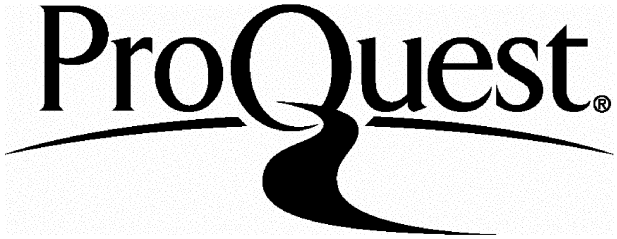
STUDIES OF THE FALLOPIAN TUBE ENVIRONMENT AND AN ASSESSMENT OF ITS ROLE IN ASSISTED REPRODUCTION

NAZAR NAJIB JARMANOS AMSO

A THESIS SUBMITTED TO THE FACULTY OF MEDICINE,
UNIVERSITY OF LONDON FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY, 1996

ACADEMIC DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY,
THE ROYAL FREE HOSPITAL SCHOOL OF MEDICINE,
UNIVERSITY OF LONDON,
LONDON, U.K.



The investigations performed resulting in the submission of this thesis were carried out in the Academic Department of Obstetrics and Gynaecology at The Royal Free Hospital, London during the four year period 1988-1991. The candidate was the principal investigator responsible for the implementation of the studies contained in this manuscript. The candidate was the major instigator in the design and implementation of the laboratory and clinical projects, as well as being responsible for the sample collection, storage, and analysis, and production of the manuscripts arising from this work.

Studies of Fallopian tube secretions were carried out at the laboratories of the Academic Department of Obstetrics and Gynaecology, The Royal Free Hospital, with Dr. Graham Burford, PhD. Steroid receptor studies and Electron Microscopy studies were carried out in collaboration with Dr. Julie Crow, Consultant Histopathologist at the Department of Histopathology, The Royal Free Hospital, London.

ABSTRACT

The role of the Fallopian tube in assisted reproduction remains unclear. The work described in this thesis was undertaken; (1) to study cyclical oestrogen and progesterone receptor changes in the endometrium and Fallopian tube, (2) to determine ultrastructural similarities and differences between endometrial and endosalpingeal epithelia at the different stages of the cycle, (3) to collect tubal fluid and attempt to isolate tubal specific proteins, and (4) to determine the clinical impact of tubal environment in assisted reproduction by conducting a randomised trial comparing tubal and uterine embryo replacements after in vitro fertilisation of oocytes.

Oestrogen (ER) and progesterone receptors were studied with specific monoclonal antibodies and employing an immunohistochemical technique. The results showed that, in the tube, both the isthmic and ampullary epithelial and stromal ER increased in the follicular phase to a peak at mid cycle, then declined in the late luteal phase whilst the fimbrial end depicted an opposite pattern of staining. Progesterone receptors persisted in all tubal wall layers and endometrial stroma throughout the cycle, but disappeared completely from endometrial gland epithelium in the late luteal phase. The ultrastructural study showed; (1) an increase in ciliated cells along the tube being highest at the fimbria, (2) late follicular phase increase in cytoplasmic fragments and cellular material within the isthmic lumen, but not in the outer tubal segments and (3) similar secretory pattern and surface epithelial changes in the endometrial gland, isthmic and ampullary tubal epithelia. Gel electrophoresis of tubal flushing demonstrated two non-serum bands appearing in the late follicular and luteal phases of the cycle.

One hundred and two women were included in the randomised controlled study. Analysis of all treatment cycles (n=227) showed that; (1) the first attempt resulted in a pregnancy rate per embryo transfer (PR/ET) of 29% for tubal and 20% for uterine replacements, and an implantation rate (IR) of 15% and 12% respectively, and (2) women with unexplained infertility benefit most following tubal transfer (PR/ET; tubal 32%, uterine 15% - IR; 14% and 7% respectively). Factors

associated with increased PR in the first attempt include previous pregnancies, absent female or male factors, unexplained infertility, and human chorionic gonadotrophin luteal support.

Despite an apparently higher PR and IR following tubal transfer, no significant differences were observed in the multiple pregnancy and live birth rates, nor in the implantation rate in cycles resulting in pregnancy. These findings suggest that the embryos' quality is not enhanced following tubal transfer.

ACKNOWLEDGEMENTS

I am most grateful to Professor Robert W. Shaw for his encouragement, guidance and support and for allowing me to include the subfertile couples under his care in the clinical trial reported in this thesis. I am also indebted to Dr. Julie Crow for her advice and guidance while conducting the steroid receptor and electron microscopy studies. I am also grateful to Dr. Graham Burford, with whom analysis of tubal secretions was carried out, Mr. Albert Bernard and Mrs. Evadney Keith who were in charge of the embryology laboratories at the Assisted Reproduction Unit at the Royal Free Hospital.

I gratefully acknowledge the contribution of my clinical colleagues in the Department (Dr. Paul Curtis, Dr. Ahmad Abdel Gadir, Dr Anne Jackson, and Dr. Sangchai Preutthipan), the anaesthetic and theatre staff at the Royal Free Hospital whose understanding and support enabled me to successfully conduct the clinical trial. I also wish to thank the nursing staff (Miss Bina Chander and Mrs. Margaret Inglis) for their assistance and counselling of the patients and Mrs. Karen Gleeson in the semenology laboratory for her role in the initial investigation of the infertile couples.

I gratefully acknowledge the technical assistance of Mr. Brian Chalk, Miss Linda Moore, and Mr. Francis Moll from the Department of Histopathology and Miss Jackie Lewin from the Department of Electron Microscopy at the Royal Free Hospital, London. I gratefully acknowledge the assistance of Dr. Andrew Phillips in the initial design of the clinical trial and randomisation of patients and Dr. Peter Kelly in the statistical analysis of the data.

I dedicate this PhD thesis to my wife and family.

CONTENTS

CHAPTER 1: LITERATURE REVIEW

CHAPTER 6(II): TRIAL DESIGN AND CLINICAL METHODOLOGY

CHAPTER 7: CLINICAL TRIAL RESULTS

CHAPTER 9: SUMMARY OF CONCLUSIONS AND FUTURE RESEARCH

LIST OF FIGURES AND THEIR LEGENDS

LIST OF TABLES AND THEIR LEGENDS

CHAPTER 1
LITERATURE REVIEW

1.1 INTRODUCTION

The oviduct holds a fundamental position in reproduction. It transports both gametes and zygotes and provides an essential environment for fertilisation and cleavage of the fertilised egg (Hamner, 1973). Oviductal fluid provides the medium in which these processes take place. Tubal fluid volume, composition, and ability to support sperm capacitation and embryo development correlate with the hormonal status of the mother (Mastroianni and Go, 1979). Moreover, certain cycle dependant oviduct specific proteins have been identified both in animals and humans. Equally important is the observation that the subsequently introduced technique for assisted reproduction of Gamete Intra Fallopian Transfer (GIFT) produced higher pregnancy rates than conventional in vitro fertilisation and uterine embryo transfer (IVF-UET) (Asch et al, 1984). Since then, it is believed that the tubal environment is a major contributor to this success. However, the accumulation of more data from properly conducted clinical trials has given conflicting results and hence the subject remains a controversial one.

The original aim of this thesis was to study one aspect of tubal environment, viz secreted tubal proteins. However, to determine whether the tubal environment is the major contributor to the higher success rates, it became apparent that comparison with the uterine environment is essential. Similarly, extensive search of the literature showed that several biological variables may account for the variation between the two environments hence the study has been widened accordingly. Investigation has been carried out into both the endometrium and Fallopian tube from the same subjects in an attempt to determine cyclical variations in Oestrogen and Progesterone receptors (ER, PR) and ultrastructural changes under different hormonal conditions. By identifying structural and functional similarities and differences between the two sites, functional differentiation may be identified.

1.2 ANATOMICAL CONSIDERATIONS

1.2.1 GENERAL DESCRIPTION

1.2.1.1 The Fallopian tube

The oviduct was originally described by Gabriele Fallopius (1523-1562) who termed it the "tuba uteri" after its resemblance to a brass musical instrument. In this thesis, the term "oviduct" will be used to refer to the mammal generally, and the term "Fallopian tube" or "tube" to the human exclusively.

The oviduct is a muscular tube with an epithelial lining. It is generally divided into 5 anatomical regions: the infundibulum, fringed by epithelial covered fimbriae and opening via the tubal ostium into the peritoneal cavity, the ampulla, the ampullary-isthmic junction (AIJ), the isthmus and the intramural or interstitial portion contained in the wall of the uterus at the uterotubal junction (UTJ). The oviduct has three muscular coats. The inner longitudinal layer is lost approximately 2.5 cm distal to the cornual end and for most of its remaining length, external longitudinal and internal circular muscle coats are only identified. The epithelial lining and mucosa are thrown into folds, also known as "rugae" or "plicae" and the proportions of muscle and mucosa vary along the length of the oviduct. The outer longitudinal layer of muscle is not as distinct as the other layers of the tubal wall. In the isthmus, the circular and outer layers are separated by a collagenous layer containing the blood vessels of the tubal wall. In vitro, the circular layer exhibits a higher contractile frequency than the outer longitudinal layer and its duration of contraction is considerably shorter. At the time of ovulation, the contractile frequency is significantly increased.

The innermost longitudinal layer also exhibits regular spontaneous muscular activity, but its frequency is significantly higher than in the other layers. It has also been suggested (David and Czernobilsky, 1968) that a sphincteric action may result from the contraction of the inner longitudinal and circular layers. In the interstitial region of the tube, the inner longitudinal layer appears to be a continuation of the subendometrial muscular layer of the uterus. The similarity between the tubal and

endometrial layers is not surprising in view of the common embryological origins of the two organs from the mullerian duct. The function of the smooth muscle is to promote transport of ova and spermatozoa, albeit in different directions, by a complex pattern of integrated movements in combination with epithelial ciliary activity. The mesosalpingeal muscle elements are probably of great importance during ovum pick-up, since the fimbriae come into close contact with the surface of the ovary and move back and forth over the ovulatory follicle to remove the viscous mass of oocyte and corona cells.

The ampulla is the longest portion of the Fallopian tube, measuring 5 to 8 cm in length. Its luminal diameter varies tenfold from 1 to 2 mm at its junction with the isthmus to more than 1 cm near its distal end. The inner longitudinal musculature is contained as scattered muscle bundles dispersed within the lamina propria of the complex epithelial folds seen in this area. More recently, Vizza et al (1995) demonstrated that the myosalpinx of the human ampulla is not organised into clear cut longitudinally, circularly or spirally arranged layers, but as multidirectional smooth muscle cell bundles probably designed to stir than push the tubal contents. The infundibulum is the trumpet shaped distal portion of the oviduct terminating in the fimbriae. It is the functional site of transition from ovum pick-up to ovum transport. The ovarian fimbria is probably essential for ovum pick-up, during which the densely ciliated fimbria are brought into close contact with the cumulus mass surrounding the ovum, and the ovum is thus conducted into the tubal ostium (Pauerstein and Eddy, 1979).

1.2.1.2 The uterus

The uterus is a thick-walled, muscular, hollow organ shaped like a pear, its tapering end being the cervix which projects into the upper vagina. It is flattened in the antero-posterior plane. Its dimensions vary according to parity, the nulliparous organ measures approximately 9 cm in overall length, 6 cm in maximum width and 4 cm antero-posteriorly in its thickest part. The wall is 1-2 cm thick and the length of the uterine cavity is usually 7.5-8.0 cm. The uterus is made up of the corpus (body),

isthmus and cervix. The part of the body situated above the level of the insertion of the fallopian tubes is described as the fundus. The areas of insertion of the Fallopian tubes are termed the cornua (horns). The opening of the cervix into the vagina is the external os and the opening of cervix into the uterine cavity is the internal os. The cavity of uterus is triangular in shape when seen from the front, but is flattened to a slit when seen from the side. It communicates with the vagina through the cervical canal, and with the lumen of each Fallopian tube at the two cornua. The main mass of the uterine wall is composed of involuntary muscle fibres which for the most part run obliquely in a criss-cross spiral fashion. The more superficial fibres, however, are arranged longitudinally and are continuous with those forming the outer coats of the tubes and vagina. Internal to the muscle layers is the endometrium which is directly applied to the muscle so that its deeper parts often interdigitate with the fibromuscular tissue. The whole of the fundus, the anterior wall as far as the isthmus, and the posterior wall as far as the attachment of the vagina to the cervix are covered with peritoneum. The sides of the uterus between the attachment of the two leaves of the broad ligament, the lower anterior uterine wall, and the whole of the cervix except for the posterior aspect of its supravaginal part are devoid of peritoneal coat (Jeffcoate, 1975).

1.2.2 VASCULAR SUPPLY

1.2.2.1 The Fallopian tube

The arterial blood supply of the Fallopian tube is derived from the uterine and ovarian arteries. Generally, the uterine artery supplies the medial two-thirds of the tube, and the ovarian artery the remainder. Each of these vessels yields two or three branches, which together furnish distinct arcades of vessels along the length of the tube. The arterioles from the tubal arcade run through the serous and subserous coats to supply the different layers of the tube. It is likely that autoregulation shunts the blood supply under varying physiological conditions. The venous drainage generally parallels the arterial supply, with interconnecting capillary networks being noted at various levels in the tubal tissue. Studies of the human tubal microvascular architecture (MVA) (Koritke and Gillet, 1967; Koritke et al, 1968) demonstrated that

fimbrial MVA differs from that of isthmic or ampullary mucosa in that arteries are more numerous and are located peripherally around a central core venule. Further, variations during the menstrual cycle were observed in that mucosal arterioles became increasingly coiled and reached a maximum at the time of menstruation with changes being most marked in the fimbriae. Mucosal venules also became markedly dilated at the time of ovulation and maximally at the time of menstruation.

More recently, examination of the MVA of the rabbit oviduct by the corrosion vascular cast-scanning electron microscope method showed characteristic variations along the length of the oviduct (Verco, 1983). The differences being observed predominantly in the subserosal and submucosal areas. The isthmus has a randomly interconnecting subserosal venous plexus surrounding the myosalpinx and a mucosa which is well supplied by arterioles. The ampulla has parallel branching of subserosal arteries and veins and a mucosa supplied by few arterioles which drain into plentiful large veins originating high in the plical core. The fimbriae are supplied by parallel branching arteries and veins. These findings suggest that the subepithelial capillary plexus of the apical region of the plicae probably contains high pressure blood in the isthmus and low pressure blood in the ampulla with consequent effects on, (1) the net fluid flow between the interstitial fluid and intravascular compartments and (2) ovum transport.

The Fallopian tube is rich in lymphatics. Three lymphatic networks drain the mucosa, muscularis and serosa respectively. Upon emerging from the intrinsic system of the tube, the lymphatic vessels combine, enter the mesosalpinx and run upward in the broad ligament to the para-aortic lymph nodes. Although there is no evidence that tubal lymphatics are involved in counter-current transfer mechanisms, such a role can not be excluded (Hunter, 1988).

1.2.2.2 The uterus

The uterine artery arises from the anterior division of the internal iliac artery. It runs forwards and inwards in the base of the broad ligament, crossing above and almost

at right angles to the ureter, and approaches the uterus at the level of the internal os. Here it gives off a descending branch to supply the lower cervix, a circular branch, and the main trunk turns upwards to supply the uterus at all levels. The main branches to the uterus each divide into an anterior and posterior arcuate artery which are disposed circumferentially in the myometrium and anastomose with those of the other side in the midline. The arcuate arteries give off serosal branches and radial arteries which penetrate the myometrium to end as basal arteries to supply the endometrium. The uterine artery ends by anastomosing with the ovarian artery to form a continuous arterial arch. The venous drainage of the uterus mirrors its arterial supply and communicates with the pampiniform plexus in the broad ligament and the vaginal plexuses. The lymphatic drainage of the lower part of the body of the uterus passes mostly to the external iliac lymph nodes, accompanying those from the cervix. From the upper part of the body, the fundus and the uterine tube the vessels accompany those of the ovary to the lateral aortic and pre-aortic lymph nodes and few pass to the external iliac lymph nodes. The region near the point of entry of the uterine tube has additional drainage by lymphatics which accompany the round ligament to the abdominal wall and thence to the superficial inguinal nodes.

1.2.3 NERVOUS SUPPLY

1.2.3.1 The Fallopian tube

The oviduct is served by both sympathetic and parasympathetic nerves which carry both sensory and motor fibres. **The sympathetic supply** of the isthmus and a portion of the proximal ampulla is by postganglionic fibres via the hypogastric plexus (T10 to L2). The distal ampulla and fimbriae are supplied by postganglionic fibres arising from the coeliac, aortic and renal ganglia (T10-11) and travel with the ovarian vessels. The isthmus and AIJ are supplied by additional postganglionic sympathetic fibres originating from peripheral ganglia near the vagina, and they also show rich distribution of adrenergic nerve terminals while in contrast the adrenergic supply to the smooth muscle of the ampulla is sparse (Brundin 1965; Owman et al, 1967). **The parasympathetic supply** of the tube is also dual. The distal portion is supplied by vagal fibres from the ovarian plexus. The sacral parasympathetic fibres (S2-4) are

conveyed to the pelvic plexuses ganglia and from which, short postganglionic fibres supply the interstitial portion of the isthmus (Pauerstein and Eddy, 1979). The nervous supply of the myosalpinx may play a role in mixing the luminal contents.

1.2.3.2 The uterus

The **sympathetic** nerves to the uterus are sensory and possibly motor and are supplied by the pelvic (inferior hypogastric) plexuses. The fundus of the uterus probably receives additional twigs from nerves which accompany the ovarian vessels. **Parasympathetic** nerve supply to the uterus except in its lower part is doubtful.

1.2.4 EPITHELIAL LINING

1.2.4.1 The Fallopian tube

Cyclical changes in the human tubal epithelium during the menstrual cycle were first described by Holtzbach (1908) and further expanded by Novak and Everett (1928). Four cell types have been described in the epithelium of the Fallopian tube: (1) ciliated cells, (2) non-ciliated "secretory" cells (3) intercalary or "peg" cells, and (4) indifferent cells (Pauerstein and Eddy, 1979). The latter two may represent exhausted secretory cells or deciliated cells.

The ciliated cells are square, with relatively large, round or oval nuclei, and are more abundant at the apices of epithelial folds. Their proportion progressively declines from the fimbriae towards the isthmus. They undergo little morphological and ultrastructural changes during the ovarian cycle. There are conflicting reports as to the degenerative changes and decrease in cell numbers during pregnancy. In the puerperium, the ciliated cells decrease in number and size, and following the menopause they persist without significant regression until the late postmenopause, when deciliation becomes apparent, particularly on the fimbriae.

Non-ciliated "secretory" cells have granular cytoplasm and oval nuclei which are orientated with their long axis parallel to the long axis of the cell. They vary in

shape and appearance with the phase of the menstrual cycle. They attain their maximum height in the late proliferative phase, and form a protruding dome or cupola. These domes may extrude cytoplasmic and nuclear contents into the lumen. Cellular integrity is repaired before the subsequent menses. Ultrastructural changes compatible with increased secretory activity also become apparent during the ovarian cycle (section 1.4.2.1).

1.2.4.2 The uterus

Cyclical changes in the uterus occur as a result of the ovarian cycle. Immediately following menstruation the endometrium is thin; narrow, straight glands are lined by cuboidal epithelium and the stroma is compact. As oestrogen levels increase in the proliferative phase, the glands become longer but remain straight; the epithelial lining becomes tall and columnar, and the nuclei occupy a basal position. The stromal cells increase in number and become more loosely packed together, the whole stroma being vascular and abundant. Following ovulation, progesterone levels begin to rise and induce secretory changes. The glands become more tortuous and corkscrew-like. The epithelial lining demonstrates a series of changes during which the nuclei become displaced from their basal position towards the centre of the cell by the formation of subnuclear vacuoles. The gland lumina are seen to contain more secretion as the days go by, until maximum secretion, rich in glycogen, is achieved about day 25 of a 28-day cycle. Stromal cells further increase in size and are loosely arranged, giving the stroma an oedematous appearance which may resemble the decidua of early pregnancy. Endometrial arterioles become increasingly coiled. With declining levels of oestrogen (E) and progesterone (P), endometrial shrinkage occurs and there is constriction of the spiral arteries, stasis, necrosis and bleeding. The stratum functionalis breaks down and is cast off as menstrual flow, leaving the deeper stratum basalis as the source of regeneration of the functional layer. Cyclical ultrastructural changes in the endometrium are described in section 1.4.2.2.

1.3 TUBAL TRANSPORT

The basic mechanisms of gamete transport are remarkably similar in all mammals. Oviductal gamete transport may be influenced by smooth muscle contraction, ciliary activity, the hydrodynamics of luminal fluids and the hormonal status of the female. Essential to the mechanism of ovum entry into the fallopian tube is the physical interaction between the ovum within its investment of follicular cells and the cilia on the surface of the fimbriated infundibulum. Blandau (1973) has shown that the ovum is literally pulled or stripped from the surface of the ovulating follicle by the action of the densely arranged cilia and is rapidly propelled into the ampulla. Ova that have been artificially denuded of their follicular cells and placed on the fimbrial surface are seen simply to rotate and not to be displaced towards the tubal ostium (Blandau, 1969). Ovum passage into the tube is also aided by the constant to-and fro movement of the ovaries relative to the fimbriated infundibulum, this being caused by the heightened muscular activity of the supporting mesenteries and ligaments. The fimbria of the mammalian oviduct are intimately applied to the ovaries at the time of ovulation, and in a number of species extend around the ovary in the form of a bursa. Complete ovarian bursae are not found in humans but the fimbriae expand at ovulation to form an effective ovarian bursa whose ciliated surface is in close contact with the ovary. In rabbits and women, ampullary transport of ova is primarily through ciliary action (Halbert et al, 1976; Pauerstein, 1978). Ciliary activity, beating towards the AIJ shortly after ovulation (Blandau and Verdugo, 1976), is responsible for ovum transport from the ovarian surface into the ampulla (Blandau et al, 1979). Waves of contractile activity in the different compartments of the myosalpinx also contribute to the transport of ova to the AIJ. The distinct circular and longitudinal muscle layers, enable segmental activity to occur in the wall of the tube with displacement of the ova in a downward direction. Such smooth muscle activity is also influenced by the ratio of ovarian steroids, and as noted many years ago (Corner, 1923; Seckinger, 1923), is normally accentuated close to the time of ovulation. This enables ovum transport to the site of fertilisation to be accomplished in a matter of 6 to 45 minutes as in cats, monkeys, pigs and rabbits (Harper, 1961; Blandau, 1969; Hunter, 1974; Blandau and Verdugo, 1976; Eddy, 1976) or up to 30

hours or more in women (Croxato and Ortiz, 1975; Pauerstein, 1975), indicating that the ampulla is probably the site of fertilisation. The slower ovum transport in women has been attributed to the absence of ciliary crowns from their tubal ciliated cells. The ovum remains in the vicinity of the AIJ for much of its tubal sojourn due to the contraction or spasm of the musculature in this area (Blandau et al, 1979). Powerful constriction is activated shortly after ovulation via the dense adrenergic innervation in this area (Brundin, 1965; Brundin et al, 1969).

Interaction of the spermatozoa with tubal epithelial cells is thought to have a number of possible functions including the formation of a sperm reservoir, the maintenance of sperm viability and the prevention of polyspermic fertilisation of oocytes (Barratt and Cooke, 1991). In vitro studies of this interaction in the human Fallopian tube (Pacey et al, 1995) showed that spermatozoa can demonstrate a strong physical interaction with epithelial cells obtained from women at all stages of the menstrual cycle, but that this contact appeared to be random and there was no evidence of any taxis toward epithelial cells. This interaction was confirmed ultrastructurally where both spermatozoa and epithelial membranes were seen to be in close apposition. Transport in the isthmus is unique because it must be capable of effecting, sequentially, movement of sperm and ova in opposite directions. Several factors may contribute to this unique property; (1) Evidence from experimental animals suggest that spermatozoa may be largely sequestrated in the isthmus in the preovulatory interval, only to be released for further pro-ovarian transport when ovulation has occurred and products of ovulation have entered the tube, (2) Jansen (1978, 1980), suggested that secretions in the isthmus are different from those in the ampullary region, and hypothesised that the presence of abundant tenacious mucus may be the essential vehicle for efficient transit of motile sperm at the time of ovulation in a direction opposite to that of ciliary beating and fluid flow and it may also protect the sperm from such ciliary activity. The presence of this mucus for 2-3 days after ovulation may be a major factor in locking the ovum in the ampulla. Subsequent alterations in fluid dynamics in the isthmic lumen lead to disappearance of the mucus allowing ovum transport to the uterus. Relaxation of the musculature, may at least in part, assist with this rapid transit of the ovum. It is believed that adrenergic

receptor activity or prostaglandins release are the mediators of such an effect (Black, 1974; Spilman, 1976), (3) Verco (1984) studied the changes in rabbit oviduct microvasculature after ovulation induction and showed dilatation of the isthmic subserosal venous plexus at 24 hours, but not at 48 hours after an ovulatory dose of human chorionic gonadotrophin injection. He speculated, that this plexus acts as a sphincter and decreases the luminal diameter which coupled with the intraluminal mucus observed by Jansen (1978), could occlude the isthmic lumen to ova, and (4) Another factor relevant to isthmic transport is the possible influence of the developing embryo on tubal function. In hamsters and rats, embryos and unfertilised oocytes enter the uterus at different times after ovulation, while in horses only embryos enter the uterine cavity (Van Niekerk and Gerneke, 1966; Betteridge and Mitchell, 1974). More recently, Freeman et al (1992) have provided evidence to support the hypothesis that the horse embryo initiates oviductal transport to the uterine cavity possibly by secreting a chemical factor. In women, Croxatto et al (1972) reported that unfertilised ova were recovered from the uterine cavity on the second or third postovulatory days, whereas fertilised eggs could be recovered on the fourth and fifth postovulatory days. In a subsequent publication (Croxatto et al, 1978), ova were recovered from the Fallopian tube (mainly in the ampullary portion) between 24 and 96 hours after the serum luteinizing hormone (LH) peak and none at later intervals while, the earliest recovery from the uterine cavity was at 96 or 120 hours after the LH surge.

1.4 THE TUBAL ENVIRONMENT

In this context, the term environment (milieu, atmosphere) is defined as all the conditions and influences affecting the development of an organism. Encompassed in the term "tubal environment", researchers must take into account all the factors (mechanical, physical and neuro-endocrine) that interact with and influence the different anatomical structures of the Fallopian tube. It is the summation of all these events that constitutes the tubal environment and thus it is constantly changing and may even vary between the different parts of the Fallopian tube at various stages of the menstrual cycle to produce a number of "micro-environments". A continuous

exchange of materials takes place not only between the fluid in the lumen and the cells of the endosalpinx, but with peritoneal, follicular and uterine fluids and when present, sperm, eggs, cumulus cells, and preimplantation embryos. Unfortunately, due to its relative inaccessibility for in-vivo examination, functional studies of the Fallopian tube continue to depend on in vitro culture of specimens removed at operations, from which attempts to extrapolate kinetic events may be made. A key factor that determines the cyclic nature of tubal changes is variation in the level of circulating steroid hormones, E and P during the ovarian cycle. The ovarian steroids exert an overwhelming impact on all segments of the Fallopian tube and uterus and affect every aspect of growth, differentiation and function, including both quantitative and qualitative responses to other hormones.

1.4.1 OESTROGEN AND PROGESTERONE RECEPTORS

Early studies in animal models demonstrated the ability of the uterus to take up and retain larger amounts of radioactively labelled oestrogen than any other organ. This was later explained by the presence of a specific, high affinity receptor protein which, after binding oestrogen, becomes activated and interacts with the genome to stimulate the synthesis of specific ribonucleic acids (RNAs). The mechanism of oestrogen action in target cells was originally hypothesised to consist of a number of steps including (1) diffusion of oestrogen into a target cell, (2) high-affinity, specific binding of oestrogen to a cytoplasmic receptor, (3) activation of the ER complex, (4) translocation of the activated complex to the nucleus and (5) binding to nuclear chromatin and (6) induction of specific RNAs. According to this hypothetical model the majority of ER protein is cytoplasmic under low oestradiol conditions (postmenopausal women) and nuclear when the receptor is saturated by high levels of oestradiol (proliferative phase) (Gorski et al, 1968; Jensen et al, 1968). This model has been challenged and recent evidence suggests that both ER and PR are located in the nucleus (Gorski et al, 1986; Garcia et al 1988; Lessey et al, 1988; Press and Greene, 1988). ER and PR interactions have been studied and characterised for several years using a number of techniques. The technique of sucrose density gradient ultra-centrifugation of fractionated cells has been used

extensively to determine receptor binding in different tissues. These steroid binding assays provide quantitative information about steroid receptor content, but the methods require disruption of the tissues and hence, do not provide any anatomical information about the cellular location or tissue heterogeneity of these receptors.

Comparative studies of immunocytochemistry assays (ICA) and steroid binding assays for ER (West et al, 1987), have shown that changes in the degree of nuclear staining revealed by ICA parallel changes in the cytosolic and total ER, but not the amount found in the nuclear fraction. ICA studies of frozen sections are most likely to reveal antigens in their native locations. Thus the above findings support the view that the so-called cytosolic ER resides in the nucleus of living cells, and that the techniques of homogenisation and centrifugation artifactually distribute the total ER into nuclear and cytosolic fractions. Other studies have compared tissue steroid binding site counts obtained by autoradiographic techniques with the levels obtained by biochemical determinations (Lindenbaum et al, 1987). In these studies, the grain counts reflect the distribution and specific cell type in which binding sites are found. The site and time at which binding sites are found reflect the target tissue response to serum hormonal events. These techniques have been replaced by ICA studies using monoclonal antibodies (MOAB) specifically developed against ER and PR (Greene et al, 1980; Press and Greene, 1988). Their specificity and sensitivity are now well documented (Greene and Jensen, 1982; Greene and Press, 1987; Greene et al, 1988). By virtue of their structure, they bind conjugated and free receptors allowing the total receptor content of the tissues as well as their location in the different cell types to be determined.

Variations in endometrial and oviductal ER and PR levels during the menstrual cycle have been described in humans and animals using biochemical and ICA techniques. Biochemical analyses of ER and PR levels in the different segments of the oviduct at the different stages of the cycle have been conflicting with some studies showing no variation in the segments investigated (Punnonen and Lukola, 1981; Pino et al, 1982) while others reporting that the receptor content varied in the different segments of the tube at the different phases of the menstrual cycle (Pollow et al, 1981, 1982).

ICA techniques using MOAB have demonstrated ER and PR in the cell nuclei of endometrial glandular and stromal cells (Garcia et al, 1988; Lessey et al, 1988) and in the mammalian oviduct (McClellan et al, 1984, Perrot-Applanat et al, 1985) with the highest receptor content seen in the uterine fundus and ampullary region (Coppens et al, 1993). Exogenous progesterone treatment was reported to modulate endometrial and oviductal ER and PR levels (West and Brenner, 1985; McClellan et al, 1986; Press et al, 1986; West et al, 1986). Differential suppression of PR in the endometrium was also reported following progesterone treatment (West et al, 1986) or during the luteal phase of a natural cycle (Garcia et al, 1988). In the rabbit oviduct, Hyde et al (1989) showed variations in PR under different hormonal conditions and that oestradiol treatment resulted in differential regulation of PR in the isthmic and ampullary epithelia.

Comparative studies of ER and PR content in the human (Pollow et al, 1981) and Cynomolgus Macaques (West and Brenner, 1983) oviducts and endometria at the different stages of the menstrual cycle using biochemical techniques were reported. To this investigator's knowledge, there were no comparative ICA studies between the different segments of Fallopian tube and the uterus in humans other than the studies reported in this Thesis (Chapter 3) and published recently (Amso et al, 1994a).

1.4.2 ULTRASTRUCTURAL CHANGES

Oestrogen and P are also known to affect endometrial and tubal epithelial ultrastructure. Interpretation of various findings with respect to the influence of E and P as reported in the literature is, however, limited by a number of factors; (1) it is not always clearly stated which region of the oviduct or uterus is being investigated, (2) it is difficult to compare results from different mammals with one another or different regions of the oviduct in different women, (3) the phase of the menstrual cycle is often determined on the basis of the number of days elapsing since menstruation though in some studies of luteal phase development of the endometrium, accurate timing with LH surge was carried out, and (4) no efforts have been made to compare the uterus and the Fallopian tube ultrastructurally within the same subject

and at different phases of the menstrual cycle.

Cyclical changes of the Fallopian tube and endometrium have been extensively studied independently by light microscopy (LM) and electron microscopy (EM) as well as histochemistry and ultracytochemistry. These studies correlate morphological changes with changes in the steroid hormones during the menstrual cycle and have led to the concept that structural modifications reflect changes in metabolic functions and that both are endocrine related. However, a degree of variability between individuals does exist, the extent of which depends on a number of factors such as (1) the definition of a normal subject, (2) adequate dating of the biopsy, (3) the position of the biopsy in relation to the uterus, (4) adequacy of sampling and orientation of the specimen, and (5) the subjective nature of current methods of analysis. Recent studies on human glandular epithelium in the luteal phase, timed from the LH surge, show relatively small intersubject variation which may indicate that the cellular events in the glandular epithelium, between ovulation and the mid-luteal phase, are precisely regulated (Dockery et al, 1988a,b).

1.4.2.1 Cyclical tubal changes

Bjorkman and Fredricsson (1962) were among the first to report the ultrastructural features of the human oviduct epithelium and since then the greater resolution of electron microscopy has confirmed and extended LM observations. Scanning electron microscopy (SEM) studies of the epithelial surface of the tubal plicae has demonstrated the different cell types (ciliated and non-ciliated secretory cells) and their distribution along the Fallopian tube as well as the cyclical variations they undergo (Ferenczy et al, 1972b). There is controversy as to the proportion of these cells in different sections of the tube. Ferenczy et al (1972b) found that the cell types occurred in approximately equal numbers and proportions along the different sections of the Fallopian tube. However, ciliated cells were more prominent on the plical plateaus than in the crypts (Ferenczy et al, 1972b; Patek et al, 1972a). Jansen (1984) reported that ciliated cells were predominant in the fimbria whereas secretory cells were found mainly in the isthmus. Distinction between the two cell types is not

absolute and occasionally differentiating cells have been found to display both cilia and secretory granules (Jansen and Bajpai, 1982). Mitosis have been rarely seen in the adult tube at any stage of the menstrual cycle and thus there is presumably little change in cell number. The secretory cells have been found to show the most conspicuous cyclical changes, especially in the isthmus. Early in the cycle, the apices of the secretory cells have prominent microvilli and the cilia appear discrete. At midcycle (high E levels), apocrine secretion occurs and secretory material obscures the cilia (Jansen, 1984). Additionally, "serous" granules have been found in the apical cytoplasm of the secretory cells, and this was reported to be more evident in the isthmus than in the ampulla of the macaque oviduct (Jansen and Bajpai, 1983). Throughout the ovulatory period, a high degree of secretory activity was found particularly in the isthmus, although it was also seen in the ampulla (Ferenczy and Richart, 1974; Ludwig and Metzger, 1976). In the second half of the cycle, secretory activity was less, granules were no longer seen, and ribosomes were found to lie freely in the cytoplasm rather than attached to granular endoplasmic reticulum (Bjorkman and Fredricsson, 1962). Cilia were then seen to be conspicuous again. With the loss of hormonal support at the end of the luteal phase, numerous lysosomes have been found in the cytoplasm (Clyman, 1966; Hashimoto, 1964) and microvilli were sparse on the non-ciliated cell surfaces.

Under the effect of exogenous E, secretory cells in the fimbria and ampulla were found to develop numerous vesicles containing material that is less electron dense than the contents of the dense granules (Fredricsson and Bjorkman, 1973). To a lesser extent these vesicles have also been demonstrated at the midcycle in spontaneous cycles. In the isthmus of the cynomolgus monkey, they constitute a major midcycle secretory phenomenon both in spontaneous cycles and hormone treated animals (Jansen and Bajpai, 1983). Progesterone has the opposite effect and its long term administration has been found to lead to atrophy and deciliation of the endosalpinx. Studies of the Fallopian tube with EM have not only described morphological changes, but were also utilised to locate calcium particles as well as alkaline and acid phosphatase in the epithelial cells of the fimbria (Lindenbaum et al, 1982, 1983). These studies have demonstrated the presence of sulphated and non-

sulphated glycoconjungates both in the rabbit oviduct (Menghi et al, 1984a,b) and human Fallopian tube (Schulte et al, 1985). More recently the transfer of glycoconjugates of oviductal origin to Hamster zona pellucida has been demonstrated after ovulation (Kan et al, 1990). Scanning EM studies have also been carried out on hydrosalpinges (Vasquez et, 1983) and in ectopic pregnancies (Samberg et al, 1983).

1.4.2.2 Cyclical endometrial changes

The cyclical ultrastructural changes in endometrial surfaces (luminal and glandular) have been described by several investigators (Ferenczy et al, 1972a; Ferenczy, 1977; Sundstrom and Ove Nilsson, 1982; Cornillie et al, 1985; Dockery and Rogers, 1989). Data on the effects of steroid hormones are essentially derived from four types of studies: (1) timed biopsies through the normal menstrual cycle; (2) biopsies in modified cycles, such as superovulation or the administration of steroids; (3) women undergoing hormone replacement therapy for premature menopause; and (4) women on contraceptive steroids. In addition, some observations were available on endometrial fragments treated in culture with oestrogen or progesterone. The cyclical changes described below were obtained from observation of a normal 28 day menstrual cycle. Unfortunately, not all authors have adjusted critically for the variables mentioned above. Interpretation of luteal phase changes (day of the cycle from the LMP) will be adjusted to accommodate recent descriptions of ultrastructural glandular changes as timed by the LH surge (Li et al, 1990a).

The glands as well as the surface epithelium of the endometrium were reported to be lined by a single layer of columnar epithelial cells, a few of which are ciliated. Proliferation of the endometrial mucosa was preceded by endometrial regeneration, a process that began during the menstrual period and continued for approximately two days during which the re-epithelialisation of the denuded basal layer took place from the free gland stumps of the stratum basalis. During the **early proliferative phase** (days 4-7), the cytoplasmic organelles involved in protein synthesis, such as free ribosomes, granular endoplasmic reticulum (GER), and mitochondria appeared poorly

developed. The nuclei were elongated with compact chromatin. The luminal surface of the cells was covered by microvilli, which increased in number as the follicular phase progressed. The lateral plasma membranes were tightly apposed. The endometrial glands were rich in acid phosphatase-positive cytolysosomes and lysosomes and numerous Golgi and lipid droplets were also noted. In the **mid-proliferative phase**, the glands became longer with segmental tortuosity. There was a considerable increase in RNA synthesis, reflected in the abundance of free ribosomes and GER in a subnuclear location. Endometrial ciliated cells could also be seen and appeared indistinguishable from the tubal ciliated cells. In the **late proliferative phase** (days 11-14) the increasingly tortuous glands were lined by tall pseudostratified columnar epithelium and the well developed microvilli on the luminal surface projected into the glandular lumen. Large subnuclear aggregates of mitochondria were associated with well-developed Golgi bodies and GER, and free ribosomes were found to be dispersed throughout the cytoplasm. Small deposits of glycogen could be seen occasionally before ovulation (Dockery and Rogers, 1989), but large basal accumulations of glycogen were characteristically seen in the postovulatory period (LH+3 to LH+5).

The **luteal phase** was divided into early (LH+1 to LH+7) and late (LH+8 to menstruation) phases (Dockery and Rogers, 1989). In the early postovulatory phase (days 14-16; LH+3/+4) and under the combined influence of E and P, aggregates of glycogen appeared in the subnuclear portions of the glandular cells. This glycogen takes the form of glycoproteins and glycolipids. The neighbouring mitochondria began to increase in number and size (Giant mitochondria) and, in close association with GER and free ribosomes, were found to be interconnected by undulating bundles of perinuclear microfilaments to the supranuclear Golgi. The lateral plasma membranes contained focal areas of convolutions and microvillus projections were noted near the basal lamina. Following ovulation, intranucleolar channel system (LH+4) of obscure significance, were seen. At LH+5, the cells appeared to mobilise the subnuclear material which was then secreted into the lumen. Around days 19-21 (LH+6 to +8), most of the secretions were expelled from the cell surface, and the epithelium became flattened. Secretory activity was preceded by the

appearance of numerous small vesicles beneath the surface plasma membrane. The apical accumulation of large aggregates of glycogen and various organelles may cause large portions of the cytoplasm to protrude and eventually rupture into the glandular lumen. This type of secretion reached maximum activity between days 19-21 of the cycle. Later, agranular vesicles surrounded by an electron dense amorphous matrix, "alveolate" structures, appeared in the cytoplasm. The lateral plasma membranes remained tightly apposed but convoluted especially near the glandular lumen.

At about day 24, the accumulated intraluminal secretions became prominent. They contained glycogen, acid and neutral mucopolysaccharides, lipids, and various enzymes. The cells themselves demonstrated features of involuting secretory activity as evidenced by regression of the Golgi body and a decrease in the number of vesicles and ribosomes. Large portions of the apical cytoplasm protruded into the glandular lumen and luminal microvilli appeared short and scant. The lateral plasma membranes were extremely convoluted and the intercellular space had numerous interdigitating microvilli. From the 25th day, most of the cells lacked glycogen and microfilamentous system. Acid phosphatase containing lysosomes and Golgi bodies became abundant. The increase in lysosomal activity (auto-cytolysosmes) is probably related to focal cytoplasmic degeneration and perhaps with autoregulation of excess secretory material. Eventually, total cell degeneration with expulsion of the cellular debris into the lumen occurred as the menstrual phase ensues.

Studies of the luminal surface epithelium have been contradictory. Sundstrom and Ove Nilsson (1982) using SEM concluded that no consistent ultrastructural changes of the endometrial surface could be detected in postovulatory biopsy specimens obtained early or late in unstimulated or stimulated cycles. This, in their opinion, might indicate that (1) the properties of the luminal cell epithelium are not continuously differentiating to reach a specific state at the expected time of implantation, (2) the surface may be ready to accept the embryo at any time within a certain range of days and (3) the crucial time for implantation may be governed by other changes in the endometrium or by the developmental stage of the embryo. Transmission EM studies confirmed that these cells did not undergo such dramatic

cyclic changes as the glandular epithelium (Cornillie et al, 1985). More recently, Psychoyos and Nikas (1994) and Nikas et al (1995) observed the appearance of fully developed uterine pinopods on the apical surface of the luminal uterine epithelium at the peri-implantation period in normal cycles and following exogenous oestradiol and progesterone therapy and postulated that their short life span of 48 hours may correspond to the period of optimal endometrial receptivity. Light microscopy morphometric analysis of luminal epithelial cells dimensions and nuclear parameters of fertile and subfertile women did not show any significant differences between the groups (Saleh et al, 1995).

None of the above studies had compared changes in the endometrium with those in the Fallopian tube in the same women. Further, two animal studies only compared the morphological features of the oviduct and endometria in the same animal (Brenner et al, 1983; Verhage et al, 1984). Accurate comparative studies are pertinent when the role of the oviduct in clinically important issues, such as differences in pregnancy or implantation rates between uterine and tubal embryo transfer require explanation. This deficiency in our knowledge led to the studies which are described in this Thesis (Chapter 4) and were published recently (Amso et al, 1994b; Crow et al, 1994).

1.5 TUBAL FLUID

Tubal fluid formation: Oviduct fluid is a complex mixture of constituents derived from the plasma together with specific proteins formed by the oviduct epithelium. Current thinking recognizes that molecular movement across epithelial surfaces is bidirectional in nature. The net flux of molecules from the fluid surrounding the serosal surface to those bathing the mucosal surface and vice versa, reflects the characteristic physiology of the epithelium and the other layers of tubal wall. The amount of oviductal fluid produced is mostly dependant on the hormonal status. In general, high circulating concentrations of E result in maximal production of oviduct fluid. High P levels or depressed oestrogen concentrations result in a large decrease in the daily production of oviductal fluid (Hamner, 1969, 1973). Thus, minimal oviductal fluid volumes occur in the postovulatory period (Oliphant, 1986). The

steady state concentrations of oviduct fluid constituents are therefore a function of the movement of molecules between a series of compartments. In the rabbit oviduct, the mucosal lining appears to be a chloride secreting epithelium and this flux is under the control of cyclic AMP (Gott et al, 1988). However, the mechanism by which ions move across the oviduct is unknown. Leese (1988) has speculated that changes in oviduct fluid volume and its control may be explained in terms of changes in ion fluxes, particularly chloride across the tubal epithelium. Such changes would undoubtedly cause changes in pH measurements at different stages of the menstrual cycle (Maas et al, 1979). The increase in calcium concentration in oviductal fluid as a function of the increase in the circulating P appears to be the most consistent change in the inorganic constituents and it is known that calcium is required for sperm acrosomal reaction (Yanagamachi and Usui, 1974). It has also been suggested that bicarbonate facilitates sperm motility (Hamner and Williams, 1964) and promotes dispersal of the corona radiata after fertilisation (Stambaugh et al, 1969). A high potassium concentration is also thought to promote the development of early embryos (Quinn et al, 1985; Roblero and Riffo, 1986).

Glucose, pyruvate, lactate and a number of amino acids are present in the Fallopian tube at a concentration much lower than that of plasma, suggesting that their overall transport across the oviduct is by diffusion rather than active transport (Leese and Gray 1985). The concentration of lactate and pyruvate increases in the rabbit oviductal fluid after ovulation (Oliphant, 1986). Ampullary pyruvate and lactate in the mouse increase by 12 hours after mating. Furthermore, in pregnant mice levels of isthmic lactate remain elevated for at least 72 hours after ovulation in contrast to an increase for only 48 hours in non-pregnant animals. This response by the oviduct to the presence of viable embryos may provide optimal conditions for early embryonic growth and development. Similarly, Leese and Barton (1985) have confirmed earlier reports of higher levels of pyruvate in the vicinity of the cumulus mass suggesting a contribution by these cells to the pyruvate pool in the lumen. Regional variation along the length of the oviduct has also been reported. In experiments in which the appearance of glucose, pyruvate and lactate was measured in fluid recirculated through the rabbit oviduct lumen, Leese (1983) found that all

three nutrients appeared in the ampulla at about 1.8 times that of the isthmus. This ratio corresponds more closely with the relative mucosal surface area of the two regions than with the proportions of ciliated and non-ciliated cells.

The protein concentration of oviductal fluid is typically much lower than that of plasma. It has also been reported (David et al, 1969) that different sections of the tube have different concentrations of protein in the fluid and secrete different amounts and types of glycoproteins (Hyde and Black, 1986). The effect of ovulation and subsequent increase in progesterone on total protein concentration in the oviduct is not known. Many of the typical serum proteins are present in oviductal fluid and their ability to gain access into the fluid appears to be dependent on their macromolecular size (Oliphant et al, 1978) as well as other as yet undetermined factors. Immunoglobulins (IgG and IgA) have also been identified in the oviductal fluid in varying concentrations. Whether albumin and IgG, the most abundant proteins in oviductal fluid, have embryo-specific functions is unknown. Preimplantation mouse embryos have been shown to take up whole ^{125}I-labelled Bovine Serum Albumin (BSA) intact, probably by an endocytotic mechanism, then degrade it intracellularly (Pemble and Kaye, 1986). Similarly, Wiley and Obasaju (1986) have shown that mouse oocytes and preimplantation embryos in culture media adsorb IgG to their cell surface. The development of a new technique involving vascular perfusion of the human Fallopian tube (Dickens et al, 1995) provided a controlled method with which to access and examine human tubal fluid and should expand our knowledge of tubal function.

Oviduct specific proteins. Secretion of oviduct specific proteins by the endosalpinx has been the subject of considerable interest for several years. Immunoelectrophoresis of oviduct fluid has suggested the presence of unique proteins, which may have been synthesized and secreted by the oviduct, e.g. specific ß-glycoproteins in humans (Moghissi, 1970), monkeys (Mastroianni et al, 1970) and sheep (Roberts et al, 1976). Immunoaffinity adsorption was used to separate these proteins from those derived from serum in human tubal fluid (Wagh and Lippes, 1989) and one protein was shown to bind to spermatozoa head (Lippes and Wagh,

1989). Studies of sulphated mucopolysaccharides and mucoproteins in oviduct fluid suggest that they may originate from granules observed in the secretory epithelium of the oviducts (Shapiro et al, 1971; Hanscom and Oliphant, 1976; Barr and Oliphant, 1981; Rapisarda, 1993), and in the rabbit they may form the mucin coat which surrounds the zona pellucida of the ovum (Greenwald, 1958). Oliphant and colleagues (1984 a,b) purified a complement inhibitor from rabbit oviduct fluid and provided evidence that it is a sulphated glycoprotein which can protect both embryos and spermatozoa from the maternal immune system. In tissue explant studies, high and low molecular weight proteins were isolated (Verhage et al, 1988; Maguiness et al, 1992 a,b) and anatomical variation in both the qualitative and quantitative nature of these proteins in the rabbit oviducts (Hyde and Black, 1986; Oliphant, 1986) and human Fallopian tube (Maguiness et al, 1993) were also noted. Kapur and Johnson (1985, 1986) also showed that a 215kDa glycoprotein was released by the mouse oviduct and selectively sequestrated into the perivitelline space of the oocytes and embryos. Others (Leveille, 1987; Kan et al, 1988; Kan, 1990) demonstrated uptake of an oviduct derived component by the zona pellucida of superovulated hamster oocytes and, furthermore, by using high resolution lectin-gold techniques, showed that these glycoconjugates are synthesized in the Golgi apparatus of the non-ciliated secretory cells in the oviduct and stored in secretory granules (Kan et al, 1990). Oviductal factors (glycoproteins) were also shown to alter the zona pellucida of the golden hamster (Yang and Yanagimachi, 1989). The use of new recombinant complementary deoxyribonucleic acid (cDNA) techniques (Donnelly et al, 1991) succeeded in isolating the mRNA responsible for the synthesis of a baboon oestrogen-dependent oviduct-specific protein and later (Arias et al, 1994) cloned, sequenced and characterised the cDNA to a human oestrogen-dependent Fallopian tube glycoprotein. Moore et al, (1992) showed that rabbit blastocysts in co-culture attached to endometrial but not to endosalpingeal monolayers. Addition of culture media from endosalpinx significantly decreased embryo attachment to endometrial cells in culture suggesting that rabbit endosalpinx secretes a factor that prevents tubal implantation.

The above discussion raises the crucial question of whether there are other factors which are contributed by the oviduct that are lacking in vitro and whether gametes

or early embryos replaced into the Fallopian tube have better chances of implantation than those replaced into the uterine cavity. During the early part of my research attachment at the Royal Free Hospital, several reports indicated that tubal co-cultures had a significant impact on embryonic growth. Later, with accumulating knowledge, doubts were raised and in this section of the introduction both views are presented. Possible factors that may contribute to embryo wellbeing may be physical and/or chemical.

(i) Physical Factors:

Oviduct explants, grafts of tissue or monolayers could simply be providing an appropriate substratum to promote embryonic development. Interest in co-culture systems resulted from the introduction of GIFT and the realisation that embryo culture systems used in IVF remain far from ideal. Several workers (Biggers et al, 1962; Whittingham and Biggers, 1967; Whittingham, 1968a,b; Gandolfi and Moor, 1987; Bongso et al, 1990) have demonstrated that oviduct organ/cell cultures, but not fibroblast feeder layer (Gandolfi and Moor, 1987), will support embryonic development into blastocyst, well beyond their observed restricted development in culture media. Contradictory studies (Walker et al, 1992) demonstrated a high rate (85%) of in vitro development of sheep zygotes to blastocyst stage using a simple medium supplemented with serum. Similarly, these reported beneficial effects of co-cultured cells on embryo development did not appear to be either species- or cell/tissue-specific. Hamster embryos were successfully cultured within the mouse ampulla (Minami et al, 1988), porcine embryos in mouse oviducts (Krisher et al, 1989), bovine embryos in rabbit oviducts, and human embryos in fetal bovine uterine fibroblast cells (Wiemer et al, 1989). Sequential human oviduct-endometrial co-culture system was also reported to increase human embryo expansion and hatching over the single oviductal co-culture system (Bongso et al, 1994). Other tissues shown to enhance embryo development, in a variety of species, include cumulus and granulosa cells, trophoblastic vesicles, monkey kidney cells and buffalo rat liver cells (Bongso et al, 1990; Bavister, 1992). Many of the early studies were criticised in that they used inappropriate or suboptimal culture media for their experiments and without reporting any control data (Bavister, 1992).

(ii) Chemical factors:

The functional importance of oviductal fluid in embryonic development has been evaluated by several methods. Embryos were transferred to ligated oviducts and evaluated for normal development. Adams (1973), showed that rabbit embryos could not develop beyond the early blastocyst stage irrespective of the endocrine environment of the oviduct and that four cell embryos developed best when the endocrine stimulation of the oviduct was in the early luteal phase. Abnormal embryonic development occurred when the embryos were transferred to the ampulla under an oestrogenic environment. This negative effect on development was not the direct effect of E but was attributed to a peptide inhibitor of embryonic development (Richardson et al, 1980). Such an inhibitor had been identified in rabbit and mice oviductal fluids and it was suggested by Oliphant (1986) that the tubal sulphated glycoproteins may act in vivo to inactivate the embryonic inhibitory peptides or to protect the embryos from the maternal humoral immune system. It was also suggested that human endometrial stromal cell co-cultures or endometrial secretory proteins isolated from conditioned media derived from endometrial stromal cells, significantly enhance mouse embryo development by secreting specific proteins (Liu et al, 1992). This non species- nor tissue-specific effect reduces the possibility that oviduct cell secretory products, such as "cycle dependent" proteins are embryotrophic and, as yet, there is no evidence that such secretory products are responsible for improvements in embryo development (generally called "positive conditioning"). Indeed, it would be remarkable if all the above mentioned different cell types were producing the same specific embryotrophic factor. Other cycle-dependant factors such as cytokines (Tabibzadeh and Sun, 1992; Tabibzadeh et al, 1995) and leukaemia inhibitory factor (Arici et al, 1995) have been demonstrated in the endometrium (glandular and luminal epithelia and stroma). Their abundant expression highlights the significant role they play in cell-cell interactions and in regulating endometrial function. Growth factors are also expressed by the early embryos of several species and contribute to the successful development of blastocysts (Harvey et al, 1995).

One way that somatic cells in co-culture might benefit embryo development is by stabilising or altering physico-chemical conditions, such as the pH of the culture

medium or oxygen/carbon dioxide concentrations or removal of detrimental components from the culture medium, as a consequence of their metabolic activities (generally called "negative conditioning"). Leese (1990) had postulated that the currents of fluid generated by ciliary beating constantly replenish oviduct fluid constituents in the immediate environment of the gametes and early embryos. Theoretical calculations by the same group predicted that in a stagnant, unstirred culture environment, the diffusion of oxygen towards the embryo would be insufficient to keep pace with maximal rates of oxygen consumption. Adjustments in the gas levels used in culture were also reported to have striking effects on embryo development (Bavister, 1992). A recent study (Kervancioglu et al, 1994) investigating the influence of co-culture systems on the survival of human spermatozoa and induction of sperm capacitation reported that the co-culture systems, but not conditioned media, promoted sperm survival for up to 48 hours of culture. The authors suggested that the presence of monolayers possibly reduced the oxidative stress damage to the spermatozoa. In addition, the presence of oviductal epithelial cells had a specific stimulatory effect on sperm capacitation. The fact that such widely different cell types were reported to be effective when used in co-culture for embryo development could well support the concept of "negative conditioning".

The ability of embryos to develop in vitro and the successful establishment of pregnancies in the human following IVF-UET make it highly improbable that the developing embryo directly requires specific secretions. However, initial experience with GIFT and tubal embryo transfer indicated that higher pregnancy rates are obtained in comparison with conventional IVF-UET and revived interest in the role of the tubal environment and its influence on the establishment of early pregnancy.

1.6 CLINICAL VALUE OF THE FALLOPIAN TUBE IN ASSISTED REPRODUCTION

1.6.1 ASSISTED REPRODUCTION: HISTORICAL BACKGROUND

Sophisticated biological knowledge gained in this century has led to major advances in human endocrinology and embryology, such that a considerable array of assisted

reproductive techniques is available to help subfertile couples. It took many years of animal experimentation and human work before human oocytes matured in vitro were successfully fertilised in simple culture media (Edwards and Craft, 1990). After several attempts of transcervical uterine embryo transfer and an ectopic pregnancy (Steptoe and Edwards, 1976), a term pregnancy using cleaving embryos grown in vitro was finally achieved by Edwards, Steptoe and Purdy (1980). Other forms of assisted reproduction were being developed in parallel with IVF, albeit somewhat later, in the hope of finding a simpler, cheaper, more efficacious, and less ethically controversial method applicable to patients with both open and closed Fallopian tubes. Unfortunately, most clinical studies were performed in isolation from IVF.

In 1979, L.B. Shettles introduced the concept of transferring gametes into the Fallopian tube as a means of achieving pregnancy. Extensive research on gamete tubal transfer followed and the first report of a successful pregnancy following tubal gamete (sperm and egg) transfer performed at the conclusion of a tubal microsurgery operation was published in 1983 (Tesarik et al) thereby introducing a potentially clinically useful technique in the human. Asch and colleagues (1984, 1986) widened the clinical applications of the technique to include women with non tubal infertility. He popularised the procedure under the acronym "GIFT", and was the first to suggest early on the nonsurgical transfer of gamete under hysteroscopic guidance as a possible alternative to laparoscopy. Several reports have appeared in the literature claiming superiority of this technique to IVF (Table 6.1). The clinical pregnancy rates per cycle or embryo transfer appear to be comparable from one national register to another (Amso and Shaw, 1992). In these registers, this rate is slightly higher for GIFT than for IVF-UET. Several factors may be responsible for this difference. One possible reason is the greater number of transferred oocytes (up to 10 and occasionally more) resulting in an increase in the frequency of multiple pregnancies in all registers (Cohen, 1991). Admittedly, the few extant randomised, controlled trials comparing IVF and GIFT failed to confirm previous reports (Leeton, 1987).

Several advantages have been proposed for the GIFT procedure; (1) It is assumed to be more physiological in that the sperm and oocytes are replaced into the Fallopian

tube where natural fertilisation and early embryonic development takes place, and (2) Its apparent reduced cost, mainly due to the absence of the IVF laboratory techniques and ET procedure. However, recent developments in transvaginal ultrasound guided oocyte collection techniques, under local analgesia and as an outpatient procedure have cut the costs of IVF dramatically. Disadvantages of the GIFT procedure include; (1) the need for general anaesthesia with its accompanying risks and costs for the laparoscopy or minilaparotomy operation, (2) it is limited to women with a normal pelvis as any tubal damage jeopardises chances of success and increases the risks of ectopic pregnancy, and (3) there is lack of information on the fertilising capacity of sperm in general and more specifically in male factor infertility. This uncertainty concerning the fertilisation of oocytes in vivo led to the development of the transfer of fertilised oocytes into the Fallopian tube instead of gametes. Devroey et al (1986) in Belgium and Blackledge et al (1986) in Australia, working independently, reported their experience with a similar methodology. The former termed the procedure zygote intrafallopian transfer (ZIFT) and the latter pronuclear-stage tubal transfer (PROST). A number of reports had proposed the superiority of this technique to either IVF or GIFT (Table 6.1).

Since the introduction of transvaginal ultrasound guided oocyte recovery, attempts were under way to eliminate laparoscopy for the transfer of gametes by developing nonsurgical techniques. Jansen et al (1988a), reported the first successful pregnancy following transfer of fertilised oocytes at the pronuclear stage and cryostored donor semen (Jansen et al, 1988b) transcervically and under ultrasound guidance into the Fallopian tube using a specially designed tubal cannulation system. Pregnancies were also reported following the transfer of gametes, using either the Jansen technique (Bustillo et al, 1988) or hysteroscopic guidance (Wurfel, 1988; Possati et al, 1991). Such rapid advances in the field have led to the introduction of these newly developed techniques into clinical practice without adequate evaluation in properly designed and conducted clinical trials. It is astonishing that retrograde GIFT or ZIFT are being proposed as state of the art treatment for infertile women with patent Fallopian tubes when in fact the exact role of the tube is still under evaluation and when laparoscopic GIFT has not been proven, beyond doubt, to be superior to conventional IVF-UET.

AIMS OF THE STUDY

1. To study the cyclical variation of oestrogen and progesterone receptors (as assessed by immunocytochemical assays) in different segments of the Fallopian tube as well as the endometrium.
2. To examine ultrastructural changes of different parts of the Fallopian tube and the endometrium in the same patients.
3. To attempt to collect tubal fluid, isolate tubal specific, cycle dependant proteins and then raise polyclonal antibodies against them. Later, to determine the location of these proteins in different parts of the tube using immuno-cytochemical techniques and investigate any cross reactivity with the endometrium in the same group of women.
4. To assess the clinical relevance of the Fallopian tube in a clearly defined group of women requiring assisted reproduction treatment, by conducting a randomised, controlled trial of IVF-uterine embryo transfer (UET) versus IVF-tubal embryo transfer (TET).

CHAPTER 2
METHODOLOGY (1): LABORATORY TECHNIQUES

2.1 INTRODUCTION

The aim of the studies in the next three chapters was (1) to investigate the presence of steroid receptors in the Fallopian tubes and endometria, (2) to observe the ultrastructural changes that occurred in the same sites at different stages of the menstrual cycle, and (3) to identify and isolate tubal specific proteins with a view to ultimately establishing the site of their production within the Fallopian tube. This section of the thesis will describe the laboratory techniques used to achieve these aims.

2.2 OESTROGEN AND PROGESTERONE RECEPTOR STUDY

2.2.1 MONOCLONAL ANTIBODIES (MOAB)

The MOAB used against the ER and PR (Abbott ER-ICA and PgR-ICA Monoclonal) were supplied by Abbott Laboratories Ltd, Maidenhead, UK. Production of MOAB against human steroid receptor proteins permitted the development of assays based on direct antigenic recognition rather than steroid binding activity (Greene et al, 1980; Weigand et al, 1986). These assays measure ER and PR molecules whether or not they are bound to endogenous steroids (Greene and Press, 1987). The Abbott Monoclonal systems employs a sensitive peroxidase-anti-peroxidase technique for visualisation of the respective receptors in frozen tissue sections through the use of MOAB directed specifically against the receptor (Figure 2.1). The specificity and assay reproducibility for these MOAB had been determined by the manufacturer (Abbott laboratories). Oestrogen and progesterone target and non-target tissues were assayed by ER-ICA and PgR-ICA (Abbott laboratories information sheets 1990 and 1989 respectively). Target tissues such as endometrium, uterine muscle, Fallopian tube, and human breast cancers known to respond to endocrine therapy exhibited ER and PR specific staining respectively. Non-target tissues such as renal carcinoma, liver carcinoma, and bladder cancer did not exhibit staining. Furthermore, tumour sections were incubated with potentially interfering substances such as oestradiol,

progesterone, androstan, diethylstilbestrol, prednisone, tamoxifen, and dexamethasone at concentrations of 10^{-7} and 10^{-9}M prior to staining with ER-ICA or PgR-ICA and did not show any interference in the assay.

Assay reproducibility for ER-ICA and PgR-ICA was determined by the manufacturer by assaying tissue sections from tumour specimens in independent runs. The "Within run coefficient of Variation (%CV)" for ER-ICA was between 1.8 and 7.0% and for PgR-ICA between 2.1 and 16.4%. The "Between run %CV" for ER-ICA was between 2.3 and 10.2% and for PgR-ICA was between 2.1 and 16.4%. The "Between technician/laboratory %CV" for ER-ICA was between 2.4% and 10.2% and for PgR-ICA between 2.1 and 24.1% (Abbott laboratories information sheets 1990 and 1989 respectively).

2.2.2 TISSUE SECTIONS PREPARATION AND FIXATION

All procedures including tissue preparation, fixation, immunostaining for ER and PR, and assessment of stained sections were performed by the principal investigator (N.N.A.) under the supervision of Dr. J.C., Department of Histopathology.

Using a cryostat, serial frozen sections (4-6 μm thick) were cut and mounted on specially prepared glass slides. The first and last section of each sample were stained with haematoxylin and eosin for conventional histological examination and endometrial dating respectively. Immunostaining for ER and PR of all sections strictly followed the methodology described by the manufacturer. The cryostat sections were fixed in 3.7% formaldehyde in 0.01M Phosphate-Bufferred Saline (PBS) at room temperature for a maximum of 15 minutes, then transferred to a PBS bath until all sections were completed. The sections were then immersed in 100% methanol at -10 to -25°C for 4 minutes and acetone at -10 to -25°C for 2 minutes to complete the fixation process. They were then washed twice in PBS solution (each for 6 minutes) and staining was commenced within 2 hours of the last wash.

2.2.3 IMMUNOSTAINING OF TISSUE SECTIONS

To prevent staining by endogenous peroxidase in the tissues, a Blocking Reagent was added to the fixed sections and incubated for 15 ± 2 minutes in a humidified chamber at room temperature. One sample from the same specimen was incubated with the primary antibody, an IgG fraction of monoclonal (rat) antibody to human ER and PR respectively, and the other was incubated with the control antibody, normal non-immune rat serum (serving as a negative control) for 30 ± 2 minutes. After the first incubation, the slides were washed in PBS twice for five minutes each and then were incubated with goat anti-rat immunoglobulin (secondary-bridging antibody) for 30 ± 2 minutes, followed by two further washes in PBS. Horseradish peroxidase-rat antihorseradish peroxidase in PBS (PAP complex) was applied to the sections for 30 ± 2 minutes, followed by two more PBS washes. The slides were flooded with freshly prepared diaminobenzidine-hydrogen peroxide solution for 6 minutes and rinsed with distilled water before counterstaining with haematoxylin, dehydration in alcohol and xylene and mounting. In the presence of hydrogen peroxide, the tissue-bound peroxidase converts diaminobenzidine to an insoluble chromogen product easily visualised by its reddish-brown colour on light microscopy. Using this method, minute quantities of receptor may be amplified through the use of the bridging antibody, PAP complex and enzyme reaction products. Positive controls were either endometrial samples known to be ER-positive, or PR-positive frozen slides provided by the manufacturer.

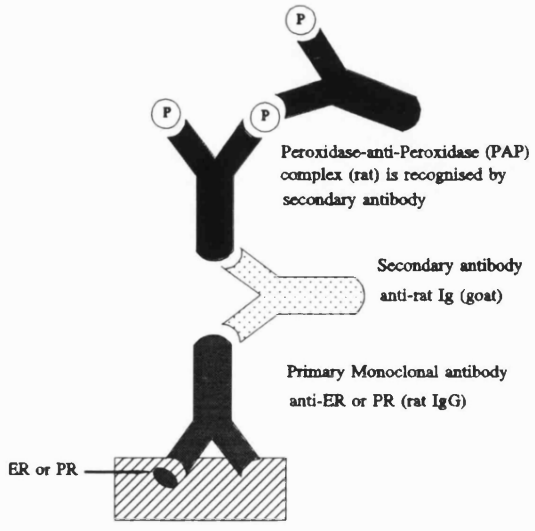


Figure 2.1
Diagrammatic representation of PAP labelling technique. (P = Peroxidase)

2.2.4 ENDOCRINE ASSAYS

All assays were performed in duplicate. FSH and LH were measured with a solid-phase radioimmunoassay (RIA) as described by Ferguson et al (1982), with inter-assay and intra-assay %CV of 8.1% and 3% for FSH and 6.4% and 1.4% for LH. E_2 was analysed with a nonextraction RIA kit (Code ER-155, Steranti Research Ltd., St. Albans, Herts, UK) with a calculated sensitivity of 18.5 pmol/l, an inter-assay %CV between 10.8 and 32.7% and intra-assay %CV between 2.9% and 7.2%. Normal E_2 value for post-menopausal women was <100 pmol/l, early follicular phase was between 110-183 pmol/l, preovulatory peak was between 550-1650 pmol/l, and mid-luteal phase was between 550-845 pmol/l.

Progesterone was measured with Coata-count progesterone ^{125}I kit (Diagnostic Products Ltd., Wallingford, Oxon, UK; imported from Diagnostic Products Corporation, Los Angeles, CA) with an inter-assay %CV between 5.1% and 10% and intra-assay %CV between 2.6% and 6.4%. Median value for follicular phase serum P in fertile women was 1.8 nmol/l (central 95% range=0.48-4.5 nmol/l), luteal phase was 22 nmol/l (central 95% range=5.1-67 nmol/l), and for post-menopausal women was 0.86 nmol/l (absolute range=0.35-2.9 nmol/l).

2.3 ULTRASTRUCTURAL STUDY

The specimens were transported immediately from the operating theatre to the histopathology department and dissected before fixation. Transverse blocks of tissue were taken from four areas (isthmus, isthmic-ampullary junction or middle section, ampulla and fimbriae) of one Fallopian tube in a standardized fashion by one pathologist (JC). A sample of endometrium with immediately underlying myometrium was taken from the mid-cavity of the uterine corpus. Adjacent slices of each tissue sample were examined with light microscopy and electron microscopy. Preparation of tissue samples for light and electron microscopy was carried out by technicians in the pathology and electron microscopy departments. Sections of the Fallopian tube and endometrium were examined jointly by the principal investigator (N.N.A.) and one pathologist (J.C.).

2.3.1 LIGHT MICROSCOPY

Tissue samples were fixed in buffered formalin, processed routinely then embedded in paraffin wax. Sections were stained with haematoxylin and eosin (H+E).

