injections (usually every third day) in order to sustain E2 production by the ovary. For spontaneous conception one should aim for an E2 level on the sixth day of E2 rise between 1000 and 3000 pmol/l. This will minimize the chance of a multiple pregnancy greater than twins. For cases having follicle aspiration, an ideal E2 level to aim for is 6000 pmol/l which would indicate the potential to retrieve between four and six mature oocytes. Where cycles are planned for spontaneous conception, if levels of E2 ≥3000 pmol/l occur, the risk of multiple pregnancy is high and couples should consider avoiding intercourse that month or converting to ovum aspiration so that a selected number of oocytes can be transferred in a GIFT treatment cycle.

The aspiration of follicles reduces the chance of hyperstimulation syndrome and enables a reduced number of oocytes to be available for fertilization. It is important to monitor P4 and LH levels along with E2 as elevated LH (≥1 s.d. above the mean) is associated with a poor prognosis (Stanger and Yovich, 1985) and indicates the need to withdraw clomiphene and, if necessary, add GnRH analogue for suppression in a subsequent cycle. Elevations of P4 indicate premature luteinization—again a poor prognostic feature if present for more than 1 or 2 days prior to hCG administration.

Ultrasound

In the early days of intensive ovarian monitoring, ovarian ultrasound provided clear information on follicle development. However, the quality of oocytes retrieved from follicles is more directly related to E2 levels, hence hormonal monitoring appears more important. It is useful to undertake an ultrasound assessment around the fourth to sixth day of E2 rise and calculate the E2 levels per follicle ≥1.5 cm. This will provide a reasonably accurate estimation of the number of oocytes which will be retrieved—generally one oocyte for every 600–700 pmol E2 on the day of trigger using the aspiration system

described in Chapter 10, which has a high rate of recovery from all follicles ≥1.0 cm.

Cervical mucus

For those patients treated within ovulation induction and insemination programmes, cervical mucus monitoring is important. Spontaneous conceptions and DI conceptions will only occur if fertile mucus (Insler score ≥6/12) appears. Cervical mucus may be inhibited by clomiphene in some women, in which case it should then be withdrawn. Stimulation with hMG always produces fertile mucus if there is a satisfactory ovarian response—often for several days before ovulation. The only exceptions arise in women with cystic fibrosis who appear to be unable to generate satisfactory mucus, and those who have had major cervical surgery, such as extensive cone biopsy or radical cauterization of the cervix. The presence or absence of cervical mucus appears to bear no relation to the prognosis for IVF-related treatments or for AIH where insemination is performed after the LH surge. Fertile cervical mucus usually disappears rapidly when luteinization occurs.

The test should be performed no later than 8–12 hours post-coitus in the preovulatory phase. The ectocervix should be cleaned, an endocervical sample of mucus is obtained with Howard–Kelly forceps and placed on a warm slide. Using magnification (×200) the following scoring system is used according to the number of progressively motile sperm seen per high power field examined: 0 sperm = negative; <10 sperm = poor; ≥10 sperm = normal.

THE LUTEAL PHASE

The diagnosis of luteal phase inadequacy or dysfunction has not found universal acceptance. This is partly because of reluctance to use endometrial biopsy as a method of diagnosis and also because of a prevailing view that normal ovulation and luteal function are a natural consequence of normal follicular development. It would also be argued that short luteal phases are only seen in non-conception cycles and are not a cause of failed conception.

The authors do not encourage the use of endometrial biopsy for diagnosis and our view follows that of Hull and his colleagues (1982) who suggest that favourable 'conception' cycles show mid luteal progesterone levels as a narrow subset within a wider range accepted for normal ovulatory menstrual cycles. Isolated luteal phase anomalies do occur and are correctable (84%) with clomiphene citrate or clomiphene citrate/hMG stimulation (Yovich et al., 1987). The chance of pregnancy is 21–32% per treatment cycle when ovarian stimulation

is combined with hCG to trigger ovulation (initially described by Radwanska and Swyer, 1974), with additional hCG support during the luteal phase (initially described with gonadotrophin therapy by Townsend et al., 1966) and later, with clomiphene citrate by Garcia et al. (1977).

Our own studies of luteal phase length have shown that where clomiphene has been used with or without hMG, luteal phase lengths are not shortened but can be further extended to more than 16 days by hCG. However, where hMG is used alone, in the absence of luteal support, the majority of cycles have luteal phase lengths of only 9 or 10 days (Fig. 4.4). With hCG given in the protocol described below, luteal phase lengths can be prolonged to more than 16 days.

The shortened luteal phase phenomenon was clearly shown by Edwards et al. (1980) who discarded gonadotrophin stimulation for IVF in 1977 as luteal phase lengths were inversely related to the total urinary oestrogen excretion during the ovarian stimulation phase.

In clinical trials of luteal phase treatments applied in a range of therapies, we have repeatedly shown that luteal phase support by either hCG boost injections or supplemental intramuscular progesterone significantly improved the chance of successful implantation and pregnancy. In the majority of cases, hCG 1000 IU on days 4, 7, 10 and 13 of the luteal phase is sufficient. For some patients, in particular in IVF-ET, the additional use of intramuscular progesterone injections (Proluton 50 mg) on luteal days 0, 1, 2, 3, and 4 also appears to confer improvement in the chance of pregnancy (Yovich, 1988, 1989).

STIMULATION OF NORMAL OVULATORY WOMEN

Ovarian stimulation in women who ovulate spontaneously has arisen as an adjunct to IVF-related procedures. The main benefit appears to be the larger number of oocytes available for fertilization, thereby increasing the chance of pregnancy. There appears to be a constant rate of implantation for each embryo transferred which is also influenced by the procedure and transfer site. The risks are those associated with multiple pregnancies when these occur (20–25%) and the occasional complication of severe ovarian hyperstimulation syndrome (OHSS). Fortunately, the various drugs used are free of major side-effects.

A typical stimulation schedule for women undergoing IVF-related procedures includes:

1 Stimulation with the standard clomiphene citrate/hMG regimen for women under 35 years (see Fig. 4.5.) with hCG given on day 6 of the E2 rise;

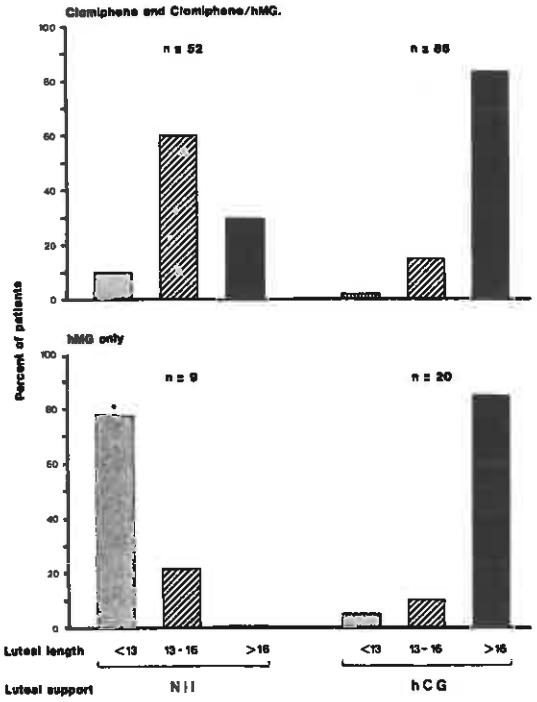

Fig. 4.4 Luteal phase length with respect to stimulation protocol and the effects of hCG support. Clomiphene and clomiphene/hMG cycles grouped together as homogeneity was demonstrated by interaction X^2 analysis. The majority of hMG/hCG luteal phases are ca. 9 days.

2 Preferential stimulation with Lucrin/hMG for all women ≥35 years or for those with specific indications such as raised LH, tendency to premature LH surge, or PCO disorder. This is commenced on day 21 of the previous cycle, which allows for the stimulation effect preceding down-regulation. hMG injections are commenced after the ensuing menstrual period when E2 levels are shown to be <200 pmol/l and both P4 (<2 nmol/l) and LH (<20 IU/l) are shown to be appropriately low (see Figs. 4.6 and 4.7);
3 Aim for 7 days of sustained E2 rise. Day 1 is often difficult to determine but day 2 is constant and readily identified as an E2 of between 800 and 1200 pmol/l;
4 hMG injections are adjusted daily to maintain a continuing rise of E2, aiming to reach a minimum level of 6000 pmol/l on day 7. The ideal range is an E2 of 6000–8000 pmol/l, providing maximum benefit with minimized risk;
5 Ultrasound is performed to coincide with the sixth consecutive day of E2 rise. If follicles have reached 1.5 cm or greater, the hCG trigger injection is given that evening. If ultrasound reveals that the dominant follicles are all less than 1.5, the hCG trigger may be withheld until the evening of day 8;
6 Oocyte recovery is scheduled for 36 h post-hCG.

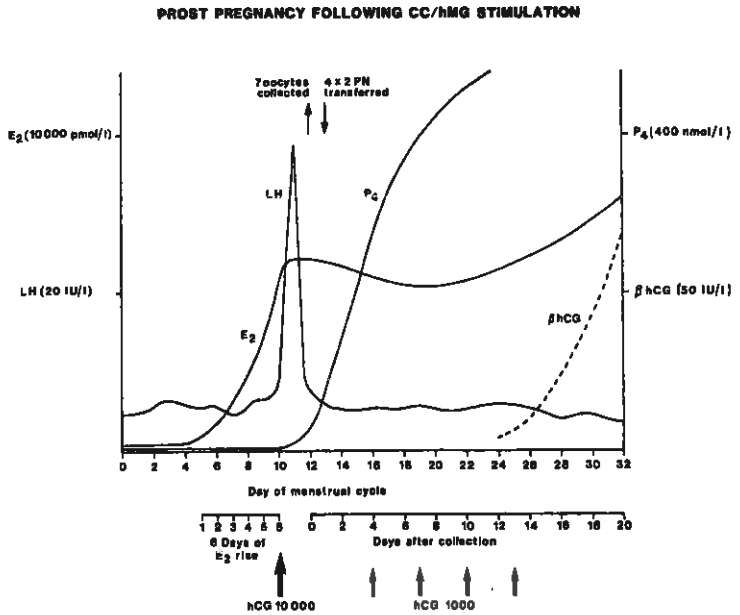

Fig. 4.5 Hormonal changes and the results of procedures in a patient stimulated with clomiphene citrate and human menopausal gonadotrophin who conceived in a PROST treatment cycle.

Women respond variably to ovarian stimulation and can be categorized into good, average and poor responders. The former may respond quite significantly to one or two ampoules of hMG per day and occasionally this may need to be reduced to one ampoule every second or third day. On the other end of the spectrum, poor responders may demon-

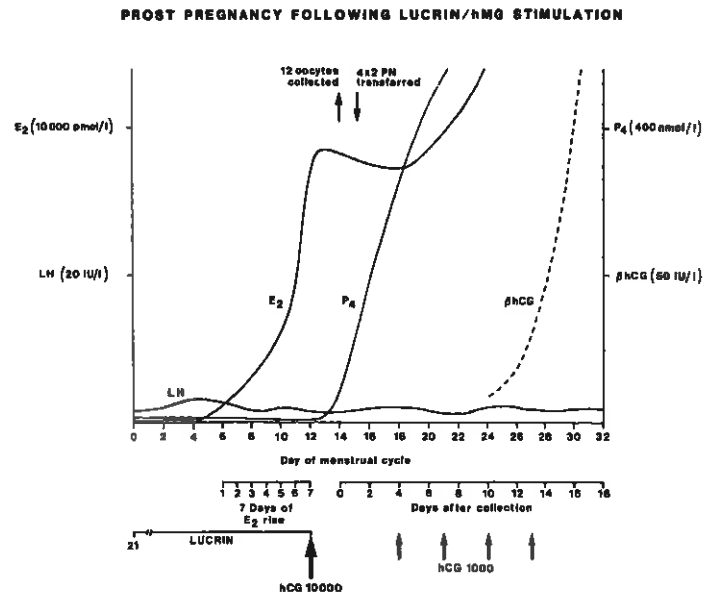

Fig. 4.6 Hormonal changes and the results of procedures in a patient who had pituitary down-regulation with leuprolide acetate (Lucrin) followed by hMG stimulation in a PROST treatment cycle. Lucrin was commenced on day 21 of the previous cycle.

strate only small rises of E2, despite being given many ampoules of hMG per day. At PIVET, pregnancies have been obtained in women who required up to 12 ampoules per day. However, the overall prognosis has been poor, hence 10 ampoules is regarded as the upper limit at this stage.

Women who have previously demonstrated poor ovarian responsiveness may often respond to the flare technique which involves commencing both the GnRH analogue and hMG together at the beginning of the cycle when the analogue will initiate pituitary release of gonadotrophins as a normal physiological effect prior to down-regulation and so supplement the exogenous hMG. An ultra-short flare regimen (Macnamee et al., 1989) has also been described which may prove equally useful and have certain cost benefits. However the poor responder group remains a difficult group to treat effectively and cancellation rates due to an inadequate response is of the order of 10%. Current research indicates the combined use of growth hormone may improve the response or at least reduce the amount of hMG required to effect suc-

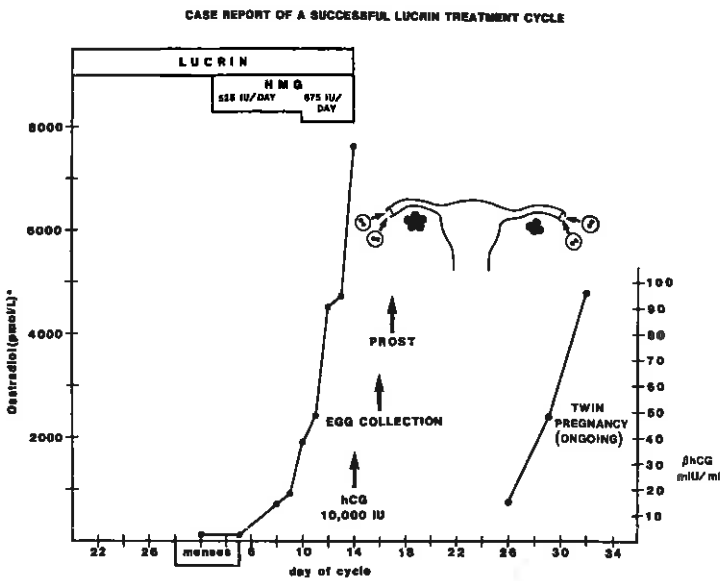

Fig. 4.7 Case summary of a 37-year-old woman treated by the PROST procedure following Lucrin/hMG in a pituitary down-regulation schedule.

cessful stimulation (Homburg et al., 1988). However the expense of such treatment is currently prohibitive for consideration in clinical service.

TREATMENT OF ANDROGEN EXCESS

Some women have raised androgens and will require preliminary treatment with spironolactone or dexamethasone. The prognosis for pregnancy is poor if there are significant elevations of A4, dehydroepiandrostenedione sulphate or testosterone (Yovich, 1988). Androgens should be measured on day 2 of observation cycles and clomiphene citrate should be avoided in women with elevated levels as androgens will rise further during stimulation, adversely affecting the chance of pregnancy. Often androgens will normalize following pituitary down-regulation with GnRH analogues, presumably as a response to diminished LH stimulation of the ovaries. Persistent androgen elevation in such cases probably indicates an adrenal source which might respond to dexamethasone suppression and spironolactone therapy to block peripheral effects at the receptor level.

LUTEAL SUPPORT

As previously discussed, benefits can be shown from luteal support therapy and the following is now used routinely at PIVET: hCG 1000 IU on days 4, 7, 10 and 13 of the luteal phase is given. Additional intramuscular progesterone (Proluton) is given from days 0 to 4 inclusive in cases of IVF-ET where embryos are transferred to the uterine cavity in the early post-operative phase.

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Chapter 5

Hormone assays

INTRODUCTION

Along with ultrasound measurement of follicular growth, the assay of hormone levels in peripheral blood or urine is helpful for the timing of egg collection.

Patients entering the infertility programme undergo an observation cycle during which the androgen status is assessed by determining A4, DHEA, DHEAS, testosterone (T), PRL, FSH and SHBG levels. These tests should be done once between days 1 and 5. Those patients with elevated androgen levels are unlikely to be successful on the infertility management programme if left untreated. E2, P4 and LH are monitored from day 8 until the commencement of the LH surge. On day 21, E2 and P4 are measured to confirm that ovulation has occurred and to assess the receptiveness of the uterus. If P4 is less than 30 nmol/l, luteal support is advised.

Following an observation cycle, patients can start one of a number of treatment cycles (ovulation induction, AIH, DI, IVF-ET, GIFT, PROST or TEST).

HORMONES OF THE REPRODUCTIVE SYSTEM

Oestradiol (17-β oestradiol)

This is a steroid hormone (18C) which circulates predominantly bound to protein. In non-pregnant women, production is mainly from the ovary; in men it is from the testis. The levels reflect the degree of follicular maturity and peak levels are reached just prior to ovulation, followed by decline during the ensuing luteal phase.

As levels reflect follicle maturity, it is necessary to measure E2 for stimulated treatment cycles daily up to hCG trigger (for ovulation). This is usually 6 days after the E2 level has risen 150% above baseline, (1000–1500 pmol/l = mature follicle). E2 levels also reflect ovarian integrity for women suspected to be menopausal (i.e. E2 < 100 pmol/l, FSH level > 10 IU/l).

Progesterone

This is a steroid hormone (20C) which has a role in the preparation of the endometrium for implantation and maintenance of pregnancy. It is produced mainly in the ovary—in the granulosa cells—and in the placenta, with small amounts also produced in the adrenal cortex.

P4 levels remain low during the follicular phase (i.e. <5 nmol/l) but increase sharply just after the LH surge and continue until a peak at 5—10 days after surge. The levels then decline sharply to the pre-ovulatory values range before the next cycle. If the women becomes pregnant, P4 levels are elevated (>50 nmol/l) until full term. P4 is monitored daily during the follicular phase to ensure that a surge is detected early and so that the timing of ovulation is more accurately predicted.

During the luteal phase in observation cycles, P4 is measured at approximately day 21 to ensure that there is a luteal response. P4 is also useful in the monitoring of early pregnancy, especially in high-risk patients for the early detection of failure or ectopic pregnancy.

Luteinizing hormone

LH is a protein hormone with two subunits—α and β. The α subunit is similar to both FSH and hCG, but the β subunit is unique. LH is secreted by the basophil cells of the anterior pituitary, under the control of GnRH.

LH levels increase sharply when the ovarian follicle reaches maturity, after which the levels quickly subside under the influence of the ovarian steroids E2 and P4, produced by the corpus luteum. Together with FSH, LH helps to complete follicular maturation and induces ovulation and the subsequent formation of the corpus luteum after ovulation.

LH levels are measured daily in observation and treatment cycles so that ovulation can be detected or in stimulated cycles to a point where the hCG trigger is administered to induce ovulation once the E2 and ultrasound indicate the presence of mature follicles.

Measurements of E2, P4 and LH are all necessary to achieve collection of the optimum number of mature, fertilizable oocytes.

Human chorionic gonadotrophin

hCG is produced by the placenta during pregnancy and is present in the maternal circulation in low levels within 10 days of conception from which time it rises to a peak at 7–12 weeks of gestation after the last menstrual period. In disorders of early pregnancy such as ectopic pregnancy or spontaneous abortions, hCG levels are abnormally low.

Given the similarities of a common α subunit among FSH, LH and hCG but a unique β subunit of hCG, then β-hCG specific immunoassays should be used for diagnostic evaluations.

β-hCG can be assayed in GIFT, PROST, or IVF treatment cycles at 3- day intervals, 10, 13 and 16 days after transfer or collection. For other treatments, hCG is assayed when a menstrual period is 3 days overdue and if these results are inconclusive, another test 3–7 days later should suffice. Positive β-hCG results (>25 IU/l) should at least double every 3 days during the first three tests before one can conclude confidently that pregnancy has occurred.

Prolactin

Human PRL is a single chain peptide hormone, which has the primary function of the growth and development of the mammary glands to produce milk. In men it is involved in the development and maintenance of testicular function.

PRL is measured to detect hyperprolactinaemia cases in women early in the treatment cycle. An elevated PRL level (> 400 mIU/l) may mean that it is the cause of the underlying infertility—or amenorrhoea or pituitary tumour—and that pregnancy is unlikely. In these cases, it is usually necessary to prescribe bromocriptine to reduce PRL synthesis and high blood levels. However transient elevations of PRL during stimulation cycles appear to be of no significance and do not require treatment (Gonen and Casper, 1989).

Follicle stimulating hormone

Human FSH is a protein hormone secreted by the basophil cells of the anterior pituitary. It comprises two subunits, α and β; the α is homologous to the α chain of LH and is very similar to α-hCG.

The function of FSH is to facilitate the development and maintenance of the ovarian follicles in females early in the menstrual cycle. The level of FSH is controlled by mid-cycle levels of E2 and the corpus luteum in the luteal phase, by negative feedback. In the menopause, FSH levels are greatly elevated due to a lack of negative feedback by E2. In males, FSH is required along with LH and testosterone to maintain spermatogenesis in the seminiferous tubules of the testes.

FSH should be measured in the early follicular phase of the observation cycle. If elevated (>10 mIU/ml), there are major implications for treatment as the patient may be in incipient ovarian failure, have ovarian resistance or be frankly menopausal. FSH is also useful for male infertility patients as raised FSH levels with raised LH are usually indicative of primary testicular failure.

Testosterone

This is an androgenic steroid hormone which is important in the development of male sexual characteristics. Therefore, much higher concentrations are found in males than in females.

In the male, T is synthesized mainly in the interstitial Leydig cells of the testis. In females, the hormone is derived from three sources in small amounts: from both the adrenals and ovaries; the majority (50–60%) is derived from the peripheral metabolism of prehormones (chiefly A4). Common causes of increased levels in women are PCOs, ovarian tumours, adrenal tumours and adrenal hyperplasia. The level of virilization in women seems to have a positive correlation with serum testosterone levels.

T should be measured in both female and male infertility patients to detect the abnormalities mentioned above. Females should have assays performed in observation cycles and have repeat assays during any subsequent PROST, GIFT, IVF or TEST cycle if previously elevated.

Dehydroepiandrosterone sulphate

DHEAS is an androgenic steroid. It originates entirely from the adrenal glands in females; only some is produced in the testis of men. DHEAS itself is only weakly androgenic, but it can be metabolized to more potent androgens (A4, T), so indirectly it can be a cause of hirsutism or virilization.

DHEAS provides a useful measurement as it is one of the androgen indices, along with free testosterone (T/SHBG ratio = free testosterone) and androstenedione. Levels are often raised in cases of clinical hyperandrogenism and correlate with an adverse outcome for infertility treatment (Yovich, 1988). DHEAS is more likely to be elevated than other androgens in the early stages of hirsutism and high levels are often found in PCO disease.

Androstenedione

This is a strongly androgenic precursor of testosterone and estrone. It is a Δ^4 steroid and are synthesized in both the adrenals and ovaries unlike DHEA and DHEAS which is synthesized from an adrenal source only. It exhibits a cyclic variation during the menstrual cycle, being highest at mid-cycle. It is also elevated during pregnancy.

A4 should be measured as it is androgenic. Increased levels are associated with hirsutism or virilization, usually with PCO disease and therefore a cause of infertility in women. Elevations are associated with a poorer outcome in treatment cycles. It is assayed early in the observation cycle and repeated during any treatment cycle involving ovarian stimulation.

Dehydroepiandrosterone

This is an adrenal steroid, similar in structure to DHEAS, and is synthesized almost entirely by the adrenal glands. It cannot be interconverted with DHEAS peripherally and is only weakly androgenic but, like DHEAS, it can serve as a precursor to more potent androgens. DHEA has a much shorter half-life.

Sex hormone binding globulin

This is a plasma glycoprotein which has a specific and high affinity for the sex steroids E2 and T. Changes in its plasma concentration therefore alter the bound and free distribution of E2 and T. It is thought that SHBG-bound steroids are not physiologically active as they cannot be internalized into the target cell.

SHBG is thought to be produced mainly in the liver and appears to be controlled by oestrogens and thyroid hormone, which cause an increase in SHBG and androgens, which cause a decrease.

SHBG is therefore a useful analyte as it indicates the active level of E2 and in particular, T. SHBG concentration is often reduced in cases of female hyperandrogenism (e.g. hirsutism) and elevated in men with gynaecomastia or hypogonadism.

LABORATORY SCHEDULE

Assay timetable

Daily assays (sample required by 9.30 a.m.)

E2; P4; quantitative β-hCG; LH.

Results by 3.00–3.30 p.m. daily.

Twice-weekly (Monday/Tuesday and Thursday/Friday)

PRL (24 h assay); FSH (2 h assay).

Samples by Monday or Thursday, 9.30 a.m.
Results by Tuesday or Friday, 3.30 p.m.

(Note: FSH may be run either Monday/Tuesday or Thursday/Friday, depending on the number of samples and the workload.)

Weekly (samples by Wednesday 9.30 a.m.)

T (3 h), run Wednesday—results that afternoon.
DHEAS (30 min.), run either Wednesday or Thursday (depending on workload—results afternoon of day of run).

SHBG (3 h), set up Wednesday, results late Wednesday or Thursday a.m.
A4 (1 h)—Thursday: assay, results Thursday p.m.
DHEA (2 h)—Wednesday: extraction, Thursday: assay, results: Thursday p.m.

Daily schedule

1 Patients are bled 7.30–9.00 a.m. into 10 ml venoject tubes (glass); the blood is allowed to clot.
2 Samples and forms arrive in lab at approximately 9.30 a.m. Samples are centrifuged for 15 minutes at >1500 rpm to separate the cellular and serum phases. During this time details of each patient are entered into the day book and storage tubes are labelled.
3 Centrifuged serum is decanted into appropriately labelled sample storage tubes (9.30–10.00 a.m.).
4 Assay tubes are labelled and the processing of assays begin (E2, P4, LH, hCG). Start incubating (10.00–11.00 a.m.). (See assay protocol below.)
5 Assays incubate for 2–3 h depending on the particular assay. Other laboratory business is carried out at this time.
6 Assays are finished, counted and data reduction is carried out on computer to give results for each assay. Results are entered in the day book and the controls are checked (1.00–3.00 p.m.).
7 Results page is photocopied and nurses then enter results into each patient's file.
8 At the results session, the day's results for E2, P4 and LH are compared with other data,—ultrasound, mucus score, etc.—to allow review of patient management (drug dose, treatment, etc.). Results session involves review by clinicians, nurses, embryologists and RIA technologists.

Note: This schedule relates to the normal daily assays. Extra assays are fitted in wherever possible. See assay timetable for days of 'extra' assays.

ASSAY PROTOCOLS (Tables 5.1–5.15)

All assay reagents are equilbrated to room temperature before measurements are commenced. Assays are done on serum only.

Quality control samples are included in each assay run to monitor the assay precision. Control values should fall within a predetermined range of variation (usually <10%) before the assays are accepted as being valid.

Table 5.1 Hormone assay specifications of a typical range of hormones in an infertility clinic

Analyte	*Manufacturer*	*Units*	*Dynamic range*
E2	DPC Coat-a-count	pmol/l	27–13200
P4	DPC Coat-a-count	nmol/l	0.25–127
LH	Amersham-Amerlax-M	IU/l	0.4–132
β-hCG	Amersham-Amerlax-M	IU/l	2.1–200*
PRL	Amersham Amerlex-M	mIU/l	16–4980
FSH	Amersham-Amerlex-M	IU/l	1.0–75
T	DPC Coat-a-count	nmol/l	0.1–104
DHEAS	DPC Coat-a-count	umol/l	0.1–27.1
A4	DSL-Active RIA†	nmol/l	0.2–35
DHEA	DPC Coat-a-count	nmol/l	0.1–104
SHBG	Farmos Diagnostica IRMA	nmol/l	0.5–200

* Undiluted samples; see assay protocol.
† Diagnostic Systems Laboratory.

Total tubes should be included in all Coat-a-count assays (1.0 ml of ^{125}I in 3 DT tubes).

ASSAY PROCESSING

Ideally a laboratory should use a dedicated gamma counter e.g. DPC Gambyt CR20, linked to a professional microcomputer. This system would satisfy all the needs of the laboratory in first counting and then processing the diagnostic RIA assay results.

Such a system comprises the following:

1 DPC Gambyt CR20 (20-well) gamma counter (Diagnostic Products Corporation).
2 Professional TI microcomputer using MS-DOS software. (TI = Texas Instruments).
   256 K RAM, two floppy disk drives.
   High resolution 34 cm colour monitor.
   Qwerty keyboard.
   Dot matrix printer with graphics card.
   Gamma counter–computer interface (RS 232).
   Microsoft disk operating system.

Basic system operation

The computer controls the gamma counter via the interface; thus the two work together as an integrated system.

Table 5.2 Oestradiol Coat-a-count assay

	Standards							*Controls (Lyphochek)*			
Tube	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*1*	*2*	*3*	*Unknown samples*
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Volume of std or unknown serum	100 µl	→									
Volume of ^{125}I Oestradiol	1.0 ml	→									

*Tubes are decanted down a sink in which sodium thiosulphate has been sprinkled. Sodium thiosulphate keeps iodine in solution during disposal.

Vortex and incubate for 3 h at room temperature.
Decant, using foam decanting rack*, drain for 2 to 3 min. and strike on absorbent paper. Count for 1 min. in gamma counter.

Table 5.3 Progesterone Coat-a count assay

	Standards							*Controls (Lyphochek)*			
Tube	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*1*	*2*	*3*	*Unknown samples*
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Volume of std or unknown serum	100 µl	→									
Volume of ^{125}I Oestradiol	1.0 ml	→									

Follow procedure as for oestradiol (Table 5.2).

Table 5.4 Amerlex-M LH

	Standards							*Controls (Lyphochek)*			
Tube	*NSB* contains A std*	*A*	*B*	*C*	*D*	*E*	*F*	*1*	*2*	*3*	*Unknown samples*
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Volume of std or unknown serum	100 µl	→									
Anti-LH serum	100 µl	→									
	Vortex. Incubate at 37°C for 30 min†.										
^{125}I-labelled LH	100 µl	→									
	Vortex. Incubate at room temp for 1 h†.										
Amerlex-M second antibody	1.0 ml	→									
	Vortex and stand for 10 min.										
	Separate for 15 min using Amerlex-M magnetic separator.										
	Decant and count for 1 min in gamma counter.										

* NSB: Non-specific binding tubes do not contain anti-LH serum. These tubes determine the small amount of ^{125}I-labelled LH which binds non-specifically to second antibody reagent. The subsequent count is subtracted from the counts accumulated in all other tubes.
† This is the shortened protocol.

Table 5.5 Amerlex-M β-hCG

	Standards							*Controls (Lyphochek)*			
Tube	*NSB* contains A std*	*A*	*B*	*C*	*D*	*E*	*F*	*1*	*2*	*3*	*Unknown samples*
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Volume of std or unknown serum	100 µl	→									
Anti-β-hCG serum	100 µl	→									
	Vortex. Incubate at 37°C for 30 min*.										
^{125}I-labelled β-hCG	100 µl	→									
	Vortex. Incubate at room temp 37°C for 45 min*.										
Amerlex-M second antibody	1.0 ml	→									
	Vortex and stand for 10 min.										
	Separate for 15 min using Amerlex-M magnetic separator.										
	Decant and count for 1 min in gamma counter.										

* Shortened protocol.
The β-hCG assay has a detectable range of 2.1–200 IU/l. Since patients who are more than a few weeks pregnant have β-hCG levels greater than 200 IU/l, patient samples are diluted in phosphate-buffered saline (PBS). These samples are assayed using neat serum, serum diluted 1/50 or serum diluted 1/1000. The 1/50 dilution is prepared using 10 µl patient serum in 490 µl PBS. Of this 1/50 dilution, 25 µl is then added to 475 µl PBS to prepare the 1/1000 dilution. By diluting pregnancy patient's serum in this manner, it effectively extends the range of the β-hCG assay from 2.1 to 200 000 IU/l.

Use 3DT clear plastic tubes.

Table 5.6 Prolactin assay

		Standards						Controls (Lyphochek)			
Tube	NSB* contains A std	A	B	C	D	E	F	1	2	3	Unknown samples
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Std. serum or control	100 µl	→									
Unknown serum											100 µl →
^{125}I labelled Prolactin	100 µl	→									
Anti-prolactin serum	100 µl	→									
		Vortex. Incubate for 18–24 h at room temperature (or 4 h at 37°C).									
Second antibody	1.0 ml	→									
		Vortex. Leave at room temperature for at least 5 min. Centrifuge for 15 min at 1500 g. Aspirate supernatant (or decant). Count radioactivity in precipitates for 1 min.									

Use 3DT clear plastic tubes.

Table 5.7 Amerlex-M FSH assay

		Standards						Controls (Lyphochek)			
Tube	NSB* contains A std	A	B	C	D	E	F	1	2	3	Unknown samples
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Std. serum or control	200 µl	→									
Unknown serum											200 µl →
Anti-FSH serum	100 µl	→									
		Vortex. Incubate at 37°C for 30 min*.									
^{125}I labelled FSH	100 µl	→									
		Vortex. Incubate at 37°C for 1 h*.									
Second antibody	1.0 ml	→									
		Vortex and stand for 10 min. Separate for 15 min using the AMERLEX-M magnetic separator. Decant. Count for 1 min in gamma counter.									

* Shortened protocol.

N.B.: Patients suspected of being menopausal, or those with oestradiol <100 pmol/l have measurement on a 1/5 in addition to an undiluted sample as the FSH level may be greater than 75 IU/l. Use 3DT clear plastic tubes.

Table 5.8 Testosterone Coat-a-count assay

	Standards								*Controls (Lyphochek)*		
Tube	*A*	*B*	*C*	*D*	*E*	*F*	*1*	*2*	*3*		*Unknown samples*
Tube nos.	1,2	3,4	5.6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Volume of std or control	50 µl →										
Volume of unknown										50 µl →	
Volume of ^{125}I testosterone	1.0 ml →										

Vortex. Incubate for 3 h in 37°C waterbath.
Decant using foam decanting rack, drain for 2–3 min and strike on absorbent paper.
Count for 1 min in gamma counter.

Use Coat-a-count tubes provided in kit.

Table 5.9 DHEAsulphate Coat-a-count assay

	Standards								*Controls (Lyphochek)*		
Tube	*A*	*B*	*C*	*D*	*E*	*F*	*1*	*2*	*3*		*Unknown samples*
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Volume of std or control	50 µl →										
Volume of unknown										50 µl →	
Volume of ^{125}I DHEAS	1.0 ml →										

Vortex. Incubate for 30 mins in 37°C waterbath.
Decant and count for 1 min in gamma counter.

Use Coat-a-count tubes provided in kit.

Table 5.10 Sex hormone binding globulin assay

	Standards								Controls		
Tube	*Totals*	*NSB contains A std*	*A*	*B*	*C*	*D*	*E*	*F*	*Low*	*High*	*Diluted test samples*
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
SHBG buffer		10 µl									
Diluted STD or control			100 µl →								
Diluted test sample											100 µl →
Anti-SHBG antiserum	100 µl →										
^{125}I labelled anti-SHBG antibody	100 µl →										
	Vortex. Incubate for 1 h at room temperature.										
Separation agent*	500 µl →										
	Vortex. Incubate for 15 min at room temp.										
	Vortex.										
0.9% saline	2.0 ml →										
	Vortex. Centrifuge for 15 min. at 200 g.										
	Decant or aspirate supernatant (not totals).										
	Count for 1 min in gamma counter.										
	(N.B.: NSB in wells 1,2, totals in 3,4 followed by stds, etc.)										

Totals are set up to determine the total counts for assay.
* Stirred with magnetic stirrer to suspend solid phase.

Table 5.11 DHEA Coat-a-count assay

Extraction

1 Label one 150 × 19 mm glass tube for each patient sample, and two each for Lyphochek controls 1, 2 and 3.
2 Pipette 0.5 ml of each patient sample and control into appropriate tubes.
3 Add 5 ml dichloromethane* to every tube and vortex each tube gently for 1 min.
4 Place in freezer until frozen to separate the two phases:

5 After thawing, remove the top layer by aspiration, being careful not to disturb the interface.
6 Transfer 3.0 ml of the lower dichloromethane phase to a clear 12 × 75 mm borosilicate tube. Evaporate to complete dryness under a gentle stream of nitrogen at 37°C. (We use a water bath with hot water to cover the bottom of the tubes.)
7 Resuspend the extracts with 0.6 ml DHEA buffer (calibrator A). Place in 37°C water bath for 10 min and then vortex thoroughly.
8 Cover the reconstituted extracts with Parafilm and store at 4°C overnight.

N.B.: Half the recommended volumes are used throughout the extraction procedure. This has been compensated for by doubling the standard values in the assay protocol.
*Dichloromethane is a dangerous solvent. All work should be carried out in a fumehood, or well-ventilated area.

The computer runs via an MS-DOS programme written by DPC, from which all assay operations and other ancillary procedures can be carried out. The system programme is contained on one floppy disk in drive A, with the data disk in drive B. All information which is stored from an assay is therefore stored on the data disk in drive B.

Note: This section will pertain mostly to the system above, but many features of operation and maintenance would be applicable to other operating systems. For this reason, it is best to refer to the operation manual concerned with the particular system in use, as this will only give a brief and basic overview.

Daily assay procedure

The computer is first 'boosted' with the system RIA programme and then set up for the day by inputting the date and time. The start-up

Table 5.12 DHEA radioimmunoassay procedure

	Non-extracted standards							*Extracted controls (Lyphochek)*			
Tube	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*1*	*2*	*3*	*Extracted test samples*
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Std or control	200 µl →										
Test serum											200 µl →
^{125}I-DHEA	1.0 ml →										
	Vortex. Incubate for 2 h in 37°C waterbath.										
	Decant using foam decanting rack, drain for 2–3 min and strike on absorbent paper.										
	Count for 1 min in gamma counter.										

Table 5.13 Androstenedione (DSL)—direct method

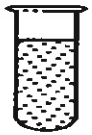
	Standards						*Controls (Lyphochek)**				
Tube	*A*	*B*	*C*	*D*	*E*	*F*	*1*	*2*	*3*		*Unknown samples*
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Volume std/ unknown	50 µl† →										
^{125}I-A_4	500 µl →										
Anti-A_4		100 µl →									
		Vortex. Incubate at 37°C for 30 min†.									
Separating reagent (mixed)	1 ml →										
	Vortex. Let stand for 15 min.										
	Centrifuge at >1500 g for 15 min.										
	Decant and aspirate, then count in gamma counter.										

* Two controls are supplied with the kit (low/high controls) and should be included as with unknowns, and the results checked in the package insert.
† Add extra 100 µl A standard to NSB to compensate for no anti-serum.
Use 3DT clear plastic tubes.

Table 5.14 Androstenedione Coat-a-count assay (alternative method)

Extraction

1 Label one 150 × 19 mm glass tube for each patient sample and two each for Lyphochek controls 1, 2 and 3.
2 Pipette 0.5 ml of each patient sample and control into appropriate tubes.
3 Add 5.0 ml diethyl ether to every tube. Vortex each tube vigorously for 1 min.
4 Place in freezer to separate the two phases:



Diethyl-ether (upper layer)

Serum phase (lower layer)

5 Allow to thaw, then transfer 3.0 ml of the upper diethyl ether phase to a clear 12 × 75 mm borosilicate tube. Evaporate to complete dryness under a gentle stream of nitrogen at 37°C. Use a water bath.
6 Resuspend the extracts with 0.6 ml androstenedione buffer (calibrator A). Place in 37°C waterbath for 10 min and vortex thoroughly.
7 Cover the reconstituted extracts with Parafilm and store at 40°C overnight.

Note: Half the recommended volumes are used throughout the extraction procedure. This has been compensated for by doubling the standard values in the assay protocol.

procedure also asks whether the gamma counter is required and whether it is a warm start, i.e. has the gamma counter been left on. The gamma counter is usually left on as a cold start requires a warm-up time and recalibration.

RIA processing

The DPC system stores the RIA protocols (i.e. instructions for a particular assay) on the data disk, as adjustment to the protocols may be required.

To begin an assay count, the assay type is called up via the 'applications' menu under 'RIA stored protocol'. The required assay number is then selected (e.g. 2 = progesterone), and the protocol details for the assay are shown. These details include the following:

1 Assay type (RIA-bound, RIA-free, IRMA).
2 Blanks.
3 Totals.
4 Standards.
5 Controls.
6 Patients.

Table 5.15 A_4 radioimmunoassay procedure—extraction method

Tube	*Non-extracted standards*							*Extracted controls (Lyphochek)*			*Extracted test samples*
	A	*B*	*C*	*D*	*E*	*F*	*G*	*1*	*2*	*3*	
Tube nos.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22,23 etc.
Std or control	200 µl	———	———	———	———	———	———	———	———	———	———
Test serum											200 µl ———
^{125}I-A_4	1.0 ml ———										———
	Vortex. Incubate for 3 h at room temperature.										
	Decant using foam decanting rack, drain for 2–3 min and strike on absorbent paper.										
	Count for 1 min in gamma counter.										

* Diethyl-ether is extremely toxic and the precautions taken for DHEA should be adhered to when using this solvent.

Other details include count rate, time, curve fit, isotope type and threshold.

The protocol also contains standard concentrations in the units in which the results are to be expressed (i.e. nmol/l), along with control concentrations. Control standard deviations and percentage binding at B ,0 (zero binding) and ED (estimated dose) 80, 50, 20 may be added.

Finally, the protocol lists the tube numbers for the controls. It is most important therefore that an assay is set up according to the protocol, i.e. if total radioactivity tubes are used, then this should be stated in the protocol. Tubes should also be in the order as shown in the protocol.

The protocol can be edited to alter the assay set up depending upon whether any alterations have been made to the normal protocol. The number of patients need only be entered if the protocol is correct. The printer will print out the completed protocol summary.

Once the assay start-up is completed, the computer directs the counter to commence counting the assay. For the DPC system, the tubes are placed 20 at a time in the plastic counting racks in order of NSB, totals (if either are used), standards, controls and then patients (in order of RIA number).

The computer prompts the operator to start counting with the message: 'Load rack and press START', followed by three beeps. The gamma counter displays the word 'READY' on the screen so the start button can be pressed. Once counting has started, it can only be stopped by pressing 'PAUSE' followed by 'CLEAR'. This will allow the assay to be aborted totally, restarted (i.e. recount standards, etc.), or to recount the rack in question.

Once the standard rack is counted, the computer will print out the counts for the standards (with CVs for the duplicates) and a standard curve. The computer draws a line of best fit for the curve. If a point is unacceptable (i.e. t is a bad tube), this tube can be rejected when asked if a tube needs rejecting ('REJECT TUBE'). If no tubes need rejecting and the curve fit is acceptable (i.e. it is straight, not curved, for IRMA methods, spline curve fits will be S-shaped), the control values will be printed out. Following this, each rack of samples will be counted, and a result calculated.

Once the assay is finished, the tubes should be left in their counting racks so that they can be checked or recounted if a problem is found when results are entered. Before results are entered, the quality control (QC) values for the QC samples should be checked against the range for that sample. Two-thirds of the QCs should be in range. The results can then be entered with confidence into the day or extra assays book.

Once the assay has been written up and no more tubes need counting, the QC results can be stored on the data disk.

It is best to keep completed assay sheets for at least 1 week, so

that if a query does arise concerning that assay, the count and results etc. are available. Once all the day's assays are finished, the computer can be turned off and disks stored, but the counter is left on.

Counter calibration

The gamma counter should be re-calibrated weekly to ensure the quality of the counting results. A ^{57}Co sealed rack—which is the same as normal counting racks—is measured by the counter via a special programme which examines the spectral peak at 122 in each head, to which the EHT voltage of the photomultiplier is adjusted for the full scale energy. For our purposes, the full scale energy is 250 keV.

If the programme detects a faulty head, the operator will be notified with the message 'WARNING, DO NOT USE DETECTOR 5'. The anode voltage will then be printed out. These should be kept and compared to those from the previous week. The anode V'o should not differ by more than 1–2 V.

'Poor' assays

Poor or unsuitable assays are those with results likely to be false for part of or all of the range of the assay. A poor assay is usually detected using the quality control sera (see below) as these give a result, for which a range of results is available, for each analyte assayed. Three controls are usually used to represent low, normal and high values for the particular analyte. By covering most of the diagnostic range, the controls can guarantee reliable results. It is therefore imperative that four of six of the controls are within a predetermined range, to accept that the assay has provided reproducible results. If two of the same control are out, then the assay may be suspect in that region of the curve.

Poor assays can also be detected before printout of QC values from visualization of the standard curve. If the duplicates of the standard are quite varied and the curve fit is not straight or does not intersect at most of the points, then the assay is likely to be unsuitable. The correlation for the assay should also be as close to 1.00 as possible. Below 0.98 is usually unacceptable.

The ED results are also useful as they give an indication of the ED in relation to the standard dose. If there is a gross discrepancy between the two, then the assay is likely to be unsuitable.

To correct a poor assay, tubes can be rejected if they are far from the line of best fit, or if this does not rectify the problem, then the whole assay should be recounted. If recounting fails to solve the problem, the assay will need to be repeated.

There are many more reasons for unsuitable assays. Table 5.16 gives details of problems, with likely solutions.

QUALITY CONTROL

The authors use the Biorad Lyphochek immunoassay (human) control serum levels I to III. These controls are prepared from pooled human serum with added constituents to achieve levels of clinical significance. The controls are supplied in lyophilized form, to which 5 ml of distilled water is added. Once reconstituted, the controls are stable at 2–8°C for 7 days, and up to 20 days at −20°C.

Supplied with the controls is a booklet giving the ranges for each analyte or control for the particular method in use. This booklet gives ranges for all three controls in SI units. However, the ranges supplied are likely to be a compromise, and it is best for the individual laboratory to calculate its own ranges.

Quality control sample ranges should be calculated for each analyte every 2–3 months, on at least 50 control results (i.e. 25 duplicates) for each daily assay. For weekly assays, this may be done at longer intervals, so as to generate enough raw data to give an acceptable 'population' of data. To calculate the QC range for a particular control and method, at least 50 control results (25 assays) for daily assays and results (10 assays) for weekly assays should be used.

The Biorad controls are provided in batches which may vary in the concentrations of analyte, so it is ideal to stock a large quantity of each batch (e.g. 25) to generate a sizeable database without fluctuations to provide appropriate laboratory QC ranges. When control batches change, then new ranges for each analyte are supplied, and these should be used until enough data is available to produce individual laboratory QC ranges.

As well as internal laboratory QC, Biorad offer an inter-laboratory QC programme where the results of the individual laboratory are compared to those of all other laboratories in the programme and the control ranges for each analyte are re-calculated monthly. Assay results are entered and plotted on to a Levy–Jenning chart supplied every month. This gives a visual indication of QC performance for each assay. The QC results for a particular method for a particular analyte are sent in every month (e.g. DPC Coat-a-count Progesterone), and from these figures the data are analysed for each control level (I–III). An individual and inter-laboratory report is received each month to show the laboratory's own statistical performance and its performance compared with other laboratories for the particular analyte method. External QC is very important in maintaining accuracy in an RIA laboratory.

STORAGE OF REAGENTS AND SERUM SAMPLES

Blood samples are centrifuged and the serum is decanted into previously labelled sample or storage (5LP) tubes. These tubes are labelled with the centre's number and the patient's name. All serum samples are retained and stored in freezers at −20°C, primarily for research purposes. The first and last samples for each day are numbered and stored in numbered racks for ease of retrieval.

All assay reagents are stored in the refrigerator at 4°C in the manufacturer's containers, with the exception of the radioactive label used in Coat-a-count assays. This is stored in 1 ml dispenser bottles (Oxford repeatable dispensers), ready for dispensing. It is recommended to store Coat-a-count tubes in a refrigerated environment; however it is possible to store them in their sealed bags in air-tight containers with a small amount of silica gel. These containers are not refrigerated as this dry environment is found to be sufficient for storage of the tubes before use in an air-conditioned environment (i.e. 22°C).

WASTE DISPOSAL

A typical RIA laboratory has four types of waste with which to contend:

1 Radioactive.
2 Human origin—blood, serum, amniotic fluid, urine, etc.
3 Sharps—needles, glassware.
4 Safe waste—paper, plastic.

Radioactive waste

This is present in both liquid form (the decanted tracer) and in solid form in tubes and paper towels. The paper towels are used to soak up the waste after most of the waste has been decanted. The liquid waste is poured into a metal sink, to which sodium industrial grade thiosulphate crystals are added to absorb the ^{125}I and to prevent vaporization in the sewage system. The tracer and sodium thiosulphate are then washed down the sink with copious amounts of tap water. This form of disposal is acceptable only if there is a low level of radioactive waste.

The solid radioactive waste is placed in large bins in a heavy-duty-liner. Any solid material in contact with radioactive tracer should be disposed of in this bin. The bin should have a tight fitting lid, which should be kept on to minimize vaporization of ^{125}I. The bin liners are tied when full, and then picked up on a regular basis by a disposal company and incinerated.