2.3.2 ELECTRON MICROSCOPY

Specimen were fixed in glutaraldehyde (3% in 0.2M cacodylate buffer and 0.2M sucrose at pH 7.4) for a maximum of 2 hours and then were stored in cacodylate buffer until processed. Specimen were cut into blocks $< 1mm^2$ and post fixation was carried out in 1% osmium tetroxide with 1.5% potassium ferricyanide in cacodylate buffer for one hour. The specimens were washed well with buffer and then dehydrated through graded alcohols. Infiltration with LEMIX resin was carried out first using 50:50 resin/alcohol solution for 8 hours and then 100% resin overnight. Blocks were embedded in fresh resin and polymerised at 60°C overnight. Thin one micron sections (1μm) were cut on a Reichert Ultracut microtome, stained with 1% toluidine blue in 1% borax and areas were selected to include epithelial lining for the Fallopian tube and glandular tissue for the endometrium. All areas selected for EM study were initially examined using 1μ toluidine blue blocks under light microscopy to determine the appropriate orientation of the tissue, assess pseudostratification, and the per centage of ciliated cells. In an individual section each cell was cut only once, and only the cells that reached the surface in this plane were counted. One hundred adjacent cells in each of the different segments of each tube were examined with a x100 microscope objective and the percentage of ciliated cells was recorded. Ultrathin sections were then cut, mounted on 300 mesh grids and stained with saturated uranyl acetate in 50% ethanol and Reynold's lead acetate. The sections were viewed with a Philips 201 transmission EM. Additional blocks from some cases were dehydrated using dimethoxypropane, dried using liquid CO_2 in a critical point dryer, mounted on aluminium stubs, sputter coated with platinum and examined in a Philips 501 scanning EM. Photographs were taken on Kodak Fine Grain Release Positive 5302-35mm film which was developed using Kodak HC110 high contrast developer and fixed using Ilford Hypam. Prints were made using Ilford Multigrade Deluxe III paper, developed and fixed using Ilford Multigrade chemicals.

2.4 FALLOPIAN TUBE PROTEIN STUDY

2.4.1 ONE-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (1-D PAGE)

1-D PAGE was performed using the discontinuous buffer system described by Ornstein (1964) and Davis (1964). Separating gels (2mm thick) of different concentrations ranging from 5% to 12% were prepared containing 2.2M Tris-HCL, 0.1% SDS at pH 8.9. Volumes of the different constituents used for the respective separating gels are shown in Table 2.1. A 4% stacking gel containing 1.25M Tris-HCL, 0.1% SDS, at pH 6.8 was used. The reservoir buffer consisted of 0.025M Tris, 0.192M glycine and 0.1% SDS at pH 8.3. Serum samples were diluted before use while tubal fluid samples were used after collection as described in 5.2.2. Sucrose and Bromophenol Blue marker were added to the sample. Electrophoresis was performed at different voltages and currents until the Bromophenol blue marker reached the end of the gel. Following 1-D PAGE the gel was fixed and stained with Coomassie Blue R-250. After destaining, the gels were photographed, and then silver stained according to the method of Marshall (1984). Oviduct flushing samples were electrophoresed alongside serum samples of the respective patients under the same conditions.

Table 2.1 CONSTITUENTS OF THE DIFFERENT CONCENTRATIONS OF SEPARATING SDS GEL SLABS

Constituent (ml)	5%	7%	12%
Acrylamide	2.0	2.8	4.8
Buffer (pH 8.9)	3.3	3.3	3.3
SDS	0.13	0.13	0.13
Sucrose	--	--	8.0
TEMED	5μl	5μl	5μl
Water	13.57	12.77	2.77
Ammonium persulphate	1.0	1.0	1.0
	20.0	20.0	20.0

2.4.2 ANTIBODY PREPARATION AND DETECTION

2.4.2.1 PREPARATION OF EMULSION FOR IMMUNISATION

Acrylamide-supported protein bands identified as being of tubal origin were cut (Figure 2.2) and minced into small pieces and placed into a 3 ml Luer-lock syringe containing 1.5 ml water. A small piece of tubing was then used to connect this syringe to a second Luer-lock syringe (without needles) and the gel was passed back and forth between the two syringes until it was broken into small, uniform pieces. The tubing was then removed and two 18-gauge needles were connected to the syringes. 1.5 ml of complete Freund's adjuvant (immunogen:adjuvant ratio = 1:1) was brought into one syringe and after the single needle was removed, the connecting-needle apparatus was securely fastened into the Luer-lock. The adjuvant was slowly drawn into the syringe and the syringes connected by tubing. The immunogen and adjuvant mixture were emulsified, as shown in Figure 2.3, by passing the mixture back and forth between the syringes until the sample was thick.

2.4.2.2 IMMUNISATION AND BLEEDING OF RABBITS

The emulsified mixture was injected into the layers of the skin of two shaved rabbits (1910-A and 1911-B) using a 22-gauge needle and enough emulsion was added to form small raised "pockets" in the skin. A total volume of approximately 0.5-1.0 ml was injected into 10-12 sites in each rabbit. After 3-4 weeks, the first boost injection (0.5-1 ml) was given subcutaneously.

Bleeding of the rabbits followed a standard practice. The rabbits were gently restrained and the ears were bled in a standard way and the blood was collected in glass tubes. The blood was allowed to clot for 2-4 hours at room temperature then centrifuged at 1000 g for 10 min, aliquoted and then frozen.

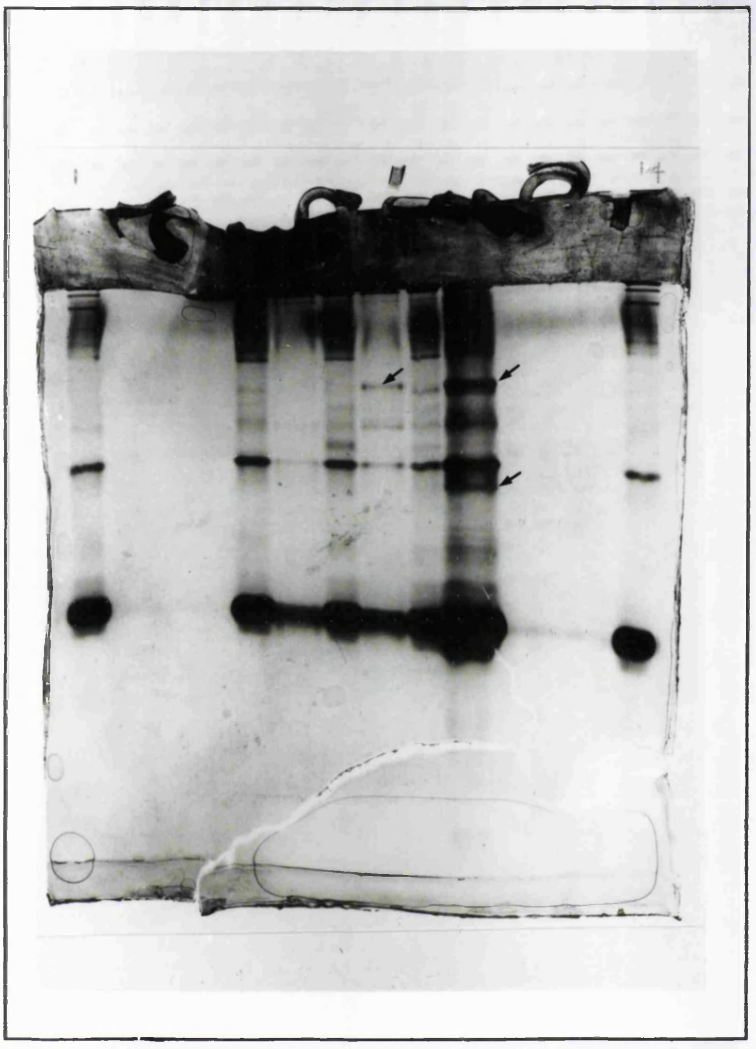


Figure 2.2 Protein bands selected to be cut and used for immunisation of Rabbits 1910-A and 1911-B.

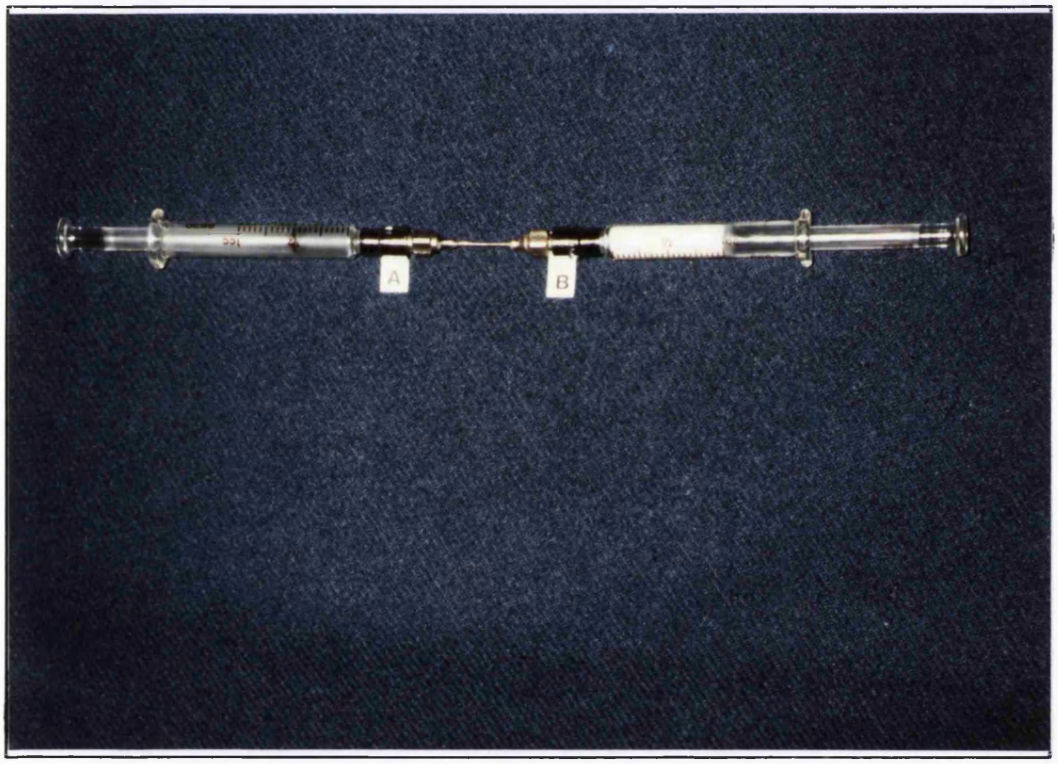


Figure 2.3 Syringe setup employed for emulsifying acrylamide pieces with adjuvant. (A) Luer-lock syringe. (B) 18-gauge needle.

2.4.3 IMMUNOPRECIPITATION METHODS

2.4.3.1 OUCHTERLONY DOUBLE-DIFFUSION METHOD

This method was first described by Ouchterlony in 1949 and is a commonly used form of immunoprecipitation. 1% Agarose plates were prepared in a standard way and wells were punched using the telescopic well punchers. 5μl of rabbits 1910-A and 1911-B antisera were placed in the central wells of rows 1 and 2 of rosettes formations. The central wells of the third row of rosette formations were filled with 5μl of "controlled" human antiserum. The surrounding wells were filled with 5μl of male and female sera and tubal fluid. After filling all wells, the plate was placed in the Humidity Chamber and left for diffusion at room temperature for 24 hours. The plate was washed and stained with Coomassie Blue R-250 according to standard procedure.

2.4.3.2 IMMUNOELECTROPHORESIS ACCORDING TO GRABAR AND WILLIAMS

Immunoelectrophoresis according to Grabar and Williams (1953) is a classical qualitative technique which is often referred to as "immunoelectrophoresis". It was carried out in two steps; (1) the antigen solution was placed in a well in the agarose gel and separated electrophoretically for one hour at 10V/cm and 4°C using LKB Multiphor Basic Unit and Power supply, and (2) after electrophoresis, troughs were cut out of the agarose gel parallel to the direction of migration and were then filled with 100 μl of rabbits 1910-A and 1911-B antisera obtained 8 weeks after immunisation of the rabbits. The plate was placed in the Humidity Chamber at room temperature for approximately 15 hours and then was pressed, dried and stained with Coomassie Blue R-250 before being photographed.

CHAPTER 3
OESTROGEN AND PROGESTERONE RECEPTORS IN THE FALLOPIAN TUBE AND UTERUS

3.1 INTRODUCTION

Oestrogens elicit their biological responses in target tissues by means of high affinity specific binding proteins, called oestrogen receptors (oestrophilin, ER) (Gorski et al, 1968; Jensen et al, 1968). The current concept of how oestrogens initiate target cell response stipulates that the nuclear receptor protein is present in at least two forms; the unoccupied form is thought to be bound to nuclear components by low affinity interactions. Oestrogens are lipophilic and therefore can diffuse through cell membranes, cytoplasm, and nuclear envelope to interact with the nuclear receptor. This interaction leads to rapid changes in the conformation of the receptor protein and as a result, this occupied form becomes tightly associated with nuclear chromatin. Oestrogen binding to its receptor causes increased rates of transcription of a variety of genes, depending on the respective target cell (Gorski et al, 1986). The importance of ER and progesterone receptor (PR) presence for endometrial and Fallopian tube structure and function has been well documented previously (Verhage and Jaffe, 1986; Hyde et al, 1989).

Although biochemical assays have been used for years to measure ER levels, they do not take into account the tissue heterogeneity which is demonstrated by the histological appearance of the sample. Measurement of ER and PR in cytosol extracts thus does not permit the identification of receptors in particular cell types. With the availability of specific monoclonal antibodies to human ER prepared against human breast cancer (Greene et al, 1980) specific and very sensitive methods for immunocytochemical assay (ICA) of ER (Greene and Jensen, 1982) and PR (Greene and Press, 1987; Press and Greene, 1988) have become feasible, thus permitting the identification of the anatomical location and content of receptors in individual cells *in situ*. Several studies have measured either or both receptors in the female genital tract. Using ICAs, both receptors have been identified in the human Fallopian tube (Press et al, 1986), the endometrium (Press et al, 1984, 1988; Lessey et al, 1988;

Garcia et al, 1988) and uterine corpus and cervix (Scharl et al, 1988). However, most of these studies did not accurately determine the stage of the menstrual cycle with particular reference to the circulating levels of serum oestradiol (E_2) or progesterone (P), did not correlate receptor variation with the associated ultrastructural changes of the respective sites, did not compare endometrial with Fallopian tube changes in the same patients, or a combination of these factors.

The aims of this study were;

(1) to determine the cyclical variations in endometrial and myometrial ER and PR receptors and

(2) to compare such variations to those in the different segments of the Fallopian tube from the same patients.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Samples of uterine and tubal tissue were obtained from 10 patients scheduled for hysterectomy and salpingectomy. Preoperative blood samples were obtained from the patients and were assayed for serum E_2 (pmol/l), P (nmol/l), LH (IU/l) and follicle stimulating hormone (FSH, IU/l). Each serum sample was analysed in duplicate and in one batch. The assay characteristics were described in section 2.2.4. Details of the patients' menstrual history, date of the last menstrual period, fertility and any medication were recorded (Table 3.1). Dating of the stage of the menstrual cycle was based on the date of the last menstrual period, endometrial histological dating using the method of Noyes et al (1950), and the levels of serum E_2, P, LH and FSH.

After resection, hysterectomy specimens were immediately transported to the histopathology department where they were examined and dissected by one pathologist (JC) and the principal investigator. Uterine samples were taken from the mid-section of the uterine cavity and included both endometrium and underlying

myometrium. The left fallopian tube was dissected from the specimen and sections were taken from the isthmus, the middle section (corresponding to the ampullary-isthmic junction), the mid-ampullary area and the fimbriae. The tissues were quickly mounted on cork plates, frozen in liquid nitrogen and then stored at -70° C until assayed.

3.2.2 MONOCLONAL ANTIBODIES

These are described in section 2.2.1

3.2.3 TISSUE SECTIONS PREPARATION AND FIXATION

This is described in section 2.2.2

3.2.4 IMMUNOSTAINING OF TISSUE SECTIONS

This is described in section 2.2.3

3.2.5 EVALUATION OF STAINING

The immunocytochemical staining for the receptors is localised in the nuclei of the cells treated with the primary antibody and the colour of staining was reddish-brown of varying intensities. Specific staining was defined as staining that was present with one of the monoclonal antibodies and absent in the negative control section (non-immune rat serum). The staining was evaluated semi-quantitatively using a double grading system. In the uterus, glandular epithelial cells, stroma and myometrium were examined, while in the Fallopian tube surface epithelial cells, stroma and smooth muscles were assessed in the different segments. The staining intensity was graded on a scale of: 0 = no stain, 1 = faint, 2 = moderate, 3 = strong, and 4 = very strong (maximum score). The percentage of positively stained cells was graded on a scale of 1 = 1-30%, 2 = 30-70%, and 3 = 70-100%. Where the staining intensity and percentage of stained cells varied from one area to another within the same specimen, an intermediate score of half a point was allocated to give a balanced

overview. A final score was obtained by multiplying the staining intensity grade by the percentage of positively stained cells. One observer (N.N.A.) who was blinded to the identity of the slides performed all the assessments. After completion of the study, 14 slides were re-examined by the same observer to ensure reproducibility of the semi-quantitative assessment.

3.3 RESULTS

3.3.1 CLINICAL AND HORMONAL CRITERIA OF SUBJECTS STUDIED

Table 3.1 summarizes the stage of the menstrual cycle, hormonal profile and clinical details of the patients who have been included in this study.

In Patient 6 no endometrial sample was obtained and in Patient 3 it was not possible to examine endometrial glandular tissue due to the poor orientation of the sample. In two patients, no isthmic samples were available due to previous surgery. A total of 93 sections were stained with MOAB (ER-ICA=47, PgR-ICA=46) and evaluated for cyclical changes in epithelial, stromal and muscular tissue. Positive staining was observed only in cell nuclei of both ciliated and non ciliated cells and none was seen in the control sections. The ER and PR staining scores of the different cell types (epithelial, stromal and smooth muscle) during the follicular and luteal phases of the menstrual cycle are depicted as bar graphs in Figures 3.1-3.3. Figure 3.4 depicts a negative control and Figures 3.5-3.14 show positive immunostaining for ER and PR in different sections at the different stages of the menstrual cycle.

3.3.2 ER STAINING DURING THE MENSTRUAL CYCLE

3.3.2.1 Uterine endometrium/myometrium

Glandular epithelial cells (Figure 3.1). There was a gradual increase in the staining score for ER in the early and mid follicular phase to a peak score just before ovulation (Figure 3.5) and then a decline to a nadir in the late luteal phase (Figure 3.13). In the early menopause strong staining for ER was observed, particularly in the basal layer and in the patient on hormone replacement therapy (HRT). Glandular

epithelium in the basal layer of the endometrium in general stained more intensely than that in the functional layer.

Endometrial stroma (Figure 3.2). The staining for ER was initially similar to that of the glandular epithelium (moderate) in the early and mid follicular phase. It then showed a slower and less marked increase than in the epithelium, plateaued near the time of ovulation and declining in the late luteal phase (Figure 3.13). In the early menopausal women, the intensity of ER staining was stronger in the patient who was not on HRT.

Myometrium (Figure 3.3). The intensity of ER staining was moderate throughout the cycle except in the late follicular phase when it was very strong. In the menopausal women, whether on HRT or not, the intensity of staining was moderate.

3.3.2.2 The Fallopian tube

Epithelial surface (Figure 3.1). The medial end of the tube (isthmic and mid sections) demonstrated a moderate degree of staining during the follicular and early luteal phases (Figure 3.7), decreasing to faint staining in the late luteal phase (Figure 3.9). In the mid-ampullary section, however, the low level of staining for ER in the early/mid follicular phase increased to moderate staining at mid-cycle and then declined again in the late luteal phase. Staining in the fimbrial end appeared to follow a different pattern. Apart from the first patient, in whom low levels of staining were observed, the other samples demonstrated strong staining in the early/mid follicular phase gradually declining to faint staining at around the time of ovulation. It then increased to moderate staining in the luteal phase. While in the endometrium the intensity of staining was highest in the late follicular phase, no such intense staining was noticed in any section of the Fallopian tube.

Endosalpingeal stromal staining (Figure 3.2). The intensity of staining in the medial half of the Fallopian tube appeared to be moderate during the follicular and early luteal phase in most samples. It then declined to faint staining in the late luteal phase. In the mid ampullary section, an increase in the intensity of staining was observed

from faint in the early follicular phase to moderate at mid-cycle and back to faint in the late luteal phase. These stromal changes were similar to those in the epithelial cells in the same section of the tube.

Muscle layer (Figure 3.3). This was more difficult to evaluate as not all sections showed enough muscle fibres for assessment especially in the fimbrial specimens. An increase in staining at mid-cycle (moderate) was noted in some sections. Within the same specimen, no regional variation in staining was observed and when the MOAB binding was present, all muscle fibres expressed the same intensity of binding.

3.3.3 PR STAINING DURING THE MENSTRUAL CYCLE

3.3.3.1 Uterine endometrium/myometrium

Glandular epithelial cells (Figure 3.1). The intensity of staining for PR was stronger in the early follicular phase compared with that for ER and increased during the follicular phase to reach a maximum immediately before ovulation (Figure 3.6). It then declined during the luteal phase, initially disappearing from the superficial layer but remaining in the basal layer (Figures 3.11 and 3.12) during the mid-luteal phase, then completely disappearing from the glandular epithelial cells in the late luteal phase (Figure 3.14). Progesterone receptor staining was very strong in the early menopause and also in the patient receiving HRT (before the onset of the progesterone component).

Stromal cells (Figure 3.2). Staining was judged to be strong or very strong throughout the menstrual cycle including the late luteal phase (Figure 3.14). Staining was moderate in the menopausal patients whether on HRT or not.

Myometrium (Figure 3.3). Contrary to the slight fluctuations seen in ER, the intensity of PR staining was strong throughout the cycle and similar findings were noticed in menopausal women whether on HRT or not.

3.3.3.2 The Fallopian Tube

Epithelial surface (Figure 3.1). The staining intensity for PR along the length of the Fallopian tube was strong or very strong during most of the cycle (Figure 3.8) until the late luteal phase when it decreased slightly (Figure 3.10). An increased degree of patchiness in staining was noticeable at the fimbrial end during all stages of the cycle. Staining for PR was faint or moderate, in the early menopause in comparison with the endometrium but increased markedly in the patient who was receiving HRT. In the woman not receiving HRT, PR staining in the tubal epithelium was generally faint and similar to the pattern of ER staining in the corresponding patient. In both menopausal women the staining for ER and PR was greater in the endometrium than it was in the tube.

Endosalpingeal stroma (Figure 3.2). In the medial half of the tube, PR staining remained moderately strong until the late luteal phase and no particular staining pattern could be identified. However, in the distal tube, PR staining showed greater variability in the follicular phase and decreased rather more in the fimbriae in the late luteal phase than in more medial segments. At the menopause, faint or moderate staining was observed in all segments of the Fallopian tube, but was stronger in the patient on HRT.

Muscle (Figure 3.3). This generally stained strongly until very late in the luteal phase (moderate staining) and with very slight variation along the different segments of the tube. The uterine muscle fibres stained more strongly than those of the tube in the late luteal phase, and remained uniformly stained rather than showing any patchy distribution.

Histological, hormonal and clinical details of the ten patients undergoing operation

Patient	CDC	HDC	Hormonal Profile LH	FSH	E2	P	Age, parity, clinical details and operative findings
Early Proliferative							
1	6/28	Prolif	(data not available)				44 P2+0 Addison's disease on steroids, leiomyomas. Normal pelvis
2	8/28	Prolif	5.0	2.8	297	0.4	42 P2+0 Old endometriosis, adenomyosis. Normal pelvis
Late Proliferative							
3	13/28	Prolif	4.6	1.3	202	4.0	44 P0+0 Not sexually active. Menorrhagia. Leiomyomas and simple right ovarian cyst. Other pelvic organs are normal
4	13/21	Prolif	4.8	2.8	459	6.4	42 P2+0 Old endometriosis. Normal pelvis
Peri-ovulatory							
5	11/28	Prolif	32	6.6	443	6.3	31 P2+1 History of dysmenorrhoea. Adenomyosis Small uterine fibroids and 3 cm left ovarian follicle
Early/Mid Luteal							
6	15/24-28	Secr	0.9	0.6	214	13.8	40 P2+0 Leiomyomas and left corpus luteum. Normal pelvis
7	18/28	Secr	8.0	3.4	392	16.6	35 P2+0 Menorrhagia. Minimal endometriosis seen at laparotomy, normal pelvis otherwise. Right corpus luteum
Late Luteal							
8	23/26	Secr	0.5	0.4	56	3.5	40 P3+1 Leiomyoma, adenomyosis and right corpus luteum
Peri-menopausal							
9	N.A.	Inactive	>range		22	4.3	56 P3+1 Six months postmenopausal, leiomyomas. Normal pelvis
10	17/28	Prolif	39	25	329	0.7	50 P3+1 Menopausal for 2 years, on Prempak-C 0.625. Bilateral benign ovarian teratomas. Normal pelvis

CDC=chronological day of cycle, HDC=histological day of cycle, N.A.=not applicable, Prolif=proliferative phase endometrium, Secr=secretory phase endometrium

Table 3.1

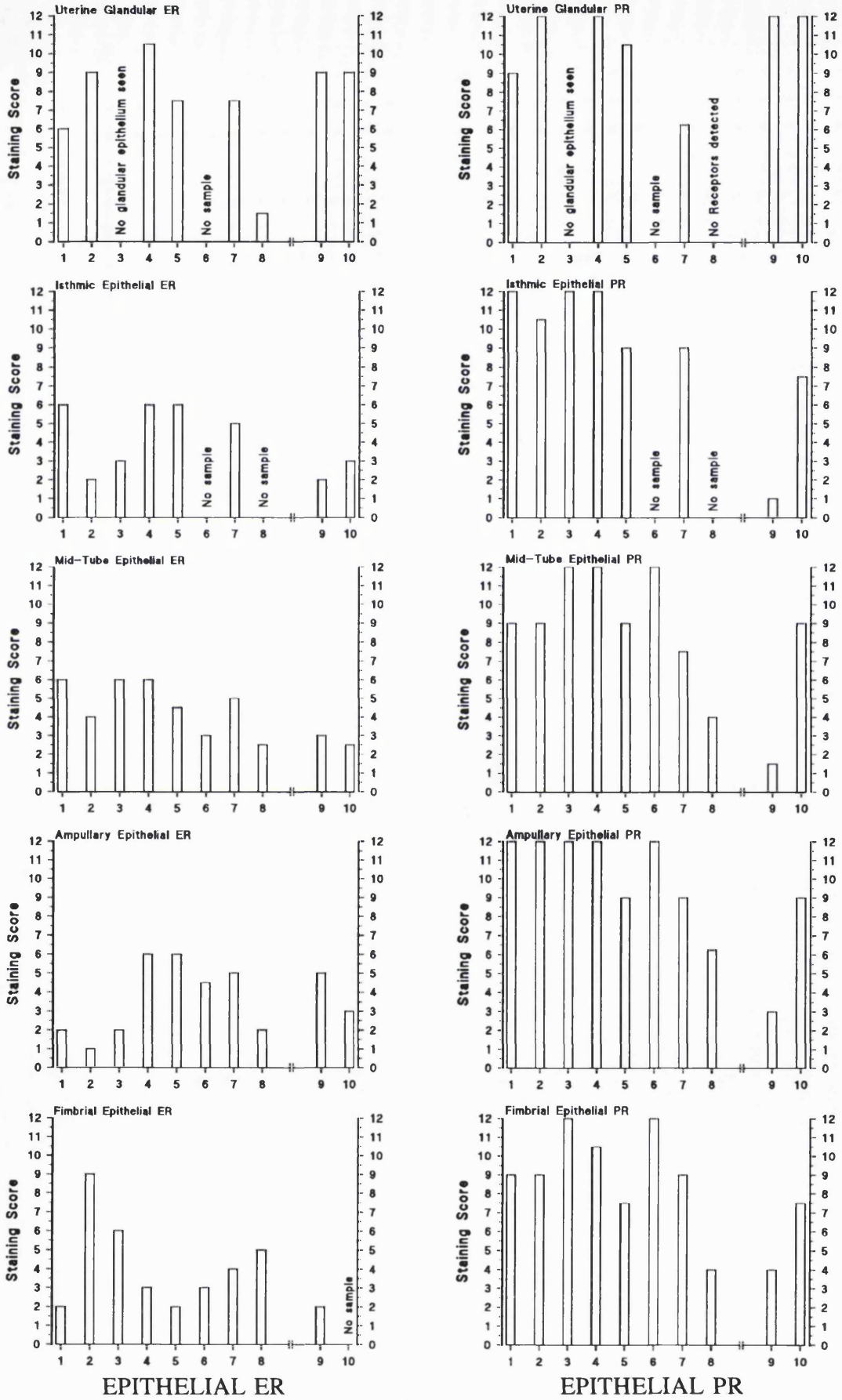


Fig. 3.1 Bar diagram depicting changes in ER and PR staining score in the endometrial and tubal epithelia during the menstrual cycle and the menopause.

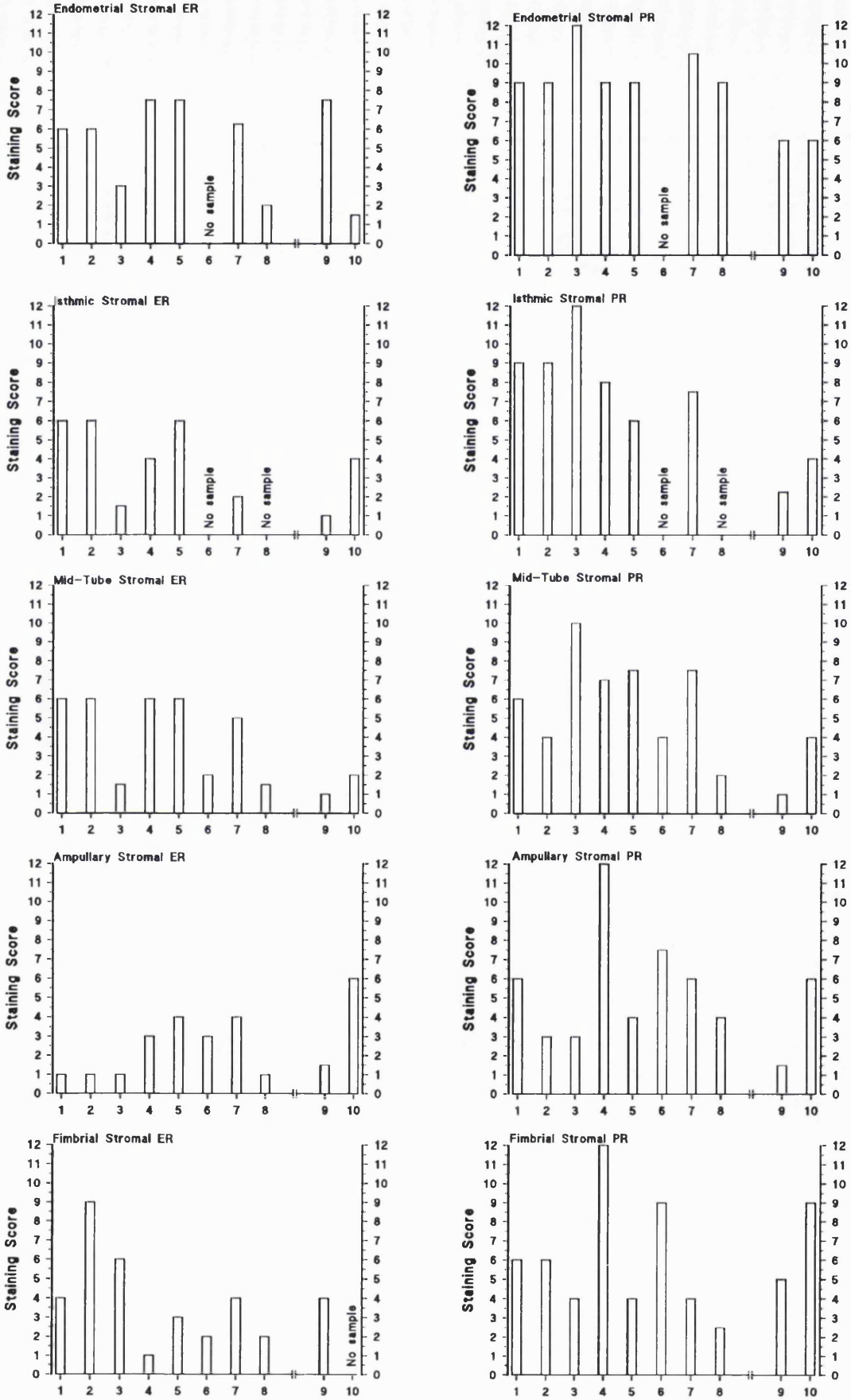


Fig. 3.2 Bar diagram showing changes in ER and PR staining score in the stroma of the endometrium and endosalpinx during the menstrual cycle and the menopause.

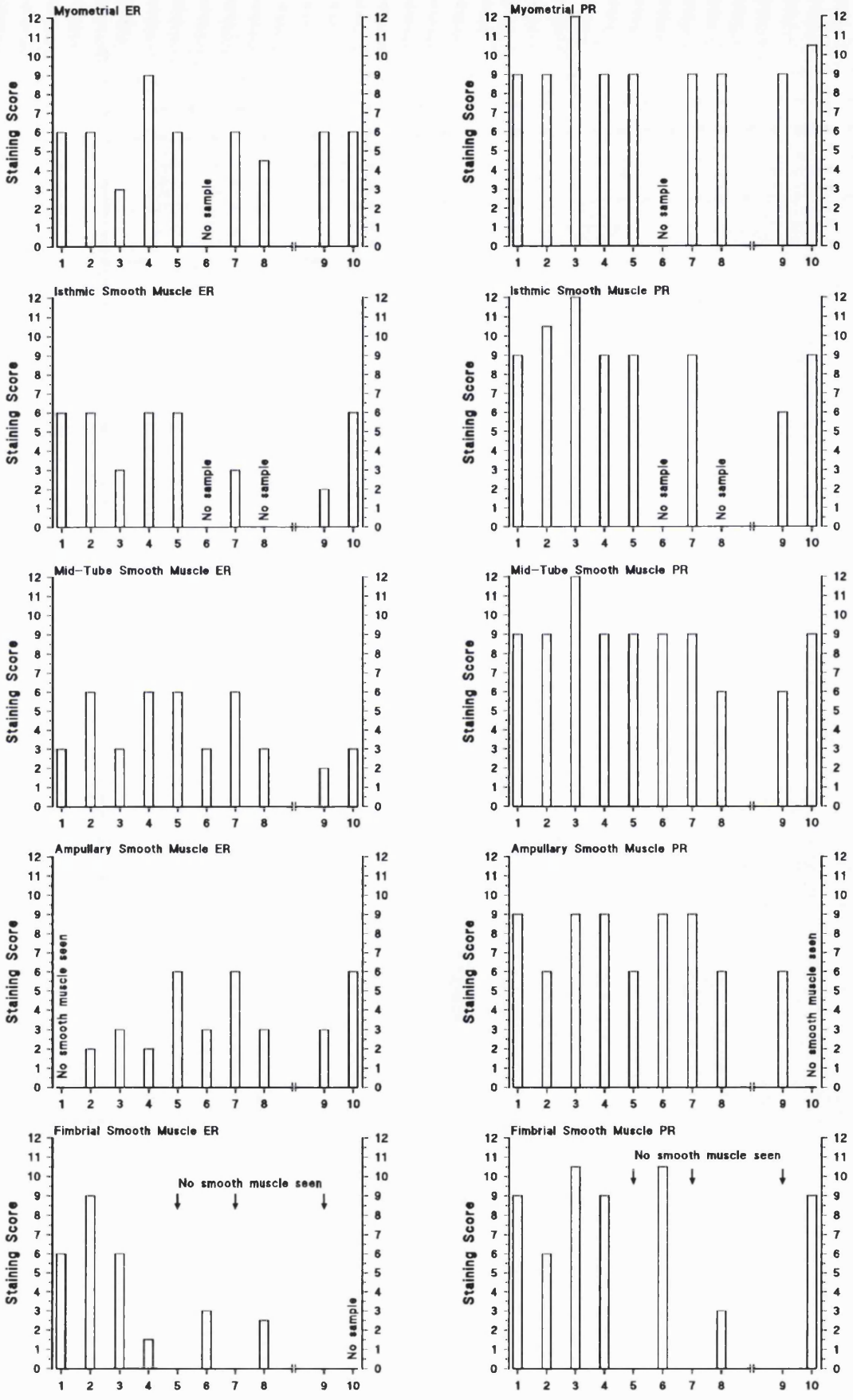


Fig. 3.3 Bar diagram showing changes in ER and PR staining score in the myometrium and tubal smooth muscle during the menstrual cycle and the menopause.

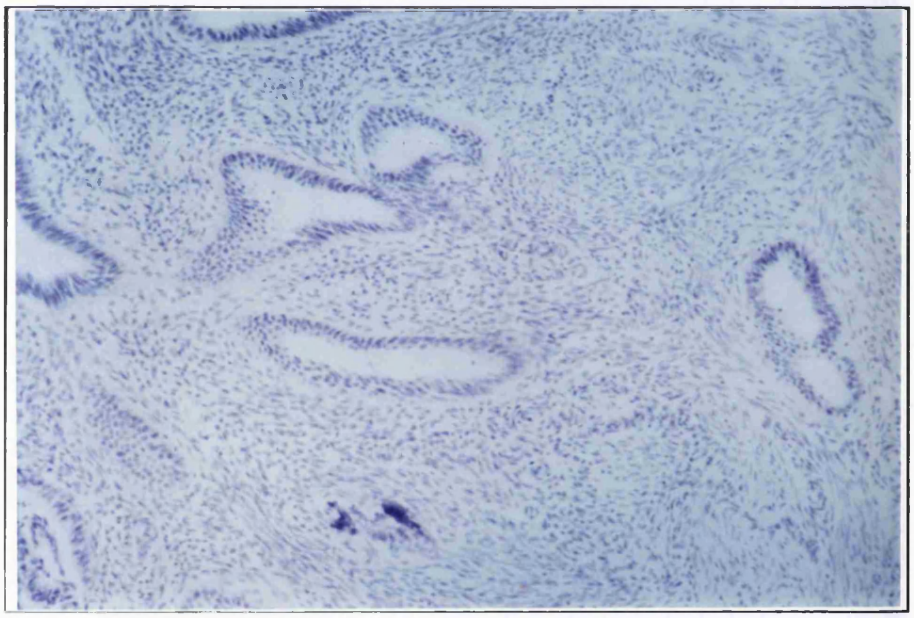


Fig. 3.4 A negative control section of the endometrium stained with normal non-immune rat serum in place of monoclonal antireceptor antibody. No brown pigmentation is seen (X150).

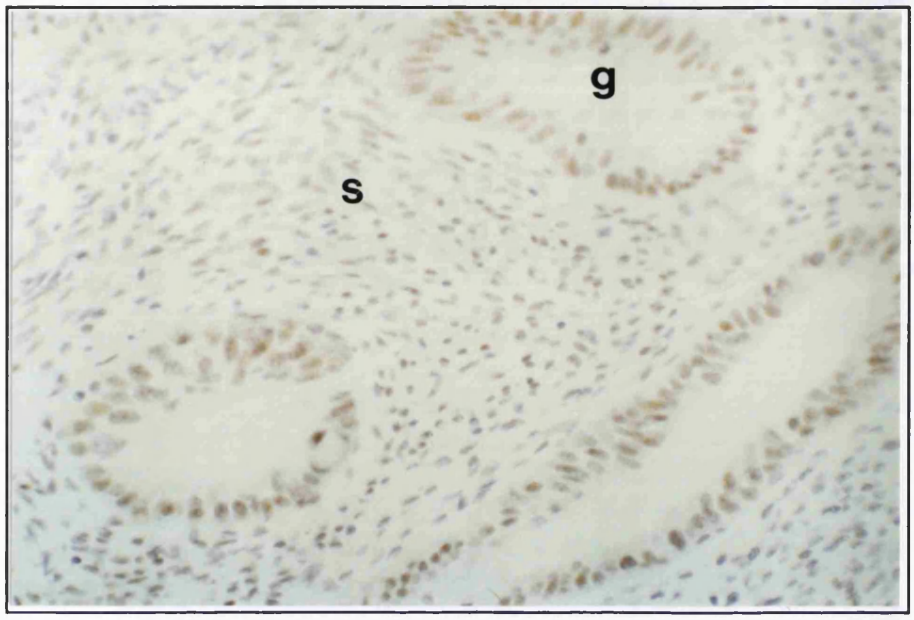


Fig. 3.5 Periovulatory phase endometrium immunostaining for ER: moderate to strong nuclear staining of the endometrial glands (g) and stromal cells (s) (X350).

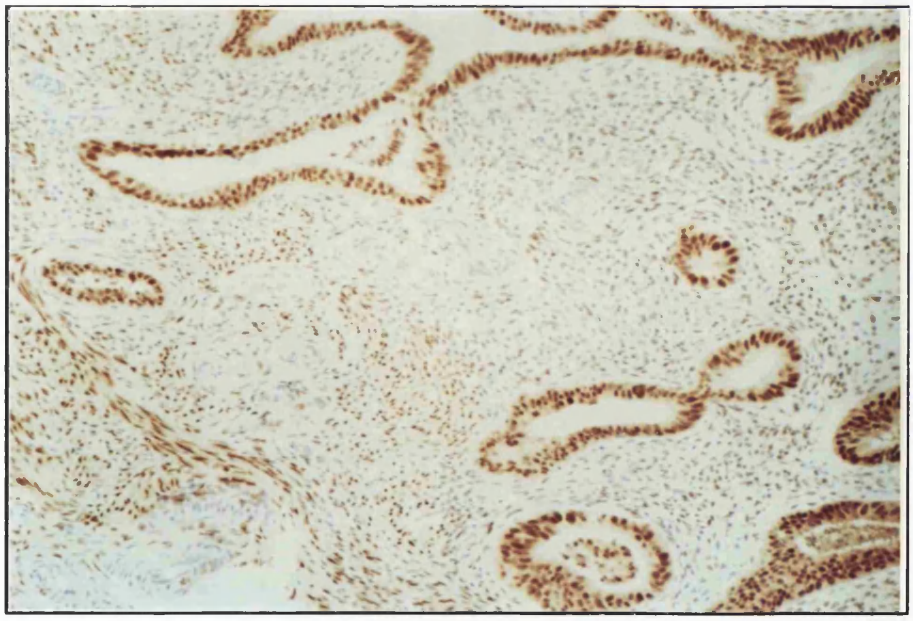


Fig. 3.6 Periovulatory phase endometrium immunostaining for PR: strong nuclear staining of the endometrial glands and stromal cells (X150).

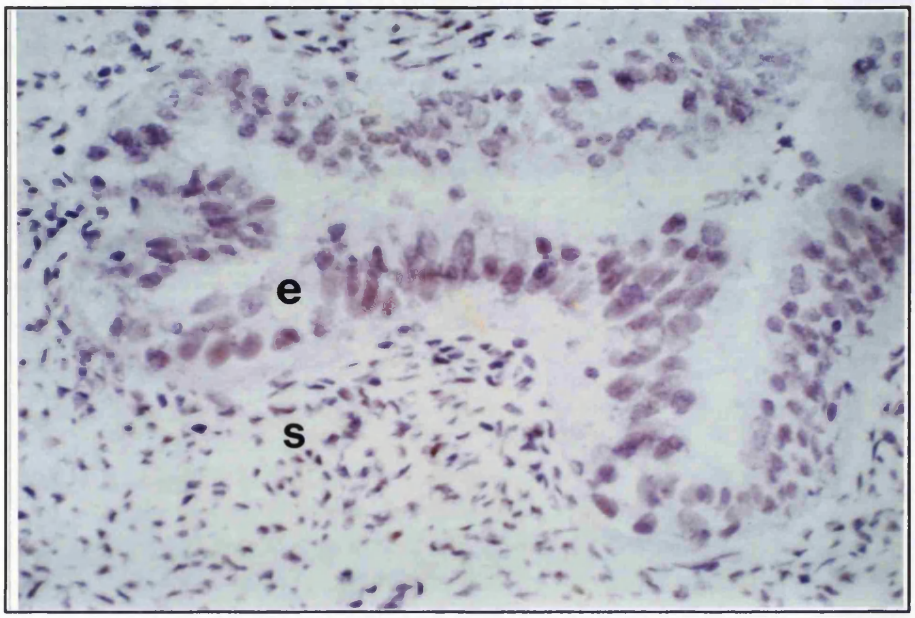


Fig. 3.7 Moderate immunostaining of the tubal isthmic epithelium (e) and stroma (s) for ER during the late proliferative phase (X350).

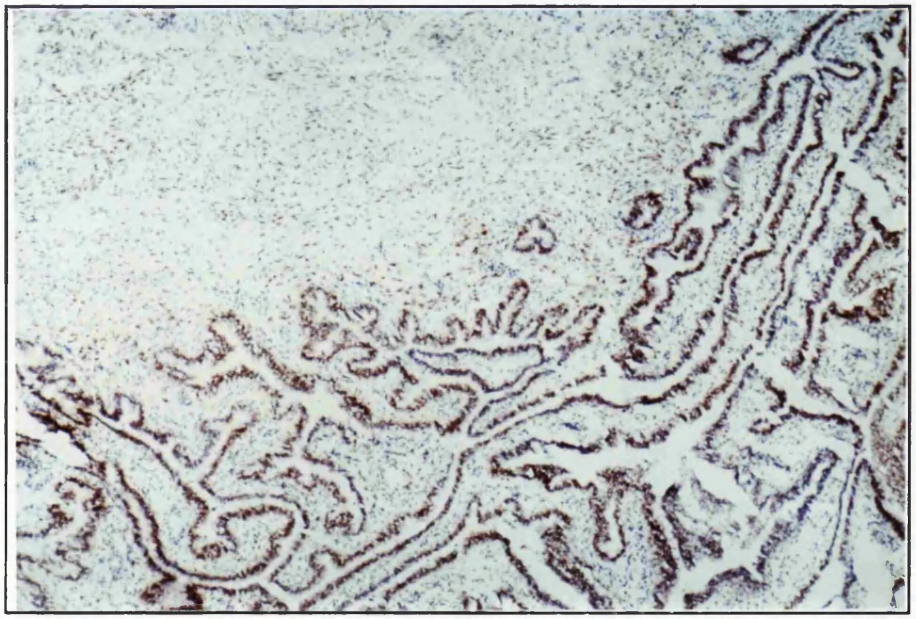


Fig. 3.8 Very strong immunostaining for PR of the epithelial and stromal cells of the mid tube during the late proliferative phase (X55).

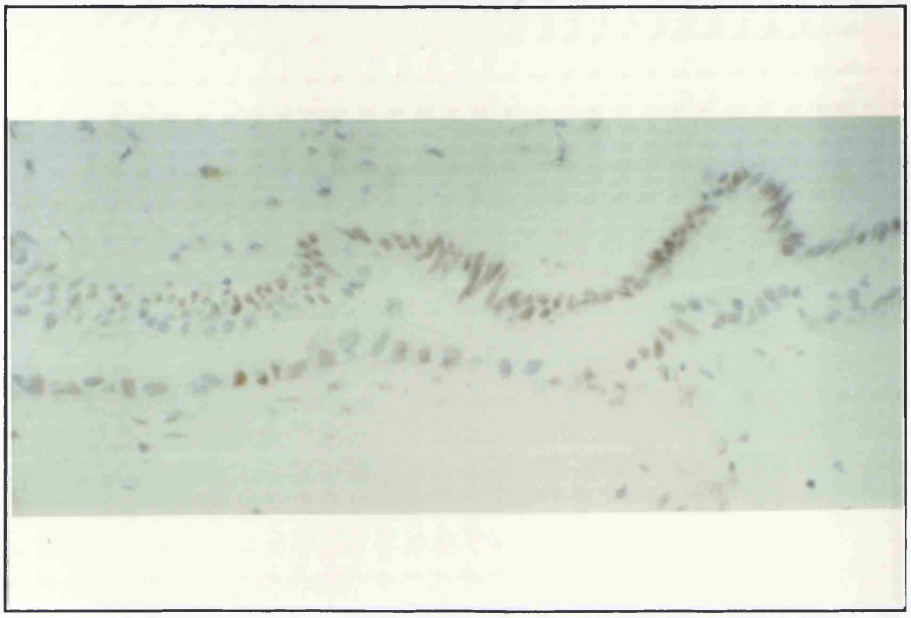


Fig. 3.9 An example of faint immunostaining of the mid tube epithelial surface for ER during the late luteal phase. The staining intensity is variable and patchy in distribution (X350).

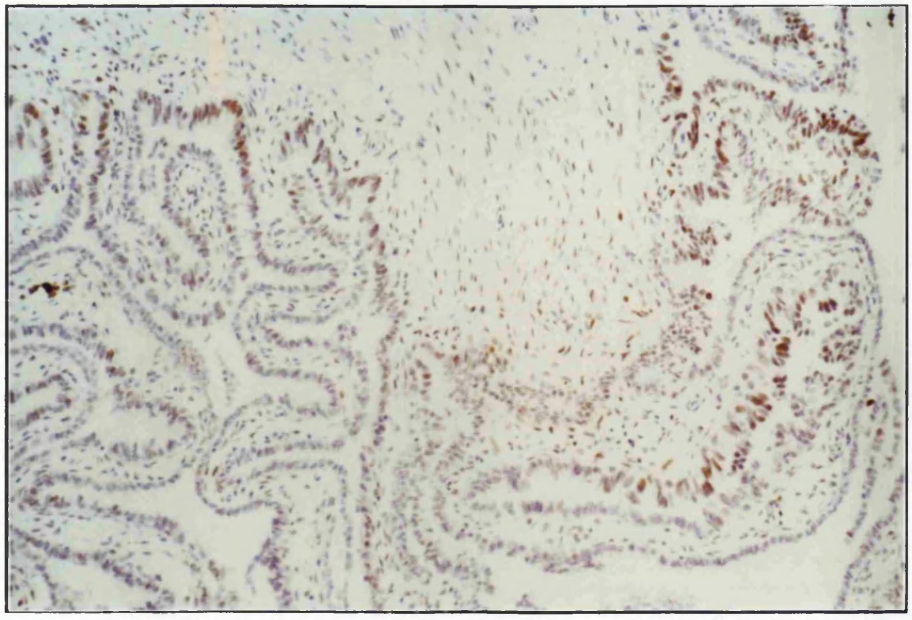


Fig. 3.10 Immunostaining of ampullary epithelial cells for PR during the late luteal phase. Staining intensity ranges from moderate to strong and is also patchy in distribution (X150).

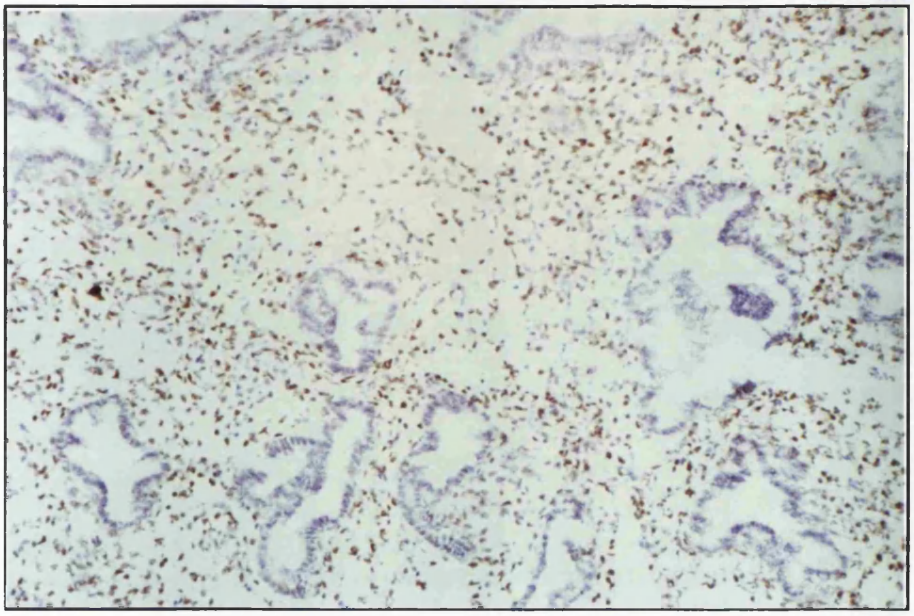


Fig. 3.11 Epithelial and stromal immunostaining for PR in the superficial layer of the endometrium during the mid luteal phase (X150).

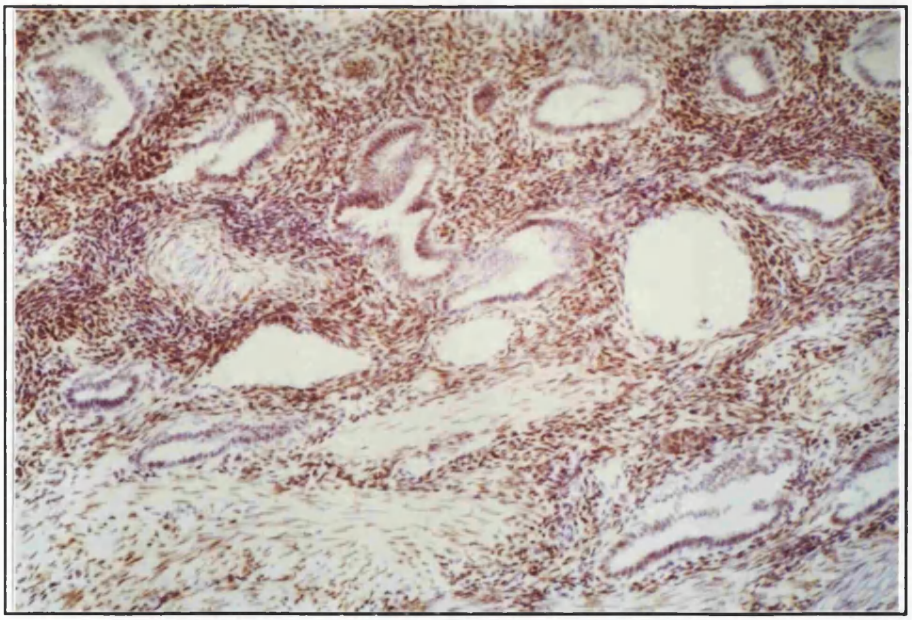


Fig. 3.12 Epithelial and stromal immunostaining for PR in the basal layer of the endometrium during the mid luteal phase. Note the stronger intensity of staining in this layer (X150).

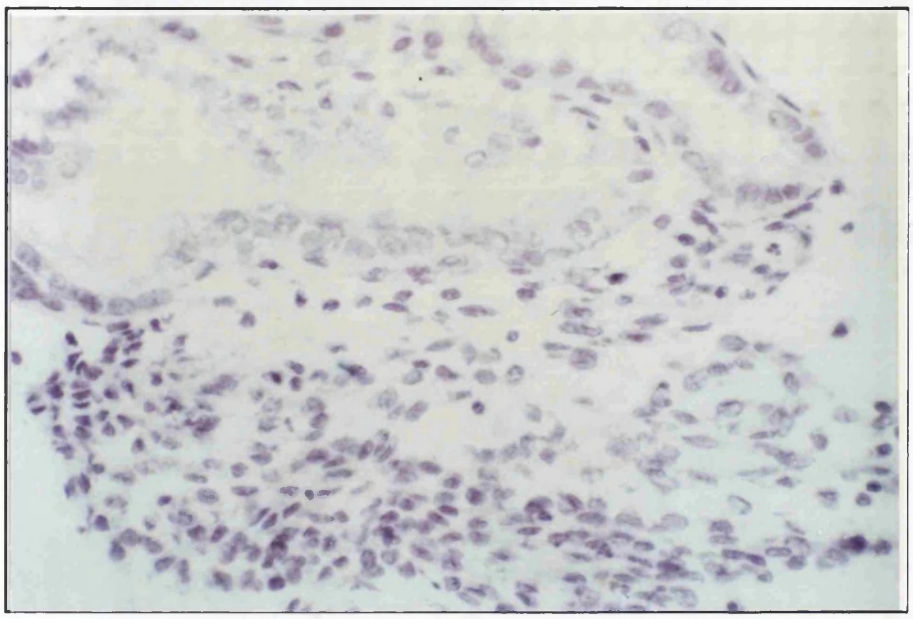


Fig. 3.13 Minimal ER staining of endometrial epithelial and stromal cells in the late luteal phase (X350).

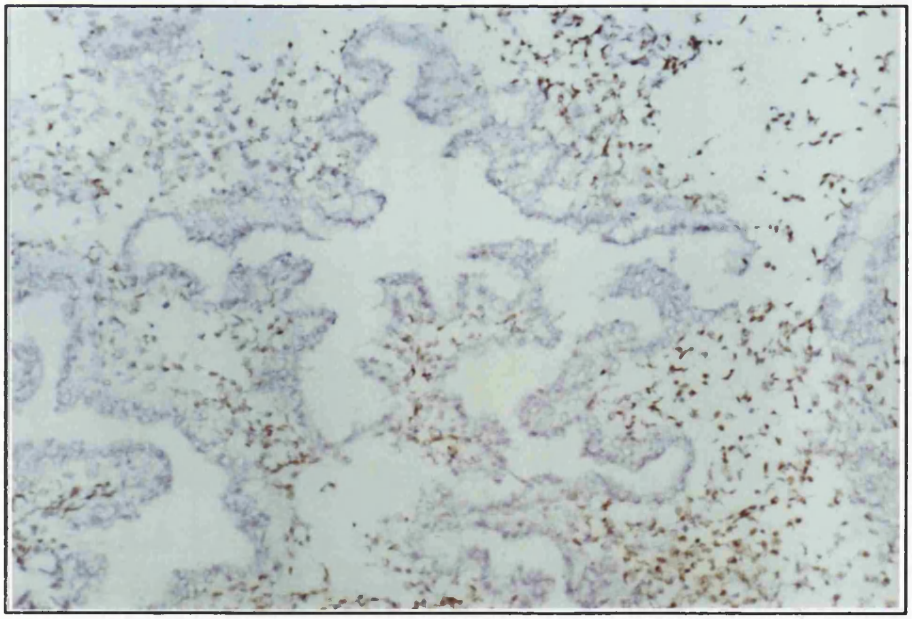


Fig. 3.14 Late luteal phase endometrium. Immunostaining for PR showing strong nuclear staining in the stromal cells but no staining in the glandular epithelial cells (X150).

3.4 DISCUSSION

Several advantages are obtained by using immunostaining with monoclonal antibodies for the receptor proteins rather than biochemical techniques for steroid binding activity. The receptors are known to be very labile and quantitative ER and PR analysis with biochemical assays requires rigorous technical quality controls for interlaboratory reproducibility and complicates interpretation of quantitative receptor results in national and international clinical trials. By contrast, ER and PR antigenic sites have been shown to be more stable than steroid binding activity, and thus immunoassays are reproducible, simpler to perform, and applicable to small biopsies and curettage specimens (DeSombre et al, 1984). These assays measure ER and PR molecules whether or not they are bound to endogenous hormone (Greene and Press, 1987; Greene et al, 1988), and enable assessment of receptor heterogeneity among the cells or regions within a tissue whereas binding assays measure an average receptor level in cell homogenate. It is recognised, however, that ICA is a semiquantitative method and thus prone to inter-observer variation, and that a study of the histological sample of one small area may not necessarily correlate well with the whole uterus or Fallopian tube.

In this study, positive staining was observed only in cell nuclei. This is consistent with other reports in which similar antibodies and staining procedure (PAP) were used (Press et al, 1984, 1986, 1988; Lessey et al, 1988; Garcia et al, 1988). Ideally, two independent observers should have examined the sections and the inter-observer variations estimated accordingly. The endometrial immunostaining for ER and PR detected in our patients is consistent with findings reported by others (Garcia et al, 1988; Lessey et al, 1988). However, the PR immunostaining was strong in both glandular epithelium and stroma in our mid-follicular samples contrary to the findings of Garcia *et al* and more consistent with those reported by Lessey et al. It is possible that the use of a different MOAB, inaccurate histological dating or some other factors that determine the presence of PR receptors in different tissue may explain these discrepancies. Both ER and PR are known to be induced by oestrogens, and therefore the intense ER and PR staining during the mid-cycle period is very likely to be correlated with the increased oestradiol secretion at this time. However, in the

study by Garcia et al, the intensity of PR immunostaining did not correlate with circulating level of plasma oestradiol. Moreover, the intensity of ER immunostaining in endometrial stroma from our post-menopausal cases appeared stronger in the patient who was not receiving HRT. The decrease and even disappearance of ER and PR from endometrial glandular epithelial cells during the late luteal phase is probably due to the effects of progesterone. In macaques, plasma progesterone levels modulate endometrial ER concentrations (West and Brenner, 1985), and immunostaining for ER disappears when animals are treated with exogenous progesterone (McClellan et al, 1986; Press et al, 1986). However, the suggestion that PR is up-regulated by oestrogen and down-regulated by progesterone may be an over simplification. PR distribution as revealed by the Abbott MOAB showed complex patterns during the menstrual cycle and differential effects of oestrogen and progesterone on glandular epithelium and stroma, as have been previously reported (Kelly et al, 1978; McCormack and Glasser, 1980; King et al, 1981; West et al, 1986; Garcia et al, 1988; Lessey et al, 1988).

The ability to reproduce previously reported cyclical endometrial changes allows validation of the cyclical changes in the Fallopian tube against a well-documented parameter. Furthermore, as all samples from each patient were stained simultaneously, thus eliminating technical variations, it is possible to make direct comparisons between the various sections. Previous studies utilising traditional biochemical methods had reported greater cytosol ER and PR concentration in the human ampulla than in the isthmus (Robertson and Landgren, 1975; Flickinger et al, 1977; Pollow et al, 1981, 1982) and that the fimbrial end, or infundibulum had the lowest concentration of steroid receptors at all stages of the cycle. Punnonen and Lukola (1981) in a study on the binding of oestradiol and progesterone in the human Fallopian tube did not detect a significant difference in the nuclear and cytoplasmic receptor concentration between the three anatomical sections of the tube (isthmus, ampullary and fimbriae). The differences in the cytoplasmic ER concentration between the luteal and follicular phases were also not significant, although they were generally elevated in the follicular when compared with the luteal phase. The nuclear PR concentration in the isthmus and fimbriae were found to be significantly higher in the follicular than in the luteal phase. Similarly, Pino et al (1982) reported very

little variation in the cytosol ER concentration of the human isthmus, ampulla and fimbriae during the menstrual cycle, though it was higher at the periovulatory phase. However, the concentration of the nuclear ER varied markedly in the isthmus and ampulla and was significantly higher in the late proliferative than in the secretory phase. Others (Pollow et al, 1981), using a steroid binding method, compared the cytoplasmic and nuclear ER and PR in the human Fallopian tube and endometrium and concluded that there was similarity in the pattern of the cyclic fluctuations in the two organs. The total (cytoplasmic and nuclear) ER concentrations were generally higher in the endometrium at all stages of the cycle, while the total PR concentrations were higher in the Fallopian tube in the early follicular phase and in both organs the concentrations decreased towards the late luteal phase. West and Brenner (1983) also demonstrated that the amounts of nuclear and cytoplasmic ER in both the oviduct and endometrium were approximately twofold greater in the follicular phase than in the luteal phase. In a subsequent publication (West et al, 1986), the same group showed differential suppression of PR in the endometrium following Progesterone treatment.

Press et al (1986), using immunocytochemical techniques for the ER localisation in the female genital tract, found no variation in ER staining intensity in the different segments of the Fallopian tube with minimal variation during the menstrual cycle. However, it is not clear from that study whether any of the Fallopian tube segments was studied in a systematic way at the different phases of the menstrual cycle, nor was a comparison made with endometrial changes in the same subjects. To this investigator's knowledge, this is the first comparative ICA study between the different sections of the Fallopian tube and the endometrium in the same subjects. The findings are broadly similar, though using a different technique, to those reported previously (Pollow et al, 1981; West and Brenner, 1983). ER staining score was generally higher in the endometrial glandular epithelial cells than tubal (isthmic, mid-tube and ampullary) epithelium. Interestingly, the pattern for the fimbrial epithelium (Figure 3.1) appears to be a mirror image or out of phase to the other regions of the tube! The stromal ER scores in the endometrium and tube were very similar and showed a decline towards the late luteal phase in all samples studied (Figure 3.2). PR scores were generally high in both the endometrial glandular epithelium and tubal epithelium in the early and late follicular phase and declined in the luteal phase. This

decline in the endometrium is in agreement with similar observations reported previously (Punnonen and Lukola, 1981; Pollow et al, 1981; Lukola and Punnonen, 1983). However, as reported in this study, there was a differential control of PR in the two organs during the luteal phase. In the glandular epithelium PR disappeared completely in the late luteal phase while moderate staining persisted in the tubal sections studied (mid-tube, ampulla, fimbria) (Figure 3.1). This depletion of PR in glandular epithelium may parallel its declining (or perhaps even undesirable) functional importance during early pregnancy. Stroma, which becomes decidualized, would logically require receptors to support its further growth and development. This may have a functional role as recent evidence suggests that human endometrial stromal cells co-cultures or endometrial secretory proteins isolated from conditioned media derived from endometrial stromal cells, significantly enhance mouse embryo development by secreting specific proteins (Liu et al, 1992). The myometrium must retain its PR, since progesterone is believed to inhibit contractility of this tissue during pregnancy. The above results suggest that the functional response of the uterine tissue to oestrogen and progesterone may be determined in part by fluctuations of the level of hormone receptors in individual cell types. The high PR score in the early follicular phase as yet is unexplained but may be viewed as a consequence of the release of the system from antagonism by Progesterone. Similar high nuclear PR concentration using biochemical methods has been reported previously (Pollow et al, 1981).

It is interesting to note that in the menopausal woman not receiving HRT, the ER and PR score was relatively high in the glandular endometrial epithelium and stroma but not in the tube. There is no explanation for this, but a similar observation was reported previously (Punnonen and Lukola, 1981). More recently, ER but not PR receptors were detected by ICA in Fallopian tubes removed at surgery for ectopic pregnancy (Land and Arends, 1992). This is in contradiction to previous observations of high cytoplasmic PR and very low ER concentrations along the length of the tube in a case of tubal ectopic pregnancy (Punnonen and Lukola, 1981).

Differences in receptor content within the same cell type in the same section (patchy distribution) has been reported previously (Perrot-Applanat et al, 1985; Hyde et al,

1989; Cano et al, 1990). This may be either a permanent difference reflecting a pattern of differentiation or a temporary event reflecting differences in receptor content according to the stage of the cell cycle. Differences between cell types (epithelial, stromal, muscular) within a target tissue and regional differences in receptor content support the concept of microenvironment within the upper genital tract, and may explain morphological and ultrastructural variations as well as secretory activity within these areas.

Any study of physiological receptor changes would require normal subjects. However, the difficulties in recruitment of normal individuals are well recognised as they are unlikely to require hysterectomy, though those undergoing sterilisation with salpingectomy are suitable for inclusion in such study. Additionally, timing of surgery may not always be possible to coincide with the desired stage of the menstrual cycle. Inclusion of larger number of patients into the study may have overcome this problem. In some cases, endocrinological findings did not coincide with the date of the cycle or the histological findings and this may question the ER and PR findings. Furthermore, precise endocrine assays and frequent sampling may assist to define the stage of menstrual cycle accurately. In case number 4, the raised progesterone in the late follicular phase may indicate the beginning of the LH surge which is not reflected in the histology due to a time-lag between endocrine parameters, the response of the receptors, and the histological picture. Similarly, in case number 6 the low FSH and LH in the early/mid luteal phase may not reflect the true values due to their pulsatile release and that more than one sample should have been obtained. In case number 9, the P level was above the menopausal range although other endocrine parameters and endometrial histology were indicative of menopausal status.

In women with luteal phase inadequacy endometrial ER and PR disturbances have been noted (Levy et al, 1980; Saracoglu et al, 1985). Additionally, the administration of drugs to induce ovulation has been reported to have an adverse effect on endometrial maturation consequent on profound modifications of endometrial receptor dynamics (Forman et al, 1989), and may interfere with endometrial responsiveness for implantation. The observed difference in the intensity

of ER staining between the endometrium and the Fallopian tube and their variation at different phases of the cycle may be of relevance.

In animal studies, the hormonal milieu of the oviduct had been reported to influence embryonic development. Adams (1973) demonstrated that when rabbit 4-cell embryos were transferred to ligated oviducts, they developed best when the endocrine stimulation of the oviduct was in the early luteal phase. Similar studies in ovariectomized rabbits (Stone and Hamner, 1977) demonstrated abnormal embryonic development under oestrogen supplementation but not when the embryos were transferred during the progesterone phase of sequential regimen of oestrogen followed by progesterone. These reports suggest that the oestrogenic phase may have a negative effect on embryonic development within the oviduct. Thus the lower oestrogen receptor content of tubal epithelium seen in natural cycles may have important implications if also seen in stimulated cycles. The supra-physiological levels of oestrogen and progesterone encountered under these conditions may influence the endometrial environment adversely to a greater extent, due to its higher ER content, than the Fallopian tube during the early and mid luteal phase. The tubal environment may therefore be more appropriate for early embryonic development than the uterine milieu and this may be reflected in differences in pregnancy or implantation rates between GIFT/TET and IVF-uterine embryo transfer. Reports which suggested greatly enhanced pregnancy and implantation rates following tubal gamete or embryo replacements were reviewed recently (Amso and Shaw, 1993), however, the evidence remains inconclusive and further studies are needed to verify these reports.

CHAPTER 4

MORPHOLOGY AND ULTRASTRUCTURE OF ENDOMETRIAL GLAND AND FALLOPIAN TUBE EPITHELIA AT DIFFERENT STAGES OF THE MENSTRUAL CYCLE AND MENOPAUSE

4.1 INTRODUCTION

The Fallopian tube has an important role in reproduction and its different sections appear specialised to perform different functions. The isthmus is capable of effecting the transport of spermatozoa and ova in opposite directions. The ampulla is believed to be the site where fertilisation normally takes place, and the fimbrial fronds are instrumental in ovum pick-up at the time of ovulation. Changes in E and P levels are known to affect endometrial and tubal function, ultrastructure, and smooth muscle motility. These effects have been observed during the menstrual cycle (Patek et al, 1972a; Ferenczy et al, 1972a,b), after the menopause (Patek et al, 1972b), following hormonal therapy (Fredricsson and Bjorkman, 1973) and during pregnancy and the puerperium (Patek et al, 1973a,b).