Table 5.16 Problems and solutions in RIA

Apparent problem	*Possible cause*	*How to find out cause*	*Suggested solution*
No (maximum) binding	No antibody added	All tubes had same counts as NSB	Devise pipetting procedure to ensure addition of all reagents
	Wrong tracer or wrong antibody added	Inventory of some reagents does not reflect assays run	Code reagents of each assay to avoid mismatching
	Separation procedure failed	No pellets in tubes	Check for pellets before decanting
Low (maximum) binding	Old tracer used	Check expiration date of tracer	Don't use tracer close to expiration date or tracer that has been stored improperly
	Incorrect dilution (too much) tracer used	Calculate cpm that should be in the assay	Count 100 μl of tracer to verify cpm before adding to assay
	Incorrect (too little) antibody used	Check dilution of antibody	When diluting make sure final volume gives right number of tubes
Flat curve with normal NSB and B_0	No standards added	All standard tubes had same counts as B_0	Devise pipetting procedure to ensure addition of all reagents
	Wrong standards added	All standard tubes had about the same counts as B_0	Code reagents of each assay to avoid mismatching
High NSB	Antibody added to NSB tubes	Cpm in NSB = B_0 and no tubes have greater cpm whereas most have fewer cpm	Devise method to isolate NSB tubes from antibody addition
	Old tracer used	Check expiration date of tracer	Don't use tracer close to expiration date or tracer that has been stored improperly
	Damaged or improperly stored tracer	Binding should be low throughout, assay out of control	
Low sensitivity	Too much tracer used per tube	Calculate cpm that should be in the assay (low B_0)	Count 100 μl of tracer to verify cpm before adding to assay
	Too much antibody used	Check dilution of the antibody (high B_0)	When diluting make sure final volume gives right number of tubes
	Old tracer used	Check expiration date of tracer	Don't use tracer close to expiration date
Too much sensitivity	Too little tracer used per tube	Calculate cpm that should be in the assay (high B_0)	Count 100 μl of tracer to verify cpm before adding to assay
	Too little antibody added	Check dilution of the antibody (low B_0)	When diluting make sure final volume gives right number of tubes
Low or high control values	Standards improperly stored (high control values)	Curve will be flatter than normal	Store standards according to protocol
	High NSB due to old tracer or improper storage	Abnormally high NSB (2 or 3 × normal)	Do not use tracer close to the expiration date
	Old and improperly stored controls used	Check expiration of control	Store reagents according to protocols and don't use after expiration date
	Standards underdiluted (low control values)	Curve will be shifted to the left; all values will be low	Double-check reconstitution instructions
No pellets	Second (precipitating) antibody not added	Inventory of Ab_2 does not reflect assays run	Devise pipetting procedure to ensure addition of all reagents
	Normal serum and second antibody mismatched	Inventory of Ab_2 and normal serum do not correspond	Code reagents of each assay to avoid mismatching
Poor duplication	Poor technique	No pattern in duplication, problem in all assays of that particular technician performs	Technician training and qualification programme should be developed
	Poor pipettors	Errors are occasional, problems occur in all assays	Pipette ^{125}I solution into 10 tubes and make sure CV is acceptable
	Old or damaged reagents used	Check expiration date and storage of all reagents	Store reagents according to protocol and don't use after expiration date
Low activity	Tracer diluted improperly	Check tracer dilution; calculate cpm that should be in the assay (B_0 high)	Count 100 μl of tracer to verify cpm before adding to the assay
	Old tracer used	Check expiration date of tracer (B_0 low)	Don't use tracer close to expiration date

Iodinated compounds are very volatile, so for this reason all waste should be disposed of properly, in order to prevent ^{125}I vaporization into the laboratory atmosphere.

Human waste

Blood, serum and other waste of human origin is disposed of by incineration as it is a potentially infectious material. All blood collection tubes etc. are placed in a lined bin once the serum has been decanted off, and then collected for incineration at least once a week.

Sharps

These are discarded into a sealed bin which is collected at the same time as the radioactive waste for careful disposal in an industrial disposal system.

Safe waste

Paper, boxes and plastic are disposed of into open bins in the laboratory, which are removed by the cleaning staff for removal by the local domestic or industrial waste disposal system.

NON-ISOTOPIC HORMONE ASSAYS

Recently a number of non-isotopic, enzyme-based assay systems have been developed for commercial use, stemming from enzyme linked immunosorbent assays (ELISA). A number of assays are currently being evaluated by infertility units and at one centre the Amerlite system (Amersham) has been evaluated and introduced into routine screening.

Basic system description

The Amerlite system is an immuno-assay-based methodology which differs from the current RIA immuno-assays in that a chemiluminescent emission is quantified rather than counts of ^{125}I gamma radiation.

This system uses microtitre tray wells, with a maximum capacity of 200 µl in which the primary antibody is covalently bound to the walls. To this is added the sample and a conjugate solution which may be either horseradish peroxidase (HRP)—labelled antibody (immunometric) or HRP-analyte (competitive) which then reacts at 37°C until equilibrium is reached. Following this, the wells are washed thoroughly in buffer using an automated washer to remove any unbound species. Next, a predetermined amount of the signal reagent (isoluminol) is added, which in the presence of enhancer (which is activated by the HRP label), produces a light emission (stable for 20 min.) which is quantified using the Amerlite reader. The complete microtitre tray (96 wells) can be measured in 2 min., giving a full printout of assay data and results via a quiet Inkjet printer set into the top of the analyser.

The assay system is not all that different in principle from the common routine RIA, but the availability of the non-isotopic label system to quantify the reaction removes the logistical concerns associated with radiation safety and storage.

Advantages over RIA

In brief, the system's major advantages over RIA are:

1 Non-isotopic. The signal reagent is isoluminol-peroxidase which is a stable, non-radiation detection system offering excellent sensitivity. Natural luminescence (bioluminescence) is rare being found only in the firefly and marine bacteria, so background luminescence is not a problem.
2 Speed. The system offers both rapid assay times (commonly less than 1 h incubation) with rapid quantification using the analyser (e.g. current RIA E2 assay—3 h incubation; Amerlite E2—1 h incubation).
3 Long shelf-life. '125I-based RIA methods have a maximum shelf-life of 60 days due to the half life of ^{125}I. The Amerlite reagents have a shelf-life of 6 months, thereby simplifying stock control and lot changes. This can allow for the running of low number assays that were previously uneconomical on the RIA system.
4 Ability to economize standards. Full standardization is not required in every run of the Amerlite system due to the stability of the system and the design of the analyser software. For every new lot, only one complete standardization need be prepared, after which only four standards (in singlet) are required until the kit lot is exhausted. The software checks the results for the four standards against the stored full curve and if satisfactory, the results are then accepted. This should allow for a vast cost saving.
5 Precision. The system shows extremely good precision, with the CVs being under 5% in most cases for intra- and inter-assay comparisons. This is exceptional as an efficient RIA system is one with inter-assay CVs around 10%
6 The overall costs of the system (including rental of all equipment) is less than that of RIA reagents for E2, P4, LH, β-hCG, PRL and FSH alone.
7 Versatility. Many non-isotopic immuno-assay systems cannot pro-

vide the wide range of analytes which are offered by RIA. However, the Amerlite has a wide range of analytes available including those mentioned above.

Correlations with RIA

The advantages over RIA can only be considered relevant if the Amerlite results (especially E2, P4 and LH) correlate successfully with the current RIA methods. Our internal studies showed this to be the case with E2, P4 and LH all having correlations of 0.97, namely the following results from 55 samples:

Oestradiol	
1 Methodology	½ incubation time
2 Data	Amerlite = 0.76 RIA (raw results) r = 0.97. Sensitivity = Excellent Precision = Superior (6.5% vs. 14.1%) inter-assay CVs
Progesterone	
1 Methodology	½incubation time
2 Data	Amerlite = 0.82 RIA (raw results) r = 0.97 Sensitivity = Excellent Precision = Superior (5.1%vs. 7.7%) inter-assay CVs
LH	
1 Methodology	½ incubation time Monocloncal IRMA (compares with current)
2 Data	Amerlite = 0.66 RIA (raw results) r = 0.97 Sensitivity = As good Precision = Comparable (5.1%vs. 5.2%) inter-assay CVs

In these three major assays, the Amerlite performed faster, providing accurate results that were as good or better than those currently seen. The major finding was the impressive precision of the E2 assays, which can result in less day-to-day fluctuation being observed in a patient's oestradiol levels. β -hCG, prolactin and FSH were also analysed in a similar fashion and results again were highly comparable.

CONCLUSIONS

At this stage, the Amerlite system is being introduced into routine clinical practice and it has been estimated that the overall costs will

be less than the RIA system. Furthermore it is expected that the various advantages described will enable a simplified approach to the complex area of hormonal monitoring of ovarian cycles and early pregnancy. Although the experience to date has been short, long-term benefits are expected.

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Chapter 6

Donor insemination (DI)

Summary: Donor insemination (DI)

Indications	Intractable azoospermia.	
	Severe asthenospermia or oligospermia.	
	Genetic carrier states	
Donor	Careful donor screening to exclude risk of transmitting infections, genetic disorders	
	Accept if sperm density >50 m/ml; >60% motility.	
Semen Preparation	Egg yolk CPM + 15% glycerol	
	Rapid freeze/thaw	
Clinical procedure	Inseminate into cervix, days 0 ± 1	
	Add ovarian stimulation after 4–6 cycles	
Results	Pregnancy rate/cycle	20%
	Average 5 cycles to pregnancy	
	Pregnancy wastage	15%
	Multiple pregnancy	5%

The first successful DI was reported by Dr Pancoast in 1884 (Leeton, 1980). However, from that time until very recently there have been only intermittent reports in the medical literature from Europe and the USA. It is difficult to determine how extensively this procedure has been used as it has been an area of controversy and it is therefore likely that many practitioners were reluctant to publish their experiences.

The successful use of frozen semen was first reported in 1953 (Bunge and Sherman) but widespread use did not begin until the mid 1970s. Nowadays, DI programmes use frozen semen exclusively as the current techniques and methodology show little reduction in the chance of pregnancy in comparison with the use of fresh semen and there is the added bonus of appropriate screening of the donor to exclude the chance of transmitting serious infections such as hepatitis B and acquired immune deficiency syndrome (AIDS). It is because of the unfortunate similarity in acronyms between AID (artificial insemination by donor) and AIDS that the Fertility Society of Australia (FSA) recommended to its members in 1986 that the term '*Donor insemination*' be used in preference to artificial insemination by donor. Based on the knowledge that four of eight women treated in an Australian clinic became seropositive after DI with an HTLVIII seropositive donor, both the FSA (1987) and the American Fertility Society (AFS, 1988)

have issued guidelines to their members, recommending a 6-month period of quarantine for all semen specimens, so that donors can be re-checked to ensure they have remained seronegative. Both societies believe that fresh semen for DI can no longer be justified.

SEMEN DONORS

The demand for donor semen has increased markedly in recent years, associated with the wider dissemination of information related to infertility treatment procedures. In Australian clinics this demand appeared to have reached its peak in 1984; most clinics now have a relatively stable demand. The most common sites for recruitment of donors are the various further education institutions and less so from organizations such as the police force, firemen and defence forces. Grateful patients are an additional source, and most donors appear to provide samples for altruistic reasons but are appreciative of a small sum given for travel expenses.

Potential donors require a medical consultation for history and physical examination. Donor history should include details of the family history to exclude inherited disorders, as well as a personal history of physical, mental and psychological disabilities. Intravenous drug users, homosexuals and those at risk of transmitting infections such as hepatitis, malaria, syphilis etc. must be excluded. All donors should sign a lifestyle declaration as required by the relevant Department of Health (Table 6.1)); this statement is similar to the one signed by blood donors. A physical examination should be undertaken and should include a check of the genital region to exclude any obvious abnormality.

SEMEN ANALYSIS

For best results in a DI programme, only those donors with sperm density >50 million/ml and at least 60% forward progression with high-grade motility and >60% normal morphology should be selected. An excessive number of polymorphonuclear leucocytes (>5/hpf) suggests genital tract infection and requires further investigation such as urethral swabs to exclude gonococcus, *Chlamydia* and other organisms. Such cases should be excluded from contributing to the donor sperm bank. Even in the absence of raised leucocyte counts, specific culture for gonococcus and a check for *Chlamydia* antigen should be performed. Screening for other organisms such as cytomegalovirus, herpes simplex, human papilloma virus, mycoplasma hominis, urea plasma urealyticum and group B *streptococcus* may also be relevant (Hummel and Talbert, 1989).

Table 6.1 Declaration signed by semen donors

I hereby declare to the best of my knowledge that:

1 I have not engaged in male-to-male sexual activity during the past 5 years.
2 I have no reason to believe I am suffering from AIDS or any disease related to it.
3 I have not injected myself with any substance not prescribed by a qualified medical practitioner within the past 5 years.
4 I am not suffering from night sweats, unintentional weight loss or persistent fever, diarrhoea, or swollen glands.
5 I have not received a blood transfusion or treatment with human blood products within the past 5 years.
6 No sexual partner of mine is a person who could not have made a declaration of this kind in respect of paragraphs 1, 2, 3, 4 or 5.
7 I have not been treated by acupuncture, had my ears or nose pierced or been tattooed within the past 6 months.
8 I have never had an attack of malaria.
9 I have not had jaundice or hepatitis in the past 12 months, and have not been in close contact in the past 6 months with a person who had jaundice or hepatitis.

The following serological tests should also be performed:

1 Blood group and rhesus status.
2 Syphilis serology.
3 Hepatitis B antigen and antibody screening.
4 Human immune deficiency virus (HIV) antibody titres.

Semen specimens should be stored for 6 months and donors screened again for syphilis serology, hepatitis B and HIV. Semen specimens should only be released from screening after quarantine at the 6-month interval has shown negative results.

Other tests may be indicated; for example, where a donor has Mediterranean or Asian background, consideration should be given to screening for a haemoglobinopathy by haemoglobin, red cell volume and haemoglobin electrophoresis examination. Where a donor is Jewish, consideration should be given to screening for Tay–Sachs disease—an autosomal recessive trait present in 1/50 Ashkenazi Jews but in only 1/100 000 in the general population.

DONOR MATCHING

In order to facilitate matching the characteristics of the donor with the recipient the following physical characteristics require detailed recording:

1 Height—tall, short or average.

2 Stature or body build—small, medium or heavy frame.
3 Eye colour.
4 Hair colour.
5 Skin complexion—fair, freckled, olive.
6 Race or ethnic background.
7 Social history.

It is also considered appropriate to record the following additional non-identifying donor information which may be of assistance to parents who wish to inform their children at a later date:

1 Age.
2 Religion.
3 Nationality.
4 Ethnic background of parents.
5 Country of birth.
6 Schooling.
7 Occupation.
8 Marital status.
9 Number of children.
10 Interests and hobbies, sports etc.
11 Comment on donor personality by interviewer.
12 Reason for assisting DI programme.

CONFIDENTIALITY

Care should be taken to avoid any disclosure of patient or donor-identifying information. It is considered appropriate that identifying information regarding donors should be retained as confidential records within the DI unit, in line with good medical practice. The clinic director must be able to have access to donor records to check any unusual situations, e.g. a recipient patient contracting a known transmissible disease or recurrent fetal abnormalities relating back to a particular donor.

SEMEN CRYOPRESERVATION

Several cryoprotective media (CPM) can be used, but the preferred medium contains fresh egg yolk and 15% v/v glycerol. The medium has been described by Richardson (1976; Table 6.2).

Table 6.2 Preparation of stock solutions

Stock solutions	*mM*	*g/l*
Sodium citrate buffer	100	29.40
Glucose solution	330	54.60
Fructose solution	330	54.60
Penicillin solution	1 million units dissolved in 10 ml citrate buffer	
Streptomycin solution	1 g dissolved in 5 ml sodium citrate buffer	

Cryopreservation technique

Semen is diluted 1:1 with the egg yolk–citrate–glycerol CPM and can be frozen stored by either a hand-held technique or within a programmable freezer. It is of interest that our results have often been better using the hand-freeze technique, possibly because the sperm straws can be supercooled more efficiently than in the programmable freezer (Planer Programmable Temperature Controller PTC300) which has proven satisfactory for embryo freezing. Spermatozoa for cryopreservation are best stored in 0.5 ml straws. These come in a range of colours which should be selected in a coding system which also includes a number on the straw and an additional code related to the storage position within the liquid nitrogen dewar vat.

Each straw is filled with 0.5 ml of the sample via the non-plugged end of the straw using a 1 ml plastic syringe or a protected mouth pipette (Plate 6.1a). The ends are then plugged immediately using coloured cement mixed with water and sealed and labelled with the same identification code as the other end. Straws are grouped together in a plastic goblet with the cement ends up and frozen by one of two methods using liquid nitrogen (N_2; Plate 6.1b). The following programmable freezer technique is one of the most successful sperm-freezing programmes.

−1°C/min–+3°C
−2°C/min–+2°C
−5°C/min–+1°C
−10°C/min––80°C
Hold for 30 min.

The goblet is then placed in the liquid N_2-containing dewars.

Another successful technique involves hand-freezing the sperm. The goblet containing the straws is placed in the refrigerator (4°C) for 10 min, then put into the deep freeze for 10 min. The straw is then held over a dewar vat within the vapour for 5 min, then placed deeper near the liquid surface for a further 5 min before it is moved to its

storage cannister and plunged into liquid nitrogen for long-term storage. An expensive automated technique enabling horizontal freezing of straws and simulating the manual method is currently undergoing trials with early favourable results.

DONOR INSEMINATION TECHNIQUES

Indications

1 Intractable azoospermia, e.g. Klinefelter's syndrome, absent vasa deferentia, Sertoli cell-only syndrome.
2 Dysfunctional spermatozoa, e.g. immotile sperm (Kartagener's syndrome—absent dynein arms), and other situations where spermatozoa repeatedly fail to fertilize oocytes in vitro. This is sometimes seen with normal morphology and motility but may be associated with round-headed sperm and abnormalities detected only by electron microscopy.
3 Genetic reasons, e.g. autosomal dominant conditions with a high risk of serious abnormality or mortality (Huntington's disease, myotonia dystrophica etc.).
4 Severe oligospermia or asthenospermia (i.e. progressively motile sperm count <5 million/ml). Until recently, this group has fared poorly with IVF-related procedures but current techniques of sperm preparation, maximizing sperm–egg interaction, improving sperm motility (e.g. with pentoxifylline) and using micromanipulation procedures have improved the prognosis for this group (see below).
5 Spinal and neurogenic disorders, e.g. quadriplegia, where satisfactory semen specimens might not be obtainable, even following vibro- or electroejaculation techniques.

In many situations couples may elect to have DI as their preferred treatment option because it is simpler to organize, less intensive and less expensive than AIH or IVF procedures.

Clinical technique

Daily intracervical inseminations are performed in the periovulatory phase. Generally two or three inseminations are carried out. Ideally, a full investigatory infertility profile should include an assessment of the female. Where this has not been performed, it may be reasonable to undertake up to three treatment cycles prior to a full evaluation which should then include a laparoscopic appraisal, check for ASABs, genital tract infection and ovulatory disorders.

It may be practical to undertake up to a maximum of four treatment cycles on cervical mucus assessment alone, followed then by periovulatory monitoring to determine the day of LH surge. If conception has not ensued within a maximum period of six cycles, ovarian stimulation is included in the management. This is introduced earlier if an ovulation disorder of any type is suspected or has been detected.

Patients attend the clinic at a time convenient to them, ranging anywhere from 7 a.m. to 6 p.m. Within this timeframe, no relationship between the time of insemination and pregnancy has been shown. Patients are inseminated in the dorsal position using a bivalve vaginal speculum to expose the cervix. The appropriate 0.5 ml straw is withdrawn from the holding dewar flask (Plate 6.1c) and allowed to thaw on a tissue in an aseptic area on the workbench at room temperature. The cement-tipped end of the straw is cut off with scissors (Plate 6.1d), the straw is then inserted into the tip of the insemination gun (Plate 6.2) and the sterile sheath is fitted over the entire inseminating apparatus. The tip of the insemination instrument is inserted between 1 and 2 cm into the cervix and the insemination gun plunger is slowly closed to release the semen. After 1 min, the insemination instrument is withdrawn, allowing the tip of the bivalve speculum gently to grip the cervix closed for a period of approximately 2 min before it is released and withdrawn. The patient remains lying on the couch for a period of 15–20 min and then leaves the clinic, usually proceeding to her work.

RESULTS

Overall, a pregnancy rate of 20% per treatment cycle is possible and more than 80% of pregnancies will proceed to the delivery of viable infants. The pregnancy rate for DI at an established clinic over an 8-year period is shown in Table 6.3. An average of five treatment cycles is usually required to achieve pregnancy but this can be reduced as certain factors were shown to be associated with the poorer results noted during the latter part of 1985 through to 1987. During that period, three factors were identified which were not present before or since:

1 A reduced glycerol concentration of 7.5% in the CPM.
2 The use of a CPM which did not utilize egg yolk (Trounson et al., 1979).
3 The use of the programmable freezer (Planer PTC300) rather than hand-freezing.

Because of the nature of this clinical work, it is not known how much importance to give to each individual factor but improved results

Table 6.3 DI procedures at the PIVET Medical Centre, Australia, 1980–1988

Period	Number of cycles	Pregnancies (percentage)	Cycles/ pregnancy
1 July 1980–12 May 1983	166	42 (24.6%)	4.0
13 May 1983–1 March 1985	157	40 (25.0%)	3.9
2 March 1985–31 December 1985	112	21 (18.7%)	5.3
1 January 1986–31 December 1986	120	16 (13.3%)	7.5
1 January 1987–31 December 1987	111	15 (13.5%)	7.6
1 January 1988–31 May 1988	40	8 (20.0%)	5.0
Total	706	142 (20.1%)	4.9

have continued by using the egg yolk with 15% v/v glycerol CPM and a rapid manual freeze or automated horizontal loading method.

With respect to pregnancy outcome (Table 6.4) the prospects are better than those for women undergoing other infertility procedures, in particular with respect to ectopic pregnancy rate (<1%, compared to 5% or more from other treatments). This undoubtedly reflects the case selection criteria for DI therapy, i.e. the DI group contains fewer cases with female-related infertility factors.

DONOR INSEMINATION FAILURES

Ovarian stimulation should be introduced as part of the clinical protocol—at an early stage if ovulation disorders are suspected or identified, but also after six treatment cycles, even in the absence of a demonstrable disorder. Table 6.5 shows a significant improvement in the chance of pregnancy following the introduction of ovarian stimulation agents, particularly those utilizing hMG in the regimen. However, care must be taken as almost 8% of the total pregnancies were multiple (see Table 6.4). In each case, hMG had been used in the ovarian stimulation regimen.

Depending upon the age of the couple and identification of additional infertility factors, treatment by PROST should be considered if pregnancy has not ensued within 6–12 treatment cycles. PROST is preferred to GIFT as it allows the identification of fertilization, hence providing diagnostic information. If successful fertilization is shown but pregnancy does not occur, the couple may continue with DI therapy. Previously, advice has included changing donors, double-dose insemination or two inseminations on the day of LH surge. These approaches can be considered but there are no scientific data to support their greater efficiency over the standard regimen. The use of PROST (or GIFT if fertilization has been shown) on every fourth or so treatment

Table 6.4 Pregnancy outcome for all DI pregnancies conceived by May 1988 at PIVET Medical Centre, Australia

Pregnancy outcome	Number of pregnancies (percentage)
Early wastage (<20 weeks)	
Preclinical	2 (1.4%)
Blighted ovum	13 (9.2%)
Miscarriage	9 (6.3%)
Ectopic	1 (0.7%)
Total	25 (17.6%)
Late pregnancy outcome (>20 weeks)	
with surviving infants	115 (81.0%)
without surviving infants	2 (1.4%)
Total	117* (82.4%)
Combined total	142 (100.0%)

* Total infants 132 (70 females, 62 males)
singleton 106
twins 10
triplets 2

Congenital abnormalities 4/117 pregnancies (3.4%); 4/132 infants (3.0%)
1 Renal abnormality.
2 Tracheo-oesophageal fistula.
3 Noonan's syndrome.
4 Combined urogenital abnormality.

Table 6.5 The chance of pregnancy per treatment cycle from DI with respect to ovarian stimulation therapy

Ovarian stimulation	Treatment cycles	Pregnancies (percentage)
Nil	312	42 (13.5%)
Clomid	215	42 (19.5%)
Clomid/hCG	103	32 (31.1%)
hMG alone	76	26 (34.2%)
Totals	706	142 (20.1%)

cycle is much more effective as the pregnancy rate will be in the order of approximately 40% for that cycle if three oocytes are transferred.

Figure 6.1 shows the insemination days for 113 DI pregnancies where the day of LH surge (spontaneous or by hCG trigger) was known. It appears that the day of LH surge (day 0) ± 1 day is the optimum time for insemination. However, at a clinical level, pregnancies will be achieved more rapidly if insemination is undertaken between days

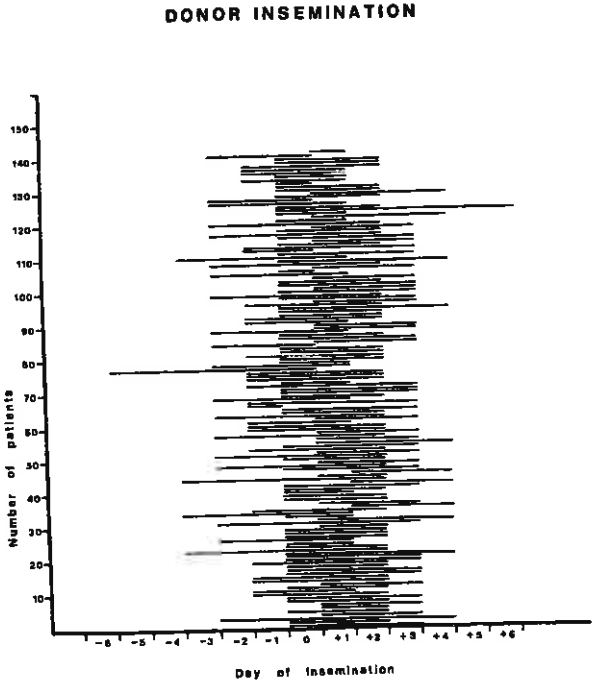

Figure 6.1 Insemination days for 113 DI pregnancies.

−2 and 0, coinciding with peak preovulatory mucus. It seems that cervical mucus can become rapidly impenetrable in many women following luteinization.

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Chapter 7

Artificial insemination by husband (AIH)

Summary: Artificial insemination by husband (AIH)

Indications	ASABs (male and female)	
	Negative post-coital tests	
	Simple oligospermia	
	Absent cervical mucus	
	Psychosexual reasons	
Semen preparation	Sperm 'washed' with T6	
	Incubate for 1–2 h	
	Motile sperm harvest	
Clinical procedure	Ovarian stimulation	
	Insemination into uterus on days +1 and +2	
Results	Pregnancy rate/cycle	15%
	Pregnancy wastage	33%
	Blighted ova	19%
	Multiple pregnancies	5%

Artificial insemination by husband's spermatozoa covers a wide range of techniques. Fundamentally, the method of insemination is concerned with the deposition of spermatozoa at various locations in the female reproductive tract to aid the successful transport of spermatozoa to the site of fertilization. At an elementary level, insemination of semen can be performed intravaginally, pericervically using a cap, intracervically or directly into the uterine cavity. However, limitations can exist with these routes as intravaginal, pericervical and intracervical inseminations appear to have no benefit over natural coitus because of the failure to bypass the cervical canal (Joyce and Vassilopoulos, 1981). Furthermore, intrauterine insemination of semen can cause severe cramps in 6–17% of patients (Allen et al., 1985). In addition, there is a theoretical risk of infection by the introduction of non-sterile material into the uterine cavity, although the incidence of demonstrable infections in practice appears to be small (Allen et al., 1985).

As with DI, ovarian stimulation has been shown to improve the pregnancy rate significantly. The greater success rate is clearly related

to the number of oocytes available for fertilization but the mechanism for the benefits of ovarian stimulation is not understood. Ovarian stimulation can be advised as a component of the AIH protocol in all cases by the fourth treatment cycle if pregnancy has not occurred. If an ovulation disorder is revealed during the observation period or there is severe oligospermia, ovarian stimulation is introduced from the outset. The regimen is similar to that previously described, beginning with clomiphene citrate in the first instance followed by clomiphene citrate/hMG and then hMG alone.

The development of laboratory techniques associated with IVF has led to the refinement of procedures for the preparation of washed motile spermatozoa. Early descriptions of washed semen techniques were provided by Halim et al. (1974) and Usherwood (1978). Such methods have been improved upon, particularly for oligospermic samples (Yovich and Stanger, 1984). Consequently, many workers are now using their experience with IVF to perform intrauterine insemination of washed motile capacitated spermatozoa (Sher et al., 1984; Yovich and Matson, 1988a; Remohi et al., 1989). It was considered that this use of prepared spermatozoa would be an improvement over the direct intrauterine insemination of semen because the seminal plasma, with contains inhibitors of fertilization (Kanwar et al., 1986) and prostaglandins is removed and the selection of motile spermatozoa improves the proportion of available spermatozoa with good morphology (Leung et al., 1984).

AIH has been used by many centres to treat infertile couples presenting with conditions such as reduced semen quality, poor cervical mucus, cervical hostility and the presence of ASABs in either partner, or unexplained infertility (Nachtigall et al., 1979; Allen et al., 1985). However, a review of the literature suggests that the probability of conception following treatment is very much dependent upon the underlying disorder. For example, we have obtained good results (>15% pregnancies per treatment cycle) in those couples whose main cause of infertility was poor sperm–mucus interaction creating negative PCTs, particularly where this was associated with ASABs in either the male or the female partner. Moderate success has been obtained in those with simple oligospermia and in some cases of unexplained infertility (9–10% pregnancies per treatment cycle), but poor results have been found in other types of infertility and no pregnancies have been achieved where there was a severe degree of asthenospermia (Yovich and Matson, 1988a).

INDICATIONS FOR AIH

1 ASABs—male or female.
2 Poor sperm–mucus interaction (negative PCT in proven preovulatory mucus).

3 Absent cervical mucus, e.g. cervical trauma, cystic fibrosis.
4 A relative option for simple oligospermia or unexplained infertility.
5 A remedy for psychosexual disorders precluding satisfactory intercourse.

Although there has been considerable controversy regarding the significance of ASABs in the literature, current studies show that combined IgA and IgC antibodies detected on indirect immunobead testing in semen are significantly associated with reduced oocyte fertilization and a reduced chance of pregnancy in IVF programmes. In the female, the presence of any clearly detected antibody in the serum will prevent fertilization of oocytes and pregnancy in virtually all cases undergoing IVF where maternal serum is used in the culture medium; this can be corrected by the replacement of maternal serum with donor serum (see Chapter 9).

LABORATORY PREPARATION OF SEMEN SAMPLES

If there are ASABs in the semen, or if the seminal fluid is highly viscous, ejaculates should be collected directly into culture medium. The semen collection rooms should be sited adjacent to the laboratory area and connected to it by a hatchway which has a small door on either side. The semen collection rooms should be accessible from outside the building but be part of the centre's structure and therefore in the same air-conditioned environment. The rooms should be soundproofed and the decor suited to the purpose of semen collection by masturbation. Samples should immediately be placed into the double-door hatchway and the patient should alert the laboratory staff to this fact by pressing a buzzer before departing. The laboratory staff can immediately retrieve the sample jar from their side of the hatchway and begin the process of repetitive pipetting of specimens collected directly into the culture medium. Semen specimens not containing antibodies and not known to be particularly viscous, are allowed to liquefy for 15 min.

The culture medium used is the modified Tyrode's preparation (T6) (Whittingham, 1971). The spermatozoal preparation is undertaken according to the technique described in Chapter 9 and will vary according to the severity of the oligospermia and asthenospermia.

CLINICAL PROCEDURE

The wife presents to the clinic for insemination approximately 2 h after her husband's appointment for semen collection. The insemina-

tion procedure is performed in the dorsal position and the cervix is exposed with a bivalve speculum. The spermatozoal preparation is contained in a small syringe (e.g. a tuberculin) which is then attached to a 38 cm FG5 polyvinylchloride (PVC) paediatric feeding tube (AHS, Australia) and the specimen is carefully injected into its tip. The catheter is inserted 4 cm using a curved Sortel or preferably, a right-angled George forcep (Plate 7.1). The PVC catheter has been shown to have no toxic effects to either embryos or spermatozoa. A dry swab is used to clean the cervical os and the catheter is threaded 4 cm into the canal. The tuberculin syringe is gently plunged 0.5 ml; this evacuates the seminal preparation from the PVC catheter. The tips of the bivalve speculum are allowed to close gently around the cervix whilst the catheter is removed. After 2 min the bivalve speculum is removed and the patient remains recumbent for 15–20 min before leaving the clinic, usually to go to work.

Inseminations are performed in the periovulatory period but this differs from DI in that treatments are performed at the time of and after the LH surge, i.e. on days +1 and +2. Occasionally a third specimen is inseminated on day +3.

TREATMENT RESULTS WITH AIH

Table 7.1 shows a typical series comprising 345 couples with long-standing infertility awaiting treatment by IVF in relation to the diagnostic category of subfertility. All women had ovarian cycle monitoring commencing on day 8 of the menstrual cycle, which included the daily analysis of serum LH, P4 and E2, together with ovarian ultrasonography, cervical mucus score (Insler et al., 1972), evaluation of sperm–mucus interaction by a PCT and mid luteal estimation of serum P4 and E2. Women with ovulation disorders were excluded from this study and offered treatment in a separate programme (Yovich et al., 1987). Such women often had poor cervical mucus which improved significantly and entered the fertile mucus range following successful ovarian stimulation, particularly with hMG. However, such cases were reconsidered for AIH if PCTs remained negative after inducing ovulation and fertile mucus.

PCTs were assessed as described in Chapter 4 (Matson et al., 1986) and ASABs were tested in the semen of the male partner and serum of the female partner. A gelatin agglutination test was used initially (Kilbrick et al., 1952), with clinically significant titres considered to be >1/8 in semen and >1/32 in serum. The indirect immunobead test was used to identify IgA, IgG and IgM (Junk et al., 1986a).

Applying WHO criteria (1980), semen samples were classified as being normospermic (>12×10^6 motile spermatozoa/ml) or oligospermic (<12×10^6 motile spermatozoa/ml). Samples were regarded as asthenospermic if the motility was ⩽30%. Pelvic endometriosis was assessed at laparoscopy and classified according to the revised AFS criteria (1985). Cases with grades I and II (mild) were classified separately from grades III and IV (severe). Most of the women with severe endometriosis and approximately a third of the mild cases had previously been treated with progestagens or danazol prior to referral; all were reclassified following acceptance to the specialist infertility centre. None of the women considered here had had specific hormonal treatment within the 6 months preceding AIH.

Table 7.1 Profile of couples treated by AIH whilst on the IVF waiting list

Group	Number of couples	Age (years)		Duration of infertility (years)
		Male	Female	
Negative PCT	88	34.4 ± 0.05	31.9 ± 0.4	5.2 ± 0.3
Male ASAB	14	34.8 ± 1.1	31.4 ± 0.8	5.4 ± 0.7
Female ASAB	19	35.6 ± 0.8	32.8 ± 0.6	6.4 ± 0.6
Oligospermia	42	33.0 ± 0.5	30.5 ± 0.5	5.0 ± 0.3
Poor cervical mucus	45	32.1 ± 0.6	30.2 ± 0.5	4.8 ± 0.5
Asthenospermia	13	33.3 ± 1.7	29.7 ± 1.0	3.5 ± 0.6
Endometriosis				
mild	33	33.5 ± 0.4	30.6 ± 0.4	5.1 ± 0.3
severe	23	34.8 ± 0.9	32.9 ± 0.9	5.2 ± 0.5
Unexplained infertility	101	33.6 ± 0.4	30.6 ± 0.3	5.1 ± 0.3
Total	345	33.9 ± 0.4	31.2 ± 0.4	5.1 ± 0.3

Because of the intensive treatment mode, the AIH treatment involved ovarian stimulation in order to maximize the chance of pregnancy. This was performed in similar fashion to the IVF programme, using clomiphene citrate, hMG or a combination of the two drugs. Ovulation was triggered by the administration of 5000 IU hCG or the defined occurrence of an endogenous LH surge. Insemination occurred over the next two or three mornings during the periovulatory period. In the usual case, an hCG injection was given in the evening (6.00–8.00 p.m.) and inseminations commenced the next day at 10.00–12.00 a.m. The results are shown in Figure 7.1, and confirm that AIH is a useful treatment option for couples with negative PCTs, ASABs, and possibly, for mild oligospermia and some cases of unexplained infertility. However, the results are disappointing for those couples with asthenospermia and this implies that spermatozoal motility is very important in ensuring successful fertilization. This is in accord with other observations in an IVF programme (Mahadevan and Trounson, 1984). Similarly, a poor pregnancy rate in women with endometriosis, and particularly in those with severe endometriosis, is consistent with

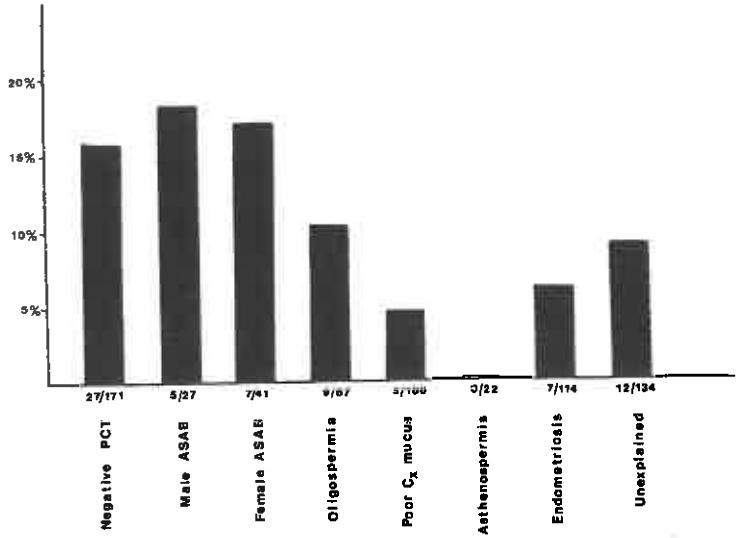

Fig. 7.1 Pregnancy rate per treatment cycle of AIH for various infertility categories.

the results obtained following IVF-ET and may relate to an impairment of implantation rather than reduced fertilization (Matson and Yovich, 1986). The lower pregnancy rate in women with poor cervical mucus is difficult to understand since bypassing the cervical canal is expected to be an effective manoeuvre. However, the results of AIH infer that the poor mucus development reflects an anomaly acting more widely in the genital tract, possibly at the level of the endometrium, providing an inadequate environment for implantation of the embryo.

The pleasing results obtained in the negative post-coital group demonstrate that once spermatozoa are beyond the cervix, gamete transport, fertilization and implantation can proceed. The pregnancy rates confirm our previous observations in the IVF programme (Matson et al., 1986) and the GIFT programme (Yovich and Matson, 1986) that the PCT is a poor marker for assessing the fertilizing ability of spermatozoa and that pregnancies can be achieved when spermatozoa are introduced to the oocyte in vitro or into the female reproductive tract at GIFT. The high pregnancy rates in those couples with ASABs suggest that the main functional defect is the failure of the spermatozoa to pass through the cervical mucus. Despite reduced fertilization rates in the presence of some ASABs (Junk et al., 1986b), the failure of the

ntibodies to totally block fertilization enables pregnancy to occur. The role of ovarian stimulation is not fully understood but the ncreased number of oocytes available increases the probability of at east one ovum being fertilized.

Results in couples with oligospermia are also encouraging. This category of patients has often shown a poor prognosis in other reports of studies applying AIH (Hull et al., 1986); however we suggest that AIH should be used, and that it is specifically those patients with severely reduced motility of spermatozoa—asthenospermia—who do not succeed with AIH.

FAILED AIH

If AIH fails to achieve pregnancies over four to six treatment cycles, couples may wish to consider the option of alternative treatments. For the negative PCT and unexplained infertility groups, GIFT is the next option. However, for those with ASABs or oligospermia, PROST is required in order to maximize the chance of fertilization before oocyte transfer. In some cases, sperm enhancement and micromanipulation techniques may be useful (see Chapter 19). Overall, the preference for AIH as opposed to GIFT or PROST relates to treatment intensity, invasiveness of therapy and the higher cost of the latter techniques.

PREGNANCY OUTCOME

The pregnancy outcome following AIH is shown in Table 7.2. The overall incidence of early and late pregnancy losses is apparently high but not significantly greater than that reported in the subfertile population conceiving after treatments involving ovarian stimulation and gamete manipulation such as IVF-ET or GIFT. The apparently high rate of occurrence of 'blighted ovum' pregnancy (20.8%) is of concern as it may reflect poor embryo quality as a consequence of the spermatozoal preparation technique. It is also possible that the technique of intrauterine insemination may disturb the endometrium and its capacity for normal implantation. It should be noted that the blighted ovum rate may also be higher with GIFT and this should be considered when choosing the therapeutic option. It is also higher than for insemination using donor spermatozoa (see Chapter 6).

The high ectopic pregnancy rate (5.2%) is not greater than that seen for subfertile women conceiving after various treatments (ovarian stimulation, reconstructive tubal surgery, AIH, IVF-ET and GIFT; Yovich and Matson, 1988b) and may bear no relationship to the AIH technique per se. Similarly, subfertile women conceiving after such treatments

Table 7.2 Pregnancy outcome in patients conceiving following intrauterine insemination of washed spermatozoa

Pregnancy outcome	*Number of pregnancies*	
Early wastage (< 20 weeks)		
Preclinical	7	(7.2%)
Blighted ovum	18	(18.6%)
Miscarriage	3*	(3.1%)
Ectopic	5	(5.2%)
Total	33	(34.0%)
Late pregnancy outcome (>20 weeks)		
with surviving infants	60†	(61.9%)
without surviving infants	4	(4.1%)
Total	64‡	(66.0%)
Combined total	97	(100%)

* One multiple pregnancy terminated completely (with five gestational sacs on ultrasound).
† Total infants 68 (one twin pregnancy had selective termination).
‡ Total infants 73.

Congenital abnormalities 2/64 (3.0%)
1 Hare lip.
2 Malformation of ear and ribs.

have a greater late pregnancy wastage, mainly from preterm delivery; some of this wastage is due to the increased number of multiple gestations following ovarian stimulation but also unexplained reasons primarily related to unknown findings in the infertile population. The late pregnancy loss (4.1%) in the AIH group was similar to that seen in the IVF-ET and GIFT programmes. Congenital abnormalities were not significantly greater than in the same wider community rate (Bower and Stanley, 1986) and recurring abnormalities were not seen.

In conclusion, the AIH technique must be considered to be a useful and relatively non-invasive therapeutic modality for treating infertility in couples with a particular range of disorders. AIH results in a reasonable chance of pregnancy, comparable to IVF-ET procedures, in couples with poor sperm–mucus interaction (negative PCT), those with ASABs (male and female), and cases of discrete oligospermia not complicated by asthenospermia.

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Chapter 8

IVF and reproductive physiology

THE FIRST PREGNANCIES

Although successful embryo transfers were described in the rabbit a century ago (Heape, 1890) the process of IVF has a much shorter history, with the first mammalian success producing viable offspring again being achieved in rabbits (Chang, 1959). It is of interest that the process of rabbit IVF remains difficult unless the spermatozoa have been resident in the female genital tract for around 5 h to achieve capacitation prior to IVF. In many ways the subsequent human work has proven to be more simple, although successful techniques have now been reported for several mammalian laboratory species including mouse, (Whittingham, 1968), hamster (Yanagimachi and Chang, 1964) cat, pig and rat (Evans et al., 1980).

The history of human IVF begins with certain observations on crude attempts to achieve fertilization undertaken during the 1940s (Rock and Menkin, 1944; Menkin and Rock, 1948) and 1950s (Shettles, 1953, 1955, 1958). During the 1960s, the IVF pioneer Robert Edwards collected surgical specimens of ovary and explored the fertilization of oocytes matured in vitro (Edwards, 1965a, b; Edwards et al., 1966, 1969). It became clear from the animal reports that oocytes matured in vitro fertilized infrequently and rarely produced live offspring. Subsequently, successful human IVF was achieved with oocytes collected at laparoscopy from maturing follicles at the preovulatory stage following hyperstimulation with hMG (Edwards et al., 1970; Steptoe and Edwards, 1970; Steptoe et al., 1971). The oocytes were fertilized in vitro in a modified Tyrode's solution, adding sodium bicarbonate, bovine serum albumin (BSA) and sodium pyruvate (Bavister, 1969). Subsequent embryo growth was in Ham's F10 solution containing added BSA. The first human pregnancy was reported in 1976 (Steptoe and Edwards, 1976). Unfortunately, it was an ectopic gestation; presumably the oocyte was flushed into the proximal tubal remnant in an excessive volume of conveying medium (0.5 ml). The same team subsequently changed their approach to oocyte collection and described a technique for monitoring natural, unstimulated follicle development (Steptoe and Edwards, 1979; Edwards et al., 1980). The first successful

pregnancy arose from a series of such cases. A total of 32 embryo transfers (ETs) were described, leading to four pregnancies, two of which were normal—a female child, Louise Brown, was born in July 1978 and later a male infant was born; both were delivered at term. Two of the four pregnancies miscarried, one in the first trimester, shown by chromosomal analysis to have a triploidy, and one in the second trimester; it contained certain chromosomal defects thought to be inherited from the father (Steptoe et al., 1980).

The first Australian success was reported from Melbourne in 1980, using the natural cycle method of oocyte collection (Lopata et al., 1980). However, at that stage of developments, the natural cycle technique was found to be wasteful of resources, as on-call medical and laboratory staff were needed because oocyte collection is often required during the night and at weekends, outside the routine working day. In addition, the pursuit of a single oocyte led to successful collection and IVF-ET less often than those cycles in which ovulation was stimulated using clomiphene citrate (McBain and Trounson, 1984). In stimulated cycles, oocyte collection could be timed to fit into routine operating theatre lists by using hCG just before the natural LH surge. Mature preovulatory oocytes could be aspirated from ovarian follicles 34–36 h after hCG. The first successful stimulated cycle pregnancies were reported from Melbourne in 1981 (Trounson et al., 1981) and other states followed soon after (Yovich et al., 1982).

In the USA, the National Institutes of Health refused to fund human IVF research and a voluntary moratorium was imposed on human IVF work through the 1970s. Following extensive debates after the British success, the Jones Institute was set up in Virginia in 1980 and their first delivery was in December 1981 (Jones et al., 1982). In Europe, clinics have been established in all countries and a number of them reported deliveries in 1982 (Seppala et al., 1985). There are now few places in the world that do not have IVF clinics but at this stage there appears to be a wide range in the results, indicating variable efficiency. This is shown from the Australian, British and American data for 1988 (Lancaster, 1990; ILA, 1990; AFS, 1990).

SPERMATOZOAL CAPACITATION

Information on spermatozoal capacitation has been available since 1951 with the independent observations of Austin (1951) and Chang (1951). Freshly ejaculated or surgically removed spermatozoa have minimal ability to penetrate the cumulus, coronal coat, zona pellucida and vitelline membrane of the ovum. Developing the capacity to do so appears to depend on the removal of certain proteins coating the

spermatozoa surface and subsequently enabling spermatozoa to undergo the acrosome reaction. Certain observations are relevant:

1 Spermatozoa are capacitated in the female reproductive tract of animal species only during certain stages of the oestrous cycle (Chang, 1958).
2 Capacitated spermatozoa are decapacitated by resuspension in seminal plasma (Bedford and Chang, 1962).
3 In vivo, capacitation is limited to the female reproductive tract (Hamner and Sojka, 1967).

Further studies suggest that a decapacitation factor exists in the fluid of the male genital tract and this may serve to prevent spermatozoa from penetrating cells and tissues. After capacitation in the uterus, spermatozoa become penetrating cells, not only penetrating ova but also other cells and tissues. The decapacitation phenomenon may also allow the more ready phagocytosis of spermatozoa attempting to penetrate the uterine wall within its lumen (Williams et al., 1967).

Some spermatozoal proteins have been shown to be immunogenic and to inhibit fertilization (Brackett, 1978). The early studies on spermatozoal capacitation highlight the importance of complex natural capacitating factors resident within the female genital tract at appropriate times. Nonetheless, it is now recognized that for many species, capacitation can be achieved by the incubation of spermatozoa in simple synthetic media such as Tyrode's or Krebs-Ringer bicarbonate solution containing NaCl, KCl, $CaCl_2$, KH_2PO_4, $MgSO_4$, $NaHCO_3$ and the presence of serum albumin. The latter does appear to be a specific requirement for capacitation (Fraser, 1984) even though a recent report (Caro and Trounson, 1986) describes successful IVF of mouse and human gametes in protein-free medium; in this case the preliminary preparation of spermatozoa was carried out in the presence of maternal serum.

THE ACROSOME REACTION

Following capacitation, changes occur to the acrosomal region of the spermatozoa prior to sperm penetration of the ovum. Initially, the interior aspect of the acrosome cap begins to swell. Membrane fusion then occurs between the outer acrosomal and inner plasma membrane resulting in vesiculation and the release of acrosomal enzymes. The phenomenon is calcium-dependent and inhibited by magnesium (Gwatkin, 1977; Bedford, 1983). It is associated with rising levels of cyclic adenosine monophosphate (cAMP), activation of the acrosomal enzymes and the movement of Ca^{++} across the membrane. The

enzymes hyaluronidase, acrosin and esterases are liberated from the acrosome, assisting spermatozoal passage through the cumulus cells which become dispersed. Spermatozoa then attach to the zona pellucida which is composed of mucopolysaccharides and glycoproteins. The interaction involves calcium (Heffner et al, 1980) and trypsin-like activity (Saling, 1981) probably provided by acrosin released from the acrosomal cap. Binding takes place at specific spermatozoal receptors on the zona pellucida and the movement of spermatozoa through the zona pellucida is assisted by enzymes such as acrosin derived from within the acrosome. Following movement through the zona pellucida, the spermatozoon then attaches to the plasma membrane of the oocyte in the post-acrosomal region and it then appears that the oocyte plays an active role in drawing the spermatozoon into the ooplasm, where the sperm head undergoes swelling and decondensation prior to the formation of the male pronucleus (Fishel and Edwards, 1982).

Following capacitation and the acrosome reaction, most spermatozoa of eutherian mammals demonstrate hyperactivated or whip-like motility but such changes are not distinctly obvious in human spermatozoa which are structurally unique (they have no perforatorium and relatively broad dimensions of the narrowest profile of the head). Activated motility therefore is probably an insignificant element of the pre-fertilization process in humans (Bedford, 1983).

OOCYTE MATURATION AND BLOCK TO POLYSPERMY

Human oocytes are contained within the ovary for many years in the dictyate or diffuse diplotene stage of meiotic prophase (Baker and Wai Sum, 1976). Natural fertilization of ovulated oocytes occurs at the metaphase II stage of meiosis in the human. During the ovarian cycle, a number of oocytes begin the meiotic maturation process and many pre-antral follicles develop. However, most undergo atresia and only the dominant one or two follicles expand under the influence of FSH. Following the LH surge, follicles prepare for dispersal, luteinization occurs (granulosa cells begin to secrete progesterone) and the oocyte resumes meiotic division, releasing its first polar body and becoming suspended in the metaphase II stage (Plate 8.1; Tsafriri et al., 1983).

The development of meiotic competence is dependent upon FSH and, at least partially, upon ovarian oestrogen synthesis (Moor and Trounson, 1977). Within the fully grown preovulatory follicles, the resumption of meiosis is prevented by an inhibitory action of the granulosa cell. This is overcome by the preovulatory surge of LH (Channing and Tsafriri, 1977). The inhibitory factor from granulosa cells is thought to be a peptide of less than 2000 Da and is described as the oocyte maturation inhibitor (OMI) (Channing et al., 1982).

Once a spermatozoon has fused to the oocyte membrane, a block to polyspermy is established. The block is associated with extrusion of cortical granules which lie at the periphery of the oocyte. It is probable that there is also a faster, electrically mediated block which involves rapid depolarization of the oocyte membrane immediately following contact of the spermatozoon (Cooper and Bedford, 1971). A third, enzymatic block may also exist at the zona pellucida level following the acrosin-assisted penetration of spermatozoa with the release of acrosomal enzymes such as neuraminidase (Gould et al., 1971). However, such blocks to polyspermy may not be fully effective in vitro where superovulated oocytes can demonstrate polyploidy, thought to be attributable to polyspermy. The phenomenon has been seen in both animal and human studies on IVF (Barros and Yanagimachi, 1972; Wolf et al., 1978; Mahadevan and Baker, 1984). It is a common occurrence in oocytes which have had the zona pellucida lysed, so removing the interspecies block to fertilization (Yanagimachi et al., 1976).