Cyclical ultrastructural changes in the endometrium at different stages of the menstrual cycle have been extensively studied (Ferenczy et al, 1972a; Ferenczy and Richart, 1973; Ferenczy, 1977; Sundstrom and Ove Nilsson, 1982; Dallenbach-Hellweg, 1987) and luteal phase changes as measured from the LH surge have been accurately described (Dockery et al, 1988a,b; Dockery and Rogers, 1989; Li et al, 1990a; Kim-Bjorklend et al, 1991). More recently, endometrial ultrastructural changes in spontaneous cycles, luteal phase deficiency (Li et al, 1990b) and gonadotrophin stimulated cycles have been compared with a view to assessing their impact on fertility and the success of assisted reproduction (Martel et al, 1987).

Similarly, cyclical ultrastructural changes in tubal epithelia have been studied in animals (Jansen and Bajpai, 1982, 1983) and humans (Bjorkman and Fredricson, 1962; Ferenczy et al, 1972b; Patek et al, 1972a,b; Jansen, 1980, 1984; Crow et al, 1994). The lining epithelium of the Fallopian tube is known to show significant morphological variations in response to changes in E and P levels (Donnez et al,

1985), and it has been suggested that ultrastructural changes which have been observed during the phases of the menstrual cycle are functionally important (Clyman, 1966). However, some studies of tubal morphology have looked only at one segment of the tube (Clyman, 1966; Brosens and Vasquez, 1976; Fadel et al, 1976; Jansen, 1980; Lindenbaum et al, 1983), others have been performed on animal oviducts (Jansen and Bajpai, 1982, 1983; Odor et al, 1983; Oliphant et al, 1984b) which may not be relevant to human reproduction, and some have been restricted either to light microscopy (Donnez et al, 1985) or to scanning electron microscopy (Patek et al, 1972a; Ferenczy et al, 1972b; Fadel et al, 1976; Jansen, 1980; Bonilla-Musoles et al, 1983).

Although each organ has been studied separately in some detail, there are no reports in the literature comparing the various changes in the two organs in the same subjects under the same hormonal conditions. Several factors may be responsible for this. Firstly, there are recognised difficulties in obtaining specimens for such work. Essentially, the women should have proven their fertility, and be undergoing abdominal hysterectomy and salpingectomy for reasons that do not adversely affect tubal or uterine function in any significant way. Secondly, most studies have used samples obtained at endometrial curettage, tubal sterilisation or salpingectomy. Thirdly, until recently there has been no link between endometrial and tubal physiology that was considered to be of clinical relevance.

Morphometric analysis of endometrial or tubal changes would have been superior to the subjective methods used in this thesis. A number of indices are usually chosen for quantitative assessment such as: (1) the number of transversely sectioned endometrial glands per mm^2, the diameter of the glandular lumen, gland cell height; (2) the number of vacuolated cells; (3) the secretion of the endometrial glands or within the tubal lumen; (4) tubal epithelial cell height; (5) volume fraction of nucleus to cell or various other cytoplasmic events such as mitochondria, membrane-bound secretory apparatus, and rough endoplasmic reticulum. Morphometric procedures can be carried out on semi-thin sections with light microscopy or ultra-thin sections with electron microscopy (Dockery et al, 1991; Kim-Bjorklund et al, 1991). Such techniques, though time consuming and require special equipment, allow objective

and reproducible comparisons and have proved to be invaluable in assessing endometrial maturity (Dockery et al, 1988a). Such facilities were not available at the Royal Free Hospital during the period that these ultrastructural studies were carried out. The results in this chapter should be interpreted with caution as they are descriptive and reflect subjective but not quantitative comparison between the different segments of the Fallopian tube or between the tube and the endometrium.

In the clinical management of the infertile couple, the successful introduction of GIFT and subsequently ZIFT/TET and their reported higher pregnancy/implantation rates compared to conventional IVF-UET has generated considerable interest in studying the role of Fallopian tube in assisted reproduction. In this chapter, the first study (section 4(I).3 Results-I) documented in detail the combined morphological and ultrastructural features of the epithelial lining along the length of healthy Fallopian tubes from human females at different stages of the menstrual cycle. Comparisons were made with the post-menopausal state with or without HRT. This provided a systematic basis of knowledge for understanding the role of the various segments of the tube. The aim of the second study (section 4(II).5 Results-II) was to compare the morphological and ultrastructural changes of the endometrial glandular epithelium to those of the tubal epithelium at different stages of the menstrual cycle in the same subjects, with a view to identifying similarities and differences which may inform the debate on the different techniques of assisted reproduction.

4.2 PATIENTS AND METHODS

4.2.1 PATIENTS

Nine women undergoing hysterectomy with salpingectomy were included in the study. In all cases the pelvis appeared normal at operation and there was no evidence of active endometriosis, pelvic inflammatory disease or adhesions. Accurate dating of the cycle phase was based on the date of the last menstrual period, the hormonal profile and the endometrial histology using the method of Noyes et al, (1950). Preoperative blood samples were obtained from the patients and were assayed in duplicate and in one batch for serum E_2, P, LH and FSH as described previously (section 2.2.4). The clinical features and cycle data are shown in Table 4.1.

4.2.2 METHODS

This is described in sections 2.3, 2.3.1 and 2.3.2.

4(I).3 RESULTS-1: MORPHOLOGICAL AND ULTRASTRUCTURAL CHANGES ALONG THE LENGTH OF THE FALLOPIAN TUBE

4(I).3.1 General morphological features

By light microscopy the lining of the Fallopian tube was seen to be a single layer of cuboidal or columnar epithelium which often appeared pseudostratified due to crowding and bunching of cells of different heights (Figures 4(I).1-4(I).5). A varying proportion of the cells showed ciliation of the luminal surface (Figure 4(I).6). The nuclei were oval or elongated with a rather open chromatin pattern and sometimes a solitary nucleolus.

A few mononuclear cells with small hyperchromatic dense or slightly "clockface" nuclei were always present within the basal epithelium and in the underlying stroma. These had the appearance of lymphoid cells (Figures 4(I).1-4(I).5).

In H+E sections the luminal surface of the tubal non-ciliated cells (NCC) was difficult to visualise. It appeared pale, wispy and basophilic suggesting the production of a mucinous secretion from the surface of the cells (Figures 4(I).1 and 4(I).2). However, examination of the one micron toluidine blue stained sections using high-magnification light microscopy clearly showed that this appearance was produced by the presence of cytoplasmic apical projections or domes at the luminal surface (Figure 4(I).6). In many sections there were some tubal epithelial cells apparently in the process of being extruded from the epithelium into the lumen (Figure 4(I).2) and in some sections, thin and dark "intercalated" cells were present (Figure 4(I).3).

Some H+E sections showed vacuolation of cytoplasm of the tubal epithelium. Comparison with the adjacent TB sections showed that this usually corresponded to accumulations of glycogen particles and occasionally to lipid droplets.

4(I).3.2 General ultrastructural features

The epithelial cells of the Fallopian tube were seen to be of two types, some having surface cilia, ciliated cells (CC) and others which were non-ciliated (Figure 4(I).7). The NCC had apical protrusions or domes of variable height projecting into the lumen whereas the CC had a relatively flat luminal surface. Occasional cells had both domes and cilia (Figure 4(I).13), and the nuclei and other cytoplasmic features of the two types appeared very similar including the presence of numerous microvilli at the luminal surface. These were seen between the cilia of the CC and over the surface of the domes in the NCC (Figure 4(I).7). A variety of cytoplasmic organelles were noted including mitochondria, free ribosomes and profiles of rough endoplasmic reticulum, Golgi areas, lysosomal and lipid bodies. Of particular interest in terms of possible secretory activity of the epithelial cells were the presence in some cases of variable numbers of dense granules (Figures 4(I).11 and 4(I).12) and particulate glycogen (Figure 4(I).14).

"Intercalated" or "peg" cells were not identified as a specific cell type but were part of the variable appearance of the NCC, some of which appeared quite narrow and some of which did not reach the luminal surface in the plane of section (Figure 4(I).7b). The scattered lymphoid cells observed by light microscopy were also identified at ultrastructural level.

4(I).3.3 Variations in appearance in different areas of the tube

The shape, size and degree of crowding of the tubal epithelial cells varied from section to section but no systematic changes along the length of the tube were identified. Similarly, although there was some variability in the exact shape, size and position of the nuclei within the epithelial cells, no systematic changes were observed in the nuclei of either CC or NCC from the different sections of the tube. The numbers of intra-epithelial lymphocytes varied slightly from section to section but showed no particular trend in relation to the site in the tube.

The major variation along the length of the tube was the proportion of CC, which

showed a systematic increase in number from the isthmus outwards, with the highest numbers in the sections from the fimbriae (Table 4.2). Glycogen was noted within the cytoplasm of some cells in many of the cases (Figure 4(I).14). It was generally more prominent in the CC than the NCC and hence more obvious in the outer reaches of the tube where CC were more common.

In the follicular phase, fragments of cytoplasmic and cellular material were seen in the lumen of the isthmic segments (Figure 4(I).8b) but not in the outer tubal segments. Similarly, the surface domes and dense granules were most prominent in the NCC of the mid-tube and ampulla and less developed in the fimbrial sections. In the fimbriae, cell surface activity, electron dense granules and intracytoplasmic glycogen become increasingly apparent towards the late luteal phase of the cycle.

4(I).3.4 Variations in appearance at different stages of the menstrual cycle

Pseudostratification. The degree of apparent multilayering of the tubal epithelium increased during the follicular phase (Figure 4(I).1) and was at its maximum just before ovulation (Figure 4(I).2). In contrast the luteal phase cases showed mainly single layered epithelium of a much more simple appearance (Figure 4(I).3). The post-menopausal case also showed a simple flat epithelium (Figure 4(I).4) but the endosalpinx of the HRT patient showed a multilayered appearance with cell bunching similar to that of the late follicular phase (Figure 4(I).5).

Nuclei. Within the natural variability of appearance, no systematic changes in the shape, size, or position of the nuclei of either CC or NCC were noted at different stages of the menstrual cycle.

Lymphocytes. No obvious variation in the number of intra-epithelial lymphocytes was detected at different stages of the cycle but in the post-menopausal case there were noticeably greater numbers of lymphocytes which were collected into small aggregates but did not form true lymphoid follicles (Figure 4(I).4).

Ciliation. No significant difference was found in the number of CC between the

follicular and luteal phases of the menstrual cycle, but there were markedly fewer CC in all sections of the post-menopausal tube (Table 4.2). In contrast, the number of CC in the sections of tube from the post-menopausal woman on HRT was very similar to the mean values of the pre-menopausal women.

Domes. The size and shape of the surface domes on the NCC varied markedly during the menstrual cycle and this appeared to be related to cell surface activity in general. In the post-menopausal state the domes were low or almost flat in shape and the microvilli were short and sparse. On scanning EM the CC were seen standing up prominently above the relatively flat surrounding surface (Figure 4(I).10). During the follicular phase the domes were more prominent and became more irregular in shape, appearing as finger-like protrusions on transmission EM (Figures 4(I).7 and 4(I).8). In the immediately pre-ovulatory phase this surface activity reached a peak with very irregular protrusions, some of which were higher than the adjacent cilia and appeared to be waisted at the base or even completely separated from the underlying cell. This release of surface cytoplasmic fragments was also accompanied by extrusion of larger cell fragments, sometimes including nuclei and greater numbers of whole cells (Figure 4(I).8). A similar picture to the late follicular phase was also seen in the HRT case. The flurry of surface activity around the time of ovulation was followed by relative quiescence at the cell surface in the early/mid luteal phase with reversion to a flat or slightly domed appearance and no decapitation secretion (Figure 4(I).9). In the late luteal phase protrusions were again becoming more prominent.

Secretory granules. Occasional dense granules were seen in the cytoplasm of both CC and NCC after the menopause and during the early follicular phase, but these were not orientated to the cell surface and it was difficult to tell if they were secretory granules or other dense bodies such as compound secondary lysosomes (Figure 4(I).13 and Figures 4(II).1b and 4(II).2b). In the late follicular phase, dense granules became more numerous and were lined up at the luminal cell surface, often within the surface protrusions (Figure 4(I).11). Although an occasional example of individual granule release was seen, more often the surface protrusions themselves appeared to be released into the tube lumen with the granules in them, as a form of

apocrine or decapitation secretion (Figure 4(I).8b). In the luteal phase the granules were no longer seen near the surface (Figure 4(II).5b). In contrast with the untreated post-menopausal case which showed little evidence of secretory activity (Figure 4(II).6b), the appearance in the HRT case strongly resembled that of the late follicular phase, with granules lined up in domes of apocrine secretion (Figure 4(I).12). Along the tube, the surface domes and dense granules were most prominent in the NCC of the mid-tube and ampulla and were less developed in the fimbrial sections.

Other organelles. No notable variation in the numbers of other cytoplasmic organelles was detected at different stages of the cycle, although lipid-containing compound lysosomes were noted to be particularly prominent in the late luteal phase and in the post-menopausal state (Figure 4(I).13). The post-menopausal endosalpingeal epithelial cells showed little evidence of surface secretory activity, while the cytoplasmic organelles still included ribosomes, Golgi areas and glycogen, indicative of continued cellular metabolic activity (Figures 4(I).13 and 4(I).14).

Histological, hormonal and clinical details of the nine patients undergoing operation

Patient	CDC	HDC	Hormonal Profile LH	FSH	E2	P	Age, parity, clinical details and operative findings
Early Proliferative							
1	6/28	Prolif	(data not available)				44 P2+0 Addison's disease on steroids, leiomyomas. Normal pelvis
2	8/28	Prolif	5.0	2.8	297	0.4	42 P2+0 Old endometriosis, adenomyosis. Normal pelvis
Late Proliferative							
3	13/28	Prolif	4.6	1.3	202	4.0	44 P0+0 Not sexually active. Menorrhagia. Leiomyomas and simple right ovarian cyst. Other pelvic organs are normal
4	13/21	Prolif	4.8	2.8	459	6.4	42 P2+0 Old endometriosis. Normal pelvis
Peri-ovulatory							
5	11/28	Prolif	32	6.6	443	6.3	31 P2+1 History of dysmenorrhoea. Adenomyosis Small uterine fibroids and 3 cm left ovarian follicle
Early/Mid Luteal							
6	18/28	Secr	8.0	3.4	392	16.6	35 P2+0 Menorrhagia. Minimal endometriosis seen at laparotomy, normal pelvis otherwise. Right corpus luteum
Late Luteal							
7	23/26	Secr	0.5	0.4	56	3.5	40 P3+1 Leiomyoma, adenomyosis and right corpus luteum
Peri-menopausal							
8	N.A.	Inactive	>range		22	4.3	56 P3+1 Six months postmenopausal, leiomyomas. Normal pelvis
9	17/28	Prolif	39	25	329	0.7	50 P3+1 Menopausal for 2 years, on Prempak-C 0.625. Bilateral benign ovarian teratomas. Normal pelvis

CDC=chronological day of cycle, HDC=histological day of cycle, N.A.=not applicable, Prolif=proliferative phase endometrium, Secr=secretory phase endometrium

Table 4.1

Table 4.2 Percentage of ciliated cells along the Fallopian tube

Case	Cycle phase	Isthmus	Mid-tube	Ampulla	Fimbriae
1	Follicular	35	34	50	26
2	Follicular	27	24	54	51
3	Follicular	45	52	51	65
4	Follicular	35	40	53	55
5	Follicular	34	39	45	60
Mean	Follicular	35.2	37.8	50.6	58.6
6	Luteal	27	34	45	58
7	Luteal	--	48	46	67
Mean	luteal	27.0	41.0	45.5	62.5
8	Post-menopausal	9	25	28	40
9	Post-menopausal +HRT	34	47	50	52

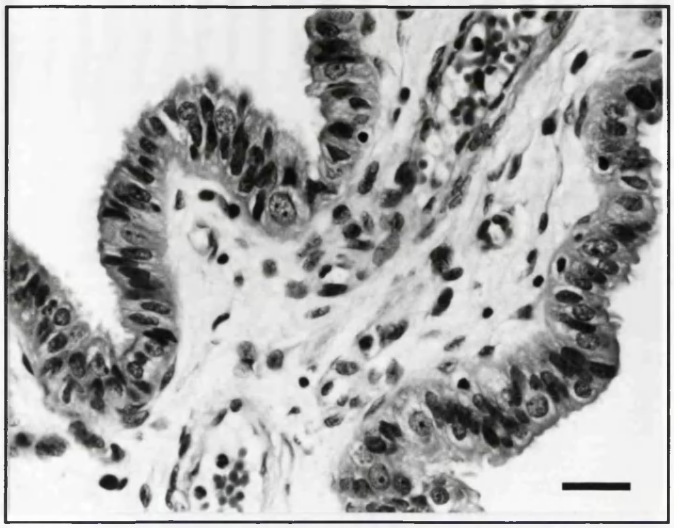


Fig. 4(I).1

Mid-follicular phase Fallopian tube epithelium showing pseudostratification of cells and "wispy" cell surface (bar=30μm).

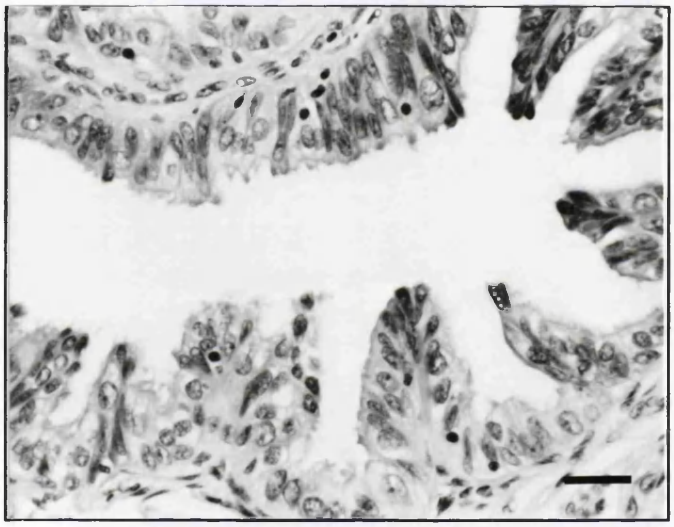


Fig. 4(I).2

Late follicular phase tubal epithelium showing marked pseudostratification due to cell crowding with extrusion of cell fragments from the luminal surface (bar=30μm).

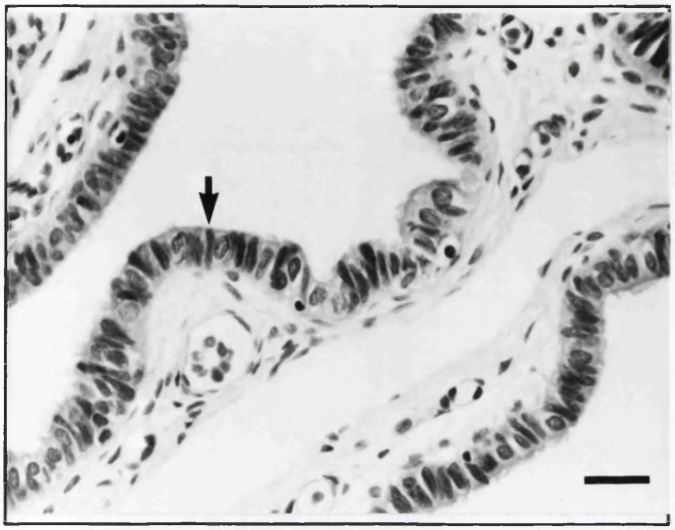


Fig. 4(I).3

Luteal phase tubal epithelium appearing flat and simplified with little surface activity. Thin dark intercalated cells are also seen (arrow) at this stage (bar=30 μm).

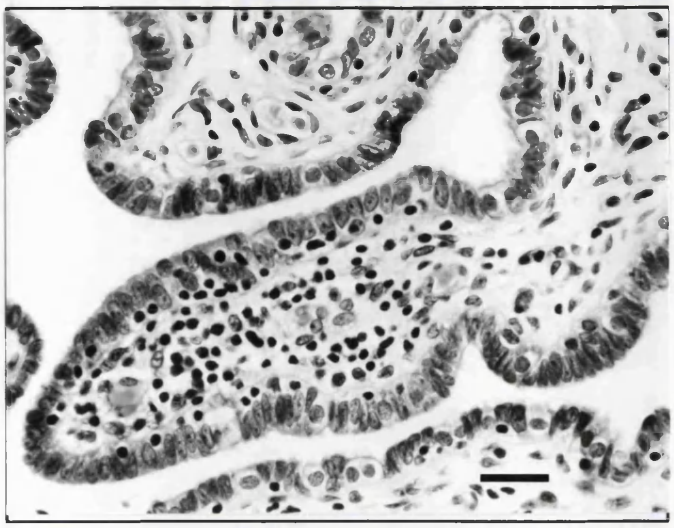


Fig. 4(I).4

Early menopausal endosalpinx showing a single epithelial layer with flat luminal surface and prominent collections of lymphoid cells in the stroma (bar=30μm).

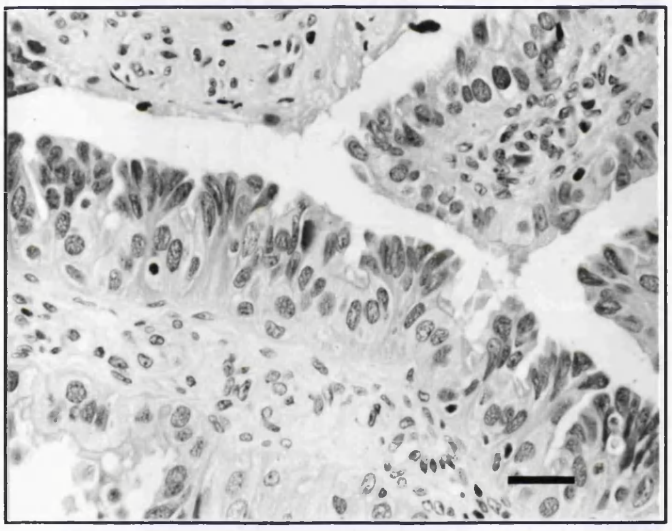


Fig. 4(I).5

Post-menopausal endosalpinx from a patient on HRT (oestrogen phase) showing prominent multilayering and cell surface activity comparable with the late follicular phase (bar=30μm).

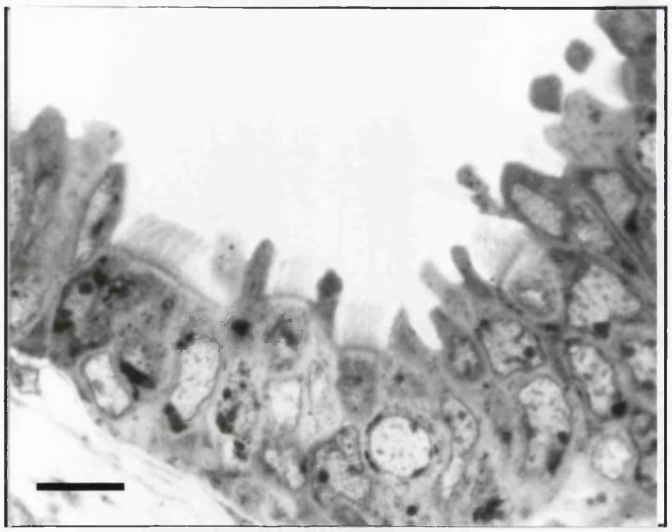


Fig. 4(I).6

One micron section from EM block showing CC with flat surface and cilia, and NCC with protruding luminal domes (bar=10μm).

Fig. 4(I).7a

Scanning EM in mid-follicular phase showing domed NCC covered with microvilli, and bundles of cilia on the surface of CC (bar=5μm).

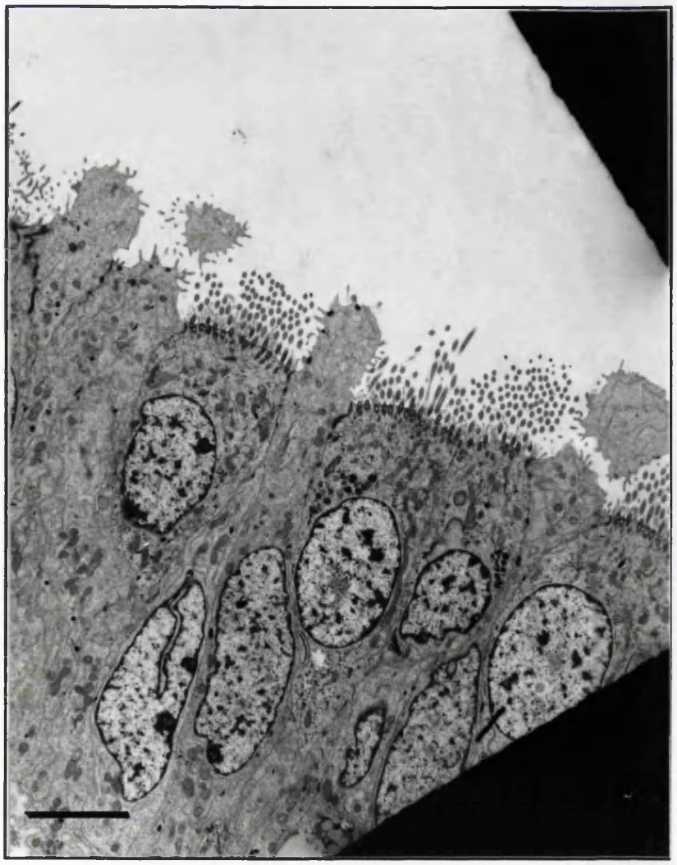


Fig. 4(I).7b

Transmission EM in mid-follicular phase showing microvilli on the surface of both CC and NCC. NCC domes are prominent and a few granules are lined up at the surface. Other cytoplasmic organelles are similar in both CC and NCC (bar=5μm).

Fig. 4(I).8a

Scanning EM in late follicular phase showing irregular shaped domes with secondary protrusions into the lumen dwarfing the adjacent cilia (bar=5μm).

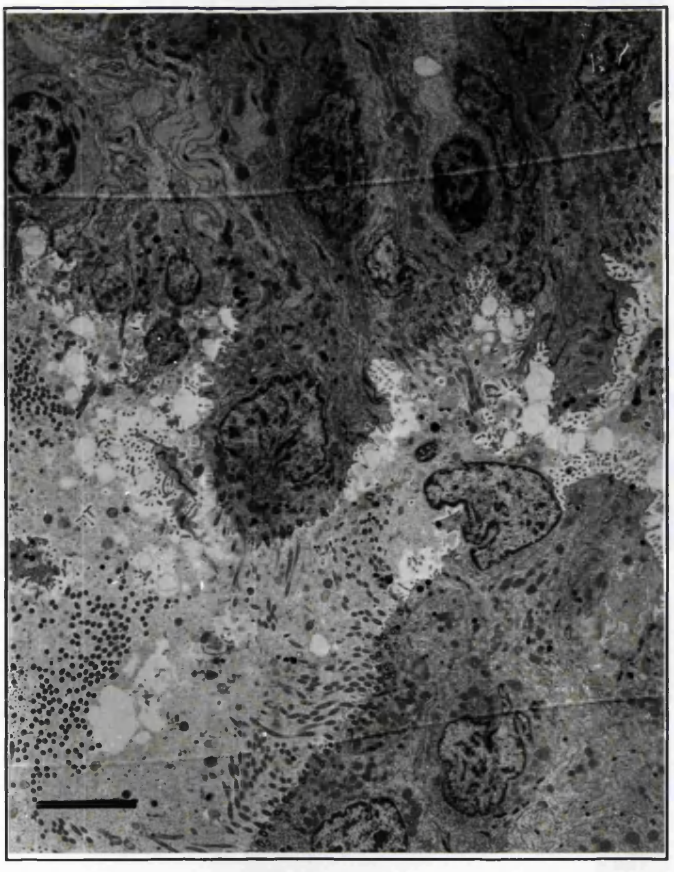


Fig. 4(I).8b

Transmission EM in the late follicular phase showing extrusion of cellular fragments from the surface and the presence of cytoplasmic debris in the lumen. Granules are seen near the cell surface and in cytoplasmic fragments within the lumen (bar=5μm).

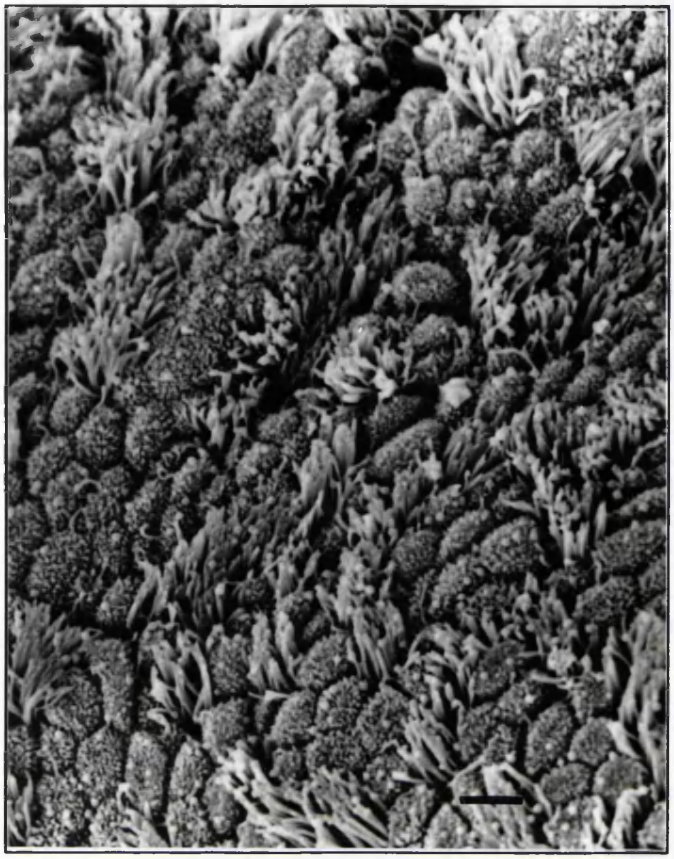


Fig. 4(I).9a

Mid-luteal phase Scanning EM showing relatively quiescent NCC surfaces with resulting greater prominence of adjacent cilia. Only a few cytoplasmic protrusions are seen (bar=5μm).

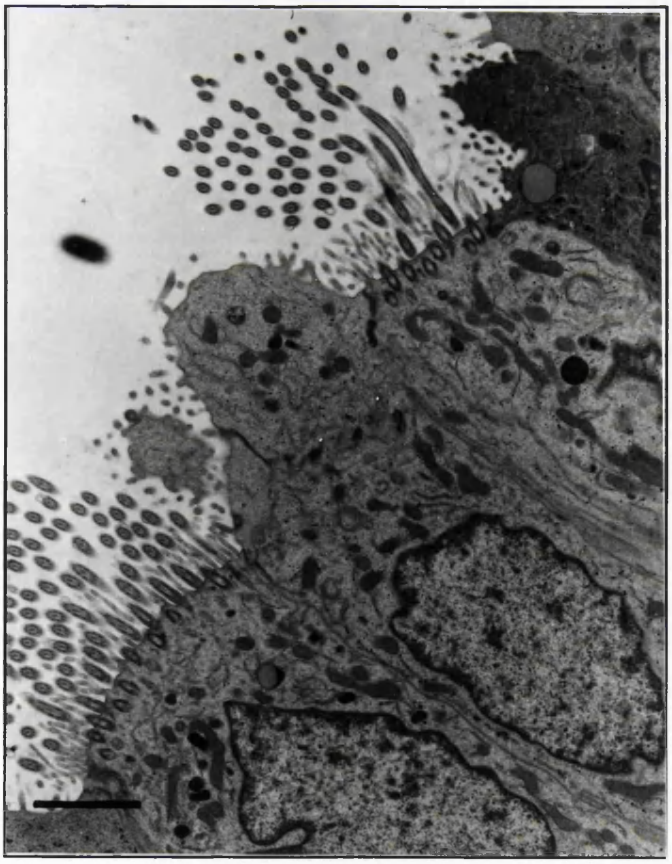


Fig. 4(I).9b

Mid-luteal Transmission EM showing mildly domed NCC with no surface granules (bar=2μm).

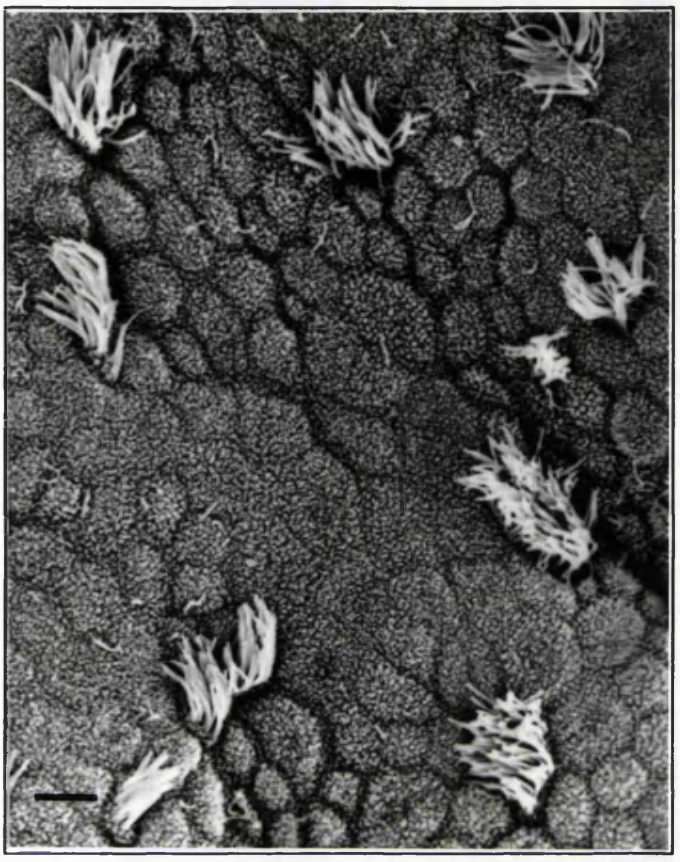


Fig. 4(I).10a

Scanning EM of post-menopausal endosalpinx showing flat NCC with no surface granules (bar=5μm).

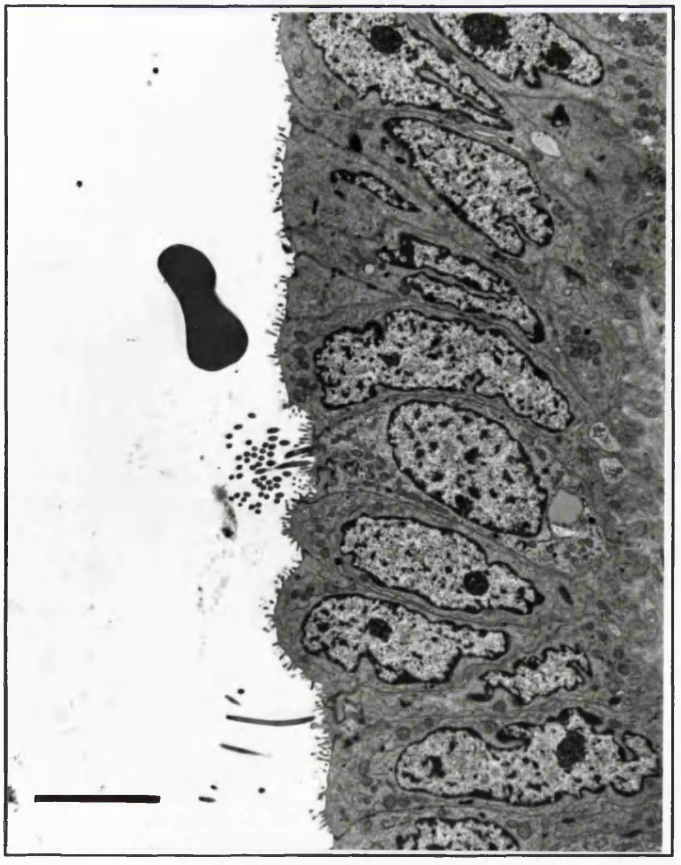


Fig. 4(I).10b

Transmission EM of post-menopausal endosalpinx showing relatively flat-surfaced NCC (bar=5μm).

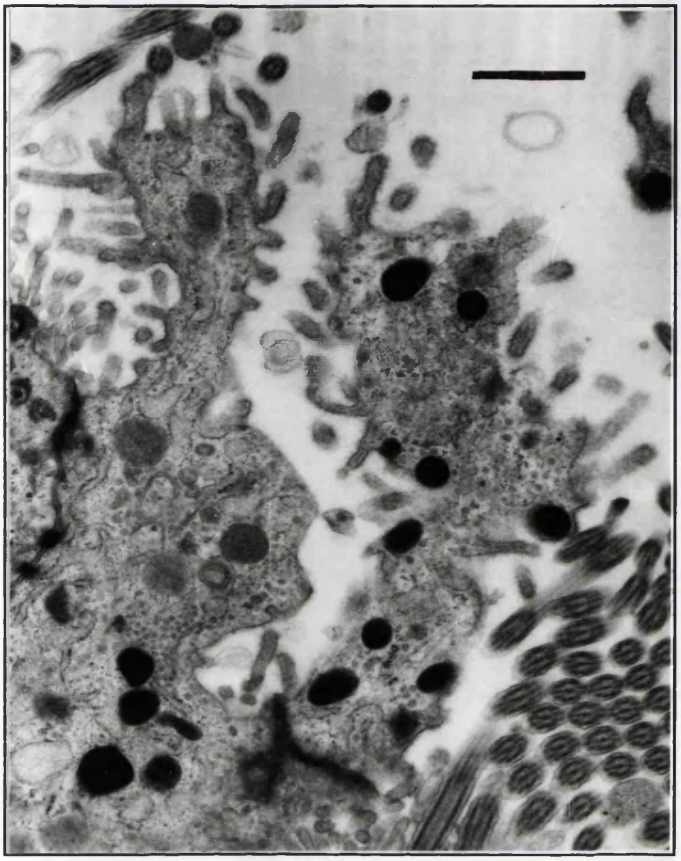


Fig. 4(I).11

Late follicular phase endosalpingeal surface with prominent electron dense granules lined up at the cell surface of irregular cytoplasmic protrusions (bar = 1μm).

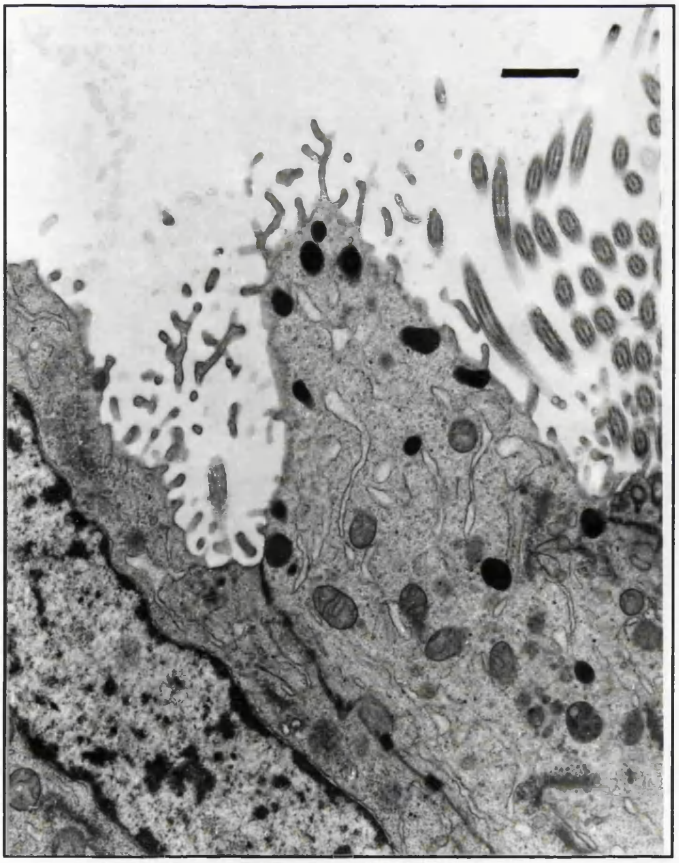


Fig. 4(I).12

Granules lined up at the protruding cell surface of endosalpingeal NCC in post-menopausal case on HRT (bar = 1μm).

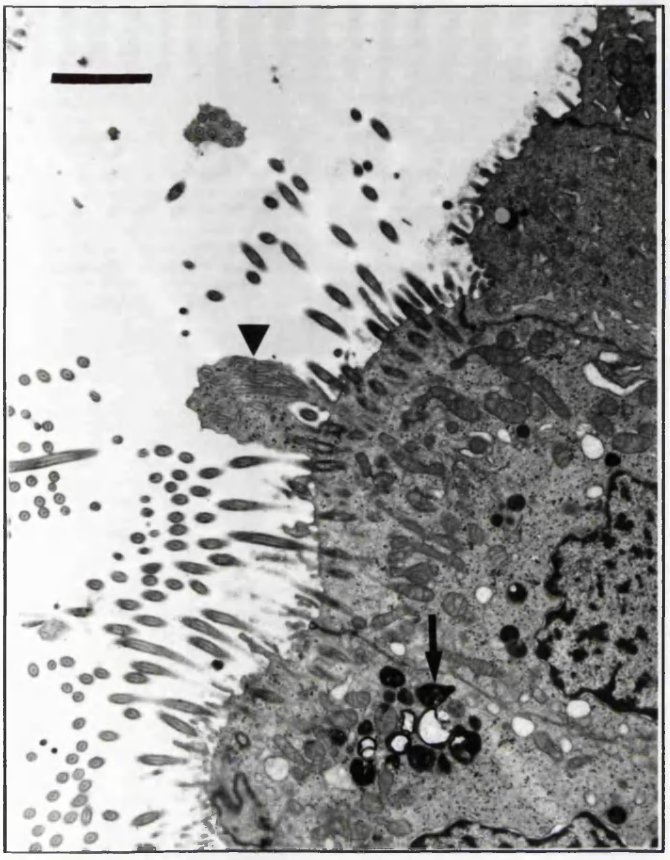


Fig. 4(I).13

Menopausal endosalpinx showing a cytoplasmic dome in a ciliated cell with engulfment of the cilia by the protruded cytoplasm (arrow head). Compound lysosomes are seen in the adjacent cell (arrow) and other electron dense granules are also present in the cytoplasm (bar=2μm).

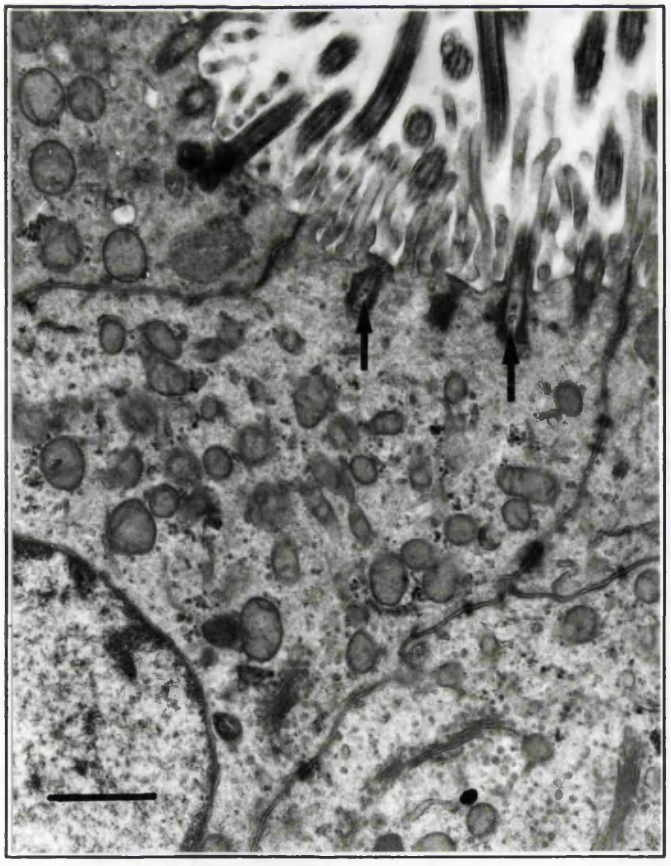


Fig. 4(I).14

Post-menopausal endosalpinx showing glycogen particles in the supranuclear cytoplasm of CC and within the rootlets of the cilia themselves (arrows) (bar=1μm).

4(I).4 DISCUSSION-1

Traditionally four cell types have been described in the Fallopian tube lining epithelium: CC, secretory cells, intercalated or "peg" cells and basal or "reserve" cells (Wheeler, 1982). The basal cells with small round dark nuclei are now recognised to be lymphoid cells (Odor, 1974) and have been shown immunocytochemically to be T-lymphocytes of predominantly the cytotoxic/ suppressor type (Morris et al, 1986; Peters, 1986). It is possible that reserve cells do also exist within the epithelium since there clearly must be some replacement of cells lost into the lumen. Mitoses have been observed in epithelial cells (Odor, 1974; Donnez et al, 1985) and it is unlikely that fully differentiated cells divide. However in this study such cells were not identified as recognisably different from other NCC cells.

Intercalated (peg) cells were originally described as slender rod-like dark cells squeezed between adjoining cells (Novak, 1947). Most authors now believe these are "exhausted" secretory cells. In addition to cell fragments being extruded from the luminal surface, we identified narrow cells within the epithelium whose upper parts sometimes did not reach the surface within the plane of the section. We did not find these intercalated cells to be a separate cell type and would concur with the suggestion that when a considerable amount of apical cytoplasm is lost into the lumen during the process of decapitation secretion, the residual NCC may appear as an intercalated cell.

There is some evidence to suggest that the two main cell types are not immutably restricted to being either ciliated or non-ciliated but may to some extent be interchangeable. Both cell types have been found to contain oestrogen and progesterone receptors (Press et al, 1986; Amso et al, 1994a) and apart from the surface specialisation the ultrastructural features appeared very similar. Occasional cells were observed which had both cilia and domes at the surface, sometimes with apparent engulfment of cilial shafts by development of domes (Figure 4(I).13).

There is a clear variation in the relative numbers of CC and NCC along the length

of the tube and it is known that the proportion of ciliated cells may be increased by the administration of E and decreased by P (Donnez et al, 1985). Donnez et al, also reported variations in the proportion of CC during the menstrual cycle with maximum ciliation around the time of ovulation, particularly in the fimbria. Verhage et al (1979) also found maximum ciliation in the fimbriae in the late follicular phase with a decrease in numbers of CC during pregnancy and the puerperium. Like Clyman (1966), Patek et al (1972a), Brosens et al (1976) and Critoph and Dennis (1977) we did not find a significant variation during the normal cycle although the fall in E levels after the menopause had a very clear effect which was apparently reversed by HRT. All these observations can be explained if the cells can change their surface differentiation under different circumstances, particularly in response to changes in hormone levels.

There has been some debate over the function of the cilia and whether their role is to aid the outward movement of the spermatozoa or the inward movement of the ovum. Since there are more CC at the fimbrial end and since they have been shown consistently to beat down towards the isthmus (Gaddum-Rosse et al, 1973; Weström et al, 1977) the latter seems most likely. The difference in the cellular distribution of glycogen between the cell types is interesting. Glycogen was seen in both cell types throughout the cycle but there was generally more in the CC than in the NCC. The presence of occasional particles actually within the cilial rootlets (Figure 4(I).14) suggested the possibility that glycogen is used as a source of energy for cilial action. One explanation for the quantitative difference however could be that some glycogen is lost from the NCC during the process of decapitation secretion, resulting in less being visibly stored in the cytoplasm of these cells.

The NCC have also been referred to in the literature as secretory cells and we confirm that during the follicular phase many of the NCC show prominent surface activity with discharge of cytoplasmic material into the lumen. Occasionally an appearance suggesting individual granule release was noted but more often whole domes became waisted at the base and eventually broken off from the underlying cell. At the peak of such surface activity, just before ovulation, this type of cytoplasmic secretion also included larger cell fragments containing nuclei and even whole cells.

Some of the cellular debris including cytoplasmic organelles and larger cell fragments, was seen in the lumen of the inner segments of the tube (Figure 4(I).8b). Thus the secretory activity at this site appears to be a combination of merocrine, apocrine and holocrine activity. Similar concentration of granules in bulging surface domes has also been described in monkeys (Jansen and Bajpai, 1983), and two types of granules have been identified in secretory tubal cells of rabbits although only those in the isthmus appeared to be exocytosed (Jansen and Bajpai, 1982).

Fallopian tube secretion is usually regarded as "serous" in nature and analogous with that found within the cystic spaces of papillary serous ovarian neoplasms which are lined by similar epithelium (Fenoglio et al, 1977) and have been shown to contain similar secretory granules (Gondos, 1971). This secretion is also considered to be composed of a combination of a selected transudate of serum components together with secretory products of the tubal epithelial cells (Leese, 1988; Wagh and Lippes, 1989). Several earlier studies had suggested that oviduct-specific proteins may originate from these granules in the secretory oviductal epithelium (Hanscom and Oliphant, 1976; Shapiro et al, 1971; Barr and Oliphant, 1981) and may be of high or low molecular weights (Verhage et al, 1988; Maguiness et al, 1992a,b). Subsequently, antibodies raised to oviductal glycoproteins were able to immunolocalise these molecules to the epithelial secretory cell granules of the rabbit isthmus and ampulla (Oliphant et al, 1984b) and in the apical cytoplasm of secretory cells in the baboon oviduct (Verhage et al, 1989). In the human Fallopian tube, preliminary results using polyclonal antibodies for localisation (Rapisarda et al, 1993), depicted the oviductal glycoprotein in the apical granules of the secretory cells only. In this Thesis, the ultrastructural observations on the pattern of tubal secretions concur with other studies reporting variations in the glycoprotein content of oviductal fluid under different hormonal stimuli in monkey (Mastroianni et al, 1970), human (Lippes, 1981) and sheep (Sutton et al, 1984) and with reports describing quantitative and "qualitative" variation in sulphated glycoproteins secretion between the isthmus and ampulla of rabbit oviducts (Oliphant et al, 1984b; Hyde and Black, 1986).

The presence of cellular debris within the isthmic lumen may be an artefact relating to the small size of the lumen at this site which enabled it to be embedded completely

in one electron microscopy block and therefore possibly to retain its luminal contents whereas the segments with larger cross-sections had to be divided for blocking and did not have an intact lumen. It could however be a genuine finding since it correlates with Jansen's observations of a mucus isthmic plug containing glycoproteins in rabbit (Jansen and Bajpai, 1982), monkey (Jansen and Bajpai, 1983) and human oviducts (Jansen, 1980). The luminal material in our studies was composed of cell cytoplasmic debris including dense granules as well as many other organelles (Figure 4(I).8b). It is possible that retention of luminal contents in the isthmus is facilitated by the smaller number of CC at this site compared to more distal sections of the tube, and the lack of any cytoplasmic material in the lumen of the distal end may be explained by relative lack of secretory activity in the cells as well as to the more expanded lumen at the fimbrial end. Jansen and Bajpai (1983), also reported the absence of any substantial precipitate secretion in the ampulla of the macaque oviduct at any stage of the cycle and in the isthmus before the mid-cycle oestrogen rise or ≥ 48 hours after ovulation. They proposed that the biochemical basis for this isthmic property may lie in the expected highly expanded state of acid muco-glycoproteins in the secretions.

The observations in this study show that the Fallopian tube epithelium, which is not generally recognised as showing follicular and luteal stage patterns, undergoes cyclical changes that are comparable with the proliferative and secretory patterns of the endometrium. Furthermore, the follicular phase is clearly a complex "secretory" phase in the tube and a similar pattern is reproduced after the menopause by HRT. The luteal phase reveals much less surface activity and appears to be a recovery stage with some evidence of intracellular housekeeping in the form of prominent secondary lysosomes. It was also shown that some differences exist between the different areas of the tube thus supporting the concept of functional differentiation. The evidence suggests that the Fallopian tube is not just a simple passage for the conveyance of germ cells. Although implantation clearly can occur following intra-uterine transfer of an ovum fertilised *in-vitro*, the release of maternal cytoplasmic components, including "secretory" granules specifically timed to coincide with the passage of the ovum, must have important effects on a conceptus that may improve its chance of implantation and ultimate survival; thus, further studies are required.

4(II).5 RESULTS-2: COMPARISON OF MORPHOLOGICAL AND ULTRASTRUCTURAL CHANGES IN THE ENDOMETRIAL GLAND AND FALLOPIAN TUBE EPITHELIA

4(II).5.1 General morphological and ultrastructural features

Despite having the overall characteristic histological features of their respective sites, there were some similarities between endometrial glandular cells and endosalpingeal NCC. In sections stained with H+E both types of cells showed a luminal border with a rather indistinct wispy surface corresponding to the presence of apical cytoplasmic domes visualised on 1 μm toluidine blue-stained sections.

By electron microscopic examination, endometrial glandular epithelium was seen to be composed mainly of NCC (Figure 4(II).1a), with only occasional CC being found at this site. These NCC had surface features in common with tubal NCC, especially the luminal cell surface which showed apical protrusions or domes of variable height projecting into the lumen and were covered by numerous microvilli (Figures 4(II).1-4(II).4). A variety of cytoplasmic organelles was noted in endometrial NCC, including mitochondria, free ribosomes and profiles of rough endoplasmic reticulum, Golgi apparatus, lysosomal and other lipid bodies (Figures 4(II).1a-4(II).7a). Variable numbers of electron-dense intracytoplasmic granules were noted (Figures 4(II).1a-4(II).4a) and glycogen particles were seen in greater quantities than in tubal epithelium, especially in the luteal phase of the cycle (Figure 4(II).5a).

4(II).5.2 Luminal contents

In the early and mid follicular phase, the endometrial glandular lumen was narrow and contained little cellular debris or other material (Figure 4(II).1a). In the tube, fragments of cytoplasmic and cellular material were seen in the lumen of the isthmic segments but not in the outer tubal segments (Figure 4(II).2b).

Later in the follicular phase, cytoplasmic fragments appeared in the endometrial glandular lumen, apparently shed from the cells by a process of cell decapitation or apocrine secretion (Figure 4(II).3a). This process increased as the follicular phase

advanced and was maximal at the time of the LH surge, before ovulation, when the lumen contained some quantities of granular and membranous material, including variably sized cell fragments and even nuclei (Figure 4(II).4a). In the tube, the isthmic lumen also showed increasing amounts of cytoplasmic debris and cellular fragments. Similar findings, but to lesser degree, were seen in the mid-section of the tube (Figures 4(II).3b and 4(II).4b) but not in the outer sections.

In the luteal phase, despite having a characteristic "secretory" pattern with eosinophilic material in the gland lumen on H+E sections, on electron microscopy examination the gland lumens contained little cellular material (Figure 4(II).5a) and the tubal lumen (isthmic and mid-sections) also showed minimal cellular contents (Figure 4(II).5b).

After the menopause there was again little luminal material present (Figure 4(II).6a) but, under the influence of exogenous oestrogen therapy, the endometrial glandular lumen was again noted to contain cytoplasmic material (Figure 4(II).7a) as seen in the late follicular phase, and the same was also true for the tubal isthmic sections.

4(II).5.3 Epithelial luminal surface

In the early and mid follicular phase, endometrial epithelial morphology appeared relatively undifferentiated and the luminal surface showed little irregularity and few dome shaped projections (Figure 4(II).1a). The domes became more obvious and more variably shaped in the late follicular phase and during the LH surge (Figures 4(II).3a and 4(II).4a). In the early/mid luteal phase, there was a lull in the cell surface activity (Figure 4(II).5a) but surface domes were again prominent in the late luteal phase.

Tubal epithelial surface changes have been reported in detail in section 4(I).3. Generally, the tubal NCC epithelial surface changes in the corresponding subjects appeared similar to those of the endometrial glands at the various stages of the menstrual cycle and in the menopausal women with or without HRT (Figures 4(II).1b -4(II).7b), although surface projections were less noticeable in the fimbriae than in

the inner sections of the tube.

4(II).5.4 Secretory granules

In the early and mid follicular phase, the cytoplasm of the endometrial glandular epithelium contained a few electron-dense granules that were quite variable in shape and size and dispersed randomly within the cytoplasm (Figure 4(II).1a). The numbers of granules increased and reached a peak in the pre-ovulatory phase, when they were seen in the supranuclear cytoplasm and sometimes lined up beneath the cell surface in the apical domes and decapitated fragments of cytoplasm (Figures 4(II).2a and 4(II).3a). Occasional granules and other cytoplasmic organelles were also apparently released individually into the lumen from the superficial cytoplasm. Glycogen particles could also be recognised in the endometrial cell cytoplasm in the late follicular phase and by the early/mid luteal phase there were dense glycogen accumulations especially prominent in the supranuclear position (Figure 4(II).5a). At this stage, there appeared to be a lull in the surface cytoplasmic decapitation secretion exactly corresponding to the appearances in the isthmic and ampullary epithelial cells at the same stage (Figures 4(II).5a and 4(II).5b).

The uterine glandular epithelium in the untreated post-menopausal case showed little evidence of secretory activity (Figure 4(II).6a), while the appearance in the HRT case strongly resembled that of the late follicular phase (Figure 4(II).7a). Similarly, the tubal epithelium in the HRT case showed secretory granules and luminal surface changes similar to those seen in the late follicular phase (Figures 4(II).6b and 4(II).7b).

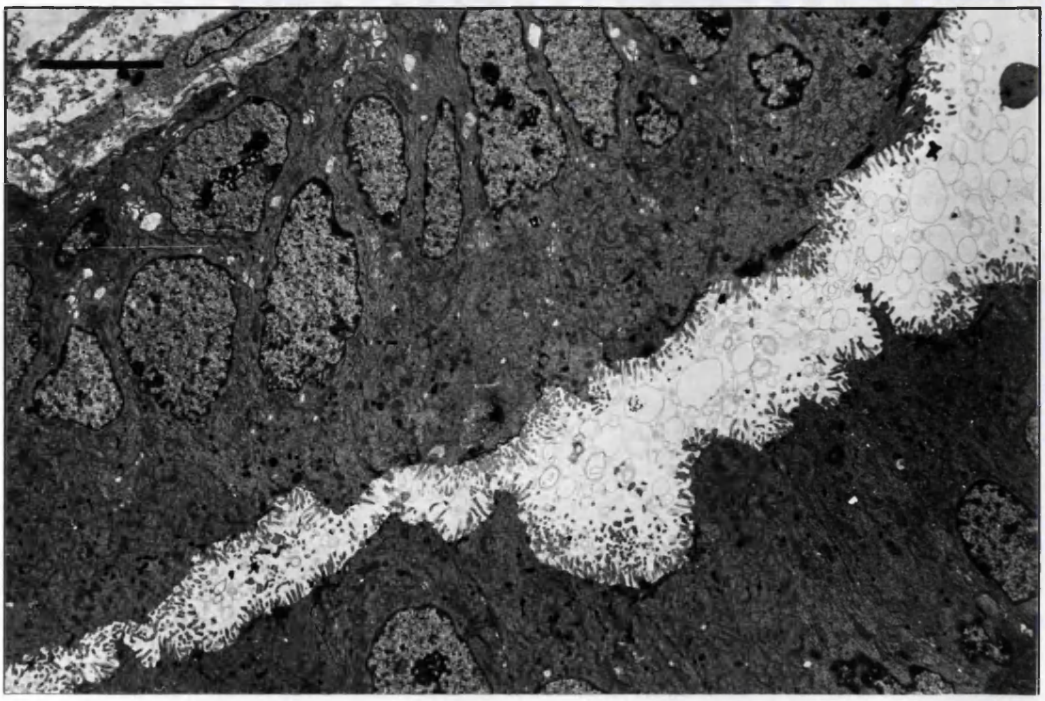


Fig. 4(II).1a Mid-follicular endometrial gland. NCC with slightly domed luminal surfaces covered by microvilli. Scattered dense granules can be seen in the supranuclear cytoplasm (bar=5μm).

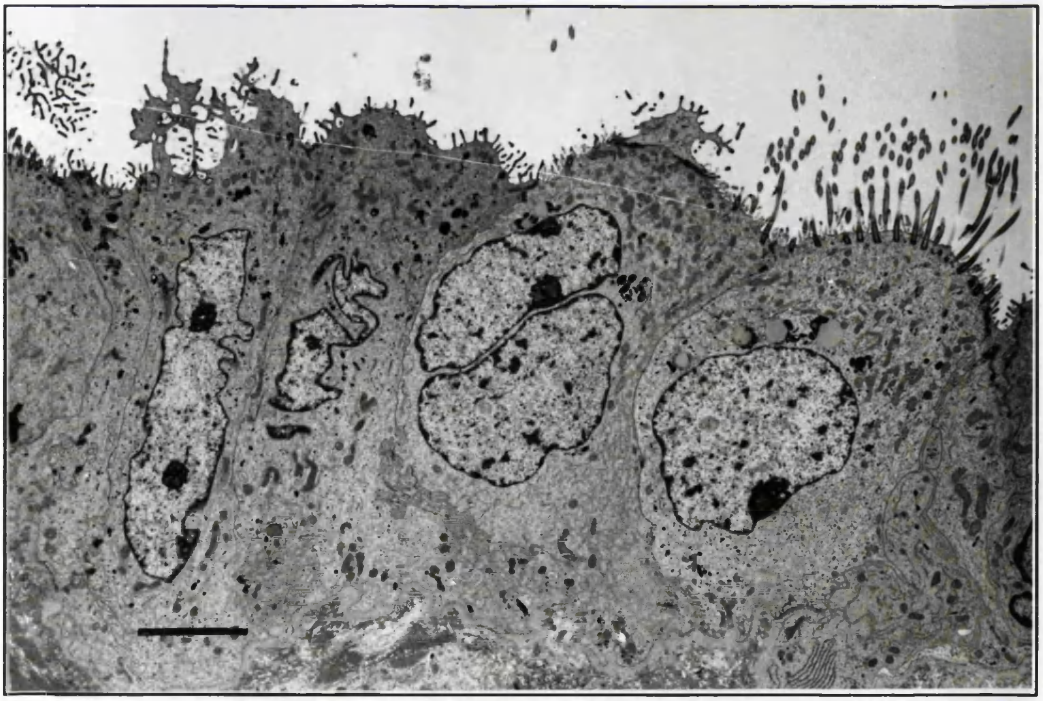


Fig. 4(II).1b Mid-follicular endosalpinx from mid-tube showing CC and NCC. The NCC have slightly domed luminal surfaces and both cell types show surface microvilli (bar=5μm).

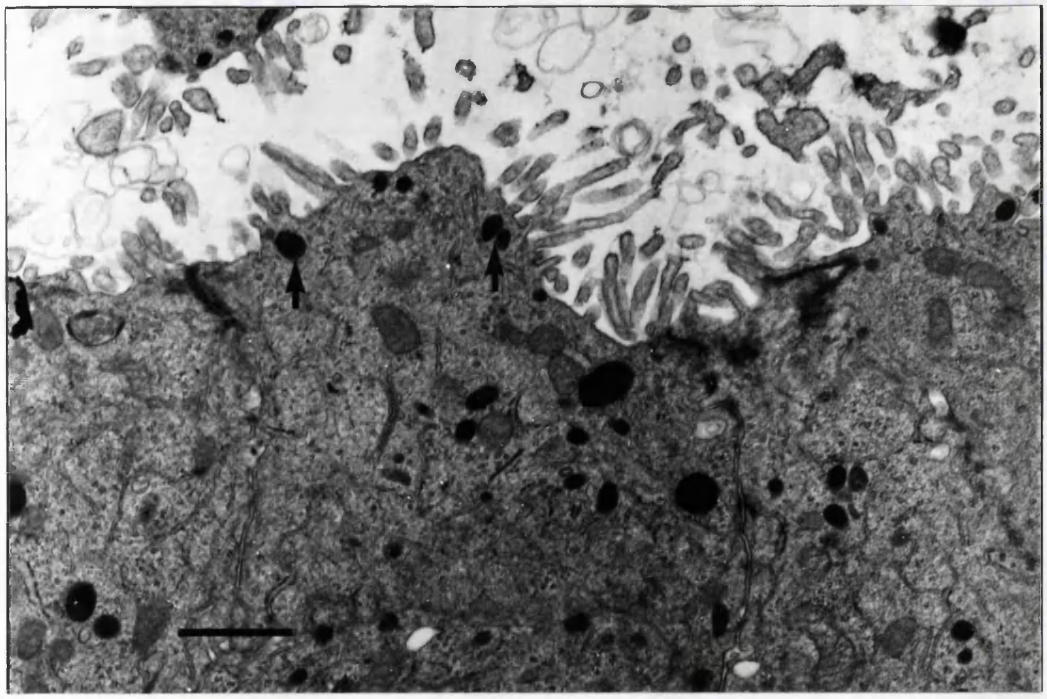


Fig. 4(II).2a Epithelial cell surface of mid-follicular endometrial gland showing electron dense granules (arrows) lining up at the luminal edge (bar = 1μm).

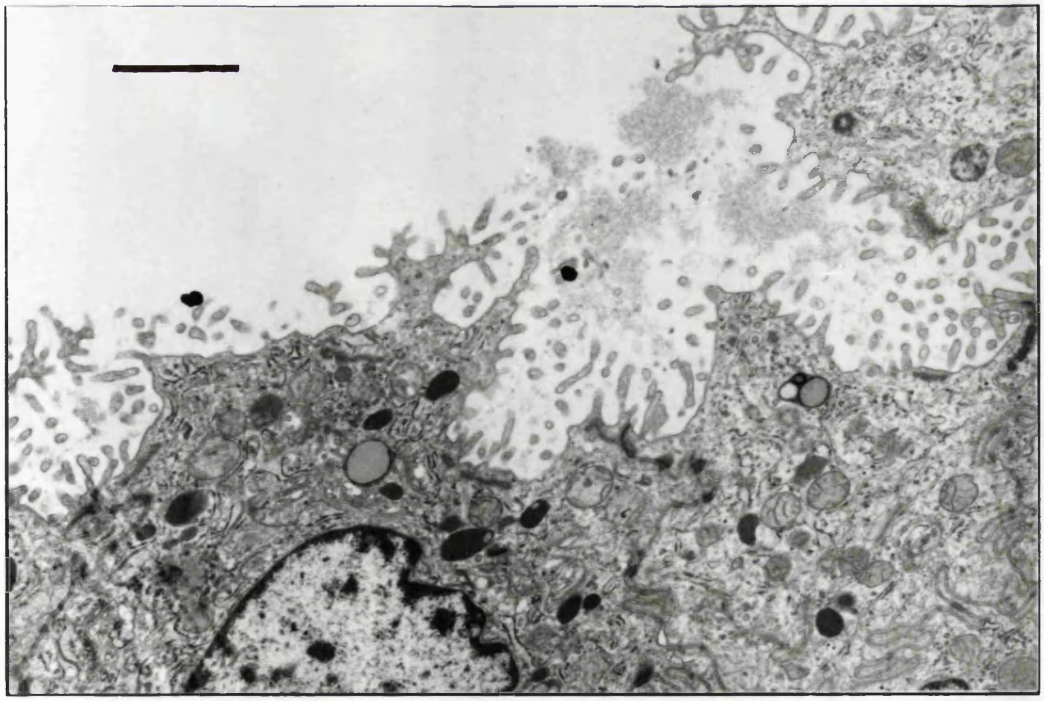


Fig. 4(II).2b Epithelial cell surface of isthmic endosalpinx in mid-follicular phase showing scattered granules and irregular surface protrusions (bar = 2μm).

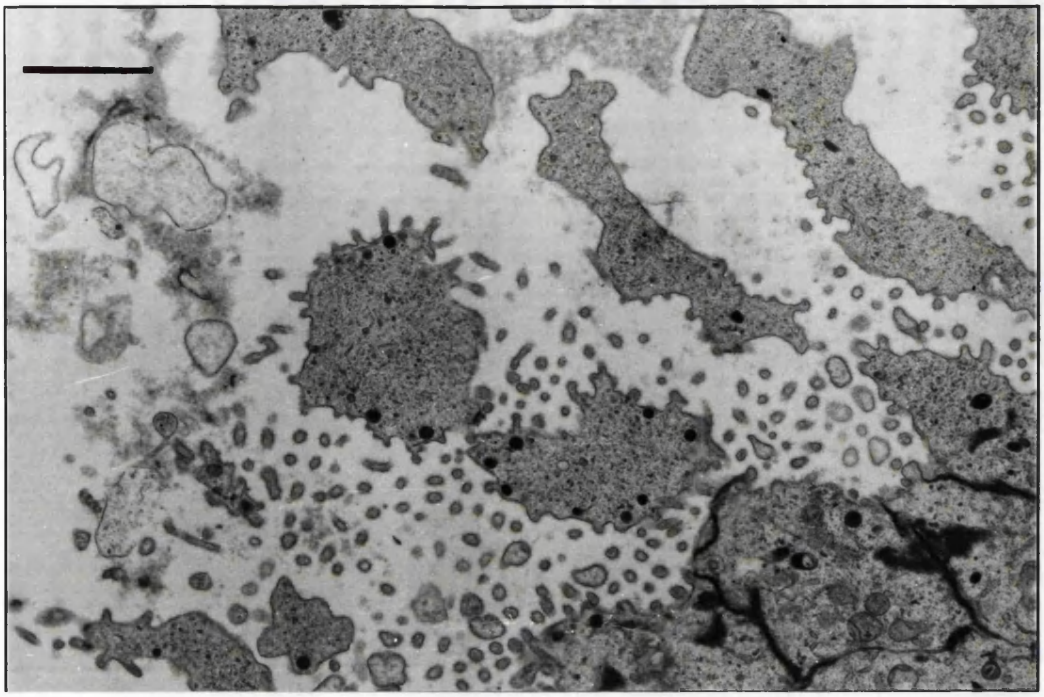


Fig. 4(II).3a Endometrial glandular luminal surface in late follicular phase showing decapitation secretion of cytoplasmic fragments containing electron dense granules (bar = 2μm).