EARLY EMBRYO DEVELOPMENT

Preimplantation development

Following successful penetration of the oocyte by the fertilizing spermatozoon, the male and female pronuclei become apparent and subsequently coalesce in a process known as syngamy. This involves the homologous pairing of chromosomes and is not a visible process at microscopy in humans. The meiotic process is then completed, providing the embryo with the full diploid status from the haploid chromosome content of each gamete. The one-cell mammalian embryo is the same diameter as the oocyte—approximately 100 μ and has a low nuclear : cytoplasmic ratio, unlike adult cells. However, the process of cleavage to the morula stage occurs without any increase in total mass and there may in fact be 'negative' growth during the early cleavage phase. The total amount of cellular material decreases by about 20% in the cow and the protein content of the mouse embryo falls by 25% during the first 3 days after fertilization (McLaren, 1982).

The embryos of lower order mammals cleave rapidly (goldfish: 20 min; frog eggs: 60 min) but eutherian mammals require 24–36 h for the first cleavage division and 10–15 h for each succeeding division (Edwards et al., 1981; Mohr and Trounson, 1984). During the first few cleavage divisions, each mitosis is followed immediately by DNA synthesis without any detectable G-1 period characteristic of adult cells. In fact, a G-1 period cannot be detected in mouse embryos before the 16-cell stage.

Until the 8-cell stage, most blastomeres are spherical but thereafter

will flatten against one another, maximizing the amount of cell-to-cell contact and developing intercellular links and junctional complexes. The process is termed compaction and coincides with the stage at which cellular differentiation occurs so that each blastomere no longer has the full potential to develop a complete embryo. Pre-compaction blastomeres are capable of full embryonic development, as shown by the experiment of Willadsen (1979) who successfully separated blastomeres from 40-cell sheep embryos and implanted each into separate, empty zonas. He has reported on monozygotic twins born from separate mothers.

At the morula stage, a fluid-filled cavity appears within mammalian embryos and subsequently the blastocyst develops with a single peripheral layer of large, flattened cells (trophectoderm or trophoblast) and an inner cell mass in one area of the cavity, from which will develop the adult organism comprising ectodermal, endodermal and mesodermal elements. Expansion of the blastocoele space leads to marked enlargement of the entire blastocyst and extreme thinning of the zona pellucida. In mice and humans, the blastocoele cavity begins to form at around the 32-cell state and hatching of the blastocyst from the zona pellucida occurs only two divisions later. In humans, the blastocyst remains reasonably spherical; in sheep and cattle there is an elongation to 20 cm and in the pig there is marked elongation to create a thread-like tube more than a metre in length prior to attachment.

One of the concerns with in vitro manipulation is that parthenogenetic activation of oocytes can occur. Such activation can be induced by electric shock and treatment of eggs with alcohol or culture medium low in calcium (Willadsen, 1979; Quinn, 1984). In most species, development usually ceases at the blastocyst stage but in the mouse, parthenogenetic activation has been followed by embryo development up to mid-gestation.

Several media can be used to support cleaving human embryos in vitro, including Earl's, Ham's F10 (see Chapter 9), Hoppe and Pitt's modification of Whitten's medium, and modifications of Tyrode's medium (Bavister, 1969; Whittingham, 1971; Kaufman, 1978). Most workers now appear to use a simple medium such as T6 (see Chapter 9), and we have described a suitable modification known as human tubal fluid medium (HTFM; see Chapter 9; Quinn et al., 1985).

Culture media contain deactivated human serum and are prepared using highly purified water which is prefiltered, multi-distilled or prepared by reverse osmosis and deionized, whilst additional procedures such as ultrafiltration and extraction through charcoal cartridges to remove volatile organic agents may also be required. The medium is prepared each week. A high bicarbonate concentration maintains the pH level at around 7.3 under anaerobic gas conditions (5% CO_2; 5% O_2; 90% N_2, which provides oxygen tensions thought to be similar

to the physiological state within the fallopian tube). Fertilization and embryo development may be performed under equilibrated paraffin oil in microdroplets or in loosely capped tubes with a saturated, humidified environment and strict control of the temperature at 37°C. This serves to avoid evaporation, as the osmotic tension must be maintained between 280 and 290 mOsmol/kg, close to the values found in follicular fluid (Purdy, 1982). It appears that 5% CO_2 in air may also be suitable and it is apparent that sodium:potassium ratios, energy sources such as pyruvate and trace metals such as magnesium may be important factors influencing subtle differences related to fertilization, cleavage and implantation success.

Post-implantation development

The duration of the pre-implantation stage varies between species, ranging from 4 to 6 days in rabbits, mice and humans, through to nearly 20 days in kangaroos and wallabies and domesticated animals. The pre-implantation phase may be extended by the suspension of blastocyst development in the phenomenon known as embryonic diapause, which is common in marsupials.

Following blastocyst hatching, the trophoblast tissue has a large area exposed to the uterine lumen and it is thought that the embryo descends into a uterine gland. In the human, implantation is interstitial within the uterus (rather than centric or eccentric within the lumen) and involves penetration of the epithelium to pass into the subepithelial connective tissue. It is essential during implantation that the blood vessels of the embryo can develop a functional communication with the maternal blood supply. This occurs within the uterine stroma and is related to special invasive properties of the trophoblast layer as well as to uterine epithelial cell degeneration.

The process of human implantation is completed to the full chorionic disc stage by day 13 or 14 (Renfree, 1982). It is thought that the proteolytic enzymes within the trophoblast break junctional complexes and lysosomes are involved in phagocytosis of stromal cells. The stromal cells of higher primates undergo a decidual cell reaction with increased cellular size, increased cytoplasmic inclusions and glycogen granules. In most species, the change is dependent upon the presence of a blastocyst, but in the human this may occur in the late luteal phase in its absence. It is known to be progesterone-dependent. In the rabbit, a uterine-specific protein called blastokinin (Renfree, 1982) or uteroglobin (Beier, 1976) appears in the uterine fluid in the peri-implantation period. However, it is not found in humans or in most other species and a complex interplay of embryonic and maternal signals are considered to be important in successful implantation (Fig. 8.1; Yovich, 1988). The signals pass between the ovary, uterus and

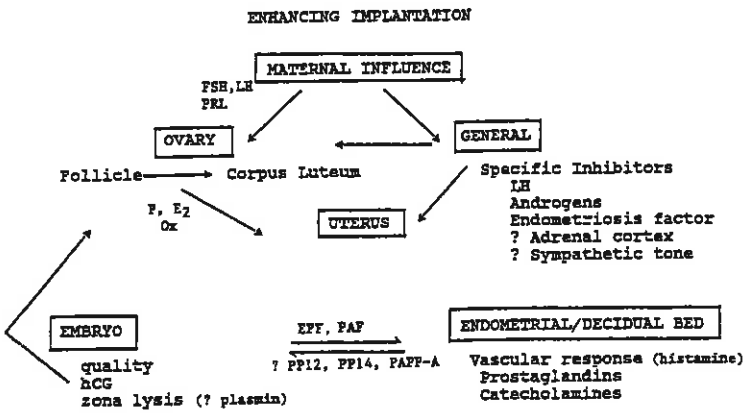

Fig. 8.1 Maternal and embryonic factors which are considered important in human implantation.

blastocyst beginning with ovulation and subsequent sensitization of the uterus and ending with hatching, attachment and implantation of the blastocyst and maintenance of the corpus luteum by hormones of embryonic origin. A comprehensive review of the systemic and local factors associated with implantation, the relevance of synchrony and timing and the pattern of implantation failure after IVF-ET has been prepared by McLaren (1985).

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Chapter 9

IVF: Laboratory aspects and local considerations

The results obtained following IVF-ET are influenced by the underlying disorder of the male and female partner being treated (Yovich et al., 1986). Other factors, such as the age of the female partner and criteria for cancellation of cases during treatment cycles, may also influence the results. Notwithstanding, optimal laboratory conditions are required to maximize the chance of pregnancy for any couple. Therefore when initiating an IVF-ET programme, aspects such as culture systems should be assessed with a view to altering the formulation of the culture medium (Quinn et al., 1985), choosing the most suitable protein supplement (Leung et al., 1984) and culture vessels (Marrs et al., 1984) and exploring the possible use of biological culture fluids such as serum (Kemeter and Feichtinger, 1984) and amniotic fluid (Gianardi et al., 1988) in order to determine the most advantageous system in the local circumstances.

A water purification system to provide quality water is an essential requirement for an active IVF-ET programme both for the routine washing up of glassware and instruments and, most importantly, for the preparation of culture medium. The embryos are far more sensitive to variations in the culture medium than other cells and tissues. When preparing medium, apart from selecting the purest clinical ingredients, one should obtain a very high quality water to reduce the contamination of medium by embryotoxic organic material, metallic ions or dissolved volatile vapours. With the introduction of water purification systems employing new technologies, tissue culture grade water is now available for human IVF work.

WATER PREPARATION

Whittingham (1971) has reported that the number of distillations for purification depends upon the water source. Scheme water requires purification to analytical grade level for its use in performing hormone RIA. Such water is also required for washing any equipment to be used in IVF procedures. However, for embryo culture, more purified and highly polished water is required to remove all particulate matter,

dissolved ionic salts, volatile hydrocarbons and pyrogens which may arise from organisms proliferating in areas of stasis within the water preparation equipment.

Distillation

Typically, scheme water available in Perth, Western Australia contains a higher concentration of salts and organic substances, requiring considerable pretreatment before it can be used in cell culture work (Yovich et al., 1988). To produce water pure enough for use in embryo culture media, rainwater is distilled eight times. The distillation apparatus consists of a heating mantle with a timer, a round-bottomed flask topped with a long baffled fractionation column and a headpiece leading to a Leibig condenser which feeds into another spherical collection flask. Glass beads are used in the distillation flasks to ensure even boiling and to prevent bumping which can fracture flasks.

A separate flask, column, condenser and connecting pieces are used for each distillation step. All the glassware is acid-washed overnight, sonicated, rinsed 20 times in Milli-Q water, dried and sterilized before use. After each distillation step the glassware is dried in a drying oven at 100°C, capped with aluminium foil and sterilized at 150°C for 2 h before it is used again.

At the beginning of each distillation step the first 50 ml or so of water that distils across is used to rinse out the collection flask and is discarded. Similarly, the last 50 ml of water remaining in the distillation flask is discarded.

After rainwater has been distilled eight times, it is either used immediately for media preparation or stored in Nunc 2-litre flasks at 4°C until needed.

Reverse osmosis/Milli-Q system

In the day-to-day running of an IVF programme, large volumes of purified water are required for making the different culture media used and the washing of laboratory glassware and theatre equipment.

The Milli-Q purification system uses a variety of methods to remove contaminants from incoming tap water. The following system is recommended. Before entering the reverse osmosis (RO) system, tap water should be pretreated by an initial passage through a 50 μ filter, a large water softener, an activated carbon filter and a final 5 μ filter. This method of pretreatment was specially designed to cope with Perth water and may not be a requirement for all locations.

Pretreated water should then be fed through a pressure regulating valve into two side-by-side RO units. This water can be drained off

for use in chemical analyses and hormone RIA or for washing equipment. Water for embryo culture requires further purification. This should be carried out by passing the double RO water through a final 'polishing' system consisting of two deionizing cartridges; one organex cartridge which removes any remaining organic material; and one pyrogard ultrafilter cartridge. Finally, the water should be passed through a 0.22 μ Millipore filter prior to use. This polishing system should be contained within a separate housing and should have a recirculation loop which continuously recirculates the water within the housing when the system is in the operating mode. When this system is in standby mode, the system recirculates water every 3 h.

On starting the system up each morning, the double RO water should be allowed to drain until its conductivity drops under 4 μΩ. The water should then be switched over to the Milli-Q system and again allowed to run to waste until its conductivity falls under 0.05 μΩ or resistivity rises above 18 μΩ. The end hose should be changed each morning and replaced with a heat-sterilized piece of silicon tubing. After use, the system should be turned back to the RO system and turned off. The end of the Milli-Q hose should be sealed with plastic film. This system is shown in Figs 9.1. and 9.2.

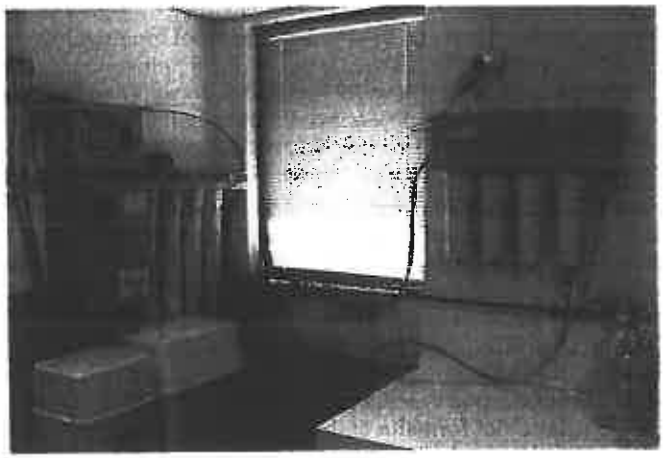

Fig. 9.1 Water purification system showing pre-filters, water softener and carbon filter prior to reverse osmosis (double unit) followed by deionization and ultrafiltration.

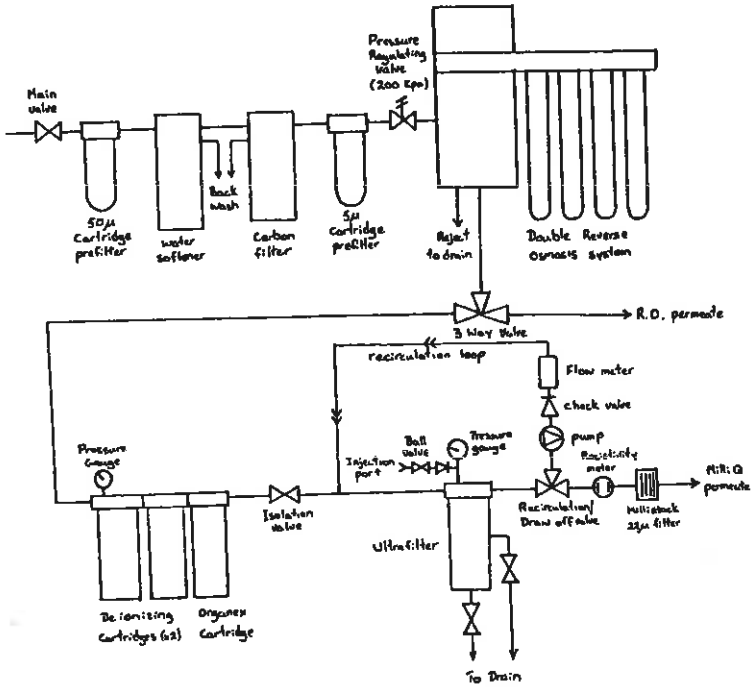

Fig. 9.2 Schematic illustration of water purification system used at PIVET.

Maintenance

The water softener regenerates itself automatically every 3 days and should only require topping up with pool salt periodically. Weekly checks should be made on the salt level to make sure that the salt is not 'bridging' over the water.

The carbon filter also backflushes itself automatically every 3 days. The initial 50 μ filter should be changed two or three times weekly and the 5 μ filter should be changed each fortnight, at the same time as the double RO unit is sterilized. This is done by filling the cartridge with a 2% solution of formalin and allowing them to stand overnight. Formalin should be flushed out in the morning. Formalin is introduced to the RO cartridges by removing the 5 μ filter from its bowl and pouring in 750 ml of formaldehyde. The RO system should be switched on for 1 min to draw the formaldehyde into the system and then shut down overnight. The following day a fresh 5 μ filter should be inserted and the system runs until all the formaldehyde is flushed from the

RO cartridges.

The deionizing and organex cartridges have a life of 4–6 weeks and should be replaced when the water quality wanes, as shown by difficulty in achieving polished water at better than 18 MΩ resistance.

The ultrafilter can be isolated and is sterilized by circulating approximately 5.0 g of free chlorine around it for 5–10 min, then flushing the chlorine out of the ultrafilter. This is followed by a thorough flushing of the Milli-Q system for 12 h on two successive days. This procedure should be performed weekly. The final 0.22 μ Millipore filter requires frequent changing (at least each week) as it has proven to be a common site for pseudomonas proliferation and the consequent release of pyrogens.

If the system is well maintained, copious high-quality water is generated to provide optimal conditions for cell culture. As an additional check of water quality, feed water, double RO water and Milli-Q water should be assayed weekly for pyrogens, using the chronogenic limulus amoebocyte lysate (LAL) test (QCL1000, Whitaker MA Products, MD, USA). This will allow the detection of bacterial growth within the system and more rigorous sterilization methods can be applied if necessary.

CHEMICALS

The chemicals used for the preparation of medium are readily available from large companies such as Sigma or Mallinckrodt. Powdered chemicals should be replaced annually and stored correctly, either in a cool dry cupboard or in the fridge-freezer as indicated by the manufacturers. Access to IVF chemicals should be limited to the person responsible for the preparation of culture media. Table 9.1 summarizes the chemicals used. Media preparations used at PIVET are Tyrode's medium (Table 9.2), Whittingham's modified Tyrode's T6 (Table 9.3), HTFM (Table 9.4), Ham's F10 (Table 9.5), modified Tyrode's T9 (Table 9.6) and PB1 (Table 9.7).

PREPARATION OF SERUM AND CULTURE MEDIUM

Human gamete preparation

Twenty millilitres of venous blood is taken from the patient 1 day before oocyte collection using 20 ml glass syringes and a 19-gauge needle (Terumo) attachment which have been put through an IVF wash. The blood is centrifuged (3500 rpm for 3 min) immediately after collection. The plasma is transferred into another tube and is allowed to stand for 1 h to clot at room temperature. The fibrin clot is removed

Table 9.1 List of chemicals used for preparation of culture media

Chemical	*Name*	*Cat. No.*	*Product*	*Conditions of storage*
$CaCl_2$	Calcium chloride dihydrate	4160	Mallinckrodt	Cool/dry shelf
NaCl	Sodium chloride	7581	Mallinckrodt	Cool/dry shelf
KCl	Potassium chloride	6858	Mallinckrodt	Cool/dry shelf
$MgCl_2 . 6H_2O$	Magnesium chloride	5958	Mallinckrodt	Cool/dry shelf
$MgSO_4 . 7H_2O$	Magnesium sulphate	6066	Mallinckrodt	Cool/dry shelf
$Na HCO_3$	Sodium bicarbonate	7412	Mallinckrodt	Cool/dry shelf
$Na_2 HPO_4$	Sodium phosphate diluxic anthydrous	7917	Mallinckrodt	Cool/dry shelf
$Na H_2 PO_4 . 2H_2O$	Sodium dihydrogen orthophosphate	—	Sigma	Cool/dry shelf
$KH_2 PO_4$	Potassium dihydrogen orthophosphate	—	Sigma	Cool/dry shelf
$C_6 H_{12} O_6$	Glucose	4912	Mallinckrodt	Cool/dry shelf
Na Lactate	DL–lactic acid	L 4263	Sigma	Fridge 0–5°C
Na Pyruvate	Sodium pyruvate	P 5280	Sigma	Desiccated 0–5°C
Na Hepes	Hepes sodium salt	H 7006	Sigma	Store with desiccant
Penicillin-G	Penicillin sodium salt	P 3032	Sigma or CSL	Fridge
Streptomycin	Streptomycin, sulphate	5711	Calbiochem or CSL	Fridge
Phenol red	Phenol sodium salt	P 5530	Sigma	Cool/dry shelf
BSA	Bovine serum albumin	A 9647	Sigma	Desiccated 0–5°C

Table 9.2 Tyrode's medium

Components	*mM*	*g/l*
NaCl	136.893	8.000
KCl	2.683	0.200
$CaCl_2 . 2H_2O$*	1.360	0.200
$MgCl_2 . 6H_2O$	0.492	0.100
$Na_2 H_2 PO_4 . 2H_2O$	0.360	0.064
Glucose	5.551	1.000
$Na HCO_3$	11.903	1.000
Phenol red		0.005

* Weigh $CaCl_2 . 2H_2O$ in a 10 ml Falcon tube and dissolve in 10 ml of H_2O volume, before adding to remaining dissolved components.
Osmolality = 280–290 mOsmol/kg.
Filter medium using 0.22 μ filter (Millipore, Sterivex) after equilibration with 90% N_2; 5% O_2; 5% CO_2. Media can be stored in 50 ml flasks (Nunc) at 4°C for up to 2 weeks.

Table 9.3 Whittingham's modified Tyrode solution (T6)

Components	*mM*	*g/l*
NaCl	97.34	5.6890
KCl	1.42	0.1060
$MgCl_2 . 6H_2O$	0.47	0.0960
$Na H_2 PO_4$	0.36	0.0497
Glucose	5.56	1.000
Na pyruvate	0.47	0.0540
Na Lactate	24.9	0.49 mls
$CaCl_2 . 2H_2O$*	1.78	0.1980
$Na HCO_3$†	25.0	1.9000
Penicillin		0.0600
Phenol red		0.005

* Weigh $CaCl_2 . 2H_2O$ in a 10 ml Falcon tube and dissolve in 10 ml of water.
† Weigh $Na HCO_3$ in a 10 ml Falcon tube and dissolve in 10 ml of water.
Weigh all the components and dissolve in remaining water before adding $CaCl_2$ and $Na HCO_3$.
Osmolality 280–290 mOsmol/kg.
Filter medium after equilibration with 90% N_2/5% O_2/5% CO_2.
pH = 7.3–7.4.
The filtration is done using a Millipore peristaltic pump through a 0.22 μ filter (Millipore, Sterivex) into 50 ml flasks (Nunc).

and the serum is centrifuged again (3500 rpm for 3 min) and is then heat-inactivated for 45 min in a water bath or stock heater set at 56°C. Once cool, serum is sterilized by filtering through a 0.22 μ filter (Sterivex, Millipore) into 5 ml Falcon tubes. Excess serum can be stored at 4°C for 24 h or at 200°C for up to 2 months.

For IVF-related procedures, the medium used for culturing oocytes and embryos and preparing spermatozoa is HTFM (Table 9.4). On the

Table 9.4 Human tubal fluid medium (HTFM)

Storage details	*Component*	*Amount*	*Volume*	*Strength*
A 3 months at 4°C	NaCl	5.931 g	all in 100 ml	101.5 mM
	KCl	0.350 g		4.69
	$MgSO_4 . 7H_2O$	0.050 g		0.20
	KH_2PO_4	0.050 g		0.37
	Na Lactate*	3.700 ml		21.4
	Glucose	0.500 g		2.78
	Penicillin	0.060 g		100 IU/ml
	Streptomycin	0.050 g		50 μg/ml
B 2 weeks at 4°C	$NaHCO_3$	1.050 g	50 ml	25 mM
	Phenol red	0.005 g		0.001%
C 2 weeks at 4°C	Na Pyruvate	0.051 g	10 ml	0.33 mM
D 3 months at 4°C	$CaCl_2 . 2H_2O$	0.262 g	10 ml	2.04 mM
E 3 months at 4°C	Hepes-Na salt	3.254 g	50 ml	†
	Phenol red	0.005		

* Added last in liquid form.
† Only added for follicle flushing medium (FFM). Adjust to pH 7.5 with NHCl before making up to final volume of 50 ml.

Final preparation of media from stocks (i.e. per 10 ml only)

	Culture medium		*FFM*	
A	1.0 ml		1.0 ml	
B	1.0		0.16	
C	0.071	In 10 ml	0.071	In 10 ml
D	0.115		0.115	
E	—		0.84	
H_2O	7.81		7.81	

Check osmolality and adjust to 280 mOsmol with water if osmolality is too high. Calculation for adjusting osmolality is as follows:

e.g. Osmolality of medium / Required osmolality = 296 / 284 — In 500 ml of medium

$$\frac{296}{284} \times 500 = 524.8$$

Therefore one needs to add 25 mls of H_2O to adjust osmolality to 284.

Gas HCO_3—HTFM with 5% CO_2; 5% O_2; 90% N_2. Filter sterilize with 0.22 μ filter (Millipore, Sterivex) into 50 ml flasks (Nunc). Media can now be stored at 4°C for up to 2 weeks. Re-gas HTFM and add serum when required.

day before oocyte recovery 30 ml of HTFM is gassed with 5% CO_2:5% O_2:90% N_2 for about 5 min and heat-inactivated; sterile maternal serum is added to give a concentration of either 10% (IVF/PROST) or 20% (GIFT). For AIH, the preferred medium for spermatozoal preparation is T6 (Table 9.3). Donor serum replaces maternal serum if circulating ASABs are detected in the woman (Yovich et al., 1984).

For IVF and PROST patients, 1 ml aliquots of the final medium are pipetted into six 5 ml Falcon tubes labelled 1 to 6 and all four wells

Table 9.5 Ham's F10 medium*

Inorganic salts			
$CaCl_2$	33.3 mg		
$CuSO_4$	0.0016 mg		
$FeSO_4$	0.456 mg		
KCl	285.0 mg		
KH_2PO_4	83.0 mg		
$MgSO_4$	74.6 mg		
NaCl	7400.0 mg		
Na_2HPO_3	156.2 mg		
$ZnSO_4 . 7H_2O$	0.0288 mg		
Other components			
Dextrose	1100.0 mg		
Hypoxanthine	4.08 mg		
Lipoic acid	0.206 mg		
Phenol red	1.24 mg		
Sodium pyruvate	110.0 mg		
Thymidine	0.727 mg		
Amino acids			
L-alanine	8.91 mg	L-leucine	13.12 mg
L-arginine HCl	210.70 mg	L-lycine HCl	29.30 mg
L-asparagine . H_2O	15.01 mg	L-methionine	4.48 mg
L-aspartic acid	13.31 mg	L-phenylalanine	4.96 mg
L-cysteine . HCl . H_2O	35.13 mg	L-proline	34.54 mg
L-glutamic acid	14.71 mg	L-serine	10.51 mg
L-glutamine	146.20 mg	L-threonine	11.91 mg
Glycine	7.51 mg	L-tryptophan	2.04 mg
L-histidine . HCl . H_2O	20.96 mg	L-tyrosine	5.44 mg
L-isoleucine	2.62 mg	L-valine	11.72 mg
Vitamins			
Biotin	0.024 mg	Nicotinamide	0.611 mg
D-Ca Pantothenate	0.715 mg	Pyrodoxine	0.206 mg
Choline chloride	0.698 mg	Riboflavin	0.376 mg
Folic acid	0.32 mg	Thiamine	1.012 mg
i-ionositol	0.541 mg	Vitamin B12	1.36 mg

* Supplied as powder by Flow Laboratories, catalogue no. 10-401.
The medium is made up by adding:
1 Penicillin G 0.075 g
Streptomycin sulphate 0.075 g
Ca Lactate (hydrate), ± Lactatic acid, calcium salt)
} freshly dissolved in 100 ml d-distilled deionized water
2 $NaHCO_3 . 2 . H_2O$ 106 g dissolved in 200 ml fresh d-distilled deionized water.
Solution is made up to 1 litre and osmolarity is adjusted to 280–290 mOsmol/kg.
Following equilibration in 90% N_2/5% O_2/5% CO_2, pH = 7.4–7.5.

of a Nunc 4-well multichamber culture dish.

For GIFT patients, two 4-well Nunc dishes with medium are prepared (labelled 'wash' and 'A' dishes) and in 'A' dish the fourth well is left empty for later addition of sperm overlay. The tubes with their caps placed loosely and the dishes are left overnight in the incubator

Table 9.6 Modified Tyrode's solution (T9)

Components	*mM*	*g/l*
NaCl	97.34	5.6890
KCl	4.78	0.3560
$CaCl_2 . 2H_2O$*	2.0	0.2940
$NaHCO_3$†	25.0	2.1000
$MgCl_2 . 6H_2O$	0.47	0.0960
Na_2HPO_4	0.34	0.0480
Na Pyruvate	0.5	0.0550
Na Lactate	17.84	2.0 ml
Glucose	5.55	1.000
Penicillin	100 μg/ml	0.0600
Hepes‡	20 mM	5.206
Phenol red	0.001% W/V	0.0050

* Weigh $CaCl_2 . 2H_2O$ in a 10 ml Falcon tube and dissolve in 10 ml H_2O before adding to other dissolved components.
† Weigh $NaHCO_3$ and dissolve in 2/3 volume of water; weigh remaining components and add to dissolved $NaHCO_3$.
‡ Weigh Hepes and dissolve in 20 ml water before adding to other components. Make up to required volume.
Osmolality 280–290 mOsmol/kg.
pH = 7.3–7.4.
Filter medium (as per 3.2.1) into 30 ml aliquots in Nunc tissue culture flasks.

Table 9.7 Composition of phosphate buffered medium (PB1)

Components	*mM*	*g/l*
NaCl	136.87	7.990
KCl	2.68	0.200
$CaCl_2 . 2H_2O$*	0.90	0.132
KH_2PO_4	1.47	0.200
$MgCl_2 . 6H_2O$	0.49	0.0996
Na_2HPO_4	8.09	1.150
Na Pyruvate	0.33	0.036
Glucose	5.56	1.000
Bovine serum albumin (BSA)†		3.000
Penicillin		0.060
Phenol red		0.005
Distilled H_2O		up to 1 litre

* Weigh $CaCl_2 . 2H_2O$ in a 10 ml Falcon tube and dissolve in 10 ml H_2O before adding to other dissolved components.
Osmolality 280–290 mOsmol/kg.
Filter medium with 0.2 μm Millipore Sterivex filter into 50 ml Nunc flasks.
† Weigh 3 mg/ml BSA, sprinkle on the medium surface and mix gently to dissolve. Refilter.
Media can be stored in 50 ml Nunc tissue culture flasks at 4°C for up to 2 weeks.

for equilibration.

Sperm preparation

It is vital to remove seminal plasma as completely as possible from an ejaculate to permit sperm capacitation in culture. This is achieved by a sperm washing procedure, where the semen is mixed with an equal volume of culture medium (T6 with 10% deactivated human serum) and centrifuged (e.g., 1500 rpm for 6 min) to separate the sperm pellet which consists of motile and dead sperm. The centrifuge rate must be calibrated to ensure that the sample is not subjected to forces greater than 200 g. After removal of the overlay, the sperm pellet is washed once again. To harvest a satisfactory motile sperm preparation with the minimum number of dead sperm for AIH and other procedures such as IVF-ET, GIFT, PROST or TEST, the following methods are used.

Overlay

The sperm pellet obtained following washing of semen (Fig. 9.3) is placed under a fresh 3 ml column of culture medium (e.g. for AIH, T6 with 10% human serum) in a 5 ml Falcon tube. An alternate method is gently to place medium over the sperm pellet. The tube, with the cap placed loosely, is incubated at 37°C in an incubator gassed with 5% CO_2 for a period depending on the quality of the original semen sample and the purpose for which the sperm is prepared, i.e. 10–15 min for normospermic, 1–2 h for oligospermic samples.

Sedimentation

The sedimentation procedure for obtaining sufficient numbers of motile sperm (Fig. 9.4) is used primarily for combined asthenospermic/oligospermic semen samples or frozen-thawed semen with increased numbers of dead sperm. In this procedure, the washed sperm pellet is placed in a 4-well Nunc dish and fresh culture medium is placed gently over it prior to incubation as above. A longer incubation period for severe oligospermic specimens allows better sedimentation and thus a suitable supernatant with motile sperm. Spermatozoa are then finally prepared in a volume of 0.5 ml medium containing from 0.5 up to 8×10^6 million motile spermatozoa. The total incubation and preparation period following liquefaction is usually around 90 min.

Recent trends

Centrifugation on Percoll separation gradients enables a visibly cleaner preparation of fewer spermatozoa, but there is no obvious effect on

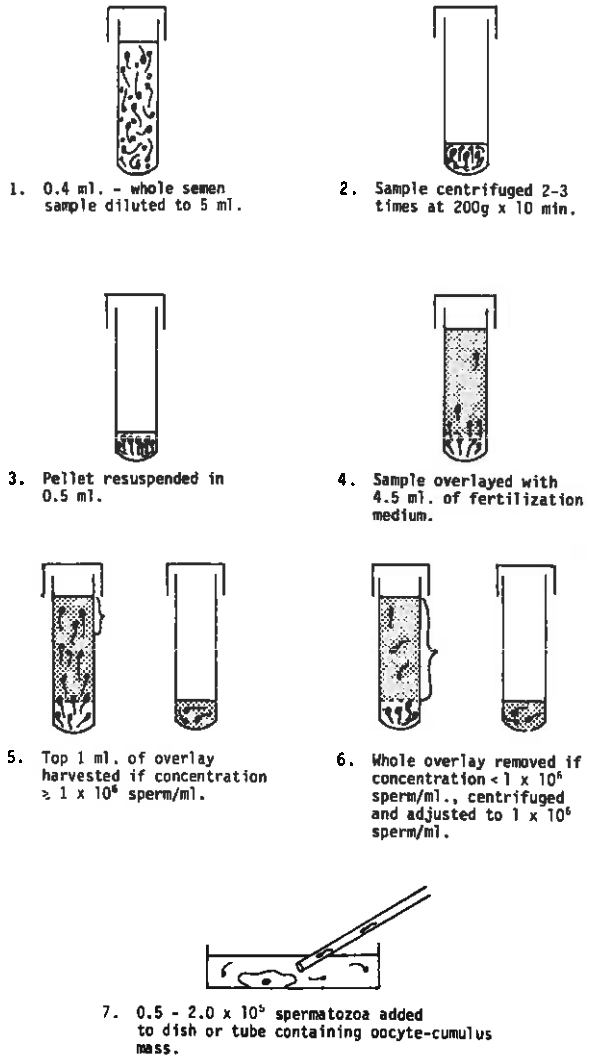

Fig. 9.3 The overlay technique of sperm preparation, including the modifications applied to oligospermic samples for use in IVF.

fertilization rates or embryo quality. For oligospermic samples, current trends indicate that centrifiguation may be detrimental and optimal

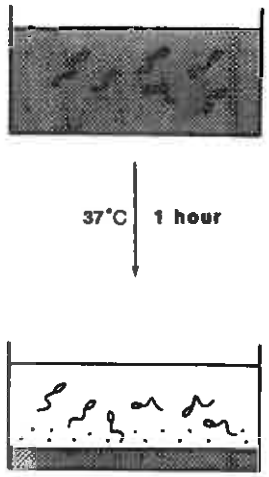

Fig. 9.4 The sedimentation technique of sperm preparation is used for severely oligospermic samples. The final preparation is not as free of seminal debris as the overlay technique.

preparations are achieved by simple sedimentation. Motility enhancement (Chapter 19) and culture in microvolumes in capillary tubes or straws (Vanderven et al., 1989) appear to be useful advances. Intravaginal culture (Ranoux et al., 1990) permits IVF as an adjunct in GIFT-alone units, particularly for transport of embryos to central IVF units for cryopreservation.

MICE

The mouse model is used for quality control assessment of techniques, equipment and culture media. The described system evaluates the rate of development of one-cell fertilized oocytes through to expanded and hatched blastocysts (should be ≥85%; plate 9.1). This is a minimal assessment system for evaluating human procedures as it is recognized that the mouse system will tolerate variations in pH, osmotic tension, temperature and water quality (George et al., 1989), more readily than the human. Improvements in quality control assessment are continuing to be evaluated, e.g. cell counts within mouse embryos, which reach defined stages, such as morula or blastocyst.

Animal husbandry

Mice for quality control and experimentation are F1 hybrids produced by mating inbred C57Bl/6J females (black) with CBA/CaH males (brown).

Some mouse lifespan statistics (which vary according to the strain) are given in Table 9.8.

Table 9.8 Mouse lifespan statistics

Life stage	*Time*
Oestrous cycle	5 days
Gestation period	18–21 days
Weaning	3 weeks old
Puberty	7–8 weeks old
Reproductive age	12–14 months
Fertilizable life of oocytes	10–15 h after ovulation

The mouse room is kept between 20°C and 25°C by a combination of a thermostatically controlled heater and an air conditioner. The lighting is controlled by a timer-switched light to provide 14 h of light and 10 h of darkness. These conditions optimize the oestrous cycles of females.

Hygiene of the colony cannot be over-emphasized and is therefore the most important consideration in all daily routines. Changing and sterilization of cages and bottles must be a regular routine.

The mice are housed in plastic cages of either 16 × 30 × 12 cm or 36 × 48 × 13 cm. The metal lids for these cages come with bottle and hopper mounts. Food and water are supplied ad libitum. Food is provided in pellet form (rat and mouse autoclavable pellets, Milne Feeds, WA). Water supplemented with vitamin (Vitastress brand made up at a rate of 2 scoops/20 l) is given to the mice in bottles fitted with sipper tubes. When necessary, mice are treated for worms and ectoparasites. This procedure involves thorough cleaning of cages with sodium hypochlorite (1%) and addition of Canex puppy suspension (4%) into the drinking water.

Grouping of mice

Breeding group

Two C57Bl/6J female mice are housed with one CBA/CaH male in one small cage to obtain C57Bl × CBA F1 hybrids. Sexing is done when litters are 1–2 weeks old. Females are used to provide oocytes for IVF studies at 4–5 weeks and at 5–8 weeks for quality control embryos.

Stud males

These mice are housed one per small cage. Either F1 hybrids or random-bred Swiss studs can be used: the latter combination will give a three-way cross embryo line. Males should be used up to 1 year of age, starting at 3 months.

Injectables

For superovulation of mice, pregnant mare's serum gonadotrophin (PMSG; Folligon Intervet) and hCG (Profasi) are used.

Injection regimen

For superovulation, 5–8-week-old female F1 hybrids are used. The first injection of 7.5 IU PMSG is given intraperitoneally in 0.1 ml of saline at 11.00 a.m. After 54 h (at 5.00 p.m.), the second injection of 10 IU hCG is given by the same route of administration. The females are placed with stud males (either one or two females per male) after the second injection, for mating. On the following morning the vaginae of the females are examined for the presence of vaginal plugs to confirm mating (day 1 of pregnancy). If stud males do not mate successfully 3–4 times in succession they should be culled.

Preparation of injections

1 PMSG—made up at 7.5 IU/0.1 ml and stored at 4°C in 1 ml plastic syringes (Terumo).
2 hCG—made up at 10 IU/0.1 ml and stored frozen in 1 ml plastic syringes (Terumo).

Collection of embryos

1 **One-cell.** F1 hybrid females (C57 × CBA) are used. The hybrids respond consistently to superovulation between the ages of 5 to 6 weeks and are mated with singly housed F1 males (3–12-months-old). The mice are killed by cervical dislocation on day 1 (between 10.00 a.m. and 1.00 p.m.) and one-cell embryos are obtained by tearing the swollen ampulla of the oviduct using a 30 gauge needle (Becton & Dickinson).

Culture

To disperse the cumulus mass, embryos are placed in hyaluronidase (0.1%). They are washed twice in PB1 prior to being placed in culture media (HTFM + 3 mg/ml BSA) in 4-well dishes (Nunc; cat. no. 64673)

and cultured under standard IVF conditions. Only morphologically normal embryos are selected, i.e. those which do not have shrunken or fragmented cytoplasm.

Assessment

On day 2 the embryos are observed for cleavage and the numbers growing to two-cell are recorded. Embryos are then cultured for a further 3 days when most of them should have reached the blastocyst stage. Less than 85% of embryos attaining blastocyst stage is considered unacceptable.

Specific media

Hyaluronidase—PB1 + 3 mg/ml BSA (Sigma) + 300 μ/ml hyaluronidase (Sigma) are mixed, filtered and frozen in 2 ml aliquots.

2 **Two-cells**. Using the same superovulation regimen, females are mated with individually housed males and checked for plugs on day 1. Mice are killed on day 2 by cervical dislocation and oviducts removed to collect two-cell embryos. To remove two-cell embryos the blunted tip of a 30 g needle (Becton & Dickinson) attached to a 1 ml glass syringe containing medium is inserted into the fimbrial end of the oviduct under a dissection microscope. The plunger is then gently depressed and embryos can be expelled. Culture and assessment are similar to one-cell embryos.

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Chapter 10

In vitro fertilization and embryo transfer (IVF-ET): current status (1988)

Summary: In vitro fertilization and embryo transfer (IVF-ET)

Indications	Infertility due to tubal disease.	
Ovarian stimulation	Clomiphene citrate/hMG	
	Lucrin/hMG	
	6–7 days of sustained E2 rise	
Oocyte recovery	36 h after hCG trigger 10000 IU or augmented LH surge	
	Transvaginal ultrasound-directed	
	PIVET-Cook Asp/Flush needle	
Laboratory procedures	Oocyte grade/4	
	Semen collected 4 h after	
	Insemination at 6–16 h	
	PN check, 12–18 h post-insemination	
	Culture: HTFM + 10% (Maternal Serum)	
Embryo transfer	40–48 h	
	2- to 8-cell embryos grade/4	
	Transfer up to 3 embryos	
	Transfer in HTFM + 50% MS	
	Double cannula transfer set	
	4 h head-down ± overnight stay	
Luteal phase	Proluton 50 mg intramuscular injection days 0–4 inc	
	hCG imi 1000 days 4, 7, 10 and 13	
Results	Pregnancy rates/transfer	20–35%
	Sacs/embryo transferred	5–13%
	Pregnancy wastage	30–35%
	Multiple pregnancies	10–20%

The birth of Louise Brown in the UK in July 1978 saw the end of an era of mounting scientific and public scepticism regarding the possibility of achieving successful pregnancies following IVF-ET (Edwards and Steptoe, 1980; Fishel, 1986). Over the ensuing 5 years (1978–1983)

a handful of centres around the world began to report their first IVF successes (Lopata et al., 1980; Testart et al., 1980; Craft et al., 1982; Jones et al., 1982; Yovich et al., 1982; Wood et al., 1982). By the time of the Third World Congress on IVF-ET held in Helsinki in April 1984, 65 teams of clinicians and scientists were pursuing IVF-ET around the world, having achieved the birth of 600 infants from 523 IVF pregnancies. The earliest cases were women with occluded or absent fallopian tubes and, at the time of the Helsinki Congress, 78% of IVF procedures were performed for tubal disease. At that time, the overall success rate was 11% of all cycles—'success' being a clinical pregnancy—and 14% of replacement cycles. The multiple pregnancy rate was 12%. At that meeting, the term 'biochemical' pregnancies was introduced and it was noted that approximately 2% of reported pregnancies were ectopic. However, there appeared to be no increased risk of fetal abnormalities over that expected after natural conception (Seppala et al., 1985).

In the next five years (1983–1988) the main developments in IVF-ET included:

1 Exploring the use of the technique in a wide range of infertility cases not due to tubal disease.
2 Recovering oocytes by ultrasound-directed techniques.
3 Improving ovarian stimulation methods to increase the number of mature oocytes.

EXTENDING THE INDICATIONS

Following the early successes in women with tubal infertility, some workers began to see the useful role of IVF in the diagnosis of unexplained infertility (Trounson et al., 1980), with the benefit of sometimes achieving pregnancy in the process. Its use for infertility due to antispermatozoal antibodies was reported for the female in 1984 (Yovich et al., 1984b) and the male in 1985 (Clarke et al., 1985). The first successful use of IVF for male infertility was also reported in 1984 (Yovich and Stanger, 1984; Yovich et al., 1985a). There were conflicting reports on the usefulness of IVF-ET for infertility due to poor sperm–mucus interaction, with a group from Bristol suggesting that negative PCTs were a poor prognostic feature (Hull et al., 1984), although in a subsequent report (Matson et al., 1986) the results of IVF-ET for this group were shown not to be significantly different from those with tubal infertility. Finally, IVF-ET was applied in cases of endometriosis; there appears to be a reduced chance of success for severe grades of untreated endometriosis managed by IVF-ET (Wardle et al., 1985; Matson and Yovich, 1986).

ULTRASOUND-DIRECTED FOLLICLE RECOVERY

Follicle aspiration and oocyte recovery commenced as a laparoscopic procedure. The first reports using ultrasound guidance were from Scandinavia, describing the transcutaneous transvesical method (Lenz et al., 1981; Wikland et al., 1983). Subsequently, the transurethral approach (Parsons et al., 1985) and finally the transvaginal ultrasound-directed follicle aspiration has proven to be the most effective and is now in popular use, largely superseding the other methods (Delenbach et al., 1984; Blackledge et al., 1986; Torode et al., 1986).

OVARIAN STIMULATION

During the developmental years, Steptoe and Edwards (1976) described the use of hMG stimulation but they attributed their poor results to a high oestrogen output causing a shortened luteal phase in those cycles. Subsequently they achieved their first successful pregnancies by monitoring the natural cycle and recovering the single oocyte from the dominant follicle in those cycles. The early reports with stimulated cycles used clomiphene citrate. Subsequently hMG was introduced in combination with clomiphene as a routine in European and Australian centres. In the USA, hMG was the preferred stimulation method and this was combined with the routine use of progesterone supplements to ensure an adequate luteal phase. Recently, pure FSH and GnRH analogues have been added to the armamentarium for general use, or in specific clinical situations.

CURRENT STATUS (1988)

Following the introduction of GIFT for infertility cases where at least one fallopian tube is accessible, further tubal transfer procedures have been introduced. They show a significant improvement in the chance of pregnancy per procedure over IVF-ET, which can now be reserved essentially for the primary indication of tubal infertility.

The term 'IVF' is widely and colloquially used to embrace all those procedures involving the handling of both gametes in vitro, regardless of whether fertilization is performed in vitro or in vivo. In that sense, GIFT, PROST, IVF-ET, TEST and similar procedures are all known as IVF-related forms of assisted reproduction.

For IVF-ET, the trend is to collect oocytes using transvaginal ultrasound-directed methods, hence for oocyte recovery and embryo transfer general anaesthesia is not essential, and patients undergo either short-stay day-care procedures or outpatient procedures.

IVF-ET PROCEDURE

Indications: tubal infertility

The indications for IVF-ET include women who on previous surgery, have had their fallopian tubes removed, whether because of ectopic pregnancy, severe pelvic inflammatory disease or other reasons, as well as those women whose degree of fallopian tube damage is too extensive to hope to achieve satisfactory results by reconstructive microsurgery. In general, this situation prevails if there is proximal cornuo/isthmic and distal occlusions (e.g. combined salpingitis isthmica nodosum and hydrosalpinges, or tuberculous salpingitis). For those patients with distal fallopian tube disease only (such as hydrosalpinges or fimbrial agglutination), preliminary reconstructive surgery should be considered. Even though the chance of spontaneous conception may be of the order of only around 20%, such cases may subsequently prove to be suitable for GIFT or PROST. However, there are limitations emerging to this approach (e.g. increased ectopic pregnancy; see Chapters 11 and 12). One must therefore balance the benefits of tubal transfer against the risks. Increasingly, IVF-ET is considered the favoured option for all forms of tubal infertility (Yovich and Matson, 1990).

Preparation

Prior to any IVF-related procedure, active pelvic conditions must be excluded or identified and treated so that the pelvis is in a 'quiescent' state. There should be no active inflammatory process, ovarian cysts should be treated definitively, and cases of pelvic endometriosis should have been brought under control as much as possible. In many situations, the control of pelvic disease may include preliminary pelvic adhesiolysis and definitive surgery. However, a previous practice of ventrosuspension and ovarian suspension in order to improve laparoscopic access of ovaries, should be abandoned. The optimal situation for transvaginal ultrasound-directed aspiration is a retroverted uterus with the ovaries residing in the Pouch of Douglas (POD). Ideally, the pelvic structures will be mobile and not densely bound down by adhesions in order to improve the prognosis for subsequent pregnancies.

The first IVF-ET implantation was an ectopic pregnancy (Steptoe and Edwards, 1976) and worldwide the documented ectopic rate from IVF-ET is approximately 5%. This may be reduced by preliminary complete salpingectomies (Steptoe and Edwards, 1979) or clip application to the cornual region of the fallopian tubes (Craft et al., 1978). Whereas these procedures were previously considered in the preliminary preparation of IVF-ET cases, they are not now recommended as they may preclude patients from alternative treatment options in the future

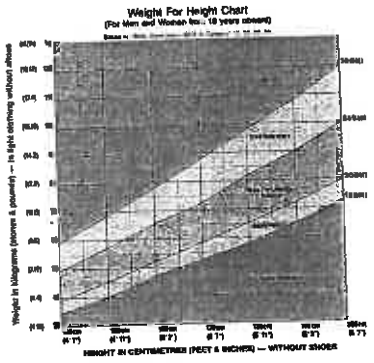


Plate 4.1 Body mass index: weight (kg)/height $(m)^2$. The healthy range (BMI 20–25) equates with optimum ovulation parameters.

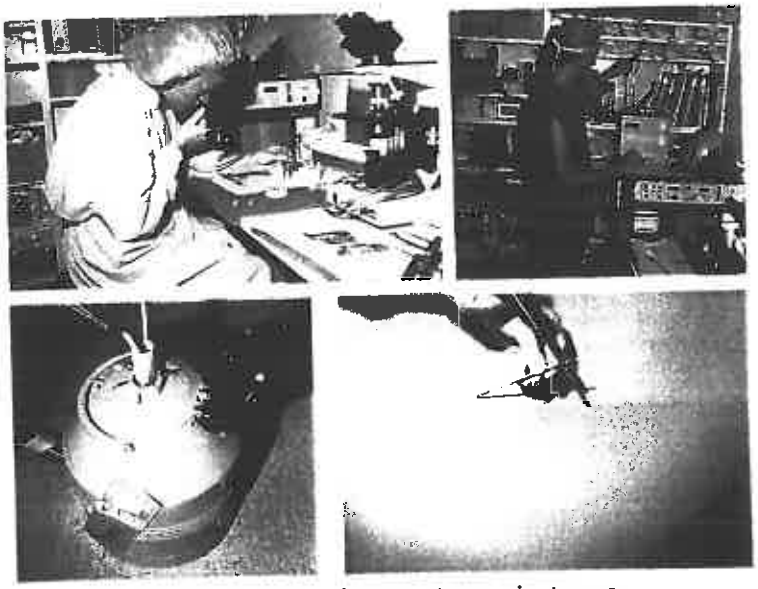
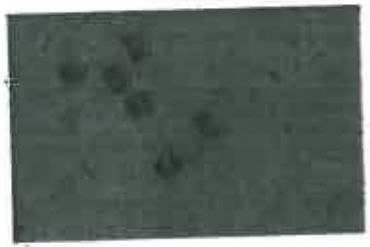


Plate 6.1 (a) The filling of semen straws prior to cryopreservation. (b) The rack of straws is inserted into the freezing chamber of the Planer programmable freezer. (c) The semen straws are withdrawn from the storage dewar containing the liquid nitrogen. (d) The cement-tipped end of the semen straw is removed with scissors after it has thawed on the bench.

Plate 6.2 The semen straw is inserted into the tip of the insemination gun.

Plate 7.1 The polyethylene AIH catheter is marked at 4 cm from the tip indicating the length to be inserted into the cervical canal.

Plate 8.1 Metaphase II stage of meiotic division of the oocyte with spermatozoa adherent to the zona pellucida.



a)

b)

Plate 9.1 In vivo fertilized mouse oocytes collected at the one-cell stage and developing through (a) the two-cell stage to (b) expanded and hatching blastocysts.

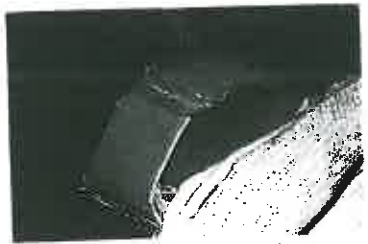


Plate 10.1 Preparation of patient for transvaginal ultrasound-guided aspiration using a tight abdominopelvic band to stabilize the ovaries.

Plate 10.2 Transvaginal ultrasound-guided oocyte recovery in progress using PIVET-COOK needle guided by General Electric 5.0 MHz vaginal probe.

a)

b)

c)

Plate 10.3 (a) Oocytes are recovered from follicular aspirates, washed and graded — (b) 2 and (c) 3 from a maximum of 4 points.

Plate 10.4 The coronal coat which remains after cumulus dispersal by spermatozoa (1 h in this figure) and requires careful dissection to reveal the pronuclear oocyte at 12–18 h post-insemination.

Plate 10.5 Oocytes are incubated with spermatozoa in tubes or multi-well dishes within a gassed, humidified desiccator in a water jacketed CO_2 incubator at 37°C.

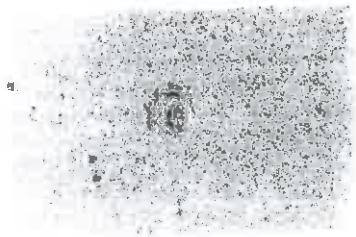


Plate 10.6 Pronuclear stage oocyte 14 h post-insemination after partial dissection of the coronal coat.

Plate 10.7 Pronuclear stage oocyte viewed by interference contrast microscopy.

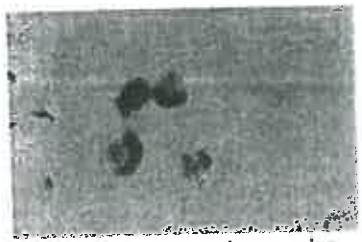
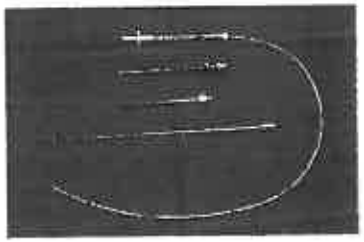


Plate 10.8 Three embryos prior to transfer at 44 h, with subsequent twin delivery.

Plate 11.1 Laparoscopic oocyte aspiration with patient in the combined lithotomy/ Trendelenburg position.

Plate 11.2 Laparoscopic oocyte aspiration with patient in mild Trendelenburg position: follicle flushing via the PIVET AN1 needle.



a)

b)

Plate 11.3 (a) Items of equipment for tubal cannulation and (b) method of use. The double mark is 4 cm from the distal end of the Teflon cannula and is the ideal position at the fimbrial orifice prior to gamete transfer.

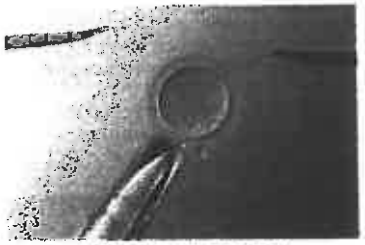


Plate 12.1 Oocyte held by suction prior to zona splitting.

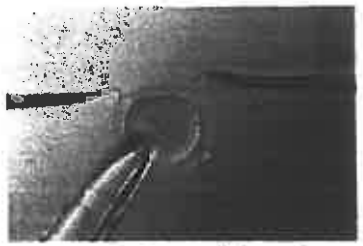


Plate 12.2 Zona splitting using fine glass hooks prepared on the microforge.

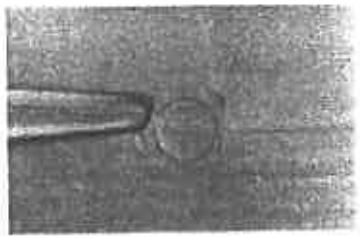


Plate 12.3 Oocyte held by suction prior to placement of acid Tyrode's medium (pH 2.0) using finely drawn micropipette.

and the risk of ectopic pregnancies may be reduced by modifying the transfer technique (Yovich et al., 1985b).

Ovarian stimulation

This has been fully described in Chapter 4 and the mode selected will depend upon the following factors:

1 Whether the woman has a normal or disordered ovulation profile demonstrated by a preliminary observation cycle and previous experience. Correction of weight factors (either too light or too heavy) and hyperprolactinaemia are preliminary requirements. Some cases may have already been shown to be unresponsive to clomiphene and require high-dose hMG for stimulation.
2 Age of the female partner: the prognosis for older women has improved significantly by using a combination of GnRH analogue for down-regulation, with superimposed hMG stimulation. At PIVET, leuprolide/hMG (as described in Chapter 4) is the preferred mode for women of 35 years or more.

   Young women (under 25 years) may be very sensitive to ovarian stimulation and may well be considered for clomiphene citrate only in the first instance, or clomiphene citrate combined with low-dose hMG. This consideration should also be given to those women who have previously demonstrated OHSS.
3 Clomiphene citrate should be avoided in those women who have demonstrated raised androgens, raised basal LH levels, or adverse reactions to the drug.
4 Severe oligospermia: where one is expecting a reduced fertilization rate, the stimulation schedule should be aimed at delivering a minimum total of six oocytes.
5 Following previous experience, if oocyte recovery has been frustrated by raised basal LH, premature LH surges or premature luteinization (creeping P4 elevation during the follicular phase), a combination of leuprolide/hMG is preferred.
6 With known cases of PCO disease or where multicystic ovaries have been demonstrated by ultrasound performed in the early follicular phase, adjustments to the stimulation schedule will need consideration—for example, some cases will be very sensitive to ovarian stimulation while others will be frustrated by raised basal LH and premature luteinization. The use of leuprolide, and in some of these cases, replacement of hMG by pure FSH (Metrodin; Serono, Italy), should be considered.

The majority of cases are women with normal cycles, aged between 30 and 35 years, and the preferred treatment has been clomiphene

citrate 50 mg b.d. on days 2 to 6, combined with hMG beginning with two ampoules given daily beginning on day 4. The serum hormonal effects should be monitored from day 6. The hMG dose may be changed after every third day to generate 6 days of sustained E2 rise. As this book goes to press, a number of clinics have introduced a routine regimen employing a GnRH agonist, mainly for the benefit of eliminating spontaneous LH surges. The advantage of this approach has to be considered against the increased costs and rates of OHSS (see Chapter 19).

LH surge: preovulatory hCG administration

The stimulation schedule should be geared to maximizing the number of collections performed following an hCG trigger, and minimizing those collected on an LH surge. To achieve this, follicular phase monitoring is performed on a daily basis from day 6 and the stimulation schedule is adjusted to create a sustained rise in E2. Generally, the hCG injection (10 000 IU) is given on the evening of the sixth day of E2 rise. This may be projected forward to day 7 if pelvic ultrasound indicates that there are no ovarian follicles $\geq$1.5 cm or the regimen includes leuprolide which prevents a spontaneous LH surge. Optimum pregnancy rates occur following an hCG trigger on day 6 (see Fig. 4.3) for clomiphene citrate/hMG or day 7 for Lucrin/hMG (see Fig. 4.4). If an LH surge is identified in the morning blood sample of day 6 and there has been a satisfactory E2 rise during the follicular phase, the surge may be augmented and the oocytes recovered that evening if P4 is $\geq$10 nmol/l, the following morning if P4 is $\geq$5 nmol/l and the following evening if P4 is <2.5 nmol/l.

The dose schedule of 10 000 IU of hCG in a single dose was selected as it was found that a previous schedule of 5000 IU is occasionally not associated with a satisfactory luteinization effect. A two-stage injection (Kerin, 1983) was not found to confer any benefits.

Oocyte recovery

We have previously described the optimization of oocyte recovery from ovarian follicles aspirated at laparoscopy (Yovich et al., 1989). However, oocyte recovery for IVF-ET is now performed exclusively by transvaginal ultrasound-directed techniques. Since 1984, ultrasound-directed methods were initially explored in women with difficult or impossible access to ovaries at laparoscopy. Initially a percutaneous transvesical method was used (Lenz et al., 1981), which was occasionally simply percutaneous transabdominal as the ovary was sometimes tethered higher than the bladder could reach. Subsequently, the transurethral approach was briefly evaluated (Parsons et al., 1985) before

the widespread acceptance of the transvaginal technique, largely due to improvements in image technique.

One of the authors' (JLY) initial experience was with a 5 MHz general purpose transducer on an Aloka 280S mechanical sector scanner. A guide was made to fit over the transducer to introduce a 16-gauge spinal needle into the side of the beam at an angle of 5° to the long axis of the transducer. The electronic calipers which are part of the biopsy software were placed across the screen in the path to be taken by the needle. Orientation was improved by top-to-bottom image inversion. The early experience led to adequate results (71% recovery rate from follicles measuring $\geq$1.5 cm and recovery rate of 37% from follicles <1.5 cm; Blackledge et al., 1986) but the following limitations were noted:

1 Very mobile ovaries could be difficult to penetrate.
2 Repeat ovarian puncture caused blunting of the needle tips which made subsequent ovarian punctures difficult.
3 Occasionally the uterus or a loop of bowel was interposed between the vaginal vault and the ovary, especially when the ovary had previously been secured to the round ligament at a previous operation to facilitate laparoscopic oocyte pick-up.
4 The flushing technique through a single-bore spinal needle was cumbersome and involved moving dead space back and forth through the needle, undoubtedly limiting oocyte recovery in some cases.

However, the following features were impressive: the resolution of images within the follicular cavity, the lack of postoperative pain experienced by patients, the minimal need for anaesthesia, no requirement for a full bladder, and the improved recovery rates of oocytes when compared with other ultrasound-guided methods. Whilst the technique was greeted as an improvement by patient and operator alike, there was concern about lowered fertilization rates until the use of vaginal antiseptics was abandoned in favour of washing the vagina with culture medium. These developments have led to similar rates of fertilization and pregnancy from both laparoscopic and transvaginal ultrasound collection of oocytes.

Transvaginal ultrasound-directed aspiration

The transvaginal oocyte recovery technique can be improved by the following:

1 A pressure cuff similar to that used for intravenous pyelogram examinations placed on the lower abdomen (Plate 10.1) has the important

effect of stabilizing the ovaries, to prevent the problem of ovaries slipping away during penetration of the ovary.

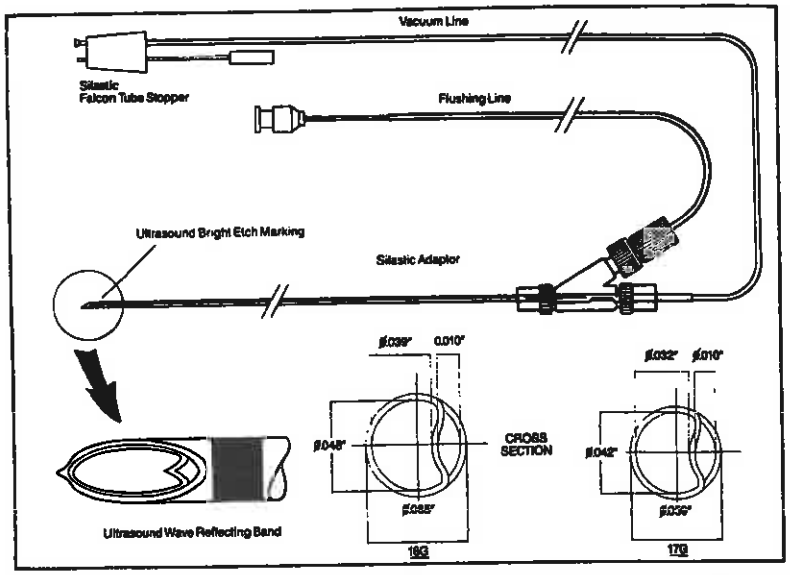


Fig. 10.1 Schematic version of PIVET-COOK double-lumen aspiration/flushing needle for oocyte recovery.

2 The use of a double-lumen needle (PIVET-Cook) permits the application of vacuum and flushing simultaneously or independently without any dead space in the cannula or vacuum system during the procedure. The double cannulation system consists of a 16-gauge needle with a 23-gauge needle sealed to one aspect of the wall within it (Plate 10.1 and Fig. 10.1). The tip of the needle must be prepared for sharpness and the length of the needle should be selected according to the vaginal probe being used. Ideally, an ovary is entered using the following procedure:
   a Aspiration of clear follicle fluid—L1 (left) or R1 (right ovary).
   b From a 10 ml syringe filled with flushing culture medium, approximately 6 ml is flushed into the follicle, which is allowed partially to fill, and then the foot pedal is depressed so as to effect a continuous flush—aspiration—L1F1 (first flush from first follicle on the left side);

   c The remaining fluid in the flushing syringe is emptied into the follicle which is flushed and aspirated as above, providing the embryologist with a third specimen—L1F2.

   If there are a small number of follicles (four or less), it is wise to wait for an indication from the embryologist that the oocyte has been identified and recovered, before proceeding to the next follicle. Occasionally further flushes may be required. However, if there is a large number of follicles, there is no value in prolonging the procedure for an occasional missed oocyte and the operator should proceed; e.g. to L2 immediately after L1F2.
   d The needle is moved from one follicle to the other, repeating the standard procedure until all follicles have been aspirated. It has been our policy to aspirate all follicles ≥ 1.0 cm. If larger numbers of oocytes are required (e.g. for oligospermia, cryopreservation, donation to research or donation to another infertile couple), all follicles ≥1 cm may contribute oocytes with an equivalent likelihood of fertilization and generation of pregnancies. The recovery rate of oocytes from follicles of varying sizes, reaching almost 100% for follicles 1.5–2.4 cm in diameter, is shown in Figure 10.2.

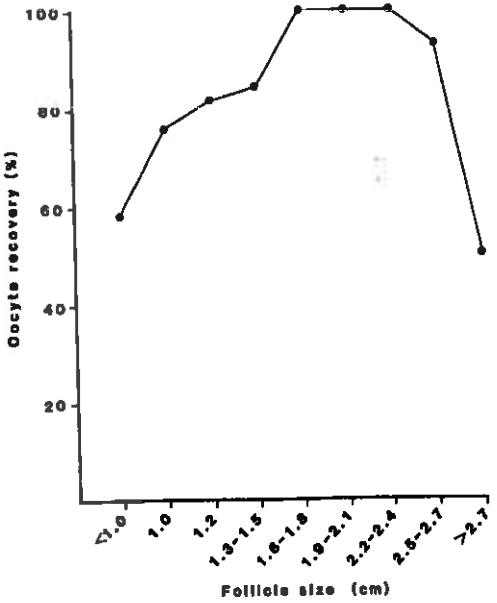


Fig. 10.2 Oocyte recovery rates from follicles of various diameters measured at the time of transvaginal ultrasound-directed collections using a routine three-tube technique comprising one clear aspirate and two flush aspirates.

e On completion of follicle aspiration from both ovaries, any fluid collected in the POD should be aspirated and examined by the embryologist.

f Ideally, the PIVET-Cook needle should be discarded after each use as some blunting of the needle tip usually occurs. In practice we have found it is generally suitable for three to five vaginal aspirations and can subsequently be used for laparoscopic collections where the requirement for sharpness is not as crucial, given the two-handed approach at laparoscopy, permitting the ovary to be held and penetrated at right angles to the surface of the follicle.

3 The aspiration pressure required for effective egg retrieval is governed by Poiseuille's law which relates to the flow of fluids through pipes:

$$\frac{V}{t} = \frac{r^4(P_1 - P_2)}{8\eta l}$$

where the rate of flow (V/t: volume of fluid flowing past a given point per unit time) is directly related to the fourth power of the radius (r) and the pressure difference between the ends ($P_1 - P_2$), and is inversely related to eight times the length (l) of the needle and viscosity (η) of the fluid. In effect, we have found that the 25 cm PIVET-Cook needle has an optimal aspirating pressure around 100 mmHg; at 30 cm it requires 140–150 mmHg and at 35 cm it requires 180–200 mmHg. The higher pressures are not transmitted to the follicle or the oocyte as the increased needle length means that the flow rate and forces applied will remain the same.

4 The General Electric RT 3600 electronic phased array sector scanner with a 5.0 MHz vaginal probe has been used by one of the authors (Plate 10.2). This instrument has certain advantages, particularly in being able to manipulate ovaries away from positions behind the uterus and yet allowing a nice snub-nosed fit up against the ovary so that the ovary does not slip off the tip. In comparison, whilst exploring the use of an alternative probe, the additional improvement in image resolution was negated by difficulties in holding the ovary in position and maintaining the image.

5 Currently, transvaginal ultrasound-guided egg recoveries are performed in the operating theatre on a day-care basis (4 h stay). Light intravenous anaesthesia is used (e.g. Diprivan), creating a neuroleptic tranquillizing effect. This can be used in combination with a light (mask/airway) general anaesthetic if desired. In certain patients the procedure can be performed under a simple pethidine/atropine/promethazine hydrochloride premedication. Discomfort during the procedure is minimal, and patients have not complained

of any significant post-collection pain, vaginal bleeding or evidence of inadvertent intrapelvic trauma during a series of more than 2000 aspirations. However, there have been two pelvic infections requiring antibiotic therapy.

During the early phase of introducing transvaginal ultrasound-guided collections, routinely laparoscopies were performed to examine the pelvis and to determine if further oocytes could be recovered. This has proven to be an unnecessary exercise and is not advised as a routine approach.

In those patients proceeding from transvaginal collections to PROST or TEST, blood-stained fluid in the pelvic cavity is drained prior to tubal transfer and rarely exceeds 80 ml. This indicates a minimal degree of fluid and blood loss from ovaries and puncture sites within the pelvic cavity following initial collection.

As a precaution against causing a possible pelvic inflammatory process, all patients undergoing IVF-related procedures have a high vaginal and endocervical swab performed on day 5 of the treatment cycle. The occasional detection of *Chlamydia* antigen or non-gonococcal bacterial infections can be treated with antibiotics; this allows oocyte recovery to proceed if there is clinical control of the infection. Again, vaginal preparation is simply the use of a vaginal douche with a small amount of culture medium. Recently the routine use of a single dose of antibiotic (cefoxitin IG) given intravenously has been introduced.

EMBRYOLOGY PROCEDURES

Laboratory layout

There should be a dedicated human IVF laboratory adjacent to the operating theatre so that the embryologists and clinicians can work in close harmony. In particular, the communication between the two areas must be direct and effective. Ideally, an open hatchway between theatre and the laboratory should be operational during procedures so that the surgeon and embryologist can speak directly with each other. If such a direct link is not possible, an open microphone or speaker system should exist between the two areas to assist communication. A video unit operating between the areas is beneficial to the patient as well as the clinical team.

If the laboratory area is some distance from the operating theatre, a working trolley is required within the theatre. This can be very efficient if there is adequate space within the operating theatre.

The dedicated IVF laboratory should be separate from the main infertility laboratory and there should be restricted access. It is best contained within the operating suite, ensuring that aseptic techniques

are applied as for operative procedures. A separate semen collection room adjacent to the theatre laboratory is also ideal. All gamete handling and standard embryo culture procedures for PROST, IVF-ET and TEST are carried out within the dedicated IVF laboratory. However, it does not need to be a large area as the preparation of culture media, cryopreservation procedures, and cleaning and sterilization of equipment are performed elsewhere as a function of the main infertility laboratory (Fig. 10.3).

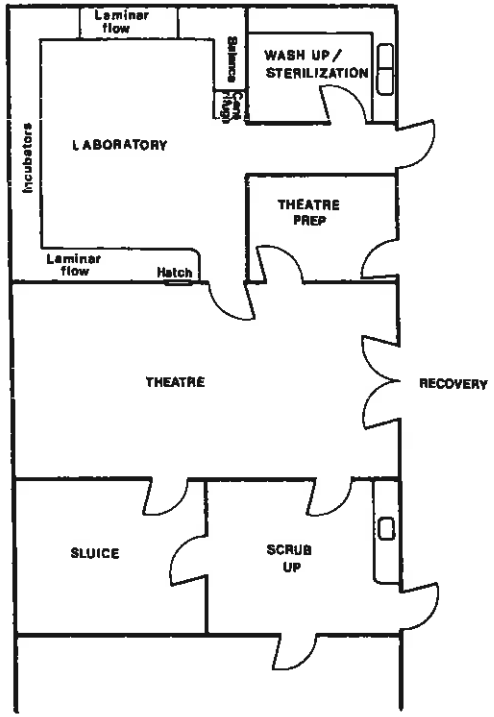


Fig. 10.3 Diagram showing PIVET IVF laboratory.

Within the theatre, follicle flushing medium (FFM) is kept at 37°C within a water bath (preferred to heat block) and the 10 ml syringes filled with FFM are held in a pre-warmed area on the theatre procedures trolley.

Similarly, aspirate tubes are rapidly labelled (e.g. R3F2, denoting the second flush from the third follicle aspirated in the right ovary)

and are transferred immediately to the embryology area where they are placed in a water bath at 37°C, awaiting examination by the embryologist.

Oocyte identification

The follicle contents are emptied into sterile medium-sized petri dishes (15 × 60 cm, Nunc) and scanned using a dissecting microscope with a magnification of × 40. In fact, the cumulus–oocyte mass can just be detected by the naked eye as a white gelatinous-clear mass. Under the microscope, oocytes are graded according to the nature of the cumulus–corona–oocyte complex (Plates 10.3 and 10.4; Table 10.1).

Table 10.1 Oocyte grading protocol

Cumulus	*Corona*	*Grade*
Loose, large mass	loose	4
Loose, large mass	tight	3
Slightly tight, small	tight	2
Very few cells	tight	1

Once the oocytes have been identified and graded they are washed, firstly in FFM (HTFM with hepes buffer) and then in IVF culture medium (HTFM with 15% maternal serum). After washing, the oocytes are placed in 5 ml Falcon tubes and incubated at 37°C in a humidified atmosphere of 5% O_2:5% CO_2:90% N_2 (Fig. 10.4; both Forma Scientific

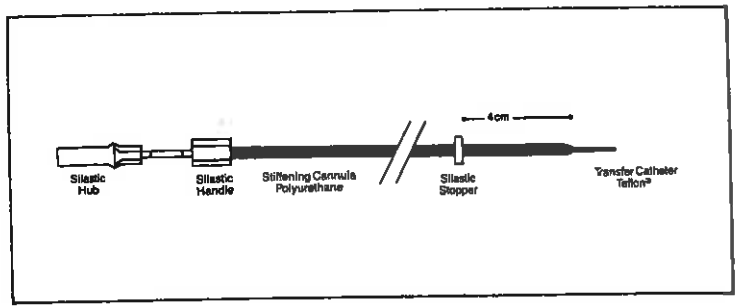


Fig. 10.4 PIVET double-cannula embryo transfer set.

and Heraeus incubators have proven satisfactory, with the latter preferred).

After 4–6 h sperm is prepared by either the overlay or sedimentation method and adjusted to give a sperm count of 1.0×10^6 ml; 0.1 ml of the dilution is added to each tube to achieve a final concentration of 50 000–100 000 sperm/ml.

Following a further 14–18 h of incubation the oocytes are examined for fertilization. Some oocytes regarded as immature (small oocyte with tight corona and dense, tight cumulus mass) may be left for periods of up to 16 h prior to insemination. Our own observations support the data of Harrison et al. (1988) which indicate that the optimum time for insemination can be from 4–16 h following oocyte recovery.

Identification of pronuclei

The oocytes (at this stage free of cumulus) are transferred from the tubes to small petri dishes (10 × 35 mm; Nunc) or a Nunc 4-well dish after incubation for approximately 14–16 h following insemination. This procedure involves identifying the oocyte at the bottom of the tube under the microscope and then using a short undrawn Pasteur pipette to aspirate the oocyte and draw up the rest of the culture media behind it. The oocyte and medium are placed in the 4-well dish. The surrounding coronal cells are then separated, using a freshly drawn Pasteur pipette with a bore size only slightly larger than the oocyte (around 120 μ). A fine 30-gauge needle may also be used to assist in peeling off the coronal cells.

The oocyte is then examined under the highest magnification of the dissecting microscope (×50) for the presence of a swollen, decondensed sperm head or two pronuclei (Plates 10.5 and 10.6). Oocytes with more than two pronuclei or with no pronuclei are separated and placed in different wells of a 4-well Nunc dish with medium which has been equilibrated over the previous night. If the unfertilized oocytes are of good quality, reinsemination with either freshly prepared sperm or sperm prepared 1 day earlier but still highly motile, is performed. All oocytes are placed back in tubes containing fresh HTFM + 10% maternal serum and cultured through to the following day. Multi-pronuclear oocytes and unfertilized oocytes are also cultured through but identified separately.

Embryo transfer

This is carried out approximately 24 h after the pronuclear check. Prior to transfer the embryos are examined under the highest magnification of the dissecting microscope and graded over a four-point scale, taking into consideration the factors shown in Table 10.2.

A maximum of three embryos with a score ≥3 may be transferred. For IVF-ET, embryos are transferred in HTFM + 50% maternal serum.

Table 10.2 Grading of embryos

Factor	*Scale*
No fragments	1
Small fragments	½
Many fragments	0
No granularity (lucid appearance)	1
Slight granularity	½
Highly granular (brown cytoplasm)	0
Discrete, even-sized blastomeres	1
Irregular blastomeres	½
Highly irregular blastomeres	0
Rate of development (4-cell at 44 h)	1
< 4-cell or ≥ 4-cell	½

They are loaded into the embryo transfer catheter (William A. Cook Australia; cat. no. MWO 11821) which is made from Teflon tubing and a metal luerlock attachment (Plate 10.7). Three markings 1 cm apart on the transfer catheter allow the clinician to gauge distances in relation to the outer catheter and thereby how far the inner catheter is within the uterine cavity in relation to the external cervical os.

ET catheters are packed in glass rods and sterilized with low heat in a dry oven. Initially, the outer catheter used was a Vialon material made by Deseret (cat. no. 38-3112-1); it is prepacked and sterilized by gamma radiation. A complete embryo transfer set (with the outer catheter made of polyurethane) prepared by Cook Australia is currently under evaluation (Fig. 10.4); it appears to be an improved system which is particularly useful for the 'difficult' cervix and acutely anteverted uterus.

Embryo transfer procedure

The patient is placed in the lithotomy position and a bivalve speculum illuminated from a cold light source is inserted into the vagina to expose the cervix. Mucus is gently wiped from the cervix with a dry swab and the external os is moistened with a small dab soaked in culture medium. The Vialon catheter is gently and slowly manipulated through the cervical canal with a long George forceps with a right-angled tip until the silicon rubber marker abuts against the external cervical os at 4 cm exactly. Once the external catheter is in position, the Teflon tube is filled with culture medium from the tuberculin syringe and the embryos are moved from the culture site in the incubator across to the stereomicroscope. A small air space is created at the end of the Teflon catheter, and up to three embryos are loaded into a 10–20 μl segment of culture medium. Again a small air space is created at the tip so that the transfer segment is discretely placed

in the final 3 cm of the transfer catheter. This is then inserted 1.5–2 cm from the tip of the outer catheter, and by this means the embryos are always transferred at a set distance of 55–60 mm from the external cervical os. Once the inner catheter is positioned, the outer catheter is withdrawn back to the luer hub and, after a settling period of 60 seconds, the embryos are transferred out of the inner catheter by the injection of 50 μl of culture medium. The catheter is then immediately slowly withdrawn. Typically the patient is advised to remain resting for up to 6 h or even 24 h with 10° head-down tilt of the bed.

The use of general anaesthesia and ultrasound control should be considered for women who have had difficult transfers on previous occasions. However, the routine use of these additional procedures has not proven to have significant benefits.

The protocol of transferring embryos on day 2 (44–48 h) is considered to be a compromise between the ideal (day 4 or 5) and the limitation of culturing embryos in vitro. Even at this stage, studies indicate that less than 30% of embryos will progress through to the blastocyst stage in continuous culture (Whittingham and Penketh, 1987). However, with current knowledge that embryos transferred to the fallopian tubes (see Chapter 13) have a significantly improved chance of pregnancy, it appears that the uterine environment is unfavourable for embryos in the immediate postovulatory days. For this reason, it is worthwhile continuing studies into the essential requirements for embryo culture in vitro, with a view to transferring embryos at a later stage.

LUTEAL PHASE

Extensive controlled studies have been performed to examine the benefits or otherwise of luteal support treatment, and our own work has shown that both intramuscular P4 administered by injection, or hCG given intermittently during the luteal phase, can improve the rate of implantation, and therefore pregnancy (Yovich, 1988; Yovich et al., 1984c, 1985c, 1990). For patients undergoing IVF-ET, it may be beneficial to enhance endometrial development, as embryos are placed in the uterine cavity 3 days before they would usually reach that site in natural conceptions. The luteal phase treatment for IVF-ET cases preferred by the authors is:

1 imi Proluton 50 mg daily for 5 days, days 0 to 4 inclusive (day 0 = day of oocyte recovery). The first injection is given immediately after follicle aspiration.
2 imi hCG 1000 IU on days 4, 7, 10 and 13 of the luteal phase.

Whilst the use of luteal support may not be considered mandatory for all patients by some IVF-ET centres, it does appear to be an absolute requirement for those cases stimulated by hMG without clomiphene as the luteal phase length is usually reduced to 9 days (Yovich, 1988a). The hCG output from embryos may not be sufficient to salvage corpus luteal function.

OUTCOME

Pregnancy rates arising from IVF-ET reported from various centres worldwide vary from 5 up to 35% of the number of cycles treated. Even within highly efficient and experienced IVF units, wide fluctuations in pregnancy rates are noted throughout the year. Furthermore, the rates of early pregnancy wastage may also fluctuate with an inverse trend to the pregnancy rates. Comprehensive studies initiated independently from IVF units, e.g. by the Voluntary Licensing Authority (VLA; 1988) in the UK, the Commonwealth Department of Health (1988) in Australia, the Health Department of Western Australia (Webb, 1988), indicated that overall the pregnancy rate is around 15% per treatment cycle with around 9% of pregnancies proceeding to the delivery of live infants.

Typically, a detailed report, such as Webb (1988) includes only those pregnancies reaching clinical detection and containing a viable fetus with confirmed fetal heart movement on ultrasound. This rate was 9.3% of ETs and live birth confinements were 9.0% per ET. Given that additional wastage occurs from blighted ovum, ectopic and biochemical stage pregnancies (27%; Yovich and Matson, 1988), the total pregnancy rate per ET was 14–15%. The most significant factor influencing the pregnancy rate was shown to be the number of embryos transferred—for four embryos, the total pregnancy rate was of the order of 24% with 17.0% containing a viable fetus at the 8-week ultrasound check. However, the multiple pregnancy rate was 25% (17% twins, 8% triplets and no higher order pregnancies). Rarely were more than four embryos transferred in any patient.

The report also recorded an increased Caesarean section rate, an increased preterm delivery rate and a lower mean birth weight of IVF infants compared with other Western Australia births. These features were largely influenced by the multiple pregnancies but there was an increased risk of preterm delivery even amongst singleton pregnancies. Perinatal mortality was fivefold that of non-IVF deliveries, again reflective of the increased risk of preterm delivery. There was no increase in the rate of fetal congenital abnormalities.

The report also detailed the early results for GIFT and PROST, revealing a statistically significant improvement in pregnancy rates, viable

pregnancies and livebirths (threefold increased chance of livebirths over IVF-ET).

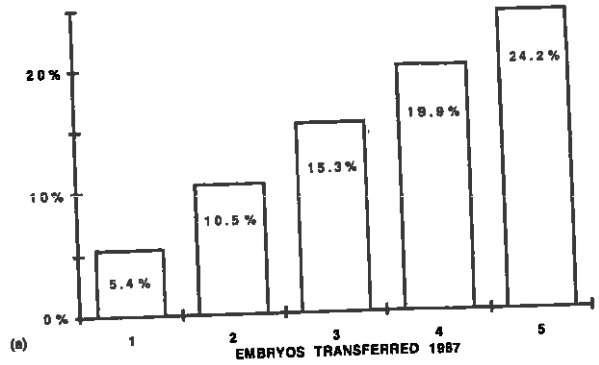
Current results in an established centre

The chance of an individual embryo implanting ranges from 4% (Yovich et al., 1988) to 12.0% (Yovich, 1985) in an established centre such as PIVET. These fluctuations are session-related and may reflect changes in:

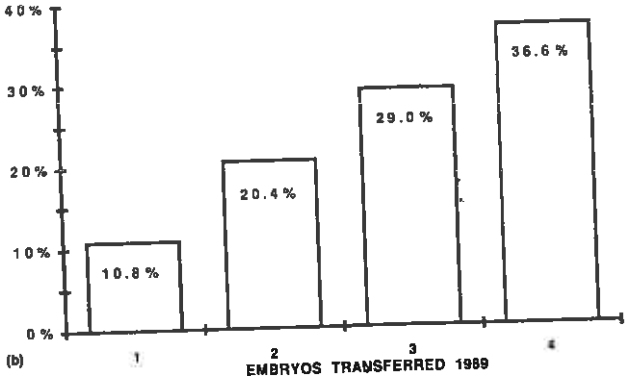
1 *Laboratory conditions*—in particular, the quality of culture medium which is in turn dependent upon water purity, maternal serum factors and the chemical ingredients.
2 *Patient factors*—categories of infertility, the proportion of poor responders, advanced age and PCO patients in the programme at the time.
3 *Procedural factors*—an adverse factor within the theatre environment (failure fully to rinse off glutaraldehyde from laparoscope equipment or the use of antiseptic agents within the vagina prior to transvaginal ultrasound aspirations), within the laboratory (bacterial contamination within the incubator), or surrounding areas (fresh paint, pesticides in the atmosphere).

The main factor influencing the chance of pregnancy is the number of high-grade embryos transferred to the uterine cavity. Depending upon the sessional results, this can range from 16% up to 40% if four embryos are transferred. The risk of multiple pregnancy is calculated as the binominal expansion of the chance of implantation for each embryo if factors which may enhance implantation are not considered (Fig. 10.5). Multiple pregnancies may occasionally arise from a single embryo (Yovich et al., 1984a) but such monozygotic twinning appears to be unrelated to the techniques of IVF. The authors noted a reduction in the rate of implantation of individual embryos from 12% (Yovich, 1985) in 1984 down to levels around 5–9% despite increasing technical experience. In 1989 the implantation rate was 11% of embryos and the improvement may relate to a return to embryo transfer techniques used in 1984. Another factor associated with this change was a rise in the mean age of the couple from 29 years in high-rate sessions to almost 34 years in the recent group.

The low chance of pregnancy noted for women who have only one or two oocytes recovered relates more to adverse hormonal events in the cycle and generally poor-quality oocytes and subsequent embryos in such cases. Women who generate six or more oocytes but subsequently have only one or two embryos transferred (e.g. severe oligospermia) conceive at the expected rate during that session. The



	1	2	3	4	5
TWIN	-	2.78%	5.39%	7.86%	10.10%
TRIPLET	-	-	0.10%	0.30%	0.58%
QUADRUPLET	-	-	-	0.01%	0.02%
QUINTUPLET	-	-	-	-	0.01%



	1	2	3	4
TWIN	-	5.71%	10.75%	15.18%
TRIPLET	-	-	0.43%	1.23%
QUADRUPLET	-	-	-	0.04%
QUINTUPLET	-	-	-	-

Fig. 10.5 Pregnancy rates and risk of multiple pregnancy for IVF-ET based on the full year's data from 1987(a) to 1989(b). Pregnancy rates and multiple pregnancy risks (as a proportion of pregnancies) increase with the number of embryos transferred according to the binomial distribution = U $\binom{n}{r}$ $E^r(1-E)^{n-r}$. The embryo factor (E) was 0.054 in 1987 and 0.108 in 1989 and the uterine factor (U) is assumed to be 1.0 in conceiving patients. n = number of embryos transferred and r = number of embryos implanting.

1987–88 data from PIVET are shown in Table 10.3. The outcome from pregnancies is shown in Table 10.4 (also see Fig. 16.2).

Table 10.3 IVF-ET data from PIVET (January 1987–June 1988)

	Embryos transferred				
	1	*2*	*3*	*4*	*5*
Pregnancy rate	0/12	2/28	4/27	8/42	9/44
(%)	—	(7.1)	(14.8)	(19.0)	(20.4)
Pregnancy sacs arising	—	2/56	4/87	8/168	15/220
(%)	—	(3.5)	(4.5)	(4.7)	(6.8)

Total collections = 168
Pregnancy rate/transfer = 23/153 (15%)
Sacs/embryo transferred = 29/537 (5.4%); complete data for 1988 showed an improved implantation rate of 33/374 (8.9%), possibly arising as a consequence of an increased proportion of patients stimulated with the Lucrin/hMG regimen.
IVF-ET < GIFT/PROST/TEST; $p < 0.001$.

Table 10.4 Pregnancy outcome in patients conceiving following IVF-ET (1983–1988)

Pregnancy outcome	*Number of pregnancies*	
Early wastage (<20 weeks)		
Preclinical	13	(9.3%)
Blighted ovum	18	(15.8%)
Miscarriage	2	(1.5%)
Ectopic	13	(10.0%)
Total	46	(35.4%)
Late pregnancy outcome (>20 weeks)		
with surviving infants	81	(62.3%)
without surviving infants	3*	(1.0%)
Total	84†	(64.6%)
Combined total	130	(100%)

* Perinatal mortality 3/109 (2.8%)
Congenital abnormalities 3 (3.6%)
1 Goldenhar syndrome
2 Microcephaly
3 Klinefelter's syndrome
† Total infants = 109.

Preclinical (biochemical) pregnancies occur more frequently in IVF-ET and similar procedures involving gamete manipulation. The rate is higher than that seen in ovarian stimulation and insemination groups (Yovich and Matson, 1988) and probably reflects an increased likelihood of poor-quality embryos implanting. The high rate of ectopic pregnancies is similar to that seen in the GIFT series and in both treatment groups underlying tubal disease is the common predisposing factor. Previously we have suggested that this is related to the ET technique, in that high cannulations may cause the embryos to be transferred directly into the tubal orifice or within the tubal lumen. The chance of an ectopic is reduced by placing the embryos in a mid fundal position within the uterus (described under ET technique). However, with the current knowledge that the tubal environment is the preferred location for placement of embryos, it is likely that the high ectopic rate arises as a combination of tubal cannulation and some form of tubal disease which inhibits embryos from returning to the uterine cavity. It is probable that, depending upon placement and uterine configuration, four-cell embryos may drift into the fallopian tubes and return later in the late morula/blastocyst stage. However, certain types of underlying tubal disease, e.g. major cornual constrictions and mucus plugs within the cornu, may limit their return. This concept is supported by the finding of a higher ectopic rate in those patients having GIFT and TEST treatments after previous tubal microsurgery. It has led to the consideration of deliberate occlusion of the cornuo/isthmic region of the fallopian tubes or removal of the fallopian tubes prior to IVF-ET, but this is not universally advised.

Pregnancies which proceed successfully have a higher risk of preterm delivery, largely related to multiple pregnancies. However, even singleton pregnancies deliver prematurely twice as often as the general rate in the community. This appears to reflect characteristics within the women rather than being related to the procedures and is discussed later (Chapter 16). There is an increased proportion of infants who are small for gestational age and again, this is mostly reflective of multiple pregnancies. The Caesarean section rate is high, at around 40%. Although a number of congenital abnormalities have been reported in IVF infants, there has been no increase in the rate of congenital abnormalities among IVF infants and recurring problems have not been recorded in local surveys. However, the National Perinatal Statistics Unit (NPSU; 1987) reports some clustering of spina bifida and transposition of the great vessels in a large series of IVF infants.

Very few studies have looked at the long-term development of IVF infants; in Western Australia IVF infants have been shown to have normal developmental quotients at 1 year of age (Yovich et al., 1986). Worldwide, the results of IVF indicate normal development of infants and children, led by Louise Brown who recently celebrated her tenth

birthday with the event happily observed and disseminated by the international media.

Further progress

This chapter has details of the status of IVF-ET in 1988. Further developments have been described in Chapter 19.

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Chapter 11

Gamete intrafallopian transfer (GIFT)

Summary: Gamete intrafallopian transfer: GIFT

Indications	Unexplained infertility	
	Poor sperm–mucus interaction	
	Endometriosis	
	Failed DI	
Ovarian stimulation	Clomiphene citrate/hMG	
	Lucrin/hMG	
	6–7 days of sustained E2 rise	
Oocyte recovery	36 h after hCG trigger 10 000 IU or augmented LH surge	
	Laparoscopic and/or transvaginal ultrasound-directed recovery	
	PIVET-Cook aspiration/flush needle	
Laboratory procedure	Semen collected 2 h before	
	Oocytes graded/4	
Gamete transfer	Transfer in HTFM + 20% MS	
	Up to 3 oocytes graded ≥3	
	Normospermic: 100 000/tube	
	Oligospermic: up to 500 000/tube	
	3 into 1 tube at laparoscopy	
Luteal phase	hCG 1000 IU days 4, 7, 10 and 13	
Results	Pregnancy rate/transfer	40%
	Sacs/oocyte transferred	11%
	Pregnancy wastage	30%
	Multiple pregnancies	20%

The technique of GIFT, first reported by Asch and his colleagues (1984), provided a timely addition in the armamentarium of workers in the area of human infertility. Although IVF-ET was being applied for a range of infertility disorders apart from tubal conditions, the overall pregnancy rates per procedure were disappointingly low, remaining under 20% in large annually rated series. The GIFT technique was applied at the PIVET Medical Centre from November 1985. It proved rapidly successful for many types of non-tubal infertility but we

reported its limited application for the treatment of male infertility (Yovich et al., 1986) and subsequently devised a modified protocol which has proved successful for many cases in that subcategory (Matson et al., 1987a). At an early stage it became obvious that GIFT was producing a significantly higher pregnancy rate when compared with IVF-ET (Yovich and Matson, 1990) and hence it was applied preferentially for all cases where there was at least one fallopian tube available. Table 11.1 shows the results of an early comparative series of GIFT and IVF-ET cases undertaken at PIVET. Table 11.2 details the main infertility categories of the GIFT and IVF-ET groups.

Table 11.1 Comparative clinical pregnancy rates for GIFT and IVF-ET in the first consecutive series from December 1980 to March 1987 (GIFT cases commenced October 1985)

	GIFT	*IVF-ET*
Pregnancy rate/collection	100/373 (27%)*	143/1120 (13%)†
Pregnancy rate/transfer	100/372 (27%)	143/956 (15%)†

* 2 minilaparotomy collections, 1 failed collection.
† χ^2, $p < 0.001$.

Table 11.2 Clinical pregnancy rates from GIFT and IVF-ET with respect to the subcategory of infertility (from December 1980 to March 1987, after which IVF-ET was reserved for primary indication of tubal infertility; GIFT cases commenced October 1985

Aetiology	*Pregnancy/case transferred*	
	IVF-ET (%)	*GIFT (%)*
Tubal	102/648 (15.7%)	21/72 (28.4%)
Unexplained	10/67 (14.9%)	20/69 (29.0%)
Male factor		
Oligospermia	13/76 (17.1%)	11/66 (16.7%)
Male ASAB	5/19 (26.3%)	2/12 (16.7%)
Endometriosis	4/96 (4.2%)	18/54 (33.3%)
Failed DI	4/19 (21.1%)	13/43 (30.2%)
Peritoneal adhesions	2/12 (16.7%)	2/8 (25.0%)
Cervical factor	3/19 (15.8%)	13/46 (28.3%)
Total	143/956 (15.1%)	100/372* (26.9%)

* IVF < GIFT; $p < 0.001$.

The early data indicated that the chance of pregnancy from GIFT was dependent upon the number of oocytes transferred, showing an improvement over IVF-ET only when three or more oocytes were trans-

Table 11.3
(a) Pregnancy rates following GIFT and IVF-ET with respect to the number of oocytes or embryos transferred (1986–87)

	Number of oocytes/embryos					
	1	*2*	*3*	*4*	*5*	*Total*
GIFT	3/22 (13.6%)	3/44 (6.9%)	18/65* (28%)	68/217* (31.3%)	8/24 (33.3%)	100/372† (27%)
IVF-ET	18/215 (8.4%)	39/282 (13.8%)	41/237 (17.3%)	32/158 (20.3%)	13/64 (20.3%)	143/956 (15.0%)

* 1 or 2 vs 3 or more: $\chi^2 = p < 0.01$.
† GIFT vs IVF-ET, $p < 0.001$.

(b) GIFT pregnancy results for the period January to December 1989 compared with IVF-ET for the same period. There were 92 collections, mostly transvaginal, and all cases had successful laparoscopic transfers

	Number of oocytes/embryos				
	1	*2*	*3*	*4**	*Total*
GIFT	0/1 (—)	2/9 (22.2%)	27/63 (42.9%)	8/19 (42.1%)	37/92 (40.2%)
IVF-ET	0/8 (—)	4/18 (22.2%)	37/122 (30.3%)	5/13 (38.5%)	46/161 (28.6%)

* Maximum of 3 oocytes transferred after February 1989.
GIFT vs IVF-ET: pregnancy rates not different ($P = 0.058$) but implantation rates (pregnancy sacs arising per oocytes transferred) are higher for GIFT (50/462; 10.8% for IVF-ET vs 46/284; 16.2% for GIFT: $P < 0.05$). The degree of significance is greater if one assumes that only 72% of GIFT oocytes are fertilized.

Table 11.4 The outcome of GIFT and IVF-ET treatments applied for the subcategory of endometriosis

Classification	*GIFT*		*IVF-ET*	
	Pregnancy/cycle	*(%)*	*Pregnancy/cycle*	*(%)*
Stage I	13/30	(43%)	3/24	(13%)
Stage II	3/19	(16%)	5/37	(14%)
Stage III	1/4	(25%)	2/36	(6%)
Stage IV	1/1	(100%)	1/52	(2%)*
Total	18/54	(33%)	11/149	(7%)†

* χ^2, $p < 0.05$ compared to groups I and II.
† $p < 0.001$ IVF-ET v. GIFT.

ferred (Table 11.3). The difference between GIFT and IVF-ET was particularly marked for the infertility subcategory of endometriosis (Table 11.4) but on the other hand remained limited for cases of severe oligospermia (Table 11.2), even using the modified preparation method (Matson et al., 1987a). With the subsequent development of PROST and TEST, certain non-tubal categories of infertility were seen to be better managed in those programmes, e.g. male factor infertility, female ASABs, ovum and embryo donation, and any procedure where IVF is preferable, including the development of only one or two follicles during ovarian stimulation.

The benefits of GIFT over IVF-ET also provided an encouragement to relook at the question of fallopian tube reconstructions, which, although they may have a relatively poor prognosis for spontaneous conception, might allow the conversion of an IVF-ET case into the GIFT programme. We have reported the results of such cases, showing a significantly improved benefit in the chance of pregnancy but a higher risk of ectopic pregnancy (11%; Yovich and Matson, 1988), mainly related to the inclusion of cases with underlying tubal disorders, e.g. previous tubal reconstructive surgery.

INDICATIONS

The indications for GIFT are:

1 Unexplained/poorly explained infertility.
2 Endometriosis.
3 Poor sperm/mucus interaction (negative PCT).
4 Ovarian stimulation patients demonstrating ≥4 follicles.
5 Failed DI (although PROST may be preferred).

In the past we have also included two other categories which are now treated by TEST or IVF-ET:

6 Moderate oligospermia (>5 million motile sperm/ml); and other cases of male factor (e.g. ASABs) without asthenospermia.
7 Cases of previous tubal reconstructive surgery where pregnancy has failed to occur but where is at least one fallopian tube remaining patent.

OVARIAN STIMULATION

This should be along precisely the same lines as for IVF-ET, with the intention of providing the trigger of hCG 10 000 IU on the sixth day

of sustained E2 rise. Where GnRH analogues are used, the hCG trigger should be given on the seventh day of E2 rise.

OOCYTE RECOVERY PROCEDURE

In GIFT, both the oocyte recovery and gamete transfer techniques are performed during the same procedure, which involves general anaesthesia with muscle relaxation and endotracheal intubation. Postoperative discomfort is minimized if the anaesthetist avoids the use of the short-acting, depolarizing muscle relaxant suxamethonium chloride and the pneumoperitoneum is induced with carbon dioxide with full evacuation on completion. Early workers used the triple gas mixture of 5%CO_2:5% O_2:90%N_2 to induce the pneumoperitoneum but many have adopted the simple expedient of using 100% CO_2 alone. The PIVET unit changed from triple gas to CO_2 in 1982 without recognizing any adverse changes in fertilization or pregnancy rates.

The optimization of laparoscopic oocyte recovery is dependent upon:

1 Timing factors (relating to the ovarian stimulation schedule and LH surge/hCG trigger).
2 The instrumentation (dual lumen aspiration/flushing needle preferred).
3 Ovarian access (preliminary pelvic adhesiolysis may be required).

Instrumentation

The PIVET-Cook double-lumen needle (Fig. 10.2) is used for both laparoscopy and transvaginal ultrasound-directed collections at PIVET.

Procedure

For laparoscopy, a three-puncture technique (Fig 11.1 and Plate 11.1) is used with the laparoscope usually placed within the lower rim of the umbilicus, Semm's grasping forcep is inserted via a trochar in the midline just within the pubic hairline and the aspiration needle trochar is placed just lateral to the edge of the right rectus muscle, a few centimetres below the umbilical level. This high location is chosen to improve the ability for subsequent tubal cannulation.

The ovaries are held in position by grasping the ovarian ligament and the needle is gently inserted into the follicle in or near the thinnest, most distended area. By depressing the foot pedal and applying an aspiration pressure of around 100 mmHg for the 15 cm needle, or

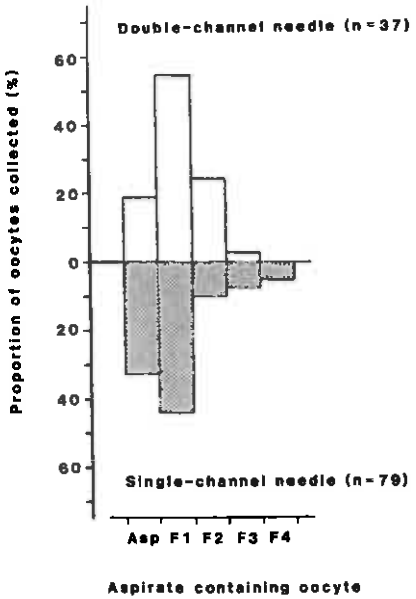


Fig. 11.1 Aspiration fraction containing oocyte as a function of a single (spinal needle; n = 79; hatched area) or double channel (PIVET-AN1; n = 37; blank area) system.