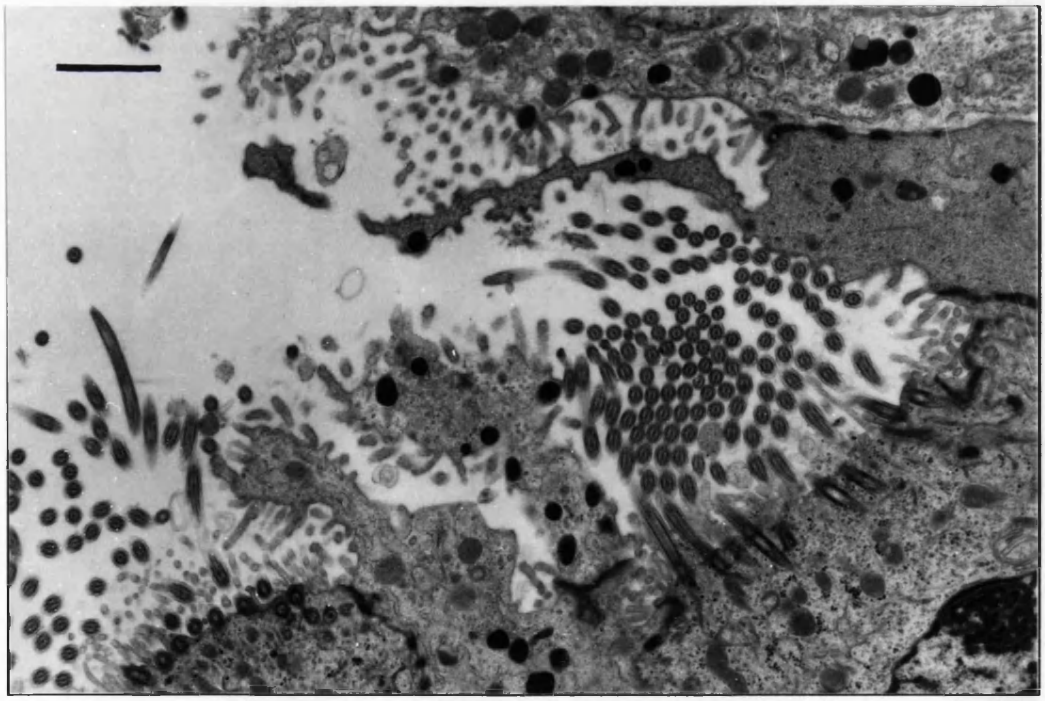


Fig. 4(II).3b Mid-tube epithelial surface in late follicular phase showing decapitation secretion of cytoplasmic fragments containing electron dense granules (bar = 2μm).

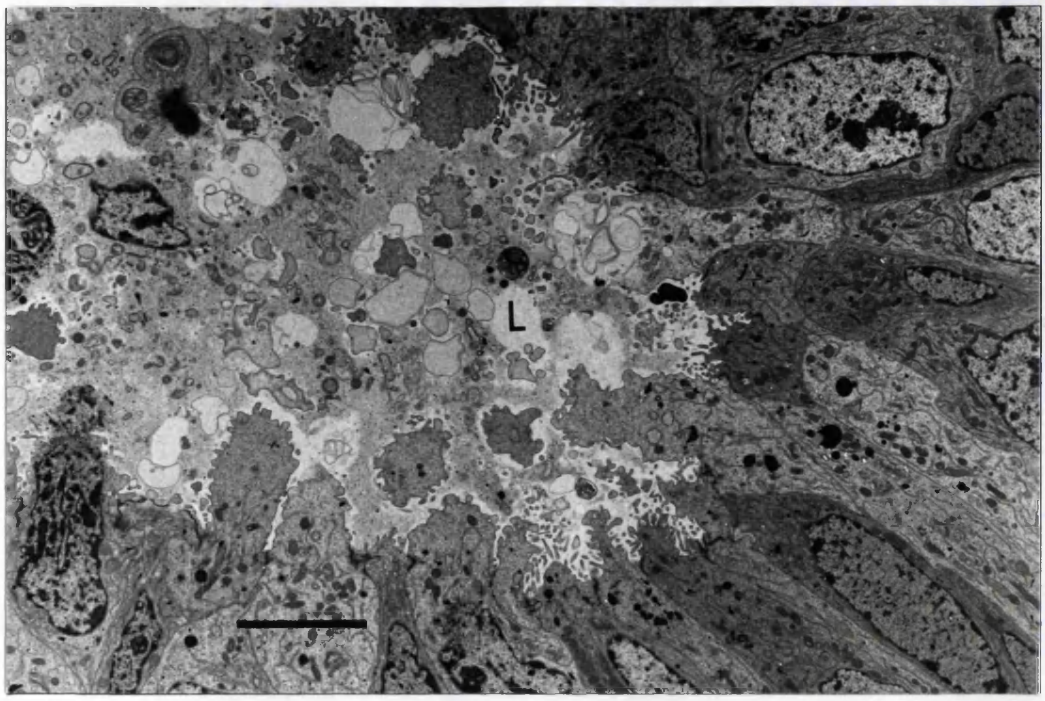


Fig. 4(II).4a Peri-ovulatory endometrium showing maximal cell surface decapitation and cell extrusion with numerous cell fragments in the lumen (L) (bar = 5 μm).

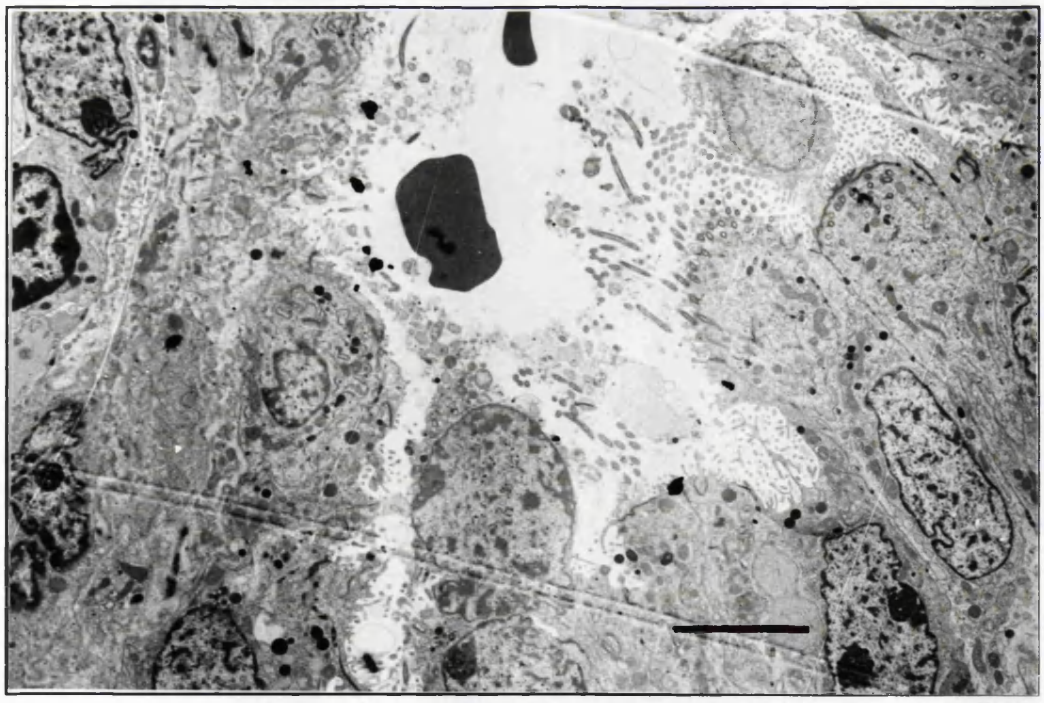


Fig. 4(II).4b Peri-ovulatory mid-tube endosalpinx showing cell surface decapitation and cell extrusion into the lumen (bar = 5μm).

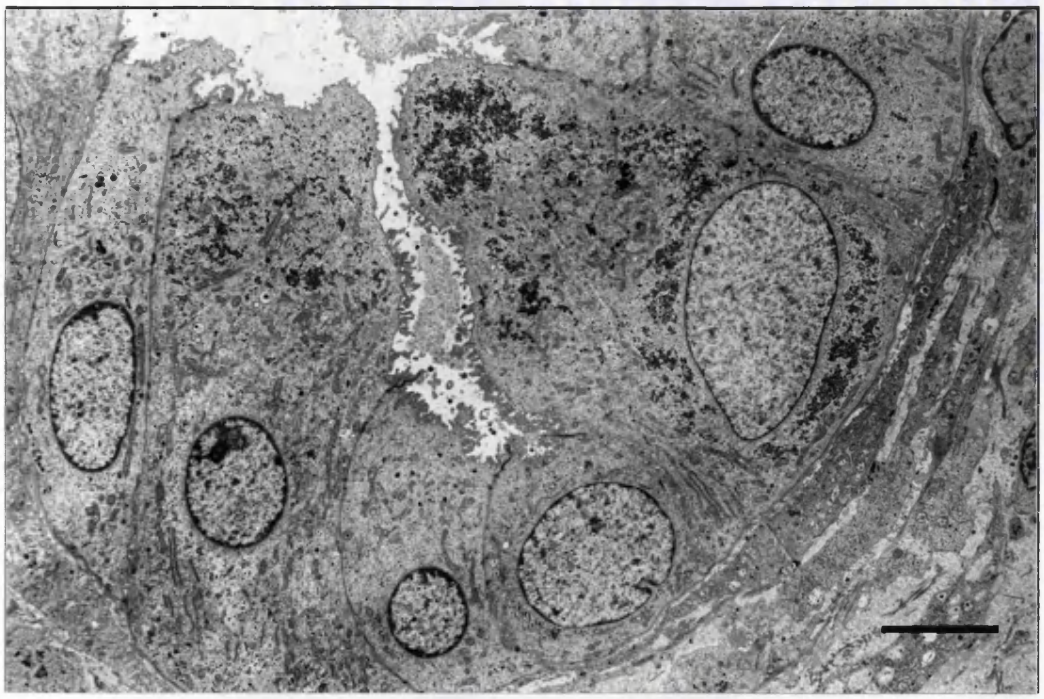


Fig. 4(II).5a Mid-luteal endometrium showing dense accumulation of glycogen particles within the cells but no cytoplasmic fragments in the lumen and little cell surface activity (bar = 5μm).

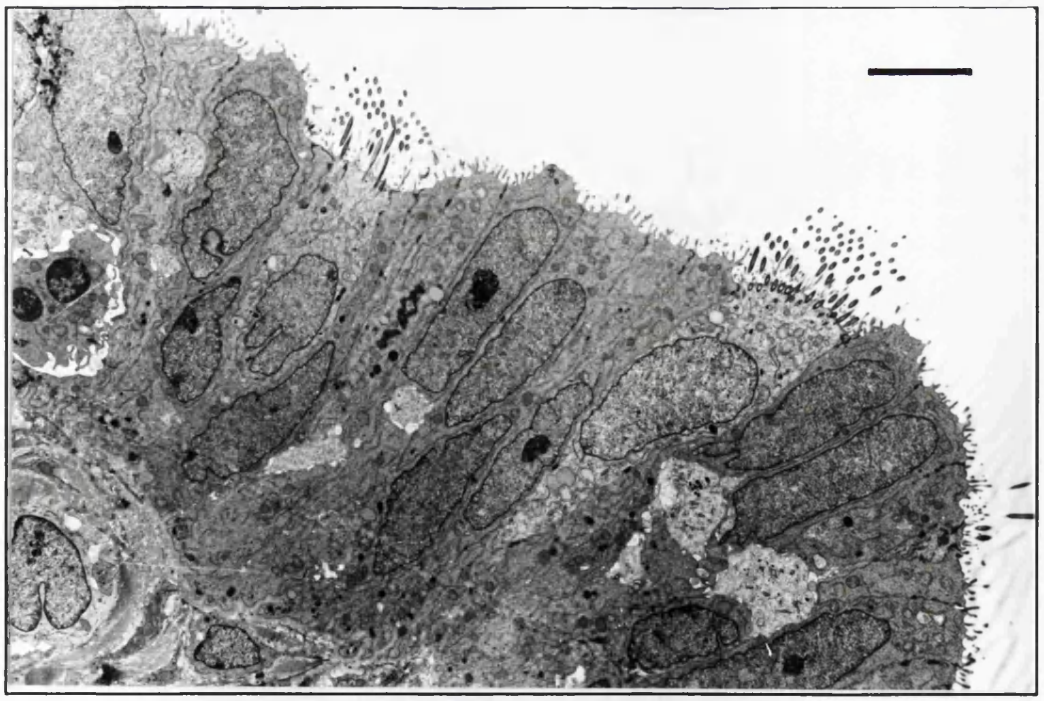


Fig. 4(II).5b Mid-luteal isthmic endosalpinx showing marked lack of cell surface activity and no cytoplasmic fragments in the lumen (bar = 5μm).

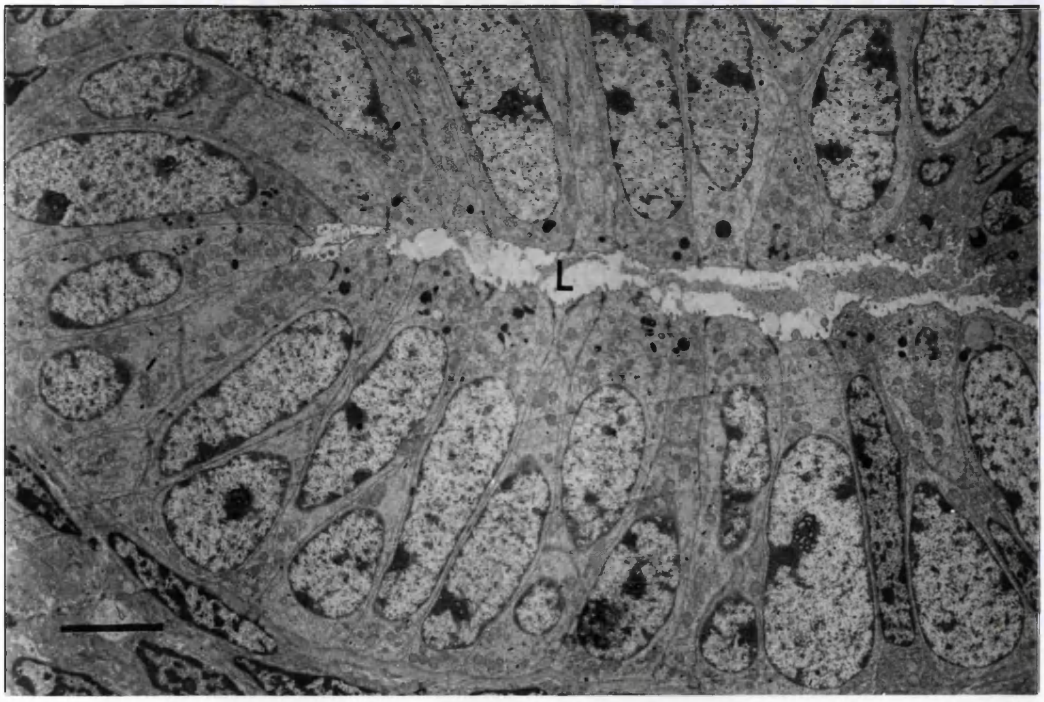


Fig. 4(II).6a Post-menopausal endometrial gland showing flat cell surfaces and little secretion in the lumen (L) (bar = 5μm).

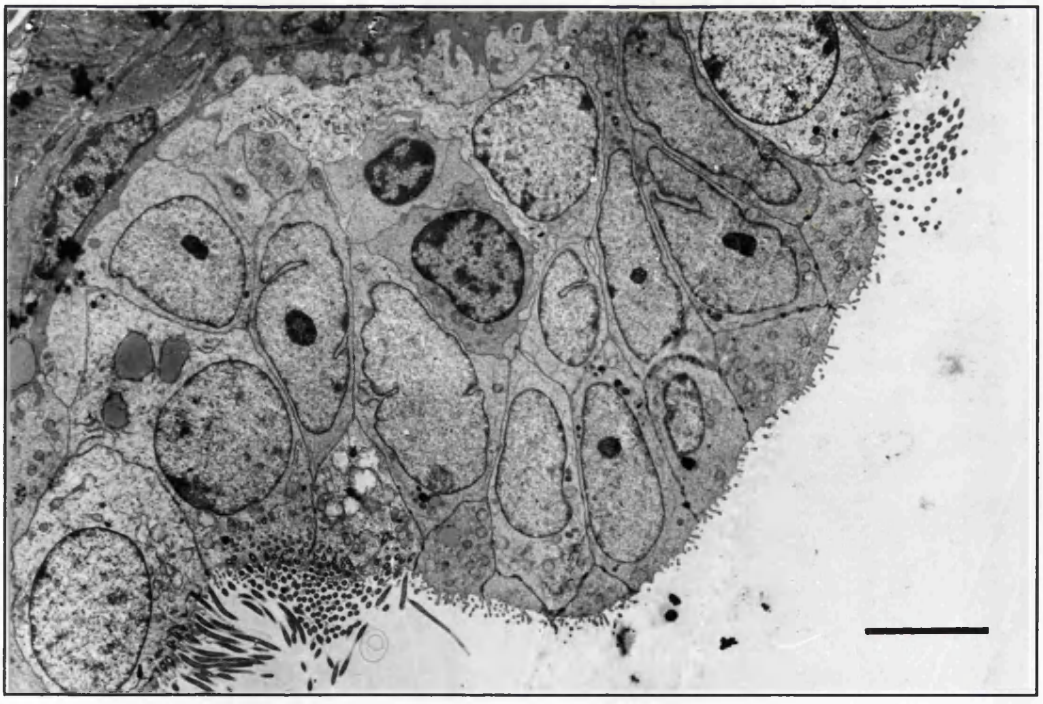


Fig. 4(II).6b Post-menopausal endosalpinx showing flat cell surfaces and no cytoplasmic fragments in the lumen (bar = 5μm).

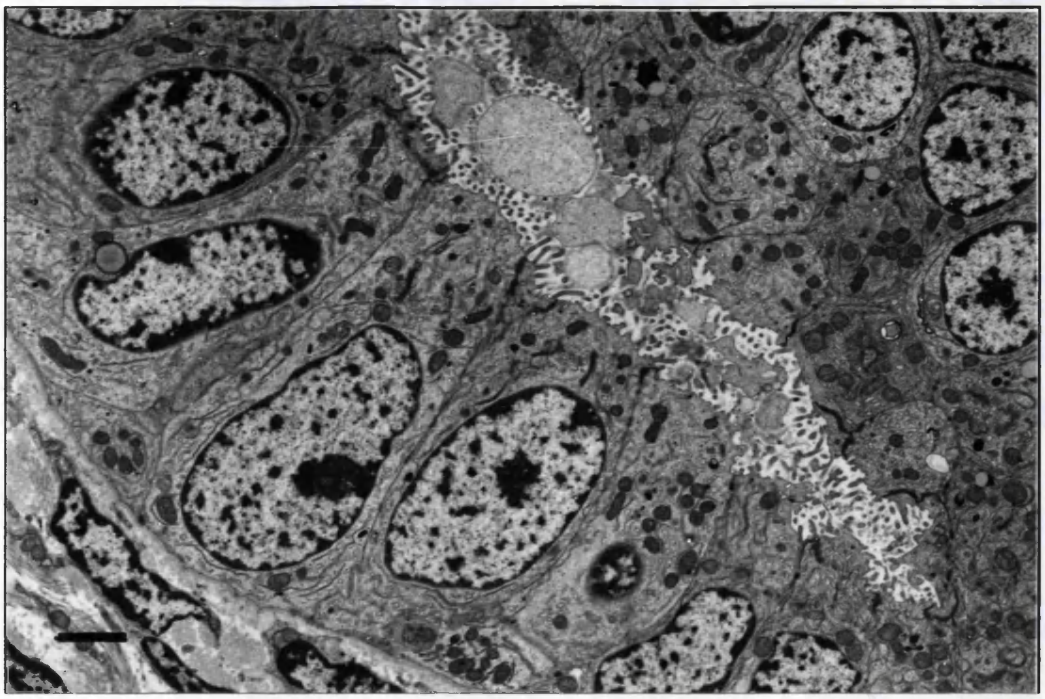


Fig. 4(II).7a Post-menopausal endometrium during HRT in oestrogen phase showing prominent cytoplasmic fragments in the gland lumen (bar = 2μm).

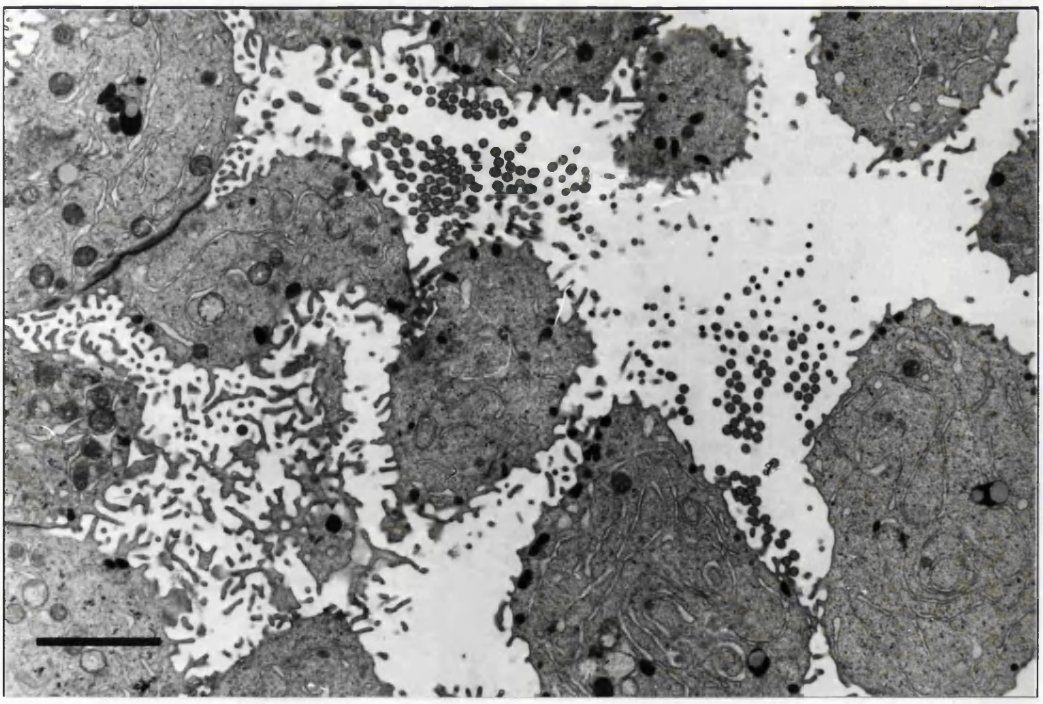


Fig. 4(II).7b Endosalpingeal surface from post-menopausal patient on HRT showing cell surface decapitation with electron dense granules lined up at the luminal surface (bar = 2μm).

4(II).6 DISCUSSION-2

The morphological features of endometrial cells as seen on H+E sections vary in a well-known fashion during the phases of the menstrual cycle (Noyes et al, 1950) and indeed are used in this study together with other information to stage the cycle for each patient. The morphological features of Fallopian tube epithelium at light- and electron-microscopic level were described in detail, including variations along the length of the tube with the menstrual cycle and after the menopause (section 4(I).3).

In one study comparing the morphology of the oviduct and endometrium of the Cynomolgus Macaques, Brenner et al (1983) demonstrated eight specific stages through which the oviduct passes in sequence during the cycle, comparable to that reported in women. The authors suggested that ciliogenesis, which usually began during menstruation, and endometrial proliferation after menses are due to the decline or withdrawal of P. The authors also showed that the fimbrial epithelium was out of phase and at a more advanced stage than the endometrial glandular epithelium in the same animal. Furthermore, the mid-cycle bulging and domes of the secretory cells were more prominent in the medial end of the oviduct than the fimbriae.

It is believed that this study is the first report making direct comparisons, in the same subjects, of ultrastructural changes in human endometrial glandular and Fallopian tube epithelia at different stages of the menstrual cycle. Comparison of luminal contents, epithelial luminal surface changes and secretory granules has revealed a number of similarities and some differences which may have significance in relation to assisted reproductive techniques.

The epithelial surface changes were generally similar in the epithelium of the endometrial glands and the NCC of the endosalpinx, although the development of domes and granules appeared slightly earlier in the endometrium and was less well developed at the fimbrial end of the tube compared to the inner segments. These similarities, suggest that the changes at both sites were directly related to the increasing and decreasing levels of circulating E and P and that variations may also occur due to changes in the ER and PR content of the different tissues at different

stages of the cycle. These changes could be correlated with different physiological functions of the different segments. The increase in isthmic and endometrial gland luminal content throughout the late follicular phase and before ovulation corresponded with the steady increase in the nuclear ER and PR immunostaining described previously (Chapter 3). In the early/mid luteal phase sample which concurs with day 18 of the menstrual cycle (Patient no. 6 in Table 4.1) the ultrastructural appearance (endometrium, isthmus, mid-tube and ampulla) of reduced luminal material, relative quiescence of cellular surface, fewer domes, and absence of surface orientation of the secretory granules corresponded to the reduction at this phase of the cycle in both ER and PR immunostaining. In the late luteal phase, the increase in epithelial surface activity and dome formation in the above regions was at variance with the markedly reduced ER content in the same areas and could be related to the decrease in the antagonistic effect of P on E driven differentiation (Brenner et al, 1983). Similar reductions of PR content and its total disappearance from the glandular epithelium were observed at this stage of the cycle. Changes in the fimbriae were at variance with those in the glandular epithelial cells and the central portions of the tube. This was probably due to the observed differences in fimbrial ER content at different stages of the cycle as compared to the other regions (Figure 3.1) and may relate to the different function of the fimbrial end of the tube.

The endometrial follicular phase is normally described as "proliferative" in pattern, in contrast to the "secretory" pattern of the luteal phase when the glycogen-rich secretions are produced and the glands become saw-toothed in outline by light microscopy. The observations in this study suggest that there were actually two phases of secretion in the endometrium. In the mid and late proliferative phase, electron dense granules and other cytoplasmic material were shed into the lumen by a process of "apocrine" or decapitation secretion similar to that occurring in the Fallopian tube at the same time. The increasing presence of cellular material within the endometrial glandular lumen in the follicular and periovulatory phases resembled that found in the isthmic lumen. After ovulation there was the "classical" endometrial secretory phase in which glycogen-rich secretion forms. In the mid-luteal phase when dense glycogen accumulations were identified in the endometrial glandular cells, there was a notable lull in surface apocrine secretory activity. Thus

the secretion of glycogen apparently involved a different and more subtle mechanism. These ultrastructural observations on endometrial glandular secretory pattern were in agreement with electrophoretic analyses of uterine secretions in which three protein patterns were noted (Beier-Hellwig et al, 1989). In this study, Beier-Hellwig and colleagues described a dynamic spectrum of appearing and disappearing bands reaching a peak between days 15 and 24 of the ideal cycle. On the basis of their study, the physiological cycle was divided into three functional phases: an "intermediate" phase of quiescent pattern of proteins (days 1-5 and 25-28; a "proliferative" phase (days 6-14) and a "secretory" phase (days 15-24) with the different groups of bands appearing at the relevant "functional" phases. Similarly, expression of a cycle-dependent secretory glycoprotein glycan corresponding to day LH+5-LH+6 was described in normal fertile women (Smith et al, 1989).

The ultrastructural appearance of the electron-dense "secretory" granules was similar in the endometrium, isthmus, mid-tube and ampulla. The granules appeared to line up at the surface slightly earlier in the endometrium and the isthmus than in the distal tube, and the granules in the cells of the distal end of the tube were also fewer in number. These differences may suggest functional differentiation. However, the biological importance of such secretory products is still speculative. Jansen (1980) suggested that there was a physical plugging of the isthmic lumen by secretions and its function was to facilitate sperm transport up the tube and hold up the entry of the fertilised ovum into the uterus to allow it to mature into a state suitable for implantation and ensure that the endometrium was at an optimal receptive stage. Unique oviductal glycoproteins and mucoproteins had been identified in humans (Moghissi, 1970), monkeys (Mastroianni et al, 1970) and sheep (Roberts et al, 1976) and one protein was shown to bind to the head of spermatozoa (Lippes and Waugh, 1989).

Animal studies have been more informative. In the rabbit, these proteins may form the mucin coat which surrounds the zona pellucida of the ovum (Greenwald, 1958). Oliphant et al (1984a,b) purified a complement inhibitor from rabbit oviduct fluid and provided evidence that it is a sulphated glycoprotein which could protect both spermatozoa and embryos from the maternal humoral immune system. A similar role

for these maternal cell fragments to protect the conceptus from being rejected by the maternal immunological system may operate in humans. Kapur and Johnson (1985, 1986) demonstrated that a glycoprotein was released by the mouse oviduct and selectively sequestrated into the perivitelline space of the oocyte and embryos. They postulated that this protein was involved in the creation of a specialised micro-environment for fertilisation and early embryonic development. Others (Leveille, 1987; Kan et al, 1988, Kan, 1990) also demonstrated uptake of an oviduct-derived component by the zona pellucida of superovulated hamster oocyte and, furthermore, by using high resolution lectin-gold cytochemistry techniques, showed that these glycoconjugates are synthesised in the Golgi apparatus of the non-ciliated secretory cells in the oviduct and stored in secretory granules (Kan et al, 1990). Oviductal factors, probably glycoproteins, have also been shown to alter the characteristics of the zona pellucida of the hamster (Yang and Yanagimachi, 1989). The zonae of oviductal oocytes or oocytes exposed *in vitro* to ampullary and/or isthmic fluids appeared "heterogeneous" in their optical density, whereas no such heterogeneity was detected in the zonae of ovarian oocytes. The biological implications of these changes and their relevance to human studies remain undetermined. More recently Moore et al, (1992) showed that rabbit blastocysts in co-culture attached to endometrial but not to endosalpingeal monolayers and addition of culture media from endosalpinx significantly decreased embryo attachment to endometrial cells in culture. These observations suggest that rabbit endosalpinx secretes a factor that prevents tubal implantation.

In summary, this study has shown that a similar process of granule secretion by cell decapitation also takes place in the endometrial glands during the late follicular phase but whether the proteins produced at the two sites are the same or tissue specific has not been determined. Immunostaining techniques with antibodies raised against the proteins isolated from the two sites could be used to elucidate the nature of these secretions and to determine the relationship between the endometrial secretion occurring in the proliferative phases with that occurring in the secretory phase. If the follicular phase proteins are the same at both sites, and if they do act to prevent implantation in the tube, by the time a conceptus had reached the uterine cavity the secretion would have changed to the classical glycogen-rich secretion, assumed to

provide beneficial conditions for implantation. Such studies will also improve our knowledge on the functional differentiation of the various segments of the tube and highlight the similarities and differences between the tube and the endometrium which may be important to fertility and assisted reproductive techniques.

CHAPTER 5
IDENTIFICATION AND ISOLATION OF FALLOPIAN TUBAL PROTEINS

5.1 INTRODUCTION

Oviductal fluid provides the optimal environment for gamete transport and the development of early embryos. The reported improved pregnancy rates associated with GIFT or ZIFT as compared with IVF-UET have raised considerable interest in the possibility that either "tubal factors" and/or "tubal proteins" may be responsible for this difference.

In both humans and animals oviductal fluid volume and composition are dependent on the stage of the menstrual cycle. Secretion of cycle specific oviductal proteins by the endosalpinx has been the subject of considerable interest for several years. Immunoelectrophoresis of oviduct fluid has suggested the presence of unique proteins, which may have been synthesized and secreted by the oviduct. Immunoaffinity adsorption has been used to separate these proteins from those derived from serum in human tubal fluid and one protein has been shown to bind to the head of spermatozoa (Waugh and Lippes, 1989). Studies of sulphated mucopolysaccharides and mucoproteins in oviduct fluid suggest that they may originate from granules observed in the secretory epithelium of the oviducts (Shapiro et al, 1971; Hanscom and Oliphant, 1976; Barr and Oliphant, 1981).

These observations and other similar studies led to the investigator's interest in studying the role of these tubal specific proteins in human reproduction.

The aims of this study were:

(1) to establish whether simple methods of collecting tubal secretions would provide adequate sample material to determine the cycle-specific changes in tubal proteins.

(2) to generate antibodies against these proteins and utilise them to localise the sites of production of these proteins within the oviduct.

5.2 CYCLICAL CHANGES OF HUMAN OVIDUCTAL PROTEINS

5.2.1 PATIENTS

Fallopian tubes were obtained from non-pregnant women (age 34-56 years) who underwent abdominal hysterectomy and salpingectomy at various stages of the menstrual cycle. The phase of the menstrual cycle was determined from the menstrual history and histology of the respective endometrium. All the women had been pregnant in the past with no known history of infertility and at laparotomy, the Fallopian tubes appeared healthy.

5.2.2 COLLECTION OF TUBAL SECRETIONS

After removal of the Fallopian tube, its external surface was cleaned of any blood and a fine cannula (Wallace Y-can 23 gauge; Figure 5.1) introduced through the medial end of the tube. Approximately 1.5-2 ml of sterile 0.9% w/v normal saline or isotonic glycine 1.5% w/v was injected and the tubal flushing allowed to trickle by gravity to be collected from the fimbrial end into a sterile container (Figure 5.2). The flushings were centrifuged (1600 g for 5 min at 4°C) to remove any cellular debris. The resultant supernatant was aliquoted and stored at -30°C until the time of analysis.

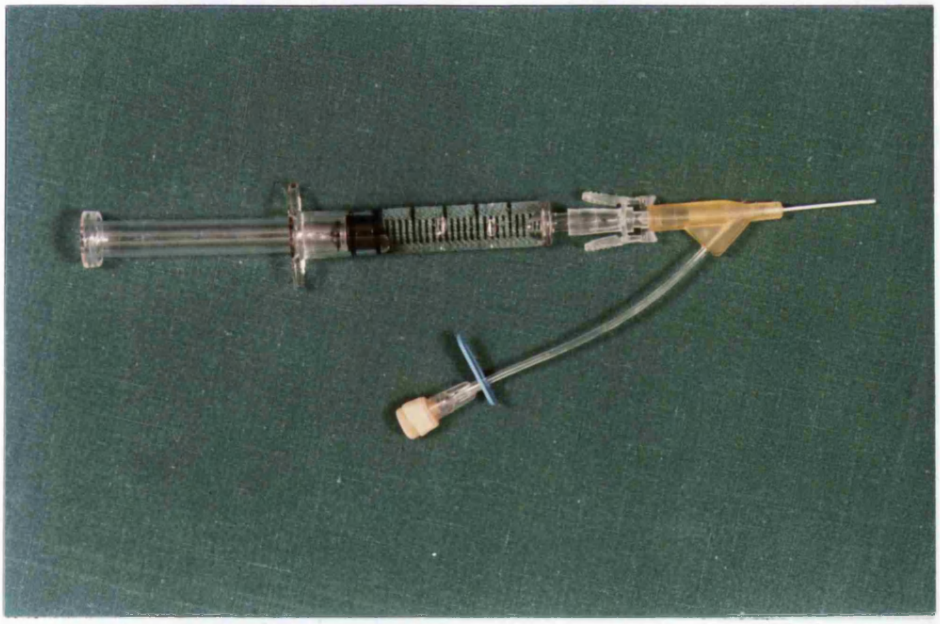


Fig. 5.1 "Wallace Y-can 23 gauge" cannula used to flush the tube from its medial end.

5.2.3 ONE-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (1-D PAGE) METHODOLOGY

This is described in section 2.4.1

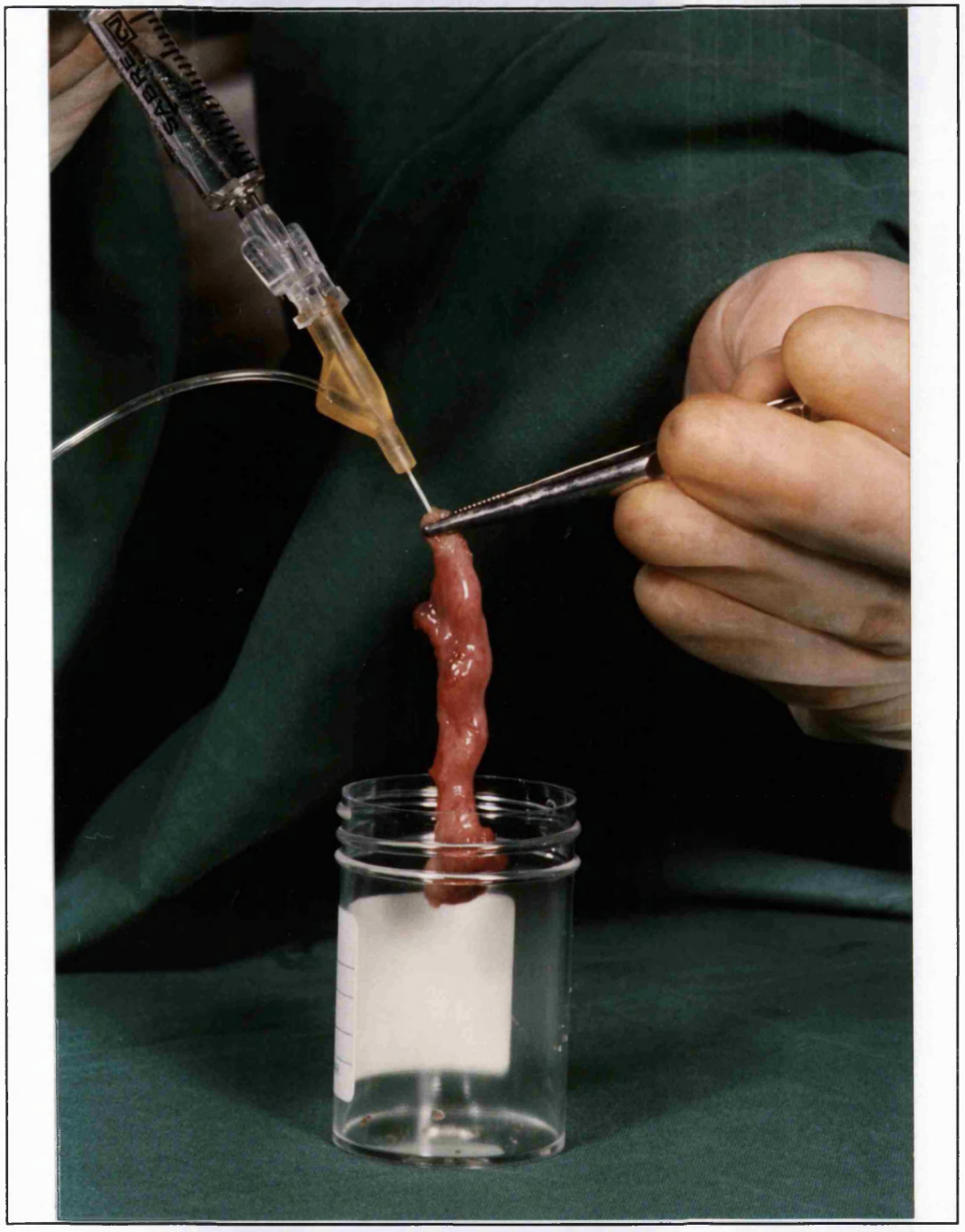


Fig. 5.2 Collection of tubal flushing from the fimbrial end into a sterile container after injecting 1.5-2 ml of normal saline or isotonic glycine into the medial end of the tube.

5.2.4 RESULTS

Between October 1988 and September 1989, a total of 26 tubal flushing samples were collected (Proliferative phase histology n= 12, Luteal phase histology n= 11, and

three women with inactive endometrium on histology). Eight samples from women with healthy Fallopian tubes at laparotomy and well documented endometrial histology were selected for 1-D PAGE analysis and are reported in sections 5.2.4.1 and 5.2.4.2.

5.2.4.1 CHARACTERISTICS OF THE WOMEN BEING INVESTIGATED

Table 5.1 summarises the age, stage of the menstrual cycle, oestrogen and progesterone levels as well as the indication for the hysterectomy in these women.

Table 5.1 DETAILS OF THE PATIENTS UNDERGOING INVESTIGATION

Pt	Age and parity	Phase of the cycle CDC	HDC	E_2 (pmol/l)	P (nmol/l)	Indication for hysterectomy and state of the pelvis
(1)	48 (p2)	4	Menstrual	211	1.5	Fibroid, normal pelvis
(2)	53 (p3)	uncertain	Prolif	6	3.7	Peri-menopausal and menorrhagia, normal pelvis
(3)	50 (p3)	9	Prolif	232	0.5	Menorrhagia, normal pelvis 12 mm follicle on left ovary
(4)	44 (p2)	16	Prolif	41	2.1	Fibroid, normal pelvis
(5)	40 (p3)	12	Mid-secretory	143	13	Fibroid and polymenorrhoea, normal pelvis
(6)	34 (p2)	19	Secretory	213	41	Menorrhagia, normal pelvis left corpus luteum
(7)	46(p0+3)	34	Secretory	282	21	Fibroid, normal pelvis left corpus luteum
(8)	56 (p1)	menstrual	Inactive	249	0.3	Fibroid, normal pelvis

CDC= Chronological date of the cycle, HDC= Histological date of the cycle,
Prolif= Proliferative, E_2= Oestradiol, P= Progesterone, p= Parity

5.2.4.2 RESULTS OF 1-D PAGE

The major protein in the oviductal flushing was apparently serum albumin (Alb) since its position on the gel was the same as that of human serum albumin. Other serum proteins also appeared in tubal flushing but at greatly reduced concentrations. In addition, certain protein bands were exclusively seen in the tubal flushing. The silver staining method which we adopted proved to be more sensitive than Coomassie staining as more protein bands were seen and with clearer outlines.

Figure 5.3 shows silver stained gels of tubal flushings from women during the proliferative phase of the cycle. Band A (arrow) appeared in tubal flushing of women in the mid/late proliferative phase and apparently increased in intensity in the mid-secretory phase sample and later disappeared in menstrual phase flushing (Figure 5.4). Band B (arrow head) appeared in secretory phase samples and a late proliferative phase tubal flushing sample. These two major bands were not identified following 1-D Gel electrophoresis of peritoneal fluid samples.

Bands A and B were consistently demonstrated when 1-D PAGE was carried out on other tubal flushing samples obtained during the respective stages of the menstrual cycle. Furthermore, repeat 1-D PAGE on the samples shown in Figures 5.3 and 5.4 after a period of nine months demonstrated the same protein bands pattern (results not shown).

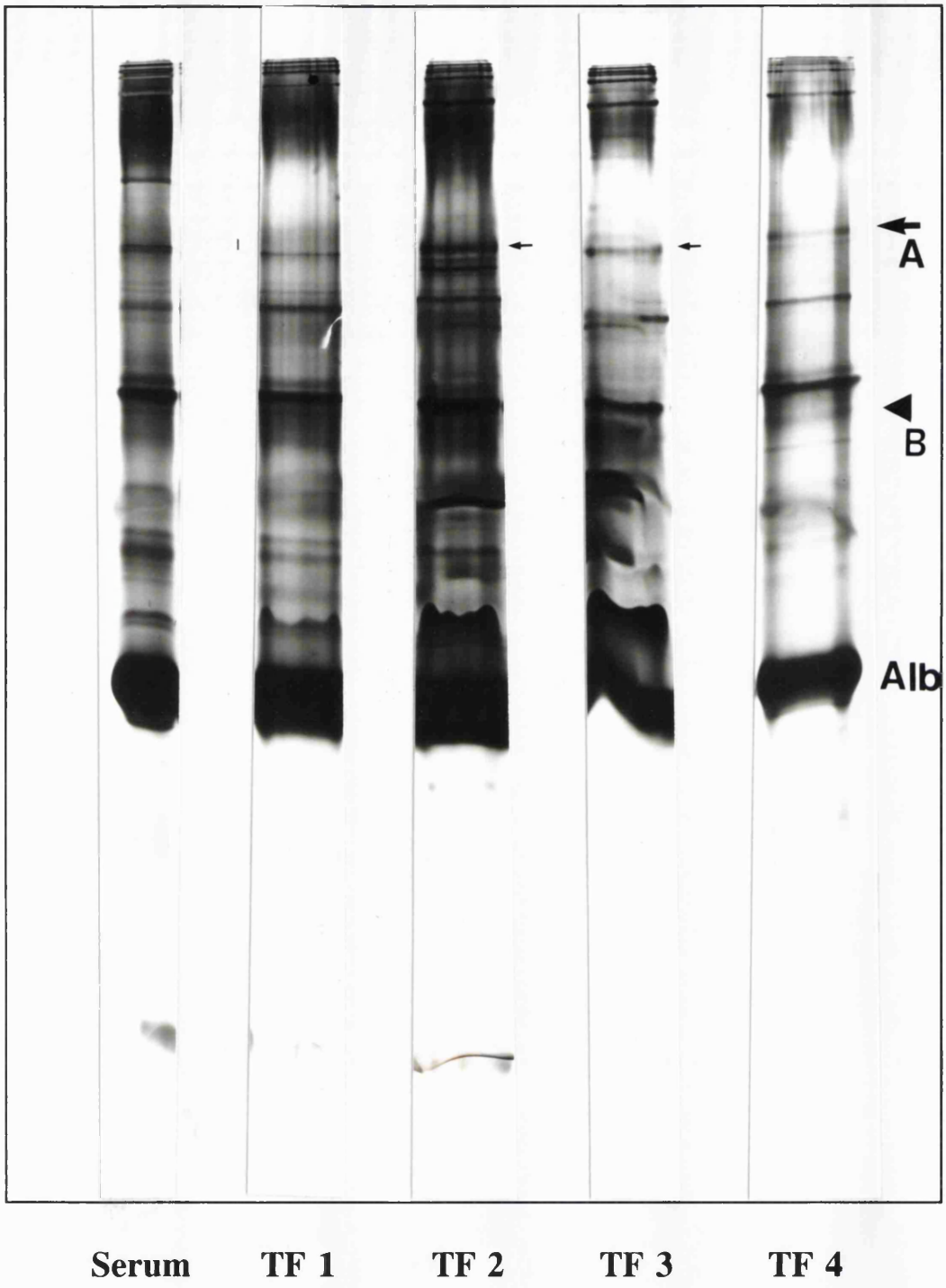


Fig. 5.3 Silver stained gel of serum and tubal flushings from women at the menstrual/proliferative phase of the menstrual cycle.

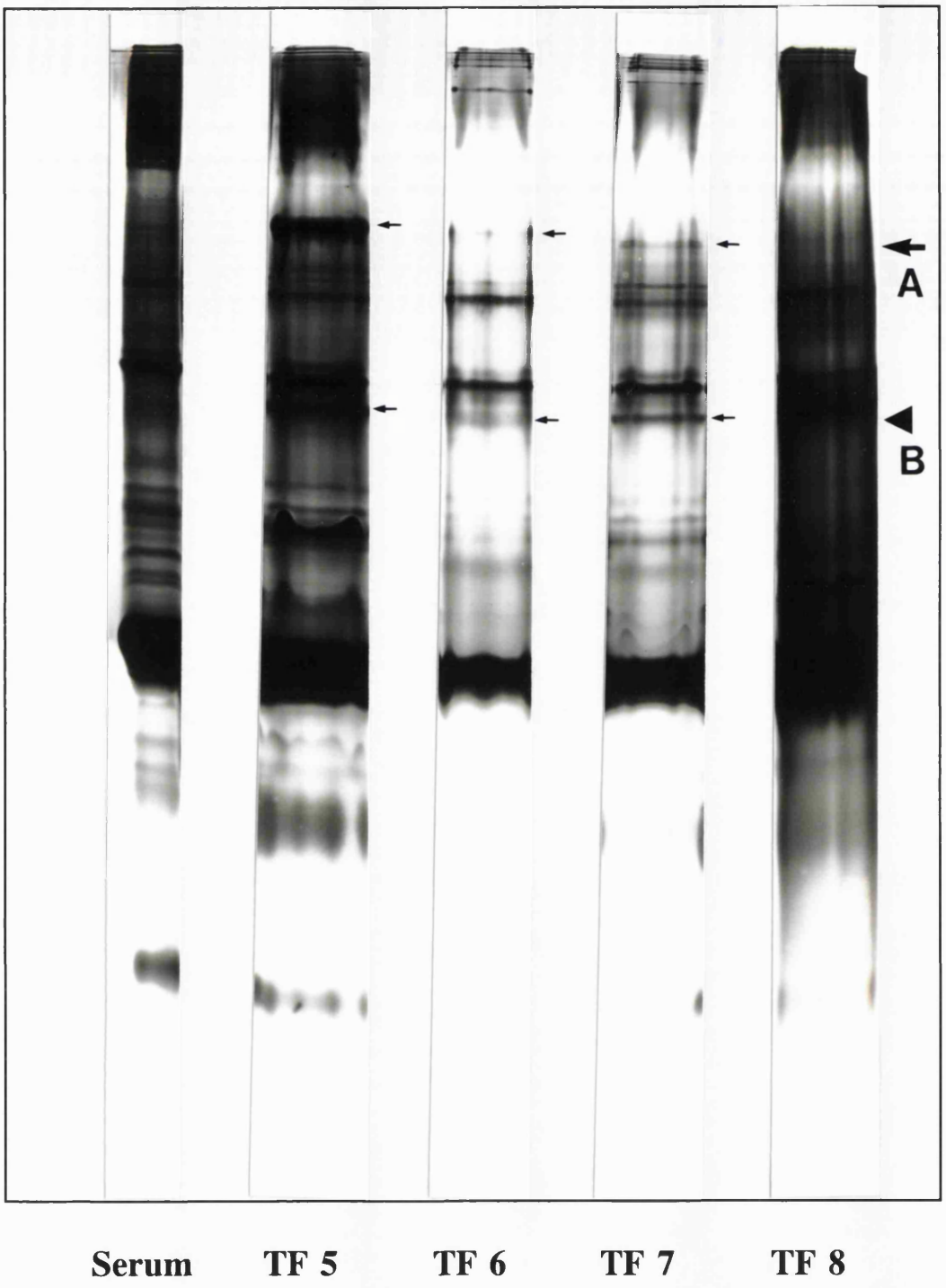


Fig. 5.4 Silver stained gel of serum and tubal flushings obtained from women at the secretory phase of the menstrual cycle.

5.3 ANTIBODY PREPARATION AND DETECTION

5.3.1 PREPARATION OF EMULSION FOR IMMUNISATION

This is described in section 2.4.2.1. Figure 2.2 depicts the acrylamide-supported protein bands identified as being of tubal origin that were cut and minced into small pieces. Figure 2.3 shows the two Luer-lock syringes connected with each other and used initially to break the gel into small uniform pieces and subsequently to emulsify the immunogen/adjuvant mixture.

5.3.2 IMMUNISATION AND BLEEDING OF RABBITS

This is described in section 2.4.2.2.

5.3.3 IMMUNOPRECIPITATION METHODS

5.3.3.1 IMMUNODIFFUSION-OUCHTERLONY DOUBLE-DIFFUSION METHOD

This method described by Ouchterlony in 1949 is the most commonly used form of simple diffusion in which the antigen is placed in one well punched in the agarose while antibody is placed in a second well. The methodology is described in detail in section 2.4.3.1. In this experiment, 5 μl of rabbits' 1910-A and 1911-B antisera, from the first and second bleed following immunisations, were placed in the central wells of rows 1 and 2 while the central wells of the third row were filled with 5 μl of human antiserum and used as a control. In Figure 5.5, wells marked (a) were filled with 5 μl of male serum, (b) were filled with 5 μl of female serum, and (c) were filled with 5 μl of tubal fluid of the reference patient whose tubal flushing demonstrated non-serum protein bands. In rows 1 and 2, the wells were filled in the same order. Note the absence of any precipitation lines between Rabbits A or B antisera and the male and female sera or tubal fluid. The controlled antiserum demonstrates precipitation lines with the corresponding sera and to a much less extent with tubal fluid samples.

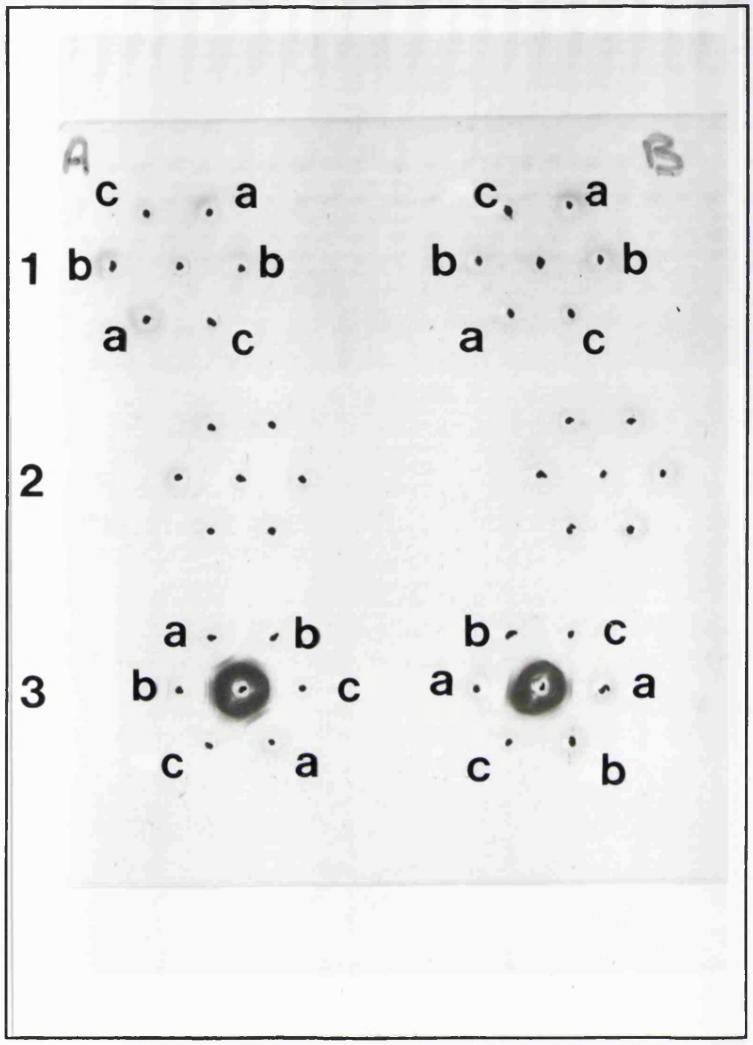


Fig. 5.5 Results of the Ouchterlony double-diffusion experiment with 5 μl of rabbits 1910-A and 1911-B antisera placed in the central wells of rows 1 and 2, and the third row filled with 5 μl of human antiserum as a control. Surrounding wells were filled with male sera (a), female sera (b), and tubal fluid (c).

5.3.3.2 IMMUNOELECTROPHORETIC TECHNIQUE ACCORDING TO GRABAR AND WILLIAMS

The methodology is described in section 2.4.3.2. In Figures 5.6 and 5.7, wells (a) and (d) were filled with 2 μl of male serum, wells (b) and (e) were filled with 2 μl of female serum and well (c) 5 μl of the reference human tubal fluid sample known to contain bands A and B. After the initial electrophoresis, troughs were cut and were filled with 100 μl of rabbits' 1910-A and 1911-B antisera. Figures 5.6 and 5.7 illustrates the absence of any precipitation lines. Due to the absence of any reaction, the rabbits were given further booster injections of immunogen, subcutaneously, and the same experiment was repeated after a third bleed, 22 weeks later. Figure 5.8 shows the results of the repeat immunoelectrophoresis. All wells contained 10 μl of tubal flushing obtained from the reference patient whose tubal fluid was known to contain non-serum protein bands on 1-D PAGE. Troughs (a) and (c) contained rabbit 1910-A antiserum and (b) contained rabbit 1911-B antiserum. Antihuman antiserum was used as a control in trough (d). No precipitation lines are seen between the tubal flushing sample and rabbits' antisera but a reaction is clearly demonstrated between the antihuman antiserum and tubal flushing sample.

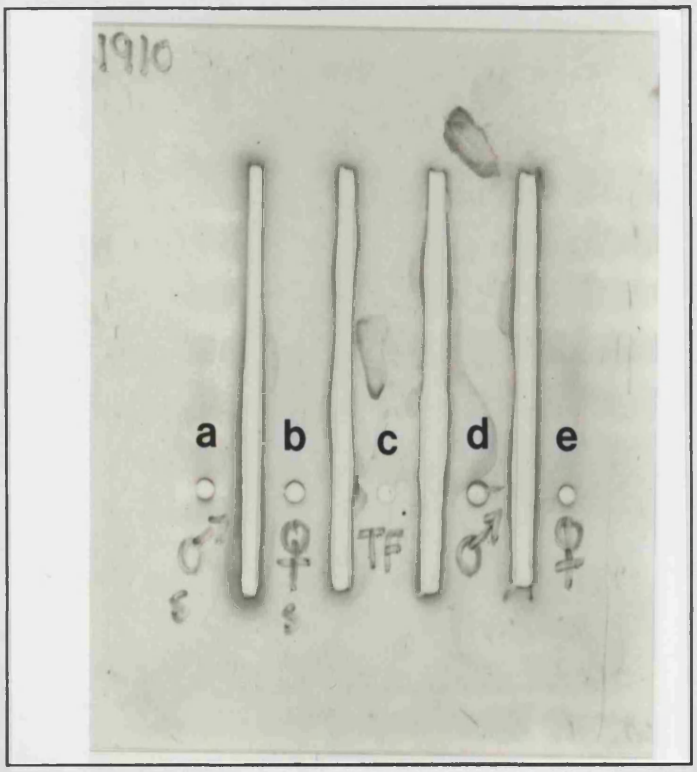


Fig. 5.6
Antiserum from rabbit 1910-A tested against male (wells a,d) and female (wells b,e) sera and human tubal flushing (well c) sample.

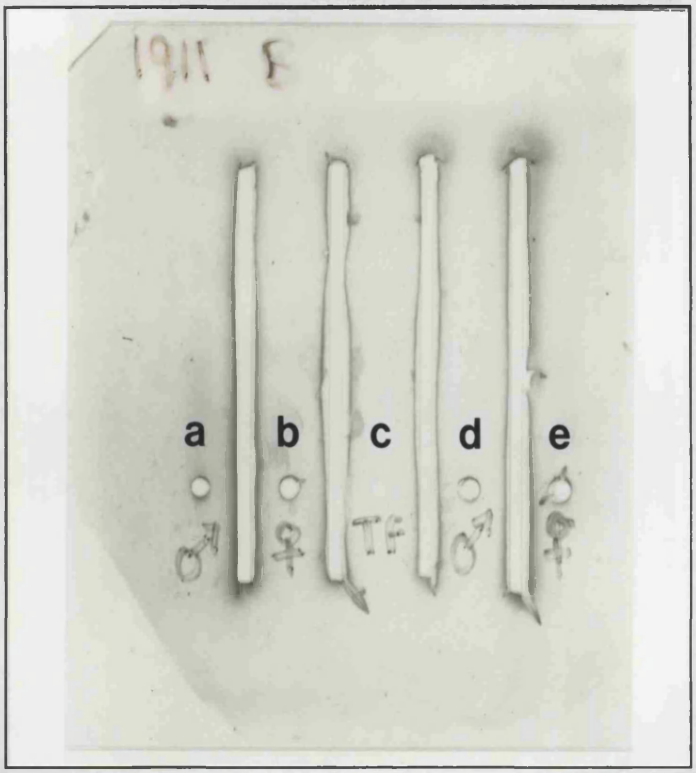


Fig. 5.7
Antiserum from rabbit 1911-B tested against male (wells a,d) and female (wells b,e) sera and human tubal flushing (well c) sample.

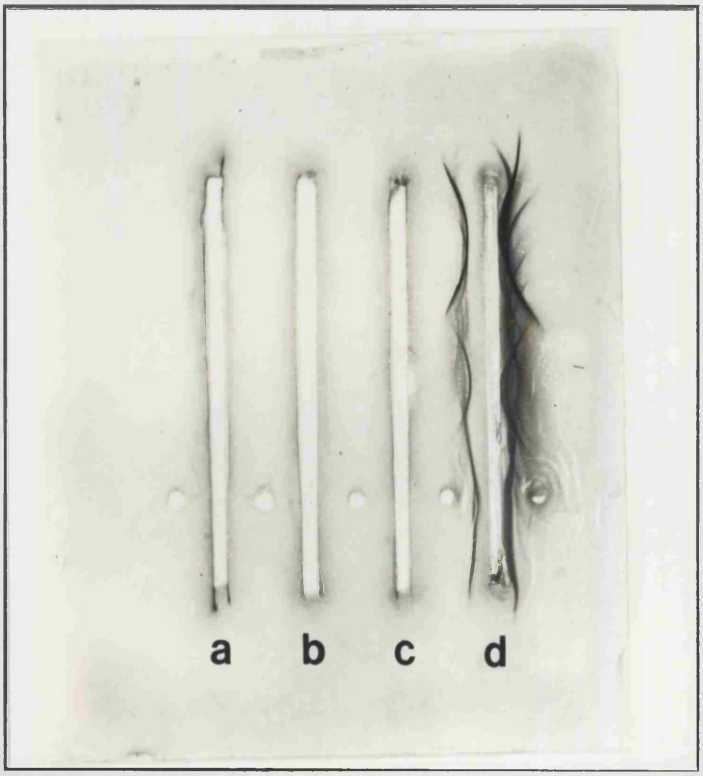


Fig. 5.8 Results of repeat immunoelectrophoresis following further immunisation of rabbits. Anti-human antiserum was used as a control.

All wells contained 10 μl of tubal flushing. Troughs (a) and (c) contained rabbit 1910-A antiserum and (b) contained rabbit 1911-B antiserum.

5.4 DISCUSSION

Human tubal fluid is notoriously difficult to obtain and this is probably the greatest obstacle for investigators in gathering data on tubal secretions and proteins. Moghissi (1970) and Lippes et al (1972) described a technique for collection of tubal fluid which involved insertion of a small catheter into the fimbrial end at the time of tubal ligation or surgery. In the latter study, the majority of patients produced 1 to 2 ml per day, but the greatest quantity of fluid was obtained near the day of ovulation from those patients with the longest Fallopian tubes. Such a technique may be difficult to employ in current gynaecological practice due to changes in patients' expectations and the introduction of laparoscopic procedures which have transformed tubal ligation into a single day procedure. Moreover, a surgical procedure would interfere with local blood supply and any negative pressure may affect the production and composition of tubal fluid. Similarly, the presence of the catheter for a prolonged period of time may lead to an inflammatory reaction in the tube and potential contamination of the tubal fluid. In addition, these techniques do not allow the investigation of each anatomical section of the tube. In the early stages of the studies described in this thesis, transcervical cannulation of the Fallopian tube under transvaginal ultrasound guidance, as described by Jansen (1987), was attempted by this investigator in order to collect tubal fluids (unpublished observations). Several problems were encountered including; (1) difficult passage of the fine cannula into the tubal ostia resulting in kinking of the fine tip, (2) patients experiencing moderately severe pain, and (3) insufficient amount of fluids being collected. This investigator abandoned this approach and attempted instead to obtain tubal fluid by flushing one end of the tube and collecting the secretions in a sterile container for analysis. This is similar to the studies of Wang and Brooks (1986) who successfully flushed uterine horns and oviducts of rats to study oviductal and uterine luminal fluid proteins.

An alternative approach would be to study oviductal protein synthesis and release or secretion by incubating tissue in a short-term explant system with a radiolabelled amino acid, which would be incorporated into newly synthesized proteins (Hyde and Black, 1986; Fazleabas and Verhage, 1986; Verhage and Fazleabas, 1988; Verhage et al, 1988; Maguiness et al, 1992a, 1993). Both approaches have their advantages

and disadvantages. The former requires relatively large quantities of protein content to quantify and determine their nature, while the latter in vitro studies require adequate control experiments and also may not reflect accurately secretory processes taking place in vivo under the effect of dynamic hormonal milieu.

In the studies carried out in this thesis, discontinuous 1-D PAGE of tubal luminal fluid demonstrated the presence of two non-serum protein bands (Bands A and B) with a suggested molecular weight in the region of 115-140kDa for Band A and 49-69kDa for Band B. Wagh and Lippes (1989) reported identification of four proteins in the human Fallopian tube fluid ranging from 44-150kDa, which appeared to bind to the body and/or head of sperm. Using short term explant studies, Verhage et al (1988) identified two proteins, 120-130kDa, at mid-cycle in the mid region of the human Fallopian tube, and 100-130kDa glycoproteins in the oviducts of oestradiol-treated ovariectomized baboons (Verhage et al, 1989). However, Maguiness et al (1992a,b), using short term explants of tubal mucosa, identified two proteins (17, 25 kDa) which were of lower molecular weight and were present during the ovarian cycle. Immunochemical reaction on Western blots had also provided evidence for the synthesis and release of PP4 and PP7 throughout the ovarian cycle with PP14 only in the late luteal phase (Verhage et al, 1988).

During the study period from August 1988 to September 1991, attempts to determine the nature of these proteins and generate antibodies for subsequent assays were essentially unsuccessful. It should be acknowledged that absence of precipitation lines with immunodiffusion or immunoelectrophoretic techniques may simply be due to an inadequate antigen:antibody ratio, and thus it may not be possible to say conclusively that no antibodies were produced. Other possible explanations include; (1) the proteins obtained from polyacrylamide gels under study were not immunogenic enough to generate antibodies in general, and more specifically in the rabbits, (2) protein conformation may have been affected after 1D-PAGE, (3) insufficient amount of protein in the tubal lumen flushings in individual samples, (4) the amount of antibodies generated was extremely low and insufficient to interact with the tubal proteins, (5) technical deficiencies in the methodology employed to generate the antibodies, or (6) a combination of all of the above. The most likely

explanation is that there was not enough protein in the bands obtained from the gels and hence the outcome of these experiments might have been enhanced by pooling all the samples together,

The above studies utilised techniques that have been in use for many years. More recent innovations include the use of monoclonal antibodies directed against specific protein molecules (Seif, 1989) or the use of recombinant complementary deoxyribonucleic acid (cDNA) techniques (Donnelly et al, 1991). Seif (1989) described a different approach to investigate cyclical secretory patterns of the human endometrium. He generated a panel of mouse MOAB following a schedule of immunisation with intact epithelial cells. The MOAB were used to demonstrate cycle-dependent and independent endometrial epithelial cells surface markers, characterise them and determine their relationship to ovulation. The information provided molecular criteria for analysis of endometrial secretory differentiation and suggested the existence of defect(s) that may be associated with female subfertility. Donnelly et al (1991) were able to prepare a recombinant cDNA library and ultimately isolated the messenger ribonucleic acid (mRNA) responsible for the production of a baboon oestrogen-dependent oviduct-specific protein. More recently, the same group (Arias et al, 1994) was able to use the same techniques to clone, sequence and characterise the cDNA to a human oestrogen-dependent Fallopian tube glycoprotein.

Adoption of such approaches in studies of the human Fallopian tube will inevitably provide valuable information on tubal specific proteins, their use as markers of tubal function, and possibly better understanding of their role in the complicated reproductive processes in the oviduct.

CHAPTER 6-I
NEED FOR THE CLINICAL STUDY

6(I).1 INTRODUCTION

Evidence of the growing importance of assisted reproduction in the treatment of infertility, and concern that such procedures should be effective is given by the increase in the number of published reports of clinical trials. Unfortunately, the increase in number of clinical trials has not been matched by an increase in the strength of research design (Tulandi and Cherry, 1989).

Several treatments for infertility have been promoted with little objective data demonstrating their therapeutic value. Once suggested, these treatments seem to generate a momentum independent of their demonstrated benefit, assisted by the lay medical press and well-meaning practitioners. Similarly, newer options such as GIFT and ZIFT or TET have been used in many clinical situations despite the absence of supporting data. There is a lack of randomised clinical trials including the use of appropriate control groups to ascertain the efficacy of these therapeutic modalities in a specific clinical setting. It is also true that many investigators are discouraged by the large number of patients required to demonstrate unequivocally the increased pregnancy or live birth rates. In spite of this, the use of sophisticated reproductive techniques **should not** alleviate the need to demonstrate efficacy.

6(I).1.1 CONFOUNDING VARIABLES

It is well known that IVF-UET initially assisted patients with damaged or occluded Fallopian tubes. Subsequent experience with the technique of oocyte and sperm preparation led to the recognition that, in some other infertility conditions with healthy Fallopian tubes, IVF was effective by simply increasing the proximity of the prepared spermatozoa to the oocytes. However, the choice of any of the assisted reproductive techniques depends on a balance of factors. The cause of infertility and the choice of treatment are seldom absolute as it is for bilaterally occluded and irreparable Fallopian tubes, and mostly include varying degrees of subfertility.

Similarly, an important facet of the diagnosis is accurate quantitative prognostication of a couple's chance of conceiving, to help them decide whether they have a realistic hope and whether treatment offers sufficient chance to make its choice worthwhile. In most cases, therefore, the choice of treatment must take into account several factors including the chance of conceiving naturally without treatment, the relative chance of success with other treatments, the relative cost and complexity of alternative methods, the age of the woman, and the diagnostic value of IVF, particularly in cases of suspected sperm dysfunction.

6(I).1.2 TO TREAT OR NOT TO TREAT

It is essential to appreciate the chance nature of fertility, and the relative inefficiency of human reproduction. The average conception rate in populations of proven fertility is only $\approx 20\%$ in each ovulation cycle (Hull, 1990). Women's fecundity and the time they require to conceive varies considerably, depending on age, cause of infertility and multiplicity of factors contributing to their infertility. It can take a young, nulliparous, fertile woman only 2-3 months to conceive, while a 35 year old patient with treated endometriosis and receiving donor spermatozoa may need up to 7 years to conceive spontaneously (Jansen, 1987).