150 mmHg for the 30 cm needle (see Chapter 9), the follicle is aspirated. It is not necessary to empty the follicle completely and risk the walls collapsing away from the needle during the initial aspiration.

The first aspirate, e.g. L1 (denoting the first follicle of the left ovary) is always a clean fraction and the collecting tube is then changed whilst the needle tip is held in the partially decompressed follicle under direct vision. The follicle is then flushed with 6–7 ml of FFM (from a 10 ml syringe of medium) and is aspirated at the same time so that it is allowed to redistend only partially. The needle tip is held still or slowly rotated within the follicle, allowing the fine spray of flushing medium to create a gentle turbulent action around the follicle wall. The collecting tube would usually be noted to contain cellular fragments and is denoted as F1 (e.g. L1F1—first flush from first follicle in left ovary).

When the follicle is decompressed the collecting tube is exchanged for a third tube and the final 3–4 ml of flushing medium is inserted into the follicle which again is allowed partially to fill prior to aspiration. For the large follicles visible on the ovarian surface, the needle

is gradually withdrawn from the follicle, rotating it to ensure complete evacuation. The needle is removed from the abdomen and washed with a small volume of flushing medium before handing the third collecting tube across to the embryologist, labelled F2W (e.g. L1F2W denoting the second flush plus wash from the first follicle of the left ovary).

The entire process of aspiration and flushing is usually completed within 2 min and is curtailed once the oocyte has been identified by the embryologist. For the smaller follicles and those contained deep in the ovarian substance, the operator may prefer to proceed through ovarian tissue to the next follicle after F2 without withdrawing to wash the needle and tubing system.

The oocyte recovery rate with this system is >90% per follicle overall and is similar to the transvaginal technique using the PIVET-Cook needle (see Fig. 10.3) which approaches 100% for follicles >1.5 cm. However, the transvaginal recovery method permits oocyte recovery from many more follicles smaller than 1.5 cm. Hence the oocyte recovery rate overall by laparoscopy is one per 1000 pmol per peak E2 compared with one per 600–700 pmol per peak E2 by the transvaginal method. The majority of oocytes are found in the first flush and are thought often to be held within the dead space in the aspiration needle or decompressed follicle and are pushed through at the very beginning of the flushing process. This finding is similar for follicles either smaller or larger than 1.5 cm. Follicles >2.7 cm rarely contain mature oocytes and are regarded as follicular cysts.

The data from Fig. 11.3 shows the highly significant improvement in oocyte recovery rate from follicles aspirated with the double-lumen needle when compared with a single-channel aspiration needle (Monash) at laparoscopy. The fertilization rates and mean embryo quality (shown in Table 11.5) are not significantly different in the three fractions (aspirate, F1 and F2/W samples) and the highest graded embryos were from oocytes in the first flush collected by either aspiration needle. However, the double-lumen needle is preferred for its greater overall efficiency and ease of handling.

Ovarian and tubal access

A descriptive preliminary laparoscopy is useful in all cases entering IVF programmes but is particularly crucial when considering GIFT. It is important to determine the ease of access to each ovary, potential hazards with the collection, the proper documentation of the case with respect to the infertility history, the consideration of preliminary adhesiolysis, the patency of each fallopian tube, the mobility of each tube and an examination of the fimbrial tip to check for fimbrial agglutination and the potential for cannulation. All such details require careful documentation. It is also important to ensure that all pelvic

Table 11.5 Fertilization rate and embryo quality related to fraction containing oocyte

	Aspirate	*1st flush*	*Flush/ wash*	*Repeat entry and flush*		
	(ASP)	*(F1)*	*(F2/W)*	*F3*	*F4*	*F5, 6*
Oocytes recovered	73	213	47	9	6	1
Oocytes fertilized						
Number	62	157	38	8	6	1
Percentage	84.9%	73.7%	80.9%	88.9%	100%	100%
Embryo grade						
Median	2½	2½	2½	2½	2	1
Range	½–3½	1–4	1–3½	2–3½	1–3	—

pathology is totally controlled, particularly pelvic inflammatory disease and endometriosis.

Following the preliminary laparoscopy one should also perform cervical and uterine sounding, an exploration of the uterine cavity, an assessment to exclude uterine pathology—particularly submucous fibroids—synechiae and endometritis.

Oocyte recovery technique with restricted access

Women who have had previous abdominal or pelvic surgery often display abdominal and intraperitoneal adhesions. The most common are bands of omental adhesions to the anterior abdominal wall, impeding access to the pelvis in varying degrees. Women who have had previous pelvic inflammatory disease or the more severe grades of pelvic endometriosis will often have immobile ovaries, adherent to the broad ligaments or bound down by adhesions deep in the POD. The ovaries are often concealed by overlying fallopian tubes and periadnexal adhesions. If previous uterine, tubal or ovarian surgery has been performed, one finds densely adherent bowel loops covering the ovaries, particularly the sigmoid colon on the left side.

During oocyte recovery, additional grasping forceps can be inserted to improve ovarian access. Sometimes, additional benefit can be obtained by an assistant digitally mobilizing the retroverted uterus on vaginal examination, with the patient in the lithotomy position. However, despite extensive experience, we have not found this manoeuvre to be reliably helpful and prefer adding a second grasping forcep into the peritoneal cavity. We therefore no longer use the lithotomy position for laparoscopies but simply have the patient in the flat Trendelenburg position after bladder catheterization with 10° head-down tilt.

In contrast to general pelvic operations, those women who have had reconstructive tubal surgery where microsurgical principles have

been applied tend to have minimal pelvic adhesions without omental attachment to the anterior scar and ovarian access is usually good. The likelihood of oocyte recovery has been assessed in relationship to the degree of visually accessible ovary (Fig. 11.2), showing a significant reduction in the ability to aspirate follicles which have been previously detected on ultrasound if the degree of ovarian access is less than 25%. Above this level, follicles can be successfully aspirated despite immobilization and partial concealment.

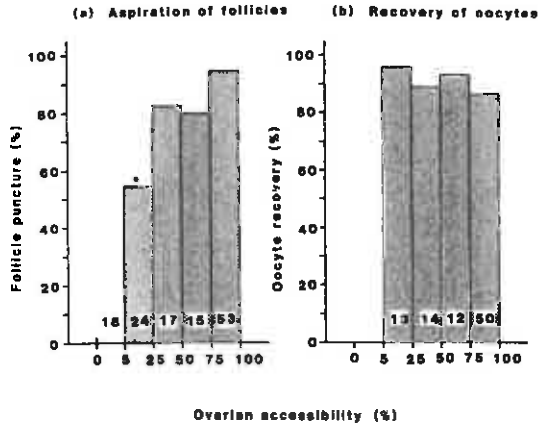


Fig. 11.2 The effect of limited ovarian access on oocyte recovery where access has been assessed as a percentage of visualized surface (* $p < 0.01$).

Occasionally, one finds that follicles have ruptured, whether spontaneously or during the laparoscopy recovery procedure. It has been shown that flushing the dispersed follicle (Stanger and Yovich, 1984) and aspirating the contents of the POD (Matson et al., 1986) leads to oocyte recovery in a high proportion of cases (Fig. 11.3). Embryos derived from oocytes freshly released into the POD appear to have a similar potential to generate pregnancies as those aspirated from follicles (Matson et al., 1986).

Pelvic adhesiolysis

Preliminary pelvic adhesiolysis can be performed to improve ovarian access. Generally, the procedure involves the use of fine needle point electrocautery to release adhesions, mobilization of bowel from the adnexae and uterus, partial resection of the omentum when involved,

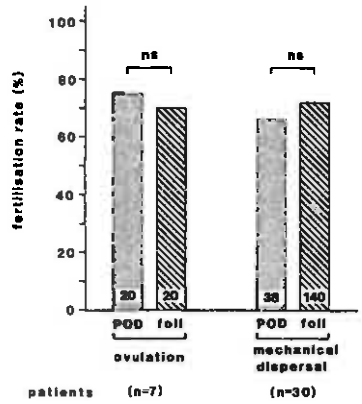


Fig. 11.3 The fertilization of oocytes from the POD is not significantly different from those aspirated from ovarian follicles, regardless of whether the oocytes were released by spontaneous ovulation or accidental mechanical dispersal during the laparoscopy procedure.

mobilization of ovaries from their embedded positions within the broad ligaments, microsurgical reconstructive tubal surgery if relevant, or complete excision of redundant fallopian tube tissue and plication of the round ligaments to correct retroversion. Plication of the ovarian ligaments (Yovich et al., 1988a) is no longer advised.

The procedures are carried out under antibiotic and parenteral steroid cover, the pelvic organs and peritoneal cavity are constantly lavaged with 6% dextran and 50 ml of 32% dextran is retained within the peritoneal cavity on completion. Microsurgical principles are applied, such as minimization of tissue handling, the gentle use of moist packs, meticulous attention to reperitonealization of all serosal surfaces using fine nylon or low reactivity polyglactin sutures, attention to complete haemostasis using bipolar coagulation and eversion of the parietal peritoneal surfaces during abdominal closure (Fig. 11.4).

Combined ultrasound-guided recovery in GIFT

Where an ovary remains less than 50% accessible, an ultrasound-guided technique of follicle aspiration is preferred. Generally, the transvaginal technique will be appropriate but occasionally an ovary tethered high may be better approached with the percutaneous transvesical or transabdominal method. In fact, this has led to the situation in many patients where an inaccessible (usually left) ovary has oocytes recovered per vaginam whilst the accessible right ovary has oocytes recovered abdominally at laparoscopy. It has been our experience that for

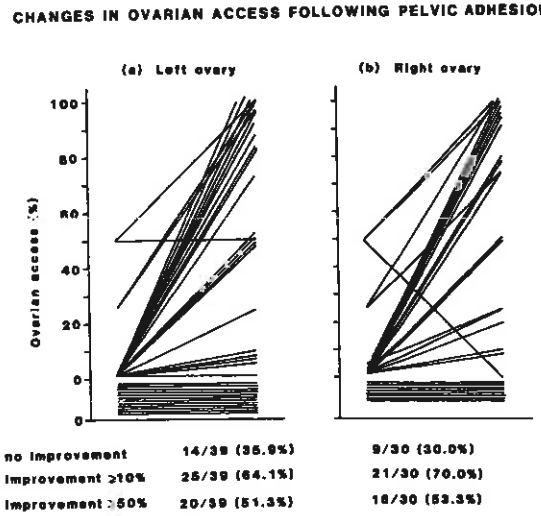


Fig. 11.4 Preliminary pelvic adhesiolysis applying microsurgical principles generally improves subsequent ovarian access at laparoscopy.

the ovary containing more than four follicles, it is easier to recover oocytes by the transvaginal technique. Hence some gynaecologists will prefer to aspirate all follicles by the transvaginal technique prior to laparoscopy for tubal cannulation in GIFT. These developments do limit the need for consideration of pelvic adhesiolysis. However, this should always be offered to the patient if there is seen to be a definitive benefit to the underlying infertility problem. This may also lead to the conversion of a case from IVF-ET to GIFT or PROST or improve the technical aspects of subsequent tubal cannulation.

Tubal cannulation

On completion of the follicle aspiration procedure, the POD is routinely aspirated prior to undertaking cannulation of the fallopian tubes for GIFT. The standard approach is to transfer two oocytes and the prepared spermatozoal fraction to each fallopian tube. It is important to know beforehand the current status of patency of the fallopian tubes; a laparoscopic assessment or hysterosalpingogram is an absolute requirement within the 12-month period prior to the GIFT attempt. If there is any suspicion of difficulty with access or cannulation of one or other fallopian tube, the difficult or suspect tube should be

attempted first. Should it prove impossible to cannulate, the three (or occasionally four) oocytes will be transferred to the more accessible tube. Therefore, in the preliminary laparoscopic assessment, one will be particularly interested in any peritubal tethering, degree of tortuosity of the fallopian tube, appearance of the fimbrial end and any nodularity along the fallopian tube, particularly if it is associated with impeded flow of dye during pertubation. GIFT cannulation of suspect fallopian tubes should be avoided if possible.

During the GIFT procedure, the distal fallopian tube is grasped immediately adjacent to the fimbrial tip at the antemesovarial border (i.e. by the 'scruff'). Semm's grasping forceps are used and we have found the grasping potential is enhanced by placing horizontal grooves in the grasping tip.

For the GIFT procedure, an 18 cm guiding trochar is inserted into the shorter abdominal trochar which was previously used for the aspiration needle. This is shown in Fig. 11.3 and Fig. 11.5. Three operator hands are required—one holding the Semm's forcep, another holding the laparoscope, and a third steering the cannula to the fimbrial tip. There are benefits in using Semm's flexible pelviscope holder with or without Semm's shoulder rest.

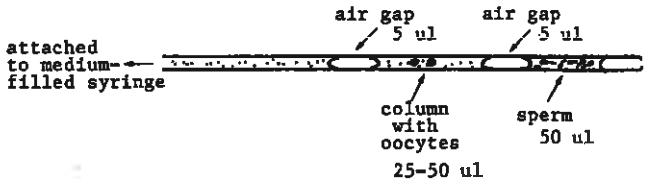


Fig. 11.5 Schematic diagram depicting method of preparation of GIFT catheter prior to transfer.

When the gynaecologist is ready, the embryologist brings the gametes in the Teflon GIFT cannula which is then inserted through the trochar and guided into the fallopian tube by the gynaecologist to a point shown by a double mark which indicates that the GIFT cannula is within the tubal lumen 4 cm from its distal end (Fig. 11.7). The gametes are transferred and the gynaecologist waits to hear from the embryologist that the gametes have been flushed from the catheter

before resting the fallopian tube against the pelvic side wall. The procedure is then repeated on the other tube. Once the tubes have been cannulated the operating table is returned to the flat position, preferably prior to removing the cannula. On completion, the pneumoperitoneum is released, a single suture is placed in the trochar wounds and the patient is woken up from anaesthesia. She would usually proceed home that evening or the following morning and no restrictions are placed on activities.

Laboratory procedures: gamete preparation

The first step of the procedure is to prepare spermatozoa from the semen which is produced approximately 2 h before ovum pick-up. An overlay or sedimentation technique is used, as previously described, to obtain a spermatozoal count of around $2–5 \times 10^6$/ml. Two 4-well Nunc dishes with medium are prepared (labelled 'wash' and 'A' dishes); the sperm preparation is placed in well 4 of 'A' dish.

Oocytes are recovered from the follicle aspirates and are washed, similar to the IVF procedure and placed either two each in well 1 and well 2, or four in well 1 of 'A' dish. The handling medium for GIFT is HTFM containing 20% deactivated maternal serum.

At the time of transfer of the gametes, sterile non-powdered rubber gloves are worn before handling the GIFT catheter and direct contact of the rubber glove with the catheter is avoided by using a piece of sterile paper packing which can slide up and down the catheter. The GIFT catheter is attached to a 1-ml tuberculin syringe which is filled with medium and the catheter is flushed with the medium before loading with gametes according to the sequence shown in Fig. 11.8.

The catheter is passed through a 30 cm (12-inch), 16-gauge blunt-ended steel cannula and guided into the oviduct under laparoscopic control. Once the GIFT catheter is 4 cm into the oviduct the gametes are released and the catheter is slightly removed. For bilateral patency two eggs per oviduct and for single tube patency up to four eggs per oviduct are transferred. Results from more than one clinic indicate pregnancy rates are maintained following single tube transfers. It is possible to load and transfer all gametes for two-tube transfer in the one manoeuvre although many embryologists will prefer to reload the catheter for the second tube. The laparoscopist must wait until the embryologist has checked the cannula after transfer to ensure that all gametes have been successfully transferred. (From early 1989, a maximum of three oocytes is transferred to one fallopian tube as a routine at PIVET.)

Remaining oocytes are treated according to the patient's wishes, i.e. fertilized with husband's spermatozoa for IVF, donated to another couple, used in an approved research project, or discarded.

Luteal phase management

There appear to be several benefits from gamete transfer into the fallopian tubes:

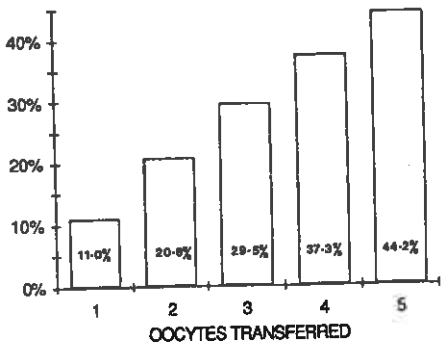
1 No restriction need be placed on the woman's activities. She may return to work the following day, play sport, enjoy normal marital relationships including sexual activity. This differs from traditional IVF-ET where there appears to be a benefit from restricting physical activity and sexual relationships for several days after transfer.
2 Luteal phase therapy is not such a crucial requirement as for IVF-ET. However, a randomized controlled study does show that the chance of pregnancy is higher if some form of luteal support is used but there is no difference for those having intermittent hCG injections, continuous Proluton injections or a combination of Proluton/hCG (Yovich, 1989). Therefore, the authors use hCG 1000 IU on days 4, 7, 10 and 13 after GIFT. Hormonal monitoring is the same as for IVF-ET—ideally, days 10, 13 and 16, which provides a useful guide to the need for additional hormonal support in early pregnancy or to assess additional requirements for subsequent GIFT attempts, e.g. identifying poor E2 and P4 levels on day 10 despite hCG.
3 There is an improved chance of pregnancy. It remains uncertain at this stage whether the tubal environment confers some specific factor which is not present in IVF cultures or whether the main benefit relates to keeping the early embryo out of the uterine cavity, which may be relatively hostile during the early postovulatory phase. Currently, it is believed that the latter factor is the most likely one operating.

Outcome

GIFT appears suitable for most types of non-tubal infertility with the following provisos:

1 For women with severe grades of pelvic endometriosis (grades III and IV), preliminary treatment with steroid suppression therapy (e.g. danazol) should be given for several months prior to undertaking GIFT. Studies have shown that the chance of pregnancy with GIFT is high for untreated grades I and II but reduced for grades III and IV, possibly due to interference with implantation (Yovich et al., 1988a). However, the rates are similar if there has been pre-treatment with danazol. It should be noted however that danazol should stop at least 4 weeks before starting ovarian stimulation for GIFT because it may cause persistent inhibition of endometrial development, despite ovarian stimulation. It also cross-reacts with testosterone on the androgen assay and may cause confusion regarding androgen status. This effect usually lasts for 2 weeks after ceasing danazol. More recently the use of GnRH analogues over a prolonged period (e.g. 6 weeks) prior to GIFT is giving favourable results.
2 Women with ASABs are unlikely to conceive by GIFT, even if donor serum replaces maternal serum. We were unable to achieve a pregnancy in a single instance of 13 cases of GIFT in this situation (Yovich and Matson, 1990). Such cases are better managed by PROST.
3 Where the husband has less than 5 million progressively motile spermatozoa/ml (severe oligospermia), there is an associated functional disturbance, so that higher than normal numbers of spermatozoa are required to effect normal fertilization. It is reasonable to undertake GIFT in such cases if 300 000 or more spermatozoa can be transferred with the oocytes (Matson et al., 1987a). However, recent data indicates a higher rate of blighted ovum pregnancies (see Chapter 19). Otherwise, treatment is best managed by PROST or TEST. Furthermore, if pregnancy fails to occur following a maximum of two GIFT attempts in oligospermic cases, again, treatment by PROST is preferred.
4 It has been our policy to offer GIFT to those couples who have had previous microsurgical reconstruction of the fallopian tubes if they do not conceive spontaneously within 6 months. With current techniques, the anatomical results of surgery are usually excellent and invariably the fallopian tubes remain accessible and can usually be cannulated for GIFT regardless of the underlying surgical procedures required (e.g. proximal reanastomoses, salpingostomy procedures, fimbrioplasties and salpingo/oolysis procedures). Subsequent failure to conceive implies additional infertility factors, poor oocyte/pick-up mechanism and underlying endosalpingeal/fimbrial defects. The chance of pregnancy following GIFT in such patients is similar to the non-tubal group as a whole, but the ectopic pregnancy rate is higher (11% as opposed to 5%). In counselling such patients, they need to consider the benefit of a significantly higher pregnancy rate against a marginal increase in the chance of ectopic pregnancy. In IVF-ET, the ectopic rate is 5–8% for those with tubal infertility.

Overall, the chance of pregnancy by GIFT is as high as 40% and the main factor contributing to pregnancy is the number and quality of oocytes transferred, ranging from around 10% if only one oocyte is transferred, up to 45% if four oocytes are transferred (Yovich et al., 1988b, 1988c). We have shown that the likelihood of an individual oocyte implanting is around 11% (Fig. 11.6). If one assumes a fertilization rate of 72% (equivalent to that noted in vitro), then it appears that approximately 15% of embryos which develop in GIFT can implant. This is a similar rate to that noted with PROST and TEST but is significantly better than the rates found with IVF-ET which



TWINS	5.93	11.15	15.24	19.61	Multiple
TRIPLETS	-	0.47	1.32	2.47	Pregnancy
QUADS	-	-	0.04	0.16	Risk %
QUINS	-	-	-	0.006	

Fig. 11.6 Pregnancy rates following GIFT are related to numbers of oocytes transferred. The multiple pregnancy rate increases with the number of oocytes transferred.

can differ from 4% (Yovich et al., 1988d) up to 12% (Yovich, 1985) over various series, and generally averaged around 8% implantation per embryo transferred.

Patients can consider one of four options regarding supernumerary oocytes:

1 Discard them.
2 Fertilize them with husband's spermatozoa and consider cryopreservation of the embryos.
3 Donate them to another infertile couple.
4 Donate them to an approved research project.

In the early stage of the GIFT programme, many couples chose option 2 but we found that the chance of fertilization of supernumerary oocytes bore no relationship to the chance of pregnancy arising in the treatment cycle (Matson et al., 1987b). This seems to be related to the selection process whereby the highest graded oocytes were transferred to the fallopian tubes. Supernumerary oocytes have a fertilization rate of around 52% which is significantly lower than the 72% rate found in IVF-ET for all oocytes inseminated (Yovich et al., 1989a).

Table 11.6 shows the pregnancy outcome for 238 GIFT pregnancies

(also see Fig. 16.2), of which 39% failed to proceed beyond 20 weeks. A high chance of ectopic pregnancy is seen with GIFT, particularly in those with underlying tubal disease, but there was also a higher risk of blighted ovum pregnancies as well as a relatively high mid-trimester pregnancy loss related to an increased chance of multiple pregnancies. The blighted ovum pregnancy rate was significantly higher than for IVF-ET or PROST and may reflect the relatively unsatisfactory oocyte selection method based on the grading technique described, and the inability to select out polyspermic fertilized oocytes. The early pregnancy wastage rate is higher than the 27% seen in a general population of infertile women who subsequently conceive (Yovich and Matson, 1988).

Table 11.6 Pregnancy outcome in patients conceiving following GIFT (1985–89)

Pregnancy outcome	*Number of pregnancies*	
Early wastage (< 20 weeks)		
Preclinical	20	(8.4%)
Blighted ovum	36	(15.1%)
Miscarriage	16	(6.7%)
Ectopic	20*	(8.4%)
Total	92	(38.7%)
Late pregnancy outcome (> 20 weeks)		
with surviving infants	139	(58.4%)
without surviving infants	7†	(2.9%)
Total	146‡	(61.3%)
Combined total	238	(100.0%)

* Includes 2 heterotopic pregnancies.
† Total perinatal mortality—12 infants (5.9%).
‡ Total infants—204.
Congenital abnormalities: 6 (2.9%)
1 Multiple anomalies of genioto-urinary and alimentary tracts
2 Periventricular leucomalacia in a 29 week twin
3 Cleft palate
4 Hydrocephalus
5 Patent ductus
6 Hemitruncus and patent ductus
Pregnancy distribution:

Singleton	111 (76.0%)
Twin	28 (19.2%)
Triplet	7 (4.8%)
Quad/Quin	0

In all, 24% of GIFT pregnancies were multiple (Table 11.6), with the majority being twins, and a small proportion (4.8%) of triplets. In addition, in one pregnancy seven gestational sacs containing a live fetus were identified on the early scan at 8 weeks even though only four oocytes were transferred (Matson et al., 1986). Two possibilities

were considered here: firstly, that early embryos divided by splitting, creating a mix of dizygotic and monozygotic fetuses; and secondly, that further ovulation may have occurred after GIFT, with additional oocytes entering the fallopian tubes and fertilizing with the transferred spermatozoa. Two selective termination procedures were undertaken in this patient to reduce the pregnancy to triplets; however, the entire pregnancy aborted spontaneously by the 24th week and all fetuses were lost.

Because a relatively high multiple pregnancy rate occurs with the transfer of four oocytes, it is considered unwise to transfer more than three, although additional oocytes of poor grading may not contribute to the pregnancy pool. Certain patients may be considered of poor receptivity, e.g. those women older than 40, high androgen females, repetitive IVF or GIFT failures; but again, caution should be maintained even in those cases, as the multiple pregnancy rate may be high when receptivity factors are optimal—when a poor prognosis patient does become pregnant, she is similarly likely to have a multiple pregnancy even though her overall chance of conceiving was lower.

For those pregnancies proceeding beyond 20 weeks gestation the pattern was the same as for IVF-ET in that a higher perinatal mortality was noted, mainly due to preterm delivery which in turn was directly related to multiple pregnancies but was also increased with singletons. Fortunately most pregnancies proceeding beyond 20 weeks had a satisfactory outcome with very few stillbirths and neonatal deaths; the perinatal mortality rate was 4.5% which is similar to other IVF-related procedures. A congenital abnormality rate of 2.9% was well within the range of abnormalities noted in the West Australian community (Bower and Stanley, 1986) and no recurring abnormalities were noted.

Addendum

Refer to Chapter 19 for current (1988–1990) developments.

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Chapter 12

Pronuclear stage tubal transfer (PROST)

Summary: Pronuclear stage tubal transfer: PROST

Indications	Male factor infertility	
	ASABs	
	Synchronized ovum donation	
	Failed GIFT	
Ovarian stimulation	Clomiphene citrate/hMG	
	Lucrin/hMG	
	6–7 days of sustained E2 rise	
	Hormone replacement therapy for ovarian failure	
Oocyte recovery	36 h after hCG trigger 10 000 IU or augmented LH surge	
	Transvaginal ultrasound-directed	
	PIVET-Cook aspiration/flush needle	
Laboratory procedure	Oocyte grade/4	
	Semen collected 4 h after	
	Insemination 6–10 hr	
	Pronuclear check 12–18 h post-insemination	
	HTFM + 10– maternal serum	
Oocyte transfer	12–18 hr	
	2 pronuclear oocytes	
	Select from high grade: up to 3	
	Cook catheter 4 cm into each tube	
	3 into 1 tube at laparoscopy	
Luteal phase	hCG 1000 IU days 4, 7, 10 and 13	
Results	Pregnancy rate/transfer	35–40%
	Sacs/embryo transferred	16%
	Pregnancy wastage	25%
	Multiple pregnancies	20%

PROST was developed at PIVET as a modification of the GIFT technique for couples in whom GIFT was possible but evidence of fertilization was important before the transfer (Blackledge et al., 1986; Yovich

et al., 1987). This procedure is also referred to as zygote intrafallopian tube transfer (ZIFT; after Devroey et al., 1986).

The main clinical categories considered to benefit from PROST included those with severe oligospermia and ASABs in either partner, particularly where the female has circulating antibodies which are likely to inhibit sperm migration in the female genital tract. It was also seen as having benefits over GIFT in the ovum donation programme for the recipient women whose cycles were synchronized with those of the donor. It is not possible to guarantee anonymity if GIFT is used in ovum donation programmes as both donor and recipient women are in hospital on the same day. However, anonymity between donor and recipient could readily be maintained in the PROST series as each patient could be hospitalized on a different day.

Oocytes are recovered as described for IVF-ET but with a preference for the ultrasound-guided transvaginal route. The gametes are treated exactly as for an IVF attempt and once the pronuclear stage has been confirmed, up to three PN oocytes are transferred into the fallopian tubes by laparoscopy. Initial results have been promising with pregnancy rates a further improvement on GIFT for selected categories of infertility and the pregnancy outcome has also been more favourable.

INDICATIONS

1 Male factor infertility, especially severe oligospermia and male ASABs.
2 Female ASABs.
3 Synchronous oocyte donation.
4 Repeated GIFT failures.

OVARIAN STIMULATION

The stimulation schedule is precisely the same as applied for IVF. However, with male factor infertility, a relatively high number of oocytes is required for the consideration of additional spermatozoal preparation and fertilization enhancement techniques including micromanipulation and split fertilization by donor and husband. One should aim to generate at least six to eight mature oocytes and this can usually be achieved by increasing the hMG dosage when required. This is facilitated by preliminary GnRH analogue down-regulation of the pituitary. Ideally, one should aim for the controlled situation of hCG trigger 10 000 IU given on the sixth day of consecutive E2 rise for clomiphene citrate/hMG and seventh day of E2 rise for leuprolide/hMG (see Figs 4.5 and 4.6).

PROCEDURES

1 *Oocyte recovery*. This is performed by the transvaginal ultrasound-directed technique previously described (see Chapter 9). Light general anaesthesia or intermittent intravenous anaesthesia (e.g. with propofol) is preferred and the patient may be discharged 4 h later or remain overnight for the transfer procedure in the morning.
2 *Pronuclear stage transfer*. The procedure is performed at laparoscopy under full general anaesthesia with endotracheal intubation. Tubal cannulation is performed precisely as for GIFT transfer and ideally three pronuclear stage oocytes are transferred 4 cm down one fallopian tube using the GIFT catheter. Patients are discharged home that evening and, as for GIFT patients, no restrictions are placed on their subsequent activities. Occasional pregnancies have been reported following transcervical catheterization of the fallopian tube (see TC-TEST, Chapter 13) and this may lead to major cost benefits.

LABORATORY ASPECTS

The first stage of the PROST technique is the same as for IVF-ET. Oocytes are recovered from the follicular aspirates, washed, graded and transferred to Falcon tubes containing 1 ml of culture medium. These are placed in the incubator and the husband produces semen 4 h later. If the husband has highly viscous semen or is known to have ASABs present in the semen, the ejaculate is collected directly into culture medium for assisted liquefaction and extraction of motile spermatozoa (see Chapters 7 and 9).

Where women have ASABs present in their serum, donor serum free of ASABs is substituted within the flushing, insemination and culture media.

For severe oligospermia, the following adaptations should be considered:

1 If the overlay method does not allow sufficient motile spermatozoa to be harvested, the sedimentation technique is used and the supernatant aspirated to obtain motile spermatozoa. The method does not permit a completely cellular- and debris-free harvest of spermatozoa but is a necessary compromise.
2 Because of the functional disturbance associated with oligospermia and asthenospermia, IVF for severe oligospermics requires

200 000–500 000 motile spermatozoa per ml (the precise number is proportional to the degree of severity of oligospermia). This may be associated with a slight increase in the proportion of multipronuclear ooctyes occurring, but remains less than 10% if cases are appropriately selected.

3 If insufficient spermatozoa are available to permit insemination of individual oocytes within individual tubes, oocytes should be grouped, preferably according to maturity, although sometimes all oocytes may have to be inseminated together because of insufficient numbers of spermatozoa.

4 The use of two ejaculates collected at an interval of 24 h or less has been explored (Check and Chase, 1985). Both short and long intervals can lead to an increase in the total number of spermatozoa available for fertilization; the best fertilization and pregnancy rates occur after short interval double-ejaculate production.

5 If there has been a history of failed fertilization at IVF, couples can be offered pentoxifylline (PF) to enhance spermatozoal motility and improve their chance of fertilization. Currently this therapy is under evaluation, with each case providing a control and experimental aliquot of sperm. However, a control may not always be available if spermatozoal numbers are severely reduced. Controls are washed with culture medium containing 10% heat-inactivated human serum and experimental samples are washed with the same medium containing 1 mg/ml PF. After 45 min in either control or PF-containing medium, the sperm suspension is centrifuged and the pellet overlaid with PF-free medium. After 1 h more, samples of the supernatant are removed for study and insemination. The preliminary observations of this approach are very promising (Yovich et al., 1988). PF is a methylxanthine phosphodiesterase inhibitor and was shown to improve the counts of total motile and total progressively motile spermatozoa in cases of oligospermia/asthenospermia. Application of this agent in the PROST programme for a series of 9 couples presenting for treatment with a history of repeated failed fertilization in vitro resulted in successful fertilizations and 5 pregnancies. No fetal abnormalities were recognized in the first 7 children born following PF-enhancement and mice studies undertaken at PIVET did not reveal any congenital abnormalities from PF exposure (Edirisinghe et al., 1988). More recently, a revised protocol has been developed (Yovich et al., 1990; see also Chapter 19).

6 A number of techniques have been proposed using micromanipulation methods to fertilize oocytes, e.g. the direct injection of spermatozoa into the oocyte or the perivitelline space, zona splitting and zona drilling. We have explored both the zona drilling and zona splitting techniques which resulted in generation of normal embryos (Odawara et al., 1988). The method requires three micromanipulator arms (Plates 12.1 and 12.2). However, the alternative technique of partial or complete zona drilling by applying acid Tyrode's medium (pH 2.5) to the zona (Plate 12.3) using a Picospritzer instrument has also enabled the successful generation of morphologically normal cleaving embryos up to the blastocyst stage. This can be achieved with motile spermatozoal counts of the order of 5000–10 000/ml for oligospermic men. It is proposed to transfer embryos generated by micromanipulation in the near future as electron microscopy failed to reveal abnormalities in embryos generated following these techniques. It is expected that the method will contribute significantly to the management of oligospermic and asthenospermic infertility. To date few successful pregnancies have been reported—several from the method of partial zona dissection (Cohen et al., 1989) and one from the technique of microinjection of spermatozoa into the perivitelline space (Ng et al., 1990).

7 Delayed fertilization is common in severe oligospermics and the usual time of pronuclear check may leave some uncertainty as to whether fertilization has taken place as pronuclei many not yet have formed fully. Reinsemination can be performed with the initial semen preparation if there is a high grade of motility. Usually, however, a fresh semen sample is obtained and prepared, often with PF enhancement, and reinsemination is performed. A pronuclear check is then undertaken the following day and such oocytes as may remain unfertilized have reached the pronuclear stage—indicating fertilization on reinsemination—or be found to have developed to the two-cell stage, indicating delayed fertilization from the initial insemination. Pregnancies have been achieved in the solitary situation where delayed fertilization and even fertilization on reinsemination has occurred, albeit only occasionally. Three such pregnancies have occurred at PIVET but have been blighted ova on each occasion. The value of continuing with reinsemination is currently under review.

8 In cases where failed fertilization is likely, the option of split fertilization can be considered. This involves dividing the harvested oocyte population into two groups so that up to four randomly selected oocytes can be fertilized by donor sperm. This provides three benefits: firstly, comparative knowledge of husband's fertilizing capacity with that of a known donor; secondly, the likely option of having at least some embryos available for transfer in that treatment cycle (however only donor or husband embryos should be transferred for ethical reasons); thirdly, the advantages for the IVF programme are of generating research knowledge concerning comparative gamete fertilization in a matched setting, and redundant embryos may become available for donation or approved research studies.

OVUM DONATION

Ovum donation oocytes are best transferred in the PROST or TEST programmes for the following reasons:

1 Preservation of anonymity between donor and recipient is possible.
2 One selects only fertilized oocytes for transfer, realizing that supernumerary oocytes from GIFT patients donating oocytes are generally of lower quality and may not have a high fertilization rate.
3 Selection of the appropriate stage for transfer (PROST or TEST) depends upon any preliminary need for cryopreservation.

Oocytes donated from GIFT cases are washed and prepared as for IVF. The husband of the recipient is notified and produces his semen sample approximately 4 h later. Insemination and fertilization are undertaken as for IVF-ET. Oocytes are transferred in synchrony if the recipient is ovulatory. Oocytes can be transferred between days 15 and 19 for those on hormone replacement therapy (see Chapter 14).

The pronuclear stage check is performed at the usual time, i.e. 12–18 h after insemination. The case will proceed to PROST if a satisfactory number of fertilized oocytes has been obtained. However, if reinsemination is contemplated, the case may be delayed until the following day for a combined TEST–PROST procedure.

LUTEAL PHASE MANAGEMENT

Management of the luteal phase for PROST is the same as for GIFT. It appears that a high P4 level in the immediate postovulatory stage is not essential for tubal transfer procedures, unlike the requirement for uterine transfer. Hence a routine of simply giving hCG 1000 units on days 4, 7, 10 and 13 of the luteal phase is considered. However, if poor luteal function is demonstrated on routine luteal phase monitoring, in subsequent cycles a combination of Proluton and hCG is given as for IVF-ET.

OUTCOME

From the outset, the pregnancy rate per transfer in PROST was noted to be high (Table 12.1), at around 36% overall, with the chance of pregnancy mainly dependent upon the number of fertilized oocytes transferred; being 14–18% per oocyte. In order to avoid high order multiple pregnancies, a limit of three pronuclear oocytes per transfer was established. However, as the case selection increased to include

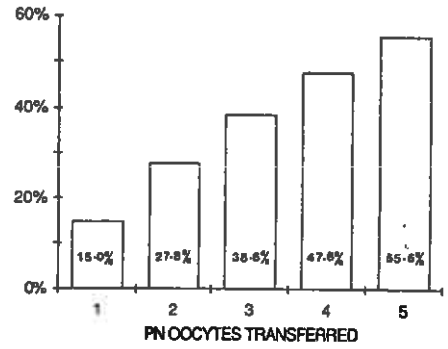
many more failed GIFT and other cases, the implantation rate per

Table 12.1 PROST date from PIVET (January 1987–June 1988)

PN oocytes transferred	*1*	*2*	*3*	*4*	*5/6*
Pregnancies	1/11	1/11	8/20	22/56	8/10
pregnancy rate	9%	9%	40%	38%	80%
Pregnancy sacs	1/11	1/22	10/60	31/224	15/52
implantation rate	9%	5%	17%	14%	28%

From June 1988, PROST programme postponed during evaluation of TEST programme.

Total collections = 138
Pregnancy rate/transfer = 39/108 (36.1%)
Preg. sacs/oocytes transferred = 58/367 (15.8%)



TWINS	7.87	13.76	19.93	23.56	Multiple Pregnancy Risk %
TRIPLETS		0.83	2.17	4.11	
QUADS	-	-	0.10	0.36	
QUINS	-	-		0.012	

Fig. 12.1 Pregnancy rates following PROST are related to the number of pronuclear (PN) oocytes transferred. The multiple pregnancy increases with the number of pronuclear oocytes transferred.

oocyte transferred decreased. It was therefore considered reasonable to transfer higher numbers of fertilized oocytes in some cases (e.g.

Table 12.2 Pregnancy outcome in patients conceiving following PROST (March 1986–June 1988)

Pregnancy outcome	*No. pregnancies (%)*	
Early wastage (< 20 weeks)		
preclinical	6	(7.0%)
blighted ovum	9	(10.5%)
miscarriage	5*	(5.8%)
ectopic	4†	11(4.7%)
Total	24	(27.9%)
Late pregnancy outcome (> 20 weeks)		
with surviving infants	60	(69.8%)
without surviving infants	2‡	(2.3%)
Total	62§	(72.1%)
Combined total	86	(100.0%)

* Includes one hydatidiform mole.
† Includes one heterotopic.
‡ Perinatal mortality—3 infants (3.4%).
§ Total infants—87.

Congenital abnormalities: nil
Pregnancy distribution:
Singleton: 45 (64.5%)
Twin: 11 (17.7%)
Triplet: 5 (8.1%)
Quin/quad: 1 (1.6%)

women of advanced age or those with repeated failures) but increased multiple pregnancies including a quintuplet occurred. The relative risk rates of multiple pregnancy per oocyte transferred is shown in Fig. 12.1.

The preliminary observations on the outcome from PROST indicates less early pregnancy wastage (around 25%) when compared with IVF-ET with almost 75% of pregnancies proceeding beyond 20 weeks gestation. Table 12.2 shows the data for the first 86 PROST pregnancies generated (also see Fig. 16.2). In particular, the ectopic pregnancy rate (4.7%) has so far been lower than that noted with GIFT or IVF-ET. This possibly relates to case selection in that there is less often any significant female factor—female ASABs not being seen as a contributory cause to ectopic pregnancies. Where ectopic pregnancy has occurred, it has usually been in women with previous tubal surgery or previous ectopic pregnancy indicating that such cases should now be excluded.

In common with pregnancies following GIFT and IVF-ET, there is a higher risk of preterm delivery, mainly due to multiple pregnancy, but this is also seen in singleton pregnancy. So far no fetal abnormalities have been reported in PROST infants at PIVET.

Addendum

See Chapter 19 for current (1988–1990) developments.

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Chapter 13

Tubal embryo stage transfer (TEST)

Summary: Tubal embryo stage transfer: TEST

Indications	Asynchronous ovum donation	
	Severe male factor (e.g. micromanipulation)	
	Embryo transfer after cryopreservation	
	Uncertain tubal access	
	Surrogacy	
Ovarian stimulation	Clomiphene citrate/hMG	
	Lucrin/hMG	
	6–7 days of sustained E2 rise	
	Hormone replacement therapy	
Oocyte recovery	36 h after hCG trigger 10000 IU or augmented LH surge	
	Transvaginal ultrasound-directed	
	PIVET-Cook aspiration/flush needle	
Laboratory procedure	Oocyte grade/4	
	Semen collected 4 h after	
	Insemination at 6–16 h	
	Pronuclear check 12–18 h post-insemination	
	HTFM + 10% maternal serum	
	PF prep/micromanipulation	
	Culture to embryo stage	
	Cryopreservation where required	
Embryo transfer	40–48 h	
	2- to 8- cell embryos grade/4	
	Transfer up to 3 embryos	
	Cook catheter 4 cm into each tube	
	3 into 1 tube laparoscopy/TC-TEST	
Luteal phase	hCG imi 1000 days 4, 7, 10 and 13	
	imi Proluton for ovarian failure cases	
Results	Pregnancy rates/transfer	20–35%
	Sacs/embryo transferred	12–17%
	Pregnancy wastage (higher ectopic pregnancy risk)	35–39%
	Multiple pregnancy	10–20%

TEST was developed as an extension of the PROST procedure (Yovich et al., 1987), with particular reference to the ovum donation programme and involved the transfer of early cleavage stage embryos into the fallopian tubes. This situation usually occurred when the embryos had previously undergone cryopreservation for variable periods to permit synchronous transfers following asynchronous fertilizations. The early experience was rather remarkable in that the first three patients conceived; 30% of the embryos implanted (Yovich et al., 1988).

These observations in humans contrast with the animal studies of Vanderhyden and his colleagues (1986) which suggested the benefits of in vivo rather than in vitro fertilization and where possible, reducing the in vitro culture period to a minimum. They showed a reduced size of implantation sites and reduced progeny after IVF, as opposed to in vivo fertilization where all gametes had been previously handled in vitro.

Their findings may still have some relevance when comparing natural single egg fertilization with IVF procedures as a whole. However, their results do not explain the differences between IVF-ET, GIFT, PROST and TEST treatment methods. It would appear that the IVF techniques for gamete preparation, insemination, fertilization, pronuclear stage handling and subsequent development up to 8-cell embryos are entirely satisfactory and there may not be a special benefit obtained by returning such gametes and embryos to the 'in vivo' situation at the earliest possible opportunity. It now seems that the early uterine environment is relatively unfavourable for several days after ovulation and that gametes, early fertilized oocytes and early cleaving embryos derive benefits from being in the tubal rather than the uterine environment. Whether the in vitro environment is better than the intrauterine up to the blastocyst stage is still to be determined. Previously, even in the best centres <30% of fertilized oocytes developed to expanded blastocysts in vitro (Whittingham and Penketh, 1987). Following the early success with TEST, a number of clinics have adopted it in preference to PROST (e.g. Dalmaceda et al., 1988).

INDICATIONS

1 Ovum donation—particularly for asynchronous fertilization where a period of cryopreservation is required to achieve syngamy.
2 Cryopreserved embryos from previous treatment cycles of GIFT or PROST.
3 Male factor infertility—with severe oligospermia and asthenospermia, a 48 h period of culture may be preferred to allow for delayed and reinsemination fertilization. Furthermore, those oocytes sub-

jected to micromanipulation procedures may be better assessed at the 4-cell stage prior to transfer.

4 TEST is also preferred for those cases having transcervical tubal transfer. In many cases the possibility of tubal cannulation remains uncertain and the procedure may need to be converted from tubal transfer to uterine transfer. It is likely that 4-cell and 8-cell embryos will fare better in the uterine cavity rather than pronuclear stage ET in that situation.

5 Similarly, TEST is preferred for attempted laparoscopic tubal cannulation in cases suspected to be difficult (e.g. after tubal reconstructive surgery). If the procedure fails, one can revert to uterine ET at the same procedure.

6 Where IVF surrogacy is practised (see Chapter 15), TEST is the preferred technique for the surrogate woman if she has normal fallopian tubes. It allows selection of the highest grade embryos to maximize the chance of conception.

7 TEST may be preferred in all situations over both GIFT and PROST by some, as it offers greater control over the quality of embryos transferred. Potentially, this may lead to increased efficiency, a reduced risk of multiple pregnancy and less early pregnancy wastage. However, it is premature at this stage to advise such an approach until larger studies have been assessed. For example, recent data indicates that implantation rates are reduced in proportion to the period of in-vitro culture (Yovich, 1990; and Chapter 19).

OVARIAN STIMULATION

The schedule is the same as previously described for IVF-related procedures. Again, as with PROST, a high number of oocytes (6–8) are preferred for male factor infertility and this can usually be achieved by adjusting the hMG dose schedule, especially if pituitary function has been down-regulated by a GnRH analogue. Ideally, follicle aspiration is based on an hCG trigger of 10 000 IU 35–36 h prior to follicle aspiration.

For transcervical TEST the Proluton/hCG schedule may be preferred as embryos may not have been confidently placed within the fallopian tubes, instead residing finally in the fundal uterine cavity.

PROCEDURES

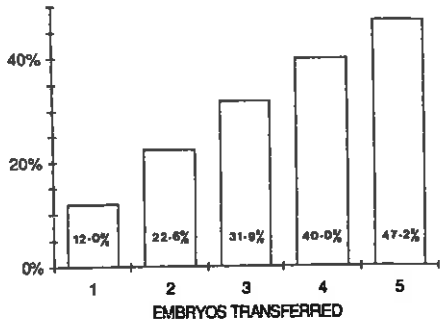
Laparoscopic TEST

1 Follicle aspiration is performed as for PROST using transvaginal ultrasound-directed oocyte recovery, as previously described (see Chapter 12).

2 After oocyte recovery, patients are discharged 4 h later and are readmitted 2 days later as a day-case TEST transfer.

3 The method of transfer is the same as for PROST, with full general anaesthesia and endotracheal intubation. Up to a maximum of four embryos (two to each side) are transferred into the fallopian tubes via the GIFT catheter.

So far, 46 pregnancies have been generated in 105 treatment cycles by laparoscopic TEST (44%). Fifty-seven gestational sacs were identified from a total of 327 embryos transferred (17%). These results are similar to those seen with PROST (Fig. 13.1); the risk of multiple pregnancy is around 25%. However, 7 of the 46 pregnancies were tubal ectopics and in all cases there was known underlying tubal disease



TWINS	-	6.27	11.74	16.46	23.23
TRIPLETS	-	-	0.52	1.47	2.74
QUADS	-	-	-	0.05	0.18
QUINS	-	-	-	-	0.005

Multiple Pregnancy Risk %

Fig. 13.1 Pregnancy rates following TEST are related to the number o embryos transferred. The multiple pregnancy rate increases with the numbe of embryos transferred.

(partial tubal occlusions, fimbrial agglutination, peritubal adhesions previous tubal reconstructions). The risk of ectopic pregnancy in th group appears inordinately high (15% of the total; 40% of those wit known tubal disorders), hence tubal transfer of embryos in such case is no longer advised. Other early embryo wastage is similar to PROS

and so far 18 of the remaining pregnancies have delivered, including pregnancies from cases of very severe oligospermia in which pentoxifylline enhancement in vitro was used. So far there have been no late pregnancy losses and the infants have been entirely normal.

Transcervical TEST

The technique of catheterization of fallopian tubes from the vagina under ultrasound control was described by Jansen and Anderson (1987) from Sydney. They had described a tubal insemination technique which was then adapted for use in the TEST programme (Yovich, 1989). It was scheduled to be applied for asynchronous ovum donation patients, some cases of very severe oligospermia and also for some patients with distal fallopian tube disease who failed to conceive with IVF-ET on several occasions but who might be considered for proximal tubal cannulation.

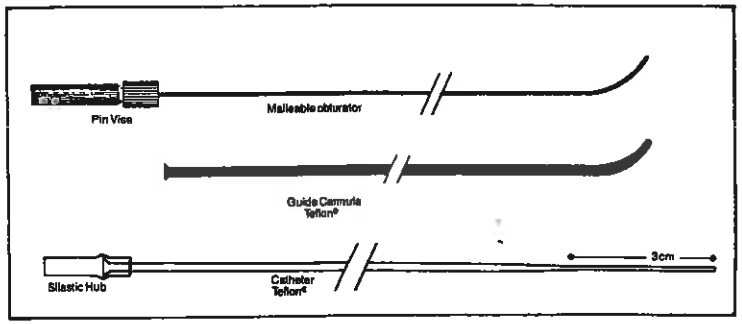


Fig. 13.2 Intratubal transfer set used for transcervical TEST cases.

The system (Fig. 13.2) consists of:

1 A soft Teflon 3 French open-ended inner catheter, 33 cm long, tapered to 2 French 0.66 mm for its distal 3 cm.
2 A firm but flexible opaque Teflon 5.5 French outer cannula 28 cm long, bearing a lateral curve for entering the uterine angle.
3 A malleable metal obturator which over-rides the outer cannula's laterally directed curve during transit of the cervix. This equipment is available from William Cook Australia.

The procedure can be performed without any anaesthesia and involves careful cannulation of the cervical canal without using a tenaculum or dilator for the cervix. In our own modification we have found it useful to follow the entry of the cannula by abdominal scanning through a partially filled bladder. Once the outer cannula is introduced to the fundal region of the uterus, the metal obturator is withdrawn. The natural memory in the outer cannula will curl it to the left or right and this should be checked prior to attempted cannulation. The inner catheter with embryos loaded (similar to the GIFT catheter) is introduced via the outer cannula, pushed through the distal opening of the outer cannula and 'felt' in the tubal ostium region until the resistance disappears. One then cannulates the fallopian tubes via the interstitial and isthmic portion to the hub of the inner catheter. An unanaesthetized patient can usually sense successful tubal cannulation with precise location to the appropriate side although the procedure is not painful. Sometimes cannulation can be visualized through the abdominal scan or by the transvaginal probe (5.0 mHz; General Electric) which can fit comfortably between the blades of a bivalve speculum. With a satisfactory fit, the inner catheter is 3–4 cm within the tubal lumen and embryos can be transferred—up to two per side.

In our experience of 19 transfers, the uterine configuration appears to dictate ease or difficulty of this procedure. A subarcuate or slightly subseptate uterus appears to have the optimum configuration as the catheter seems to be directed readily towards the inner ostium. However, a pear-shaped cavity may cause one to be 'feeling' for the tubal ostium for quite some time, creating some mild trauma to the uterine wall. The T-shaped planar uterus may be impossible to cannulate as the uterine shoulder around which one has to negotiate may not allow the cannula to reach the tubal orifice and this can create minor trauma within the cavity by trying to feel for position (Yovich et al., 1990). If one fails to cannulate the tubal orifice readily, the embryos should be deposited in the uterine fundal region as for IVF-ET.

The first two patients who had transcervical TEST were both cases of occlusive distal tube disease who had failed to conceive by IVF-ET on at least three previous occasions. Neither could be considered for a laparoscopic tubal procedure. In both cases the procedure appeared relatively easy and there was minimal discomfort to the unanaesthetized women who proceeded home 1 h later. The second woman conceived and has now delivered a healthy infant. Nineteen procedures have now been completed, resulting in three pregnancies (16%). A total of 57 embryos were transferred in the group and six pregnancy sacs have been identified (two singleton and one quadruplet), i.e. 11% of embryos have implanted. The pregnancy rate is between the results of IVF-ET and PROST, probably indicating that tubal cannulation was not achieved successfully in some cases, thus leading to an outcome which was closer to that of IVF-ET. One of the pregnancies was a

tubal ectopic, which occurred in a woman with known underlying tubal disease. The increasing experience with TEST (both laparoscopic and transcervical) indicates a high risk of ectopic pregnancy where there is known underlying tubal disease, hence we are now reluctant to consider the method in this situation. The other two pregnancies are ongoing; one has already had a healthy female infant with a spontaneous vaginal delivery.

LABORATORY PROCEDURES

The techniques have been described fully in the sections on IVF and PROST. Cryopreservation methods, including the thaw procedure are described in Chapter 14. Embryos are loaded into the GIFT catheter in precisely the same way as described for PROST and the total group can be loaded into separate segments for a single cannulation transfer, depositing two embryos into each fallopian tube at a point exactly 4 cm from the fimbrial end. The loading is similar for the transcervical cannula, as previously described.

OUTCOME

The early results from TEST were very encouraging with a pregnancy rate >40% (Yovich, 1988) but the rate has not persisted. The results from TEST are directly influenced by the indications for the procedure; e.g. TEST after cryopreservation is dependent on the success of the cryopreservation procedure and quality of embryos after thawing; it is also dependent upon the proportion of male factor cases in the series and the number performed by the transcervical method. However, even after expanding its indications, the overall pregnancy rate for laparoscopic TEST is 28% and 12% of embryos have implanted (Table 13.1), although 11% of pregnancies occurred within damaged fallopian tubes. Transcervical TEST has not been so successful, with a pregnancy rate of only 16.7%. Whether one persists with transcervical TEST will depend upon a clear definition of those cases for which the procedure might be suitable, and the improvements in instrumentation and its localization by ultrasound.

To date, early pregnancy wastage from TEST has been high from ectopic pregnancies and only 61% of pregnancies have proceeded beyond 20 weeks (Table 13.2; see also Fig. 16.2). A quadruplet pregnancy has occurred in one of the transcervical TEST cases, indicating the danger of transferring more than 3 embryos. The risk of preterm labour is also probably increased, in common with other IVF-related procedures.

Table 13.1 Pregnancy rates and implantation rates from TEST (January–December 1989)

Embryos transferred	1	2	3	4*
Pregnancies	1/6	5/18	29/102	2/8
pregnancy rate	16.7%	27.8%	28.4%	25.0%
Pregnancy sacs	1/6	5/36	48/306	2/32
implantation rate	16.7%	13.9%	15.7%	6.3%

* Maximum of 3 embryos transferred after February 1989.

Total collections = 156
Pregnancy rate/transfer = 37/134 (27.6%)
Preg. sacs/oocytes transferred = 46/380 (12.1%)

Table 13.2 Completed pregnancy outcomes in patients conceiving following TEST (January 1987–December 1989)

Pregnancy outcome	*No. pregnancies (%)*	
Early wastage (< 20 weeks)		
preclinical	3	(3.8%)
blighted ovum	15	(18.8%)
miscarriage	4	(5.0%)
ectopic	9	(11.3%)
Total	31	(38.8%)
Late pregnancy outcome (> 20 weeks)		
with surviving infants	46	(57.5%)
without surviving infants	3*	(3.8%)
Total	49†	(61.3%)
Combined total	80	(100%)

* Perinatal mortality—4 infants (5.7%).
† Total infants—70 (includes 20 pentoxifylline infants).

Congenital abnormalities: nil
Pregnancy distribution:
Singleton: 30 (61.2%)
Twin: 18 (36.7%)
Triplet: 0
Quad/quin: 1 (2.0%)

All infants have been born normal and in good condition. This is particularly encouraging as it includes five infants following cryopreservation, the first of which was born at PIVET in October 1987 and was the first frozen-TEST infant born worldwide. Within the programme such cryopreservation-TEST procedures are known as FROST (frozen embryo salpingo-transfer).

FUTURE DIRECTIONS FOR TEST

The experience to date reveals three important messages. Firstly, the procedure of TEST permits the selection of high-grade embryos for transfer to the tubal site where the chance of pregnancy is much higher than following uterine transfer. IVF groups should consider exploring the advantages of TEST in those cases currently being considered for GIFT or PROST. There are also certain disadvantages with regard to the two-stage procedure and wide time separation from the date of collection to the date of transfer.

Secondly, the accumulating data from all tubal transfer procedures indicate that the transfer of gametes, pronuclear stage oocytes and cleaving embryos to the fallopian tubes carries a high risk of ectopic pregnancy if there is known to be *any* degree of underlying tubal disease. Such cases can therefore only be relatively safely treated by conventional IVF-ET with the transfer of cleaving embryos to the uterine cavity.

This leads to a third message which begs the question of how to improve the results of conventional IVF-ET so that the implantation rate is equivalent to that achieved with GIFT and PROST procedures, i.e., at around 40%, and which is significantly higher than the rate for IVF-ET. It is likely the answer will lie in the improvement of in vitro culture methods, permitting the transfer of embryos at a later stage, e.g. morula or blastocyst at day 5.

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Chapter 14

Ovum donation

The first successful case of ovum donation was reported in a woman with primary ovarian failure, by the Monash group (Lutjen et al., 1984). Lutjen and his colleagues described a regimen of ovarian steroid replacement with oestradiol valerate and intravaginal progesterone pessaries for the woman over two preliminary cycles. During the third cycle another woman undergoing IVF-ET donated one oocyte which was fertilized by the recipient patient's husband, and subsequently a two-cell embryo was transferred to the recipient woman's uterus on day 16. In that cycle the progesterone pessaries were replaced by intramuscular progesterone, and hormone replacement was continued, to be withdrawn gradually between the 12th and 19th weeks. A healthy male infant was delivered at 38 weeks' gestation. Following this report, ovum donation programmes were added to the range of services provided by established IVF-ET centres.

INDICATIONS

1 Primary ovarian failure.
2 Absent ovaries (congenital or surgically removed).
3 Safe access to ovaries denied.
4 Poor response to ovarian stimulation regimes.
5 Incipient ovarian failure.
6 High risk of transmitted genetic disease to offspring.

It is of interest that the authors did not proceed with ovum donation in group number 3 (above) as the ultrasound-directed techniques of follicle aspiration have obviated the need for ovum donation in virtually all cases in that category. Also, in recent months, the use of GnRH analogues for down-regulation of pituitary function and superimposed stimulation with high dose gonadotrophins have permitted many patients from group 4 to re-enter IVF programmes, again using their own oocytes. In some of these women, the quality of oocytes has been poor and others remain poorly responsive to stimulation and are therefore categorized in group 5—incipient ovarian failure.

Women in group 6 include many with autosomal dominant conditions such as Huntington's disease, dystrophia myotonica, fragile

X disease and hereditary microcephaly, and some with X-linked diseases such as muscular dystrophy and haemophilia. In some of these women the indications may disappear as the techniques of embryo biopsy and preimplantation embryo diagnosis using recombinant DNA techniques will enable preselection of embryos prior to transfer.

DONOR OOCYTES

There are three potential sources of oocytes:

1 *Supernumerary oocytes following GIFT procedures.* Approximately 50% of couples in the GIFT programme agree to donate supernumerary oocytes. These are largely derived from those with unexplained infertility; those having PROST procedures for oligospermia require additional oocytes to effect fertilization for themselves. However, if more than eight oocytes are recovered, such patients will sometimes donate the excess.
2 *During sterilization procedures.* Although many women at first express a desire to assist infertile couples and donate oocytes, in practice only a small number appear willing to undergo the inconvenience of stimulation, monitoring and uncertain scheduling of their case for the procedure.
3 *Relatives or friends.* In many cases, a close relative (e.g. sister or mother) or a close friend wishes to donate oocytes to a specific woman. Currently this approach is not favoured by major institutional ethics committees although occasional reports in which a known donor is involved have been published (Leeton et al., 1986; Craft et al., 1987).

RECIPIENT PREPARATION

For recipients who are ovulating, it is simply a matter of maintaining a track of the patient's cycle and offering donor oocytes to those women who happen to be in synchrony with the recipient. For those women who might be considered, close periovulatory monitoring should be performed in order to synchronize with the late LH surge, i.e. selecting those recipients who are closest to the donor. In practice, we have found that pronuclear stage oocytes and early cleaving embryos can be transferred between days 15 and 19 of the recipient's cycle where day 13/14 identifies the LH surge and concomitant progesterone rise. It appears that donated embryos can be up to 24 h ahead of the recipient's cycle without compromising the chance of pregnancy—these comments pertain to tubal transfer. For uterine transfer, closer synchrony may be required.

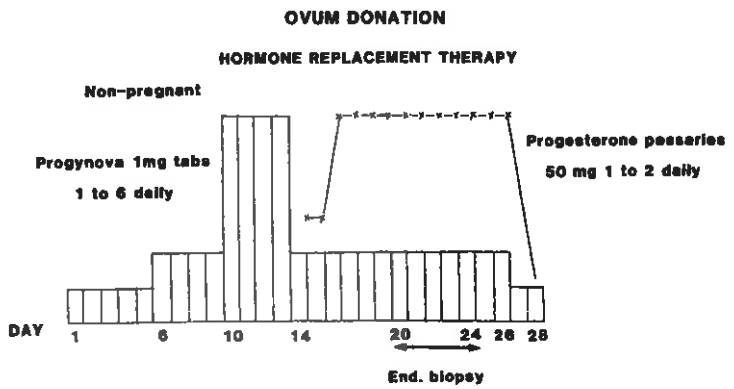

Fig. 14.1 Hormone replacement protocol during preliminary cycles for women with primary ovarian failure. Endometrial biopsy is performed on the second treatment cycle to assess adequacy of the endometrial response. From Lutjen et al. (1984).

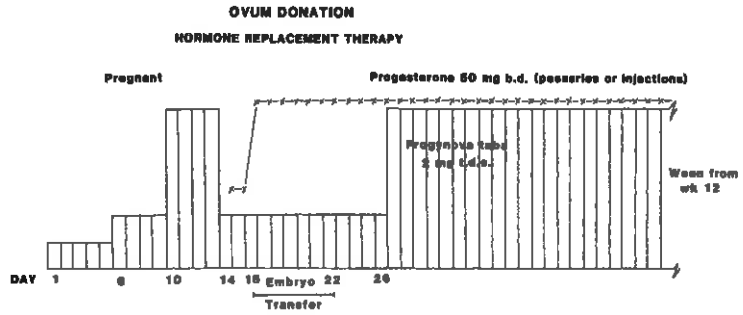

Fig. 14.2 Hormone replacement protocol during ovum donation treatment cycle whereby pronuclear oocytes or cleaving embryos are transferred to the fallopian tubes, generally between days 15 and 19. From Lutjen et al. (1984).

Women in ovarian failure or incipient ovarian failure require hormonal replacement therapy. A modification of the Lutjen regimen (Fig. 14.1) can be used, with intramuscular progesterone injections replacing vaginal pessaries during the transfer cycle (Fig. 14.2). An effective flexible schedule currently applied at Monash (Leeton et al., 1989) and now used at PIVET treats patients in a 33-day regimen using oestradiol valerate (Progynova) from days 1 to 33 with progesterone pessaries 100 mg b.d. from days 23 to 33 inclusive. One can consider embryo transfer from days 10 to 25 simply by adjusting the progesterone pessaries to begin on the day when donor oocytes become available. It is still advisable to check an endometrial biopsy during a preliminary cycle to determine that a satisfactory endometrial response has occurred. Occasionally, higher-dose Progynova is required. In addition, PROST and TEST have proven far superior to uterine transfer of early cleaving embryos.

LABORATORY ASPECTS

Cryopreservation of embryos

Preparation for freezing using DMSO

Human embryos are frozen at the 4-cell to 8-cell stage while mouse embryos are best frozen at the 2-cell, 4-cell or 8-cell stage. Embryos are frozen and thawed in a medium called PB1 (see Chapter 9) which is actually a modified version of Dulbecco's buffered medium. This medium is phosphate-buffered and does not require carbon dioxide to control its pH, which permits working at room temperature (approximately 22–24°C) on the bench. Using media less than 2 weeks old, 3 mg/ml of BSA is added to PB1 as a protein supplement when freezing and thawing mouse embryos. Heat deactivated human serum (10% HS) is generally used for freezing and thawing of human embryos. Until recently, the cryoprotective agent used was dimethylsulphoxide (DMSO), but lately the use of propanediol (PROH) has been explored.

A 3 mol stock solution of DMSO is prepared in a 10 ml Falcon tube by adding 2.2 ml of DMSO to 7.8 ml of PB1 + BSA (or PB1 + 10% heat-inactivated maternal serum). This is mixed well by inverting the tube. Six Nunc petri dishes (10 × 35 mm) are labelled 0.25, 0.75, 1.0, 1.25 and 1.5 mol on the lid and side. Dilutions of DMSO are made up as shown in Table 14.1. This is mixed thoroughly by gently agitating or swirling dishes until the DMSO has been distributed evenly. Embryos are removed from the tube and placed in the dishes of PB1 + BSA and examined under the inverted microscope. The morphology and grade of the embryos is assessed using the grading system shown in Table 14.2.

Table 14.1 Dilutions of DMSO

Dilutions	*3 mol DMSO*	*PB1 + BSA*
0.25 mol	0.25 ml	2.75 ml
0.5 mol	0.5 ml	2.5 ml
0.75 mol	0.75 ml	2.25 ml
1.0 mol	1.0 ml	2.0 ml
1.25 mol	1.5 ml	2.0 ml
1.5 mol	1.5 ml	1.5 ml

Table 14.2 Method for grading human embryos before freezing

	Yes	*No*
Does the embryo contain four or eight blastomeres?	0.5	0
Are all the cells regular and spherical?	0.5	0
Are all the blastomeres of similar size?	0.5	0
Are there any cytoplasmic fragments present?	0	0.5
Are the cytoplasmic contents even and translucent?	0.5	0
Are the blastomere membranes smooth, glossy and distinct?	0.5	0
Do the blastomeres fill the zona, i.e. the embryo is not contracted?	0.5	0
Is the embryo		
a 4-cell less than 48 h PI age?	0.5	0
a 6–8-cell at 48 h PI age?	0.5	0

PI = post-insemination.
Points are allocated to the embryo based on answers to these questions. Maximum score = 4 points. *All* embryos scoring 2 or more points are frozen.

Freezing procedure

Immediately after scoring, the embryos are removed and placed in 0.25 mol DMSO for 10 min, after which they are moved stepwise through the DMSO dilutions at 10-min intervals, using a pulled glass pipette and mouthpiece. The mouthpiece offers better control in the handling of embryos.

While embryos are in 0.25 mol DMSO, the freezing machine (Planer PTC300) is set up and the dewar pressurized. Embryo scores are calculated and written up on the patient's cryopreservation sheet.

While embryos are in 0.5 mol DMSO, ampoules are labelled and approximately 0.5 ml of 1.5 mol DMSO is placed into each ampoule. Tags are made from surgical silk and masking tape. Canes are labelled and tagged appropriately.

When embryos are in 1.0 mol DMSO, the plastic caps are put on to boil. The freezing programme is checked while the embryos are in 1.25 mol DMSO to ensure it has not been deleted.

After 5 min in 1.5 mol DMSO, the embryos are loaded into Wheat glass ampoules (one embryo per ampoule) and a check is made to ensure that the embryo is in the ampoule, by looking down the microscope into the ampoule. Caps are placed on the ampoules and carefully checked to ensure there are no splits. The ampoule is placed on the cane at the third level from the top and tags are tied around the neck, just below the cap. Silk is wrapped around the folded tag and secured at the top of the cane with a small piece of masking tape. This should take no longer than 5 min. The canes are then placed in the freezing chamber.

Freezing programme number 1 (for glass ampoules)

−2°C/min to −6°C
Hold at −6°C for 30 min

After 10 min holding at −6°C, the ampoules are seeded by touching the top of the media (meniscus) with cold forceps dipped in liquid nitrogen until ice can be just seen to form. The ampoule is replaced in the freezing chamber and checked to ensure that seeding has started in 10 min. Ice streaks (crystals) should be seen forming down the media in the ampoule; if no ice can be seen, the ampoule should be re-seeded. Complete seeding should take approximately 5–10 min, otherwise large crystals may form which can damage the embryos. Programme:

1 −0.3°C/min to −80°C
2 −10°C/min to −110°C to liquid N_2
3 Hold at −110°C for 1 h
4 Plunge into liquid N_2

One should always have a 1- or 2-hour hold programme in at −110°C. The canes are placed in the allocated canister of the dewar and the freezing chamber is warmed up to 20°C and depressurized after turning the machine off. The canister and dewar numbers where embryos are stored are then recorded in the notes.

Thawing of embryos

The freezing machine is turned on and the dewar pressurized. Once the pressure is up, the thawing program is checked and machine cooling down is commenced. Programme:

1 −6°C/min to −80°C
2 Hold at −80°C for 10 min
3 +8°C/min to +4°C

4 Hold at +4°C

While the freezing machine is cooling down, a small sperm transport container is filled with liquid nitrogen and canes with ampoules on them are removed from the storage unit and placed in the container. Using a pair of scissors, the tops of the plastic caps on the ampoules are cut (the Medishield face mask should be worn at all times when handling frozen embryos in ampoules). This prevents the ampoule from exploding during the thawing process should any liquid nitrogen get into the ampoule. Once the machine has reached −80°C, the plastic tops of the freezing machine are clipped on to the canes which are quickly moved into the freezing chamber.

During the thawing process, the DMSO concentrations are set up in the dishes as for freezing.

Once the machine has reached 4°C, it is left for approximately 5 min before the ampoules are checked. The canes are removed once all the ice crystals have melted and ampoules are placed on the bench at room temperature for 10 min. Ampoules are gently agitated to ensure media has not concentrated on the bottom.

After 10 min, embryos are removed from the ampoules and placed in 1.5 mol DMSO and the appearance of each embryo is recorded in the patient's notes in the laboratory. Embryos are left for 10 min in 1.5 mol DMSO. Thawing of embryos is simply the reverse procedure of freezing; however, it is more important that the embryos spend the 10 min in each concentration of DMSO as it has been noted that the zonae will split if moved too quickly through the different concentrations.

After embryos have been moved through final concentration of DMSO in 0.25 mol, they are washed in PB1 + BSA for 10 min each. Embryo quality and appearance are assessed and embryos are transferred to fresh HTFM + 10% human serum for culture overnight before transfer to the patient. Fig. 14.3 shows the before-freeze and post-thaw appearance of an 8-cell embryo cryopreserved in CPM with DMSO.

All notes should be filled out correctly and embryo quality recorded along with any other notes concerning transfer etc. Consent forms should be sent out to each patient on the day the embryos are frozen. It is the responsibility of the laboratory technician involved to follow up the forms and make sure they have been signed and returned. All embryos frozen and thawed are recorded in the records book in the laboratory.

Preparation for freezing using PROH

Recent advances in cryopreservation of embryos include the use of Propane 1–2 diol as a cryoprotectant. Embryos are best frozen at the

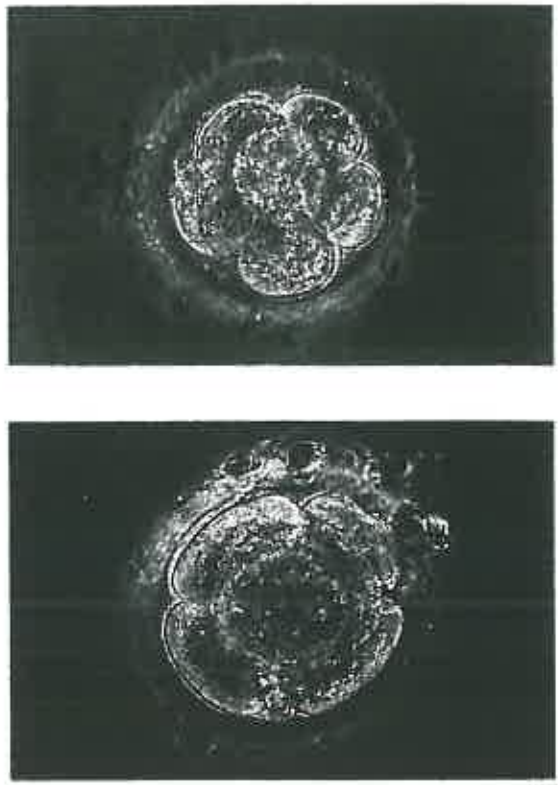

Fig. 14.3 Human 8-cell embryo (a) prior to cryopreservation (a) using DMSO and (b) post-thaw. This was followed by a successful single embryo transfer; and resulted in the delivery of a healthy infant in October 1986.

2–4-cell stage using this technique, although stages from 2 pronuclei through to 8-cell have produced successful results. When freezing mouse embryos the medium used is PB1 supplemented with 3 mg/ml BSA; however for human embryos, heat-inactivated human serum (20% HS) is added.

A solution of 0.2 mol sucrose is prepared in a 10 ml Falcon tube by the addition of 0.6846 g sucrose (S) into 10 ml PB1. This is well mixed. Two Nunc petri dishes (10 −35 mm) are labelled 1.5 mol (solution 1) and 1.5 mol + S (solution 2) on lid and base. The dilution of PROH is made up as shown in Table 14.3. Embryos are graded as previously described.

Freezing procedure

Immediately after scoring, embryos are placed in 1.5 mol PROH for 15 min, using a glass pipette and mouthpiece.

Table 14.3 Constitution of PROH

	Dilution	*PB1 + 20% PROH*	*serum*	*Sucrose (0.2 mol)*	*Time*
1.	PROH 1.5 mol	0.22 ml	1.78 ml	—	15 min
2.	PROH 1.5 mol + sucrose 0.1 mol	0.22 ml	0.78 ml	1.00 ml	15 min

When embryos are in 1.5 mol PROH (solution 1), the cane and goblets are labelled and the goblets attached with masking tape. Straws are labelled using a tag of masking tape at the cotton plugged end. The freezing programme should be checked and a cryopreservation sheet filled in with patient details.

Embryos are transferred into 1.5 mol PROH + sucrose (solution 2) and after 5 min loaded into straws. This is done using a 1 ml syringe (Terumo) to which a 2 cm piece of silastic tubing (cat. no. 602–265) is attached. The straw is attached to the tubing, flushed with medium and loaded with the embryos in the sequence shown in Fig. 14.4.

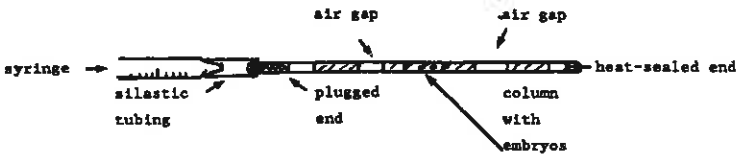

Fig. 14.4 Loading of straws prior to embryo cryopreservation.

The open end is then heat-sealed. It is usual procedure to load one embryo per straw. One should check that the embryo is in the straw and aim at placing it in the centre of the column. The sealed straw can be placed in the goblet and secured with a small piece of tissue stuffed into the goblet. It is then placed in the freezing chamber.

Freezing programme number 2 (for plastic straws)

1 −2°C/min to −7°C
2 Held at −7°C for 10 min

3 After 5 min holding, straws are seeded by touching the top of the meniscus of the column loaded with the embryo, using cold forceps dipped in liquid N_2. The straw is replaced and checked for seeding after a further 5 min
4 0.3°C/min to −30°C
5 25°C/min to −110°C
6 Plunged into liquid N_2

Canes are then put into liquid nitrogen and canister and dewar numbers recorded in the notes. The details are summarized in Fig. 14.5.

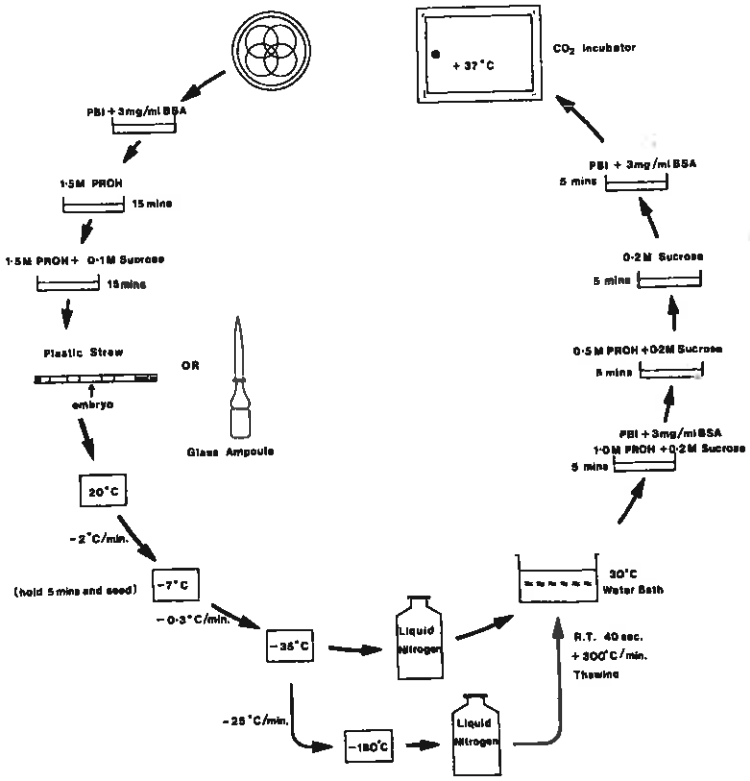

Fig. 14.5 A summary of the protocol for cryopreservation using propanediol as the cryoprotectant.

The PROH regimen has now been applied to a large number of human embryos at various stages between two pronuclei and blastocyst. In all, 62% have survived with >50% of cells intact; of these, 50% developed to fully expanded blastocyst in culture. More recently, we have explored a rapid programme in which the embryos are plunged into liquid nitrogen at −35°C (rather than −81°C), resulting in a survival rate of 80% with 65% growing to the expanded blastocyst stage. These developments have been associated with an improved pregnancy rate in the ovum donation programme.

LUTEAL PHASE/EARLY PREGNANCY

For women who ovulate, the PROST and TEST transfers can be managed in the routine manner, i.e. with hCG boosts on days 4, 7, 10 and 13. However, for those on hormone replacement schedules, intramuscular Proluton is continued (50 mg imi per day) until the 16th week. These cases also require continuation of oestradiol valerate (1 mg); 2 tablets to be given three times a day until 12 weeks' gestation, decreasing to 1 tablet three times a day until 14 weeks', 1 tablet twice a day over the ensuing week and finally 1 tablet daily, ceasing altogether at the end of the 16th week. It is known that many women can continue their pregnancies if the Proluton is ceased by week 12 or even earlier—however, this appears inadequate for some who will demonstrate a fall in progesterone and bleeding on withdrawal of Proluton or transfer from Proluton to progesterone pessaries prior to week 12.

PREGNANCY OUTCOME

Case report

The 26-year-old patient, TM, had a normal thelarche at 13 years and developed secondary sexual characteristics. However, she had primary amenorrhoea when she presented to her general practitioner with pelvic pains at 18 years of age. A gynaecologist was consulted, and noted that she was 158 cm tall, had normal adult female features and an appropriate height–span relationship. No obvious abnormality was detected on pelvic examination apart from mild bilateral tender ovarian enlargement.

The patient had a laparoscopic appraisal followed by laparotomy to excise bilateral cystic gonadoblastomas from streak ovaries. Subsequently, serum gonadotrophins were shown to be persistently elevated (both LH and FSH >50 IU/l) and chromosome assessment on cultured white cells revealed a normal female XX karyotype. Hormone replacement therapy was offered but declined, and she remained amenorrhoeic.

She was married at age 20 years and established normal coital activity. Two years later she wished to explore the possibility of pregnancy and was referred to the PIVET Medical Centre for consideration of possible ovum donation. Further investigations excluded underlying pathology or endocrinopathies. Serum gonadotrophins remained elevated between 50 and 100 IU/l and serum prolactin was normal at 120 mIU/l. At laparoscopic review, the cervix was noted to be mature, the uterine cavity was 5 cm and no curettings were obtainable. Within the pelvis, bilateral streak ovaries were noted and the fallopian tubes were described as thin and juvenile in appearance. The husband was investigated and found to be healthy and normospermic.

Mrs TM had two cycles of hormone replacement therapy using oestradiol valerate and progesterone pessaries. In the second cycle an endometrial biopsy was undertaken on the 22nd day and this revealed normal secretory endometrium. No adjustments were required to the hormone replacement schedule and it was decided to maintain the patient on that regimen until donor oocytes became available.

Between August 1984 and June 1986, a total of five ET procedures were carried out. In all cases oocytes were fertilized in vitro by spermatozoa from the patient's husband and between two and four cleaving embryos were transferred to the uterus. On all but one occasion, the embryos had been cryopreserved for periods of up to 6 weeks to enable synchronous transfers, matching donor embryo stage with the recipient's artificial luteal stage, between days 16–19 (Mohr et al., 1985). On the last two occasions the progesterone pessaries were substituted with intramuscular Proluton, however conception did not ensue.

At review in August 1986, it was decided to undertake further treatments using our recently developed method of PROST. A preliminary hysterosalpingogram was undertaken, which determined that both fallopian tubes were patent. The first attempt was performed in September 1986 and two pronuclear oocytes were transferred, both to the one tube. Pregnancy did not ensue and hence in October 1986 a further PROST treatment was carried out. On this occasion five pronuclear oocytes were transferred at laparoscopy, two to the left tube and three to the right. On both occasions, luteal hormone therapy was provided by Proluton imi 50 mg per day. On day 16 of the luteal phase the serum β-hCG level was 200 IU/l and this reached 1030 IU 3 days later. An ultrasound in the seventh week showed three separate gestational sacs within the uterus with a definable fetus and fetal heart action identified in each. The hormone replacement schedule during pregnancy was as follows: Progynova 2 mg three times a day through to 16 weeks and Proluton 50 mg imi through to 20 weeks. A cervical suture was inserted at 13 weeks' gestation and MPA (Pro-

Table 14.4 Summary of ovum donation at PIVET 1984–1989

(a) *Underlying aetiology of cases treated*

	Cases	*Preg.*	*Cycles*
Premature ovarian failure	13	9	38
Incipient ovarian failure*	32	14	61
Genetic factor	8	2	15
Anatomical	1	0	1
	54	25	115

(b) *Outcome of frozen v fresh embryos with respect to uterine (ET) or tubal (TEST) transfers*

		Cycles	*Preg.*	(%)
Tubal	Fresh	35	13	(37.1%)
(1986–89)	Frozen	5	3	(60.0%)
Uterine	Fresh	24	3	(12.5%)
(1984–89)	Frozen	51	6	(11.8%)

(c) *Pregnancy rate and implantation rates (gestational sacs arising per embryo transferred)*

		Preg.	(%)
Uterine ET:	Pregnancy rate	9/75	(12.0%)
(1984–89)	Implantation rate	10/229	(4.4%)
TEST:	Pregnancy rate	16/40	(40%)
(1986–89)	Implantation rate	23/143	(16.1%)

(d) *Pregnancy outcomes*

Blighted ovum	4	(16%)
Miscarriage	1	(4%)
Ectopic	2	(8%)
Births	19	(76%)

vera) tablets were prescribed 20 mg qid as part of a study on multiple pregnancies, to evaluate the role of such treatments in preventing preterm delivery (Yovich et al., 1988).

Spontaneous labour ensued in the 32nd week and emergency Caesarean section was performed in May 1987 with the delivery of three infants—two males (1700 g and 1755 g) and one female (1655 g)—requiring minimal care in the neonatal nursery. Their examination details were entirely normal and they have subsequently thrived, being partially breastfed during the first 6 weeks of the post-natal period.

Overall outcome

The above case highlights a number of features described earlier in relation to ovum donation. The pregnancy outcome for this group is a reflection of the techniques applied and is therefore similar to the PROST and TEST series. So far no abnormalities have been noted in any of the infants. This is particularly encouraging, given the fact that a number are infants who began life as frozen embryos. A summary of the overall experience at PIVET (1984–89) is detailed in Table 14.4.

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Chapter 15

Surrogacy

The concept of surrogacy may provide the only 'therapeutic' solution for women with:

1 Absent uterus
   Congenital: 46XX, e.g. Rokitansky–Küster–Haüser (RKH) syndrome; 46XY, e.g. testicular feminization syndrome.
   Acquired: hysterectomy, e.g. for the increasingly common condition of invasive cervical cancer in younger women.
2 Uterine malfunction
   Non-receptive hypoplasia, atrophy, Asherman's syndrome.

Most people are not opposed to surrogacy for medical indications. However, the general public (80%) is opposed to the consideration of surrogacy for non-medical reasons such as occupation (on behalf of a woman who is busy establishing her career), lifestyle (on behalf of a woman who is busy in a sporting or social-life career), and cosmetic (on behalf of those women who may wish to preserve cosmetic body features, e.g. for a modelling career; New South Wales Law Reform Commission, 1987).

Surrogacy arrangements are available in the USA and recently the Infertility Center of New York declared the 300th surrogate baby delivered by arrangements within their clinics. These have been achieved by artificial insemination of the surrogate woman by semen from the commissioning couple. It is estimated that 800 surrogacy cases have been arranged in North America to the end of 1988.

The widely publicized Baby M case was notable because the surrogate mother changed her mind about releasing the infant after delivery. It was the first instance where the father (William Stern) successfully obtained custody of the infant from the surrogate mother (Mary Beth Whitehead) by court action. One State (New Jersey) has ruled that surrogate mother contracts are not valid.

Only isolated instances of surrogacy have been reported in other countries. The Baby Cotton case was well publicized in the UK in 1985; in which a High Court decision granted an adoption order to a commissioning couple with the surrogate mother's consent.

In Australia, one commercial surrogacy arrangement was reported in the popular press (*New Idea*, May 21, 1983), following which advertising for the purpose of surrogacy was made illegal in the State of Victoria by the Infertility Medical Procedures Act of 1984. In April 1988 the first Australian case of IVF surrogacy involving a 35-year-old woman acting as surrogate mother for her sister was reported from Melbourne, Victoria in the media.

The issue of surrogacy has divided professionals considering the clinical, legal, ethical and moral issues. Despite prominent supporters of the concept (Kirby, 1987), there is currently a trend of anti-surrogacy legislation being introduced into most States of Australia.

THE SURROGACY CONCEPT

There are two broad areas of surrogacy for consideration:

1 Where a woman allows herself to be inseminated, using the semen from the husband of an infertile couple, generally where the wife has one of a range of infertility causes which have not proven readily remediable. In this situation the surrogate woman has contributed genetically to the pregnancy via her own oocyte. Such surrogacy arrangements have been undertaken in the USA and the UK, generally as commercial undertakings, and have led to widespread publicity in the situation where the surrogate mother has been reluctant to relinquish the infant to the commissioning couple.
2 Where both gametes or the embryos are provided by the commissioning (infertile) couple and the surrogate woman therefore has no genetic (biological) association with the infant. This situation generally arises where a woman has become infertile as a result of congenital absence of the uterus, or having had her uterus removed because of serious disease such as uterine or cervical cancer. This problem is becoming more common due to the increasing prevalence of early invasive cervical cancer in young women and its treatment by extended hysterectomy. In such cases, the commissioning couples often wish to use a nominated woman (generally a close relative or friend) who would carry the pregnancy for no commercial consideration. This concept has been termed 'compassionate family surrogacy' (Yovich and Hoffman, 1989).

THE SURROGATE WOMAN

In general, infertility services involving donor gametes (either sperm or oocytes) have required the procedures to be performed with due consideration of confidentiality so that neither donor nor recipient can identify the respective person receiving or donating the gametes.

However, we believe that such confidentiality is not relevant to the situation of surrogacy as gametes are not being transferred. Since pronuclear oocytes (i.e. fertilized eggs) or cleaving embryos are transferred in the procedures known as PROST, IVF-ET, or TEST, the surrogate woman does not contribute her own oocytes and therefore has no genetic attachment to the developing fetus. In its simplest context, her uterus provides a harbour for the embryo to implant and develop as a fetus. Following delivery of the infant, it is proposed that the infertile commissioning couple should undertake a formal adoption contract, for which legislation exists in some countries.

When selecting the surrogate woman the following conditions should be satisfied:

1 The woman is providing the service for altruistic reasons and may therefore be a relative or close friend of the infertile commissioning couple.
2 Medical costs and travelling expenses may be covered by the commissioning couple but there is to be no commercial fee for providing surrogate services.
3 The surrogate woman is in the reproductive age range and has children of her own, has completed her own family and does not have an adverse past obstetric history.
4 The surrogate woman understands the need to comply with medical advice regarding good obstetric care, and any specific management advised by the attending medical practitioner.

EXPENSES

The recipients (commissioning couple) will be expected to cover all their own medical expenses involved in gamete collection, fertilization and embryo preparation. They will also be expected to cover reasonable fees encountered by the surrogate woman. There is to be no commercial fee paid to the surrogate woman who will be providing her service for altruistic reasons.

MEDICOLEGAL ASPECTS

The legal implications of medical practices in relation to surrogacy are not universal. At present, it is the presumption at common law that the surrogate mother is the lawful mother of any child born, irrespective of the origin of the genetic material involved. This is enshrined in the Artificial Conception Act (1985) (Western Australia), which

specifies that the birth mother is the legal mother. However, under the Adoption of Children Act (1896–1985) there is allowance of a preferred adoption of a child by a relative or the natural father. This appears to allow for a preferred adoption of a child in a surrogacy arrangement which has a known biological (genetic) attachment to the potential adopting parents.

A research report by the New South Wales Law Reform Commission published in 1987 is one of a series in the area of artificial conception, which indicated that Australian public opinion is not opposed to surrogacy arrangements and that the large majority of Australians support disclosure of the surrogate's identity to the child. However, several deficiencies were noted in this report, including the fact that it did not cover the IVF-surrogacy situation where embryos from a commissioning couple are transferred to a surrogate female who therefore has no genetic attachment. Also, it did not cover the possibility of a friend or relative being the surrogate mother, in which situation the identity of the surrogate woman would always be known to the child who in fact may have a close association with that woman (e.g. an aunt), and therefore the principle of maintaining confidentiality is irrelevant (Yovich, 1987). This is a similar opinion to that noted in the *Report of the Committee Appointed by the Western Australian Government Inquiring into the Social, Legal and Ethical Issues Relating to In Vitro Fertilization and its Supervision* (1986).

CONSENT FORM

A consent form for use in IVF-related surrogacy arrangements is shown in Table 15.1.

Table 15.1 Consent form for use in IVF-related surrogacy procedures

Date

Name of surrogate mother

Name of husband of surrogate mother

Name of father

Name of mother

I/We, the above-named, each hereby consent to the IVF procedure and authorize Dr _____ and whomever he/she may designate as his/her assistants, to perform the following operations:

1 Transvaginal egg pick-up procedure from the mother/(or donor oocytes).
2 In vitro fertilization of the egg/s by sperm provided by the father/(or donor sperm).
3 Transfer by laparoscopy of up to four embryos to the surrogate mother.
4 Deep freezing of _____ other embryos (other than those transferred)

if they continue normal development but only for the purposes of implantation into the womb of a woman.

5 The administration of local anaesthesia, intravenous sedation and/or general anaesthesia where required for these operation/s.

And if any unforeseen condition arises in the course of the operation/s calling in his/her judgement for procedures in addition to or different from those now contemplated I further request and authorize him/her to do whatever he/she deems advisable and understand and agree as follows:

1 I am over the age of 18 years.

2 The nature and purpose of each operation, possible or alternative methods of treatment and options, the risks involved and the possibility of side-effects and complications including prematurity, multiple pregnancy and spontaneous abortion have been fully explained to and understood by me.

3 The above-mentioned risks, side-effects and complications occur in frequencies that range from occasional, as in the case of infection, to extremely rare, as in the case of anaesthetic death and most others.

4 The intention of every doctor involved in the operation/s is to treat the disease of infertility and the benefits of the intended treatments far outweigh the potential risks, side-effects and complications as outlined above. It is in this spirit that I have voluntarily presented myself to the above-mentioned doctors for such treatment, have signed this consent for treatment and agree to hold harmless each of the doctors for any infection, failure to achieve fertilization or pregnancy, death of sperm, egg/s or embryo/s or mental or physical defects in any fetus or child.

5 I shall comply with all medical instructions given and undergo all tests and investigations ordered from time to time by the above-named doctors.

6 I, the surrogate mother, shall not smoke, drink any alcoholic beverages, or take into my body any illegal drugs or prescribed medications without written authority or prescription from a personal physician or from one of the above-named doctors.

7 I, the surrogate mother, shall follow an antenatal medical examination schedule consisting of no fewer visits than:
- a one visit per month during the first seven months of pregnancy.
- b two visits, each to occur at fortnightly intervals, during the eighth month of pregnancy.
- c weekly visits commencing from the beginning of the ninth month of pregnancy.

8 I, the surrogate mother, shall use my best endeavours to carry, until birth, every embryo, fetus and child transferred to me. Confinement for delivery shall occur at _____ in the State of _____.

9 I/We, the father and mother, wish to adopt (and I/we the surrogate mother and her husband wish the above-named father and mother to adopt) every child born under this arrangement and by each of our signatures hereto intend this document to be conclusive evidence of those wishes.

10 Where the father, or the mother, is a relative of any child born under this arrangement, I/we the surrogate mother and her husband shall:
- a within five (5) days of birth of that child, or as soon thereafter as medically possible:
  - (i) sign a form of consent under Section 4E of the *Adoption of Children Act* 1896 and amendments (Western Australia) to the adoption of that child by the said relative/s *and* will not revoke or contradict that consent.

  - (ii) give the notice prescribed by Section 5B of the said Act.
- b not less than thirty (30) nor more than forty (40) days after giving the said notice, file in the Family Court of Western Australia an application under the said Act for the adoption of that child by the said relative/s.

I/We the surrogate mother and her husband shall sign all documents deemed by the mother or the father necessary or desirable for the adoption of that child and in order to have the names of the father and the mother placed as parents on that child's birth certificate *and hereby irrevocably appoint* the father and the mother jointly as my/our attorneys to do all things and sign all such documents.

11 No fee of any kind shall be paid to the surrogate mother or her husband relating in any way to this arrangement but I/we the father and the mother shall jointly and severally indemnify the surrogate mother's expenses of pregnancy and confinement beginning on the day when pregnancy is verified and continuing for one (1) month after the birth, these expenses being defined as:
- a her medical, hospital, pharmaceutical, laboratory and therapy expenses incurred in the pregnancy, not covered or allowed by Medicare or her present health and hospital insurers, including all extraordinary medical expenses but excluding any expenses for emotional/mental conditions/problems relating to the pregnancy, loss of wages or other incidentals.
- b her travel expenses incurred by road, rail or economy return ticket on a scheduled routine flight between her home and Perth for embryo transfer, and the usual place for antenatal visits, treatment and testing.
- c single hotel accommodation of up to $_____ per night for up to three (3) nights whilst she is in Perth for embryo transfer, and for up to three (3) further nights after her release from hospital after the birth.
- d her meals during her travels between home and Perth for embryo transfer and antenatal visits and confinement all up to $_____ per day.
- e the cost of paternity testing, if requested.

12 I/We the father and mother jointly and severally agree to:
- a pay the premium for a term life assurance policy in the sum of $_____ payable to a named beneficiary of the surrogate mother and agree that the said policy shall remain in effect for six (6) weeks after the said birth.
- b make a will providing for the support of every child born under this arrangement in the event of either of us dying before the said birth.
- c insure, and throughout the pregnancy keep insured each of our lives under term assurance policies each in the sum of $_____ payable in trust for all children born under this arrangement.

I/We all agree that if any party is asked to produce any policy of assurance for inspection, that party shall do so within seven (7) days afterwards.

13 If I/We the surrogate mother or her husband shall commit any breach of any promise/s made by either of us in this Consent Form then I/we shall indemnify the father and the mother for all and any monies which the father or the mother is required to pay for support of any child born under this arrangement and the pregnancy expenses and all legal costs and disbursements incurred by the father and the mother (on the basis of solicitor-and-client) for advice and representation in and about enforcement or attempted enforcement of any promise or any obligation under this arrangement and/or this Consent Form.

14 I/We the father and the mother jointly and severally accept all legal responsibility for every child born under this arrangement whether the said child may possess physical and/or mental defect/s or otherwise.

15 I/We agree that I/we will not provide or allow any agent to provide any information to the public, news media, or any other individual, group or corporation relating to the existence of any egg pick-up, in vitro fertilization, embryo transfer, embryo freezing, surrogate pregnancy or the identity of any party to or child born under this arrangement.

16 Where any provision herein is held to be invalid or unenforceable, the same shall be deemed severable from the remainder of this consent and shall not cause the invalidity or unenforceability of the remaining provisions.

Definitions

'Surrogate mother'	the woman who receives the embryo/s and carries the pregnancy.
'Father'	the infertile man whose spermatozoa (sperm cells) are used to generate the embryo/s.
'Mother'	the infertile woman whose oocytes (eggs) are used to generate the embryo/s.
'Donor'	spermatozoa or oocytes matched to the respective infertile partner and used on their behalf to generate the embryo.

Signature of surrogate mother

Signature of husband of surrogate mother

Signature of father

Signature of mother

CASE HISTORY OF A SURROGATE PREGNANCY ARRANGEMENT

A report by Egarter and Huber (1988) described the successful stimulation, retrieval and IVF of oocytes from a woman with Rokitansky–Küster–Haüser syndrome. Those authors preferred the term 'Mayer–Rokitansky–Küster syndrome. In the absence of a suitable surrogate mother, they subsequently froze the fertilized oocytes at the pronuclear and 2-cell stages. We reported a similar case (Yovich and Hoffman, 1988) with transfer of embryos to the uterus of the woman's sister who successfully conceived and subsequently delivered healthy triplet infants.

Case report

The infertile woman was aged 26 years and has the characteristic features of Rokitansky–Küster–Haüser syndrome, i.e. normal female phenotype with vaginal aplasia, rudimentary cornua uteri and morphologically normal fallopian tubes and ovaries sited on the pelvic sidewall (diagnosed at preliminary laparoscopy). She was shown to have a normal female karyotype and although she had amenorrhoea, a normal ovarian cycle was demonstrated on daily hormonal and ultrasound assessment. Her husband was normospermic and her sister, a 30-year-old married woman with two children, who had undergone laparoscopic sterilization in the past, agreed to act as surrogate mother for the pregnancy. A consent form was developed, consistent with the recommendations of the Ontario Law Reform Commission of 1985 and signed by the mother (infertile woman who provided the eggs), father (husband of the infertile woman who provided the sperm), the surrogate woman (woman who agreed to carry the pregnancy), and husband of the surrogate woman. The basis of the surrogacy agreement was that the infertile couple would cover the medical costs and travelling expenses of the surrogate woman. There was to be no surrogacy fee and the consent form included statements that the surrogate woman would abide by medical advice regarding management of the pregnancy and the infertile couple agreed that they would arrange to adopt the infant/s after delivery, regardless of the presence of any physical or mental defects in any of the infants.

Following ovarian stimulation of the infertile woman with clomiphene citrate combined with hMG, five oocytes were recovered by a transvaginal ultrasound-directed technique. Following insemination with her husband's prepared spermatozoa, three high-grade embryos and two poor-quality embryos developed (Cummins et al., 1986) and all were transferred to the sister's uterus by conventional ET. The sister was monitored by daily hormonal assays and fortuitously was shown to be 5 days post LH surge on the day of transfer, hence preliminary cryopreservation was avoided. Subsequently three gestational sacs (each containing a viable fetus) were identified at 7 weeks from the last menstrual period. A further scan at 16 weeks revealed satisfactory development of each fetus and the anatomical features were within normal ultrasonic limits. Delivery occurred in October 1988 by elective Caesarean section under epidural anaesthesia. The infertile couple were present and have subsequently undertaken a formal adoption of the infants (two females and one male).

This case of IVF surrogacy fulfils two essential criteria for acceptable surrogacy arrangements, namely that the surrogate woman does not allow her ova to be used (or her husband's spermatozoa), and there is no commercial trade. The surrogate woman must act altruistically in this regard, hence a sister, relative or close friend would generally be the relevant surrogate woman. Such close, open arrangements are termed compassionate family surrogacy and are not likely to lead to problems with relinquishment of the infants or identity confusion by the developing children.

EXPERIENCE WITH COMPASSIONATE FAMILY SURROGACY

During 1987 and 1988, a total of 38 cases had surrogacy approved at PIVET. These were all cases of infertility due to absent or diseased uterus and a small number of cases included repeated IVF failure, recurrent pregnancy loss or advanced maternal age. Some cases of medical indications were considered but only one fulfilled a requirement of the Ethics Committee that a substantiated medical opinion be given that quality maternal survival was expected to be 18 or more years, i.e., to cover the child's developmental years. Some cases of genetic indication were considered but these were largely resolved by the use of donor gametes or embryos and no cases were approved where the basis of the application was for social reasons. The outcome of five couples who had a total of seven treatment cycles is within the 'compassionate family surrogacy' and IVF programme as summarized in Table 15.2. The programme has now been terminated following withdrawal of ethical approval in the light of impending state legislation designed to prohibit surrogacy arrangements. At this stage the matter remains under continuing debate.