Unfortunately and until recently, there has been no clear indication from published reports of the place of assisted reproduction techniques in the general context of infertility medicine, because the results of individual treatments are reported selectively and because of rapid historical changes. It is essential when attempting to ascertain whether or not treatment is required that time-specific or cycle-specific conception rates are used. Crude pregnancy rates per couple are almost meaningless. Pregnancy rates per cycle can also be misleading if limited to the first cycle or two because the rate may fall in subsequent cycles. The cumulative conception (CCR) or live birth rates (CLBR) should be used whenever possible. A recent review (Hull, 1992) compared CCR for some of the commonest causes of infertility in the untreated population or following treatment with conventional methods. It demonstrated that in some conditions such as amenorrhoea and oligomenorrhoea or in those being treated with donor insemination, the CCR following conventional therapy is almost

the same as in normal women. However, in other circumstances, such as salpingostomy for distal tubal occlusion or the use of high dose glucocorticoids for seminal antisperm antibodies, the prognosis is worse and the CCR are lower than what can now be expected in a single cycle of IVF treatment.

It remains to consider the balance of choice between various treatments in different conditions with due consideration to the factors leading to infertility. Most likely, the only unfavourable conditions when deciding on a treatment are the woman's age, 40 years or more (Hull et al, 1992), and sperm dysfunction (Sharma *et al.*, 1988; Craft, 1990).

When sperm dysfunction is strongly suspected, IVF serves both diagnostic and therapeutic purposes and is the treatment of choice. Similarly, in women with blocked tubes, the choice is clearly IVF with uterine embryo transfer. In other circumstances such as minimal endometriosis and unexplained infertility, where the pelvis and Fallopian tubes are essentially normal, the need for treatment and the choice of a treatment modality becomes more difficult. Here, the duration of the couple's subfertility, the factor(s) contributing to it and their overall wishes will influence the decision on whether or not to treat.

6(I).2 CHOICE OF TREATMENT

There is no treatment of proven benefit to help couples with unexplained infertility **to conceive naturally**, apart from benefit of using ovarian stimulation with clomiphene (Fisch et al, 1989; Glazener et al, 1990). There is also conflicting evidence on the effectiveness of ovarian stimulation with or without artificial insemination. Dodson and colleagues (1987) reported that ovarian stimulation combined with intrauterine insemination (SO-IUI) yielded a cycle fecundity which approaches that of normal women and equals or exceeds that reported with the uterine transfer of embryos during IVF and with GIFT. Serhal et al (1988) in a non randomised study reported beneficial effect of SO-IUI in women with unexplained infertility. However, others (Martinez et al, 1991), suggested that IUI was not beneficial when human menopausal gonadotrophin (hMG) was used for stimulation

and advised that IUI in natural cycles is the treatment of choice. More recently, Crosignani et al (1991) in a comparative, controlled, randomised study of five methods for the treatment of unexplained infertility (ovarian stimulation alone, intra-uterine insemination, intra-peritoneal insemination-IPI, GIFT, and IVF-UET), reported much improved pregnancy rates from each method compared to spontaneous pregnancy rates, but there was no evidence showing the superiority of any of the individual invasive methods. Variation in methodology among the various participating centres in this last study may have seriously affected the outcome and thus renders interpretation of the results difficult (Amso and Shaw, 1992a).

Several factors may account for the lack of uniformity among these reports, including variation in the selection criteria and the intensity of investigations performed before a diagnosis of unexplained infertility was achieved, differences in the study design, ovulation induction techniques, sperm preparation, timing and number of inseminations, and finally non uniformity of end points and reporting methodology.

The only treatments of proven and documented value in unexplained infertility are the assisted reproduction methods. In a retrospective study, Simon et al (1991), compared superovulation with gonadotrophins with or without artificial insemination and IVF-UET in the treatment of couples with unexplained infertility. He reported a pregnancy rate for one cycle of IVF-UET to be equivalent to CCR following three treatment cycles by ovulation induction. However, the CLBR of three treatment cycles of ovulation induction was greater than the one cycle of IVF-UET. A more recent retrospective analysis (Hull et al, 1992) reported higher pregnancy rate following GIFT as compared to ovarian stimulation and intrauterine insemination in women under 40 years of age with unexplained infertility (36% and 18%, respectively). In the same study the corresponding CCR and CLBR after two treatment cycles were equally higher for GIFT. Others have also reported significantly better results for GIFT than SO-IUI (Kaplan et al, 1989) and three treatment cycles offered a greater chance of pregnancy achieving a CCR of 0.56 (Iffland et al, 1991). Similarly high CCR (0.52) after three cycles of GIFT when compared with a spontaneous CCR of 0.30 after 24 months without treatment were reported by Murdoch et al (1991). The same authors also demonstrated a

significantly higher "average" monthly fecundity in the treated group (0.14 versus 0.01, $P<0.001$). Although other studies have reported similar rates of spontaneous pregnancies among couples with unexplained infertility (Lenton et al, 1977; Templeton and Penney, 1982; Collins and Rowe, 1989), Murdoch's (1991) study is the only one which demonstrates benefits from treatment versus no treatment within the same group of patients. Clearly, given enough time, the spontaneous CCR would be similar to three attempts of GIFT, but since many women are >30 years of age at the time of presentation, "no treatment" would be unacceptable advice for most couples.

6(I).3 EVALUATION TECHNIQUES: CLINICAL TRIALS

Several of the assisted reproductive techniques have been suggested to be superior to others. Many investigators report considerably higher pregnancy rates following GIFT and even higher pregnancy and implantation rates following ZIFT or TET than after UET or GIFT (Table 6.1). Unfortunately, many of these reports are either non-randomised studies or retrospective analyses, have no clearly defined inclusion criteria, have no controls or use only historical controls, or suffer from a combination of the above faults. It would be meaningless, for example, to compare women with tubal infertility undergoing IVF and women with unexplained infertility having GIFT. Similarly, it is impossible to draw any conclusions from comparing GIFT and ZIFT or TET, as the establishment of fertilisation and its normality are impossible to verify in the former. Thus, with the rapid proliferation of treatment modalities, it is necessary to evaluate objectively any new treatment in a properly designed and conducted clinical trial.

Randomised controlled trials remain the hallmark of a scientific evaluation in clinical medicine. However, acceptance of the need for more rigorously planned and executed investigations has been slow. Recent surveys (Olive, 1986; Tulandi and Cherry, 1989) found that only a small proportion of therapeutic fertility trials published in a specialist journal were randomised and controlled! Several concerns persist while designing a clinical trial. These include; (1) clear objectives and proper design with relevant end points (measures of outcome), (2) well defined inclusion and

exclusion criteria to minimize the influence of different pathologies on the outcome, (3) consideration of the size of the clinically important difference between the test population and the control group, and (4) efficient statistical designs in order to improve the power of detecting a significant difference.

6(I).4 CLINICAL TRIAL

In the design of the current clinical study, the above issues as well as other concerns related to a "crossover" design were taken into consideration. The main features of the trial and the methodology employed will be described in detail in section 6.II.

Table 6.1. **Review of published reports (1987-1992) comparing pregnancy and implantation rates for IVF-UET, GIFT and TET**

Author	Study design			Controls			Patient selection & (number)#	Pregnancy rate (%)§¶				Implantation rate (%)			
	Pr	Re	Ra	None	Conc	Hist		UET	GIFT	TET	p	UET	GIFT	TET	p
Leeton '87	X		X		X		UI (64)	20%	19%	---	NS				
							male (11)	28%	33%	---	NS				
Balmaceda '88	X			X			male (10)	---	---	60%		---	---	28%	
Hamori '88	X			X			failed IVF	---	---	28%		---	---	12%	
							UI, male & endometriosis (42)								
Yovich '88	X				X		none (377)	12%*	36%*	37%*	<0.001	4%*	11%*	17%*	<0.001
Deveroey '89	X			X			UI (54)	---	---	48%		---	---	24%	
Palermo '89	X			X			male (42)	---	---	48%		---	---	22%	
MRI-SART '92	X				X		none (18,923)	21%	30%	27%					
Hammitt '90	X				X		none (172)	28%*	54%	54%	<0.05	11%*	18%	20%	<0.05
Tanbo '90	X		X		X		UI, male & endometriosis (106)	46%	26%	38%	NS				
Pool '90		X			X	X	none (281)	21%*	32%	40%	<0.001	8%*	11%	17%	<0.05
Bollen '91		X			X		none (1064)	28%	19%*	38%	<0.05	14%	8%*	18%	<0.01
Tournaye '91		X			X		male (193)	29%	18%	33%	NS				
Amso '91	X		X		X		UI, male & endometriosis (97)	17%	---	29%	NS	9%	---	16%	NS
Asch '91	X				X		Tubal	22%	---	53%	<0.001	9%	---	18%	<0.001
							male, immune & failed GIFT (358)								
Balmaceda '92	X		X		X		Ovum donor (42)	55%	---	58%	NS	17%	---	21%	NS
Tournaye '92b	X		X		X		male (80)	22%	---	27%	NS	10%	---	12%	NS
Toth '92	X				X		nontubal (78)	34%	---	29%	NS	11%	---	10%	NS

Pr= Prospective, Re= Retrospective, Ra= Randomised, Conc= Concurrent, Hist= Historical, UI= Unexplained infertility, §= pregnancy rate per embryo transfer (UET/TET), ¶= pregnancy rate per oocyte replacement (GIFT), p= significance level, *= denotes a value that is significantly different from the other two values, #= total number of all replacement cycles reported in the study

CHAPTER 6-II
TRIAL DESIGN AND CLINICAL METHODOLOGY

6(II).1 CLINICAL TRIAL DESIGN

6(II).1.1 AIM

This trial's principal aim was to test the hypothesis that following IVF, tubal embryo transfer was more conducive to the establishment of pregnancy than uterine transfer. We utilised a newly introduced, and presumably highly successful therapeutic modality "ZIFT/TET" (zygote intrafallopian transfer/tubal embryo transfer) for this randomised, controlled trial.

6(II).1.2 MAIN FEATURES OF THE TRIAL

A randomised, controlled crossover trial was planned to test the above hypothesis. The following is an outline of the main features of the trial;

(1) All couples recruited for the trial had been thoroughly investigated and found to be suitable for both modalities of treatment. Those with purely unexplained infertility had been trying to achieve a pregnancy spontaneously for > 2 years before entry into the study.

(2) The estimated required number of treatment cycles under each arm of the study was based on an anticipated improvement of $\geq 20\%$ in pregnancy rate between the two treatments as judged by the initial reports of pregnancy rate of 48% per transfer (Devroey et al, 1989; Palermo et al, 1989) for ZIFT and an average pregnancy rate of $\approx 18\%$ per embryo transfer for conventional IVF-UET treatment in our unit during the preceding two years (Shaw, unpublished data). The test size (significance level) and power were set at 5% and 80% respectively. Details of the protocol are outlined in Appendix 1

(3) Ethical approval was obtained from the Royal Free Hospital ethical committee.

6(II).2 PATIENTS

6(II).2.1 SUBJECTS RECRUITMENT

Patients with history of infertility, referred by their General Practitioners or Consultant Gynaecologists, attended the infertility clinic under the care of Professor R W Shaw at the Royal Free Hospital. Appropriate history was taken, followed by physical examination of both partners. All details were entered in special pre-printed forms. The following investigations were carried out for both partners:

Female partner;

- Baseline hormone profile (menstrual phase FSH, LH and Prolactin)
- Mid-luteal serum progesterone (day 21 of a 28 day cycle)
- Laparoscopy and dye hydrotubation
- Hysterosalpingography
- Serum antisperm antibodies
- Rubella, HIV status
- Post coital test

Male partner;

- Semen analysis
- Serum antisperm antibodies
- Hamster egg penetration test (in selected cases only)

The cause of infertility was classified according to the outcome of the above investigations as follows;

(1) idiopathic infertility; where all the tests proved to be negative,

(2) male factor infertility; as defined by the W.H.O. (1987) criteria of normal sperm analysis with count of $\geq$20 million/ml, progressive motility of $\geq$40%, and normal morphology $\geq$40% at the time of preliminary analysis.

(3) Endometriosis (mild, moderate, severe) as determined by Laparoscopy (Revised AFS stages II-IV). The patients recruited into this study included women with lesions not involving the Fallopian tubes (mild or moderate endometriosis) whether requiring prior treatment or not.

(4) Immune factors (presence or absence of antisperm antibodies in the male or female partner on two occasions). Males or females with antisperm antibodies were included in the corresponding category (e.g male or female) rather than a separate subgroup.

(5) Multiple factors (any combination of the above).

6(II).2.2 PATIENT ASSESSMENT FOR ASSISTED REPRODUCTION THERAPY

All couples underwent thorough assessment before they were entered into the trial. This included detailed explanation of the proposed treatment, the experimental nature of the trial, risks associated with superovulation protocols, the low success rate of IVF in general, and the psychological and emotional stresses associated with the treatment. All couples agreed to be screened for hepatitis B, human immuno deficiency Virus and venereal disease. All couples signed consent forms agreeing to participate in the proposed trial (Appendix 1).

6(II).3 MANAGEMENT OF THE TREATMENT CYCLE

6(II).3.1 STIMULATION PROTOCOL

A standardised stimulation protocol utilising a gonadotrophin releasing hormone agonist (GnRH-a), buserelin (Hoechst, Germany) and gonadotrophin injections (pure FSH or hMG) was adopted. In all patients the GnRH-a was commenced in the mid-luteal phase (Day 22) of the preceding menstrual cycle (=Day 1 of treatment) and continued until desensitisation of pituitary activity was achieved (long desensitisation protocol). The onset of menses and quiescence of the ovaries on ultrasound scanning (regression of all follicles to less than 10 mm and absence of endometrial echo) indicated pituitary/ovarian suppression and marked the commencement of gonadotrophin injections. This period of hypogonadotrophic hypogonadism (down-regulatory phase) was varied to enable a set number of treatment cycles to be started on a predetermined day and facilitate programmed oocyte retrieval. Gonadotrophin injections were usually started on a Wednesday or Thursday (Day +1 of treatment) and the concomitant administration of buserelin nasal spray was continued until the

time of the human chorionic gonadotrophin (hCG) injection (Serono, UK). Oocyte collections usually took place on a Monday, Tuesday or Wednesday 13-15 days after commencement of the injections.

6(II).3.2 MONITORING OF THE TREATMENT CYCLE

The principal method for monitoring ovarian response was by vaginal ultrasound scans. A baseline scan was carried out prior to commencement of gonadotrophin injections to confirm quiescence of the ovaries, otherwise the GnRH-a was continued and the treatment deferred until the cysts disappeared. Subsequently, scans were repeated on day +6 or +8 of stimulation and the gonadotrophin dosage was adjusted according to ovarian response. HCG (5000 IU) was administered intramuscularly 34-37 hours prior to oocyte collection, when three or more follicles reached 18 mm in diameter. A sample follicular growth chart (folliculogram) is depicted in Figure 6.1.

6(II).4 VAGINAL EGG COLLECTION (VEC)

6(II).4.1 EQUIPMENT

The ultrasound machine (Diasonics DRF250) was equipped with a slim line vaginal probe (1.5 cm diameter, 7.5 MHz crystal). The ultrasound transducer (Figure 6.2) was enclosed in a special sterile condom and plastic sleeve prior to insertion into the vagina. The aspiration needles had double lumina to enable aspiration and flushing through different routes, a very sharp tip to enable easy puncture of mobile ovaries and a roughened distal 2 cm to enhance ultrasound visualisation. The needle was connected by tubing to a test tube to which suction was applied by a foot operated pump.

6(II).4.2 ANAESTHESIA

Vaginal egg collections were performed under general anaesthesia or under intravenous sedation with Diazemuls (diazepam 5 to 12.5 mg, Kabi Vitrum) and pethidine (75 to 125 mg, Roche products). The dose of the latter varied according to the patient's tolerance and time taken to complete the operation.

6(II).4.3 TECHNIQUE

All patients received metronidazole as antibiotic prophylaxis, 1g per rectum (May and Baker) one hour before oocyte recovery. In the operating theatre, the patient was placed in the lithotomy position and the legs draped. The vagina was cleaned carefully with 0.5% solution of chlorhexidine acetate B.P. and then rinsed with sterile water. The assembled transducer and needle guide was gently introduced and the ovaries were carefully scanned to determine the plane which will enable the best access to the largest follicles. The needle was introduced via the needle guide and then pushed through the lateral vaginal vault and into the first follicle by a single, firm thrusting movement. Suction was applied as soon as the needle tip was seen to be within the follicle which collapsed as the follicular fluid was aspirated (Figure 6.3). During aspiration, the needle was rotated and moved gently in all directions within the follicle to increase the chance of oocyte retrieval. If the oocyte was not retrieved in the first aspirate, the follicle was flushed several times with Earle's Balanced Salt Solution (Gibco Ltd, UK) supplemented with benzyl penicillin, streptomycin, pyruvate sodium bicarbonate, HEPES and heparin. Once the egg was obtained, the needle was aligned against a neighbouring follicle without removing it from the ovarian surface. Unanesthetized patients experienced pain when the external follicular surface was entered. However, the procedure was generally well tolerated and usually completed in about 30 minutes. At the end, any remaining fluid in the pouch of Douglas was aspirated, the vaginal vault was inspected to exclude bleeding from puncture sites and the ultrasound transducer was thoroughly cleaned with a damp cloth. The patients usually recovered quickly, and left the hospital after a few hours.

6(II).5 LABORATORY TECHNIQUES

6(II).5.1 PROTOCOL FOR MEDIA PREPARATION

All glassware and equipment were cleaned in "7X" solution and rinsed several times in sterile water then baked at 150°C for 7 hours. Aspiration needles and tubing sets were cleaned as above then autoclaved at 126°C for 3 minutes.

The embryo culture and flushing media were prepared from a basic IVF media recipe as follows:

(A) Basic IVF media

1. Dissolve 8.65 g of Earle's balanced salt -EBS- (GIBCO, UK) in 200 ml of sterile water, then add 1.0 mg of Sodium Bicarbonate, 60 mg Benzyl Penicillin, 11 mg Sodium Pyruvate and 50 mg Streptomycin. The colour changes from yellow to red.
2. Add sterile water to one litre mark and mix well.

(B) Culture media

1. Dissolve 0.22 g of Sodium Bicarbonate in 200 ml of basic IVF media and mix well.
2. Determine osmolarity using an Osmometer (Osmette A, U.K.) and adjust to 282-285 m mol/kg.
3. Store in refrigerator in 200 ml Falcon flask.
4. On the eve of the egg collection, filter 50 ml of media into a 50 ml flask using an 0.2 μm Acrodisc PF syringe filter (Gelman Sciences, USA) and gas with 5% CO_2 until there is a purple colour change.
5. Add 2 ml of thawed 20% human serum albumin (HSA).
6. Aliquot 1 ml into 5 ml test tubes and incubate at 37°C until oocyte retrieval.

(C) Flushing media

1. Add 16 ml of HEPES buffer solution 1M (GIBCO, U.K.) and 1.6 ml of heparin to 800 ml of basic IVF media and mix well.
2. Filter through a 0.2 μm filter and store in 200 ml flasks in a refrigerator at

4°C. Place the flasks in an incubator on the eve of the VEC.

6(II).5.2 SPERM PREPARATION

The semen sample was produced by masturbation and prepared according to the following protocol;

(1) The sample was allowed to liquify at room temperature for 30 minutes. Sperm density and total percent motility were determined using a Makler counting chamber (X100) while the percentages of abnormal forms and sperm progression were determined using a microslide (X400) mounted on a Zeiss compound microscope.

(2) One ml of sperm sample was added to 4 ml of Earle's balanced salt solution, EBS (GIBCO, U.K.) and 20% HSA. The sample was mixed by inversion and centrifuged for 15 minutes at 1200 revolutions per minute. The supernatant was discarded and the pellet re-suspended. Four ml of fresh media were added and the procedure was repeated again. The pellet was gently layered with 1 ml of media and the tubes were placed in the incubator. The sperm were allowed to swim up for 30 minutes before further analysis.

(3) Sperm parameters of the swim-up were determined (density, motility and progression) and the final concentration was adjusted to $1X10^6$/ml. The sample was kept in the incubator until the oocytes were inseminated (100,000 sperm or 0.1 ml per oocyte).

6(II).5.3 OOCYTE/EMBRYO HANDLING

The follicular aspirate/flush was poured into a culture dish and examined under a Stereo Zoom microscope at X25 magnification. The oocytes were identified and then washed with flushing media. They were classified into mature, intermediate and immature according to cumulus dispersion, appearance of the corona radiata, and nuclear status. The oocytes were transferred into clearly labelled test tubes containing 1 ml of EBS + 20% HSA and placed into a heated block in a warmed portable incubator until the oocyte collection was completed. The test tubes were

transferred to the main incubator for further incubation for approximately 5 hours at 37°C, depending on their maturity, then inseminated as described above.

The oocytes were examined 16-20 hours after insemination for signs of fertilisation. The cumulus cells were removed by gently steering the oocytes in and out of a finely drawn pipette and the presence of two or more distinct pronuclei was regarded as evidence of fertilisation (Figure 6.4). The oocytes or zygotes were transferred back to test tubes containing fresh culture media and returned to the incubator. Embryonic development was assessed again at 48 hours after insemination and the embryos showing the best morphological features (evenly divided blastomeres, absent anucleate fragmentation) were selected for transfer (Figure 6.5).

6(II).6 EMBRYO TRANSFER

6(II).6.1 TRANSFER MEDIA AND LOADING OF THE CATHETERS

A special transfer medium was prepared for each patient using 1 ml of EBS and 2 ml of the patient's own heat deactivated serum. The solution was mixed, filtered and then incubated at 37°C for 30 minutes to allow for equilibration (pH, electrolytes, proteins) before the zygotes or embryos were added.

The embryos were loaded into a Wallace embryo transfer catheter (Wallace, London) for uterine embryo transfers or into a GIFT catheter (Rocket of London Ltd) for Fallopian transfers. The catheters were rinsed thoroughly with the special transfer medium and then loaded in the following sequence, (1) 0.1 ml of air, (2) embryo(s) in 0.1 ml of media, and (3) 0.01 ml air.

6(II).6.2 UTERINE EMBRYO TRANSFER TECHNIQUE

All uterine embryo transfers took place approximately 52-56 hours after the egg collection. The patient was placed in the dorsal position. The legs were covered and a sterile speculum was introduced into the vagina to expose the cervix. A warm and sterile water solution was used to clean the vagina and cervix. The catheter was gently introduced into the cervical canal and through the internal os into the uterine

cavity. When the tip of the catheter touched the uterine fundus the patient could experience a slight degree of discomfort. The catheter was then withdrawn by 1 cm and the embryos were injected. The catheter was removed and checked under the microscope to ensure that all embryos had been transferred. The patient rested for approximately 2-3 hours and was then allowed to go home.

6(II).6.3 PELVIC ASSESSMENT AND FALLOPIAN EMBRYO TRANSFER TECHNIQUE

All transfers were carried out at laparoscopy and under General Anaesthetic. At operation, the pelvis was examined thoroughly for signs of trauma, haematoma formation or other findings that had not been previously reported. Particular attention was paid to the presence of peritubal or periovarian adhesions that could have been attributed to repeated trauma during egg collections. Residual fluid collection in the pouch of Douglas was aspirated, the volume measured and two samples were sent for (1) microscopy, general culture/sensitivity and (2) chlamydia culture. The samples were also cultured in blood agar for aerobic organism and in selective anaerobic agar, New York City medium, McConkey agar and Trichomonas medium (37°C overnight). Chlamydia culture was performed by a standard technique (Ripa and Mardh, 1977).

Fallopian transfer (maximum 3 zygotes/embryos) took place the day after the egg collection (approximately 30 hours) if three or more oocytes had shown evidence of fertilisation (Day one transfer). Otherwise, the oocytes were examined again the following day and a maximum of three embryos were transferred (Day two transfer). If the tubes appeared normal and free, the fimbrial end was grasped from the antimesenteric surface with an atraumatic grasping forceps and the tube was catheterised using a " GIFT- catheter introducer set " (Rocket of London Ltd). The set consisted of a cannula and an obturating component to ensure smooth tubal cannulation. The zygotes/embryos were deposited in the mid ampullary region approximately 3-4 cm from the fimbrial end. Post operative care followed the standard practice and the patients were discharged home the following day.

All patients were given luteal phase support. Generally, progestogen support in the form of Cyclogest (Progesterone) 200 mg rectal suppositories (Hoechst, Germany) was given twice daily for 18 days. If pregnancy was established, the suppositories were continued until the fourteenth week. On other occasions, depending on the clinician reviewing the patient on the day of embryo transfer, hCG (2,000 IU) was administered intramuscularly on the day of ET, and two further doses at three days' interval were given for luteal support.

6(II).7 PREGNANCY

The patients were instructed to report any delay of their periods after a treatment cycle. If the period was 3-4 days late (ie. 18-19 days after embryo transfer), a highly sensitive urinary ß-hCG test was performed (detection rate >25 IU ß-hCG). A positive test was followed by a transvaginal ultrasound scan 2-3 weeks later (ie. 6.5-7.5 weeks gestation) to confirm the presence or absence, number and site of the gestation sac(s). In this study, a diagnosis of clinical pregnancy was verified by visualising a gestation sac within the uterine cavity at 6.5-7.5 weeks, or by obtaining histological evidence of an extra uterine pregnancy.

6(II).8 STATISTICAL METHODS

Randomisation. Randomisation cards were prepared by an independent statistician from the Department of Clinical Epidemiology at the Royal Free Hospital and were placed in sealed envelopes. The envelopes were opened after the couples' recruitment into the programme and just before starting their treatment.

Data collection and analysis. The data relating to ovarian stimulation and response, oocyte collection and embryology were collected prospectively and entered into special pre-printed forms that were in use in the IVF unit and were subsequently utilised to abstract any information into two specially designed computer sheets (Appendix 2) for data analysis. Epidemiological and clinical data relating to all treatment cycles are listed in Appendix 3 and the laboratory details of the corresponding treatment cycles are listed in Appendix 4.

The hardware consisted of an IBM-compatible 386-SX notebook computer equipped with a math co-processor. Data were entered into a specially written file for the personal computer version of the Statistical Package for Social Sciences (SPSS-PC). Minitab statistical programme was also used for some statistical procedures. Whenever appropriate, the following statistical procedures were used; (1) analysis of variance (ANOVA), (2) cross-tabulation and the construction of frequency tables with ***r*** rows and ***c*** columns - the 'r x c' table, (3) odds ratios and confidence intervals, and (4) logistic regression analysis of the dependent variable with other independent variables (sections 7.4.1.5 and 7.4.1.6).

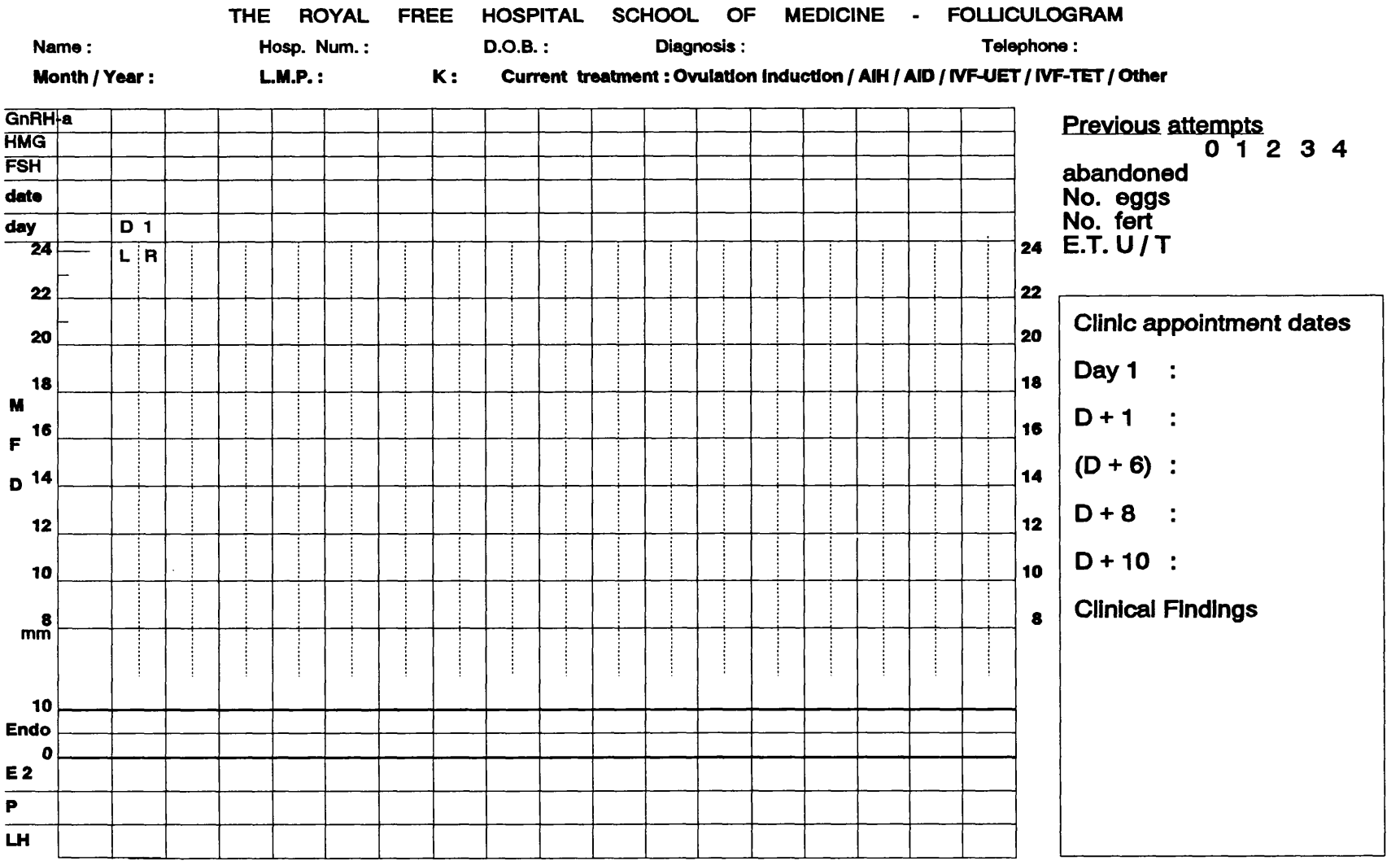


Fig. 6.1 Follicular growth chart-Folliculogram

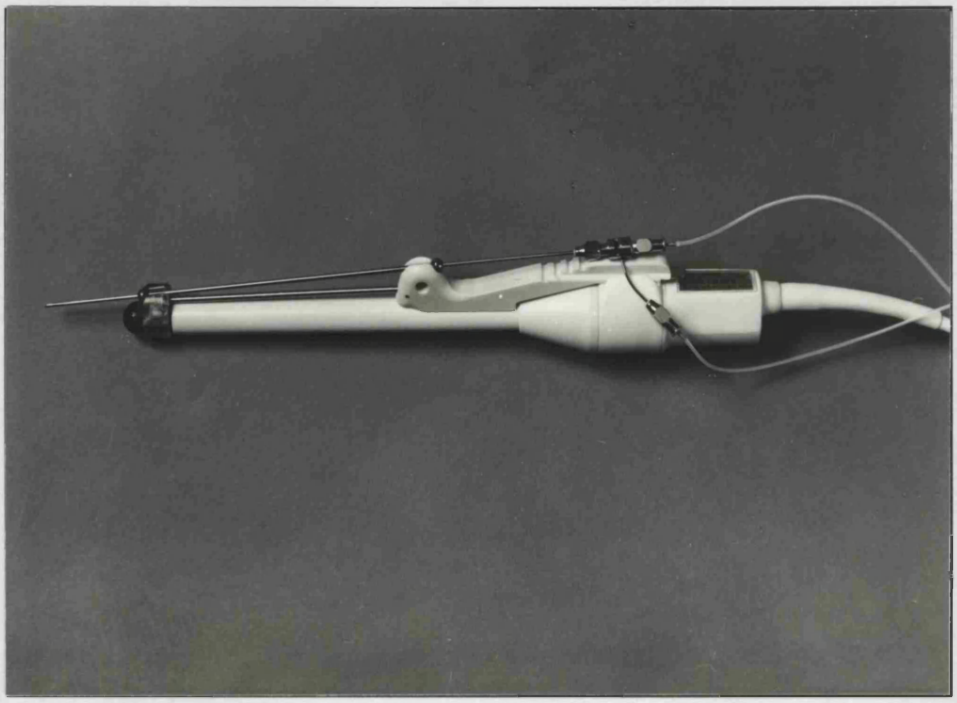


Fig. 6.2 Vaginal ultrasound transducer with needle bracket and guide. The needle tip is protruding from the proximal end of the probe.

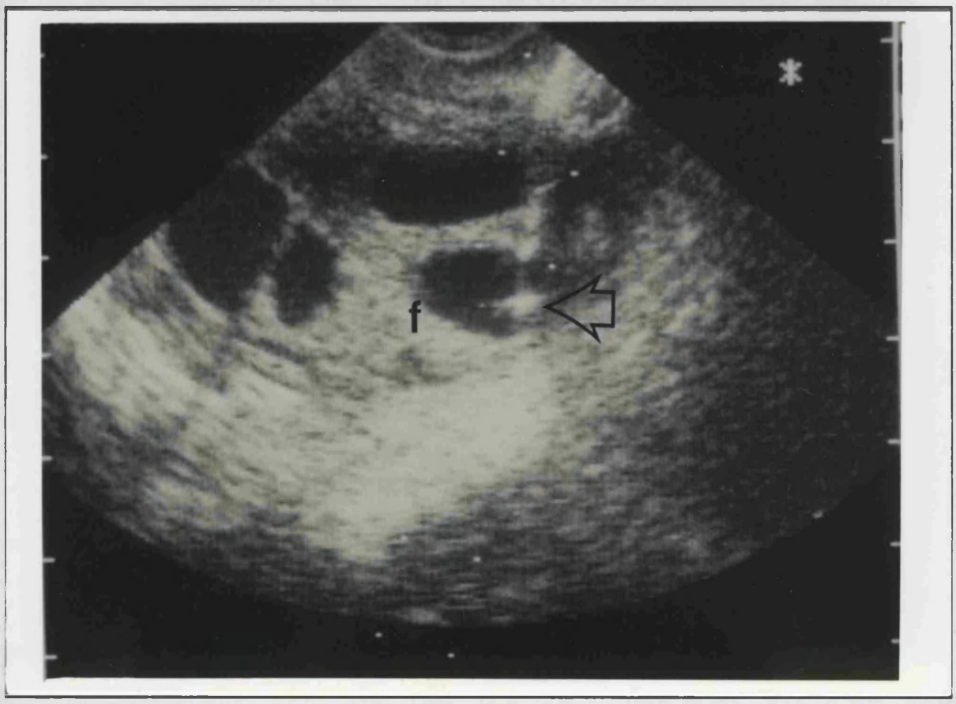


Fig. 6.3 Ultrasound picture during vaginal egg recovery. The tip of the needle (arrow) is seen within the follicle (f). The dots are 1 cm apart.

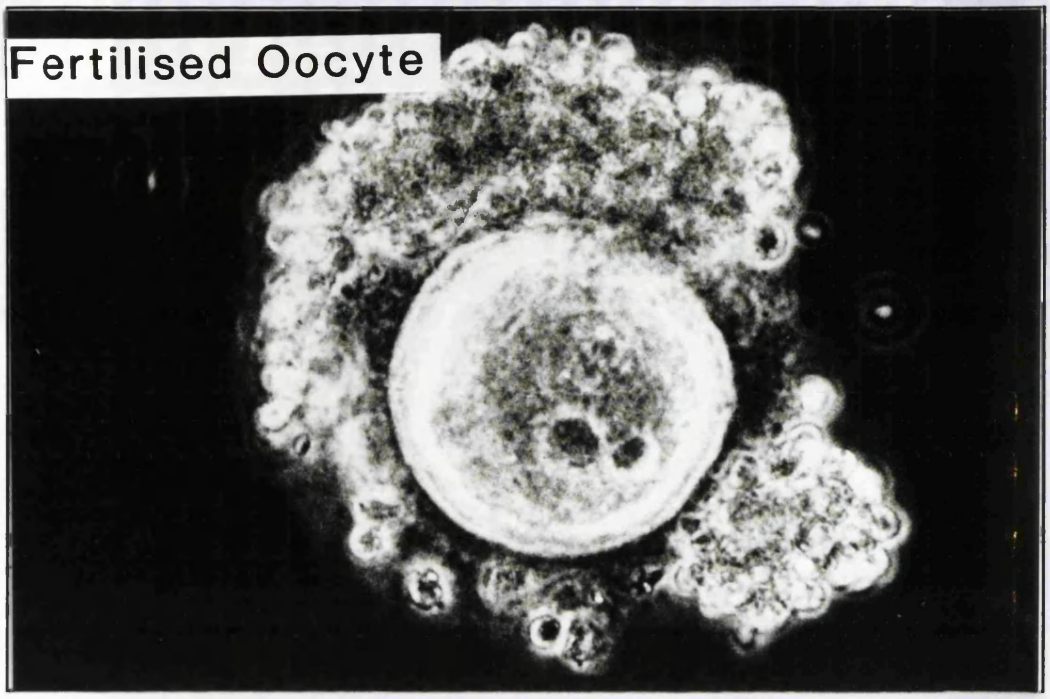


Fig. 6.4 Pronuclear embryo 18 hours post insemination. The two pronuclei are clearly visible.

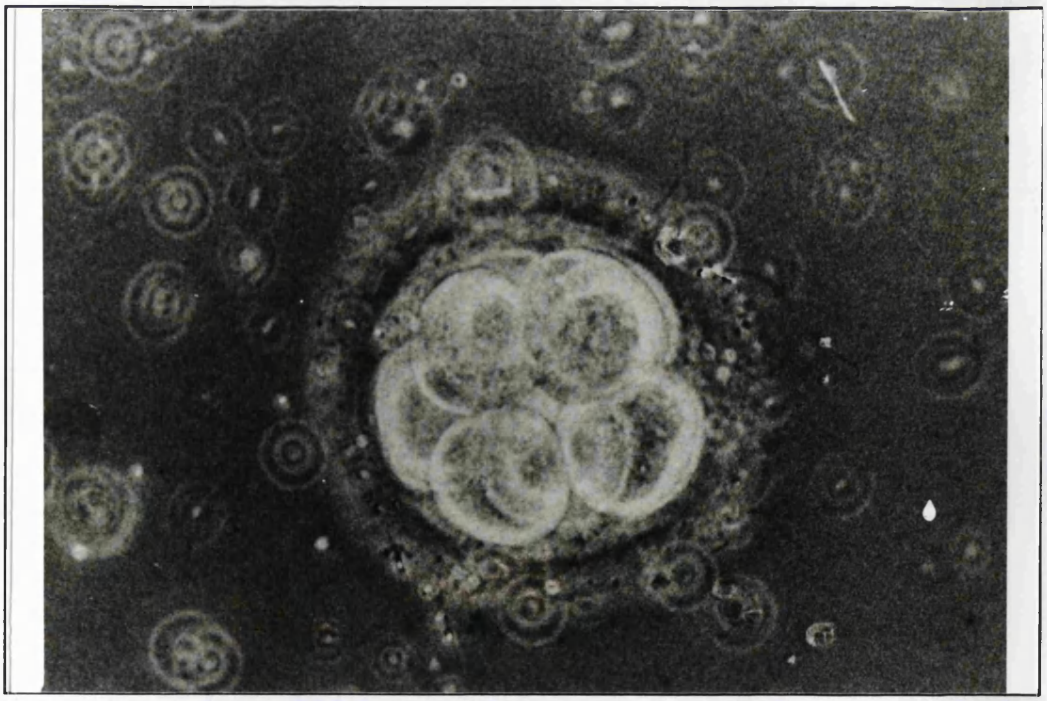


Fig. 6.5 An 8-cell stage pre-embryo. The blastomeres are evenly divided.

CHAPTER 7
CLINICAL TRIAL RESULTS

7.1 INTRODUCTION

Reporting of pregnancy success rates after assisted reproduction techniques has been the subject of considerable debate (Saunders et al, 1989; Wilcox, 1993). The outcome of interest or numerator for pregnancy success rates has several definitions including, biochemical or clinical pregnancy, implantation and live delivery. Similarly several measures of assisted reproduction procedures or denominators are employed but examining their usefulness is more complex. The number of ovarian stimulation procedures, oocyte collections and embryo transfer procedures are all important clinical and research measures of the efficiency of the various steps of an assisted reproduction process in a clinic. Comparison of success rates between different treatment modalities is commonly described as either pregnancy rate of completed cycles and using the number of embryo transfer procedures as the denominator, or the number of live deliveries per 100 embryo transfer procedures. All the above definitions have the important limitations of being summary rates that do not reflect variations in patient characteristics nor the number of embryos transferred per procedure. Such restrictions may be overcome by reporting these rates stratified by clinical and demographic characteristics and detailed descriptions of pregnancy data. This approach has in recent years been employed in the reporting of assisted reproduction results by national registers (USA, Australia, UK) or retrospective analyses of several years experience in individual clinics (Hull et al, 1992; Tan et al, 1992). Others have used life-table analysis with cumulative pregnancy rates in the reporting of success rates in couples undergoing several treatment procedures.

In this chapter, the results of a randomised controlled "cross-over" trial comparing uterine with tubal embryo transfers are reported. The analysis will be stratified by the stimulation attempt that each couple underwent, infertility factors (unexplained, male, female and combined factors), age and duration of infertility at recruitment into the study. In the original design of the trial, it was anticipated that at completion of

all intended treatment cycles there will be sufficient patients completing two arms of the study to obtain cumulative conception rates. This was not achieved due to a number of limitations mainly related to recruitment into the trial of adequate number of suitable couples accomplishing their treatment within the time limitations required to complete this thesis. The first stimulation cycle represents a true parallel design of a randomised controlled trial and thus will be subjected to more detailed analysis. However, all the treatment cycles will be utilised when reporting pregnancy and delivery rates stratified by clinical and demographic characteristics.

7.2 PATIENTS AND METHODS

7.2.1 SUBJECTS RECRUITMENT

These are described in sections 6(II).2.1 and 6(II).2.2.

7.2.2 STIMULATION PROTOCOL

This is described in section 6(II).3.1.

7.2.3 MONITORING OF THE TREATMENT CYCLE

This is described in section 6(II).3.2.

7.2.4 VAGINAL EGG COLLECTION (VEC)

This is described in section 6(II).4.

7.2.5 LABORATORY TECHNIQUES

These are described in section 6(II).5.

7.2.6 UTERINE AND TUBAL EMBRYO REPLACEMENT

These are described in section 6(II).6.

7.2.7 STATISTICAL ANALYSIS METHODS

These are described in section 6(II).8.

7.3 RESULTS-1: OUTCOME OF ALL STIMULATION CYCLES

7.3.1 CHARACTERISTICS OF WOMEN INCLUDED INTO THE TRIAL

Table 7.1 summarises the characteristics of women (n=102) included into the trial and who underwent a total of 227 stimulation cycles.

Table 7.1: Characteristics of women included into the trial

		UET	TET	p	Total[1]
Age (mean ± s.d)	=	32.0±3.0	32.0±3.4	0.85	32.0±3.2
range		27-42	24-40		24-42
Duration of infertility (mean ± s.d)/(range)	=	4.4±2.2 2-12	5.0±2.6 2-17	0.20	4.7±2.4 2-17
Parity:					
nulliparous (%)	=	37 (71.2%)	34 (69.4%)	))0.85	72 (70.6%)
parous (%)	=	15 (28.8%)	15 (30.6%)	)	30 (29.4%)
Factors contributing to infertility:				0.33	
none (unexplained)	=	17	14		31 (30.4%)
male factors	=	22	18		40 (39.2%)
female factors	=	4	10		14 (13.7%)
male and female factors	=	9	7		17[1](16.7%)
Total number	=	52	49		102[1]

[1] One patient conceived after commencing GnRH-a and before randomisation to either UET or TET group

7.3.2 OVERALL PREGNANCY RATES

Table 7.2 summarises the outcome of all stimulation cycles, embryo transfers, and pregnancy rates in each group.

Table 7.2: Outcome of all stimulation cycles (n=227) included in the trial

CYCLES (no.)	%A	%C	%D	%E
A- Stimulation started (227)	---	---	---	---
TET = 111	48.9%			
UET = 115	50.7%			
undetermined = 1				
B- Abandoned (36)*	15.9%	---	---	---
TET = 23	20.7%			
UET = 12	10.4%			
C- Successful VEC (190)**	83.7%	---	---	---
TET = 88	79.3%			
UET = 102	88.7%			
	p=0.055			
D- With fertilisation (131)‖	57.7%	69%	---	---
TET = 67	60.4%			
UET = 64	55.7%			
	p=0.47			
Fertilisation Rate:				
All cycles = 44% (538/1223)		Transfer cycles = 63.3% (519/820)		
TET = 48.3% (276/571)		TET = 64.4% (266/413)		
UET = 40.2% (262/652)		UET = 62.2% (253/407)		
p=0.003		p=0.50		
E- Embryo transfers (124)	54.6%	65.3%	94.7%	---
TET = 62#	55.9%			
UET = 62	53.9%			
	p=0.77			
F- Clinical pregnancies (28)	12.3%¶	13.7%§	19.9%§	20.6%§
TET = 17	15.3%			27.4%
UET = 9	7.8%			14.5%
after GnRH-a = 2	0.9%			

* Two stimulation cycles were abandoned as the patients conceived following GnRH-a initiation and before commencing gonadotrophin injections; the first patient conceived before randomisation to either TET or UET group, and the other pregnancy occurred in a patient randomised to undergo TET

**One additional attempted VEC was unsuccessful and no oocytes were obtained

‖ Seven cycles did not undergo embryo transfer; in four only one fragmenting embryo formed in each, in two other cycles, the tubes did not appear healthy and in the seventh, the patient was at risk of ovarian hyperstimulation syndrome

\# In four cycles randomised to undergo TET the patients decided to have UET. The analysis of pregnancy and implantation rates was based on the original randomisation and intention to treat.

¶ All pregnancies are divided by all stimulation cycles started

§ Pregnancies resulting from UET and TET only are divided by the relevant denominator

Tables 7.3 and 7.4 summarise the characteristics and outcome of all embryo transfer cycles, pregnancy, implantation and live birth rates in each group.

Table 7.3: Characteristics and treatment description of ALL embryo transfer cycles (n=124)

		UET	TET	p	Total
Age (mean ± s.d)	=	31.9±2.6	31.7±3.8	p=0.74	31.8±3.3
range		(25-38)	(24-40)		(24-40)
Duration of infertility	=	4.8±2.4	4.6±2.1	p=0.50	4.7±2.3
(mean ± s.d)/(range)		(2-12)	(2-12)		(2-12)
Parity:					
nulliparous (%)	=	42 (67.7%)	43 (69.4%)	p=0.85	85 (68.5%)
parous (%)	=	20 (32.3%)	19 (30.6%)		39 (31.5%)
Factors contributing to infertility:				p=0.67	
none (unexplained)	=	27	22		49 (39.5%)
male factors	=	18	20		38 (30.6%)
female factors	=	7	11		18 (14.5%)
male and female factors	=	10	9		19 (15.3%)
Forwardly motile sperm[§]	=	3.6±2.8	4.6±4.4	p=0.21	4.1±3.7
(mean±s.d.)/(range)		(0-15)	(0-20)		(0-20)
No. oocytes collected	=	8.1±4.0	8.7±5.3	p=0.46	8.4±4.7
(mean±s.d.)/(range)		(1-19)	(3-27)		(1-27)
Fertilisation rate	=	253/407	266/413	p=0.50	519/820
		62.2%	64.4%		(63.3%)
No. embryos replaced	=	2.7±0.6	2.6±0.8	p=0.69	2.6±0.7
(mean±s.d.)					
Luteal support:				p=0.72	
hCG	=	28	30		58
Progesterone	=	34	32		66
Total number	=	62	62		124

[§] Post coital tests results are available in 48 cycles in the UET group and 43 in the TET group

Table 7.4: Outcome of ALL embryo replacements according to the original randomisation and intention to treat

	Uterine (Pregnant)	Tubal (Pregnant)		
		Day 1	Day 2	Total
One embryo	4(1)	....	10(1)	10(1)
Two embryos	14(2)	1(0)	4(1)	5(1)
Three embryos	44(6) 13.6%[§]	35(11)	12(4)	47(15) 31.9%[§]
TOTAL	62(9)	36(11)	26(6)	62(17)
Pregnancy rate (PR)	**14.5%**[¶*]	31%[¶]	23.1%	**27.4%**[*]
[§]p=0.04 [*]p=0.08 [¶]p=0.06				
Multiple pregnancy rate p=0.28	44% 4/9			24% 4/17
Patients miscarried ≤12 weeks >12 weeks	1 (n=1)			6 (n=4) (n=2)
Patients delivered p=0.19	8			11
Live birth rate/ET p=0.46	12.9%			17.7%
Live birth rate/cycle started p=0.42	7%			9.9%

Four patients analysed under tubal transfer had uterine embryo transfers. Three of whom received one embryo each resulting in a singleton pregnancy and delivery. The fourth patient received three embryos resulting in a singleton pregnancy and delivery. The overall number of embryos transferred was six resulting in two gestation sacs.

7.3.3 IMPLANTATION RATES

Tables 7.5 and 7.6 summarise the implantation rates obtained following UET and TET in all transfer cycles and in replacement cycles resulting in pregnancy respectively.

Table 7.5: Implantation rates following intended uterine and Fallopian embryo transfers in ALL transfer cycles

	Uterine (%)		**Tubal (%)**	
		Day 1	Day 2	Total
One embryo	1/4 (25%)	....	1/10	1/10 (10%)
Two embryos	3/28 (11%)	0/2	1/8	1/10 (10%)
Three embryos	9/132 (7%)*	16/105	5/36	21/141(15%)*
TOTAL	13/164	16/107	7/54	23/161
Implantation rate	**7.9%**[1]	15%	13%	**14.3%**[1]

*p=0.03 [1]p=0.07

Table 7.6: Implantation rates following intended uterine and Fallopian embryo transfers in cycles resulting in pregnancy only

	Uterine (%)		**Tubal (%)**	
		Day 1	Day 2	Total
One embryo	1/1 (100%)	....	1/1	1/1 (100%)
Two embryos	3/4 (75%)	....	1/2	1/2 (50%)
Three embryos	9/18 (50%)*	16/33	5/12	21/45 (47%)*
TOTAL	13/23	16/33	7/15	23/48
Implantation rate	**56.5%**[1]	48.5%	46.7%	**47.9%**[1]

*p=0.83 [1]p=0.50

7.3.4 INTERIM ANALYSES AND OUTCOME OF NON-TRIAL IVF

Tables 7.7 and 7.8 depict the outcome of two interim analyses with the final results and the overall results following IVF-UET in non-trial patients during a comparable period of time (1989-1990) at the Royal Free Hospital.

Table 7.7: Comparison of two interim analyses with the final analysis of the clinical trial

	September 1989-December 1990				September 1989-July 1991				Final analysis			
	n(%A)	%C	%D	%E	n(%A)	%C	%D	%E	n(%A)	%C	%D	%E
A- Stimulation cycles	140	-	-	-	177	-	-	-	227	-	-	-
B- Abandoned cycles	22 (16%)	-	-	-	30 (17%)	-	-	-	36 (16%)	-	-	-
C- Successful VEC	117 (84%)	-	-	-	146 (83%)	-	-	-	190 (84%)	-	-	-
D- Cycles with fertilisation	83 (59%)	71%	-	-	103 (58%)	71%	-	-	131 (58%)	69%	-	-
E- Cycles with embryo transfer	77 (55%)	66%	93%	-	97 (55%)	66%	94%	-	124 (56%)	65%	95%	-
F- All clinical pregnancies	17 (12%)	15%	21%	22.1%	22 (12%)	15%	21%	22.7%	28[1] (12%)	14%	20%	21%
Clinical pregnancies resulting from transfers	UET (pregnant)		TET[‡] (pregnant)		UET (pregnant)		TET (pregnant)		UET (pregnant)		TET* (pregnant)	
Number of transfers	39 (6)		38 (11)		51 (8)		46 (14)		62 (9)		62 (17)	
PR per UET or TET	15.4%		29%		15.7%		30.4%		14.5%		27.4%	

[1] Two pregnancies resulted after commencing gonadotrophin releasing hormone analogue (GnRH-a)

[‡] One patient in this group had UET (3 embryos) resulting in a singleton pregnancy and delivery. The analysis of PR was based on the randomised intention to treat

* Three patients in this group had UETs (one embryo each) resulting in one singleton pregnancy and delivery. The analysis of PR was based on the randomised intention to treat

Table 7.8: Results of uterine embryo transfers in non-trial patients undergoing in vitro fertilisation treatment at the Royal Free Hospital during 1989 and 1990. Data are used for comparison with figures obtained following UET in trial patients

CYCLES (no.)	%A	%C	%D	%E
A- Stimulation started (442)	100%	---	---	---
B- Abandoned (94)	21.3%	---	---	---
C- Successful VEC (336)	76.0%	---	---	
D- With fertilisation (257)	58.1%	76.5%	---	---
Fertilisation Rate: All cycles = 45.9% (1357/2957)				
E- Embryo transfers (236)	53.4%	70.2%	91.8%	
F- Clinical pregnancies (33)[1]	7.5%	9.8%	12.8%	14.0%

[1] Including one confirmed tubal pregnancy

Table 7.9: Pregnancy and implantation rates in non-trial patients undergoing IVF-UET treatment at the Royal Free Hospital during 1989 and 1990 subdivided by the number of embryos replaced

	Pregnancies (PR %)			**Implantation rate %**	
	Single	Multiple*	(PR %)	All cycles	Pregnancy cycles
One embryo (n=32)	2	-----	6.3%	6.3%	100%
Two embryos (n=40)	2	-----	5.0%	2.5%	50%
Three embryos (n=164)	20	8	17%	7.3%	43%
TOTAL (n=236)	**24**	**8**	**14%**	**6.6%**	**44.4%**

* All multiple pregnancies were twin gestation sacs

7.4 RESULTS-2: OUTCOME BY STIMULATION ATTEMPT RANK

7.4.1 FIRST STIMULATION CYCLE

7.4.1.1 CHARACTERISTICS OF WOMEN UNDERGOING TREATMENT IN THE FIRST CYCLE

Tables 7.10 and 7.11 summarise the clinical characteristics of all women undergoing their first stimulation cycle. These women also represent the total cohort of subjects that underwent treatment following recruitment into this study.

Table 7.10: Characteristics of women undergoing their FIRST stimulation cycle which also represent the characteristics of the women included in the whole study

		UET	TET	p	Total[1]
Age (mean ± s.d)	=	32.0±3.0	32.0±3.4	0.85	32.0±3.2
range		27-42	24-40		24-42
Duration of infertility	=	4.4±2.2	5.0±2.6	0.20	4.7±2.4
(mean ± s.d)/(range)		2-12	2-17		2-17
Parity:					
nulliparous (%)	=	37 (71.2%)	34 (69.4%)	)	72 (70.6%)
				)0.85	
parous (%)	=	15 (28.8%)	15 (30.6%)	)	30 (29.4%)
Factors contributing to infertility:				0.33	
none (unexplained)	=	17	14		31 (30.4%)
male factors	=	22	18		40 (39.2%)
female factors	=	4	10		14 (13.7%)
male and female factors	=	9	7		17[1](16.7%)
Total number	=	52	49		102[1]

[1] One patient conceived after commencing GnRH-a and before randomisation to either UET or TET group

Table 7.11: Details of male and female factors contributing to infertility

	UET	TET	Total[1]
Male factors	31	25	57[1]
Azoospermia	3	1	4
Oligospermia	1	3	4
Asthenospermia	1	-	1
Teratospermia	15	17	32
Oligoasthenospermia	2	1	3
Oligoteratospermia	5	-	5
Asthenoteratospermia	2	2	4
Antibodies	1	1	2
Antibodies + other	1	-	1
Female factors	13	17	31[1]
Treated endometriosis	7	11	18
Untreated endometriosis	4	4	8
Other	2	2	4
Total number of patients	35	35	71[1*]

[1] One patient with combined male and female factors conceived after commencing GnRH-a and before randomisation to either UET/TET group

[*] Seventeen patients had combined male and female factors contributing to their infertility

7.4.1.2 OUTCOME OF THE FIRST ATTEMPT OF TREATMENT

Tables 7.12 and 7.13 summarise the outcome of the first stimulation cycle attempt. In total 102 stimulation cycles were started proceeding to 53 embryo transfers (52%) and resulting in 13 pregnancies (24.5% of all transfers). One patient conceived after commencing GnRH-a therapy and before randomisation to either arm of the trial and was excluded from further analysis of replacement cycles. The outcome of the stimulation cycles, vaginal egg collections and the results of embryo transfers are outlined in Table 7.12. The number of embryos replaced, single and multiple pregnancies, implantation rates, and live birth deliveries are outlined in Table 7.13.

Table 7.12: Outcome of the FIRST stimulation attempt cycles

CYCLES	%A	%C	%D	%E
A- Stimulation started (n=102)	100%			
TET = 49	48%			
UET = 52	51%			
undetermined = 1	1%			
B- Abandoned (n= 21)[§]	20.6%			
TET = 12	24.5%			
UET = 8	15.4%			
C- Successful VEC (n= 81)	79.4%			
TET = 37	75.5%			
UET = 44	84.6%			
	p=0.25			
D- With fertilisation (n= 54)	52.9%	66.7%		
TET = 28	57.1%			
UET = 26	48.1%			
	p=0.47			
Fertilisation rate:				
All cycles = 44.8% (232/518)		Transfer cycles = 64.6% (228/353)		
TET = 49.4% (123/249)		TET = 63.4% (123/194)		
UET = 40.5% (109/269)		UET = 66.0% (105/159)		
p=0.04		p=0.61		
E- Embryo transfers (n= 53)	52.0%	65.4%	98.1%	
TET = 28	57.1%			
UET = 25	48.1%			
	p=0.36			
F- Clinical pregnancies (n= 14)	13.7%[¶]	16.05%*	24.1%*	24.5%*
TET = 8	16.3%			28.6%
UET = 5	9.6%			20.0%
after GnRH-a = 1				

[§] One cycle was abandoned before randomisation to either UET or TET as the patient conceived after commencing GnRH-a

[¶] All pregnancies divided by all stimulation cycles started

* Pregnancies resulting from UET and TET only divided by the relevant denominator

Table 7.13: Outcome of embryo replacements of patients in their FIRST attempt

	Number of embryo transfers			
Embryo(s) per transfer	**UET** (pregnant)	Day 1 TET (pregnant)	Day 2 TET	**Total TET** (pregnant)
One embryo	1 (1)	....	3 (0)	3 (0)
Two embryos	5 (1)	1 (0)	2 (1)	3 (1)
Three embryos	19 (3)	14 (4)	8 (3)	22 (7)
TOTAL	25 (5)	15 (4)	13 (4)	28 (8)
Pregnancy rate (PR) *p=0.47	**20%***	26.7%	30.8%	**28.6%***

	Number of embryos replaced			
Embryo(s) per transfer	**UET** (gestation sacs)	Day 1 TET (gestation sacs)	Day 2 TET	**Total TET** (gestation sacs)
One embryo	1 (1)	....	3 (0)	3 (0)
Two embryos	10 (1)	2 (0)	4 (1)	6 (1)
Three embryos	57 (6)	42 (6)	24 (4)	66 (10)
TOTAL	68 (8)	44 (6)	31 (5)	75 (11)
Implantation rate (IR) *p=0.85	**11.8%***	13.6%	16.1%	**14.7%***
Patients miscarried	0	2	3	5
Patients delivered	5	2	1	3
Live birth rate/ET *p=0.45	**20%***			**10.7%***

7.4.1.3 CHARACTERISTICS AND GENERAL DATA DESCRIPTION OF 53 WOMEN RECEIVING EMBRYOS IN THE FIRST CYCLE

Table 7.14 summarises the characteristics and treatment cycle variables in the fifty three women who reached the stage of embryo transfer (UET= 25; TET= 28). These variables have been selected due to their potential effect on the outcome of treatment and will be further evaluated in sections 7.4.1.4 and 7.4.1.5.

Table 7.14: Characteristics and treatment description of women undergoing their FIRST embryo transfer (n=53)

		UET	TET	p	Total
Age (mean ± s.d)	=	31.1±1.9	31.6±4.1	p=0.58	31.4±3.3
range		(27-34)	(24-40)		(24-40)
Duration of infertility	=	3.6±1.3	5.0±2.3	p=0.013	4.3±1.86
(mean ± s.d)/(range)		(2-7)	(2-12)		(2-12)
Parity:					
nulliparous (%)	=	19 (76.0%)	19 (67.9%)	p=0.51	38 (71.7%)
parous (%)	=	6 (24.0%)	9 (32.1%)		15 (28.3%)
Factors contributing to infertility:				p=0.75	
none (unexplained)	=	8	9		17 (32.1%)
male factors	=	9	7		16 (30.2%)
female factors	=	3	6		9 (17.0%)
male and female factors	=	5	6		11 (20.8%)
Forwardly motile sperm[§]	=	4.2±3.3	4.3±4.4	p=0.94	4.2±3.9
(mean±s.d.)/(range)		(0-15)	(0-20)		(0-20)
No. oocytes collected	=	8.8±5.1	9.2±6.0	p=0.77	9.0±5.5
(mean±s.d.)/(range)		(1-19)	(3-27)		(1-27)
Fertilisation rate	=	105/159	123/194	p=0.61	228/353
		66%	63.4%		(64.6%)
No. embryos replaced	=	2.7±0.5	2.7±0.7	p=0.81	2.7±0.6
(mean±s.d.)					
Luteal support:				p=0.18	
hCG	=	8	14		22
Progesterone	=	17	14		31
Total number	=	25	28		53

[§] Post coital tests results are available in 17 women in the UET group and 22 in the TET group

7.4.1.4 OUTCOME OF EMBRYO REPLACEMENTS IN THE FIRST ATTEMPT: THE INFLUENCE OF PATIENT CHARACTERISTICS AND TREATMENT VARIABLES ON THE OUTCOME

The overall pregnancy rates following UET and TET of the first attempt are reported in Table 7.13 and are 20.0% and 28.6% respectively. There is no significant difference in these rates (p=0.47, chi-squared). The ODDS RATIO (OR)= 1.6 with 95% CI 0.44, 5.74. The influence of parity, fertility factors and luteal phase support on pregnancy rates are reported in Tables 7.15.i to 7.15.v.

Table 7.15.i: Influence of parity on pregnancy rates following TET and UET

No previous pregnancies	TET	UET	Total	
Not Pregnant	14	15	29	**OR[$] = 1.34**
Pregnant	5	4	9	**95% C.I.[¶]**
Total	19	19	38	**0.30, 6.02**
% PREGNANT	**26%**	**21%**		

[$] OR= Odds Ratio
[¶] CI= Confidence interval

≥1 previous pregnancies	TET	UET	Total	
Not Pregnant	6	5	11	**OR = 2.50**
Pregnant	3	1	4	**95% C.I.**
Total	9	6	15	**0.19, 32.20**
% PREGNANT	**33%**	**17%**		

These results indicate that parous women were more likely to benefit from TET than nulliparous women (OR = 2.5 versus 1.34 respectively). However, this difference did not reach statistical significance.

Table 7.15.ii: Influence of female factors on pregnancy rates following TET and UET

No Female factors present (Code "/G" 1)	TET	UET	Total	
				OR = 2.12
Not Pregnant	11	14	25	
				95% C.I.
Pregnant	5	3	9	
				0.41, 10.88
Total	16	17	33	
% PREGNANT	**31%**	**18%**		

Female factors present (Codes "/G" 2,3,4)	TET	UET	Total	
				OR = 1.00
Not Pregnant	9	6	15	
				95% C.I.
Pregnant	3	2	5	
				0.13, 7.89
Total	12	8	20	
% PREGNANT	**25%**	**25%**		

These results show that women without female factors were more likely to conceive following TET than those with female factors (with or without male factors) (OR = 2.12 versus 1.0 respectively). However, this difference did not reach statistical significance.

Table 7.15.iii: Influence of male factor on pregnancy rates following TET and UET

No Male factors present (Code "/H" 0)	TET	UET	Total	
Not Pregnant	10	9	19	**OR = 2.25**
Pregnant	5	2	7	**95% C.I.**
Total	15	11	26	**0.35, 14.61**
% PREGNANT	**33%**	**18%**		

Male factors present (Codes "/H" 1-9)	TET	UET	Total	
Not Pregnant	10	11	21	**OR = 1.10**
Pregnant	3	3	6	**95% C.I.**
Total	13	14	27	**0.18, 6.76**
% PREGNANT	**23%**	**21%**		

These figures indicate that couples with no male factor infertility were twice as likely to conceive following TET than those who had any male problem (with or without female factor) in their aetiology (OR 2.25 versus 1.10 respectively). This difference was not significant.

Table 7.15.iv: Influence of a diagnosis of unexplained infertility on pregnancy rates following TET and UET

DIAGNOSIS

Known (male, female, combined) (Code "/F" 1,2,3)	TET	UET	Total	
Not Pregnant	15	13	28	**OR = 0.90**
Pregnant	4	4	8	**95% C.I.**
Total	19	17	36	**0.19, 4.14**
% PREGNANT	**21%**	**24%**		

Idiopathic (Codes "/F" 4)	TET	UET	Total	
Not Pregnant	5	7	12	**OR = 5.60**
Pregnant	4	1	5	**95% C.I.**
Total	9	8	17	**0.47, 66.50**
% PREGNANT	**44%**	**13%**		

These figures indicate that couples with unexplained infertility were more than five times as likely to become pregnant following TET than those who had any identifiable factor in their aetiology (OR 5.60 versus 0.90 respectively). This difference was not significant.

Table 7.15.v: Influence of luteal phase support on pregnancy rates following TET and UET

LUTEAL SUPPORT

"hCG"	TET	UET	Total	
Not Pregnant	8	7	15	**OR = 5.25**
Pregnant	6	1	7	**95% C.I.**
Total	14	8	22	**0.50, 54.9**
% PREGNANT	**43%**	**12.5%**		

Progesterone	TET	UET	Total	
Not Pregnant	12	13	25	**OR = 0.54**
Pregnant	2	4	6	**95% C.I.**
Total	14	17	31	**0.08, 3.51**
% PREGNANT	**14%**	**24%**		

In the hCG group more women became pregnant following TET than UET (OR= 5.25) while in the progesterone supplementation group, the OR for TET was 0.54 only. Although this difference in the hCG group is marked, it does not reach significance level.

7.4.1.5 LOGISTIC REGRESSION ANALYSIS OF OUTCOME OF TREATMENT WITH TREATMENT AS THE MAIN VARIABLE

Logistic regression analysis allows dichotomous 'response' or dependent variables, such as pregnant/not pregnant, to be analysed in a regression model with independent variables. The main variable of interest here is treatment. However, there are also several confounding variables. Unfortunately as there are very few pregnancies only one confounding variable can be included at a time to obtain 'adjusted' estimates of treatment effect (adjusted for the confounding variable). **All** OR's are expressed as TET:UET; ie ratios >1 are in favour of TET. The outcome of this analysis is shown in the following Tables (7.16.i - 7.16.ix) and summarised in Table 7.18.

Table 7.16 Logistic regression analysis of confounding variables and their effect on the outcome of treatment

7.16.i

Variable	OR	se	95% CI	p-value
Treatment	1.6	1.04	0.43, 5.92	0.47

7.16.ii

Variable	OR	se	95% CI	p-value
Age	0.88	0.09	0.71, 1.07	0.20
Treatment	1.65	1.09	0.44, 6.22	0.46

7.16.iii

Variable	OR	se	95% CI	p-value
Duration of infertility	0.68	0.16	0.43, 1.09	0.11
Treatment	2.44	1.72	0.59, 10.05	0.21

7.16.iv

Variable	OR	se	95% CI	p-value
Previous pregnancies	1.12	0.79	0.27, 4.60	0.87
Treatment	1.58	1.04	0.43, 5.90	0.49

7.16.v

Variable	OR	se	95% CI	p-value
Female factors	0.99	0.65	0.26, 3.75	0.99
Treatment	1.60	1.05	0.43, 5.98	0.48

7.16.vi

Variable	OR	se	95% CI	p-value
Male factors	0.81	0.52	0.22, 2.95	0.74
Treatment	1.57	1.03	0.42, 5.84	0.50

7.16.vii

Variable	OR	se	95% CI	p-value
Forwardly motile sperm	1.06	0.10	0.87, 1.28	0.56
Treatment	2.80	2.51	0.46, 17.21	0.26

7.16.viii

Variable	OR	se	95% CI	p-value
No. oocytes collected	0.85	0.08	0.70, 1.03	0.10
Treatment	1.72	1.16	0.44, 6.68	0.43

7.16.ix

Variable	OR	se	95% CI	p-value
Luteal phase support	0.55	0.36	0.15, 2.04	0.36
Treatment	1.44	0.96	0.39, 5.49	0.59

The above logistic regression tables analyse the relative role played by some factors on the pregnancy rate following TET. The factors found to increase the OR for TET (>1.6) are the women's age (OR=1.65), duration of infertility (OR=2.44), forwardly motile sperm in a PCT (OR=2.8), and number of oocytes recovered (OR=1.72). Factors which lower the OR or have no effect are parity (OR=1.58), female factors (OR=1.6), male factors (OR=1.57), and the type of luteal support (OR=1.44).