Table 15.2 Surrogacy cases treated at PIVET Medical Centre during 1987–1988 prior to the programme being halted due to withdrawal of ethical committee approval

(a) *Cases treated*

Indication	*Surrogate*	*Techniques*
Rokitansky-Küster-Haüser syndrome	Sister, interstate	CC/HCG for synch TEST: 5 embryos Pregnant: triplets
Asherman's syndrome	Sister-in-law, overseas	HRT* TEST: 1–2 embryos TEST: 2–3 embryos
Hysterectomy (cancer)	Sister, sterilization	CC/HCG IVF-ET: 4 embryos
Hysterectomy (ruptured uterus)	Sister	CC/HMG/HCG TEST: 4 embryos Pregnant: twins
Hysterectomy (ruptured uterus)	Close friend, 9 years	Nat. cycle/no coitus IVF-ET: 2 embryos

(b) *Pregnancies*

Indication	*Gametes*	*Surrogate*	*Method*	*Outcome*
Rokitansky-Küster-Haüser syndrome	H&W†	Sister, sterilization married, 3 children	ET	Triplets born October 1988
Hysterectomy	H&W†	Sister, married, 5 children	TEST	Twins born April 1989

* Hormone replacement therapy schedule (see Chapter 13).
† Husband and wife provided the gametes (i.e., genetic parents) and have formally adopted the resulting children.

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Chapter 16

Pregnancy management and outcome

The management of infertility aims to overcome the problem of childlessness. In practical terms, this means developing treatments and techniques to achieve conception followed by successful implantation, as well as nurturing pregnancies through to the delivery of healthy live infants. The experience of infertility clinics has reinforced the belief that the management of pregnancies arising in infertile patients after assisted conception techniques is an equally complex and challenging matter. Areas of particular endeavour include the diagnosis of pregnancy, the early detection of abnormal pregnancies, preventing fetal wastage, and attending to late pregnancy complications such as preterm delivery, placental insufficiency and complications associated with multiple pregnancies.

PREGNANCY DIAGNOSIS

Currently, there appears to be a lack of uniformity among units reporting pregnancy data, so that some are reporting higher rates of preclinical (biochemical) pregnancies and others appear to be reporting higher than expected rates of clinical pregnancies. Furthermore, the categorization of early pregnancy outcomes does not always differentiate between losses of anembryonic pregnancies as opposed to fully formed fetuses in whom a fetal heart action had been demonstrated previously.

The main problem of pregnancy diagnosis may relate to uncertainties regarding the definition as well as variability among units with respect to the diagnostic test applied, the reference standard adopted, and the timing and interpretation of such tests. This may be particularly confusing in cases having exogenous hCG injections, which may be used to trigger ovulation, boost the luteal phase, or as a therapy throughout the first trimester.

Diagnostic assays

hCG tests vary in both specificity and sensitivity. The majority of units appear to be using commercially available RIA kits although some

units are relying on in-house assays or using enzyme-linked immunosorbent assay (ELISA). Regardless of the test used, it is important to equate the result with a reference standard in the appropriate diagnostic range.

It is suggested that the diagnostic level of β-hCG above which pregnancy may be diagnosed should be 25 mIU/ml, set against the second IS (International Standard) 61/6. If the alternative standard (first international reference preparation 75–537) is used, the corresponding level is 52 mIU/ml.

Therefore, the diagnosis of pregnancy is made on the finding of a β-hCG elevation ≥25 mIU/ml (2nd IS 61/6) performed on or after day 16 after oocyte recovery, and a further test performed no less than 3 days thereafter must demonstrate a further rise in the hCG level. A demonstrable rise excludes any lingering effect of exogenous hCG if an hCG trigger has been used to activate ovulation. Where hCG injections have been given throughout the luteal phase, the first diagnostic test should be performed no less than 3 days after the injection and there should be no injections given in the interval period before the second diagnostic hCG test.

A number of reports of clinical pregnancies show that the concomitant P4 level is ≥37 nmol/l and E2 is ≥670 pmol/l. Lower levels of E2 and P4 cast doubt on the significance of an hCG elevation (Yovich et al., 1986a,b). Alternative assays, e.g. Schwangerschaftsprotein 1 (SP1), placental protein 14 (PP14) and pregnancy-associated placental protein-A (PAPP-A) are not yet suitable for the early and accurate diagnosis of pregnancy. β-hCG assays remain the most specific and sensitive to date (Yovich et al., 1986b).

Clinical pregnancy

Abdominal ultrasound examinations are usually performed in the seventh or eighth week, when a viable fetus (visible movements and the fetal heart action) can be identified. More recently, vaginal ultrasound examination can detect viable intrauterine pregnancies as early as the sixth week of pregnancy or 4 weeks after conception.

Pregnancy wastage categories

1 *Preclinical (biochemical) pregnancy*: any case which meets the criteria for diagnosis of pregnancy but which does not reach completion of week 6, i.e. bleeding ensues when the β-hCG level shows a fall, with subsequent levels <25 mIU/ml. This may occur after the completion of week 6 but if the event began at a prior stage, it should be categorized as a preclinical pregnancy loss.
2 *Blighted ovum (anembryonic)*: intrauterine pregnancy with gesta-

tional sac defined. There are no clear fetal movements or fetal heart action. This will need to be differentiated from the pseudo-sac sometimes associated with ectopic pregnancies (Nyberg et al., 1983).

3 *Spontaneous miscarriage:* viable fetus demonstrated within the gestational sac, with positive fetal movements and fetal heart action demonstrated but subsequently miscarrying before the 20th week.

4 *Ectopic pregnancy (extrauterine pregnancy loss):* as previously noted, this may be associated with an intrauterine pseudo-sac. Vaginal ultrasound scanning is more often able to pick up the intratubal gestational sac than abdominal scanning. An additional aid to diagnosis is the absence of circulating PAPP-A in most cases (Stabile et al., 1988).

5 *Advanced pregnancy losses:* perinatal deaths which occur beyond 20 weeks from adjusted last menstrual period (LMP), i.e. 18 weeks after egg recovery.

Early pregnancy wastage may be suspected during the first trimester

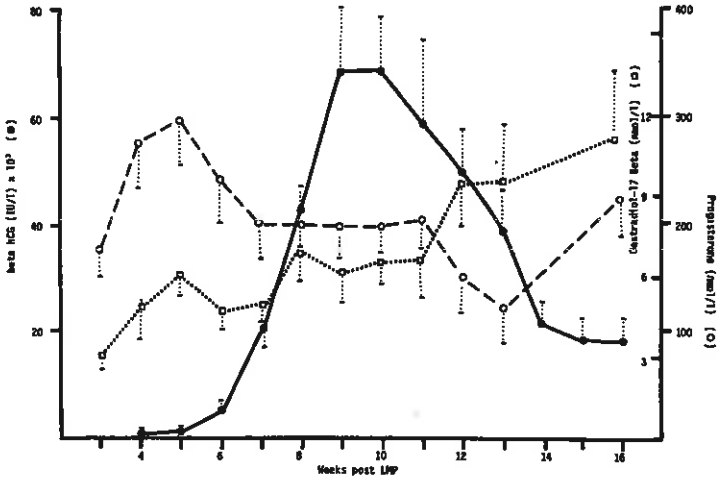

Fig. 16.1 Hormonal profiles of women conceiving after IVF-ET, showing β-hCG, E2 and P4 levels throughout the first trimester of those pregnancies which proceeded to delivery at or near term.

(Fig. 16.1) by the observation of hCG levels below the 10th centile, combined with P4 <37 nmol/l and E2 <670 pmol/l if weekly monitoring

is performed. In ectopic pregnancies, the P4 and E2 levels may be extremely low whilst high β-hCG levels persist.

Those pregnancies miscarrying before the completion of week 6 usually do not require curettage, hence definitions based on the presence of trophoblast tissue are generally not relevant in early cases.

Pregnancy wastage following gamete handling

The outcome of pregnancy following a range of assisted conception procedures is summarized in Fig. 16.2. A total of 65% of the 400 pregnancies (or 70% of clinical pregnancies) progressed beyond 20 weeks' gestation. In this series a significantly higher rate of early pregnancy wastage due to blighted ovum was seen in the GIFT pregnancies and

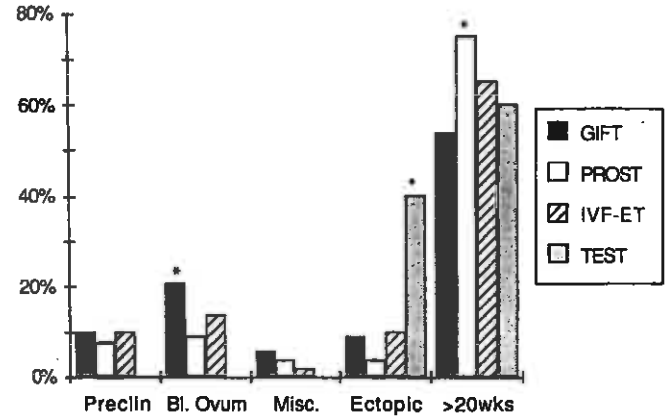

Fig. 16.2 The outcome of pregnancies achieved in relation to the techniques of assisted reproduction used, showing the various causes of early pregnancy wastage as a proportion to the pregnancies. Preclin = preclinical pregnancy; Bl. ovum = blighted ovum; Misc. = miscellaneous.

there was a higher rate of wastage in the TEST patients due to ectopic pregnancy. Similar patterns of pregnancy outcome are seen in women treated by AIH and PROST with DI (Yovich and Matson, 1988). The higher incidence of blighted ovum pregnancies from AIH has been discussed in Chapter 7 and the better pregnancy outcome for PROST and DI relate to the fact that both series contain fewer patients with female factor infertility; treatment is mostly provided because of male factors.

EARLY PREGNANCY MANAGEMENT

For comprehensive pregnancy management, the following schedule of monitoring is recommended:

1 Weekly quantitative β-hCG, E2 and P4 through to week 8.
2 Routine diagnostic scan in week 7.
3 Early vaginal scan if hormonal levels are below the 10th centile.
4 Routine fetal anomaly scan at 16–18 weeks.

Hormonal support

There is still no clear explanation for the underlying causes of early pregnancy wastage in the subfertile population and it is possible that a large proportion may be due to causes other than fetal chromosome abnormalities, the commonest observation in spontaneous pregnancy (Boué et al., 1975; Simpson, 1980). However, while the overall rate of spontaneous abortions is generally considered to be 10–15%, subfertile women who conceive following therapy are much more prone to abort their pregnancies (Yovich and Matson, 1988) and habitual aborters can be shown to have abnormal ovulatory cycles, and in particular, luteal phase defects (35%; Jones, 1976). The value of progesterone support therapy in the form of either P4 or synthetic progestagens has been controversial (Shearman and Garret, 1963; Goldzieher, 1964; Klopper and MacNaughton, 1965; Jones et al., 1974; Soules et al., 1977; Fainstat and Bhat, 1983; Tognoni et al., 1983) and an evaluation of this therapy continues. The authors favour progesterone support therapy using MPA (Provera tabs 10 mg). This substituted progestagen has been shown to be a potent progestational agent, with little or no oestrogenic or androgenic properties (Greenblatt and Barfield, 1959). Its effectiveness in maintaining pregnancies in the rabbit (Wu, 1961) and rat (Stucki and Glenn, 1961) has been demonstrated without untoward effects. MPA is considered to have 50 times the progestational activity of natural progesterone (Stucki and Glenn, 1961; Wu, 1961); a reduction in pregnancy wastage due to spontaneous miscarriage has been reported by us following MPA administration (Yovich et al., 1983; 1985; Yovich, 1988). Furthermore, a recent clinical study concluded that MPA had no embryopathic risk and was not likely to retain an abnormal fetus that might otherwise abort (Yovich, 1988). MPA can be used in the following categories:

1 History of recurrent miscarriage.
2 Uterine bleeding in the early weeks of pregnancy.
3 Those cases with reduced P4 and/or E2 who otherwise have evidence of a normal pregnancy.

4 A current trial suggests that MPA may be useful in later stages of pregnancy to reduce the chance of late spontaneous miscarriage and preterm delivery in high-risk situations, e.g. a previous history and multiple pregnancies (Devine and Yovich, 1989; Yovich et al., 1989).

For recurrent aborters, treatment is initiated at the time of pregnancy diagnosis (usually day 16 of the luteal phase) and consists of two tablets taken orally four times per day, providing a total dose of 80 mg/day. For those with threatened abortion, the regimen used in the acute phase is two tablets given 4-hourly, providing a total dose of 120 mg per day. As vaginal bleeding settles, the 2.00 a.m. dose is ceased, and 1 week later the regimen is converted to 20 mg q.i.d., the same dose administered to the recurrent aborter group. In most cases the therapy is continued to 16 weeks' gestation and then weaned slowly over the next 2 weeks so that all treatment is ceased by 18 weeks gestation. In those patients considered to be at higher risk because of recurrent bleeding or recurrent uterine irritability after reducing MPA, therapy is continued through to 35 weeks gestation.

An alternative form of treatment for recurrent miscarriage is the use of hCG 5000 IU by injection twice a week up to week 12. This has been shown to be effective but it is unlikely to contribute much after the eighth week. Therefore, an improved protocol for recurrent aborters may be a combination of hCG and MPA.

Recurrent miscarriage

Women with recurrent miscarriage should be carefully screened for an underlying cause, and investigations should consider:

1 *Genetic causes:* detailed banded chromosome analysis of leucocytes from both partners.
2 *Anatomical causes:* hysterogram and hysteroscopy to identify septate uterus, uterine synechiae, submucous fibroids, evidence of cervical incompetence, and the diethylstilboestrol (DES) uterus/cervix. In addition, laparoscopy is useful to exclude active pelvic endometriosis and active pelvic inflammatory disease.
3 *Infective causes:* antibodies: serology for syphilis, brucellosis, toxoplasmosis and rubella.
Culture: cytomegalovirus, *Chlamydia trachomatis*, herpes virus, *Listeria monocytogenes* and *Mycoplasma hominis*, aerobic and anaerobic bacteria on endocervical swabs which should also be repeated at the onset of pregnancies.
4 *Maternal disorders:* systemic lupus erythematosus and/or lupus coagulation inhibitor—antinuclear factor and coagulation profile; diabetes—fasting blood sugar; vascular/renal disease—clinically

screened and cholesterol/triglycerides; thyroid disease—thyroid function tests including thyroid stimulating hormone (TSH); and evidence of luteal insufficiency.

Lymphocyte immunotherapy

Currently, the above screening profile is required for consideration of paternal lymphocyte immunotherapy which has been proposed as an effective treatment mode for women with recurrent spontaneous miscarriage (Mowbray et al., 1985). If the above screening is negative and a cross-match study indicates that there are no detectable antibodies against the paternal lymphocytes, women are currently being offered paternal lymphocyte immunotherapy in a double-blind randomized trial at PIVET. Women who are rhesus(D)-negative with rhesus(D)-positive husbands can be treated after giving anti-D.

Lymphocytes are prepared by:

a Ficoll-Isopaque density gradient separation of the buffy coat.
b Hydroxyethyl starch sedimentation of contaminating red cells.
c Three washes in normal saline solution.
d Resuspension in 4 ml of normal saline.

At PIVET the routes of administration are subcutaneous and intradermal; 0.5 ml in two different sites and an intravenous injection of the remaining 3 ml. Patients are advised to avoid conception for 1 month, to allow adequate time for development of an immune response.

Ectopic pregnancies

At least 5% of pregnancies arising after infertility treatment with assisted conception techniques will be ectopic. The majority will occur in cases of known or suspected tubal disease (Fig. 16.3) and such high-risk patients should be counselled before treatment and cautioned at the time of pregnancy diagnosis. Ectopic pregnancy can be diagnosed before symptoms arise by hormonal and ultrasonic means and should be suspected where the hormonal levels are below the 10th centile, and in particular if the E2 and P4 levels are very low. The relative risk of this condition is also higher if very low levels of PAPP-A are seen in the presence of β-hCG. Vaginal ultrasound will exclude the presence of an intrauterine gestational sac after 6 weeks' gestation and will often identify precisely the tubal ectopic gestation. The possibility of heterotopic pregnancies must also be considered.

Current treatment is generally by conservative tubal surgery, using microsurgical techniques, or the use of direct injections of drugs (e.g. methotrexate) or chemicals (e.g. KCl) into the gestational sac.

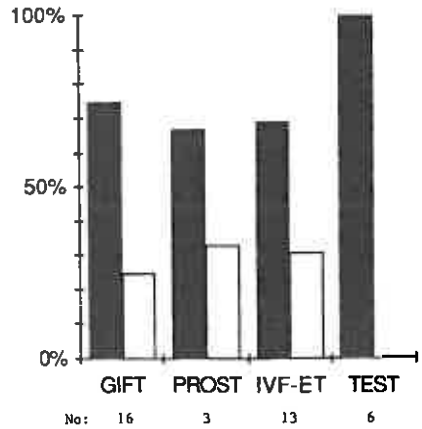

Fig. 16.3 The occurrence of tubal disease in women with ectopic pregnancy following an assisted conception procedure. Hatched area = tubal disease; blank area = normal tubes.

Blighted ovum or anembryonic pregnancy

This condition can be diagnosed by the detection of an empty gestational sac (>3 ml volume) and reduced hCG levels. The cause of early embryonic arrest leading to blighted ovum is still uncertain and chorionic villus (CV) sampling has revealed a normal chromosome arrangement in the majority of the small series of patients with this condition sampled. Once the diagnosis is made, the pregnancy can be allowed to expel itself at or under 6 weeks' gestation. If the pregnancy is further advanced, a dilatation and curettage should be undertaken.

Multiple pregnancies

Overall, 20% of pregnancies arising after assisted conception techniques will be multiple—either twins or triplets (Fig. 16.4); the rate is influenced by the number of gametes or embryos replaced. Occasionally, a higher-order pregnancy is seen: seven gestational sacs have been seen although only four oocytes were transferred in a GIFT patient (Matson et al., 1988). Multiple pregnancy of high order can be reduced by a process of selective termination, but the procedure remains controversial and the medicolegal implications are uncertain. Fetal demise reduction can be achieved by direct needling of the fetal heart at transvaginal ultrasound, using a 16-gauge needle within the needle guide. Fetal blood can be aspirated and air or potassium chloride injected. Following fetal demise, the products of conception are generally fully

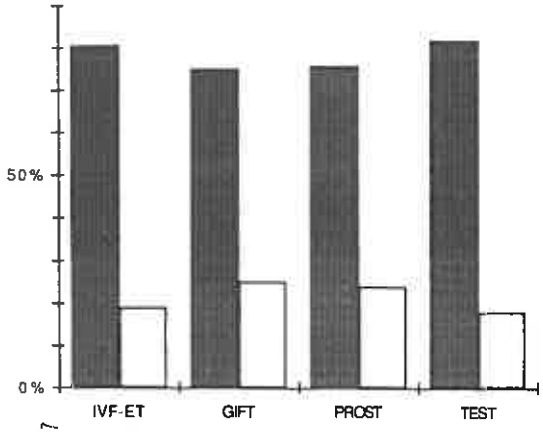

Fig. 16.4 The occurrence of multiple pregnancy in relation to the technique of assisted reproduction. Multiple pregnancies are expressed as a proportion of on-going pregnancies. Hatched area = singleton; blank area = multiple births.

resorbed over a 6–8-week period if the procedure is performed at the tenth week.

All multiple pregnancies are managed actively at the PIVET Medical Centre, as follows:

1 Provera 20 mg q.i.d. to 35 weeks.
2 Cervical suture at 12 weeks.
3 Hospital bed rest between weeks 18 and 26, allowing weekend leave if favourable.
4 The delivery mode for twins is decided on obstetric grounds, but all triplets and greater have elective Caesarean section by 36 weeks.

In a comparative study of cases there is evidence that this approach has led to a significant improvement in pregnancy outcome (Devine and Yovich, 1989).

Genetic diagnosis

Chorionic villus sampling (CVS) and amniocentesis are offered to women at high risk of chromosome abnormality, including all women over 35 years. Generally, CVS is performed, and is preferred by most women. Amniocentesis is indicated for those who do not wish to accept the 2–3% risk of CVS or where there is a suggestion of an open

neural defect on routine serum alpha-fetoprotein measurement or ultrasound assessment at 16 weeks' gestation.

LATE PREGNANCY OUTCOME

Subfertile women whose pregnancies advance beyond 20 weeks are a higher-risk group whose perinatal mortality rate is four- to fivefold that of the general population (National Perinatal Statistics Unit, 1987; Commonwealth Department of Community Services and Health, 1988). The majority of problems relate to preterm delivery, secondary to multiple pregnancy. However, even singleton pregnancies deliver preterm at a rate twice that of the general population (Fig. 16.5; Commonwealth Department of Community Services and Health, 1988). One-third of infants will weigh less than 2500 g.

The congenital abnormality rate in infants delivered in Western Australia is 3.5% and this is not higher than that seen in the general population (Bower and Stanley, 1986; Commonwealth Department of Community Services and Health, 1988). Recurring abnormalities have not been noted in Western Australia but the total data from Australia, reported by the NPSU (1987) have shown a small cluster of infants with spina bifida and translocation of the great vessels in the IVF group. At this stage it is not known whether the finding relates to the infertility treatment or some extraneous factor. A more recent report of the NPSU data (Lancaster, 1990) shows that the above findings have persisted.

Follow-up studies on IVF (Yovich et al., 1986c) and other infants from infertility management indicate a normal developmental profile.

Modern infertility treatments have captured worldwide interest and much scientific as well as public attention is directed toward the outcome of those treatments. Already there are more than 20 000 IVF-related infants worldwide and other infertility treatments described in this text have been responsible for generating well over 100 000 infants. Much attention is focused on improving the efficiency of treatment methods but it is abundantly clear at this stage that techniques of assisted reproduction are beginning to make a significant impact to the total contribution of healthy individuals within the population of most countries.

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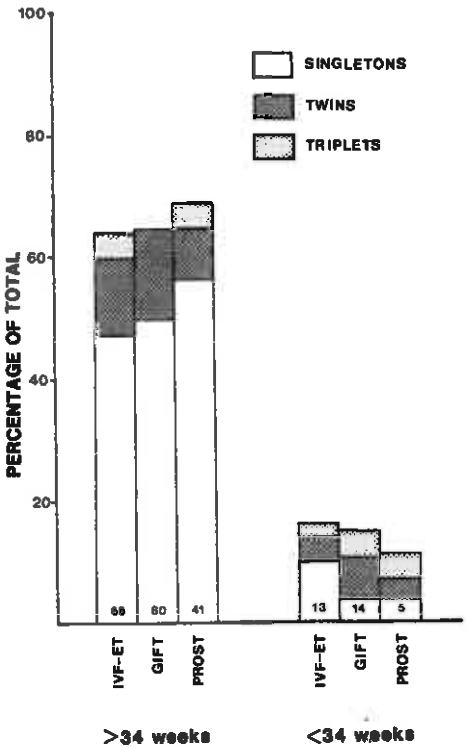

Fig. 16.5 The occurrence of premature (<34 weeks) delivery in singleton and multiple pregnancies in relation to the assisted conception procedure used.

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Chapter 17

Complications and hazards of assisted reproduction

Considering the diverse and often complex background of infertile couples and the potential for serious sequelae from infertility treatments, relatively few problems are encountered. However, it is important that infertility clinics should be conscious of the potential hazards and ensure their staff have the appropriate specialized expertise. Internal audit and quality control assessment should be integral components in the organizational management of infertility units.

DIAGNOSTIC EVALUATION

In a series of more than 10 000 diagnostic laparoscopies at PIVET, inadvertent bowel perforation has occurred on only two occasions. In both cases the women had previous episodes of at least two abdominopelvic operations and the patients were forewarned of the possibility. On both occasions, the complication was recognized immediately and corrected at laparotomy without any long-term sequelae.

Other less life-threatening complications include an occasional case of inadvertent perforation of the uterus during diagnostic curettage or hysteroscopy, and an acute flare-up of pelvic inflammation secondary to a hysterosalpingogram study.

ANAESTHETIC HAZARDS

A handful of women around the world have died during IVF-related procedures. The majority have been anaesthetic accidents. For example, two cases in Australia were due to accidental dislocation of anaesthetic tubing during laparoscopy for oocyte recovery and the inadvertent administration of the wrong gas during another case for GIFT. These cases highlight the need for alert systems in the operating theatre, including the use of the pulse oximeter, a relatively inexpensive but important monitoring device. Anaesthetic complications can be reduced by applying procedures requiring less complex anaesthesia (for example, transvaginal oocyte collections) and perhaps, removing

the need for anaesthesia totally, e.g. with transcervical TEST and IVF-ET.

OVARIAN STIMULATION

Almost 2000 cycles of ovarian stimulation are performed each year at the PIVET clinic. The known reversible side-effects of clomiphene citrate are seen, including scotomata, but these are unusual if the common dosage of 50 mg b.d. for 5 days is not exceeded. However, one patient developed partial blindness 2 weeks after ceasing clomiphene citrate in a non-conception cycle. The consultant ophthalmologist reported a coincidental optic neuritis and the patient has a persisting visual field defect. The main life-threatening condition associated with ovarian stimulation is that of severe ovarian hyperstimulation syndrome (OHSS) which occurs in up to 3% of cycles, particularly those in which conception occurs.

Ovarian hyperstimulation syndrome

OHSS is an iatrogenic disorder first reported in 1964 in which induction of ovulation was followed by a wide spectrum of clinical symptoms and signs. A useful classification put forward by Lunenfeld (1976) describes three grades of severity. In its severe form there is massive ovarian enlargement, ascites, pleural effusions, haemoconcentration, oliguria, electrolyte imbalance and hypercoagulability. These changes can potentially lead to severe respiratory embarrassment, renal failure and disseminated intravascular coagulation—all life-threatening conditions. To date, the pathophysiological mechanisms have not been elucidated and studies have concentrated on plasma renin activity, changes in aldosterone and the renin angiotensin cascade (Navot et al., 1987). Recently high CA125 (cell surface antigen) levels were demonstrated in the serum of women with OHSS after follicle aspiration in an IVF programme.

We have previously reported on the diagnosis and treatment of disordered ovulatory cycles (Yovich et al., 1987). Table 17.1 documents the rates of multiple pregnancy and OHSS noted in that study series. The total number of women receiving hMG was 261 and one woman had two separate episodes of OHSS, providing an incidence of 4.2% of women treated with hMG. The pregnancy rate was 83% in the OHSS group. We have continued to observe severe OHSS including those having stimulation with Lucrin/hMG; the incidence of hospital admissions for OHSS is approximately 3% of non-IVF cases and 1.5% of IVF treatment cycles. The lower incidence in IVF treatment cycles may possibly relate to a beneficial effect of follicle aspiration. The

majority of cases exhibiting OHSS are those who display rapidly rising E2 levels in response to low doses of hMG. In general therefore they are the younger patients, some of whom have PCOs disease.

At PIVET approximately one severe case is managed each month and the treatment routine involves intravenous hydration, management of any electrolyte imbalance, careful attention to fluid balance and paracentesis with continuous drainage of the ascitic fluid. Such patients should not receive hCG injections during the luteal phase but may continue with progesterone support if luteal therapy is favoured. Abdominal drainage is continued over 3–5 days and 7–12 litres of proteinaceous fluid may drain. If the patient is not pregnant, the condition resolves spontaneously prior to the menstrual period; otherwise it recedes slowly by the eighth week of pregnancy.

OOCYTE RECOVERY

At PIVET, over 4000 oocyte recovery procedures have been performed and no serious life-threatening complication has occurred in any patient. In the early phase, two cases of oocyte recovery for GIFT were converted from laparoscopy to mini-laparotomy because the gynaecologist had difficulty mobilizing the fallopian tubes satisfactorily at laparoscopy. Also, in the early series, occasional transvaginal oocyte collections were converted to laparoscopy collections due to mobile ovaries and incomplete transvaginal collections. This no longer occurs since the techniques described in Chapter 10 have been introduced. On very rare occasions, arterial bleeding has occurred from one of the cannula sites in the abdominal wall—this has always been corrected by the placement of a through-and-through abdominal suture tied around a small compression pack for 48 h. Similarly, an occasional bleeding vessel in the mesosalpinx or mesovarium has been stemmed by laparoscopic diathermy or pressure. No cases required laparotomy. Two cases of pelvic infection after transvaginal oocyte recovery (0.001% incidence) have lead to the routine use of antibiotic cover during the procedure.

PREGNANCY

For those patients who conceived, the high-risk nature of both early and late pregnancy has been discussed (Chapter 16). It is important to perform a blood test on days 13–16 in all cases, even for those who describe the onset of menses. On two occasions, women who did not return for arranged blood tests subsequently presented as emergency cases in peripheral hospitals with ruptured ectopic pregnancies. A careful screening is described in Chapter 16, enabling ectopic pregnancies to be detected early when they can be treated conservatively. Other major complications relate to multiple pregnancies (Table 17.1), particularly high order multiple pregnancies with the attendant problems of hyperemesis, hydramnios, abdominal discomfort, preterm delivery, etc.

Table 17.1 Incidence of multiple pregnancies and severe OHSS (including those requiring paracentesis) following ovarian stimulation

	Pregnancies			*OHSS*		
Stimulation	*No.*	*Pregnancy*	*Multiple*	*No.*	*%*	*Paracentesis*
Clomiphene	657	56	2 (3.6%)	0	0%	0
Clomid/hMG	288	41	6 (14.6%)	8	2.8%	3
hMG	126	22	6 (27.3%)	4	3.2%	3
Total	1071	119	14 (11.8%)	12*	1.1%	6

* 10 pregnant, all singleton.

Early pregnancy wastage

Current data indicate that 30% of pregnancies diagnosed after assisted conception will not reach 20 weeks' gestation. This indicates that women in subfertile marriages have up to double the risk of early pregnancy wastage. This may not constitute a significant hazard for preclinical or early blighted ovum pregnancies but is a very important consideration with respect to ectopic pregnancies and miscarriage of more advanced stages. Patients should be counselled about their risk levels when pregnancy is achieved and should be forewarned once the diagnosis of pregnancy is made. At PIVET, pregnancies are monitored each week by hormonal evaluations (quantitative β-hCG, P4 and E2) until formal pelvic ultrasound in the seventh week. At this stage the diagnosis will be clear in most cases and the appropriate management can be planned accordingly.

Occasionally heterotopic pregnancies will not be obvious once the ultrasound scan reveals a normal gestational sac/s within the uterine cavity. Heterotopic pregnancies should be suspected by persistent bleeding from the uterine cavity and the gradual development of persistent pelvic pain.

Transvaginal scans may improve the direct diagnosis of ectopic and heterotopic pregnancies in the future. It has been repeatedly shown that ectopic pregnancies are more likely to occur where underlying tubal disease has been diagnosed or treated.

Late pregnancy complications

The risk of preterm delivery is raised in all groups following assisted reproduction and the severity is proportional to the number of gestational sacs within the uterine cavity. Multiple pregnancies will inevitably deliver preterm but one study has shown that neonatal outcome is significantly improved when a strict regimen of bedrest, cervical suture and Provera therapy is given (Devine and Yovich, 1989; Yovich et al., 1989). The Caesarean section rate is raised for a variety of reasons and various uterine problems (e.g. fibroids, previous myomectomy, previous uteroplasty, uterine abnormalities, and uterine synechiae) increase the risk of complications such as uterine rupture, placenta accreta and preterm delivery. As the main risk for late pregnancy complications relates to multiple pregnancies, this can be reduced by limiting the number of oocytes or embryos transferred. Given the recent improvements in pregnancy rates and pregnancy outcome, many clinics have reduced the number of oocytes in GIFT and PROST to three, and embryos in TEST and IVF-ET to two or three respectively.

EMOTIONAL SEQUELAE

Over the past 20 years, infertile couples have expressed a range of emotional responses to their infertility and its management. Infertility per se causes a sense of anguish in most couples but in the past this has been compounded by a lack of adequate explanation of the underlying reasons for the infertility. Furthermore, treatment methods in the past have not been very effective and many couples protected themselves by denial or rejection mechanisms. However, in recent days, the development of clinics specializing in infertility management have improved diagnostic aspects, provided increased knowledge and information, and offered a wider range of effective treatment options. This has caused many more couples to seek advice regarding their infertility and its potential management. However, this has sometimes led to new frustrations with some couples having to face the problems of cancellation during stimulation cycles because of poor follicle development or premature LH surges, the shattering experiences of failed oocyte recovery or failed fertilization and, for the majority of cycles, inevitable failure of that treatment cycle to result in pregnancy. For many, these frustrations have been compounded by the high out-of-pocket expenses resulting as a consequence of the necessity for clinics to establish privately, with very few governments around the world being prepared to fund infertility services fully. Further emotional sequelae may arise after pregnancy is diagnosed, with the failure on one or more occasions for pregnancy to proceed to the stage of livebirth.

All the above concerns can be met by three main areas of endeavour:

1 *Appropriate and up-to-date informed counselling.* Generally this requires the careful consideration of the facts pertaining to the individual case and the likelihood of successful treatment within the particular infertility clinic. Medical counselling should also be supplemented by psychological counselling and attention to social welfare needs as required.
2 *Improvement of results.* Primarily, patients seek the attention of infertility clinics to assist them in their quest to produce children. Emotional sequelae will be minimized by efficient handling of the couple and offering treatments which carry a reasonable prognosis to treat their particular case. Clinics which have operated for around 10 years have noted the improved sense of satisfaction of patients over recent years as pregnancy rates have improved and a number of research developments have been put into clinical practice to lessen the chance of cancelled cycles, failed egg recovery, failed fertilization and poor pregnancy outcome.
3 *Stress management.* Specific psychological counselling, stress support, individual and group psychotherapy and arts therapies are useful complements to the sympathetic approach required of the clinical staff towards their patients (Jennings, 1988; 1991).

Emotional problems still exist in infertility management but are nowadays not as severe, due to the improved potential for specific technical solutions for the infertility, along with wider dissemination of up-to-date information provided by counselling. Both factors have contributed significantly to a reduction in the incidence and the severity of emotional sequelae.

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Chapter 18

Ethics in reproductive technology

The acronym IVF has only been in use for a little over a decade but it has already achieved a broader definition due to common international use covering all those procedural techniques contributing to assisted reproduction.

In considering the ethical debate one has to appreciate the paradox (Dunstan, 1986) whereby, on the one hand, practitioners have developed techniques for infertile couples, proceeding from self-evident obligations, while on the other hand, moralists have examined the nature of the work and proceeded from the position of what they see as self-evident truths.

CONCEPT OF THE PRE-EMBRYO

Part of the ethical confusion in reproductive technology is caused by differences in the understanding of the nature of human germ cells, fertilization and early human development (Table 18.1) by scientists, moralists and the public.

Scientists see a continuum in the process of germ cell production, sperm–egg interaction and the stages leading up to reconstitution of the diploid chromosome number at the stage of syngamy when cells develop a new identity (around 24 h). This is followed by the early cleavage stages which proceed to a compacted spherical package of cells known as the morula stage. This package is held within the zona pellucida membrane which is crucial for development up to that stage. Of relevant interest, most of those cells are destined to become trophoblast forming placental structures. Only two or three in the innermost region of the sphere are destined to become the inner cell mass which is first recognized at the expanded blastocyst stage. Even then, the majority of the cells of that inner cell mass are destined to form the amnion, chorion, umbilical cord, yolk sac and other non-fetal parts.

Scientists have only slowly realized that the ethical debate has been affected by differences in the public perception of what constitutes an embryo. For example, most cells within the early preimplantation conceptus or pre-embryo are destined to form tissues which are not

Table 18.1 Timetable of early embryonic development from fertilization to implantation

Stage	*Site*	*Time after ovulation/ insemination*
Sperm-egg binding	Ampulla	4–6 h
Pronuclear stage	Ampulla	14–18 h
Syngamy*	Ampulla	20–24 h
2-cell embryo	Ampullary isthmic region	30 h
4-cell embryo	Ampullary isthmic region	42 h
8-cell embryo	Ampullary isthmic region	54 h
Morula*	Isthmus	72 h
Blastocyst	Uterine cavity	96 h
Expanded blastocyst	Uterine cavity	5 days
Hatched embryo	Uterine cavity	6 days
Differential inner cell mass	Uterine gland	9 days
Yolk sac/amniotic sac	Decidua	11 days
Embryonic disc* (Primitive streak)	Decidua	14 days

* Significant scientific events relating to identity.
Suggested terminology: pre-embryo or conceptus for first 14 days.

going to be part of the fetus. In addition, the pre-embryo (the term is gaining increasing international usage at this stage) bears no morphological similarity to the adult human being. The human form emerges only after day 14 when the entire pre-embryo is implanted within the decidua of the uterus and a primitive streak begins to form from a few of the cells of the inner cell mass. This is now the stage when specialized cells begin to appear and segmentation occurs so that the early embryo proper can be oriented and different tissue types delineated.

Four weeks after fertilization the embryo has a human appearance. Fetal movement and a clear heart beat can be readily detected on ultrasound scanning. From this stage (also traditionally known as 6 weeks' gestation, dated from the LMP), the embryo is deemed to be a fetus.

ASSISTED REPRODUCTION

Routine procedures

Following the birth of Louise Brown in the UK in 1978, a number of IVF-related procedures have become established as part of routine infertility management. They range from simple techniques associated

with individual gamete handling, e.g. artificial insemination with husband's prepared sperm and egg transfers from the ovary to the fallopian tube or to the nearby region, techniques of putting sperm and eggs together into the fallopian tube and others which actually involve the fertilization process in vitro and subsequent culture (Table 18.2).

Table 18.2 Assisted reproduction: routine IVF-related procedures

Sperm preparation from masturbation specimens	AIH
Egg transfers (ovary to tube)	
Sperm and egg transfers to tube	GIFT
In vitro fertilization	
Pronuclear stage to tube	PROST
Embryo to uterus	IVF-ET
Embryos to tube	TEST
Transfer after cryopreservation	FROST

Whilst these procedures are now routine around the world and accepted in all western cultures, ethical debate still rages, particularly following the so-called Papal Decree which was issued in 1987 by the Congregation for the Doctrine of the Faith within The Vatican in a statement entitled *The Instruction on the Respect for Human Life in its Origin and on the Dignity of Procreation* (Table 18.3).

Table 18.3 The Catholic position according to the Papal Decree issued 1987 by the Congregation for the Doctrine of the Faith: *Instruction on Respect for Human Life in its origin and on the dignity of procreation*

No masturbation
No IVF
No heterologous gametes
No research on conceptus (at any stage)
No donors
No surrogates

The Ethics Committee of American Fertility Society (1988) issued a counter-statement on each point.

That instruction presents 'the values and rights of the human person' as the centre of its concern. Whilst acknowledging the potentially constructive role that science and medicine can play in helping to achieve the good of human beings, it regards some applications of technology to be demeaning to human beings and therefore concludes that children must only be conceived through an act of love and indeed, of sexual intercourse. It also sees no moral distinction to be considered

between zygotes, pre-embryos, embryos or fetuses. These papal instructions mean that the official Catholic position is that it does not condone assisted reproductive techniques of the sort described here as routine. Nonetheless, it appears not to have diminished the demand on those services by members of the Catholic faith, although it has certainly increased the moral conflict faced by participants.

The Ethics Committee of the American Fertility Society has recently (1988) published its *Ethical Considerations of the New Reproductive Technologies* in the light of the papal instruction. Whilst agreeing with the fundamental tenets regarding the rights of the human person, it disagrees with all the conclusions and particularly those instructional calls asking nations to bring in legislation to make it a crime to engage in:

1 Artificial insemination with one's husband's sperm.
2 In vitro fertilization.
3 Research on the conceptus (except that which is therapeutic to the potential child and not disproportionately risky).
4 Donor or surrogate aid in reproduction.

Current research

Current research is being undertaken in a number of IVF units around the world in order to improve results obtained from the routine procedures (Table 18.4).

Table 18.4 Current research to improve routine procedures of assisted reproduction

Enhance fertilization
Physical (microinjection, zona drilling)
Chemical (to enhance motility)
Improve embryo quality
Research on embryo's metabolic requirements
Research on embryo's physical environment (temperature, pH, osmotic forces)
Cryopreservation
Sperms, embryos, eggs
To develop an efficient technique without internal ice crystals forming

These include methods to enhance fertilization and involve both physical and chemical techniques to achieve sperm penetration into the egg in cases where the underlying spermatozoa have a significant weakness. This is a common problem in clinical practice and research workers would want to examine the resulting embryos after some of these experimental treatments to determine if normal embryos could

be generated. This would be the ideal principle before considering the return of such embryos to the uterus in a therapeutic trial.

However, many IVF units have not obtained ethical approval to destroy such generated embryos for the purpose of subsequent examination, so research workers may consider a direct clinical trial and examine resultant infants for abnormalities. Unfortunately, if such a technique had a 5% chance of causing fetal abnormalities, the ability to recognize this as a statistically significant finding against the normal background of a 3–5% abnormality rate would require over a thousand delivered infants. Additional research in the routine area includes methods to improve embryo quality and methods to perfect cryopreservation.

Non-routine procedures (donor gametes and surrogacy)

On the other side of the picture of reproductive technologies are a number of non-routine procedures, some of which have found their way into fairly common practice but which remain controversial (Table 18.5).

Table 18.5 Non-routine procedures of assisted reproduction

Donor semen
Conventional donor insemination (DI)
Non-conventional
Single woman having DI
Surrogacy arrangements
Donor eggs
Conventional ovum donation
Non-conventional
Age limit
Surrogacy arrangements

The papal instruction specifically opposes the use of donor gametes; this is also the stated Jewish position. Some Islamic countries which have accepted IVF have specifically opposed the use of donor gametes (Malaysia), whilst others have accepted sperm donation but not ovum donation (Singapore).

There are several other non-routine situations which need to be considered. A number of single women ask for DI for different reasons. Increasingly common is the professional career woman who is approaching 40 years of age and is heterosexual by nature but is not yet contemplating marriage. She may wish to conceive before the complete loss of her reproductive potential. Other requests come from

lesbian couples. So far most infertility centres refrain from including such women, electing to direct treatment to couples within a legal marriage or stable de facto relationship.

Surrogacy arrangements are also frequently requested, particularly from young women who have lost their uterus from surgery performed because of increasingly common diseases such as early invasive cervical cancer. Donor eggs are commonly used now where women may have suffered premature menopause or have ovarian failure. Some couples may have an underlying carrier state for genetic disease and therefore donor semen or donor eggs may be required to avoid transmitting the disorder to their children. A number of women wish to donate eggs to a specifically elected recipient (e.g. to a sister or friend) and others wish to consider surrogacy arrangements which involve the transfer of an entire embryo formed from the gametes of an infertile couple, to be simply carried and nurtured by a surrogate female, rather than the traditional surrogacy arrangement which involves fertilization of the surrogate's own egg.

As the potential to treat infertile couples by donor gametes or surrogate arrangements increases, the consideration of an upper age limit is one which faces ethics committees.

Ethical and legal considerations

There has been considerable debate about embryo experimentation over the last 10 years since the first IVF infant was delivered. Opposing moralists held the view that this was a slippery slope.

The philosophical view defending IVF (Singer and Wells, 1984) applies a utilitarian argument which can be thought to be lacking in moralist sympathy. This is possibly the main reason why the Roman Catholic stance remains strongly against IVF.

It is unfortunate that the ethical debate in Australia appears to have become bipolar. This was avoided in the UK. The Committee of Enquiry into Human Fertilisation and Embryology, chaired by the philosopher Dame Mary Warnock (1984), exposed weak arguments on both sides of the debate and won stronger and wider acclaim. In addition, IVF workers throughout the UK have conducted forums for major specific ethical debates over the past decade and have been particularly careful to improve the understanding on both sides. Many theologians and Anglican ministers have expressed a position of moral comfort with IVF procedures and ethically approved experimentation on embryos.

Under British law, the human child is not a bearer of rights until it is born live (Table 18.6). It does however have moral claims which protect it by law. Therefore the mother has certain obligations and attending medical practitioners have duties to the mother, all of which

Table 18.6 Embryo status under British Law

The human child is not a bearer of *rights* until it is born alive
It does however have *moral claims* which protect it by law e.g. the mother has obligations doctors have duties
The embryo therefore enjoys a presumption in favour of life, even though it has no personality or rights

indirectly protect the fetus. The embryo therefore enjoys a presumption in favour of life even though it has no legal personality or rights. In the UK the Warnock Committee considering IVF produced its report in 1984 and in the following year the VLA was established to accredit IVF units around Britain. The British and Australian position on embryo experimentation is outlined in Table 18.7.

Table 18.7 Ethical considerations on embryo experimentation

Status of preimplantation embryos
UK
Warnock Committee, 1984
VLA, 1985
allows experimentation up to day 14
Australia
Assisted reproduction techniques controlled at local ± State level
Victoria: only State to have legislated on IVF activities
Local ethics committees: varied approval (nil, syngamy, day 11)
National Health and Medical Research Council (NH&MRC)
Guidelines, 1983 (NH&MRC, 1983)
FSA accredits IVF units
Reproductive Technology Accreditation Council (RTAC) for monitoring and control
Ethical Aspects of Research on Human Gene Therapy, 1987 (NH&MRC, 1987)
Embryo experimentation bill (Harradine, 1985)
Senate select enquiry (GPO, 1986)

Gamete manipulation: genetic techniques

It is not appropriate to describe here the types of embryo experimentation which are considered important. Earlier, mention was made of some techniques which are essentially designed to improve the effectiveness of routine IVF procedures. However, during the last decade,

alongside the developments in IVF technology, the ability to manipulate DNA in the laboratory has increased rapidly, giving rise to a new major discipline and body of knowledge within biology known as molecular genetics. We are just beginning to see the first useful effects of this discipline in that pure DNA 'designer' drugs, hormones and enzymes are being produced. These will create dramatic breakthroughs for immunotherapy and hormone replacement in a range of diseases, and become major weapons in the struggle to control a wide variety of disorders. The next stage is the use of DNA diagnostic techniques (Penketh and McLaren, 1987). This has implications for IVF in that it will soon be possible to diagnose genetic disorders within one or two cells of an early pre-embryo. In the future it will be possible to select those embryos which will be used for subsequent transfer (see Chapter 19).

A further possible extension of this work is DNA therapy which involves the insertion of genetic material (DNA) into the nucleus of a cell which has a deficiency, thereby correcting the potential underlying disorder—for example, Huntington's disease, muscular dystropy, haemophilia, etc. The processes are complex but have now been achieved in animals and we are on the threshold of applying them in the human arena. The main issues within this area of embryo experimentation include obtaining the conceptus, biopsy techniques, storage of pre-embryos and culture of cells (Fig. 18.1).

The conceptus may be obtained by uterine flushing or IVF of donated eggs. Donated eggs may be the excess ones in GIFT or IVF attempts. The various IVF-related techniques often generate excess oocytes or embryos and the options regarding excess oocytes or embryos are given in Table 18.8. They may also be donated by women undergoing other procedures such as sterilization.

The biopsy techniques for the segregation and removal of blastomeres also require consideration—previously it was noted that the very early cells of the embryo are totipotential (capable of becoming an embryo in their own right) but that later, the majority of cells differentiate and are destined to become parts of the placenta and membranes. Several techniques of embryo biopsy have already been described, involving either removal of blastomeres or removal of cells from the edge of the blastocyst. The ethical considerations will require some understanding of the scientific principles behind the technique (Brumby, 1986).

Storage of pre-embryos also becomes an important issue, depending on the type of diagnostic technique applied. A quick karyotype may allow the remaining pre-embryo to be transferred back to the patient but a slower DNA diagnosis on cultured cells may require cryopreservation of the remaining pre-embryo for transfer in a subsequent cycle.

The culture of biopsied cells also needs ethical consideration and

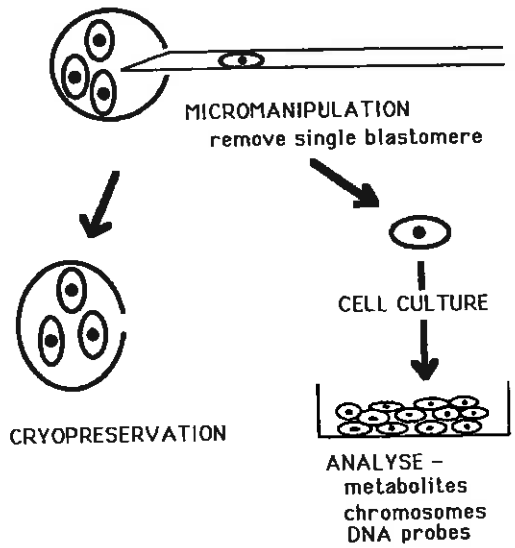

Fig. 18.1 Schematic diagram showing the techniques of embryo biopsy using micromanipulation procedures, the necessity for cryopreservation of the biopsied embryo and the need to develop suitable in vitro culture techniques to develop sufficient blastomeres for genetic diagnoses. However, current developments applying the polymerase chain reaction may enable clinical diagnosis to be performed rapidly on a single cell.

Table 18.8 Use of excess gametes and embryos generated as a natural consequence of IVF-related procedures (one-third of women generate more oocytes than will be subsequently transferred)

Options
Fertilize and store embryos for the couple's later use
Donate for use by another couple
Utilize for ethically approved research project
Discard

will depend upon whether one is dealing with totipotential cells or non-fetal differentiated cells.

IVF centres around Australia are obliged to be accredited by the national body, the Fertility Society of Australia (FSA). This requires them to have an acceptably constituted formal institutional ethics committee and to abide by the National Health and Medical Research

Council (NH&MRC) guidelines of 1983. Similar approaches are in force in other countries; the appointment of an ethics committee to each centre is mandatory. The composition of the committee is an important consideration, as are the terms of reference (for example, see Appendix 1).

An example of the standards of practice recommended by national bodies is shown in Appendix 2.

With respect to DNA treatment of patients with inherited diseases, two conclusions were drawn: firstly that certain forms of somatic cell gene therapy are ethically acceptable; secondly, the possibility of introducing pieces of DNA into human germ cell lines is ethically unacceptable at this stage. This in particular refers to the therapeutic approach and does not necessarily limit laboratory experimentation. The reasons for this opposing view are twofold. Firstly, when a diagnostic test system is developed, it will enable preselection of embryos. Secondly, this is preferable to allowing insertion of genetic material into germ cells because there is no guarantee at this stage that the gene replacement proposed will actually replace the defective gene in its normal location in the chromosomes. Any adverse effect of improper location will potentially create generations of problems rather than simply affecting the one individual.

CONCLUSIONS

We have described the historical and future developments which highlight the prevailing climate of controversy. The issues must be faced and will not go away. Currently there are over 20 000 IVF infants around the world. Scientists are highly motivated to explore the fundamental issues further and medical practitioners are keen to find solutions to the individual problems of their patients. Whilst the bipolarity of views regarding the practitioners on the one side and moral opponents on the other provides a clear picture of the extreme views, it is necessary that the two factions should find some common ground.

Currently many countries and states around the world have proposed legislative control over assisted reproduction. The four areas where such legislation often embraces are as follows:

1 Protocols and standards related to clinical and laboratory aspects.
2 The matter of accountability to the general community.
3 Specific considerations related to embryo research.
4 Property consideration with respect to stored gametes.
5 Consideration of the welfare resulting children primarily and other participants in assisted reproduction programmes secondarily.

While governments often feel impelled to control activities with as wide as possible embrace, it is the general feeling of workers in assisted reproduction that the first three issues are clearly a matter for self-regulation and that legislation should be confined to property considerations with respect to stored gametes; and the protection of arrangements concerning donors, surrogates and resulting children.

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APPENDIX 1: ETHICS COMMITTEES FOR IVF CENTRES—GUIDELINES

An Ethics Committee has as its duty the protection of both patients and public. By being seen to ensure that a centre maintains appropriate ethical standards it also protects the centre from unjustified criticism.

The objectives of an Ethics Committee are:

(i) to protect the interests of patients and of any children resulting from the use of assisted conception in particular; and those of society in general; and to provide reassurance to the public that this is being done;
(ii) to consider and, where appropriate, approve medical research and the development of new clinical procedures in the fields of embryology and infertility.

Research

When considering research projects an Ethics Committee should follow the guidelines proposed by the World Health Organization and Council for International Organizations of Medical Sciences as set out by the Royal College of Physicians (1984). Before commending a project to the Voluntary Licensing Authority (VLA) the Committee members should satisfy themselves that the project is within the current guidelines of the VLA and, in particular, that

(i) the required information cannot be obtained from animal models or that the appropriate animal work has already been undertaken;
(ii) there is no intention to replace in utero any pre-embryo resulting from or used for research procedures unrelated to its own preparation for replacement.

Clinical procedures

An Ethics Committee will also be expected to approve the centre's proposed clinical programme and to discuss any changes later introduced into that programme.

Centres are expected to follow the current VLA guidelines and any proposed departure from these guidelines must be approved by the Ethics Committee and reported to the VLA.

In approving such a proposed departure the Committee members should assure themselves that

(i) no alternative procedure exists for the case in question—the fact that a clinic may have difficulty in meeting the requirements of the VLA is not a basis for circumventing its guidelines;
(ii) the prospective patients have been fully counselled with regard to the possible consequences of the proposed procedure;
(iii) the interests of the potential offspring as well as those of the parents are taken into account when assessing the risks and benefits of the procedure;
(iv) the interests of any third party are taken into account;
(v) the effects of the procedure on the wider interests of society in terms of its norms and obligations, and of the adequacy of its provision for consequent after-care, have been given consideration.

Membership

(i) It is not necessary that those who become members of the Committee are experts in moral philosophy or in particular disciplines; they need to be reflective people of goodwill, with a high

regard for the human personality, for truthfulness and for the continued advance of reproductive medicine and medical science. Those who are totally opposed to work involving the human pre-embryo should be left to attack the system from outside; but neither should individuals be included who are acquiescent and likely to give automatic approval. Of the medical and scientific members there should be a majority who are employed in providing clinical care. It is also important that there will be individuals who will look at applications critically from the patient's points of view.

The membership should comprise the following interests:

(a) *medical and scientific*—these members should include a general practitioner and someone with experience related to infertility;
(b) *nursing*—a nurse who is in active practice with patients;
(c) *lay*—there should be not less than two lay members and they should provide effective representation of community rather than sectional interests. A lay member with legal training can be of value but his or her role should not be restricted to answering questions of law.

The committee should include women amongst its members.

(ii) The Committee should elect its own Chairman and also a secretary who should keep a record of business. It is suggested that either the chairman or the vice-chairman be chosen from amongst the lay members to emphasise lay involvement.
(iii) The Chairman of an Ethics Committee should have no financial interest in the centre for which that Committee is responsible. Any committee members who have such an interest should declare it.

Frequency of meeting

(i) An Ethics Committee should meet at least twice per year or more frequently if matters arise.
(ii) All members should visit regularly the centre for which they are responsible—this should be at least once a year.

Mode of working

(i) Business should be conducted at meetings of the Committee and not by post.
(ii) It is useful to circulate to the Committee publications relevant to the ethical conduct of assisted reproduction or pre-embryo research which may appear from time to time such as publications from the Government, the Royal Colleges or the VLA.

(iii) If the Committee finds a project unacceptable, reasons for the decision should be given to the Director of the Centre in writing. It may be possible for projects to be modified in discussion with the Director. Where the Committee finds itself uncertain about the ethics of a particular project or procedure, referral may be made to the VLA.
(iv) While it is impracticable for an Ethics Committee to monitor approved projects or procedures in detail, some follow-up is desirable to ascertain progress. Committees may consider requesting reports of work completed, or an annual report from clinical centres.
(v) In the event of an Ethics Committee discovering that its advice is unheeded or that projects or procedures are being carried out which infringe the guidelines of the VLA, these facts should be reported to the Authority.
(vi) Confidentiality should be preserved, not only for the protection of individuals but also because the issues considered are often complicated and delicate and uninformed or unbalanced publicity could raise emotions that are damaging to all concerned, especially the patients.
(vii) It is undesirable to take important decisions in the absence of, for instance, a lay member and the Committee should define the composition of a Quorum as well as its number.

Business

(i) As well as keeping a watch on the practice of its own centre one of the main duties of an IVF Centre's Ethics Committee is to see that the guidelines of the VLA are observed.
(ii) Not all centres undertake research projects; Ethics Committees should also provide guidance for centres on their clinical programme. In the current field of IVF it is often difficult to distinguish between experimental and therapeutic clinical treatment, which is where the role of the Ethics Committee becomes critical.
(iii) IVF and other techniques for assisted reproduction are areas of practice in which there is rapid development both in terms of clinical and of scientific advance. Their impact on society requires constant assessment. Legislation is in prospect which would affect both clinical services and research. Local Ethics Committees should keep themselves informed of all advances and take the opportunity to discuss together their implications whenever necessary. It may be advisable to delegate members with a particular knowledge or interest to report on such items at meetings of the Committee.

(iv) The record of business should be made available to the VLA upon request.

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APPENDIX 2: GUIDELINES TO THE CODE OF PRACTICE FOR UNITS USING IN VITRO FERTILIZATION AND RELATED REPRODUCTIVE TECHNOLOGIES

In 1986 the Fertility Society of Australia (FSA) promulgated a series of standards as a guide to the code of practice of IVF and related technologies. The Reproductive Technology Accreditation Committee (RTAC) established by the FSA in 1987 has added a series of explanatory notes to many of the original standards drawn up by the FSA. These are intended to be used as guidelines by those centres involved in IVF and related technologies (hereafter referred to as IVF).

It is appreciated that there may be differences in detail between these guidelines and those Acts and the associated regulations relevant to IVF which have already been proclaimed by some States.

The unit

1 The Medical Director of the IVF programme should be a recognized specialist skilled in infertility management. IVF should only be offered within the context of a broad clinical expertise in infertility management.

Responsibility for the conduct of the clinical affairs of the centre should be vested in a specialist medical practitioner (the Medical Director) who has knowledge, skills and experience in the management of infertility.

The Medical Director should have access to other specialist medical, surgical and nursing personnel who also have skills in the particular reproductive technology that is being carried out.

The centre must be able to provide for emergency treatment and have access to specialist anaesthetist services.

Nursing staff with special training in infertility practice should be responsible for the co-ordination, nursing supervision and comprehensive care of patients at all stages of their IVF treatment.

2 Successful IVF requires the technical and scientific supervision of suitably qualified reproductive physiologists or biologists to maintain the necessary standards of quality control. Laboratory staff should hold qualifications appropriate to their areas of responsibility.

The centre should have scientific directors who possess the appropriate tertiary qualifications relevant to their area of responsibility, e.g. embryology, biochemistry.

The centre must have laboratory facilities for the examination of gametes prior to fertilization and the pre-embryo after fertilisation. The laboratory should be located in close proximity to the clinical facility so that there is no impediment to the transfer of gametes or pre-embryos between the two facilities.

The remaining laboratory staff should possess qualifications and training relevant to their responsibilities. The laboratory staff comprises two groups: embryologists, who are responsible for the preparation of gametes for fertilization and for the subsequent culture of the pre-embryos; and biochemists who are responsible for the measurement of those hormones in body fluids that are essential (among other things) for the evaluation of ovum development in response to hormonal treatment. The biochemistry laboratory should be accredited by NATA.

The embryology laboratories should provide the necessary facilities for pre-embryo culture and microscopy under aseptic conditions using no-touch techniques. Ideally, for all major centres laboratory facilities should be available seven days each week.

3 Each unit should have access to ultrasound monitoring facilities on a daily basis.

The doctor responsible for ultrasonography should possess the relevant diploma or training in obstetric ultrasound.

4 The transport and identification of ova, sperm and embryos must conform to standards required to minimise the chance of accident, loss or confusion as to the donors' or owners' identification.

If an oocyte from a female partner is either unavailable or unsuitable for fertilization, ovum donation is possible provided that both the donor and recipient couple give their informed consent. The same applies to the donation of pre-embryos.

Although the aim of IVF is to maximize the chances of pregnancy, consideration must also be given to reducing the risks of a large multiple pregnancy. Up to three, and in exceptional circumstances four, pre-embryos may be transferred at any one cycle.

5 Gametes or embryos of different parental origin should never be mixed so as to confuse the biological parentage of the conceptus.

Patient services

6 Patients should be made aware, before commencing treatment, of the likely financial, social and medical implications of IVF and related procedures. Patients should be made aware of the availability of patient support groups.

7 Informed consent is an integral part of the processes of IVF. It is essential that each Institution provides a statement in plain language to inform potential recipients about that particular IVF programme.

An important function in the overall monitoring of the clinical practice of IVF is to ensure that patients are adequately informed so that they are able to consent to any procedure with knowledge and understanding of that procedure. This is best achieved by making sure that the staff are aware of the procedures being used and providing the patients with a plain language statement which the couple can take away and study at their leisure before being asked to give consent to any procedure or set of procedures.

The consent form should be simple and as free as possible from complex medical terms or jargon. Subjects to be dealt with in a plain language statement are outlined in Attachment A. A list of the topics that should be dealt with in a consent form is provided in Attachment B.

8 Patients should be given all the appropriate consent forms at the time of counselling prior to the beginning of the first IVF treatment, and written consent to all IVF procedures must be obtained prior to beginning any aspect of IVF or related treatment. Patients should be given their own copy of any written consent as their permanent record.

There must be a comprehensive counselling service available so that the patients know and understand the likely financial, psychological, social and medical implications that the particular reproductive technology may have on the subsequent quality of their lives. The two necessary aspects to counselling in reproductive technology programmes are information exchange and supportive counselling.

Information exchange should provide comprehensive details about treatment options, what the treatment regimen entails, possible side effects and complications, current clinic success rates and what that particular patient's chances of success may be. It is the medical practitioner's overall duty and responsibility to ensure that free and comprehending consent is obtained prior to commencing any treatment. This aspect of counselling may be delegated to a nurse or counsellor with adequate medical knowledge. Informed consent means more than a patient's signature on a consent form. When

donor gametes are to be used, counselling should include a discussion of the interests of the child, what to tell the child, relatives and friends.

The use of written material and audiovisual aids is recommended. Information given should be comprehensible to the patient. Special attention may be required for those patients from a non-English speaking background. Sometimes the educational background of the couple may make it essential for a more detailed explanation than usual. Access to an interpreting service should be available.

Supportive counselling should readily be available for all infertile patients, particularly at times of crisis such as: at initial diagnosis, while awaiting treatment, after a treatment cycle, deciding to stop treatment or after an unfavourable outcome of a pregnancy.

Supportive counselling should be provided by counsellors who are professionally trained and experienced in infertility counselling. Ideally each IVF centre should have a resident counsellor who should have training, experience and professional qualifications in psychology, social work, medicine or nursing and should in addition have had a period of training in the medical aspects of reproductive technology. Information and support counselling should be undertaken by resident counsellor(s) who should be responsible for the training of other staff in communication skills and facilitating communication within the clinic.

Records

9 A permanent record must be kept of all procedures identifying the patients, donors and recipients of all gametes involved in fertilization and embryo formation and the final outcome of any attempted fertilization and the final destination of any conceptions formed by IVF techniques.

10 Results must be made available to the associated or governing institution and to the National Perinatal Statistics Unit. It is essential that a national data base be maintained to compile and analyse the results of IVF. Results should be compiled as in Attachment C. These same results should be kept as a permanent record. Notwithstanding the need to record and report it is essential that confidentiality be maintained.

Record keeping is essential in the conduct of the affairs of the centre. The records should be both clinical and should include results of laboratory studies undertaken before, during and after treatment cycles.

In the recording and reporting of results each centre should strive to report results in a way that maintains patient confidentiality. One objective of record keeping is a long-term follow up to determine whether children born of IVF have a higher incidence of birth defects or are more susceptible to illness than their peers who have been conceived naturally. In order to achieve this it may be necessary to identify these particular individuals. Assistance with the compilation of epidemiological data may be derived from the NHMRC Supplementary Note 6: Epidemiological Research.

11 Specific records should be kept on semen quality. It is recommended that these records conform to the WHO guidelines for male infertility. These records should enable a retrospective analysis of the criteria necessary to accept men with infertility for IVF and to advise patients of the likely success rates given their semen profile.

Donors of sperm are best recruited from men in a normal heterosexual relationship; other donors should be prepared to testify that they have had no male to male sexual relationships in the last 10 years nor have they been using intravenous drugs of addiction for a similar period. Selection of donors should conform to guidelines laid down by the Fertility Society of Australia (Attachment D).

Donor insemination with fresh semen is unacceptable because of the potential risks to the recipient and to the staff of any centre which is involved in such a practice.

Ethics and research

12 IVF, whether therapeutic or experimental must only be practised within the ethical guidelines established by the NHMRC. In addition, every IVF programme must have all aspects of the programme monitored by the Ethics Committee of the hospital or the institution concerned and conform to the regulations laid down by individual State legislation.

The roles of the Institutional Ethics Committee in monitoring the activities of those centres that practise IVF have been the subject of an NHMRC publication, Supplementary Note 4: In Vitro Fertilisation and Embryo Transfer.

Every centre offering IVF and related reproductive technologies should have all research aspects of their programme approved by an Institutional Ethics Committee. All units practising IVF are encouraged to have an active research programme. The objective of the programme should be to increase basic knowledge in the field of reproductive biology, to develop new and to improve existing procedures. The research programme should be based on sound principles and be within the ethical guidelines laid down by the NHMRC.

Every endeavour should be made to publish results of research findings in authoritative peer reviewed journals. Any new technical procedure developed as part of the research programme should only be adopted as normal clinical practice if it falls within ethical guidelines and is considered to be a suitable medical practice by RTAC.

An IVF unit without an active research programme can still receive accreditation provided it meets the other criteria laid down by the RTAC. Units that do not have their own research programmes are encouraged to participate in epidemiological studies of IVF. These units should also ensure that members of their staff receive the necessary training in new procedures developed in other laboratories before these are incorporated into the routine practice of their own units.

The uses of pre-embryos for research must have the written consent of the donors. Any pre-embryo should not be permitted to develop beyond the stage at which implantation would normally occur.

13 Any experimentation involving fertilization or embryos must be approved by the governing body or the associated Institutional Ethics Committee and confined to ethical guidelines established by the NHMRC and the relevant State Government.

The Institutional Ethics Committee must be satisfied that means exist for the couple to consent to the use of sperm, spare oocytes or pre-embryos for research purposes and to indicate that excess pre-embryos may be stored and when these should be disposed of.

14 Regular interdisciplinary meetings should be held to discuss IVF procedures and patient management and should be attended by the clinical and scientific staff of the programme.

15 All programmes will be subject to scientific and medical audit by RTAC.

Attachment A: Contents of plain language statement

The committee believes that it is not possible to prepare a plain language statement that may be used uniformly. The committee suggests however that every effort be made to ensure that the statement is written in language that is free from jargon and technical terms which patients may not comprehend. It is important to remember that some patients may be too nervous or reticent to ask about terms that they may not understand.

The committee believes that the statement should at least address the following issues:

* The likely costs of the procedures: financial and emotional.
* An explanation of the terminology: IVF, GIFT, PROST, DI.
* Why one of these methods needs to be used for that particular couple.
* The various procedures that may be used during a treatment cycle:

  the need to collect blood (or urine)
  cycle tracking
  ovulation induction and the drugs used
  laparoscopy
  ultrasound
  egg retrieval
  production of a semen sample
  fertilization
  pre-embryo transfer
  the number of pre-embryos to be transferred
  spare embryos: donation or storage?

Attachment B: Consent forms

The committee suggests the following guidelines for the use and preparation of Consent Forms.

1 There should be a request by the patients for a procedure to be carried out.
2 Only one procedure is to be consented to on each form. Use a separate form (and a separate signature) for each extra option which may be consented to, for example: IVF, GIFT, PROST, donor oocytes, donor sperm, donor pre-embryos, pre-embryo freezing, oocyte freezing, oocyte donation to another woman, oocyte donation for research, semen donation to another couple, pre-embryo donation to research, pre-embryo donation to another couple. We suggest that you use a standard form and standard wording where possible and fill in the blank space for the specific procedure.
3 The form is to be signed by both partners, witnessed and dated.
4 There should be an acknowledgement that there has been an exchange of information regarding the procedure between the patients and the medical practitioner and that the patients have had adequate time and opportunity to ask questions about the procedure and its risks, and that all questions have been answered to the patients' satisfaction.
5 There should be an acknowledgement that the patients have been given detailed written information about the procedure.

6 There should be an acknowledgement of the risks and possible side-effects or complications of the procedure.
7 There should be an acknowledgement that the procedure may be cancelled or unsuccessful.
8 There should be a clear statement as to whether the clinic views the procedure as experimental, innovative therapy, a clinical trial or a standard therapeutic procedure. This is particularly important when new techniques are being introduced, for example, micromanipulation.
9 There should be an acknowledgement that the patients are free to withdraw their consent at any time.
10 In the case of cryopreservation of gametes or pre-embryos, a record of the options consented to for their fate and the conditions of storage including the wishes of the couple in the event of their death, separation or divorce.

The committee would like to draw attention to a particular publication on IVF which provides a clear and comprehensive account of the processes of reproduction, what can go wrong and how these may be overcome by IVF or related reproductive technologies: *The chance of a lifetime—infertility and IVF*, Carol Dettmann and Douglas Saunders, Penguin, 1987.

Attachment C: Information to be provided annually to the National Perinatal Statistics Unit/Fertility Society of Australia

National data base on IVF and related techniques

1 Number of patients who began a treatment cycle (include both those beginning treatment in the year of the report, and those previously treated returning for further cycles).
2 Number of cycles of treatment for women in (1).
3 Number of patients admitted for:
  (a) Laparoscopic oocyte recovery.
  (b) Ultrasound guided oocyte recovery.
4 Number of treatment cycles with admission for:
  (a) Laparoscopic oocyte recovery
  (b) Ultrasound guided oocyte recovery.
5 Number of patients admitted for GIFT.
6 Number of treatment cycles with GIFT.
7 Number of patients with embryos transferred (not cyropreserved).
  (a) Transferred to uterus.
  (b) Transferred to tubes.
8 Number of cycles with embryo transfer (not cyropreserved).
  (a) Transferred to uterus.
  (b) Transferred to tubes.

9 Number of patients with:
  (a) Donated oocytes.
  (b) Donated embryos.
10 Number of patients having embryos frozen.
11 Number of cycles with embryos frozen.
12 Number of patients receiving thawed embryos.
13 Number of cycles with thawed embryo transfer.
14 Number of embryos frozen.
15 Number of embryos thawed.
16 Number of embryos disposed of.
17 Number of embryos subjected to experimentation.
18 Number of pregnancies
  (a) Biochemical.
  (b) Miscarriages less than 12 weeks, miscarriages greater than 12 weeks.
  (c) Number of single, twin, triplet etc. pregnancies.
  (d) Number of single, twin, triplet etc. births.
  (e) Number of neonatal abnormalities.
  For:
    (i) IVF.
    (ii) GIFT.
    (iii) Tubal embryo transfer procedure.
    (iv) Frozen embryo transfer.

Attachment D: Screening for donor insemination

The Council of the Fertility Society of Australia consider the following as the minimum criteria for screening donors for donor insemination programmes. Please note that these criteria are for the screening of donors and are not meant to be guidelines for the organization of a donor insemination programme.

1 History. Donor history to include:
  1.1 Family history of inherited disorders.
  1.2 Personal history of physical, mental or psychological disabilities.
  1.3 All donors should sign a life-style declaration as required by the relevant State Department of Health.
2 Physical examination. Physical examination to exclude any obvious abnormality.
3 Semen analysis. Detailed microscopic examination of semen for:
  3.1 Potential fertility status.
  3.2 Signs of potential infection requiring further investigation.
4 Serology. The following serological tests to be performed:
  4.1 Blood group and Rh (and any other blood group antibodies).

  4.2 Syphilis serology— VDRL or similar.
  4.3 Hepatitis B surface antigen and anti-hepatitis core screen.
  4.4 HIV antibody titres.
  NB. Repeat serology for syphilis, hepatitis B and HIV antibodies should be performed on a regular basis.

The Fertility Society of Australia, in the light of current information considers that sero-conversion to HIV will occur within three months of infection. Thus, it is necessary to test donors three months after each donation to 'clear' that donation of HIV infectivity. Opportunity should be taken to carry out repeat screens for syphilis and hepatitis B. Units may prefer to hold material for longer periods before re-testing.

5 Other tests
  5.1 Thalassaemia—where a donor has Mediterranean or Asian background consideration should be given to screening for Thalassaemia trait by the measurement of the haemoglobin and mean corpuscular volume.
  5.2 Tay-Sachs Disease—where a donor is Jewish consideration should be given to screening for this autosomal recessive trait present in 1:50 Ashkenazi Jews (in America) and 1:100,000 in the general population.
6 Bacteriology. It is considered appropriate that donated semen be periodically cultured including specific culture for gonococcus.
7 Recording of screening information. Good medical practice requires that the above screening information regarding the donor be retained.

It is considered that the following information form part of the donor record.

8 Physical characteristics
  8.1 Height.
  8.2 Build.
  8.3 Eye colour.
  8.4 Hair colour.
  8.5 Skin colour.
  8.6 Race.
9 Social History. It is considered appropriate to record the following non-identifying donor information which may be of assistance to parents of children at a later date.
  9.1 Age.
  9.2 Religion.
  9.3 Nationality.
  9.4 Race.

9.5 Country of birth.
9.6 Schooling.
9.7 Occupation.
9.8 Marital status.
9.9 Number of children.
9.10 Interests (hobbies, sports, etc.).
9.11 Comment on donor personality by interviewer.
9.12 Reason for assisting donor insemination programme.

10 Confidentiality

10.1 Care should be taken to avoid any disclosure of patient or donor identifying information.

10.2 It is considered appropriate that identifying information regarding donors be retained as confidential records with the donor insemination unit in line with good medical practice.

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Chapter 19

Conclusions and future directions

The world applauded the birth of Louise Brown 10 years ago as a triumph of science over nature. Since then IVF has functioned as a flagship, introducing an array of techniques to assist in the management of human infertility. The development has been timely, as the demand for such services has increased in modern societies even though the underlying causes in previous generations have been largely cured in developed countries.

CURRENT TRENDS IN IVF PRACTICE

Although IVF clinics have now been established widely, so that the communities of virtually all countries can potentially avail themselves of modern treatments for infertility, independent reports from around the world, largely on data up to the end of 1986, showed that the live birth rate per IVF treatment cycle was only 9–10% for those who reached the stage of oocyte recovery (CDCS&H, 1988; VLAHIVFE, 1988; COTA, 1988). In order to improve IVF-related outcomes, a number of new approaches have been pursued by various clinics.

Developments on the conventional model

This implies that current concepts in assisted reproduction are based on a firm foundation and that by serial improvements in the various technical aspects, the outcome of treatments will consequently be increasingly successful. At the PIVET Medical Centre, several changes have been introduced progressively over the past two years as follows:

Tubal transfer procedures

IVF-ET is reserved for tubal factor infertility and GIFT is applied for the indications of unexplained infertility, endometriosis, poor sperm/mucus interaction and failed DI. PROST and TEST (both laparoscopic and trans-cervical) were introduced for male factor infertility, failed

GIFT, ovum donation, IVF surrogacy and post embryo cryopreservation cases.

Changes in ovarian stimulation schedule

Essentially this has meant the diminishing use of clomiphene citrate and the increasing use of the GnRH analogue leuprolide acetate (Lucrin; Abbott Australasia) using a down regulation schedule (Figure 4.4). The latter was shown to have significant benefits in patients of advanced age, underlying PCO, raised androgens, raised basal LH, previous premature LH surges and some poor responders (Cummins et al., 1989). The flare method (commencing both HMG and analogue at the beginning of the menstrual cycle) is also occasionally used for poor responders. However, the poor responder group remains a difficult group to treat effectively. The use of growth hormone as an adjuvant in ovarian stimulation therapy may improve the response (Homburg et al., 1989) or at least reduce the amount of HMG required to effect successful stimulation (Owen *et al.*, 1989). Unresponsive cases may be due to a state of incipient ovarian failure and should consider ovum donation.

Technical developments

Transvaginal oocyte recovery is now preferred for virtually all cases using the PIVET-Cook double lumen aspiration/flushing needle (William A. Cook Australia Pty Ltd). For ET an improved catheter system was developed as well as the introduction of an earlier protocol involving transfer in the lithotomy position, followed by 4 hours of absolute bedrest in a head-down position. Tubal transfers utilize the Molloy style Teflon catheter for the laparoscopic approach and the original version of the Jansen-Anderson catheter system for the trans-cervical approach (both by William Cook Australia Pty Ltd).

Luteal support

Following the demonstration of significant benefits in establishing pregnancy (Yovich, 1988), luteal support is now used routinely. The regimen utilises Proluton 50mg imi days 0, 1, 2, 3 and 4 and HCG 1000 IU imi days 4, 7, 10 and 13 dating from oocyte recovery.

Excluding ovum donations for ovarian failure, Figure 19.1 shows the outcome for all IVF-related treatment cycles commenced at PIVET during 1988. The proportion which proceeded to oocyte retrieval, successful fertilization and implantation with at least one surviving infant was 18% overall or 20% of those undergoing an oocyte retrieval procedure. There were also ten pregnancies following 26 ovum donation

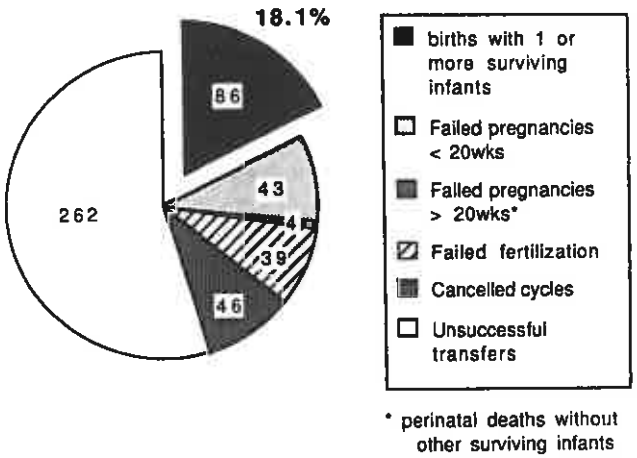

Fig. 19.1 Outcome of all treatment cycles commenced at PIVET during 1988, including cycles cancelled for various reasons.

cycles, eight of which progressed to livebirths. The total effectiveness of IVF during 1988 was therefore 33% per collection (142/434) and 34% per transfer (142/421). This provided a birth rate (i.e., deliveries after 20 weeks) of 24% per transfer procedure (16% of IVF-ET and 27% for tubal transfers), which is a 2.5 fold improvement over a 3 year period. Total pregnancy wastage (before and after 20 weeks) was 35%, and 34% of births were multiple (mean of 1.4 infants per delivery).

The relative chance of pregnancy of the respective IVF-related treatments is shown in Figure 19.2. The data includes ovum donations and post-cryopreservation transfers which are performed primarily by TEST (denoted as 'others' in the figure). During a 17-month period, 476 couples had 649 treatment cycles reaching the stage of oocyte recovery. The overall pregnancy rate was 32% per retrieval and 35% per transfer. Previously uterine receptivity has been shown to be higher for tubal transfers than for IVF-ET (Yovich et al., 1988a) but the difference has reduced on recent data, possibly as a consequence of the changes in ovarian stimulation regimens. Essentially this has meant a reduction in the use of clomiphene citrate and the increasing use of leuprolide acetate. During 1989 the maximum transfer number for both oocytes and embryos has been reduced to three and the data for the first 5 months shows a continuing trend of high pregnancy rates but with fewer high order multiple pregnancies. It is believed that each of the four treatment changes introduced have contributed to the improved results and further benefits may be achieved by applying

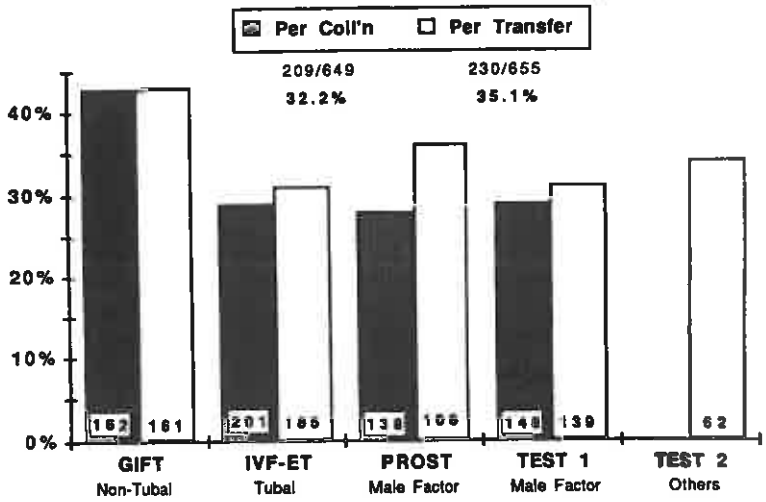

Fig. 19.2 The relative chance of pregnancy per IVF related treatment cycle proceeding to oocyte collection or oocyte/embryo transfer. TEST 1 refers to procedures performed for mainly male factor reasons; TEST 2 refers to cases having oocyte or embryo transfers following donation or previous cryopreservation. PROST was used January to June 1988 only with few cases only treated with Pentoxifylline; TEST had a high proportion of cases of male factor treated with Pentoxifylline.

advanced laboratory techniques such as sperm motility enhancement for male factor cases (see below).

Additional IVF-related techniques

The success of GIFT for unexplained infertility implies that the infertility disorder was due to the failure of oocyte release, the failure of oocyte pickup by the fallopian tubes or the failure of spermatozoa to reach the distal fallopian tube and undergo the appropriate changes of capacitation, acrosome reaction and penetration in order to achieve fertilization. A number of other procedures have been described following on the GIFT experience which have the potential to correct for one or several such dysfunctions. The first was described as DIPI (direct intraperitoneal insemination) (Forrler *et al.*, 1986). This involves the injection of spermatozoa into the pouch of Douglas, usually by the transvaginal technique, after in vitro sperm preparation. A further step on from this is the procedure known as POST (peritoneal oocyte and sperm transfer) (Mason *et al.*, 1987) which also involves the collection of oocytes and the transfer of both gametes into the pouch of Douglas,

again using the transvaginal route. The main benefit of these procedures is to avoid inpatient care involving anaesthesia and laparoscopy. Another modification of GIFT known as FREDI (fallopian replacement of eggs and delayed insemination) (Leung et al., 1989) has also been described; it involves oocyte recovery and laparoscopic placement of oocytes into the fallopian tubes but allows the interaction of spermatozoa and cervical mucus in a more natural selection process for fertilization. However, none of these procedures has yet been shown to be superior to the IVF related group of procedures (GIFT, PROST, IVF-ET and TEST).

Male infertility

For male infertility IVF provided a promising treatment method, but the original techniques were found to be of limited benefit (Yovich et al., 1984).

A number of laboratory techniques have recently been developed to improve fertilization rates in vitro for males with sperm disorders, particularly those with oligo/asthenospermia (Moderate <10 million progressively motile sperm/ml; severe <5 million/ml; very severe <1 million/ml) and teratospermia (<50% normal morphology). These levels are adapted from the WHO Manual (1987). In addition, epididymal sperm collections can be performed on cases of congenital absence of the vas (Patrizio et al., 1988) or vaso-epididymal obstructions. Such specimens usually contain considerable debris, blood cells and degenerate spermatozoa hence sperm preparation using Percoll may be preferred. Semen containing antispermatozoal antibodies (ASABs), particularly the presence of both IgA and IgG together, comprise another significant male-factor group (Matson et al., 1986).

Cases of male factor infertility which have failed to respond to AIH by intrauterine insemination may be treated in the IVF programme by TEST. The laboratory protocols include ejaculation directly into culture medium (for ASABs), using a sedimentation technique for sperm preparation, the use of pentoxifylline for motility enhancement and the use of micromanipulation (zona drilling and zona cutting). Of 269 IVF-related treatment cycles performed in a 5 month period from January to May 1989, 88 cases were specifically for male factor infertility treated in the TEST programme. The results are shown in Table 19.1 which compares all the IVF data for the period.

It can be seen that 91% (80 cases) achieved fertilization, including one case where the ejaculate had only 0.2×10^6 motile sperm/ml. The pregnancies include several cases from men categorized as having very severe oligospermia and two pregnancies following very high epididymal sperm collection in cases of obstructive azospermia. Previously we have shown a significantly higher pregnancy rate with TEST

Table 19.1 Male infertility managed by TEST and compared with general results of GIFT and IVF-ET during a comparable period in 1988–1989

Procedure	*Collections*	*Transfers*	*Pregnancies*	*Pregs/ coll'n*	*Pregs/ transf*
GIFT	50	50	21	42%	42%
IVF-ET	100	92	35	35%	38%
TEST:					
male factor	88	80	22	25%	28%
ovum don.	—	14	4	—	29%
frozen emb.	—	17	6	—	35%

(Yovich et al., 1988b) compared with IVF-ET, but this is not noted in this series (no significant differences in pregnancy rates), possibly for the aforementioned reason relating the changes in the ovarian stimulation regimens.

Pentoxifylline

Partial or total fertilization failure is common in cases of severe oligospermia, especially when associated with asthenospermia. Sperm stimulants have been used successfully to improve motility in such cases, and pentoxifylline (PF) has increased the incidence of pregnancy in cases with previous fertilization failure. (Yovich et al., 1988c; 1990). In a recent study results presented for two different PF-treatment protocols (PF1 and PF2) were applied with 57 couples who had underlying male factor infertility (61 treatment cycles) and were undergoing PROST or TEST procedures (Yovich et al., 1990).

Semen samples were split into two portions and oocytes were randomly divided into two groups to enable insemination by PF-treated or controlled sperm. Control aliquots were washed twice in HTFM with 10% deactivated human serum and motile sperm harvested by overlay or the sedimentation technique. In PF1, sperm were washed once and resuspended in medium containing 1mg/ml PF (Hoechst) for 30 minutes. Following a second centrifugation, the sperm pellet was sedimented under PF-free medium and motile sperm recovered. Sperm were washed, counted and used 1–1.5 hours later for insemination. In PF2, sperm were washed once, motile sperm harvested first and then incubated with PF for 30 minutes before washing a second time just before insemination. PF2 thus ensured that sperm were treated as closely as possible to the time of insemination while minimizing direct contact between PF and oocytes. Pronuclear oocytes (by PROST) or cleaved embryos (by TEST) were transferred to the fallopian tubes. Pregnancies were diagnosed by rising βhCG levels and confirmed by subsequent ultrasound observation of embryonic sacs. PF2 gave a signi-

ficant improvement in fertilization rates (Table 19.2) compared with untreated sperm and significantly fewer patients experienced fertiliza-

Table 19.2 Comparison of two pentoxifylline protocols for treating sperm in severe male factor infertility (1988–1989)

Fertilization rates		*Embryos transferred*		*Pregnancy outcome*	
Control	*PF*	*Control*	*PF*	*Per transfer*	*Per coll'n*
Protocol 1					
33/91 (36.3%)	52/154 (33.8%)	21	34 (8)†	7/19 (36.8%)	7/31 (22.6%)
Protocol 2					
21/103 (20.4%)	81/171 (47.4%)*	10	64 (18)†	9/27 (33.3%)	9/30 (30.0%)

* $P < 0.001$ control v. PF.
† Number of patients with only PF generated embryos transferred.

tion failure (PF1 12/31 failures: PF2 3/30; $P < 0.02$). Overall, sixteen pregnancies ensued and four were from semen samples with a mean of less than 1 million/ml progressively motile sperm; one being as low as 0.2 million/ml. A total of sixteen healthy infants have been delivered to date. Pentoxifylline treatment of sperm in cases of oligo-asthenospermia appears to be a valuable treatment option in IVF related procedures.

Micromanipulation

A range of micromanipulation techniques have been described which include microinjection (Ng et al., 1988) zona drilling (Gordon et al., 1988), zona opening (Odaware et al., 1989) and zona cutting (Cohen, et al., 1988) but to date only occasional pregnancies have been reported. At PIVET we have explored the latter three techniques. The methodology involves cumulus removal with 50 IU/ml hyaluronidase in the presence of trypsin inhibitor. Zona drilling involves the application of acid medium (pH 2.5) directly to the zona using a glass microinjection pipette attached to a Picospritzer instrument and zona opening uses glass hooks. Five pregnancies achieved following micromanipulation have each involved the additional transfer of at least one control embryo so at this stage the role of the techniques remains uncertain.

Epididymal sperm collections

Epididymal sperm collected from males with congenital absence of the vas can fertilize oocytes and the resulting embryos can implant

successfully with resulting healthy live offspring (WHO, 1987). A percoll sperm preparation technique was described by Asch's colleagues in association with such cases but its relevance is uncertain at this stage (Patrizio et al., 1988). Improved fertilization results are being achieved by avoiding centrifigation and using the sedimentation technique. We have performed high epididymal sperm collections from cases of epididymal duct obstructions as well as congenital absence of the vas. In 9 cases, the fertilization rates have been low but embryos have been generated and two early pregnancies achieved to date (Jequier et al., 1990).

Sperm function studies

Semen samples from cases of asthenospermia and unexplained failed fertilization may benefit from evaluation further than routine semen analysis (Yovich et al., 1984). Some laboratories have included automated semen analysis (Mortimer et al., 1988) and the zona free hamster penetration (HOP) test (Yanagimachi et al., 1976; Burkman et al., 1988), but standardization is difficult and the clinical value of both techniques remains uncertain. Zamboni (1987) has revealed a range of ultrastructural abnormalities of the spermatozoon and has shown the electron microscope to have an invaluable role in such cases. Others can be tested for the ability to undergo an acrosome reaction following challenge with calcium ionophore (A23187 (ARIC); Cummins et al., 1990). Acrosomal morphology and acrosome reactions can be monitored using FITC-conjugated pisum sativum lectin on ethanol permeabilized sperm (Cross et al., 1986). The same preparation can be used to evaluate sperm binding capacity to the zona pellucida of discarded oocytes (Liu et al., 1988) but using separate fluochromes (FITC and TIRTC) to differentiate test and control sperm. On occasions it may still prove difficult to determine if the cause of fertilization failure rests with the oocytes or the spermatozoa. Crossed insemination studies in vitro may well provide the answer, i.e., testing the husband's sperm against donated oocytes (e.g., supernumerary from a GIFT programme; although this has limitations as such residual oocytes have a reduced fertilization potential at around 50%) (Yovich et al., 1989) and testing the wife's oocytes with donor sperm during a split fertilization study in IVF. Such testing poses ethical problems concerning the fate of embryos derived from cross-insemination.

Male factor prognosis

Improved techniques in IVF are enlarging the options for the management of male factor infertility. In particular, improved sperm separation methodology, sperm motility enhancement and

micromanipulation can improve the chance of fertilization. Transfer of embryos into the fallopian tubes by PROST or TEST has been the preferred technique to maximise the chance of pregnancy but this may require re-evaluation given the improving results from the less invasive procedure of IVF-ET over recent months. Further improvements are anticipated with improved diagnostic methods for evaluation of sperm function and related research e.g. eliciting the acrosome reaction in vitro.

Alternative approaches

Alternative approaches currently being explored by some experienced IVF clinics are:

1 Cryopreservation of embryos arising from stimulated cycles and subsequent transfer during:
   (a) a natural cycle; or
   (b) an artificial cycle created by hormone replacement,
2 Collection and transfer during a natural, unstimulated cycle.

These approaches are based on the belief that ovarian stimulation cycles have an abnormal luteal phase hormonal milieu and this is corrected by reverting to the natural cycle or an 'ideal' artificial cycle. Large data are not yet available but an early French study (Frydman et al., 1988) has reported pregnancy rates of 7–12% when one or two embryos were transferred in natural cycles after cryopreservation. However, these rates were not better than the conventional techniques but improved the pregnancy rate with reference to the single oocyte recovery procedure. Another study from separate French workers on 80 natural cycles generated eighteen pregnancies (22.5%) with fourteen ongoing (17.5%) in a selected group (Foulet et al., 1989). These results are more encouraging and may well lead to renewed use of natural cycles in cases with normal ovulatory profiles and without adverse features with respect to fertilization potential.

EMBRYO BIOPSY AND GENETIC ASPECTS

The full potential of the technology of IVF is far greater than the small steps that have been taken over the past decade. Current research in the area of micromanipulation has resulted in successful embryo biopsy of pre-implantation mouse embryos from the 4-cell (Wilton et al., 1989) and 8-cell (Monk et al., 1987) stages; and the trophectoderm from blastocysts of various animal species including human (Edwards, 1988). The procedures are considered to be eminently feasible for the respective stages of human embryos thus enabling potential genetic diagnoses to be performed by:

1 The analysis of chromosomes e.g. for fetal sex and Down's syndrome.
2 The application of DNA probes for genetic diseases, e.g. for muscular dystrophy, cystic fibrosis, thalassaemia, haemophilia and an increasing array of others, as well as sexing by a Y-chromosome specific DNA probe.
3 To assay for enzymes and metabolic products in culture solutions, e.g. hypoxanthine phosphoribosyl transferase deficiency in Lesch-Nyhan disease.

The advances in this area have been dependent upon progress in subculturing biopsy blastomeres in order to increase the number available for assays on the one hand; and the rapid improvements in recombinant DNA technology on the other. In particular DNA amplification involving the polymerase chain reaction is potentially enabling the application of DNA probe methods (e.g., Y-probe and RFLP analysis) to be applied on fewer cells. Recent reports indicate that the techniques will be applicable to a single cell (Handyside et al., 1989; Holding et al., 1989).

The two preferred methods of pre-embryo biopsy are:

1 Removal of one or more blastomeres from the two-cell to eight-cell embryo,
2 Biopsy of the abembryonic trophectoderm at the blastocyst stage.

With the development in DNA probe techniques, some genetic diagnoses may be possible on the biopsy specimen itself, whilst others may require variable periods of subculture. If extended periods of culture are required then cryopreservation technology assumes an increasing importance. Pre-embryos will need to be stored for variable periods whilst awaiting the results of diagnostic genetic testing prior to selecting non-affected embryos for transfer.

Currently there is worldwide interest in sequencing the human genome, hence it is likely that within a few years the mutant sequences for a very large number of human conditions currently known or suspected to be genetically related, will have been identified. For example the gene sequence for cystic fibrosis has now been identified and successfully cloned (Riordan et al., 1989; Kerem et al., 1989). Following on this achievement is a proposal to undertake comprehensive community screening (Goodfellow, 1989). At-risk couples will then have a range of options to consider i.e. avoid pregnancies; undergo pre-natal diagnosis e.g. by chorionic villus sampling with a view to terminating

an affected fetus; or consider preimplantation embryo diagnosis following IVF with a view to selecting unaffected embryos for transfer.

More futuristic but no less possible is the application of recombinant DNA technology to correct a genetic defect in an entire line of cells. To practise gene therapy it is necessary, having identified the defective gene, to have a plentiful source of the correct DNA. This can already be achieved by gene cloning. In this technique the actual gene or a specific 'copy' DNA molecule (cDNA) complimentary to the 'messenger' (mRNA) that is made from the required gene is inserted into a vector (plasmid or virus) which can carry the DNA into a bacterium. Replication of that DNA sequence is achieved along with bacterial culture. The cDNA used for cloning is prepared by using a retroviral enzyme, reverse transcriptase, to make the DNA copy from an mRNA molecule. The cDNA is not precisely the same as the corresponding gene as it contains no introns but it does have all the exons which contain the active regions. DNA manufactured by gene cloning can successfully be incorporated into mammalian cells. This can be achieved by direct transfer of the cDNA (gene construct) into the nucleus of a stem cell, using microinjection techniques. DNA can also be carried into somatic cells using a viral vector, e.g. monkey tumor virus (SV40) or one of the retroviruses. Although the use of such viruses which are known to cause cancer may appear hazardous, it is possible to manipulate these viruses to excise the genes controlling replication while retaining those genes required for DNA insertion. Hence the procedure should be safe. As far as the manipulation of DNA into fertilized oocytes of animals is concerned, this has been successfully achieved, e.g. a cDNA gene construct for human growth hormone linked to the protein metallothionein has been injected into the nucleus of a fertilized mouse ovum to produce a giant mouse strain. Mettallothionein detoxifies a number of metals and can be induced by injections of zinc. Its induction leads to the concomitant expression of growth hormone and higher rates of growth can be induced in such mice. Other genes have also been incorporated into the gene line of mouse embryos, including a transgenic strain which produces human tissue plasminogen activator into milk. A recent review of the technical progress towards human gene therapy considers the potential applications (Friedman, 1989).

The views of various societies about these developments is still unclear but in Australia the NH & MRC (1987) has endorsed the concept of somatic cell gene therapy but has advised that germ cell manipulation in humans is not appropriate at this stage. There is concern about the possible consequences of the alteration of genes which will be passed on to future generations. For example, the gene construct experiments in mice have led to progeny with an increased risk of congenital disorders, thought to have arisen due to surrounding genetic disturbances during the insertion of the DNA sequences. At this stage, the NH&MRC advises that it is simpler and more expedient to implant an early embryo, preselected after having been shown to be normal, rather than attempt to correct an abnormal pronuclear oocyte. This argument currently extends to the insertion of DNA into gametes prior to fertilization.

RELEVANCE OF IVF TO THE WIDER COMMUNITY

In just a little over 10 years, IVF technology has changed from a subject attracting worldwide scepticism, to the application of highly effective treatment modes for a range of disorders in reproductive medicine, as well as opening a potential arena for the application of new knowledge in genetics. By mid-1989, it is estimated that there are approximately 20,000 infants worldwide arising from IVF related procedures. The technology has arisen at a time when developed countries have recognized a problem in maintaining their populations by natural regeneration. Many European countries, Australia and the United States are documenting net reproduction rates which are less than 1.00 (the level required to maintain the population without immigration). This is related to the fact that populations are largely city-based with declining fecundity (Table 19.3) as couples are delaying the age at which they elect to have children. When reproduction is desired, there is often only a short time-frame in which to achieve this and hence the demand for infertility and IVF services is increasing in those countries. Currently in Western Australia it has been estimated that approximately 2.5% of total births are derived from infertility treatments (approximately half each from IVF and non-IVF related infertility treatments).

Table 19.3 Characteristics of the Australian community derived from Australian Bureau of Statistics data 1987

1	Population is largely city-based with increased education		
2	Declining marriage rate per 1000 population		7.0
3	Current births	Australia	240,000
		West Australia	24,000
4	Declining fecundity	Children/women	1.85
	Births per 1000		15.0
5	Average family size		3.2
6	Pregnancies delayed/age at 1st child	Mother	27 years
		Father	30 years
7	Net reproduction rate		0.88

While concerns have been expressed about the proliferation of IVF

units around the world, the cost per treatment cycle is diminishing markedly with the application of modern methods and greater efficiency. Currently it is estimated that the total cost per infant achieved by IVF is less than $A15,000 if one excludes the subsequent obstetric costs which, it may be argued, are a separate matter. However, pregnancies generated from sub-fertile marriages have a higher morbidity than the general population with increased early pregnancy wastage (around 30%) and increased late pregnancy complications (in particular multiple pregnancies, pre-term labour, low birth weight infants and complex deliveries). Overall, the perinatal mortality is double that of the general population at 30.4/1000 singletons (NPSU, 1987) and there is a concomitantly increased requirement for neonatal intensive care facilities.

However, serious maternal complications are uncommon. The main concern is that of psychological stresses which often relate to the underlying infertility problem, poor responses during IVF treatments and financial costs. Occasionally the life-threatening problem of ovarian hyperstimulation syndrome will occur (approximately 1%) (Borenstein et al., 1989) and occasional reports document anaesthetic complications and inadvertent bowel or vascular damage during oocyte recovery procedures.

RELEVANCE OF CRYOPRESERVATION

Cryopreservation techniques are seen to have increasing importance in IVF—not only for the storage of excess embryos following an IVF attempt, but also as a definitive procedure (Frydman *et al.*, 1988) to avoid repetitive egg collections and reduce the risk of multiple pregnancy. Cryopreservation may also become increasingly important for the preservation of fecundity as it is becoming increasingly apparent (e.g., from ovum donation data) that younger oocytes are more likely to fertilize and form embryos suitable for successful implantation. Gamete banking, including both spermatozoa and oocytes, may therefore be of increasing relevance to developed communities if the current socio-reproductive trends continue.

CONCLUSIONS

Modern methods in reproductive medicine are proving increasingly effective for the management of many types of infertility. Certain conditions are treated by specific management, e.g. hormonal and surgical treatments for endometriosis, specific therapies to induce ovulation in anovulatory women and tubal microsurgery to correct mechanical

pelvic disorders. However, the majority of cases will benefit by ovarian stimulation often accompanied by one or other of the gamete manipulation procedures (insemination methods or IVF related techniques). A few patients may require gamete donation and rare cases may be solved only by surrogacy methods or adoption where this is available. The techniques have opened up a wide range of options for consideration and the efficiency is improving rapidly. However the developments in reproductive medicine have wider implications both in the area of reducing congenital abnormalities and in fecundity preservation within communities where social changes have led to significant falls in the net reproduction rate. Ethical considerations must address the issues while being attuned to the real needs of individuals and society. At this stage of development inhibitory legislation should be avoided but IVF clinics should be allowed to proceed within a framework of rational guidelines.

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