7.4.1.6 LOGISTIC REGRESSION ANALYSIS OF OUTCOME OF PREGNANCY WITH TREATMENT AS THE MAIN VARIABLE

The outcome of pregnancies following TET and UET is summarised in Table 7.13. The live birth rate per embryo transfer is 11% and 20% for the two treatments respectively resulting in an overall OR for delivery following TET (TET:UET) of 0.48. The difference between the two rates is not significant (Fisher's exact test; p=0.45). Tables 7.17.i to 7.17.ix depict the logistic regression analysis to obtain adjusted OR's for treatment with the outcome as pregnant ≥20 weeks or delivered versus not pregnant combined with miscarriages. The overall results are summarised in Table 7.18.

Table 7.17: Logistic regression analysis of confounding variables and their effect on the outcome of pregnancy

7.17.i

Variable	OR	se	95% CI	p-value
Treatment	0.48	0.38	0.1, 2.34	0.36

7.17.ii

Variable	OR	se	95% CI	p-value
Age	1.10	0.15	0.84, 1.45	0.48
Treatment	0.43	0.36	0.08, 2.25	0.31

7.17.iii

Variable	OR	se	95% CI	p-value
Duration of infertility	0.64	0.21	0.34, 1.23	0.18
Treatment	0.70	0.58	0.13, 3.70	0.58

7.17.iv

Variable	OR	se	95% CI	p-value
Previous pregnancies	1.81	1.48	0.35, 9.40	0.47
Treatment	0.45	0.36	0.09, 2.25	0.33

7.17.v

Variable	OR	se	95% CI	p-value
Female factors	1.08	0.87	0.21, 5.42	0.93
Treatment	0.48	0.38	0.10, 2.35	0.36

7.17.vi

Variable	OR	se	95% CI	p-value
Male factors	0.89	0.69	0.19, 4.24	0.88
Treatment	0.47	0.37	0.10, 2.34	0.35

7.17.vii

Variable	OR	se	95% CI	p-value
Forwardly motile sperm	0.93	0.16	0.66, 1.32	0.69
Treatment	0.74	0.79	0.09, 6.38	0.78

7.17.viii

Variable	OR	se	95% CI	p-value
No. oocytes collected	0.87	0.09	0.69, 1.08	0.20
Treatment	0.49	0.39	0.10, 2.44	0.37

7.17.ix

Variable	OR	se	95% CI	p-value
Luteal phase support	1.07	0.86	0.21, 5.42	0.94
Treatment	0.49	0.39	0.10, 2.43	0.37

Logistic regression analysis has also been applied to the live delivery per ET data to analyse the relative role played by the different factors. In this model, the OR for TET is 0.48 indicating that a lower proportion of women, undergoing their first embryo replacement, delivered following TET than UET. Factors found to be associated with an improved delivery OR for TET are; duration of infertility (OR=0.70), the number of forwardly motile sperm (OR=0.74). The number of oocytes collected (OR=0.49), type of luteal phase support (OR=0.49), presence of female factors (OR=0.48), presence of male factors (OR=0.47), parity (OR=0.45), and age of the women (OR=0.43) do not enhance the delivery rate following TET.

Table 7.18: Summary of logistic regression analysis of factors affecting outcome of tubal embryo replacements

Comparison TET:UET	Pregnancy per transfer				Delivery per transfer			
adjusted for confounding factor	OR	SE	95% C.I.	P value	OR	SE	95% C.I.	P value
Treatment (TET:UET) No factor	1.6	1.04	0.43, 5.92	0.47	0.48	0.38	0.10, 2.34	0.36
TET:UET adjusted for:								
Age	1.65	1.09	0.44, 6.22	0.46	0.43	0.36	0.08, 2.25	0.31
Duration of infertility at recruitment	2.44	1.72	0.59, 10.05	0.21	0.70	0.58	0.13, 3.70	0.58
Previous pregnancies	1.58	1.04	0.43, 5.90	0.49	0.45	0.36	0.09, 2.25	0.33
Female factors	1.60	1.05	0.43, 5.98	0.48	0.48	0.38	0.10, 2.35	0.36
Male factors	1.57	1.03	0.42, 5.84	0.50	0.47	0.37	0.10, 2.34	0.35
Forwardly motile sperm (Post coital test)	2.80	2.51	0.46, 17.21	0.26	0.74	0.79	0.09, 6.38	0.78
Number of oocytes recovered	1.72	1.16	0.44, 6.68	0.43	0.49	0.39	0.10, 2.44	0.37
Luteal phase support	1.44	0.96	0.39, 5.49	0.59	0.49	0.39	0.10, 2.43	0.37

7.4.2 SECOND STIMULATION CYCLE

7.4.2.1 CHARACTERISTICS OF WOMEN UNDERGOING TREATMENT IN THE SECOND CYCLE

Table 7.19 summarises the clinical characteristics of all women undergoing their second stimulation cycle.

Table 7.19: Characteristics of women undergoing their SECOND stimulation cycle

		UET	TET	p	Total[1]
Age (mean ± s.d) range	=	31.9±2.9 25-38	31.3±2.9 24-36	0.4	31.7±2.9 24-38
Duration of infertility (mean ± s.d)/(range)	=	5.2±2.4 2-12	4.6±2.7 2-17	0.28	4.9±2.5 2-17
Parity:					
nulliparous (primary)	=	28 (66.7%)	25 (71.4%)	))0.65	53 (68.8%)
parous (secondary)	=	14 (33.3%)	10 (28.6%)	)	24 (31.2%)
Factors contributing to infertility:				0.48	
none (unexplained)	=	15	9		24 (31.2%)
male factors	=	15	17		32 (41.6%)
female factors	=	4	5		9 (11.7%)
male and female factors	=	8	4		12 (15.6%)
Total number	=	42	35		77

7.4.2.2 OUTCOME OF THE SECOND ATTEMPT OF TREATMENT

Tables 7.20 and 7.21 summarise the outcome results of the second stimulation attempt. In total, seventy seven stimulation cycles were started proceeding to 45 embryo transfers (58.4%) and resulting in 9 clinical pregnancies (20% of all transfers). One patient conceived after commencing GnRH-a therapy. The outcome of the stimulation cycles, vaginal egg collections and the results of embryo transfers

are outlined in Table 7.20. Details of the number of embryos replaced, single and multiple pregnancies and live birth deliveries are outlined in Table 7.21.

Table 7.20: Outcome of the SECOND stimulation cycles

CYCLES	%A	%C	%D	%E
A- Stimulation started (n= 77)	100%			
TET = 35	45.5%			
UET = 42	54.5%			
undetermined = 0				
B- Abandoned (n= 6)	7.8%			
TET = 4	11.4%			
UET = 2	4.8%			
C- Successful VEC (n= 70)	90.9%			
TET = 31	88.6%			
UET = 39	92.9%			
	p=0.70			
D- With fertilisation (n= 50)	64.9%	71.4%		
TET = 23	65.7%			
UET = 27	64.3%			
	p=0.90			
Fertilisation rate:				
All cycles = 42.1% (181/430)		Transfer cycles = 60.4% (172/285)		
TET = 42.5% (74/174)		TET = 61.1% (66/108)		
UET = 41.8% (107/256)		UET = 59.9% (106/177)		
p=0.88		p=0.85		
E- Embryo transfers (n= 45)	58.4%	64.3%	90.0%	
TET = 19[§]	54.3%			
UET = 26	61.9%			
	p=0.49			
F- Clinical pregnancies (n= 10)	13.0%[¶]	12.9%*	18.0%*	20.0%*
TET = 6	17.1%			31.6%
UET = 3	7.1%			11.5%
after GnRH-a = 1				

[§] In four cycles analysed under TET, the patients opted to have UET contrary to their randomisation. Three women received one 4-cell embryo each resulting in one singleton pregnancy and delivery. The fourth woman received three embryos (2,2,3 cells) resulting in a singleton pregnancy and delivery.

[¶] All pregnancies divided by all stimulation cycles started

* Pregnancies resulting from UET and TET only divided by the relevant denominator

Table 7.21: Outcome of embryo replacements of patients in their second attempt according to the original randomisation and intention to treat

	Number of embryo transfers			
Embryo(s) per transfer	**UET** (pregnant)	Day 1 TET	Day 2 TET (pregnant)	**Total TET** (pregnant)
One embryo	2 (0)	....	5 (1)	5 (1)
Two embryos	7 (1)	....	2 (0)	2 (0)
Three embryos	17 (2)	10 (4)	2 (1)	12 (5)
TOTAL	26 (3)	10 (4)	9 (2)	19 (6)
Pregnancy rate (PR) *p=0.10	**11.5%***	40.0%	22.2%	**31.6%***

	Number of embryos replaced			
Embryo(s) per transfer	**UET** (gestation sacs)	Day 1 TET	Day 2 TET (gestation sacs)	**Total TET** (gestation sacs)
One embryo	2 (0)	....	5 (1)	5 (1)
Two embryos	14 (2)	....	4 (0)	4 (0)
Three embryos	51 (2)	30 (6)	6 (1)	36 (7)
TOTAL	67 (4)	30 (6)	15 (2)	45 (8)
Implantation rate (IR) *p=0.048	**6%***	20%	13.3%	**17.8%***
Patients miscarried	0	1	0	1
Patients delivered	3	3	2	5
Live birth rate/ET *p=0.21	**11.5%***			**26.3%***

Four patients analysed under tubal transfer had uterine embryo transfers. Three of whom received one embryo each resulting in a singleton pregnancy and delivery. The fourth patient received three embryos resulting in a singleton pregnancy and delivery. The overall number of embryos transferred was six resulting in two gestation sacs.

Table 7.22 compares the outcome of embryo transfers (TET and UET combined) in the second attempt between women who reached the stage of embryo replacement in their first treatment but did not conceive and those whose first treatment cycles were abandoned for one reason or another before the embryo transfer stage.

Table 7.22: Outcome of the second TET and UET treatment in women who received embryos in the first treatment cycle but did not conceive (group A) and those whose first cycles were abandoned (group B).

First cycle	Second cycle PREGNANT			% PREGNANT
	NO	YES	Total	
Group A; n= 30	22	8	30	27%
Group B; n= 15	14	1	15	7%
Total	36	9	45	

Table 7.23 summarises the outcome of second attempt embryo transfers (UET and TET) in 30 women whose first embryo replacement did not result in a pregnancy.

Table 7.23: Outcome of second treatment cycle in women with unsuccessful first embryo transfer

First treatment cycle		Second treatment cycle			
Unsuccessful embryo transfer		PREGNANT NO	PREGNANT YES	Total	% PREGNANT
TET (n=19)	→ TET	4	1	5	20%
	UET	11	3	14	21%
	Total	15	4	19	
		PREGNANT NO	PREGNANT YES	Total	% PREGNANT
UET (n=11)	→ TET	1	3	4	75%
	UET	6	1	7	14%
	Total	7	4	11	

7.4.3 THIRD STIMULATION CYCLES

7.4.3.1 CHARACTERISTICS OF WOMEN UNDERGOING A THIRD STIMULATION CYCLE

Table 7.24 outlines the characteristics of women undergoing their third stimulation cycle.

Table 7.24: Characteristics of women undergoing their third stimulation cycle

		UET	TET	p	Total
Age (mean ± s.d) range	=	32.9±2.5 (29-37)	32.1±3.1 (25-38)	0.44	32.4±2.9 (25-38)
Duration of infertility (mean ± s.d)/(range)	=	5.4±2.5 (3-12)	4.9±2.5 (2-12)	0.56	5.1±2.5 (2-12)
Parity:					
nulliparous (%)	=	8 (34.8%)	15 (65.2%)	)0.73	23 (65.7%)
parous (%)	=	5 (41.7%)	7 (58.3%)	)	12 (34.3%)
Factors contributing to infertility:					
none (unexplained)	=	6	7	0.19	13 (37.1%)
male factors	=	6	6		12 (34.3%)
female factors	=	1	5		6 (17.2%)
male and female factors	=	---	4		4 (11.4%)
Total number	=	13	22		35

7.4.3.2 OUTCOME OF THE THIRD ATTEMPT OF TREATMENT

In total, thirty five stimulation cycles were started proceeding to twenty nine egg collections and resulting in nineteen embryo transfers. Four pregnancies in the two groups are reported. Table 7.25 details the outcome of the stimulation cycles and Table 7.26 details the outcome of embryo transfers, pregnancy and implantation rates, and live birth rates for the two groups.

7.4.4 OUTCOME OF FOURTH AND FIFTH STIMULATION ATTEMPTS

None of patients undergoing their fourth or fifth stimulation attempts conceived. Ten patients underwent a fourth stimulation attempt resulting in six embryo transfers (TET=2; UET=4) without any pregnancies. Three patients underwent a fifth stimulation attempt resulting in one transfer only (UET). In view of the small numbers involved in the fourth and fifth attempts, these patients will be incorporated in the overall analysis of the treatment cycles only.

Table 7.25: Outcome of the THIRD stimulation cycles

CYCLES	%A	%C	%D	%E
A- Stimulation started (n= 35)	100%			
TET = 22	62.9%			
UET = 13	37.1%			
undetermined = 0				
B- Abandoned (n= 6)	17.1%			
TET = 5	22.7%			
UET = 1	7.7%			
C- Successful VEC (n= 29)	82.9%			
TET = 17	77.3%			
UET = 12	92.3%			
	p=0.38			
D- With fertilisation (n= 20)	57.1%	69.0%		
TET = 13	59.1%			
UET = 7	53.9%			
	p=0.76			
Fertilisation rate:				
All cycles = 42.3% (88/208)		Transfer cycles = 64.9% (85/131)		
TET = 47.7% (63/132)		TET = 65.6% (63/96)		
UET = 32.9% (25/76)		UET = 62.9% (22/35)		
p=0.037		p=0.77		
E- Embryo transfers (n= 19)	54.3%	65.5%	95.0%	
TET = 13	59.1%			
UET = 6	46.2%			
	p=0.68			
F- Clinical pregnancies (n= 4)	11.4%	13.8%	20.0%	21.1%
TET = 3	13.6%			23.1%
UET = 1	7.7%			16.7%

Table 7.26: Outcome of embryo replacements of patients in their third stimulation cycles

	Number of embryo transfers			
Embryo(s) per transfer	**UET** (pregnant)	Day 1 TET (pregnant)	Day 2 TET	**Total TET** (pregnant)
One embryo	1 (0)	....	2 (0)	2 (0)
Two embryos	2 (0)	....	....	....
Three embryos	3 (1)	9 (3)	2 (0)	11 (3)
TOTAL	6 (1)	9 (3)	4 (0)	13 (3)
Pregnancy rate (PR) *p=1.0	**16.7%***	33.3%	....	**23.1%***

	Number of embryos replaced			
Total number of embryos replaced	**UET** (gestation sacs)	Day 1 TET (gestation sacs)	Day 2 TET	**Total TET** (gestation sacs)
One embryo	1 (0)	....	2 (0)	2 (0)
Two embryos	4 (0)	....	....	....
Three embryos	9 (1)	27 (4)	6 (0)	33 (4)
TOTAL	14 (1)	27 (4)	8 (0)	35 (4)
Implantation rate (IR) *p=0.65	**7.1%***	14.8%	....	**11.4%***
Patients miscarried	1	0	....	0
Patients delivered	...	3	....	3
Live birth rate/ET p=0.52	...	...	....	23%

7.4.5 DISCUSSION

In the design of this prospective trial several issues were considered. Firstly, it was acknowledged that randomisation to either modality was of paramount importance. Secondly, the more commonly employed parallel design in which a group of carefully selected subjects receiving a particular treatment was compared with another group of similar characteristics receiving a different treatment might be optimal, but would require a longer period of recruitment and subsequent completion of the trial. Based on a recent document (Effective Health Care, 1992) and provided that there were adequate referrals from neighbouring General Practitioners and Consultants, it would have required a period of more than three years to complete recruitment of adequate number of couples in the categories included in this study (male, unexplained, endometriosis, and combined). Furthermore, additional period would have been necessary to perform their treatment and thus rendering conclusion of such a study prohibitive during my attachment as research fellow at the Royal Free Hospital between 1988 and 1991. Alternatively, a crossover study design was considered as each subject would act as her own control, thereby reducing the sample size required and shortening the duration of the study. Several potential problems were considered including; (1) occurrence of pregnancy in the first treatment would exclude the subject from exposure to the second modality, (2) the effect of subjects who drop out of the study, especially after the first period or those who decide, contrary to their randomisation, to undergo the opposite treatment modality and its effect on the validity of the study design as well as the overall data, (3) the possible influence of a carry-over effect of therapeutic response from the first treatment into the time period of the second treatment, and (4) the stability or instability of disease state during the study period and its effect on the response to treatment. Indeed, the place of crossover design in infertility trials has been recently questioned due to the above concerns (Daya, 1993). Due to the prohibitive numbers required with a parallel study design, and after obtaining statistical advice, it was decided that a crossover study was more feasible. In view of the above concerns, it was decided that extensive analysis of the first attempt would be carried out and the data would then be compared with the overall results to determine the validity of this approach. The analysis also attempted to address the influence of the different confounding variables

on outcome and endeavoured to explain the differences between the various reports as to the effectiveness of TET. The "overall" first treatment results were generally reflected in the final results and similarly the results were comparable in the unexplained infertility, male factor groups and in women who received hCG luteal phase support, but were dissimilar in women with female factors.

Several reports had claimed considerable advantage following ZIFT or TET as compared to UET (Hamori et al, 1988; Devroey et al, 1989; Pool et al, 1991; Palermo et al, 1989), whilst more recently, others (Tanbo et al, 1990; Toth et al, 1992; Tournaye et al, 1992; Fluker et al, 1993) had failed to demonstrate any beneficial effect. Many of these reports were either non-randomised studies or retrospective analyses, had no clearly defined inclusion criteria, had no controls or suffered from a combination of the above. Furthermore, many had methodological pitfalls such as the use of more than one stimulation protocol in the study (Palermo et al, 1989; Tanbo et al, 1990; Toth et al, 1992; Fluker et al, 1993) or introduced changes in the protocols during the study period (Tanbo et al, 1990) or did not attempt to report the results by the cause of infertility. A full review of these studies was reported recently (Amso and Shaw, 1993).

Pregnancy rates following TET or UET in the first treatment attempt are comparable with rates reported in national registers (AFS, 1993; FIVNAT, 1993; HFEA, 1993). The results of the first attempt also represent a randomised comparison between two modalities of treatment in a parallel and controlled study. In the study design, a total of 124 replacements under each arm were planned. However, due to a number of factors these targets were not achieved and 124 replacements only (TET n=58; UET n=66) are available for analysis. Based on the pregnancy rates per transfer achieved in the first attempt (TET 28.6% and UET 20%) and assuming persistence of a *true difference* of 10% between the two pregnancy rates, approximately 410 transfer cycles for each group would have been required to demonstrate a significant difference at 5% level and 90% power. This would have been prohibitive for most centres to achieve singlehandedly in a relatively short period of time.

The two groups randomised for the first attempt are comparable in age, duration of

infertility, parity and infertility factors (Table 7.10). Attention to these criteria at recruitment only could however be misleading. Table 7.14 depicts the characteristics and treatment description of women undergoing their first embryo transfer and reveals a significantly longer duration of infertility in women undergoing TET. Similarly, a significantly higher fertilisation rate per stimulation cycle started for the TET group, had disappeared when transfer cycles only are compared (Table 7.12). Attention to the characteristics of women that are receiving embryos is crucial when two treatment modalities involving different routes of transfer, such as TET and UET, are evaluated.

Analysis of factors that may influence the outcome of the first treatment cycle showed that parity, unexplained infertility, and luteal phase support with HCG were associated with increased pregnancy rates following TET, whilst in others, such as nulliparity, presence of male or female factors, the outcome of both treatments was very similar. None of these differences reached the significance level. This is probably due to the relatively small numbers in the subgroups and the trial. Logistic regression analysis of the dependent variable (Treatment) with a number of confounding independent variables showed an increase in the odds ratio in favour of TET when adjusted for the duration of infertility, number of forwardly motile sperm in a PCT and total number of oocytes collected. This statistical approach to obtain 'adjusted' estimates of treatment effect (adjusted for the confounding variable), has been used in other fertility studies comparing different treatment modalities (Abdalla et al, 1993; FIVNAT, 1993). In the present study, the live birth rate per TET after the first attempt was lower due to the higher pregnancy loss in this group. Two miscarriages occurred at approximately 20 weeks gestation (multiple pregnancy n=1, premature rupture of membranes and chorioamnionitis n=1), while the remaining women miscarried under 12 weeks gestation. These figures are in contrast to reports in the literature claiming lower miscarriage and superior live birth rates following TET.

In the second treatment attempt, the pregnancy rates following TET and UET were 32% and 12% respectively (p=0.10) (Table 7.21, analysis by intention to treat). However, women who had achieved fertilisation of oocytes and received embryos but

did not become pregnant in their first treatment cycle appear to constitute a more favourable group of patients (Table 7.22). In these patients, a high pregnancy rate was achieved following TET (44.4%) compared to UET (19%) (Table 7.23) and was most apparent when women had UET in the first attempt. It is impossible to determine the precise factors that led to this difference, which does not reach significance, but is substantially higher than the outcome between the two modalities in the first treatment cycles (TET 28.6% and UET 20%). Unfortunately, the number of transfers is small and hence it is not possible to construct a life-table chart or cumulative conception rates. It is the precise understanding of these factors which influences the overall outcome of treatment that may explain the inability of some studies to demonstrate "significant" difference and it is further compounded when the overall numbers in a prospective study are small whilst retrospective analyses benefit from accumulated treatment cycles.

It has been postulated that in vitro culture conditions adversely influence pregnancy outcome and hence improved pregnancy rates following tubal transfer may be explained by the shorter incubation period (Palermo et al, 1989). In the current clinical trial, the pregnancy and implantation rates following TET in the first attempt and in couples with unexplained infertility were very similar whether transfers were carried out on the first or second days following VEC. Balmaceda et al (1988) reported high IR following TET at 48 hours which suggested that the prolonged in vitro incubation of embryos had not influenced the outcome adversely. The authors also argued that transfers performed with embryos of more advanced stages of development may even increase the total number of embryos available for transfer per patient and thus enabled the selection of morphologically superior embryos. In some groups poor pregnancy rates were noted following the second day TET. This is most likely due to poor or delayed fertilisation in these women rather than adverse culture conditions.

During this study, two pregnancies occurred in women following the administration of GnRH-a (PR/stimulation started, 2/227=0.9%). Inadvertent pregnancies have been reported after starting GnRH-a at mid-cycle or in the mid-luteal phase (Martinez et al, 1988; Ron El et al, 1990; Jackson et al, 1992). So far, there have been no

reports of major congenital abnormalities in the fetus following early exposure to GnRH-a, but in recent reviews (Jackson et al, 1992; Balasch et al, 1993), two of the 37 reported births in the literature were reported to have minor defects including cleft palate and bilateral talipes. The two pregnancies reported in this study ended in deliveries of normal infants at term.

No ectopic pregnancies were reported following either TET or UET. This was probably due to strict selection criteria of patients and readiness to withhold embryo transfers when the Fallopian tubes appeared unhealthy during TET.

In this study, one treatment cycle only (incidence 1/227=0.44%) was considered to be at greatly increased risk of OHSS and the oocytes were aspirated, fertilised and all normally developing embryos were cryopreserved for transfer at a later date. This approach was proposed for the management or prevention of predicted OHSS (Amso et al, 1989; Amso et al, 1990) and frozen-thawed embryo replacement may take place in natural, stimulated or hormone replacement cycles with equally satisfactory results (Amso et al, 1990; Salat-Baroux et al, 1990; Amso and Shaw, 1991; Wada et al, 1992).

7.5 RESULTS-3: OUTCOME BY INFERTILITY FACTOR

7.5.1 UNEXPLAINED INFERTILITY TREATMENT CYCLES

The diagnosis of unexplained infertility was reached after thorough assessment and completion of all the tests as described in section 6(II).2. Table 7.27 depicts the characteristics of women with unexplained infertility who were enrolled into the study. Thirty one women underwent the first stimulation cycle, twenty four of whom proceeded to a second stimulation cycle, thirteen to a third cycle, five to a fourth cycle and three to a fifth stimulation cycle resulting in the total of seventy six stimulation cycles being analysed. No significant difference was demonstrated in the age, duration of infertility and parity of women undergoing the five attempts.

Tables 7.28 and 7.29 summarise the outcome of all stimulation cycles in women with unexplained infertility and the outcome of embryo transfers, pregnancy and implantation rates and live births in each group.

Table 7.27: Characteristics of the cohort of women with unexplained infertility who underwent the first stimulation cycle (n=31) in comparison with all unexplained infertility stimulation cycles (n=76)

		First stimulation cycle	All cycles
Age (mean ± s.d)	=	32.0±3.3	32.2±3.1
range		(25-42)	(25-42)
Duration of infertility (mean ± s.d)	=	4.7±2.9	4.8±2.5
range		(2-17)	(2-17)
Parity:			
nulliparous (%)	=	20 (64.5%)	48 (63.2%)
parous (%)	=	11 (35.5%)	28 (36.8%)
Total number	=	31	76

Table 7.28: Outcome of all the stimulation cycles in patients with Unexplained Infertility

CYCLES	%A	%C	%D	%E
A- Stimulation started (n= 76)	100%			
TET = 33	43.4%			
UET = 43	56.6%			
B- Abandoned (n= 10)	13.2%			
TET = 6	18.2%			
UET = 4	9.3%			
C- Successful VEC (n= 66)	86.8%			
TET = 27	81.8%			
UET = 39	90.7%			
	p=0.26			
D- With fertilisation (n= 55)	72.4%	83.3%		
TET = 25	75.8%			
UET = 30	69.8%			
	p=0.56			
Fertilisation rate:				
All cycles = 49.7% (225/453)		Transfer cycles = 63.3% (209/330)		
TET = 61.6% (109/177)		TET = 68.2% (101/148)		
UET = 42% (116/276)		UET = 59.3% (108/182)		
p<0.0001		p=0.10		
E- Embryo transfers (n= 49)	64.5%	74.2%	89.1%	
TET = 22	66.7%			
UET = 27	62.8%			
	p=0.73			
F- Clinical pregnancies (n= 11)	14.5%	16.7%	20.0%	22.5%
TET = 7	21%			31.8%
UET = 4	9%			14.8%

Table 7.29: Outcome of embryo replacements in patients with Unexplained Infertility

	Number of embryo transfers			
Embryo(s) per transfer	**UET** (pregnant)	Day 1 TET (pregnant)	Day 2 TET	**Total TET** (pregnant)
One embryo	2 (0)	....	3 (0)	3 (0)
Two embryos	7 (2)	....	4 (1)	4 (1)
Three embryos	18 (2)	13 (4)	2 (2)	15 (6)
TOTAL	27 (4)	13 (4)	9 (3)	22 (7)
Pregnancy rate (PR) *p=0.16	**14.8%***	30.8%	33.3%	**31.8%***

	Number of embryos replaced			
Embryo(s) per transfer	**UET** (gestation sacs)	Day 1 TET (gestation sacs)	Day 2 TET	**Total TET** (gestation sacs)
One embryo	2 (0)	....	3 (0)	3 (0)
Two embryos	14 (3)	....	8 (1)	8 (1)
Three embryos	54 (2)	39 (5)	6 (2)	45 (7)
TOTAL	70 (5)	39 (5)	17 (3)	56 (8)
Implantation rate (IR) *p=0.19	**7.1%***	12.8%	17.7%	**14.3%***
Patients miscarried	1	0	2	2
Patients delivered	3	4	1	5
Live birth rate/ET *p=0.27	**11.1%***			**22.7%***

7.5.2 MALE FACTOR INFERTILITY TREATMENT CYCLES

Table 7.30 depicts the characteristics of women whose partners suffered from male factor infertility who were enrolled into the study. Forty women underwent the first stimulation cycle, thirty two of whom proceeded to a second stimulation cycle, twelve to a third cycle, and three to a fourth stimulation cycle resulting in the total of eighty seven stimulation cycles being analysed. No significant difference in age or parity of women undergoing the four treatment cycles was noted. Women undergoing their fourth stimulation cycle showed a significantly higher duration of infertility (8.7±3.5 years, p=0.04; degrees of freedom=3) compared to this cohort of women recruited to the study.

Tables 7.31 and 7.32 summarise the outcome of all stimulation cycles in couples with male factor infertility and the outcome of embryo transfers, pregnancy and implantation rates and live births in each group.

Table 7.30: Characteristics of the cohort of women whose partners suffered from male factor infertility and who were recruited into the study (n=40) in comparison with characteristics of all male factor stimulation cycles (n=87)

		First stimulation cycle	All cycles
Age (mean ± s.d)	=	32.1±3.1	32.2±3.1
range		(25-38)	(25-38)
Duration of infertility (mean ± s.d)	=	4.3±2.3	4.7±2.7
range		(2-12)	(2-12)
Parity:			
nulliparous (%)	=	29 (72.5%)	61 (70.1%)
parous (%)	=	11 (27.5%)	26 (29.9%)
Total number	=	40	87

Table 7.31: Outcome of the stimulation cycles in the group with Male Factor infertility

CYCLES	%A	%C	%D	%E
A- Stimulation started (n= 87)	100%			
TET = 41	47.1%			
UET = 46	52.9%			
B- Abandoned (n= 14)	16.1%			
TET = 7	17.1%			
UET = 7	15.2%			
C- Successful VEC (n= 72)	82.8%			
TET = 34	82.9%			
UET = 38	82.6%			
	p=0.97			
D- With fertilisation (n= 40)	46.0%	55.6%		
TET = 22	53.7%			
UET = 18	39.1%			
	p=0.17			
Fertilisation rate:				
All cycles = 34.6% (150/433)		Transfer cycles = 61.9% (148/239)		
TET = 35.3% (78/221)		TET = 60.3% (76/126)		
UET = 34.0% (72/212)		UET = 63.7% (72/113)		
p=0.77		p=0.59		
E- Embryo transfers (n= 38)	43.7%	52.8%	95.0%	
TET = 20[§]	48.8%			
UET = 18	39.1%			
	p=0.37			
F- Clinical pregnancies (n= 7)	8.1%[¶]	8.3%*	15.9%*	15.8%*
TET = 4	9.8%			20%
UET = 2	4.3%			11.1%
after GnRH-a = 1	1.1%			

[§] In three cycles analysed under TET, the patients opted to have UET contrary to their randomisation. Two women received one embryo each and did not conceive. One woman received three embryos resulting in a singleton pregnancy and delivery.

[¶] All pregnancies divided by all stimulation cycles started

* Pregnancies resulting from UET and TET only divided by the relevant denominator

Table 7.32: Outcome of embryo replacements of the patients with Male Factor infertility according to the original randomisation and intention to treat

	Number of embryo transfers			
Embryo(s) per transfer	**UET** (pregnant)	Day 1 TET	Day 2 TET (pregnant)	**Total TET** (pregnant)
One embryo	2 (1)	....	5 (0)	5 (0)
Two embryos	4 (0)	1 (0)	....	1 (0)
Three embryos	12 (1)	8 (3)	6 (1)	14 (4)
TOTAL	18 (2)	9 (3)	11 (1)	20 (4)
Pregnancy rate (PR) *p=0.46	**11.1%***	33.3%	9.1%	**20%***

	Number of embryos replaced			
Embryo(s) per transfer	**UET** (gestation sacs)	Day 1 TET	Day 2 TET (gestation sacs)	**Total TET** (gestation sacs)
One embryo	2 (1)	....	5 (0)	5 (0)
Two embryos	8 (0)	2 (0)	....	2 (0)
Three embryos	36 (2)	24 (3)	18 (1)	42 (4)
TOTAL	46 (3)	26 (3)	23 (1)	49 (4)
Implantation rate (IR) *p=0.76	**6.5%***	11.5%	4.4%	**8.2%***
Patients miscarried	0	2	....	2
Patients delivered	2	1	1	2
Live birth rate/ET *p=0.92	**11.1%***			**10%***

7.5.3 FEMALE FACTOR TREATMENT CYCLES

Table 7.33 depicts the characteristics of women with female factors. Fourteen women were treated in the first stimulation cycle, nine of whom proceeded to a second stimulation cycle, six to a third cycle, and two to a fourth stimulation cycle resulting in the total of thirty one stimulation cycles being analyzed. No significant difference in the age, duration of infertility and parity of women undergoing the four attempts was noted.

Tables 7.34 and 7.35 summarise the outcome of all stimulation cycles in couples with female factor and the outcome of embryo transfers, pregnancy and implantation rates and live births in each group.

Table 7.33: Characteristics of the cohort of women with Female Factor infertility and who were recruited into the study (n=14) in comparison with characteristics of all stimulation cycles (n=31) in this group

		First stimulation cycle	All cycles
Age (mean ± s.d)	=	31.1±4.1	30.8±3.3
range		(24-40)	(24-40)
Duration of infertility (mean ± s.d)	=	4.8±1.7	4.7±1.5
range		(3-9)	(3-9)
Parity:			
nulliparous (%)	=	11 (78.6%)	25 (80.6%)
parous (%)	=	3 (21.4%)	6 (19.4%)
Total number	=	14	31

Table 7.34: Outcome of the stimulation cycles in women with Female Factors only

CYCLES	%A	%C	%D	%E
A- Stimulation started (n= 31)	100%			
TET = 22	71%			
UET = 9	29%			
B- Abandoned (n= 9)	29%			
TET = 9	41%			
UET = 0	---			
C- Successful VEC (n= 22)	71%			
TET = 13	59.1%			
UET = 9	100%			
	p=0.023			
D- With fertilisation (n= 19)	61.3%	86.4%		
TET = 11	50%			
UET = 8	88.9%			
	p=0.10			
Fertilisation rate:				
All cycles = 49.7% (77/155)		Transfer cycles = 59.8% (76/127)		
TET = 57.5% (50/87)		TET = 61.7% (50/81)		
UET = 39.7% (27/68)		UET = 56.5% (26/46)		
p=0.03		p=0.57		
E- Embryo transfers (n= 18)	58.1%	81.8%	94.7%	
TET = 11	50%			
UET = 7	77.8%			
	p=0.24			
F- Clinical pregnancies (n= 4)	12.9%	18.2%	21%	22.2%
TET = 3	13.6%			27.3%
UET = 1	11.1%			14.3%
after GnRH-a = 0				

Table 7.35: Outcome of embryo replacements in patients with Female Factors only

	Number of embryo transfers			
Embryo(s) per transfer	**UET** (pregnant)	Day 1 TET (pregnant)	Day 2 TET	**Total TET** (pregnant)
One embryo	....	....	1 (0)	1 (0)
Two embryos	3 (0)	....	....	....
Three embryos	4 (1)	7 (2)	3 (1)	10 (3)
TOTAL	7 (1)	7 (2)	4 (1)	11 (3)
Pregnancy rate (PR)	**14.3%***	28.6%	25%	**27.3%***

*p=1.0

	Number of embryos replaced			
Embryo(s) per transfer	**UET** (gestation sacs)	Day 1 TET (gestation sacs)	Day 2 TET	**Total TET** (gestation sacs)
One embryo	....	....	1 (0)	1 (0)
Two embryos	6 (0)	....	....	....
Three embryos	12 (2)	21 (4)	9 (2)	30 (6)
TOTAL	18 (2)	21 (4)	10 (2)	31 (6)
Implantation rate (IR)	**11.1%***	19%	20%	**19.4%***
Patients miscarried	...	...	1	1
Patients delivered	1	2	...	2
Live birth rate/ET	**14.3%***			**18.2%***

*p=0.45

*p=1.0

7.5.4 COMBINED FEMALE AND MALE FACTOR TREATMENT CYCLES

Table 7.36 depicts the characteristics of women with combined female and male factors. Seventeen women were treated in the first stimulation cycle, twelve of whom proceeded to a second stimulation cycle, and four to a third cycle resulting in the total of thirty three stimulation cycles being analysed. No significant difference in age, duration of infertility, or parity of women in the three stimulation attempts was noted.

Tables 7.37 and 7.38 summarise the outcome of all stimulation cycles in couples with combined female and male factors and the outcome of embryo transfers, pregnancy and implantation rates and live births in each group.

Table 7.36: Characteristics of the cohort of women with combined female and male factor infertility and who were recruited into the study (n=17) in comparison with characteristics of all stimulation cycles (n=33) in this group

		First stimulation cycle	All cycles
Age (mean ± s.d)	=	32.2±2.3	32.3±2.3
range		(27-35)	(27-35)
Duration of infertility (mean ± s.d)	=	5.6±2.0	5.7±2.1
range		(3-10)	(3-10)
Parity:			
nulliparous (%)	=	12 (70.6%)	22 (66.7%)
parous (%)	=	5 (29.4%)	11 (33.3%)
Total number	=	17	33

Table 7.37: Summary of the stimulation cycles with combined Male and Female factors

CYCLES	%A	%C	%D	%E
A- Stimulation started (n= 33)	100%			
TET = 15	45.5%			
UET = 17	51.5%			
undetermined = 1*	3.0%			
B- Abandoned (n= 3)*	9.1%			
TET = 1	6.7%			
UET = 1	5.9%			
C- Successful VEC (n= 30)	90.9%			
TET = 14	93.3%			
UET = 16	94.1%			
	p=0.95			
D- With fertilisation (n= 19)	57.6%	63.3%		
TET = 9	60.0%			
UET = 10	58.8%			
	p=0.95			
Fertilisation rate:				
All cycles = 47.3% (86/182)		Transfer cycles = 69.4% (86/124)		
TET = 45.4% (39/86)		TET = 67.2% (39/58)		
UET = 49.0% (47/96)		UET = 71.2% (47/66)		
p=0.63		p=0.63		
E- Embryo transfers (n= 19)	57.6%	63.3%	100%	
TET = 9§	60.0%			
UET = 10	58.8%			
	p=0.98			
F- Clinical pregnancies (n= 6)	18.2%¶	16.7%**	26.3%**	26.3%**
TET = 3	20.0%			33.3%
UET = 2	11.8%			20.0%
after GnRH-a = 1	3.0%			

* One cycle was abandoned before randomisation as the patient conceived following GnRH-a treatment

§ In one cycle analysed under TET, the patient opted to have UET contrary to the randomised treatment. One embryo was replaced resulting in a singleton pregnancy and delivery

¶ All pregnancies divided by all stimulation cycles started

** Pregnancies resulting from UET and TET only divided by the relevant denominator

Table 7.38: Outcome of embryo replacements in patients with combined Male and Female factors according to the original randomisation and intention to treat

	Number of embryo transfers			
Embryo(s) per transfer	UET (pregnant)	Day 1 TET (pregnant)	Day 2 TET	Total TET (pregnant)
One embryo	....	....	1 (1)	1 (1)
Two embryos	....	....	....	....
Three embryos	10 (2)	7 (2)	1 (0)	8 (2)
TOTAL	10 (2)	7 (2)	2 (1)	9 (3)
Pregnancy rate (PR) *p=0.51	**20%***	28.6%	50%	**33.3%***

	Number of embryos replaced			
Embryo(s) per transfer	UET (gestation sacs)	Day 1 TET (gestation sacs)	Day 2 TET	Total TET (gestation sacs)
One embryo	....	....	1 (1)	1 (1)
Two embryos	....	....	....	....
Three embryos	30 (3)	21 (4)	3 (0)	24 (4)
TOTAL	30 (3)	21 (4)	4 (1)	25 (5)
Implantation rate (IR) p=0.30	**10%***	**19.0%**	**25%**	**20%***
Patients miscarried	0	1	....	1
Patients delivered	2	1	1	2
Live birth rate/ET *p=0.91	**20%***			**22.2%***

In one cycle analysed under TET, the patient opted to have UET contrary to the randomised treatment. One embryo was replaced resulting in a singleton pregnancy and delivery

7.5.5 DISCUSSION

UNEXPLAINED INFERTILITY

In vitro fertilisation is an established and successful therapy for couples with unexplained infertility. Pregnancy rates reported in this study compare favourably with results in other series, in which in vitro fertilisation and uterine transfer (Leeton et al, 1987; Navot et al, 1988; Audibert et al, 1989) or Fallopian transfer (Devroey, 1989) have been used. Differences in pregnancy rates between the unexplained and tubal infertility groups have been reported previously (Navot et al, 1988; Yovich et al, 1988). In women with unexplained infertility, the fertilisation rate was reported to be significantly lower (Fishel and Edwards, 1982; Mahadevan et al, 1983; Leeton et al, 1984), while the cleavage rate either lower than (Navot et al, 1988) or similar to (Mackenna, 1992) tubal infertility. The factors that may contribute to the success of assisted reproduction in unexplained infertility include greater chance of obtaining fertilisation when more than one oocyte is inseminated, as defective fertilisation has been suggested as a cause of infertility in these women (Trounsen et al, 1980), and/or due to by-passing possible impaired sperm transport or interaction in the upper genital tract (Cefalu et al, 1988; Templeton and Mortimer, 1982). Equally, endometrial receptivity may also be affected and hence contributes to the couple's unexplained infertility (see below).

In this study, the overall fertilisation rate in the unexplained infertility group was; (1) lower than that reported in some studies (Sharma et al, 1988; Navot et al, 1988; Leeton et al, 1984) but comparable to rates reported by Audibert et al (1989), Hamori et al (1988) and very similar to the fertilisation rate in non-trial patients who were concurrently undergoing IVF-UET at the Royal Free Hospital during 1989 and 1990 (Tables 7.8 and 7.9). Despite randomisation, the fertilisation rate per stimulation cycle started was significantly higher in the TET group (Table 7.28). However, closer examination of the fertilisation rate in cycles reaching embryo transfer stage, a better index when comparing two replacement modalities, revealed considerably less and statistically not significant difference. The clinical PR/UET and IR of women with unexplained infertility in this trial and the overall non-trial UET were equally comparable (PR/UET; UI=14.8%, non-trial=14% and IR;

UI=7.1%, non-trial=6.6%), whilst the pregnancy rate was twice as high following TET. This higher PR following TET was observed after the first treatment attempt (TET=44%, UET=13%) and when all unexplained infertility treatment cycles (TET=31.8%, UET=14.8%) were analysed (Tables 7.15.iv and 7.29). These differences do not reach statistical significance and hence may lead to the assumption that there is no advantage from TET. However, the results are inconclusive because of the lack of statistical power, and persistence of a difference throughout the study period is an indicator of a better outcome with TET. On the basis of the above pregnancy rate differences, a confirmation study would require approximately 50 replacements under each arm for a one attempt per patient, parallel study design to reach statistical significance at 5% level and 80% power. A pregnancy rate of 44% per TET after the first attempt of treatment is very similar to the 48% that was reported by Devroey et al (1989) in a comparable group of women.

Such indicators, if confirmed in a larger series, would signify a potential benefit in the unexplained infertility group. It has been postulated that the serum of women with unexplained infertility may impair blastocyst formation and result in failure to achieve pregnancy (Dokras et al, 1993). In the IVF unit at the Royal Free Hospital, patients' own heat-inactivated sera were used in the transfer media (section 6(II).6.1). If this practice had any adverse effects on the pregnancy rate, then both tubal and uterine replacements would have been affected equally. It is also possible that the small volume used in the replacement medium and the shorter period of exposure of embryos to the patient's own serum had minimal effect on subsequent embryo development and chances of implantation. Similarly, there was little difference in the pregnancy and implantation rates following TET on day one or two suggesting little adverse effect of the extended culture on embryo potential for implantation.

Although it had been disputed previously (Mahadevan et al, 1983), endometrial receptivity may be altered in women with unexplained infertility. Seif (1989), using immunohistochemistry with monoclonal antibody D9B1, had reported the secretion, in fertile women, of an oligosaccharide epitope produced by endometrial gland cells between two and seven days after the LH surge (peri-implantation period) and postulated a potential role of the molecules carrying this epitope in the implantation

of the blastocyst. Graham et al (1990), reported that in women with unexplained infertility, the synthesis and secretion of this epitope was significantly reduced and delayed even in the presence of normal concentration of circulating progesterone. Similarly, Li et al (1990a), using morphometric techniques, had reported retarded endometrial development in 27% of women with unexplained primary infertility. Although, the functional role of the above factor and its influence on early pregnancy and implantation remains speculative, it is possible that exposure of embryos to tubal environment and their delayed arrival into the uterine cavity may offset this factor's deficiency at the time of UET.

MALE FACTOR INFERTILITY

In this study, male factor infertility constituted the largest group of couples seeking treatment and higher in proportion than reported in other studies. This is due to; (1) the unit at the Royal Fee Hospital was a referral centre and not a District General Hospital, (2) women with tubal factor were excluded from the study, and (3) IVF was utilised in this group for its diagnostic value as well as for therapy. IVF-UET was proposed in 1983 as a treatment for male infertility (Mahadevan et al, 1983). Since then various other assisted reproduction techniques have been applied e.g GIFT (Asch et al, 1986), intraperitoneal insemination (Guastella and Cimino, 1986); ZIFT (Devroey et al, 1986; Yovich et al, 1987) or TET (Balmaceda et al, 1988). Despite increasing interest, the possibilities and limitations of these techniques for establishing pregnancies in cases involving subfertile men are still not well established. Information regarding fertilisation is of utmost importance in male factor infertility thus tubal embryo transfer is preferable to GIFT for this reason alone. Indeed, in 32 out of 72 cycles (44%) fertilisation failed to occur and thus such information would have been unavailable if these couples were treated with GIFT. The fertilisation rate in this group of male factor infertility patients was lower than in other categories in this study (unexplained infertility, female factors) whilst comparable with reduced fertilisation rates reported for male factor infertility by others (Mahadevan and Baker, 1984; Hirsch et al, 1986; Hamori et al, 1988; Tournaye et al, 1991, 1992a,b). The causes for this low rate or failure of fertilisation could be related to sperm dysfunction, oocyte maturity, or less likely in vitro culture conditions. Coates et al (1992) evaluated couples with failure of fertilisation in vitro and highlighted the

inconsistency of semen parameters in identifying the suboptimal sperm sample, and that concomitant oocyte factor may play a part in this respect. The authors concluded that failure to fertilise in a given cycle did not necessarily predict failure to fertilise in a subsequent cycle, but did predict a poor fertility outcome unless donor gametes were used.

In this study, the results following the first treatment attempt did not show any significant benefit from tubal transfer (PR/UET= 21%; PR/TET= 23%) and were different from those when all male infertility treatment cycles were analysed (PR/UET= 11%; PR/TET= 20%; p=0.46). This is because in the overall analysis, three transfer cycles resulting in a singleton pregnancy and delivery were included under TET according to the intention to treat although the women opted to have UET contrary to their randomisation. Similarly, there was no difference in the live birth rate/ET between the two groups (UET=11%; TET=10%; p=0.92). These findings confirm other reports (Tournaye et al, 1992a) and would suggest that once fertilisation has taken place, and an embryo is available for transfer, the pregnancy rate is similar to the other categories irrespective of the place of transfer. Higher pregnancy rate was observed in patients having day one TET. This may not represent a true beneficial effect as these cycles may have sperm with superior fertilising capacity. Tournaye and colleagues (1992b), reported that 11% of all cycles with male factor are complicated by delayed fertilisation (>24 hours) and no pregnancies resulted from transfer of these embryos indicating their low potential for implantation. It is also possible that limiting the number of transferred zygotes/embryos to a maximum of three may have affected both PR and IR. The implantation rate in this group (Table 7.32) was similar whether the patients received UET or TET (6.5% and 8.2% respectively). In spite of initial heightened expectations for ZIFT treatment for male factor infertility (Palermo et al, 1989), subsequent prospective randomised studies (Tournaye et al, 1992b) failed to demonstrate any improvement in pregnancy and implantation rates from tubal transfer.

The number of treatment cycles in couples with female causes only or combined male and female factors is small and hence the results should be interpreted with caution.

Equally, the precise diagnostic category depended on the presence of infertility factors (e.g. endometriosis or high abnormal forms in several semen analyses) during the investigation phase irrespective of their status at the time of treatment which may limit the impact or adverse effect of any subsequent changes in these disorders on oocyte quality, fertilisation rate and/or implantation potential. The trial reported in this thesis is the first attempt to address the influence of the different confounding variables on outcome and endeavour to explain the differences between the various reports as to the effectiveness of TET.

7.6 RESULTS-4: ANALYSIS OF OUTCOME BY AGE AND DURATION OF INFERTILITY AT RECRUITMENT

All stimulation cycles are divided into 3 groups according to the age of the female partner at recruitment: under 30 years, 30-34 years, and 35 or above years. These age categories are the same as those used by the IVF Registry for reporting age-related outcomes of IVF-ET with the exception that women under 25 years or over 40 years of age are included in the nearest age groups due to small numbers. Similarly, all stimulation cycles are divided into 3 groups according to the couple's duration of infertility at recruitment: under 4 years, 4 years, and above 4 years. Four years of infertility is selected as the cut off point because it includes the mean (4.7), median (4.0), and mode (4.0) of the 102 couples included in the first stimulation cycle and who constitute the source of the 227 stimulation cycles under analysis. Tables 7.39 and 7.40 depict the characteristics of women in the two subcategories who were recruited into the study.

7.6.1 OUTCOME BY AGE AT RECRUITMENT

Table 7.41 summarises the characteristics of all treatment cycles undergoing UET and TET in the three age subgroups and Table 7.42 summarises the outcome of these treatment cycles (UET and TET).

7.6.2 OUTCOME BY DURATION OF INFERTILITY

Table 7.43 summarises the characteristics of all treatment cycles undergoing UET and TET in the three subgroups and Table 7.44 summarises the outcome of the stimulation cycles and embryo replacements (UET and TET).

Table 7.39: Characteristics of the women in the three age subgroups (<30 years, 30-34 years and ≥35 years) who were recruited into the study (n=102)

	< 30 years (n=21)	30-34 years (n=60)	≥ 35 years (n=21)
Duration of infertility[¶] (mean±s.d.)/(range)	4.5±1.9	4.6±1.8	5.4±4.0
Parity:[§]			
nulliparous	18	42	12
parous	3	18	9
Infertility factors:[*]			
none (Unexplained infertility)	6	20	5
male factors	8	22	10
female factors	5	7	2
male and female factors	2	11	4

[¶] Between groups of patients p=0.31
[§] Between groups of patients p=0.13
[*] Between groups of patients p=0.70

Table 7.40: Characteristics of the women in the three subgroups according to the duration of infertility (<4, 4 and >4 years) who were recruited into the study (n=102)

	< 4 years (n=32)	4 years (n=26)	≥ 4 years (n=44)
Age at inclusion[¶] (mean±s.d.)/(range)	31.6±3.7 (24-42)	32.2±2.8 (25-40)	32.1±3.0 (26-38)
Parity:[§]			
nulliparous	25	20	27
parous	7	6	17
Infertility factors:[*]			
none (Unexplained infertility)	11	8	12
male factors	16	9	15
female factors	3	4	7
male and female factors	2	5	10

[¶] Between groups of patients p=0.71
[§] Between groups of patients p=0.20
[*] Between groups of patients p=0.48

Table 7.41: Characteristics of all treatment cycles in the three age subgroups (<30 years, 30-34 years and ≥35 years)

	< 30 years (n= 42)			30-34 years (n= 139)[¶]			≥ 35 years (n= 46)		
(No.)	UET (19)	TET (23)	p	UET (72)	TET (66)	p	UET (24)	TET (22)	p
Duration of infertility[§] (mean±s.d.)	4.9 ±1.9	4.1 ±1.5	0.13	4.6 ±1.9	4.8 ±1.7	0.59	5.8 ±3.5	5.7 ±4.6	0.93
Parity:[*]			0.054			0.04			0.78
nulliparous	18	16		44	51		14	12	
parous	1	7		28	15		10	10	
Infertility factors:[**]			0.88			0.03			0.32
none (Unexplained infertility)	6	6		32	20		5	7	
male factors	8	9		22	23		16	9	
female factors	3	6		5	15		1	1	
male and female factors	2	2		13	8		2	5	

[¶] One patient conceived after starting GnRH-a and before being randomised to either group. The pregnancy ended in normal delivery
[§] Significantly longer duration of infertility in ≥35 years group both treatments (p=0.016)
[*] Significantly more nulliparous in the <30 years UET group (p=0.015)
[**] No significant difference among the subgroups for both UET and TET

Table 7.42: Outcome of ALL stimulation cycles and treatment in the three age subgroups (<30, 30-34, and ≥35 years)

	< 30 years (n= 42)			30-34 years (n= 139)[¶]			≥ 35 years (n= 46)		
	UET	TET	p	UET	TET	p	UET	TET	p
Stimulation cycles started	19	23		72	66		24	22	
Abandoned cycles	1	1		2	19[§]		9	3	
Successful VEC	18	22		69	47		15	19	
No. of oocytes (mean±s.d.)	7.6±4.3	8.9±5.8	0.45	7.6±4.2	9.4±5.6	0.04	9.5±6.2	7.1±4.2	0.19
Cycles with fertilisation	9	17		49	32		8	18	
Fertilisation rate-All cycles	36.6%	47.2%	N.S.	44.2%	44.6%	N.S.	27.9%	61.1%	<0.001
Transfer cycles	59.3%	62.1%	N.S.	62.1%	68.5%	N.S.	62.5%	62.0%	N.S.
Cycles with embryo transfer*	9	17		46	28		7	17	
No. of pregnancies (PR/ET)	1 (11.1%)	9 (52.9%)	0.037	8 (17.4%)	4 (14.3%)	0.73	-	4 (23.5%)	0.28**
Total no. of embryos replaced	25	48		119	72		20	41	
No. of sacs (IR)	2 (8.0%)	14 (29.2%)	0.04	11 (9.2%)	5 (6.9%)	0.58	-	4 (9.8%)	0.29**
No. of deliveries (LBR/ET)	1 (11.1%)	4 (23.5%)	0.45	7 (15.2%)	4 (14.3%)	0.92	-	3 (17.7%)	0.53**

ET= embryo transfer (uterine= UET, tubal= TET), PR= pregnancy rate, IR= implantation rate, LBR= live birth rate

[¶] One patient conceived after starting GnRH-a and before being randomised to either group. The pregnancy ended in normal delivery

[§] One patient conceived after starting GnRH-a and before hMG injections. The pregnancy ended in normal delivery

* In total, four cycles were analysed under TET but the patients opted to have UET contrary to their randomisation. One woman under 30 years did not conceive. One woman aged 33 years conceived and delivered, and of two women aged 35 years, one conceived and delivered.

**Fisher's exact test

Table 7.43: Characteristics of all treatment cycles according to the duration of infertility (<4, 4 and >4 years)

	< 4 years (n= 62)			4 years (n= 61)[¶]			> 4 years (n= 104)		
(n)	UET (29)	TET (33)	p	UET (35)	TET (25)	p	UET (51)	TET (53)	p
Age at inclusion[§] (mean±s.d.)	31.8 ±3.2	30.9 ±3.5	0.31	32.4 ±2.5	31.96 ±3.3	0.56	32.4 ±3.1	32.3 ±2.7	0.80
Parity:[*]			0.69			0.07			0.87
nulliparous	24	26		22	21		30	32	
parous	5	7		13	4		21	21	
Infertility factors:[**]			0.69			0.28			0.053
none (Unexplained infertility)	12	9		16	7		15	17	
male factors	14	19		9	8		23	14	
female factors	2	3		4	7		3	12	
male and female factors	1	2		6	3		10	10	

[¶] One nulliparous patient with combined male and female factors conceived after starting GnRH-a and before being randomised to either group. The pregnancy ended in normal delivery

[§] No significant difference in age between the three subgroups controlling for treatment

[*] No significant difference in parity between the three subgroups controlling for treatment

[**] No significant difference in infertility factors between the three subgroups controlling for treatment

Table 7.44: Outcome of All stimulation cycles and treatment by duration of infertility (<4, 4, and >4 years)

	< 4 years (n= 62)			4 years (n= 61)[¶]			> 4 years (n= 104)		
	UET	TET	p	UET	TET	p	UET	TET	p
Stimulation cycles started	29	33		35	25		51	53	
Abandoned cycles	3	6		5	7[§]		4	10	
Successful VEC	26	27	0.38	30	18	0.19	46	43	0.19
No. of oocytes (mean±s.d.)	6.8±3.6	7.4±4.4	0.59	8.7±4.98	9.7±5.5	0.55	7.9±4.6	9.3±5.8	0.20
Cycles with fertilisation	18	23	0.53	20	14	0.93	28	30	0.86
Fertilisation rate-All cycles	47.3%	52.3%	N.S.	29.6%	44.8%	<0.01	43.9%	47.8%	N.S.
Transfer cycles	69.3%	61.2%	N.S.	46.2%	58.7%	N.S.	68.3%	69.3%	N.S.
Cycles with embryo transfer	18	22*		17	13		27	27	
No. of pregnancies (PR/ET)	5 (27.8%)	9 (40.9%)	0.39	1 (5.9%)	3 (23.1%)	0.29	3 (11.1%)	5 (18.5%)	0.44
Total no. embryos replaced	47	53		43	31		74	77	
No. of sacs (IR)	8 (17%)	12 (22.6%)	0.49	1 (2.3%)	3 (9.7%)	0.30	4 (5.4%)	8 (10.4%)	0.26
No. of deliveries (LBR/ET)	4 (22.2%)	6 (27.3%)	0.72	1 (5.9%)	1 (7.7%)	1.0	3 (11.1%)	4 (14.9%)	0.69

ET= embryo transfer (uterine= UET, tubal= TET), PR= pregnancy rate, IR= implantation rate, LBR= live birth rate

[¶] One patient conceived after starting GnRH-a and before being randomised to either group. The pregnancy ended in normal delivery

[§] One patient in the TET group conceived after starting GnRH-a and before hMG injections. The pregnancy ended in normal delivery

* Four cycles were analysed under TET but the patients opted to have UET contrary to their randomisation. The transfers resulted in two pregnancies and term deliveries.

Table 7.45: Summary of pregnancy and implantation rates of ALL treatment cycles (n=227) and when stratified by stimulation attempt rank, diagnosis, age and duration of infertility. All analyses were according to intention to treat

Subgroup (no. of cycles)	Pregnancy rate/ET UET	TET	p	Implantation rate UET	TET	p
All cycles	14.5%	27.4%	0.08	7.9%	14.3%	0.07
Stimulation attempt rank:						
First attempt (102)	20%	29%	0.47	12%	15%	0.85
Second attempt (77)	12%	32%	0.10	6%	18%	**0.048**
Third attempt (35)	17%	23%	1.0	7%	11%	0.65
Cause of Infertility:						
Unexplained (76)	15%	32%	0.16	7%	14%	0.19
Male factor (87)	11%	20%	0.46	7%	8%	0.76
Female factor (31)	14%	27%	1.0	11%	19%	0.45
Male & Female (33)	20%	33%	0.51	10%	20%	0.30
Age at recruitment:						
< 30 years (42)	11%	53%	**0.037**	8%	29%	**0.04**
30-34 years (139)	17%	14%	0.73	10%	6%	0.33
≥ 35 years (46)	--	24%	0.28	--	10%	0.29
Duration of Infertility:						
< 4 years (62)	28%	41%	0.39	17%	23%	0.49
4 years (61)	6%	23%	0.29	2%	10%	0.30
> 4 years (104)	11%	19%	0.44	5%	10%	0.26

7.6.3 DISCUSSION

The effect of age and duration of infertility on the chance of conceiving naturally has been reviewed by Hull (1992). In women with unexplained infertility and minor endometriosis, age and more importantly, the duration of infertility are the main factors that determine the chance of spontaneous pregnancy. Most likely, the only unfavourable conditions when deciding on a treatment are the woman's age, 40 years or more (Hull et al, 1992) and sperm dysfunction (Sharma et al, 1988; Craft, 1990).

In this clinical trial, few women over 40 years were recruited and with the exception of significantly higher PR and IR following TET in women recruited under 30 years, other PR and LBR were not significantly different. The relatively small number of stimulation and transfer cycles in this age group (n=42 and n=26 respectively) does not allow firm conclusions to be drawn from these results.

In assisted reproduction treatment (IVF and GIFT) the influence of age on the success rate has been the subject of intensive studies (Craft et al, 1988; Sharma et al, 1988; Padilla and Garcia, 1989; Hull et al, 1992; Tan et al, 1992). Craft et al (1988), reported a significant decline in PR in women at or above the age of forty and treated with GIFT even though all oocytes recovered were replaced. In women treated with IVF-UET, Sharma et al (1988) reported that the highest number of clinical pregnancies per embryo transfer was obtained in the 26 to 30 years age group, whilst the lowest was in the 36 to 40 year group. The overall fertilisation and cleavage rates did not vary significantly with age, nor was there any significant change in the cumulative conception rate (CCR) within the age groups. In a subsequent retrospective analysis based on 5055 consecutive IVF cycles undertaken on 2735 patients from the same assisted conception centre (Tan et al, 1992), both CCR and cumulative live birth rate (CLBR) after five treatment cycles, were shown to decline with age from 54% and 45% respectively, at 20-34 years to 39% and 29% at 35-39 years and further to 20% and 14% at $\geq$ 40 years. These results in the under 34 years group compared favourably with spontaneous conception rates in the natural menstrual cycle and were in agreement with similar findings reported by Hull et al (1992). Tan et al (1992) also reported that the probability of pregnancy and live birth

rates declined with repeated treatment cycles and that couples with male infertility or multiple infertility factors had the lowest CCR. Similarly, in a prospective study comparing the CCR and CLBR after 3 cycles of IVF-UET, Check et al (1994) showed that there was an interaction effect of age and infertility factor on the cumulative probability of pregnancy. Women over the age of 35 years with multiple factors had the lowest CCR and CLBR (14% and 12% respectively) whilst those ≥ 35 years and with tubal factor only had the best results (CCR=33%, CLBR=30%).

National registers (FIVNAT, 1993; MRI-SART, 1992; MRI-SART-AFS, 1991; HFEA, 1992, 1993) have consistently showed decline in the pregnancy rates with increasing age of the female partner especially at or above the age of 40 years. The reduction in fecundity rate in older women has been attributed in part to a relative decrease in the number of oocytes having the potential for normal fertilisation and development or implantation. It is interesting to note that pregnancy and live birth rates in the older women (≥ 40 years) using donated oocytes are consistently higher than those when the women's own oocytes are used (HFEA, 1992, 1993; Abdalla et al, 1993) suggesting that this decline is associated with the age of the oocytes rather than the age of the uterus.

None of the studies mentioned above examined the influence of duration of infertility, at the time of recruitment, on the success rate of assisted reproduction techniques. The highest pregnancy and live birth rates in the present study were noted when the duration of infertility at recruitment was less than 4 years and then declined with increasing duration of infertility. No significant differences in the pregnancy and implantation rates between tubal and uterine replacements were noted, but in all subgroups tubal transfers resulted in a higher pregnancy rate. This effect was independent of age, parity or infertility factors as demonstrated in Table 7.43 where no significant differences in the characteristics of all treatment cycles in the three subgroups after controlling for treatment were noted. These observations would indicate that when deciding on treatment, clinicians should take the couples' duration of infertility into consideration in addition to the age of the female partner and infertility factors and that the appropriate advice should be offered to the patients.

It may be concluded that (1) the pregnancy rates and implantation rates are significantly higher following TET only in women under the age of 30 years, and (2) pregnancy and live birth rates are inversely related to the duration of infertility and are highest when the couple's infertility is of less than four years duration.

7.7 GENERAL DISCUSSION

In this trial, the analysis was carried out according to the original randomisation and intention to treat. The findings would lead to the assumption that there is no statistically significant difference between the pregnancy and live birth rates per replacement between TET and UET with the exception of a significantly higher PR and IR in women under the age of 30 years (Table 7.42) and when all cycles which underwent the transfer of three embryos were analysed (Tables 7.4 and 7.5). However, the results should be interpreted as inconclusive because of insufficient numbers and the lack of statistical power. Nevertheless, a number of indicators suggestive of a better outcome with TET were noted in the overall results and other subgroups as outlined previously. It is possible that persistence with the trial or recruitment of a larger number of patients, to achieve the previously outlined targets, might have demonstrated significant benefit in the subgroups where the difference is most noticeable. This assumption, though may not be absolutely correct, is based on, (1) consistency in the results after the first treatment attempt and all treatments in several subgroups, and (2) concordance of the results during two interim analyses with the final analysis (Table 7.7).

Assessment of efficacy in assisted reproduction is extremely difficult. Objective evaluation of treatment modalities requires other parameters such as cumulative conception rate (CCR) and cumulative live birth rate (CLBR) as well as implantation rates and early pregnancy loss rate. However, the use of the life table method to calculate CCR and CLBR has been questioned due to the selection bias that may be introduced in the method of calculation (Hershlag et al, 1992; Te Velde et al, 1992). In addition to selection bias at the point of entry to an assisted reproduction programme, the dropout population may be determined by physicians, counselling or other criteria for continuation of treatment. Thus the use of the life table method is only justified if couples deciding to refrain from further participation in the treatment have the same chance of success as the couples deciding to continue. Similar problems arise when two treatment modalities such TET and UET are compared which further complicates the issues of randomisation as discussed previously. Variation in the pregnancy rates for TET reported by the several studies may have

been influenced by factors associated with the couple's subfertility, methodological variation or number of embryos transferred. Hence these results may not represent an accurate comparison of GIFT or TET versus UET.

The implantation rate, however, may provide a better parameter in the assessment of two or more treatment modalities. Implantation is influenced by the quality of transferred embryos, endometrial receptivity, and efficiency of transfer (Paulson et al, 1990a,b) and thus any beneficial effect of a treatment modality will manifest itself easily. Another factor that may account for the differences in pregnancy or implantation rates between IVF-UET, GIFT and IVF-TET in the various studies is the influence of improvement in practice over a period of time in the individual institution. Such effect, as has recently been reported (Hull et al, 1992), may be greater on IVF-UET results than on GIFT or TET thereby greatly narrowing the gap.

Comparison of the pregnancy and implantation rates in Table 6.1, indicates that significant differences in pregnancy rates between UET versus GIFT or TET (Yovich et al, 1988; Hammitt et al, 1990; Pool et al, 1990), GIFT and UET versus TET (Bollen et al, 1991) or UET versus TET (Asch, 1991) are achieved only when the respective implantation rates are also significantly different. This implies that differences in patient selection, quality of embryos or endometrial receptivity are probably more critical to the establishment of pregnancy than simply the place of transfer. It is also interesting to note that when implantation rates for the different treatment modalities are compared, there is slight overlap between the TET and UET figures while those for GIFT are in an intermediate zone. Implantation rates for GIFT are only derived by assumption and are difficult to compare with others because no proof of fertilisation or its normality can be obtained. Indeed, it is likely that the number of embryos available for implantation in GIFT cycles is the single most important factor for the lower implantation rates in comparison to TET. On the other hand, comparison of the mean implantation rates for UET and TET (derived from Table 6.1) reveals a much greater difference (mean$\pm$sem; UET= 10.3 ± 1.2 and TET= 16.6 ± 1.2). The consistency of the higher rate for TET in the different groups of patients such as unexplained infertility may indicate that these embryos, irrespective of their genetic origin have a higher chance of implanting either because;

(1) the tubal environment contributes actively to embryo quality, or (2) the embryos reach the uterine cavity at a later stage of development and are more suitable for implantation compared to UET, or (3) encounter a more receptive endometrium at the time of their entry, or (4) because tubal replacement is a more efficient modality of transfer than uterine transfer.

Balmaceda et al (1992), in a prospective, randomised comparison of uterine versus tubal transfers of frozen-thawed embryos in an oocyte donation programme, found similar pregnancy (50% versus 58%) and implantation rates (15.5% versus 19.3%) in the respective groups. The embryos were transferred at the same stage of development (2-4 cell) and on the third day of progesterone administration during hormone replacement cycles. Similarly, uterine transfer of embryos at a more advanced stage of development (morula or blastocyst) did not result in a higher implantation rate (Bolton et al, 1991; Bolton, 1992). Thus in HRT cycles (uniform endometrial stimulation) and stimulation cycles, embryo quality and endometrial receptivity are more important than the time of entry of the embryo to the uterine cavity in determining its chances of implantation.

It has been reported that controlled ovarian stimulation protocols, including those in which GnRH-a is used, exert an adverse effect on the process of endometrial maturation in up to 50% of women (Ben-Nun et al, 1992). Tur-Kaspa et al (1990) estimated that implantation of singleton pregnancies occurred between days 7 and 9 after UET in gonadotrophin only cycles, but was delayed to days 10/11 in up to 40% of singleton pregnancies when GnRH-a was added to the stimulation protocol. More recently, Soltes et al (1994) reported a noticeable 24 hour lag in the rate of rise of mean ß-hCG levels following TET as compared with UET, which persisted up to 14 days, and suggested a slightly earlier implantation after UET.

It is possible that in some women, TET results in entry of the developing embryo to the uterine cavity at a more synchronised time with the implantation window and hence the higher IR in all transfer cycles. In this clinical trial, in spite of higher pregnancy and implantation rates following TET, the LBR/ET or per cycle started were similar (Table 7.4). Equally, the implantation rates in pregnancy cycles (Table

7.6) were similar following triple transfers (UET=50%; TET=47%) and in the overall comparison between UET and TET (UET=56.5%; TET=47.9%). Comparable IR in pregnancy cycles have been reported recently (Wang et al, 1994) following fresh (43%) and frozen-thawed UET (40%). These results suggest that under optimal endometrial conditions, the implantation potential of the embryo remains the same following uterine or tubal transfer. The role of the Fallopian tube may thus be "passive", providing the early embryo with a natural incubator environment, rather than "active" improvement of embryonic quality.

It is notable that these reported higher pregnancy and implantation rates following laparoscopic tubal transfer, have not been reproduced on a wide scale nor in a consistent manner when retrograde transcervical tubal embryo transfer is attempted (Friedler et al, 1993). Lower pregnancy rates have been reported for both retrograde GIFT or TET with a higher than average ectopic rate (Risquez et al, 1990; Yovich, 1990) unless gametes are placed near the mid section of the tube (Possati et al, 1991). These early reports were further confirmed by recently published case-control retrospective comparison of transvaginal versus laparoscopic GIFT (Jansen and Anderson, 1993) and a randomised trial comparing transcervical ZIFT with UET (Scholtes et al, 1994) which showed a statistically significant higher PR and IR following UET.

Several explanations have been proposed for the lower PR following transcervical tubal transfer; (1) the type of catheter being suboptimal, (2) tubal trauma, (3) subclinical salpingitis, (4) tubal spasm, (5) embryo damage, (6) incorrect catheter position and (7) excessive pressure and/or volume at injection (Risquez et al, 1990). With the retrograde route, the gametes/embryos are deposited at the isthmo-interstitial junction approximately two centimetres from the tubal ostia as compared to the mid ampullary region when the laparoscopic route is used. It is possible that the exact site of placement is of great importance and may adversely influence the outcome. Further refinement of the methodology may maximise the pregnancy and implantation rates (Woolcott et al, 1994).

The different segments of the fallopian tube are known to undergo cyclical changes

at different phases of the menstrual cycle and their correlation to endometrial changes have been studied in this thesis. In Chapter 3, immunocytochemical techniques showed wide variation in the intensity and distribution of staining of ER and PR in the different segments of the tube and the endometrium at different stages of the menstrual cycle. These differences were reflected in the ultrastructural appearance of these various segments (Chapter 4). It should be acknowledged that these studies were conducted in natural cycles and hence may not be fully representative of the events taking place in stimulated cycles.

On the basis of similar implantation rates in pregnancy cycles, it may be concluded that embryo quality is not enhanced following TET. However, the observed higher overall implantation rate following TET in a number of categories points to endometrial receptivity and/or factors as the most likely cause responsible for these enhanced results.

CHAPTER 8
ASSESSMENT OF THE IMMEDIATE AND LATE EFFECTS OF TRANSVAGINAL OOCYTE COLLECTIONS ON PELVIC ORGANS AND THEIR RELEVANCE TO TUBAL FUNCTION

8.1 INTRODUCTION

Vaginal egg collection (VEC) using the transvaginal ultrasound probe, is now the most commonly used method for follicular aspiration in assisted reproduction programmes. Since its introduction in 1984, the technique has gained popularity, and with advances in instrument technology, it has virtually replaced other methods of oocyte retrieval. This has been largely due to enhanced image quality, shorter puncture route, ease of learning and greater tolerance of the technique by patients. The procedure is usually performed under intravenous analgesia and sedation, although occasionally supplementation with local anaesthesia is required. Post-operative recovery is rapid and is associated with minimal patient discomfort (Russell et al, 1987), although symptoms such as low abdominal discomfort and/or distension or vaginal bleeding are occasionally experienced. All this has transformed oocyte retrieval into a day case procedure thereby reducing the overall costs of treatment.

Initial concerns regarding the effect of VEC on oocyte yield, fertilisation rate and outcome of treatment were quickly dispelled by consistently high fertilisation and pregnancy rates compatible with other methods (Feichtinger and Kemeter, 1986; Baber et al, 1988). Potential hazards associated with the procedure include operative morbidity and post-operative complications as well as the effect of ultrasound coupling gels on embryo development (Carver Ward et al, 1987). Accidental puncture of vessels (Seifer et al, 1988), bowel (Yuzpe et al, 1989), appendix (Van Hoorde et al, 1992) and other pelvic structures have been reported. The possible introduction of vaginal bacteria into the peritoneal cavity remains the single most important threat in view of the immediate and long term consequences. Such a complication will be unjustifiable in women with essentially normal pelvic organs or unexplained infertility undergoing assisted reproduction treatment. The reported incidence of clinical pelvic infection varies from 3% (Howe et al, 1988) to 0.6%

(Van Hoorde et al, 1992) in women not given prophylactic antibiotics before VEC. Pelvic infection may present with the usual symptoms and signs, but subclinical bacterial colonisation may have serious consequences on the current or future IVF treatment cycles. Similarly, other long term effects such as the formation of pelvic adhesion or deleterious effects on tubal function which may affect the establishment of subsequent spontaneous pregnancy in women with unexplained infertility remain unknown.

The aims of this study are (1) to investigate the immediate risks of vaginal egg collection (pelvic trauma, infection), (2) to determine the delayed effects (pelvic or peritubal adhesions formation) as assessed by laparoscopy or laparotomy, and (3) to assess the prospects of subsequent conception either spontaneously or following assisted reproduction treatment.

8.2 MATERIALS AND METHODS

8.2.1 PATIENTS

Women participating in a prospective, randomised trial comparing IVF-UET with IVF-TET were entered into this study. The main features of the trial, its design, stimulation protocols used and monitoring of the treatment cycle are described in detail in chapter 6(II). The clinical details of the patients and outcome of treatment are reported in chapter 7.

8.2.2 METHOD OF VAGINAL EGG COLLECTION

This is described in section 6(II).4.

8.2.3 POUCH OF DOUGLAS SAMPLES AND ASSESSMENT OF THE PELVIS

This is described in section 6(II).6.3

8.3 RESULTS

8.3.1 PERITONEAL FLUID SAMPLES

The results of both the general culture and sensitivity (n=55) and chlamydia culture (n=45) are summarised in Table 8.1. The majority of the samples studied for microscopy, general culture and sensitivity (n=51) were reported to be normal. Three showed growth of coagulase-negative Staphylococcus from the enrichment broth but not from the normal culture media. None of the patients developed any symptoms or signs of pelvic infection following embryo transfer and only one was treated with appropriate antibiotics empirically. One sample showed growth of ß-haemolytic Streptococcus. The patient had developed mild dysuria two days after TET which resolved three days later. She was apyrexial and asymptomatic at the time of her review and thus was not prescribed antibiotics. In one sample microscopy suggested the possible presence of Gram-negative rods but no organism was cultured and thus the final report was regarded as normal. Two treatment cycles, where coagulase-negative Staphylococcus was isolated resulted in pregnancies and both have since delivered.

Culture of peritoneal fluid for Chlamydia trachomatis was also negative in the majority of the patients (n=38). Six samples were reported to be toxic to tissue culture and hence no result was obtained. In one patient, Chlamydia trachomatis was cultured. The patient remained completely asymptomatic during a prolonged period of follow-up. The treatment cycle was successful and two intrauterine gestation sacs, each containing a viable fetus, were confirmed by transvaginal ultrasound scans. The patient was treated with erythromycin 500 mg twice daily for 2 weeks at 24 weeks gestation to prevent any sequelae to the fetuses. The pregnancy progressed uneventfully and spontaneous vaginal delivery of twin girls with no evidence of infection occurred at 37 weeks.

Table 8.1 Results of peritoneal fluid microbiology assessment

I-	Microscopy, culture and sensitivity		(Total=55)
	n=51	Negative	
	n=3	Coagulase negative Staphylococcus	
	n=1	ß-haemolytic Streptococcus	
II-	Chlamydia culture		(Total=45)
	n=38	Negative	
	n=6	Unsuccessful tissue culture	
	n=1	Chlamydia positive	

8.3.2 PELVIC ASSESSMENT

The pelvis was assessed in fifty nine patients undergoing laparoscopy with a view to carrying out TET. There was no evidence of pelvic trauma or haematoma formation in any of these patients. Thirty two patients were undergoing their first attempt and twenty six had had one or more VEC preceding the current attempt (one VEC n=14; two VEC n=12; three VEC n=1). In two patients, previously reported to have normal pelvic findings, (one in her first attempt and the other had had one previous VEC two months earlier but no oocyte had fertilised) pelvic and peritubal adhesions were noted and thus judged to be unsuitable for TET. Both patients were excluded from the study. In a further patient undergoing her first treatment, the left tube was found to be slightly tethered to its corresponding ovary but no peritubal adhesions or any other pathology in the pelvis were noted, hence three embryos were transferred to the right tube uneventfully. The pelvic findings in the other patients undergoing their first tubal transfer were not different from those documented during previous laparoscopies. Similarly, there was no evidence of delayed abnormal findings (peritubal, periovarian or pouch of Douglas adhesions) in patients who had more than one VEC over a period of 3 to 18 months. Additionally, the pelvic organs appeared normal during Caesarean section in two patients (n=1, spontaneous conception following one unsuccessful UET; n=1, two previous egg collections and conceived following UET).

8.3.3 PREGNANCIES

Details of the outcome of treatment are reported in Chapter 7. Eight patients conceived following their first VEC-TET attempt and seven pregnancies occurred after TET in patients who had one or more VEC previously. Eight spontaneous pregnancies were reported in the study population up to February 1993 (Table 8.2). Six pregnancies occurred within six months of an unsuccessful attempt (cancelled cycle; absent, delayed or abnormal fertilisation of oocyte with no embryo transfer; not pregnant following TET or UET). One patient whose spontaneous pregnancy ended in miscarriage, conceived spontaneously for a second time one year later.

Table 8.2 Clinical details and pregnancy outcome in patients conceiving spontaneously following previous assisted reproduction treatment

Pt	Age (yrs)	Cause and Duration of infertility (yrs)	Spontaneous pregnancy Date of LMP (Outcome)	Date and outcome of previous treatments (1)	(2)	(3)	(4)
SD	32	Unexplained (4)	Oct 1990 (Delivered)	Feb 1990; No fert	Aug 1990; Poor response, Cycle cancelled		
SK	35	Unexplained (5)	Dec 1990 (Miscarried)	Oct 1990; TET-NP			
CH	32	Endometriosis (6)	Jan 1991 (Delivered)	July 1990; UET-NP	Nov 1990; No fert		
LG	30	Endometriosis (5)	Jan 1991 (Delivered)	Nov 1990; UET-NP			
CM	28	male factor (3)	Feb 1991 (Delivered)	May 1990; Late fert	Sept 1990; Late fert		
VD[§]	26	male factor (3)	May 1991 (>21 weeks)	July 1990; UET-NP	Jan 1991; TET-EPL		
SK	35	Unexplained (5)	Jan 1992 (Overseas) (outcome unknown)	Oct 1990; TET-NP	June 1991; UET-NP		
SS	34	Unexplained (6)	May 1992 (Delivered)	April 1990; TET-NP	Nov 1990; TET-NP	June 1991; UET-NP	Oct 1991; UET-NP

[§] lost to follow-up after 21 weeks

TET= tubal embryo transfer, UET= uterine embryo transfer, Fert= fertilisation, NP= not pregnant, EPL= early pregnancy loss

8.4 DISCUSSION

Assisted reproduction techniques are now widely used for the treatment of couples with unexplained infertility. Many investigators report considerably higher pregnancy and implantation rates following ZIFT or TET than after UET or GIFT. The merits of assisted reproduction treatment and the choice of any particular technique have been recently reviewed (Hull, 1992; Amso and Shaw, 1993). This prospective randomised trial of IVF-UET and IVF-TET provided a unique opportunity to evaluate the immediate and delayed risks of the vaginal egg collection method on the internal pelvic organs in asymptomatic women. Since the introduction of VEC, the potential for intraoperative complications and intraperitoneal bacterial contamination has been acknowledged (Feichtinger and Kemeter, 1986; Evers et al, 1988). Recognised intraoperative complications include anaesthetic associated side effects, puncture of major vessels, pelvic haematoma, bowel injury and bleeding from the puncture points in the vaginal vault (Evers et al, 1988). The risk of postoperative pelvic infection and abscess formation, however, remains the most serious with potentially long term consequences. In the first 250 VEC procedures in the IVF unit at the Royal Free Hospital during 1988-1990, only one patient developed clinical features of pelvic infection (Amso and Shaw, 1992b). Reports of similar incidents of overt infection and abscess formation (Howe et al, 1988; Baber et al, 1988) have resulted in the widespread use of prophylactic antibiotics even though there is no strong evidence to substantiate such practice. At present, there is no consensus as to the type of antibiotic cover, the timing or duration of therapy or whether it is required at all (Meldrum, 1989).

In the clinical trial reported in chapter 7, no cases of overt pelvic infection or abscess formation were reported postoperatively. However, this does not necessarily exclude occult, sub-clinical bacterial or chlamydia colonisation which may influence the outcome of treatment or may become a reservoir of infection which flares up later. Indeed, there were occasions where microorganisms were grown from peritoneal fluid aspirates collected at the time of tubal embryo replacements. Coagulase-negative Staphylococci are not a recognised source of salpingitis and it is possible that their presence represents skin contamination, during laparoscopy, rather than introduction

through the vaginal route. However, ß-haemolytic Streptococcus is known to be a vaginal commensal and thus is likely to have been introduced during vaginal egg collection. Chlamydia infection may be an important cause of subsequent pelvic damage or even failure of an IVF attempt. It is reassuring that, in spite of the isolation of Chlamydia trachomatis and coagulase-negative Staphylococci in peritoneal fluid samples, the patients conceived and the outcome of their pregnancies was satisfactory.

It is possible that the low rate of clinical infection or the low rate of positive cultures from peritoneal fluid may have resulted from thorough vaginal cleaning with chlorhexidine and/or the prophylactic use of an antibiotic (metronidazole) in these patients. It is well documented that antimicrobials are detected in the various tissues of the female reproductive tract and peritoneal fluid (Brihmer et al, 1982; Daschner et al, 1983; Berthelot et al, 1986) following intravenous or oral administration with steady decline in the serum level as well as tissue concentrations with time noted (Nishino et al, 1983). Higher concentration of "Cefoxitin-mefoxin, MSD, UK" was detected in the Fallopian tube than in the endometrium (Daschner et al, 1982; Just et al, 1984), while lower levels of "Aztreonam-azactam, Squibb, UK", a monocyclic beta-lactam antibiotic with potent bactericidal activity, occurred in the peritoneal fluid (Berthelot et al, 1986). It has not been determined whether the distribution of metronidazole in the tissues of the female pelvic organs and peritoneal fluid follows the same pattern as the above mentioned antimicrobials. Pharmacokinetic studies following intravenous administration of a single dose of metronidazole (1 g) showed wide distribution in body tissues, a mean elimination half-life of 8 hours and rapid decline at 24 hours after the infusion (Kling and Burman, 1989). Following suppository administration, the bioavailability of metronidazole is 60-80% with effective blood concentrations being achieved 5-12 hours after the first suppository are maintained by the 8 hourly regimen (ABPI Data Sheet Compendium, 1994, DATAPHARM Publications).

Alternatively, this cohort of women with essentially normal pelvic organs represent a favourable group for VEC and a previous history of pelvic inflammatory disease may imply a higher risk of pelvic reinfection (Wren and Parsons, 1989; Ashkenazi

et al, 1987). Indeed, a history of severe pelvic disease (Howe et al, 1988) or endometriosis (Baber et al, 1988) was prominent in patients who developed pelvic infection or abscess formation following VEC. Dicker et al (1993) reported 14 patients developing an acute abdomen among 3,656 undergoing vaginal egg collections, an incidence of 0.38%. Nine of the patients, with a history of past healed pelvic inflammatory disease, developed tubo-ovarian or pelvic abscesses presenting 1 to 6 weeks after the procedure and requiring laparotomy or culdocentesis and drainage. It has also been suggested that appropriate preoperative vaginal preparation and minimizing the number of repeated vaginal wall punctures may serve to lower the risk of infection (Borlum and Maiggard, 1989).

As demonstrated in this longitudinal follow up study, the absence of any evidence of long term deleterious effects in the pelvis or any impediment to the chances of subsequent pregnancy is very reassuring. Indeed the occurrence of *intrauterine pregnancies* in patients who have had up to four VEC with or without tubal manipulation not only reflects minimal adverse effects on pelvic organs but also persistence of normal tubal function.

It should be recognised that the overall number of patients in this study is relatively small and it is possible that with larger numbers and less stringent inclusion criteria, ectopic pregnancies and pelvic infections may have been encountered. Moreover, the assessment of the Fallopian tube was restricted to examination of the exterior surface with no information about its lumen. Salpingoscopic examination of the ampullary lumen and infundibulum is a more accurate mode of assessment and has been proposed as part of the laparoscopic procedure (Brosens et al, 1987). Several features of the tubal lumen have been described in control groups, infertile patients and at different stages of the ovarian cycle (Maguiness and Djahanbakhch, 1992). The observations made by Maguiness and Djahanbakhch (1992) suggest that there is variation in the endoscopic appearance of the tubal lumen in women with a normal reproductive career and that such appearances do not seem to be related to the use of any method of contraception, the outcome of previous intrauterine pregnancies, or the time since the last pregnancy. The authors concluded that further studies in apparently normal women were needed to determine the influence of various luminal

features with respect to fertility and pregnancy. Similarly, salpingoscopic observations were consistent with histological findings only when endotubal disease was severe. Moderate pathological changes as documented by light microscopy and transmission electron microscopy were frequently not diagnosed salpingoscopically, even with magnification (Hershlag et al, 1991). In this cohort of patients salpingoscopic assessment of the tubal lumen at the initial pre-treatment laparoscopy and further evaluation after one or more VEC might have provided more information on the risks of severe tubal damage following these procedures.

In summary, the postoperative findings and long term observations in this group of women lead to the conclusion that one or more vaginal egg collections with or without tubal manipulation is not associated with significant morbidity and is essentially safe.

CHAPTER 9
SUMMARY OF CONCLUSIONS AND FUTURE RESEARCH

9.1 OESTROGEN AND PROGESTERONE RECEPTOR STUDY

Specific monoclonal antibodies against ER and PR were used to demonstrate the distribution of these receptors along the Fallopian tube and the endometrium. The ICA method used had been shown to be specific and sensitive to demonstrate the presence of these receptors in the tissues of interest. In this study, ER increased in the endometrial glands and stroma during the follicular phase and declined considerably in the late luteal phase. In the Fallopian tube, the isthmus and ampullary regions (epithelium and stroma) demonstrated moderate staining at mid-cycle and decreased to low levels in the late luteal phase. Conversely, the fimbrial end showed moderate staining in the early/mid follicular phase, declined to low levels at mid-cycle and then increased to moderate staining in the luteal phase. In the glandular epithelium, PR increased in the follicular phase to a maximum at mid-cycle and then disappeared from the superficial layer in the mid-luteal phase and from all layers in the late luteal phase. PR in the endometrial stroma remained high throughout the menstrual cycle. In the Fallopian tube, PR were present throughout the menstrual cycle in the epithelium and stroma, but the fimbriae showed greater variability in the follicular phase and less staining in the late luteal phase than the medial segments.

These data suggest that:

i) Variation in the levels of ER and PR along the tube during the different stages of the menstrual cycle support the concept of functional differentiation and microenvironments within the Fallopian tube.

ii) There is a differential control of ER in the different segments of the tube during the menstrual cycle. Similarly, differential control of PR in the endometrial

glandular epithelium and stroma as well as between the tube and endometrium during the luteal phase is present. These latter observations reflect differences in the functional importance of these sites during the luteal phase and early pregnancy.

Future studies:

These should aim to increase our understanding of ER and PR changes at closer intervals around the time of ovulation and in the peri-implantation period (up to LH+8) in the luminal surface of the endometrium, glandular epithelium, endometrial stroma and the Fallopian tube.

Studies are warranted to determine the origin and nature of stromal cells that display ER and PR at all stages of the menstrual cycle especially during the luteal phase.

9.2 MORPHOLOGY AND ULTRASTRUCTURAL STUDY

In this study, there was a systematic increase in the number of ciliated cells from the isthmus outwards with higher numbers in the fimbriae, but no significant variation was demonstrated at the different stages of the menstrual cycle. The following results were subjective assessments and morphometric techniques were not used, hence, the conclusions should be interpreted with care. In the early follicular phase, endometrial gland lumen and the isthmus, but not the other tubal regions, showed small quantities of secretory material. In the late follicular/pre-ovulatory phase, cytoplasmic fragments and cellular material increased greatly in the endometrial glandular lumen and the isthmus but to a lesser extent in the mid-tube and there were none in the fimbriae. Similarly, the surface domes and dense granules were most prominent in the mid-tube and ampulla and less in the fimbriae. In the luteal phase, glandular lumen, isthmus and mid-tube contained greatly reduced cellular material. The glandular epithelial cells demonstrated the classical dense glycogen accumulations in the supranuclear position, while in the tube more glycogen particles were seen in the ciliated cells. Similar luminal surface changes and electron dense granules were noted in the endometrial and tubal epithelia at the different stages, though the

fimbriae were generally less active than the other tubal regions. The postmenopausal tube demonstrated a flat surface, reduced ciliation and minimal secretory activity, while following HRT, the epithelial appearance resembled that of the late follicular phase.

These data suggest that:

i) Along the Fallopian tube, differences in luminal secretory appearance, cell surface activity and ciliated cell numbers, corroborate the concept of functional differentiation of the different segments of the tube.

ii) The appearance of electron dense secretory granules in the endometrium coincides with those in the Fallopian tube and a similar process of granule secretion by cell decapitation is noted in both areas. However, whether the proteins produced at the two sites are the same or tissue specific has not been determined.

Future studies

These should compare endometrial luminal epithelium with glandular and tubal epithelia in the late follicular and peri-implantation period (up to LH+8). Scanning and Transmission EM may demonstrate cell surface changes and secretory processes that contribute to the interaction between the embryo and the endometrium and which may facilitate implantation.

Further studies are warranted to determine the nature of these secretory products and granules in both the endometrium and tube using electron microscopic immunocytochemical techniques. Similar techniques may be used to localise the presence of specific receptors in the endometrium and tube for factors such as Insulin-like growth factor-I and Retinol. These factors are known to contribute to early embryonic development.

9.3 FALLOPIAN TUBE PROTEIN STUDY

In this study, two non-serum protein bands were identified. Band "A" with a suggested MW 115-140KDa and band "B" with a suggested MW 49-69KDa. Standard procedures were used to prepare immunogen and immunise rabbits. Immunoprecipitation techniques were unsuccessful in demonstrating production of antibodies in rabbits.

These data suggest that:

i) Tubal-specific, non serum proteins may be identified using gel separation of tubal flushing material.

ii) The quantity or immunogenic properties of proteins in these gels may have been insufficient to generate antibodies in rabbits, and other approaches, such as pooling of all tubal flushing material should have been attempted.

Future studies

Studies should use new techniques to isolate tubal proteins, generate specific poly- or monoclonal antibodies to localise the site of production of such proteins and further assess their role in human reproduction.

9.4 CLINICAL TRIAL RESULTS

9.4.1 ALL TRANSFERS RESULTS

All analyses were carried out according to the original randomisation and intention to treat. The number of treatment cycles planned in the study design (124 under each arm) was not reached and thus the overall results are inconclusive because of the lack of statistical power. The overall PR/ET following 124 replacements only were 27.4% for TET and 14.5% for UET ($p=0.08$), but when three embryo replacement cycles only were analysed, the figures were 31.9% and 13.6% respectively ($p=0.04$).

The overall LBR/ET following TET and UET were 17.7% and 12.9% respectively (p=0.46). Similarly, the IR of all cycles following TET and UET were 14.3% and 7.9% (p=0.07) respectively and 15% and 7% (p=0.03) when only three-embryo transfer cycles were compared. In pregnancy cycles only, the IR were 56.5% for UET and 47.9% for TET. Uterine and tubal embryo transfer cycles were comparable in age, duration of infertility, parity, infertility factors, fertilisation rate, and number of embryos replaced. PR and IR following UET in the study group were comparable to patients not included in the study and undergoing UET at the Royal Free Hospital during the same period. PR results in two interim analyses were concordant with the final analysis.

These data suggest that:

i) The LBR per cycle started or embryo transfer is similar in the two groups and that tubal transfer does not reduce the early pregnancy loss rate.

ii) Higher IR is achieved when all tubal TET cycles are evaluated and the difference is statistically significant when three-embryo transfer cycles only are compared.

iii) In pregnancy cycles only and when factors responsible for implantation are optimal, the implantation potential of the embryos is the same irrespective of the place of transfer.

iv) Although PR following TET are higher, though not significantly, than those following UET in this cohort of women, the results are inconclusive because of the lack of statistical power. Larger number of treatment cycles would be needed to demonstrate significant benefit following tubal transfer.

9.4.2 OUTCOME ACCORDING TO STIMULATION ATTEMPT RANK

In the first attempt, PR following TET and UET were 28.6% and 20%, the IR were

14.7% and 11.8%, and the LBR/ET were 10.7% and 20% respectively. Analysis of factors that may affect outcome showed that women are more likely to benefit from TET if they were parous, with idiopathic infertility, or if hCG was used for luteal support. Logistic regression analysis allowed the outcome of treatment to be adjusted for a number of confounding variables. The odds ratio for TET increased when the duration of infertility, number of forwardly motile sperm or oocytes collected were taken into account. The difference in PR and IR between TET and UET persisted after the second and third attempts. The IR was significantly higher after TET in the second attempt. The overall multiple pregnancy rate was 44% following UET and 24% following TET. Only one pregnancy was lost following UET while a total of 6 were lost following TET.

These data suggest that:

i) The tendency for higher pregnancy rates following TET was maintained after each of the attempts.

ii) Parity and unexplained infertility were favourable factors for tubal replacements.

iii) Neither multiple pregnancy rate nor early pregnancy loss rate improved after tubal replacements.

9.4.3 OUTCOME ACCORDING TO INFERTILITY FACTOR

Unexplained infertility demonstrated the largest gap between TET and UET in both the first cycle of treatment (PR/TET=44%, PR/UET=13%) and when all cycles in this group were analysed (PR/TET=31.8%, PR/UET=14.8%). The overall IR (14.3% vs 7.1%) and LBR per transfer (22.7% vs 11.1%) were equally higher following TET.

In male factor infertility, the PR following TET was slightly higher than UET in the

first attempt (23% vs 21%) but more in the overall cycles (20% vs 11.1%). The implantation rates were also similar (TET=8.2%, UET=6.5%) and in view of two miscarriages following TET, the LBR per transfer following TET was 10% only compared to 11.1% following UET.

There were few transfer cycles in both the female factor only (TET=11, UET=7) and the combined male and female factor (TET=9, UET=10) groups and hence it is not possible to extrapolate from the results.

These data suggest that:

i) Unexplained infertility may contribute adversely to implantation and the establishment of pregnancy following uterine transfer while tubal transfer may enhance the outcome.

ii) No advantage (PR and IR) is obtained from tubal transfers in the male factor infertility group.

9.4.4 OUTCOME ACCORDING TO AGE AND DURATION OF INFERTILITY AT RECRUITMENT

PR and IR were significantly higher following TET (PR=53% and IR=29%) than UET(PR=11% and IR=8%) in women under 30 years at recruitment. In the other age groups (30-34 and ≥35 years) the differences were not significant. However, the number of cycles in the under 30 years group was low (n=42) and hence no firm conclusions could be drawn from these results.

The highest PR and IR following TET and UET were in women whose duration of infertility was under 4 years (PR-TET=41%, UET=28%; IR-TET=23%, UET=17%). No significant difference in the pregnancy rate between tubal and uterine replacements was noted, but in all subgroups PR were higher rate following TET. Four or more years of infertility resulted in a marked drop of PR and IR

following both TET and UET.

These data suggest that:

i) Tubal transfer may significantly increase PR and IR in women recruited for treatment under 30 years.

ii) There is an inverse relationship between the duration of infertility and outcome of treatment (PR and IR) and that the benefit of shorter duration of infertility is manifested following both TET and UET.

Future studies

Based on the overall PR per transfer achieved in the first attempt (TET=28.6% and UET=20%) and assuming persistence of a true difference of 10% between the two pregnancy rates, approximately 410 transfers for each group would be necessary to demonstrate significant benefit at 5% level and 90% power. This would almost certainly require a multicentre trial.

Alternatively, a clinical trial should aim to demonstrate benefit in the group where maximum difference was observed i.e. couples with unexplained infertility and shorter duration (< 4 years) of infertility. A confirmation parallel study in couples with unexplained infertility would require approximately 50 transfers under each arm to demonstrate significance at 5% level and 80% power.

9.5 ASSESSMENT OF IMMEDIATE AND LATE EFFECTS OF VAGINAL EGG COLLECTIONS ON PELVIC ORGANS

The vast majority of peritoneal fluid samples obtained at TET did not grow any microorganisms. Chlamydia culture was positive in only one sample from an asymptomatic patient who conceived during that treatment cycle. Healthy twin girls

were born with no evidence of infection. Pregnancies also occurred in cycles where coagulase-negative Staphylococcus was isolated.

Assessment of the pelvis at TET did not show any evidence of trauma or haematoma formation subsequent to VEC, and similarly there was no evidence of substantial delayed abnormal findings in patients who had more than one VEC up to 18 months earlier. Furthermore, eight spontaneous pregnancies were reported up to 7 months after an unsuccessful attempt.

These data suggest that:

i) In women with no previous history of pelvic inflammatory disease, the risk of introducing infection following VEC is low. The presence of any microorganisms does not appear to affect the establishment and progress of pregnancy.

ii) Repeated vaginal egg collections do not increase the risk of pelvic damage and adhesion formation nor adversely affect tubal function as shown by the subsequent establishment of spontaneous pregnancies.

REFERENCES

1992. Effective Health Care: The management of subfertility. A bulletin on the effectiveness of health service interventions for decision-makers, Number 3, School of Public Health, University of Leeds, UK.

Abbott laboratories information sheet, Diagnostics division. 1989 ABBOTT PgR-ICA Monoclonal: Immunocytochemical assay for the detection of human Progesterone receptor. North Chicago, IL 60064.

Abbott laboratories information sheet, Diagnostics division. 1990 ABBOTT ER-ICA Monoclonal: Immunocytochemical assay for the detection of human Estrogen receptor. Abbott Park, IL 60064.

Abdalla HI, Burton G, Kirkland A, Johnson MR, Leonard T, Brooks AA, Studd JWW. 1993 Age, pregnancy and miscarriage: uterine versus ovarian factors. Hum Reprod 8:1512-1517.

ABPI Data Sheet Compendium. 1994 pp.1248-1251, Datapharm Publications Ltd., London.

Adams CE. 1973 The development of rabbit eggs in the ligated oviduct and their viability after re-transfer to recipient rabbits. J Embryol Exper Morphol 29:133-144.

Amso N, Shaw RW. 1991 Polycystic ovaries and problems in assisted reproduction programmes. In: Polycystic ovaries: a disorder or a symptom. Shaw RW (Ed) pp.203-216, Parthenon Publishing Group, New Jersey and Lancs.

Amso N, Shaw RW. 1992a Clinical trials in assisted reproduction (letter). Hum Reprod 7:580-581.

Amso NN, Shaw RW. 1992b New frontiers in assisted reproduction. In: Gynaecology. Shaw RW, Soutter P, Stanton S (Eds) pp.231-248, Churchill Livingstone, London.

Amso NN, Shaw RW. 1993 A critical appraisal of assisted reproduction techniques. Hum Reprod 8:168-174.

Amso NN, Ahuja K, Morris N, Shaw RW. 1989 Elective pre-embryo cryopreservation in ovarian hyperstimulation syndrome. J In Vitro Fertil Embryo Trans 6:312-314.

Amso NN, Ahuja KK, Morris N, Shaw RW. 1990 The management of predicted ovarian hyperstimulation involving gonadotropin-releasing hormone analog with elective cryopreservation of all pre-embryos. Fertil Steril 53:1087-1090.

Amso N, Curtis P, Preutthipan S, Keith E, Bernard A, Shaw, RW. 1991 A randomized controlled trial of in vitro fertilisation and uterine embryo transfer vs tubal embryo transfer in the management of idiopathic and male infertility. In: Proceedings of the 7th World Congress on In Vitro Fertilization and Assisted Procreation, Paris, June 30-July 3. Hum Reprod 6 (suppl 1):128-129.

Amso NN, Crow J, Shaw RW. 1994a Comparative immunohistochemical study of oestrogen and progesterone receptors in the Fallopian tube and the uterus at different stages of the menstrual cycle and the menopause. Hum Reprod 9:1027-1037.

Amso NN, Crow J, Lewin J, Shaw RW. 1994b A comparative morphological and ultrastructural study of endometrial gland and Fallopian tube epithelia at different stages of

the menstrual cycle and the menopause. Hum Reprod 9:2234-2241.

Arias EB, Verhage HG, Jaffe RC. 1994 Complementary deoxyribonucleic acid cloning and molecular characterization of an oestrogen-dependent human oviductal glycoprotein. Biol Reprod 51:685-694.

Arici A, Engin O, Attar E, Olive DL. 1995 Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. J Clin Endocrinol Metab 80:1908-1915.

Asch RH. 1991 Uterine versus tubal embryo transfer in the human. Ann N Y Acad Sci 626:461-466.

Asch RH, Ellsworth LR, Balmaceda JP, Wong PC. 1984 Pregnancy after translaparoscopic gamete intrafallopian transfer. Lancet 2:1034-1035.

Asch RH, Balmaceda JP, Ellsworth LR, Wong PC. 1986 Preliminary experiences with gamete intrafallopian transfer (GIFT). Fertil Steril 45:366-371.

Ashkenazi J, Ben-David M, Feldberg D, Dicker D, Goldman JA. 1987 Abdominal complications following ultrasonically guided percutaneous transvesical collection of oocytes for in vitro fertilization. J In Vitro Fert Embryo Trans 4:316-318.

Audibert F, Hedon B, Arnal F, Humeau C, Badoc E, Virenque V, Boulot P, Mares P, Laffargue F, Viala JL. 1989 Results of IVF attempts in patients with unexplained infertility. Hum Reprod 4:766-771.

Baber R, Porter R, Picker R, Robertson R, Dawson E, Saunders D. 1988 Transvaginal ultrasound directed oocyte collection for in vitro fertilization: successes and complications. J Ultrasound Med 7:377-379.

Balasch J, Martinez F, Jove I, Cabre L, Coroleu B, Barri P, Vanrell JA. 1993 Inadvertent gonadotrophin-releasing hormone agonist (GnRHa) administration in the luteal phase may improve fecundity in in-vitro fertilization patients. Hum Reprod 8:1148-1151.

Balmaceda JP, Alam V, Roszjtein D, Ord T, Snell K, Asch RH. 1992 Embryo implantation rates in oocyte donation: a prospective comparison of tubal versus uterine transfers. Fertil Steril 57:362-365.

Balmaceda JP, Gastaldi C, Remohi J, Borrero C, Ord T, Asch RH. 1988 Tubal embryo transfer as a treatment for infertility due to male factor. Fertil Steril 50: 476-479.

Barr SH, Oliphant G. 1981 Sulfate incorporation into macromolecules produced by cultured oviductal epithelium. Biol Reprod 24:852-858.

Barratt CLR, Cooke ID. 1991 Sperm transport in the human female reproductive tract-a dynamic interaction. Int J Androl 14:394-411.

Bavister BD. 1992 Co-culture for embryo development: is it really necessary? Hum Reprod 7:1339-1341.

Beier-Hellwig K, Sterzik K, Bonn B, Beier HM. 1989 Contribution to the physiology and pathology of endometrial receptivity: the determination of protein patterns in human uterine secretions. Hum Reprod 4:115-120.

Ben-Nun I, Jaffe R, Fejgin MD, Beyth Y. 1992 Therapeutic maturation of endometrium in in vitro fertilization and embryo transfer. Fertil Steril 57:953-962.

Bentick B, Shaw RW, Iffland CA, Burford G, Bernard A. 1988 A randomized comparative study of purified follicle stimulating hormone and human menopausal gonadotropin after pituitary desensitization with Buserelin for superovulation and in vitro fertilization. Fertil Steril 50:79-84

Berthelot G, Bergogne Berezin E, Vernant D, Ravina JH. 1986 Diffusion of aztreonam in the tissues and biological fluids of the female genital tract. Pathol Biol (Paris) 34:339-341.

Betteridge KJ, Mitchell D. 1974 Direct evidence of retention of unfertilized ova in the oviduct of the mare. J Reprod Fert 39:145-148.

Biggers JD, Gwatkin RBL, Brinster RL. 1962 Development of mouse embryos in organ cultures of Fallopian tubes on a chemically defined medium. Nature 194:747-749.

Bjorkman N, Fredricsson B. 1962 Ultrastructural features of the human oviduct epithelium. Int J Fertil 7:259-266.

Black DL. 1974 Neural control of oviduct musculature. In: The Oviduct and its Functions. Johnson AD, Foley CW (Eds) pp.65-118, Academic Press, New York.

Blackledge DG, Matson PL, Willcox DL, Yovich JM, Turner SR, Richardson PA, Yovich JL. 1986 Pronuclear stage transfer (PROST) and modified gamete intrafallopian transfer (GIFT) techniques for oligospermic cases (letter). Med J Aust 145:173-174.

Blandau RJ. 1969 Gamete transport-comparative aspects. In: The mammalian oviduct. Hafez ESE, Blandau RJ (Eds) pp.129-162, University of Chicago Press, Chicago, Illinois.

Blandau RJ. 1973 Gamete transport in the female mammal. In: Handbook of Physiology. Greep RO, Astwood EB (Eds) pp.153-163, American Physiological Society, Washington D.C.

Blandau RJ, Verdugo P. 1976 An overview of gamete transport-comparative studies. In: Symposium on Ovum Transport and Fertility Regulation. Harper MJK, Pauerstein CJ (Eds) pp.138-146, Scriptor, Copenhagen.

Blandau RJ, Bourdage R, Halbert S. 1979 Tubal Transport. In: The biology of the fluids of the genital tract. Beller FK, Schumacher GFB (Eds) pp.319-333, Elsevier North Holland, Amsterdam.

Bollen N, Camus M, Staessen C, Tournaye H, Devroey P, Van Steirteghem,AC. 1991 The incidence of multiple pregnancy after in vitro fertilization and embryo transfer, gamete, or zygote intrafallopian transfer. Fertil Steril 55:314-318.

Bolton VN. 1992 Controversies and opinions in embryo culture: two- to four-cell transfer vs blastocyst. J Assist Reprod Genet 9:506-508.

Bolton VN, Wren ME, Parsons JH. 1991 Pregnancies after in vitro fertilization and transfer of human blastocysts. Fertil Steril 55:830-832.

Bongso A, Ng SC, Ratnam S. 1990 Co-cultures: their relevance to assisted reproduction. Hum Reprod 5:893-900.

Bogso A, Fong C-Y, Ng S-C, Ratnam S. 1994 Human embryonic behaviour in a sequential human oviduct-endometrial coculture system. Fertil Steril 61:976-978.

Bonilla-Musoles F, Ferrer-Barriendos J, Pellicer A. 1983 Cyclical changes in the epithelium of the Fallopian tube. Scanning electron microscopy. Clin Exp Obstet Gynecol 10:79-86.

Borlum KG, Maiggard S. 1989 Transvaginal oocyte aspiration and pelvic infection (letter). Lancet, 2:53.

Brenner RM, Carlisle KS, Hess DL, Sandow BA, West NB. 1983 Morphology of the oviducts and endometria of Cynomolgus Macaques during the menstrual cycle. Biol Reprod 29:1289-1302.

Brihmer C, Brundin J, Dornbusch K. 1982 Levels of trimethoprim and sulfamethoxazole in human oviduct mucosa and plasma. Acta Obstet Gynecol Scand 61:149-152.

Brosens IA, Vasquez G. 1976 Fimbrial microbiopsy. J Reprod Med 16:171-178.

Brosens IA, Boeckx W, Delattin P, Puttemans P, Vasquez G. 1987 Salpingoscopy: a new pre-operative diagnostic tool in tubal infertility. Br J Obstet Gynaecol 94:768-773.

Brundin J. 1965 Distribution and function of adrenergic nerves in the rabbit Fallopian tube. Acta Physiol Scand 66 (Suppl) 259:1-57.

Brundin J, Fredricsson B, Norberg KA, Swedin G. 1969 The sympathetic innervation of the oviduct in the rat. Acta Physiol Scand 75:69-72.

Bustillo M, Munabi AK, Schulman JD. 1988 Pregnancy after nonsurgical ultrasound-guided gamete intrafallopian transfer (letter). N Engl J Med 319:313.

Cano A, Serra V, Rivera J, Monmeneu R, Marzo C. 1990 Expression of estrogen receptors, progesterone receptors, and an estrogen receptor-associated protein in the human cervix during the menstrual cycle and menopause. Fertil Steril 54:1058-1064.

Carver Ward JA, DeVol EB, Evers JL. 1987 A method to prevent arrest of embryo development by ultrasound coupling gels after transvaginal ultrasound-guided oocyte retrieval. Hum Reprod 2:611-614.

Cefalu E, Cittadini E, Balmaceda JP, Guastella G, Ord T, Rojas F, Asch RH. 1988 Successful gamete intrafallopian transfer following failed artificial insemination by donor: evidence for a defect in gamete transport. Fertil Steril 50:279-282.

Check JH, Lurie D, Callan C, Baker A, Benfer K. 1994 Comparison of the cumulative probability of pregnancy after in vitro fertilization-embryo transfer by infertility factor and age. Fertil Steril 61:257-261.

Clyman MJ. 1966 Electron microscopy of the human Fallopian tube. Fertil Steril 17:281-301.

Coates TE, Check JH, Choe J, Nowroozi K, Lurie D, Callan C. 1992 An evaluation of couples with failure of fertilization in vitro. Hum Reprod 7:978-981.

Cohen J. 1991 The efficiency and efficacy of IVF and GIFT. Hum Reprod 6:613-618.

Coppens MT, De Boever JG, Dhont MA, Serreyn RF, Vandekerckhove DA, Roels HJ. 1993 Topographical distribution of oestrogen and progesterone receptors in the human endometrium and Fallopian tube. An immunocytochemical study. Histochemistry 99:127-131.

Collins JA, Rowe TC. 1989 Age of the female partner is a prognostic factor in prolonged unexplained infertility: a multicenter study. Fertil Steril 52:15-20.

Corner GW. 1923 Cyclic variation in uterine and tubal contraction waves. Am J Anat 32: 345-351.

Cornillie FJ, Lauweryns JM, Brosens IA. 1985 Normal human endometrium. An ultrastructural survey. Gynecol Obstet Invest 20:113-129.

Craft I. 1990 Factors affecting the outcome of assisted conception. Br Med Bull 46:769-782.

Craft I, Ah-Moye M, Al-Shawaf T, Fiamanya W, Lewis P, Robertson D, Shrivastav P, Simons E, Brinsden P. 1988 Analysis of 1071 GIFT procedures-the case for a flexible approach to treatment. Lancet 1:1094-1098.

Critoph FN, Dennis KJ. 1977 The cellular composition of the human oviductal epithelium. Br J Obstet Gynaecol 84:219-221.

Crosignani PG, Walters DE, Soliani A. 1991 The ESHRE multicentre trial on the treatment of unexplained infertility: a preliminary report. Hum Reprod 6:953-958.

Crow J, Amso NN, Lewin J, Shaw RW. 1994 Morphology and ultrastructure of Fallopian tube epithelium at different stages of the menstrual cycle and menopause. Hum Reprod 9:2224-2233.

Croxatto HB, Ortiz ME. 1975 Egg transport in the Fallopian tube. Gynecol Invest 6:215-225.

Croxato HB, Diaz S, Fuentealba B, Croxato H-D, Carrillo D, Fabres C. 1972 Studies on the duration of egg transport in the human oviduct. I. The time interval between ovulation and egg recovery from the uterus in normal women. Fertil Steril 23:447-458.

Croxatto HB, Ortiz ME, Diaz S, Balmaceda J, Croxatto H-D. 1978 Studies on the duration of egg transport by the human oviduct. II. Ovum location at various intervals following luteinizing hormone peak. Am J Obstet Gynecol 132:629-634.

Dallenbach-Hellweg G. 1987 Histopathology of the endometrium. Fourth edition. Springer-Verlag, Berlin.

Daschner F, Petersen E, Langmaack H, Trennhauser M. 1982 Antibiotic prophylaxis in gynecology: cefoxitin concentrations in serum, myometrium, endometrium and salpinges. Infection 10:341-342.

Daschner FD, Petersen EE, Just HM, Hillemanns HG. 1983 Penetration of ceftazidime into serum, myometrium, endometrium, salpinges and subcutaneous tissue. J Antimicrob Chemother 12 Suppl A:247-249.

David A, Czernobilsky B. 1968 A comparative histologic study of the uterotubal junction in the rabbit, rhesus monkey, and human female. Am J Obstet Gynecol 101:417-421.

David A, Brackett BG, Garcia C-R, Mastroianni l Jr. 1969 Composition of rabbit oviduct fluid in ligated segments of the Fallopian tube. J Reprod Fertil 19:285-289.

Davis BJ. 1964 Disc Electrophoresis-2. Method and application to human serum. Ann N Y Acad Sci 121:404-427.

Daya S. 1993 Is there a place for the crossover design in infertility trials. Fertil Steril 59:6-7.

DeSombre ER, Greene GL, King WJ, Jensen EV. 1984 Estrogen receptors, antibodies and hormone dependent cancer. Prog Clin Biol Res 142:1-21.

Dicker D, Ashkenazi J, Feldberg D, Levy T, Dekel A, Ben-Rafael Z. 1993 Severe abdominal complications after transvaginal ultrasonographically guided retrieval of oocytes for in vitro fertilization and embryo transfer. Fertil Steril 59:1313-1315.

Dickens CJ, Maguiness SD, Comer MT, Palmer A, Rutherford AJ, Leese HJ. 1995 Human tubal fluid: formation and composition during vascular perfusion of the Fallopian tube. Hum Reprod 10:505-508.

Devroey P, Braeckmans P, Smitz J, Van Waesberghe L, Wisanto A, Van Steirteghem AC. 1986 Pregnancy after translaparoscopic zygote intrafallopian transfer in a patient with sperm antibodies. Lancet 1:1329.

Devroey P, Staessen C, Camus M, De Grauwe E, Wisanto A, Van Steirteghem AC. 1989 Zygote intrafallopian transfer as a successful treatment for unexplained infertility. Fertil Steril 52:246-249.

Dockery P, Li TC, Rogers AW, Cooke ID, Lenton EA. 1988a The ultrastructure of the glandular epithelium in the timed endometrial biopsy. Hum Reprod 3:826-834.

Dockery P, Li TC, Rogers AW, Cooke ID, Lenton EA, Warren MA. 1988b An examination of the variation in timed endometrial biopsies. Hum Reprod 3:715-720.

Dockery P, Rogers AW. 1989 The effects of steroids on the fine structure of the endometrium. Bailliere's Clinical Obstetrics and Gynaecology 3(2):227-248.

Dockery P, Tidey RR, Li TC, Cooke ID. 1991 A morphometric study of the uterine glandular epithelium in women with premature ovarian failure undergoing hormone replacement therapy. Hum Reprod 6:1354-1364.

Dodson WC, Whitesides DB, Hughes CL, Easley HA, Haney AF. 1987 Superovulation with intrauterine insemination in the treatment of infertility: a possible alternative to gamete intrafallopian transfer and in vitro fertilisation. Fertil Steril 48:441-445.

Dokras A, Sargent IL, Redman CW, Barlow DH. 1993 Sera from women with unexplained infertility inhibit both mouse and human embryo growth in vitro. Fertil Steril 60:285-292.

Donnelly KM, Fazleabas AT, Verhage HG, Mavrogianis PA, Jaffe RC. 1991 Cloning of a recombinant complementary DNA to a baboon (Papio anubis) estradiol-dependent oviduct-specific glycoprotein. Mol Endocrinol 5:356-364.

Donnez J, Casanas-Roux F, Caprasse J, Ferin J, Thomas K. 1985 Cyclical changes in ciliation, cell height, and mitotic activity in human tubal epithelium during reproductive life.

Fertil Steril 43:554-559.

Eddy CA, Garcia RG, Kraemer DC, Pauerstein CJ. 1976 Ovum transport in non-human primates. In: Symposium on Ovum Transport and Fertility Regulation. Harper MJK, Pauerstein CJ (Eds) pp.390-403, Scriptor, Copenhagen.

Edwards RG, Craft I. 1990 Development of assisted conception. British Medical Bulletin 46:565-579.

Edwards RG, Steptoe PC, Purdy JM. 1980 Establishing full-term human pregnancies using cleaving embryos grown in vitro. Br J Obstet Gynaecol 87:737-756.

Evers JL, Larsen JF, Gnany GG, Sieck UV. 1988 Complications and problems in transvaginal sector scan-guided follicle aspiration. Fertil Steril 49:278-282.

Fadel HE, Berns D, Zaneveld LJD, Wilbansk GD, Brueschke EE. 1976 The human uterotubal junction: a SEM study during different phases of the menstrual cycle. Fertil Steril 27:1176-1186.

Fazleabas AT, Verhage HG. 1986 The detection of oviduct-specific proteins in the baboon (Papio anubis). Biol Reprod 35:455-462.

Feichtinger W, Kemeter P. 1986 Transvaginal sector scan sonography for needle guided transvaginal follicle aspiration and other applications in gynecologic routine and research. Fertil Steril 45:722-725.

Fenoglio CM, Castodot MJ, Ferenczy A, Cottral GA, Richart RM. 1977 Serous tumours of the ovary. Gynecol Oncol 5:203-218.

Ferenczy A. 1977 Surface ultrastructural response of human uterine lining to hormonal environment. A scanning microscopic study. Acta Cytologica 21:566-572.

Ferenczy A, Richart RM. 1973 Scanning and transmission electron microscopy of the human endometrial surface epithelium. J Clin Endocrinol Metab 36:999-1008.

Ferenczy A, Richart RM. 1974 Female reproductive system: dynamics of scan and transmission electron microscopy, pp.213-254, Wiley, New York.

Ferenczy A, Richart RM, Agate FJ Jr, Purkerson ML, Dempsey EW. 1972a Scanning electron microscopy of the human endometrial surface epithelium. Fertil Steril 23:515-521.

Ferenczy A, Richart RM, Agate FJ Jr, Purkerson ML, Dempsey EW. 1972b Scanning electron microscopy of the human Fallopian tube. Science 175:783-784.

Ferguson KM, Hayes M, Jeffcoate SL. 1982 A standardised multicentre procedure for plasma gonadotrophin radio-immunoassay. Ann Clin Biochem 19:358-362.

Fisch P, Casper RF, Brown SE, Wrixon W, Collins JA, Reid RL, Simpson C. 1989 Unexplained infertility: evaluation of treatment with clomiphene citrate and human chorionic gonadotropin. Fertil Steril 51:828-833.

Fishel SB, Edwards RG. 1982 Essentials of fertilization. In: Human Conception in Vitro. Edwards RG, Purdy J (Eds) p157, Academic Press, London.

FIVNAT (French In Vitro National). 1993 French national IVF registry: analysis of 1986 to 1990 data. Fertil Steril 59:587-595.

Flickinger GL, Elsner C, Illingworth DV, Muechler EK, Mikhail G. 1977 Estrogen and progesterone receptors in the female genital tract of humans and monkeys. Ann N Y Acad Sci 286:180-189.

Fluker MR, Zouves CG, Bebbington MW. 1993 A prospective randomized comparison of zygote intrafallopian transfer and in vitro fertilization-embryo transfer for nontubal factor infertility. Fertil Steril 60:515-519.

Forman RG, Eychenne B, Nessmann C, Frydman R, Robel P. 1989 Assessing the early luteal phase in in vitro fertilization cycles: relationships between plasma steroids, endometrial receptors, and endometrial histology. Fertil Steril 51:310-316.

Fredricsson B, Bjorkman N. 1973 Morphologic alterations in the human oviduct epithelium induced by contraceptive steroids. Fertil Steril 24:19-30.

Freeman DA, Woods GL, Vanderwall DK, Weber JA. 1992 Embryo-initiated oviductal transport in mares. J Reprod Fert 95:535-538.

Friedler S, Lewin A, Schenker JG. 1993 Reproductive health care policies around the world: Methodology of human embryo transfer following assisted reproduction. J Assist Reprod Genet 10:393-404.

Gaddum-Rosse P, Blandau RJ, Thiersch JB. 1973 Ciliary activity in the human and Macaca nemestrina oviduct. Amer J Anat 138:269-275.

Gandolfi F, Moor RM. 1987 Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fert 81:23-28.

Garcia E, Bouchard P, De Brux J, Berdah J, Frydman R, Schaison G, Milgrom E, Perrot Applanat M. 1988 Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. J Clin Endocrinol Metab 67:80-87.

Glazener CMA, Coulson C, Lambert PA. 1990 Clomiphene treatment for women with unexplained infertility: placebo-controlled study of hormonal responses and conception rates. Gynecol Endocrinol 4:75-83.

Gondos B. 1971 Electron microscopic study of papillary serous tumours of the ovary. Cancer 27:1455-1464.

Gorski J, Toft D, Shyamala G, Smith D, Notides A. 1968 Hormone receptors: studies on the interaction of estrogen with the uterus. Recent Prog Horm Res 24:45-80.

Gorski J, Welshons WV, Sakai D, Hansen J, Walent J, Kassis J, Shull J, Stack G, Campen C. 1986 Evolution of a model of estrogen action. Recent Prog Horm Res 42:297-329.

Gott AL, Gray SM, James AF, Leese HJ. 1988 The mechanism and control of rabbit oviduct fluid formation. Biol Reprod 39:758-763.

Grabar P, Williams CA Jr. 1953 Methode permettant l'etude conjuguee des proprietes electrophoretiques et immunochimiques d'un melange de proteins. Application au serum sanguin. Biochim Biophys Acta 10:193-194.

Graham RA, Seif MW, Aplin JD, Li TC, Cooke ID, Rogers AW, Dockery P 1990 An endometrial factor in unexplained infertility. Br Med J 300:1428-1431.

Greene GL, Jensen EV. 1982 Monoclonal antibodies as probes for estrogen receptor detection and characterization. J Steroid Biochem 16:353-359.

Greene GL, Press MF. 1987 Immunochemical evaluation of estrogen receptor and progesterone receptor in breast cancer. In: Immunological Approaches to the Diagnosis and Therapy of Breast Cancer. Ceriano R (Ed) p.119, Plenum Publishing Co, New York.

Greene GL, Nolan C, Engler JP, Jensen EV. 1980 Monoclonal antibodies to human estrogen receptor. Proc Natl Acad Sci USA 77:5115-5119.

Greene GL, Harris K, Bova R, Kinders R, Moore B, Nolan C. 1988 Purification of T47D human progesterone receptor and immunochemical characterization with monoclonal antibodies. Mol Endocrinol 2:714-726.

Greenwald GS. 1958 Endocrine regulation of the secretion of mucin in the tubal epithelium of the rabbit. Anat Rec 130:477-496.

Guastella G, Cimino C. 1986 Intraperitoneal insemination (IPI) in couples with infertility unrelated to female organic pelvic disease. Acta Eur Fertil 86:377-379.

Halbert SA, Tam PY, Blandau RJ. 1976 Egg transport in the rabbit oviduct: the roles of cilia and muscle. Science 191:1051-1053.

Hammitt DG, Syrop CH, Hahn SJ, Walker DL, Butkowski CR, Donovan JF. 1990 Comparison of concurrent pregnancy rates for in-vitro fertilization-embryo transfer, pronuclear stage embryo transfer and gamete intra-Fallopian transfer. Hum Reprod 5:947-954.

Hamner CE. 1969 Biochemistry of oviductal secretions. In: The mammalian oviduct. Hafez ESE, Blandau RJ (Eds) pp.333, University of Chicago Pres, Chicago.

Hamner CE. 1973 Oviductal fluid-composition and physiology. In: Handbook of physiology. Greep RO (Ed) pp.141-151, American physiological society, Washington D.C.

Hamner CE, Williams WL. 1964 Identification of sperm stimulating factor of rabbit oviduct fluid. Proc Soc Exper Biol Med 117:240-243.

Hamori M, Stuckensen JA, Rumpf D, Kniewald T, Kniewald A, Marquez MA. 1988 Zygote intrafallopian transfer (ZIFT): evaluation of 42 cases. Fertil Steril 50:519-521.

Hanscom DR, Oliphant G. 1976 Hormonal regulation of incorporation of ^{35}S into macromolecules of oviduct fluid. Biol Reprod 14:599-604.

Harper MJK. 1961 The mechanisms involved in the movement of newly ovulated eggs through the ampulla of the rabbit Fallopian tube. J Reprod Fertil 2:522-524.

Harvey MB, Leco KJ, Arcellana-Panlilio MY, Zhang X, Edwards DR, Schultz GA. 1995 Roles of growth factors during peri-implantation development. Hum Reprod 10:712-718.

Hashimoto M, Shimoyama T, Kosaka M, Komori A, Hirasawa T, Yokoyama Y, Kawase N, Akashi K. 1964 Electron microscopic studies on the epithelial cells of the human Fallopian

tube (report II). J Jap Obstet Gynec Soc [Eng] 11:92-100.

Hershlag A, Seifer DB, Carcangiu ML, Patton DL, Diamond MP, DeCherney AH. 1991 Salpingoscopy: light microscopic and electron microscopic correlations. Obstet Gynecol 77:399-405.
(published erratum appears in Obstet Gynecol 77:809-810, 1991).

Hershlag A, Kaplan EH, Loy RA, DeCherney AH, Lavy G. 1992 Selection bias in in vitro fertilization. Am J Obstet Gynecol 166:1-3.

HFEA. 1992 Human Fertilisation and Embryology Authority. First Annual Report, London, U.K.

HFEA. 1993 Human Fertilisation and Embryology Authority. Second Annual Report, London, U.K.

Hirsch I, Gibbons WE, Lipshultz LI, Rossavik KK, Young RL, Poindexter AN, Dodson MG, Findley WE. 1986 In vitro fertilization in couples with male factor infertility. Fertil Steril 45:659-664.

Holtzbach E. 1908 Vergleichende anatomische Untersuchungen uber die Tubenbrunst und die Tubenmenstruation. Z Geburtsh Gynaek 61:565-580. (on cyclical changes of epithelium)

Howe RS, Wheeler C, Mastroianni L Jr, Blasco L, Tureck R. 1988 Pelvic infection after transvaginal ultrasound-guided ovum retrieval. Fertil Steril 49:726-728.

Hull MGR. 1990 Indications for assisted conception. Br Med Bull 46:580-595.

Hull MGR. 1992 Infertility treatment: relative effectiveness of conventional and assisted conception methods. Hum Reprod 7:785-796.

Hull MGR, Eddowes HA, Fahy U, Abuzeid MI, Mills MS, Cahill DJ, Fleming CF, Wardle PG, Ford WCL, McDermott A. 1992 Expectations of assisted conception for infertility. Br Med J 304:1465-1469.

Hunter RHF. 1974 Chronological and cytological details of fertilisation and early embryonic development in the domestic pig, *Sus scrofa.* Anat Rec 178:169-186.

Hunter RHF. 1988 Development of the Fallopian tubes and their functional anatomy. In: The Fallopian tubes: their role in fertility and infertility. Hunter RHF (Ed) pp.12-29, Springer-Verlag, Berlin.

Hyde BA, Black DL. 1986 Synthesis and secretion of sulphated glycoproteins by rabbit oviduct explants in vitro. J Reprod Fertil 78:83-91.

Hyde BA, Blaustein JD, Black DL. 1989 Differential regulation of progestin receptor immunoreactivity in the rabbit oviduct. Endocrinology 125:1479-1483.

Iffland CA, Reid W, Amso N, Bernard AG, Buckland G, Shaw RW. 1991 A within-patient comparison between superovulation with intra-uterine artificial insemination using husband's washed spermatozoa and gamete intrafallopian transfer in unexplained infertility. Eur J Obstet Gynecol Reprod Biol 39:181-186.

Jackson AE, Curtis P, Amso N, Shaw RW. 1992 LHRH agonists, exposure in early

pregnancy following the commencement of mid-luteal buserelin for IVF stimulation. Hum Reprod 7:1222-1224.

Jansen RPS. 1978 Fallopian tube isthmic mucus and ovum transport. Science 201:349.

Jansen RPS. 1980 Cyclic changes in the human Fallopian tube isthmus and their functional importance. Am J Obstet Gynecol 136:292-308.

Jansen RPS. 1984 Endocrine response in the Fallopian tube. Endocr Rev 5:525-551.

Jansen RPS. 1987 The clinical impact of in-vitro fertilization. Part 1. Results and limitations of conventional reproductive medicine. Med J Aust 146:342-253.

Jansen RPS, Bajpai VK. 1982 Oviduct acid mucus glycoproteins in the estrous rabbit: Ultrastructure and histochemistry. Biol Reprod 26:155-168.

Jansen RPS, Bajpai VK. 1983 Periovulatory glycoprotein secretion in the macaque Fallopian tube. Am J Obstet Gynecol 147:598-608.

Jansen RPS, Anderson JC. 1987 Catheterisation of the Fallopian tube from the vagina. Lancet 2:309-310.

Jansen RPS, Anderson JC. 1993 Transvaginal versus laparoscopic gamete intrafallopian transfer: a case-controlled retrospective comparison. Fertil Steril 59:836-840.

Jansen RPS, Anderson JC and Sutherland PD. 1988a Nonoperative embryo transfer to the fallopian tube. N Engl J Med 319:288-291.

Jansen RPS, Anderson JC, Radonic I, Smit J, Sutherland PD. 1988b Pregnancies after ultrasound-guided Fallopian insemination with cryostored donor semen. Fertil Steril 49:920-922.

Jensen EV, Suzuki T, Kawashima T, Stumpf WE, Jungblut PW, DeSombre ER. 1968 A two-step mechanism for the interaction of estradiol with rat uterus. Proc Natl Acad Sci USA 59:632-638.

Just HM, Petersen EE, Bassler M, Frank U, Daschner FD. 1984 Penetration of cefotetan into serum, myometrium, endometrium and salpinges. Chemotherapy 30:305-307.

Kan FW. 1990 High-resolution localization of hyaluronic acid in the golden hamster oocyte-cumulus complex by use of a hyaluronidase-gold complex. Anat Rec 228:370-382.

Kan FW, St Jacques S, Bleau G. 1988 Immunoelectron microscopic localization of an oviductal antigen in hamster zona pellucida by use of a monoclonal antibody. J Histochem Cytochem 36:1441-1447.

Kan FW, Roux E, St Jacques S, Bleau G. 1990 Demonstration by lectin-gold cytochemistry of transfer of glycoconjugates of oviductal origin to the zona pellucida of oocytes after ovulation in hamsters. Anat Rec 226:37-47.

Kaplan CR, Olive DL, Sabella V, Asch RH, Balmaceda JP, Riehl RM, Groff TR, Burns WN, Schenken RS. 1989 Gamete intrafallopian transfer vs superovulation with intrauterine insemination for the treatment of infertility. J In Vitro Fert Embryo Trans 6:298-304.

Kapur RP, Johnson LV. 1985 An oviductal fluid glycoprotein associated with ovulated mouse ova and early embryos. Develop Biol 112:89-93.

Kapur RP, Johnson LV. 1986 Selective sequestration of an oviduct fluid glycoprotein in the perivitelline space of mouse oocytes and embryos. J Exp Zool 238:249-260.

Kelly PA, Morrison C, Green B. 1978 Effect of progesterone treatment on the uptake of estradiol-17 beta by uterine tissues of ovariectomised rats. Mol Cell Endocrinol 10:319-325.

Kervancioglu ME, Djahanbakhch O, Aitken RJ. 1994 Epithelial cell coculture and the induction of sperm capacitation. Fertil Steril 61:1103-1108.

Kim-Bjorklund T, Landgren BM, Hamberger L, Johannisson E. 1991 Comparative morphometric study of the endometrium, the fallopian tube, and the corpus luteum during the postovulatory phase in normally menstruating women. Fertil Steril 56:842-850.

King RJ, Lane G, Siddle N, Taylor RW, Townsend PT, Whitehead MI. 1981 Assessment of oestrogen and progestin effects on epithelium and stroma from pre- and postmenopausal endometria. J Steroid Biochem 15:175-181.

Kling PA, Burman LG. 1989 Serum and tissue pharmacokinetics of intravenous metronidazole in surgical patients. Acta Chir Scand 155:347-350.

Koritke JG, Gillet JY. 1967 Microvascularisation of oviduct in women. Acta Anat 68:612-613.

Koritke JG, Muller P, Gillet JY. 1968 Vascularisation of the oviduct in the woman. Bulletin of the Federal Societies of Gynaecol Obstet 20:405-406.

Krisher RL, Petters RM, Johnson BH, Bavister BD, Archibong AE. 1989 Development of porcine embryos from the one-cell stage to blastocyst in mouse oviducts maintained in organ culture. J Exp Zool 249:235-239.

Land JA, Arends JW. 1992 Immunohistochemical analysis of estrogen and progesterone receptors in fallopian tubes during ectopic pregnancy. Fertil Steril 58:335-337.

Leese HJ. 1983 Studies on the movement of glucose pyruvate and lactate into the ampulla and isthmus of the rabbit oviduct. Quart J Exp Physiol 68:89-96.

Leese HJ. 1988 The formation and function of oviduct fluid. J Reprod Fert 82:843-856.

Leese HJ. 1990 The environment of the preimplantation embryo. In: Establishing a successful human pregnancy. Edwards RG (Ed) pp.143-154, Raven Press, New York.

Leese HJ, Gray SM. 1985 Vascular perfusion: a novel means of studying oviduct function. Am J Physiol 248: E624-632.

Leese HJ, Barton AM. 1985 Production of pyruvate by isolated mouse cumulus cells. J Exp Zool 234:231-236.

Lenton EA, Weston GA, Cooke ID. 1977 Long term follow-up of the apparently normal couple with a complaint of infertility. Fertil Steril 28:913-919.

Leeton J, Mahadevan M, Trounson A, Wood C. 1984 Unexplained infertility and the

possibilities of management with in vitro fertilization and embryo transfer. Aust NZ J Obstet Gynaecol 24:131-134.

Leeton J, Rogers P, Caro C, Healy D, Yates C. 1987 A controlled study between the use of gamete intrafallopian transfer (GIFT) and in vitro fertilization and embryo transfer in the management of idiopathic and male infertility. Fertil Steril 48:605-607.

Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KS Jr. 1988 Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. J Clin Endocrinol Metab 67:334-340.

Leveille MC, Roberts KD, Chevalier S, Chapdelaine A, Beau G. 1987 Uptake of oviductal antigen by the hamster zona pellucida. Biol Reprod 36:227-238.

Levy C, Robel P, Gautray JP, De Brux J, Verma U, Descomps B, Baulieu EE. 1980 Estradiol and progesterone receptors in human endometrium: normal and abnormal menstrual cycles and early pregnancy. Am J Obstet Gynecol 136:646-651.

Li TC, Dockery P, Rogers AW, Cooke ID. 1990a A quantitative study of endometrial development in the luteal phase: comparison between women with unexplained infertility and normal fertility. Br J Obstet Gynaecol 97:576-582.

Li TC, Lenton EA, Dockery P, Cooke ID. 1990b A comparison of some clinical and endocrinological features between cycles with normal and defective luteal phases in women with unexplained infertility. Hum Reprod 5:805-810.

Lindenbaum ES, Beach D, Peretz BA. 1982 Ultrastructural localization of alkaline and acid phosphatases in the human Fallopian tube epithelium during the menstrual cycle. Anat Rec 203:67-72.

Lindenbaum ES, Peretz BA, Beach D. 1983 Menstrual cycle-dependent and -independent features of human Fallopian tube fimbrial epithelium: an ultrastructural and cytochemical study. Gynecol Obstet Invest 16:76-85.

Lindenbaum ES, Beach D and Peretz BA. 1987 Steroidal binding sites in the ampulla of the human Fallopian tube-autoradiographic and biochemical study. Eur J Obstet Gynecol Reprod Biol 24:201-209.

Lippes J, Wagh PV. 1989 Human oviductal fluid (hOF) proteins. IV. Evidence for hOF proteins binding to human sperm. Fertil Steril 51:89-94.

Lippes J, Enders RG, Pragay DA, Bartholomew WR. 1972 The collection and analysis of human Fallopian tube fluid. Contraception 5:85-103.

Lippes J, Krasner J, Alfonso LA, Dacalos ED, Lucero R. 1981 Human oviductal fluid proteins. Fertil Steril 36:623-629.

Liu HC, Mele C, Noyes N, Rosenwaks Z. 1992 Endometrial secretory protein enhances early embryo development. Abstracts of the scientific oral and poster sessions, 48th annual meeting of the American Fertility Society, November 2-5, 1992. Fertil Steril S40 (Abstract).

Ludwig H, Metzger H. 1976 The human female reproductive tract. In: A scanning electron microscopic atlas, pp.79-105, Springer-Verlag, Berlin.

Lukola A, Punnonen R. 1983 Estrogen and progesterone receptors in human uterus and oviduct. J Endocrinol Invest 6:179-183.

Mackenna AI, Zegers-Hochschild F, Fernandez EO, Fabres CV, Huidobro CA, Prado JA, Roblero LS, Altieri EL, Guadarrama AR, Lopez TH. 1992 Fertilization rate in couples with unexplained infertility. Hum Reprod 7:223-236.

Maguiness SD, Djahanbakhch O. 1992 Salpingoscopic findings in women undergoing sterilization. Hum Reprod 7:269-273.

Maguiness SD, Shrimanker K, Djahanbakhch O, Teisner B, Grudzinskas JG. 1992a Evidence for the in-vitro de-novo synthesis of immunoglobulin and a previously undescribed 17 kDa protein (TEP-2) by the mucosa of the Fallopian tube. Hum Reprod 8:1199-1202.

Maguiness SD, Shrimanker K, Djahanbakhch O, Grudzinskas JG. 1992b Oviduct proteins. Contemp Rev Obstet Gynaecol 4:42-50.

Maguiness SD, Shrimanker K, Djahanbakhch O, Deeks JJ, Teisner B, Gudzinskas JG. 1993 In-vitro synthesis of total protein and placental protein PP14 by the Fallopian tube mucosa: variation in relation to anatomical site, the ovarian cycle and the menopause. Hum Reprod 8:678-683.

Mahadevan MM, Baker G. 1984 Assessment and preparation of semen for in vitro fertilization. In: Clinical in Vitro Fertilization. Wood C, Trouson A (Eds) p.63, Springer-Verlag, New York.

Mahadevan MM, Trounson AO, Leeton JF. 1983 The relationship of tubal blockage, infertility of unknown cause, suspected male infertility, and endometriosis to success of in vitro fertilization and embryo transfer. Fertil Steril 40:755-762.

Marshall T. 1984 Detection of protein in polyacrylamide gels using an improved silver stain. Anal Biochem 136:340-346.

Martel D, Frydman R, Glissant M, Maggioni C, Roche D, Psychoyos A. 1987 Scanning electron microscopy of postovulatory human endometrium in spontaneous cycles and cycles stimulated by hormone treatment. J Endocrinol 14:319-324.

Martinez AR, Bernardus RE, Voorhorst FJ, Vermeiden JP, Schoemaker J. 1991 Pregnancy rates after timed intercourse or intrauterine insemination after human menopausal gonadotropin stimulation of normal ovulatory cycles: a controlled study. Fertil Steril 55:258-265.

Martinez F, Barri PN, Coroleu V. 1988 Accidental GnRH agonist administration during early pregnancy. Hum Reprod 3:669.

Mass DHA, Storey BT, Mastroianni L Jr. 1979 Hydrogen ion and Carbon dioxide content of the oviductal fluid. Fertil Steril 28:981-985.

Mastroianni L Jr, Go KJ. 1979 Tubal secretions. In: The biology of the fluids of the genital tract. Beller FK, Schumacher GFB (Eds) pp.335-344, Elsevier/North-Holland, Amsterdam.

Mastroianni L Jr, Urzua M, Stambaugh R. 1970 Protein patterns in monkey oviductal fluid before and after ovulation. Fertil Steril 21:817-820.

McClellan M, West NB, Tacha D, Greene G, Brenner R. 1984 Immunocytochemical localization of estrogen receptors in the macaque reproductive tract with monoclonal antiestrophilins. Endocrinology 114:2002.

McClellan M, West NB, Brenner RM. 1986 Immunocytochemical localization of estrogen receptors in the macaque endometrium during the luteal-follicular transition. Endocrinology 119:2467-2475.

McCormack SA, Glasser SR. 1980 Differential response of individual uterine cell types from immature rats treated with estradiol. Endocrinology 106:1634-1649.

Medical Research International, The Society for Assisted Reproductive Technology, The American Fertility Society (MRI, SART). 1991 In vitro fertilization-embryo transfer (IVF-ET) in the United States: 1989 results from the IVF-ET Registry. Fertil Steril 55:14-23.

Medical Research International, The Society for Assisted Reproductive Technology, The American Fertility Society (MRI, SART). 1992 In vitro fertilization-embryo transfer (IVF-ET) in the United States: 1990 results from the IVF-ET Registry. Fertil Steril 57:15-24.

Meldrum DR. 1989 Antibiotics for vaginal oocyte aspiration editorial. J In Vitro Fert Embryo Trans 6:1-2.

Menghi G, Bondi AM, Accili D, Materazzi G. 1984a Fine localization of sulphated and non-sulphated glycoconjugates in the rabbit oviduct during the estrous cycle. Acta Histochem (Jena) 74:121-132.

Menghi G, Bondi AM, Accili D, Vitaioli L. 1984b Glycoconjugates in the reproductive system of female rabbits in physiological estrogenic conditions. Anat Anz 156:125-128.

Minami N, Bavister BD, Iritani A. 1988 Development of hamster two-cell embryos in the isolated mouse oviduct in organ culture system. Gamete Res 19:235-240.

Moghissi KS. 1970 Human Fallopian tube fluid. 1. Protein composition. Fertil Steril 21:821-829.

Moore G, Eddy CA, Pauerstein CJ. 1992 Rabbit endosalpinx inhibits implantation in vitro. Fertil Steril 57:902-907.

Morris H, Emms M, Visser T, Timme A. 1986 Lymphoid tissue of the normal Fallopian tube-a form of mucosal associated lymphoid tissue (MALT). Int J Gynec Pathol 5:11-22.

Murdoch AP, Harris M, Mahroo M, Williams M, Dunlop W. 1991 Is GIFT (gamete intrafallopian transfer) the best treatment for unexplained infertility? Br J Obstet Gynaecol 98:643-647.

Navot D, Muasher SJ, Oehninger S, Liu H-C, Veek LL, Kreiner D, Rosenwaks Z. 1988 The value of in vitro fertilization for the treatment of unexplained infertility. Fertil Steril 49:854-857.

Nikas G, Drakakis P, Loutradis D, Mara-Skoufari C, Koumantakis E, Michalas S, Psychoyos A. 1995 Uterine pinopodes as markers of the 'nidation window' in cycling women receiving exogenous oestradiol and progesterone. Hum Reprod 10:1208-1213.

Nishino H, Nagamatsu M, Ishii S, Takamatsu S, Saito J, Yamanaka K. 1983 Tissue

distribution of cefotetan in the field of obstetrics and gynecology. Jpn J Antibiot 36:398-407.

Novak E. 1947 Histology of Fallopian tubes. In: Gynecological and Obstetrical Pathology (2nd Edition). Novak E (Ed), pp. 242-271, WB Saunders, London.

Novak E, Everett HS. 1928 Cyclical and other variations in the tubal epithelium. Am J Obstet Gynecol 16:499-530.

Noyes RW, Hertig AT, Rock J. 1950 Dating of the endometrial biopsy. Fertil Steril 1:3-25.

Odor DL. 1974 The question of basal cells in oviductal and endocervical epithelium. Fertil Steril 25:1047-1062.

Odor DL, Gaddum-Rosse P, Rumery RE. 1983 Secretory cells of the oviduct of the pig-tailed monkey Macaca nemestrina during the menstrual cycle and after oestrogen treatment. Amer J Anat 166:149-172.

Oliphant G. 1986 Biochemistry and immunology of oviductal fluid. In: The fallopian tube: Basic studies and clinical contributions. Siegler AM, Mount Kisco MD (Eds), Futura Publishing company Inc. New York.

Oliphant G, Bowling A, Eng LA, Keens S, Randall PA. 1978 The permeability of the rabbit oviduct to proteins present in the serum. Biol Reprod 18:516-520.

Oliphant G, Cabot C, Ross P, Marta J. 1984a Control of the humoral immune system within the rabbit oviduct. Biol Reprod 31:205-212.

Oliphant G, Reynolds AB, Smith PF, Ross PR, Marta JS. 1984b Immunocytochemical localization and determination of hormone-induced synthesis of the sulfated oviductal glycoproteins. Biol Reprod 31:165-174.

Olive DL. 1986 Analysis of clinical fertility trials: a methodologic review. Fertil Steril 45:157-171.

Ornstein L. 1964 Disc Electrophoresis-1. Background and theory. Ann N Y Acad Sci 121:321-349.

Ouchterlony O. 1949 Antigen-antibody reactions in gels. Acta Path et Microbiol Scandinav 26:507-515.

Owman C, Rosengren E, Sjoberg NO. 1967 Adrenergic innervation of the human female reproductive organs: a histochemical and chemical investigation. Obstet Gynecol 30:763-773.

Pacey AA, Hill CJ, Scudamore IW, Warren MA, Barratt CLR, Cokke ID. 1995 The interaction *in vitro* of human spermatozoa with epithelial cells from the human uterine (Fallopian) tube. Hum Reprod 10:360-366.

Padilla SL, Garcia JE. 1989 Effect of maternal age and number of in vitro fertilization procedures on pregnancy outcome. Fertil Steril 52:270-273.

Palermo G, Devroey P, Camus M, De Grauwe E, Khan I, Staessen C, Wisanto A, Van Steirteghem AC. 1989 Zygote intra-fallopian transfer as an alternative treatment for male infertility. Hum Reprod 4:412-415.

Patek E, Nilsson L, Johannisson E. 1972a Scanning electron microscopic study of the human Fallopian tube. Report I. The proliferative and secretory stages. Fertil Steril 23:459-465.

Patek E, Nilsson L, Johannisson E. 1972b Scanning electron microscopic study of the human Fallopian tube. Report II. Fetal life, reproductive life, and postmenopause. Fertil Steril 23:719-733.

Patek E, Nilsson L, Johannisson E, Hellema M, Bout J. 1973a Scanning electron microscopic study of the human fallopian tube. III. The effect of midpregnancy and of various steroids. Fertil Steril 24:31-43.

Patek E, Nilsson L, Hellema M. 1973b Scanning electron microscopic study of the human fallopian tube report. IV. At term gestation and in the puerperium. The effect of a synthetic progestin on the postmenopausal tube. Fertil Steril 24:832-843.

Pauerstein CJ. 1975 Clinical implications of oviductal physiology and biochemistry. Gynecol Invest 6:253-264.

Pauerstein CJ. 1978 From Fallopius to fantasy. Fertil Steril 30:133-140.

Pauerstein CJ, Eddy CA. 1979 Morphology of the Fallopian tube. In: The biology of the fluids of the female genital tract. Beller FK, Schumacher G (Eds), Elsevier North Hollands, Amsterdam.

Paulson RJ, Sauer MV, Lobo RA. 1990a Embryo implantation after human in vitro fertilization: importance of endometrial receptivity. Fertil Steril 53:870-874.

Paulson RJ, Sauer MV, Lobo RA. 1990b Factors affecting embryo implantation after human in vitro fertilization: a hypothesis. Am J Obstet Gynecol 163:2020-2023.

Pemble LB, Kaye PL. 1986 Whole protein uptake and metabolism by mouse blastocyst. J Reprod Fert 78:149-157.

Perrot-Applanat M, Logeat F, Groyer-Picard MT, Milgrom E. 1985 Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. Endocrinology 116:1473-1484.

Peters WM. 1986 Nature of "basal" and "reserve" cells in oviductal and cervical epithelium in man. J Clin Pathol 39:306-312.

Pino AM, Devoto L, Soto E, Castro O, Sierralta W. 1982 Changes in cytosolic and nuclear estradiol receptors of normal Fallopian tube throughout the menstrual cycle. J Steroid Biochem 16:193-197.

Pollow K, Inthraphuvasak J, Manz B, Grill HJ, Pollow B. 1981 A comparison of cytoplasmic and nuclear estradiol and progesterone receptors in human fallopian tube and endometrial tissue. Fertil Steril 36:615-622.

Pollow K, Inthraphuvasak J, Grill HJ, Manz B. 1982 Estradiol and progesterone binding components in the cytosol of normal human fallopian tubes. J Steroid Biochem 16:429-435.

Pool TB, Ellsworth LR, Garza JR, Martin JE, Miller SS, Atiee SH. 1990 Zygote intrafallopian transfer as a treatment for nontubal infertility: a 2-year study. Fertil Steril 54:482-488.

Possati G, Seracchioli R, Melega C, Pareschi A, Maccolini A, Flamigni C. 1991 Gamete intrafallopian transfer by hysteroscopy as an alternative treatment for infertility. Fertil Steril 56:496-499.

Press MF, Greene GL. 1988 Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor. Endocrinology 122:1165-1175.

Press MF, Nousek-Goebl NA, King WJ, Herbst AL, Greene GL. 1984 Immunohistochemical assessment of estrogen receptor distribution in the human endometrium throughout the menstrual cycle. Lab Invest 51:495-503.

Press MF, Nousek-Goebl NA, Bur M, Greene GL. 1986 Estrogen receptor localization in the female genital tract. Am J Pathol 123:280-292.

Press MF, Udove JA, Greene GL. 1988 Progesterone receptor distribution in the human endometrium. Analysis using monoclonal antibodies to the human progesterone receptor. Am J Pathol 131:112-124.

Psychoyos A, Nikas G. 1994 Uterine pinopods as markers of uterine receptivity. Assist Reprod Rev 4:26-32.

Punnonen R, Lukola A. 1981 Binding of estrogen and progestin in the human fallopian tube. Fertil Steril 36:610-614.

Quinn P, Kerrin JF, Warnes GM. 1985 Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 44:493-498.

Rapisarda JJ, Mavrogianis PA, O'Day-Bowman MB, Fazleabas AT, Verhage HG. 1993 Immunological characterization and immunocytochemical localization of an oviduct-specific glycoprotein in the human. J Clin Endocrinol Metabol 76:1483-1488.

Richardson LL, Hamner CE, Oliphant G. 1980 Some characterestics of an inhibitor of embryonic development from rabbit oviductal fluid. Biol Reprod 22:553-559.

Ripa KT, Mardh PA. 1977 Cultivation of Chlamydia trachomatis in cycloheximide-treated Mccoy cells. J Clin Microbiol 6:328-331.

Risquez F, Boyer P, Rolet F, Magnani M, Guichard A, Cedard L, Zorn JR. 1990 Retrograde tubal transfer of human embryos. Hum Reprod 5:185-188.

Roberts GP, Parker JM, Symonds HW. 1976 Macromolecular components of genital tract fluids from the sheep. J Reprod Fert 48:99-107.

Robertson DM, Landgren BM. 1975 Oestradiol receptor levels in the human fallopian tube during the menstrual cycle and after menopause. J Steroid Biochem 6:511-513.

Roblero LS, Riffo MD. 1986 High potassium concentration improves preimplantation development of mouse embryos in vitro. Fertil Steril 45:412-416.

Ron El R, Golan A, Herman A, Raziel A, Soffer Y, Caspi E. 1990 Midluteal gonadotropin-releasing hormone analog administration in early pregnancy. Fertil Steril 53:572-574.

Russell JB, DeCherney AH, Hobbins JC. 1987 A new transvaginal probe and biopsy guide for oocyte retrieval. Fertil Steril 47:350-352.

Salat-Baroux J, Alvarez S, Antoine JM, Cornet D, Tibi C, Plachot M, Mandelbaum J. 1990 Treatment of hyperstimulation during in-vitro fertilization. Hum Reprod 5:36-39.

Saleh MI, Warren MA, Li TC, Cooke ID. 1995 A light microscopical morphometric study of the luminal epithelium of human endometrium during peri-implantation period. Hum Reprod 10:1828-1832.

Samberg I, Degani S, Zilberman A, Eibschitz I, Nir I, Scharf M. 1983 Scanning electron microscopy of human Fallopian tube in ectopic pregnancy. Gynecol Obstet Invest 16:65-75.

Saracoglu OF, Aksel S, Yeoman RR, Wiebe RH. 1985 Endometrial estradiol and progesterone receptors in patients with luteal phase defects and endometriosis. Fertil Steril 43:851-855.

Saunders DM, Quigley MM, Cohen J. 1989 Uniform assessment of success rates with assisted reproductive technology. J In Vitro Fertil Embryo Trans 6:315.

Scharl A, Vierbuchen M, Graupner J, Fischer R, Bolte A. 1988 Immunohistochemical study of distribution of estrogen receptors in corpus and cervix uteri. Arch Gynecol Obstet 241:221-233.

Scholtes MCW, Roozenburg BJ, Verhoeff A, Zeilmaker GH. 1994 A randomized study of transcervical intrafallopian transfer of pronucleate embryos controlled by ultrasound versus intrauterine transfer of four- to eight-cell embryos. Fertil Steril 61:102-104.

Schulte BA, Rao KP, Kreutner A, Thomopoulos GN and Spicer SS. 1985 Histochemical examination of glycoconjugates of epithelial cells in the human Fallopian tube. Lab Invest 52:207-219.

Seckinger DL. 1923 Spontaneous contractions of the Fallopian tube of the domestic pig with reference to oestrous cycle. Bulletin of the Johns Hopkins Hospital 34:236-239.

Seif M. 1989 Progress in Immunohistochemical analysis of the Endometrial cycle. A Thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medicine.

Seifer DB, Collins RL, Paushter DM, George CR, Quigley MM. 1988 Follicular aspiration: a comparison of an ultrasonic endovaginal transducer with fixed needle guide and other retrieval methods. Fertil Steril 49:462-467.

Serhal PF, Katz M, Little V, Woronowski H. 1988 Unexplained infertility-the value of Pergonal superovulation combined with intrauterine insemination. Fertil Steril 49:602-606.

Shapiro SS, Jentsch JP, Yard AS. 1971 Protein composition of rabbit oviductal fluid. J Reprod Fert 24:403-408.

Sharma V, Riddle A, Mason BA, Pampiglione J, Campbell S. 1988 An analysis of factors influencing the establishment of a clinical pregnancy in an ultrasound-based ambulatory in vitro fertilization program. Fertil Steril 49:468-478.

Shettles LB. 1979 Ova harvest with in vivo fertilisation. Am J Obstet Gynaecol 133:845.

Simon A, Avidan B, Mordel N, Lewin A, Samueloff A, Zajicek G, Schenker JG, Laufer N. 1991 The value of menotrophin treatment for unexplained infertility prior to an in-vitro fertilization attempt. Hum Reprod 6:222-226.

Smith RA, Seif MW, Rogers AW, Li TC, Dockery P, Cooke ID, Aplin JD. 1989 The endometrial cycle: the expression of a secretory component correlated with the luteinizing hormone peak. Hum Reprod 4:236-242.

Society for Assisted Reproductive Technology, The American Fertility Society (SART, AFS). 1993 Assisted reproductive technology in the United States and Canada: 1991 results from the Society for Assisted Reproductive Technology generated from The American Fertility Society Registry. Fertil Steril 59:956-962.

Soltes B, Rawlins RG, Binor Z, Radwanska E. 1994 Human chorionic gonadotropin levels in conception cycles after uterine and tubal embryo transfer. Fertil Steril 62:823-825.

Spilman CH. 1976 Prostaglandins, oviductal motility and egg transport. In: Symposium on Ovum Transport and Fertility Regulation. Harper MJK, Pauerstein CJ (Eds) pp.197-211, Scriptor, Copenhagen.

Stambaugh R, Noriega C, Mastroianni L Jr. 1969 Bicarbonate ion; the corona cell dispersing factor in rabbit tubal fluid. J Reprod Fertil. 18:51-58.

Steptoe PC, Edwards RG. 1976 Reimplantation of a human embryo with subsequent tubal pregnancy. Lancet 1:880-882.

Stone SL, Hamner CE. 1977 Hormonal and regional influences of the oviduct on the development of rabbit embryos. Biol Reprod 16:638-646.

Sundstrom P, Ove Nilsson B. 1982 Postovulatory endometrium. In: Atlas of Human Reproduction by Scanning Electron Microscopy. Hafez ESE, Kenemans P (Eds) pp.61-69, M.T.P., Lancaster.

Sutton R, Nancarrow CD, Wallace ALC, Rigby NW. 1984 Identification of an oestrus-associated glycoprotein in oviductal fluid of the sheep. J Reprod Fertil 72:415-422.

Tan SL, Royston P, Campbell S, Jacobs HS, Betts J, Mason B, Edwards RG. 1992 Cumulative conception and livebirth rates after in-vitro fertilisation. Lancet 339:1390-1394.

Tabibzadeh S, Sun XZ. 1992 Cytokine expression in human endometrium throughout the menstrual cycle. Hum Reprod 7:1214-1221.

Tabibzadeh S, Kong QF, Babaknia A, May LT. 1995 Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window. Hum Reprod 10:2793-2799.

Tanbo T, Dale PO, Abyholm T. 1990 Assisted fertilization in infertile women with patent fallopian tubes. A comparison of in-vitro fertilization, gamete intra- fallopian transfer and tubal embryo stage transfer. Hum Reprod 5:266-270.

Te Velde ER, Koudstaal J, Emers JM. 1992 Assisted conception for infertility. Br Med J 305:1097-1098.

Templeton AA, Mortimer D. 1982 The development of a clinical test for sperm migration

to the site of fertilization. Fertil Steril 37:410-415.

Templeton AA, Penney GC. 1982 The incidence, characterestics, and prognosis of patients whose infertility is unexplained. Fertil Steril 37:175-181.

Tesarik J, Pilka L, Dvorak M, Travnik P. 1983 Oocyte recovery, in vitro insemination, and transfer into the oviduct after its microsurgical repair at a single laparotomy. Fertil Steril 39:472-475.

Toth TL, Oehninger S, Toner JP, Brzyski RG, Acosta AA, Muasher SJ. 1992 Embryo transfer to the uterus or the Fallopian tube after in vitro fertilization yielda similar results. Fertil Steril 57:1110-1113.

Tournaye H, Camus M, Khan I, Staessen C, Van Steirteghem AC, Devroey P. 1991 In-vitro fertilization, gamete- or zygote intra-Fallopian transfer for the treatment of male infertility. Hum Reprod 6:263-266.

Tournaye H, Devroey P, Camus M, Staessen C, Bollen N, Smitz J, Van Steirteghem AC. 1992a Comparison of in-vitro fertilization in male and tubal infertility: a 3 year survey. Hum Reprod 7:218-222.

Tournaye H, Devroey P, Camus M, Valkenburg M, Bollen N, Van Steirteghem AC. 1992b Zygote intrafallopian transfer or in vitro fertilization and embryo transfer for the treatment of male-factor infertility: a prospective randomized trial. Fertil Steril 58:344-350.

Trounson AO, Leeton JF, Wood C, Webb J, Kovacs G. 1980 The investigation of idiopathic infertility by in vitro fertilization. Fertil Steril 34:431-438.

Tulandi T, Cherry N. 1989 Clinical trials in reproductive surgery: randomization and life-table analysis. Fertil Steril 52:12-14.

Tur Kaspa I, Confino E, Dudkiewicz AB, Myers SA, Friberg J, Gleicher N. 1990 Ovarian stimulation protocol for in vitro fertilization with gonadotropin-releasing hormone agonist widens the implantation window. Fertil Steril 53:859-864.

Van Hoorde GJ, Verhoeff A, Zeilmaker GH. 1992 Perforated appendicitis following transvaginal oocyte retrieval for in-vitro fertilization and embryo transfer. Hum Reprod 7:850-851.

Van Niekerk CH, Gerneke WH. 1966 Persistence and parthenogenetic cleavage of tubal ova in the mare. Onderstepoort J Vet Res 31:195-232.

Vasquez G, Winston RM, Boeckx W, Gordts S, Brosens IA. 1983 The epithelium of human hydrosalpinges: a light optical and scanning electron microscopic study. Br J Obstet Gynaecol 90:764-770.

Verco CJ, Gannon BJ, Jones WR. 1983 Fallopian tube microvasculature in the rabbit. Aust J Exp Biol Med Sci 61:127-138.

Verco CJ, Gannon BJ, Jones WR. 1984 Variations in rabbit oviduct microvasculature architecture after ovulation induced by hCG. J Reprod Fertil 72:15-19.

Verhage HG, Jaffe RC. 1986 Regulation of estradiol and progesterone receptor concentrations in cat uteri following chronic progesterone administration. J Steroid Biochem

24:587-590.

Verhage HG, Fazleabas AT. 1988 The in vitro synthesis of estrogen-dependent proteins by the baboon (Papio anubis) oviduct. Endocrinology 123:552-558.

Verhage HG, Bareither ML, Jaffe RC, Akbar M. 1979 Cyclic changes in ciliation, secretion and cell height of oviductal epithelium in women. Amer J Anat 156:505-521.

Verhage HG, Murray MK, Boomsma RA, Rehfeldt PA, Jaffe RC. 1984 The postovulatory cat oviduct and uterus: Correlation of morphological features with progesterone receptor level. Anat Rec 208:521-531.

Verhage HG, Fazleabas AT, Donnelly K. 1988 The in vitro synthesis and release of proteins by the human oviduct. Endocrinology 122:1639-1645.

Verhage HG, Boice ML, Mavrogianis P, Donnelly K, Fazleabas AT. 1989 Immunological characterization and immunocytochemical localization of oviduct-specific glycoproteins in the baboon (Papio anubis). Endocrinology 124:2464-2472.

Vizza E, Correr S, Muglia U, Marchiolli F, Motta PM. 1995 The three-dimensional organization of the smooth musculature in the ampulla of the human Fallopian tube: a new morpho-functional model. Hum Reprod 10:2400-2405.

Wada I, Matson PL, Troup SA, Hughes S, Buck P, Lieberman BA. 1992 Outcome of treatment subsequent to the elective cryopreservation of all embryos from women at risk of the ovarian hyperstimulation syndrome. Hum Reprod 7: 962-966.

Wagh PV, Lippes J. 1989 Human oviductal fluid proteins III. Identification and partial purification. Fertil Steril 51:81-88.

Walker SK, Heard TM, Seamark RF. 1992 In vitro culture of sheep embryos without co-culture: successes and perspectives. Theriogenology 37:111-126.

Wang RX, Brooks DE. 1986 Protein composition of the luminal fluid and protein synthesis in vitro by the oviducts and uteri of ovariectomized, pro-oestrus and 5-day pregnant rats. Aus J Exp Biol Med Sci 64:257-269.

Wang XJ, Ledger W, Payne D, Jeffrey R, Matthews CD. 1994 The contribution of embryo cryopreservation to in-vitro fertilization/gamete intra-Fallopian transfer: 8 year experience. Hum Reprod 9:103-109.

Weigand RA, Cotter DL, Dunn RA, Nolan C, Greene G, Przywara LW. 1986 Quantitation of progesterone receptor (PgR) in human breast tumors by double monoclonal enzyme immunoassay (PgR-EIA). Breast Cancer Res and Treatment 8:87.

Weimer KE, Cohen J, Amborski GF, Wright G, Wiker S, Munyakazi, Godke RA. 1989 In-vitro development and implantation of human embryos following culture on fetal bovine uterine fibroblast cells. Hum Reprod 4:595-600.

West NB, Brenner RM. 1983 Estrogen receptor levels in the oviducts and endometria of Cynomolgus Macaques during the menstrual cycle. Biol Reprod 29:1303-1312.

West NB, Brenner RM. 1985 Progesterone-mediated suppression of estradiol receptors in cynomolgus macaque cervix, endometrium and oviduct during sequential

estradiol-progesterone treatment. J Steroid Biochem 22:29-37.

West NB, Hess DL, Brenner RM. 1986 Differential suppression of progesterone receptors by progesterone in the reproductive tract of female macaques. J Steroid Biochem 25:497-503.

West NB, McClellan MC, Sternfeld MD, Brenner RM. 1987 Immunocytochemistry versus binding assays of the estrogen receptor in the reproductive tract of spayed and hormone-treated macaques. Endocrinology 121:1789-1800.

Weström L, Märdh PA, von Mecklenburg C, Häkansson CH. 1977 Studies on ciliated epithelia of the human genital tract. II. The mucociliary wave pattern of Fallopian tube epithelium. Fertil Steril 28:955-961.

Wheeler JE. 1982 Pathology of the Fallopian tube In: Pathology of the female genital tract. Blaustein A (Ed) pp.393-415, Springer-Verlag, New York.

Whittingham DG. 1968a Development of zygotes in cultured mouse oviducts. I. The effect of varying oviductal conditions. J Exp Zool 169:391-397.

Whittingham DG. 1968b Development of zygotes in cultured mouse oviducts. II. The influence of the estrous cycle and ovarian hormones upon the development of the zygote. J Exp Zool 169:399-405.

Whittingham DG, Biggers JD. 1967 Fallopian tube and early cleavage in the mouse. Nature 213:942-943.

Wilcox LS, Peterson HB, Haseltine FP, Martin MC. 1993 Defining and interpreting pregnancy success rates for in vitro fertilization. Fertil Steril 60:18-25.

Wiley LM, Obasaju MF. 1986 Immunoflourescent localization of immunoglobulins on the cell surface of mouse oocytes and preimplantation embryos. J In Vitro Fertil Embryo Trans 3:319

Woolcott R, Stanger J, Cohen R, Silber S. 1994 Refinements in the methodology of injection for transvaginal gamete intra-Fallopian transfer. Hum Reprod 9:1466-1468.

World Health Organisation (WHO). 1987 Laboratory manual for the examination of human semen and semen-cervical mucus interaction. Second Edition. Cambridge University Press, Cambridge, UK

Wren M, Parsons J. 1989 Ultrasound directed follicle aspiration in IVF. In: Recent advances in the management of infertility. Chen C, Tan SL, Cheng WC (Eds) pp.165-181, McGraw-Hill, New York.

Wurfel W, Krusmann G, Rothenaicher M, Hirsch P, Krusmann W Sr. 1988 Pregnancy following in vitro fertilization and intratubal embryo transfer. Geburtshilfe-Frauenheilkd 48:179-181.

Yanagamachi R, Usui N. 1974 Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp Cell Res 89:161-174.

Yang C-H, Yanagimachi R. 1989 Differences between mature ovarian and oviductal oocytes: a study using the golden hamster. Hum Reprod 4:63-71.

Yovich JL. 1990 In: The Management of infertility. A Manual of Gamete Handling Procedures. Yovich J, Grudzinskas G (Eds), Heinemann Medical Books, Oxford.

Yovich JL, Blackledge DG, Richardson PA, Matson PL, Turner SR, Draper R. 1987 Pregnancies following pronuclear stage tubal transfer. Fertil Steril 48:851-857.

Yovich JL, Yovich JM, Edirisinghe WR. 1988 The relative chance of pregnancy following tubal or uterine transfer procedures. Fertil Steril 49:858-864.

Yuzpe AA, Brown SE, Casper RF, Nisker J, Graves G, Shatford L. 1989 Transvaginal, ultrasound-guided oocyte retrieval for in vitro fertilization. J Reprod Med 34:937-942.

LIST OF ABBREVIATIONS

μm	=	micron
1-D PAGE	=	One-dimensional polyacrylamide gel electrophoresis
AIJ	=	Ampullary-isthmic junction
Alb	=	Albumin
ß-hCG	=	Beta subunit-human chorionic gonadotrophin
BSA	=	Bovine serum albumin
°C	=	Degree Celsius
CC	=	Ciliated cell
CCR	=	Cumulative conception rate
CDC	=	Chronological day of the cycle
cDNA	=	Complementary deoxyribonucleic acid
CI	=	Confidence interval
CLBR	=	Cumulative live birth rate
CV	=	Coefficient of variation
E	=	Oestrogen
E_2	=	Oestradiol
EBS	=	Earle's balanced salt
EM	=	Electron microscopy
ER	=	Oestrogen receptor
ER-ICA	=	Oestrogen receptor monoclonal immunocytochemical assay
FSH	=	Follicle stimulating hormone
GIFT	=	Gamete intrafallopian transfer
GnRH-a	=	Gonadotrophin releasing hormone agonist
H + E	=	Haematoxylin and eosin
hCG	=	Human chorionic gonadotrophin
HDC	=	Histological day of the cycle
hMG	=	Human menopausal gonadotrophin
HRT	=	Hormone replacement therapy
HSA	=	Human serum albumin
ICA	=	Immunocytochemical assay
Ig	=	Immunoglobulin
IR	=	Implantation rate
IPI	=	Intraperitoneal insemination
IVF	=	In vitro fertilisation

kD	=	Kilo Dalton
LBR	=	Live birth rate
LH	=	Luteinising hormone
LM	=	Light microscopy
LMP	=	Last menstrual period
mfd	=	Mean follicular diameter
ml	=	Millilitre
mg	=	Milligram
MOAB	=	Monoclonal antibody
mRNA	=	Messenger ribonucleic acid
MVA	=	Microvascular architecture
NCC	=	Non-ciliated cells
OR	=	Odds ratio
%	=	Percentage
P	=	Progesterone
p <	=	Probability of null hypothesis less than
p >	=	Probability of null hypothesis more than
PAGE	=	Polyacrylamide gel electrophoresis
PAP	=	Peroxidase-rat antihorseradish peroxidase
PBS	=	Phosphate-buffered saline
PgR-ICA	=	Progesterone receptor monoclonal immunocytochemical assay
PR	=	Progesterone receptor
PR	=	Pregnancy rate
PROST	=	Pronuclear-stage tubal transfer
s.d	=	Standard deviation
SEM	=	Scanning electron microscopy
sem	=	Standard error of the mean
SO-IUI	=	Superovulation and intrauterine insemination
TB	=	Toluidine blue
TEM	=	Transmission electron microscopy
TET	=	Tubal embryo transfer
UET	=	Uterine embryo transfer
UTJ	=	Utero-tubal junction
VEC	=	Vaginal egg collection
WHO	=	World Health Organisation
ZIFT	=	Zygote intrafallopian transfer

APPENDIX 1: PROTOCOL FOR IVF-UET VS. IVF-TET TRIAL

1. Purpose of the trial

Determine the influence of tubal environment on early embryonic development and the establishment of pregnancy using tubal embryo transfer versus in-utero transfer techniques after vaginal egg collection and in-vitro fertilisation .

2. Study protocol

Background and general aims:

(1) The influence of the tubal environment on fertilisation and early embryonic development is important. The improved success rate for GIFT seems to support this concept. However, reports appearing in the literature indicate that these studies are poorly conducted and compare patients of different categories, thus invalidating these reported successes.

(2) Our aim is to study the influence of the tubal factor on the establishment of pregnancy in a selected group of woman in which the transfer of embryos can be carried out either into the uterine cavity or the Fallopian tube.

(3) The objective is to carry out a controlled, randomised cross over trial in this group of women.

(4) We aim to recruit up to 90 couples to the study to give us up to 250 treatment cycles (both arms of the trial).

Patient inclusion

(1) Couples with unexplained infertility, male factor infertility (treated with husband's or donor sperm), mild/moderate or treated endometriosis will be included.

(2) The couples should have been trying unsuccessfully for more than 2 years at the time of their first attempt and/or had been treated unsuccessfully in the past.

(3) The diagnosis of unexplained infertility will be made after completing the following investigations. The women will have basic hormone profile, luteal phase progesterone, laparoscopy and dye hydrotubation +/- endometrial biopsy, hysterosalpingogram, serum antisperm antibodies and a post coital test. The male partner should have a normal semen analysis and 24 hour swim-up in culture media, and antisperm antibody testing (a hamster egg penetration testing is optional).

(3) The women should have apparently healthy, and accessible Fallopian tube(s) to enable

tubal embryo transfer.

(4) Couples should not have received any treatment for infertility within the preceding three months.

(5) Informed consent must be obtained from the couple prior to inclusion on the trial.

Patient exclusion

The couple will be excluded from the trial if;

(1) Women who at the time of tubal embryo transfer are found to have tubal damage, pelvic adhesions of inflammatory origin (other than few filmy adhesions) or extensive endometriosis will be excluded from the trial.

(2) The oocytes fail to fertilise in two consecutive treatment cycles.

(3) The couple wish to discontinue treatment.

Methodology:

(1) Ovarian stimulation protocols/monitoring

Ovarian stimulation:

The ovaries will be stimulated by using a combination of a GnRH-analogue and gonadotrophin injection. After complete downregulation the starting dose of gonadotrophins(Pergonal/ Metrodin) in the first treatment cycle will be 150 iu/day. This dose will be increased if the response is inadequate as judged by ultrasound scans either in the same treatment cycle or in subsequent cycles.

Monitoring:

Primary monitoring will be carried out by using serial ultrasound scans on the following days. Day 1 (prior to downregulation); Day +1 (prior to starting gonadotrophin injections); Day +8, Day +10 until a satisfactory response is obtained. Hormone assays (serum E_2, P and LH) will be carried out whenever possible. The treatment cycle will be abandoned if the E2 levels are low (< 500 pmol/l per mature follicle > 15mm diameter) or the pre-HCG serum LH level exceeds 10iu/l. HCG injection (5,000 iu will be given I.M. 34-37 hours prior to oocyte collection) when 3 or more follicles are $\geq$ 18 mm in diameter.

(2) Method of egg collection

Vaginal egg collection will be the principal method of egg collection for the patients entered into this study and will be carried out under general anaesthetic or local analgesia. Local analgesia will be the method of choice for all the ZIFT treatment cycles in order to avoid the administration of general anaesthetic on two consecutive days.

(3) Laboratory techniques

Egg grading, semen assessment, insemination protocols, examination of pre-embryos and their evaluation will be according to those practiced in the department.

(4) Timing of tubal/uterine transfer

A maximum of three pre-embryos will be transfered either into the uterine cavity or the Fallopian tube.

Uterine ET will be carried out 48-56 hours after egg collection at the 2-8 cell stage.

Laparoscopic intra-Fallopian embryo transfer will be carried out approximately 24-30 hours after egg collection at the pronuclear or 2 cell stage. If fertilisation can not be verified prior to the transfer or there is an inadequate number of embryos for transfer (<3 embryos), a further examination of the oocytes will be carried out after another 24 hours and tubal embryo transfer will take place accordingly.

(5) Luteal phase support

Progesterone (Cyclogest pessaries) 200mg, twice daily rectally or vaginally and started one day after oocyte collection and continued for 12 days or,
HCG injections; 2000 iu given IM on day of ET/ET+3 and +6.

(6) Administration

The patients will be admitted to the ward for their vaginal egg collection on the morning of the operation. They will have an ultrasound scan immediately prior to their operatation.

The patients who will have the ZIFT procedure will be kept in the ward overnight and depending on the fertilisation report will have the tubal embryo transfer accordingly.

(7) Evaluation methods

Definition of pregnancy is generally based on a positive urine ß-hCG test 15 to 18 days after embryo transfer (biochemical pregnancy). However, the primary end point is the establishment of clinical pregnancy. This will be determined by the appearance of gestational sac(s) on ultrasound scans at 6 to 7 weeks gestation using abdominal or vaginal techniques. Live birth that includes one or more infants will be also used in the reporting of this trial as another primary indicator of the effectiveness of either tubal or uterine embryo transfers.

Other end points

i. the number of gestational sacs as diagnosed with ultrasound scans at 6/7 weeks gestation. This will enable determination of the implantation rate (number of sacs/total number of embryos transfered)

ii. the ongoing pregnancy rate

iii. the early pregnancy loss rate

iv. the site of implantation (fundal,lower segment)

Registration and randomisation of patients

Randomisation of patients will be carried out by Dr. Andrew Phillips (Department of Clinical Epidemiology at the RFH) and sealed envelopes determining the sequence of the proposed treatments will be prepared for each patient entered into the study. The envelops will be numbered 1 to X and will be allocated to the patients when seen at the stimulation clinic accordingly. The patients will be seen in the clinic according to their order on the waiting list at the department.

An interim analysis of the results will be carried out after completion of 125 treatment cycles to determine any observed value of either treatment modality. The trial may have to be terminated if one modality appears to be far superior than the other (pregnancy rate/transfer $P<0.001$). The chi-squared test will be used for tests of significance. It may also be necessary to stratify the analysis by the number of previous attempts on either or both treatment modalities.

ZYGOTE INTRAFALLOPIAN TRANSFER (ZIFT) AND IN-VITRO FERTILISATION AND UTERINE EMBRYO TRANSFER (IVF-UET) INFORMATION AND CONSENT FORM

Until recently, in-vitro fertilisation and uterine embryo transfer (IVF-UET) was the main method of treatment for couples requiring advanced assisted reproduction therapy. The introduction of the gamete intra-Fallopian transfer (GIFT) operation in 1986 and the reported improvement in success rates associated with this procedure highlighted the very important role of the Fallopian tube in reproduction. The tube not only acts as a natural and physiological incubator for the eggs/sperm and embryo but in addition, as has been shown in animal studies, secrete important substances that coat the egg/embryo and provide the nourishment they require for early development. The GIFT procedure is unfortunately not suitable for all couples with open Fallopian tubes especially when fertilisation needs to be confirmed and IVF-UET must be performed.

There is increasing evidence from work in other centres that transferring fertilised eggs into the healthy Fallopian tube (ZIFT procedure) gives far better results than either transferring them to the uterine cavity (IVF-UET) or transferring the unfertilised eggs to the tube (GIFT). We would like to study this new technique in a scientific way by conducting a properly designed trial involving both types of treatment (IVF-UET versus ZIFT). Each couple will be offered two attempts of each type in a randomised sequence. The two procedures will be identical in all respects except the place where the embryos are placed. In the ZIFT procedure, the eggs will be collected under local anaesthetic and three embryos at most will be replaced into the Fallopian tube via the laparoscope under general anaesthetic.

We hope that your participation will help us to improve our knowledge on the role of the Fallopian tube and equally we hope to offer you a better chance of success. Please do not hesitate to ask any question if some aspects of this project remain unclear to you.

We ..... (Couple's name)
have thoroughly discussed the ZIFT/IVF-UET treatments with Mr. Nazar Amso and agree to participate in the proposed trial.

Signature (female partner) ..... Date .....

(male partner) ..... Date .....

Witnessed by (name and signature) ..... Date .....

OPERATION SHEET

Name : Hosp. no.

Date of operation : .....

Operation : IVF / GIFT / Combined

ZIFT / Other .....

Surgeon : .....

Anaesthetist : .....

Anaesthesia : .....

Time started operation : .....

Time finished operation : .....

METHOD OF OOCYTE RECOVERY : Laparoscopic / Abdominal U.S. /

Vaginal U.S. / Perurethral U.S. / Combined (specify)

LAPAROSCOPY NOTES :

Gas volume : CO2

Complications :

Closure : Clips / suture / None

ULTRASOUND PROCEDURE NOTES :

	No. of stabs	No. of follicles aspirated
Rt. Ovary :		
Lt. Ovary :		

LAPAROSCOPY DIAGRAM	ULTRASOUND DIAGRAM
	side : R L  draw ovarian position in relation to the uterus please

Laparoscopy findings of significance (use laparoscopy page if indicated)	U.S. findings of significance and complications

APPENDIX 2: DATA SHEET- I: EPIDEMIOLOGICAL AND CLINICAL DETAILS

/A Hospital num. [_ _ _ _ _ _]

/B This treatment cycle no [_]

/C Age at recruitment [_ _]

/D Duration of infertility at recruitment [_ _]

/DE Date first seen in Assisted Reproduction [_ _ _ _ _ _]

/E Previous pregnancies (0) none [_] yes {give no.}

/F Diagnosis (1) male (2) female (3) ♂+♀ (4) idiopathic

/G Female factors (1) none (2) treated endometriosis
(3) not treated endometriosis (4) other

/H Male factors (0) none (1) azoo (2) oligo (3) astheno
(4) terato (5) oligoastheno (6) oligoterato (7) asthenoterato
(8) antisperm antibodies only (9) antibodies + other factors

/I PCT [_ _] no. forwardly motile sperm (0, 01, 05, etc)

/J Intended treatment (0) ZIFT (1) Uterine

/K Date gonadotrophins started [_ _ _ _ _ _] eg.010689

/L Duration of analogue treatment in days [_ _]

/M Total days of gonadotrophin treatment [_ _]

/N Total number of gonadotrophin ampoules [_ _]

/O Cycle (0) abandoned (1) successful VEC (2) unsuccessful VEC

/OP Day of VEC +[_ _]

/P Total no. of follicles ≥15 mm on day preceeding hCG [_ _]

/Q = = = ≥18 mm = = = [_ _]

/R Total number of oocytes collected [_ _]

/S Total number of embryos replaced [_] e.g. {0-3}

/T Development stage of embryo (1) [_](0=not transferred)

/U = = = (2) [_](1=PN, 2=2 cells)

/V = = = (3) [_]

/W Transfer (0) none (1) tubal D-1 (2) tubal D-2 (3) uterine

/X Luteal support (0) none (1) hCG (2) P4

/Y Outcome (0) not preg, [_] no. of sacs, (5) biochem Preg

/Z Pregnancy outcome [_]

8= not applicable 1= first trimester M/C

2= second trimester M/C 3= del. or ongoing ≥ 20 wks

Name:

_____

_____

DATA

[99] not availabe

[98] not applicable

DATA

[9] not available

[8] not applicable

DATA SHEET- II: LABORATORY AND ENDOCRINE DATA

/A Hospital num. [_ _ _ _ _ _]

/B This treatment cycle no [_]

/C hCG - first egg retrieval in hours-minutes [_ _._ _]

/D No. of mature eggs [_ _] **Mature:**

/E No. intermediate [_ _] **Intermediate:**

/F No. immature [_ _] **Immature:**

/G No. inseminated (partner or donor sperm) [_ _]

/H = = = and fertilised [_ _]

/I No. inseminated with donor {diagnostic test} [_]

/J = = = and fertilised [_]

Name:

_____

_____

AA, AB, BB

CC, BC, CB

CD, DD, DE

[98] not applicable

MISSING DATA:

[9] / I J

[99] / D E F G H

[9999] / C

SEMEN PARAMETERS ON OPERATION DAY;

/JK semen source (1) Husband (2) Donor

/K volume [_] ml

/L density [_ _ _] X10^6/ml

/M motility % [_ _]

/N progression 1-4 [_]

/O abnormal forms % [_ _]

/P viscosity (1) normal (2) viscous

/Q agglutination (1) normal (2)slight (3)very

MISSING DATA

[0] JK, K, N, P, Q, T, U

[00] M, O

[000] L, R, S

SEMEN PARAMETERS AFTER WASH AND SWIM UP;

/R density [_ _._] X10^6/ml

/S motility % [_ _ _]

/T progression 1-4 [_]

/U survival at 24 hrs; (0) no record (1) yes (2) no

Endocrine parameters;

Day preceeding hCG; **[date:** / /]

E2 [_ _ _ _ _]; LH [_ _]; P [_ _]

Pre hCG blood sample; **[date** / /]

E2 [_ _ _ _ _]; LH [_ _]; P [_ _]

APPENDIX 3: Epidemiological and clinical details of all treatment cycles included in the study

/A	/B	/C	/D	/DE	/E	/F	/G	/H	/I	/J	/K	/L	/M	/N	/O	/OP	/P	/Q	/R	/S	/TUV	/W	/X	/Y	/Z
477177	1	31	04	091089	0	1	1	2	02	0	100190	62	11	24	1	14	07	04	06	1	200	2	2	0	8
477177	2	31	04	091089	0	1	1	2	02	1	031090	36	10	19	1	13	08	02	06	0	888	8	8	8	8
328189	1	26	05	240889	0	2	4	0	98	0	111089	30	10	19	1	13	15	04	27	3	444	2	2	0	8
470521	1	32	07	050689	0	3	2	8	99	1	081189	26	10	20	1	13	09	00	10	3	664	3	2	0	8
608478	1	32	03	031089	0	4	1	0	20	1	100190	29	11	21	1	13	06	02	11	0	888	8	8	8	8
476930	1	25	03	280989	0	1	1	8	20	0	120990	29	10	20	1	13	05	01	07	3	111	1	1	1	1
476930	2	25	03	280989	0	1	1	8	20	0	270391	33	11	22	1	13	05	01	05	3	111	1	1	0	8
572417	1	30	04	230890	0	4	1	0	04	1	020190	65	08	16	1	10	06	04	04	3	344	3	1	0	8
572417	2	30	04	230890	0	4	1	0	04	0	260691	37	11	22	1	13	09	04	05	3	111	1	2	1	3
573021	1	30	05	030590	1	1	1	7	03	0	010890	34	11	21	1	14	10	04	05	0	888	8	8	8	8
372684	1	32	05	310889	0	2	3	0	99	0	151189	28	11	22	1	14	13	03	22	8	888	8	8	8	8
478986	1	33	06	280989	0	4	1	0	98	0	030190	36	10	19	1	13	05	04	06	0	888	8	8	8	8
478986	2	33	06	280989	0	4	1	0	98	0	280390	35	11	22	1	14	04	03	04	1	400	2	1	0	8
478986	3	33	06	280989	0	4	1	0	98	0	180790	19	05	20	0	98	98	98	98	8	888	8	8	8	8
478986	4	33	06	280989	0	4	1	0	98	0	211190	68	11	44	1	13	04	02	08	3	111	1	1	0	8
478986	5	33	06	280989	0	4	1	0	98	1	120691	74	11	42	1	13	05	04	09	3	444	3	2	0	8
479989	1	32	04	251089	1	4	1	0	05	1	280290	40	10	20	1	13	11	03	12	0	888	8	8	8	8
479989	2	32	04	251089	1	4	1	0	05	1	130690	36	10	20	1	13	06	01	15	3	432	3	2	0	8
479989	3	32	04	251089	1	4	1	0	05	1	270391	32	11	21	1	13	12	03	19	8	888	8	8	8	8
476581	1	29	07	260989	0	4	1	0	00	1	110490	35	13	38	1	15	09	05	03	0	888	8	8	8	8
476581	2	29	07	260989	0	4	1	0	00	1	250790	27	11	22	1	14	10	06	08	3	233	3	1	0	8
476581	3	29	07	260989	0	4	1	0	00	0	211190	41	10	20	1	13	05	02	05	3	111	1	1	1	3
476573	1	31	03	220290	0	1	1	4	05	1	020590	43	15	35	1	16	03	01	03	0	888	8	8	8	8
476573	2	31	03	220290	0	1	1	4	05	1	150890	31	13	33	1	15	13	05	12	0	888	8	8	8	8
570935	1	29	03	150390	0	1	1	2	02	0	060690	31	09	25	1	13	06	04	06	3	333	2	1	0	8
570935	2	29	03	150390	0	1	1	2	02	1	020191	66	12	25	1	15	07	02	06	2	260	3	1	0	8
570935	3	29	03	150390	0	1	1	2	02	1	220591	66	11	22	1	13	05	02	05	0	888	8	8	8	8
478247	1	33	03	221189	0	1	1	4	06	0	090590	41	11	21	0	98	98	98	98	8	888	8	8	8	8
478247	2	33	03	221189	0	1	1	4	06	0	180790	36	11	34	1	13	06	03	01	0	888	8	8	8	8
478247	3	33	03	221189	0	1	1	4	06	0	090191	57	10	35	1	13	08	02	13	3	111	1	2	0	8
574013	1	34	05	140690	2	4	1	0	01	1	220890	46	09	43	1	16	02	00	08	0	888	8	8	8	8
574013	2	34	05	140690	2	4	1	0	01	1	060291	45	12	44	1	15	11	06	11	3	444	3	2	1	3
574147	1	35	02	020890	0	1	1	4	05	1	241090	44	07	14	0	98	98	98	98	8	888	8	8	8	8
574147	2	35	02	020890	0	1	1	4	05	1	999999	51	98	98	0	98	98	98	98	8	888	8	8	8	8
577717	1	34	04	080491	0	1	1	4	06	0	210891	56	08	14	0	98	98	98	98	8	888	8	8	8	8
478977	1	32	04	080988	2	4	1	0	03	1	070290	26	10	20	1	13	05	01	06	0	888	8	8	8	8
478977	2	32	04	080988	2	4	1	0	03	1	130690	37	10	30	0	98	98	98	98	8	888	8	8	8	8
571714	1	32	04	021189	0	1	1	4	00	0	140290	33	11	30	1	14	05	02	04	1	400	2	2	0	8
571714	2	32	04	021189	0	1	1	4	00	0	241090	29	10	30	1	13	11	03	13	0	888	8	8	8	8
575340	1	35	02	210690	0	1	1	4	05	0	101090	32	09	24	0	98	98	98	98	8	888	8	8	8	8
575340	2	35	02	210690	0	1	1	4	05	0	270291	33	11	74	1	13	03	02	03	1	400	3	1	0	8
575340	3	35	02	210690	0	1	1	4	05	0	100791	33	10	76	1	13	06	04	03	1	200	2	2	0	8
573455	1	35	04	041090	0	1	1	6	99	1	230191	33	12	32	0	98	98	98	98	8	888	8	8	8	8
572561	1	27	02	120490	0	1	1	4	07	1	270690	43	10	19	1	13	11	06	17	3	644	3	2	0	8
572561	2	27	02	120490	0	1	1	4	07	0	090191	47	10	20	1	13	13	08	11	3	111	1	2	1	1
477007	1	32	03	281189	0	3	3	6	99	1	210390	38	10	20	1	13	09	03	07	3	444	3	1	2	3
570381	1	31	05	141289	0	4	1	0	02	0	210390	29	10	20	1	13	14	02	16	3	111	1	2	0	8
570381	2	31	05	141289	0	4	1	0	02	1	250790	36	10	20	1	13	07	01	11	3	422	3	2	0	8
570381	3	31	05	141289	0	4	1	0	02	1	120691	41	12	30	1	14	99	99	14	0	888	8	8	8	8
575168	1	34	02	311090	1	1	1	4	06	1	091091	50	10	20	1	13	10	04	01	1	400	3	2	1	3
572164	1	36	02	050490	1	4	1	0	02	0	110790	42	12	30	1	13	07	04	05	2	220	2	1	1	3
573454	1	36	02	111090	0	1	1	4	05	0	230191	38	11	22	1	13	05	02	03	2	110	1	1	0	8
573454	2	36	02	111090	0	1	1	4	05	1	120691	45	12	34	1	15	05	04	10	3	444	3	2	0	8
573454	3	36	02	111090	0	1	1	4	05	0	231091	32	12	40	1	14	09	02	15	3	444	2	2	0	8
420501	1	35	05	120790	2	3	2	4	04	0	300191	47	13	32	0	98	98	98	98	8	888	8	8	8	8
420501	2	35	05	120790	2	3	2	4	04	0	120691	36	11	46	1	13	05	01	08	3	111	1	1	0	8

/A	/B	/C	/D	/DE	/E	/F	/G	/H	/I	/J	/K	/L	/M	/N	/O	/OP	/P	/Q	/R	/S	/TUV	/W	/X	/Y	/Z
372072	1	33	05	250489	1	1	1	1	98	0	230889	29	10	19	1	13	22	13	17	0	888	8	8	8	8
372072	2	33	05	250489	1	1	1	1	98	0	280290	31	10	18	1	13	19	10	09	0	888	8	8	8	8
376071	1	29	07	170889	0	3	2	1	98	1	041089	26	12	35	1	15	03	01	05	0	888	8	8	8	8
376071	2	29	07	170889	0	3	2	1	98	1	280290	34	11	44	1	14	04	02	06	0	888	8	8	8	8
474463	1	29	05	060290	0	1	1	7	00	1	020590	38	11	21	1	13	03	00	04	0	888	8	8	8	8
474463	2	29	05	060290	0	1	1	7	00	1	170491	37	07	22	1	10	04	04	05	0	888	8	8	8	8
574269	1	32	03	100590	0	1	1	6	04	1	010890	41	11	30	1	14	05	02	03	0	888	8	8	8	8
325304	1	30	04	120490	1	3	2	4	99	1	311090	43	12	32	1	15	08	02	18	3	442	3	2	0	8
377046	1	37	12	021189	0	1	1	5	98	0	140290	39	10	20	1	13	99	99	07	3	442	2	2	0	8
377046	2	37	12	021189	0	1	1	5	98	1	120990	30	11	26	1	14	03	01	06	3	234	3	1	0	8
377046	3	37	12	021189	0	1	1	5	98	1	100491	45	13	38	1	15	06	03	06	0	888	8	8	8	8
377046	4	37	12	021189	0	1	1	5	98	1	091091	47	11	44	1	13	05	01	12	3	422	3	2	0	8
475363	1	32	06	220889	3	2	2	0	99	0	100190	74	13	38	0	98	98	98	98	8	888	8	8	8	8
475363	2	32	06	220889	3	2	2	0	99	0	270690	38	11	56	1	14	06	02	07	3	111	1	1	0	8
475363	3	32	06	220889	3	2	2	0	99	0	141190	44	11	54	1	14	06	02	06	0	888	8	8	8	8
572195	1	30	04	150390	0	4	1	0	00	1	130690	44	11	22	1	14	10	03	11	2	230	3	2	1	3
470993	1	31	05	051089	0	3	2	4	99	1	100190	96	10	19	1	13	12	08	10	0	888	8	8	8	8
470993	2	31	05	051089	0	3	2	4	99	1	010890	99	10	20	1	13	07	06	11	0	888	8	8	8	8
572710	1	30	03	220390	0	4	1	0	06	1	230590	29	10	21	1	13	03	01	10	3	222	3	2	0	8
572710	2	30	03	220390	0	4	1	0	06	0	220890	25	11	24	1	13	02	02	06	3	111	1	1	0	8
572710	3	30	03	220390	0	4	1	0	06	0	080591	54	10	20	0	98	98	98	98	8	888	8	8	8	8
573477	1	33	06	010290	0	1	1	4	20	0	300590	27	09	18	0	98	98	98	98	8	888	8	8	8	8
573477	2	33	06	010290	0	1	1	4	20	0	050990	33	10	30	1	13	05	03	03	0	888	8	8	8	8
479857	1	34	04	090890	0	3	2	4	99	1	141190	33	10	20	1	13	05	01	17	3	422	3	2	0	8
479857	2	34	04	090890	0	3	2	4	99	1	030491	46	11	22	1	13	07	01	04	3	442	3	2	0	8
479857	3	34	04	090890	0	3	2	4	99	0	210891	37	11	26	1	14	05	02	09	3	111	1	2	0	8
577067	1	33	03	111090	0	4	1	0	02	1	060291	53	11	22	1	14	04	02	03	3	444	3	1	0	8
577067	2	33	03	111090	0	4	1	0	02	0	020791	36	10	29	1	13	10	03	19	0	888	8	8	8	8
353565	1	42	02	200391	1	4	1	0	99	1	100791	45	10	40	0	98	98	98	98	8	888	8	8	8	8
473290	1	30	04	140989	0	2	2	0	03	0	151189	27	11	35	0	98	98	98	98	8	888	8	8	8	8
473290	2	30	04	140989	0	2	2	0	03	0	140290	25	10	70	0	98	98	98	98	8	888	8	8	8	8
473290	3	30	04	140989	0	2	2	0	03	0	060990	98	11	58	0	98	98	98	98	8	888	8	8	8	8
478374	1	31	05	070989	1	4	1	0	03	1	291189	33	10	19	1	13	08	04	08	3	999	3	2	0	8
478374	2	31	05	070989	1	4	1	0	03	1	110490	36	10	19	1	13	09	03	07	1	200	3	1	0	8
478374	3	31	05	070989	1	4	1	0	03	0	050990	41	11	22	1	14	10	04	09	3	111	1	2	2	3
477872	1	32	02	221189	0	4	1	0	20	0	110490	36	10	32	0	98	98	98	98	8	888	8	8	8	8
477006	1	33	07	121289	0	3	2	7	99	0	110490	54	11	22	1	14	08	03	12	3	111	1	2	0	8
477006	2	33	07	121289	0	3	2	7	99	1	230191	38	10	20	1	13	09	06	11	3	232	3	2	0	8
477006	3	33	07	121289	0	3	2	7	99	0	250791	62	11	22	1	13	05	00	08	0	888	8	8	8	8
047357	1	31	02	110789	0	1	1	9	99	1	111089	32	11	23	1	14	05	01	06	3	444	3	2	2	3
575835	1	35	05	260490	0	4	1	0	99	0	110790	56	09	22	0	98	98	98	98	8	888	8	8	8	8
575835	2	35	05	260490	0	4	1	0	99	0	101090	37	11	43	1	14	06	04	09	3	111	1	2	0	8
575835	3	35	05	260490	0	4	1	0	99	1	120691	46	11	52	1	14	04	01	04	3	722	3	2	0	8
479991	1	35	05	180190	2	1	1	4	02	0	090590	31	09	22	1	13	03	03	04	3	111	1	1	0	8
479991	2	35	05	180190	2	1	1	4	02	0	260990	38	10	20	1	13	07	03	05	3	111	1	1	0	8
479991	3	35	05	180190	2	1	1	4	02	1	100491	42	10	39	0	98	98	98	98	8	888	8	8	8	8
479991	4	35	05	180190	2	1	1	4	02	1	310791	27	12	60	1	14	03	01	06	3	142	3	1	0	8
475569	1	33	04	050989	0	3	4	4	11	8	989898	98	98	98	0	98	98	98	98	8	888	8	8	1	3
478762	1	27	03	121289	0	4	1	0	99	1	090590	43	10	20	1	13	06	04	10	0	888	8	8	8	8
478762	2	27	03	121289	0	4	1	0	99	1	031090	33	10	20	1	13	09	02	08	0	888	8	8	8	8
009127	1	34	02	170590	0	4	1	0	03	1	101090	36	10	20	1	13	10	02	11	3	332	3	2	0	8
009127	2	34	02	170590	0	4	1	0	03	1	310791	40	11	21	1	13	03	02	02	2	420	3	1	0	8
376487	1	40	04	270988	1	2	4	0	06	0	101090	43	10	38	1	13	03	02	09	3	111	1	2	0	8
370124	1	34	09	220290	0	2	3	0	99	0	150890	38	12	46	0	98	98	98	98	8	888	8	8	8	8
370124	2	34	09	220290	0	2	3	0	99	0	020191	66	12	57	0	98	98	98	98	8	888	8	8	8	8
571707	1	31	08	021189	1	4	1	0	07	0	210390	51	11	22	1	14	04	02	06	3	111	1	1	0	8
571707	2	31	08	021189	1	4	1	0	07	1	071190	38	13	32	1	15	05	04	06	2	220	3	1	0	8

/A	/B	/C	/D	/DE	/E	/F	/G	/H	/I	/J	/K	/L	/M	/N	/O	/OP	/P	/Q	/R	/S	/T	/U	/V	/W	/X	/Y	/Z
479702	1	31	08	230890	1	3	2	4	07	0	020191	71	10	30	1	13	05	03	09	3	111	1	1	0	8		
479702	2	31	08	230890	1	3	2	4	07	1	080591	54	10	30	1	13	14	06	12	3	444	3	2	1	3		
475941	1	31	05	130790	2	3	3	4	07	0	190990	26	10	20	1	13	06	01	08	3	111	1	1	0	8		
475941	2	31	05	130790	2	3	3	4	07	1	250591	57	10	20	1	13	08	05	06	3	432	3	2	0	8		
574277	1	29	03	130990	0	1	1	4	01	0	130391	49	13	26	1	15	11	07	05	0	888	8	8	8	8		
574277	2	29	03	130990	0	1	1	4	01	0	250691	44	12	36	1	14	07	01	08	1	400	3	2	0	8		
574311	1	32	04	181090	0	1	1	7	00	1	130291	40	12	29	1	15	06	03	03	0	888	8	8	8	8		
574311	2	32	04	181090	0	1	1	7	00	1	170791	58	12	36	1	14	99	99	07	0	888	8	8	8	8		
477310	1	24	03	210690	0	2	2	0	99	0	050990	34	11	26	1	14	02	02	06	3	442	2	1	2	2		
477310	2	24	03	210690	1	2	2	0	99	0	050691	44	11	44	1	14	05	01	07	3	111	1	1	1	3		
278210	1	28	03	010390	1	1	1	4	02	0	160590	37	11	22	1	14	14	09	12	0	888	8	8	8	8		
278210	2	28	03	010390	1	1	1	4	02	0	220890	37	11	22	1	14	07	01	15	0	888	8	8	8	8		
574081	1	38	12	240590	1	1	1	1	98	1	290890	39	11	21	1	14	09	02	18	0	888	8	8	8	8		
574081	2	38	12	240590	1	1	1	1	98	1	160191	38	10	20	1	13	09	02	09	3	442	3	1	0	8		
574081	3	38	12	240590	1	1	1	1	98	0	290591	54	10	20	1	12	05	02	16	3	111	1	2	0	8		
474462	1	32	04	220390	0	1	1	4	99	0	270690	36	09	18	0	98	98	98	98	8	888	8	8	8	8		
474462	2	32	04	220390	0	1	1	4	99	0	989898	19	98	98	0	98	98	98	98	8	888	8	8	1	3		
473658	1	31	05	160890	1	1	1	6	02	1	281190	49	12	34	1	15	05	04	04	2	460	3	2	0	8		
473658	2	31	05	160890	1	1	1	6	02	1	270391	33	13	52	1	15	04	02	04	0	888	8	8	8	8		
575032	1	32	04	280690	0	2	2	0	04	1	050590	31	10	22	1	13	05	02	04	2	320	3	1	0	8		
575032	2	32	04	280690	0	2	2	0	04	1	230191	38	10	20	1	13	07	04	09	3	443	3	1	0	8		
575032	3	32	04	280690	0	2	2	0	04	0	120691	38	11	22	1	13	07	04	06	3	111	1	1	0	8		
575032	4	32	04	280690	0	2	2	0	04	0	301091	40	10	24	0	98	98	98	98	8	888	8	8	8	8		
475836	1	31	04	190490	1	1	1	1	98	1	010890	68	10	19	1	13	13	07	15	3	222	3	2	0	8		
475836	2	31	04	190490	1	1	1	1	98	1	020191	65	11	22	1	14	08	02	09	0	888	8	8	8	8		
475836	3	31	04	190490	1	1	1	1	98	1	070891	94	11	22	1	13	06	01	06	0	888	8	8	8	8		
474546	1	27	05	081188	0	2	2	0	98	0	090889	25	10	19	1	13	08	04	19	3	112	2	2	0	8		
474546	2	27	05	081188	0	2	2	0	98	1	240190	26	10	19	1	13	10	07	09	3	444	3	2	0	8		
474546	3	27	05	081188	0	2	2	0	98	0	200291	50	10	20	1	13	08	05	09	3	111	1	1	0	8		
477578	1	33	03	250190	0	1	1	4	02	1	270690	54	10	20	1	13	05	01	06	3	443	3	1	0	8		
477578	2	33	03	250190	0	1	1	4	02	0	171090	40	10	18	1	13	15	02	08	3	223	3	2	1	3		
479926	1	29	03	090890	0	2	4	0	99	1	281190	43	10	20	1	13	10	02	05	3	443	3	2	2	3		
574301	1	36	02	070690	1	1	1	6	98	1	260990	38	11	30	1	14	03	01	06	0	888	8	8	8	8		
476211	1	33	07	240889	0	4	1	0	03	0	200989	20	12	34	1	14	04	00	26	3	111	1	2	0	8		
476211	2	33	07	240889	0	4	1	0	03	1	070290	26	10	20	1	13	12	02	08	2	240	3	1	2	3		
570757	1	38	06	010290	0	1	1	4	04	1	020590	43	10	20	0	98	98	98	98	8	888	8	8	8	8		
576942	1	31	04	220291	0	1	1	4	03	0	190691	42	12	34	1	15	09	01	19	0	888	8	8	8	8		
576942	2	31	04	220291	0	1	1	4	03	0	161091	34	11	33	1	13	04	01	25	3	444	2	2	0	8		
377863	1	31	05	040889	0	1	1	2	05	0	011189	30	11	22	1	14	06	02	11	0	888	8	8	8	8		
377863	2	31	05	040889	0	1	1	2	05	0	180490	43	11	22	1	13	08	03	11	0	888	8	8	8	8		
577288	1	31	03	180789	0	4	1	0	04	1	060989	33	10	46	1	13	05	01	04	3	444	3	2	0	8		
577288	2	31	03	180789	0	4	1	0	04	1	210390	25	11	54	1	14	06	04	03	2	220	3	1	0	8		
577288	3	31	03	180789	0	4	1	0	04	0	250790	28	09	54	0	98	98	98	98	8	888	8	8	8	8		
377188	1	31	09	310889	4	1	1	4	04	1	100190	30	11	24	1	14	03	00	03	0	888	8	8	8	8		
377188	2	31	09	310889	4	1	1	4	04	1	040790	39	10	32	2	13	06	04	00	8	888	8	8	8	8		
377188	3	31	09	310889	4	1	1	4	04	1	120990	39	11	44	1	13	06	01	01	1	400	3	1	0	8		
377188	4	31	09	310889	4	1	1	4	04	1	180691	37	12	62	1	14	08	04	01	0	888	8	8	8	8		
220276	1	31	04	010390	0	1	1	4	00	1	230590	37	09	22	0	98	98	98	98	8	888	8	8	8	8		
220276	2	31	04	010390	0	1	1	4	00	1	211190	50	11	36	1	13	03	02	06	2	620	3	1	0	8		
575917	1	36	17	050490	2	4	1	0	99	0	200690	32	13	32	1	15	04	01	03	0	888	8	8	8	8		
575917	2	36	17	050490	2	4	1	0	99	0	290890	32	09	36	1	13	07	03	05	8	888	8	8	8	8		
378488	1	36	07	240889	1	2	2	0	04	1	251089	47	10	20	1	13	16	05	24	0	888	8	8	8	8		
571826	1	34	10	310889	1	3	3	2	99	1	151189	26	10	32	1	13	03	01	03	0	888	8	8	8	8		
571826	2	34	10	310889	1	3	3	2	99	1	160590	37	11	44	1	14	03	01	05	3	248	3	1	0	8		
571826	3	34	10	310889	1	3	3	2	99	0	060291	34	10	40	1	13	05	00	08	0	888	8	8	8	8		
476133	1	27	06	030590	0	1	1	3	05	1	110790	34	11	22	1	14	08	02	19	3	233	3	2	0	8		
476133	2	27	06	030590	0	1	1	3	05	1	020191	71	10	20	1	13	03	00	10	3	444	3	1	0	8		

/A	/B	/C	/D	/DE	/E	/F	/G	/H	/I	/J	/K	/L	/M	/N	/O	/OP	/P	/Q	/R	/S	/TUV	/W	/X	/Y	/Z
574064	1	29	03	260790	0	2	4	0	15	1	191090	36	10	20	1	13	12	05	07	2	220	3	1	0	8
574064	2	29	03	260790	0	2	4	0	15	0	170491	44	11	20	1	14	04	00	04	3	111	1	2	3	3
574137	1	29	05	070990	0	1	1	5	99	1	090191	61	11	22	1	14	05	02	03	0	888	8	8	8	8
574137	2	29	05	070990	0	1	1	5	99	0	250691	37	10	24	1	13	06	03	08	0	888	8	8	8	8
571627	1	31	04	281189	0	3	3	4	02	1	180490	45	13	26	1	15	06	05	06	3	222	3	1	0	8
571627	2	31	04	281189	0	3	3	4	02	0	311090	52	12	24	1	15	02	01	02	0	888	8	8	8	8
571627	3	31	04	281189	0	3	3	4	02	0	170491	47	11	44	1	13	04	01	07	0	888	8	8	8	8
570933	1	27	07	060990	0	3	2	4	06	0	130291	66	10	20	1	13	03	01	04	3	111	1	1	3	2
570933	2	27	07	060990	1	3	2	4	06	0	131191	38	11	41	1	13	03	00	03	0	888	8	8	8	8
572543	1	34	06	221189	0	4	1	0	04	0	040490	45	12	24	1	14	06	05	08	3	111	1	2	0	8
572543	2	34	06	221189	0	4	1	0	04	0	311090	36	11	22	1	13	02	00	06	2	220	2	1	0	8
572543	3	34	06	221189	0	4	1	0	04	1	290591	52	10	30	1	13	06	03	08	3	443	3	2	0	8
572543	4	34	06	221189	0	4	1	0	04	1	161091	47	10	40	1	13	08	03	10	3	442	3	2	0	8
478422	1	32	04	121089	0	4	1	0	98	1	280290	37	11	22	1	14	10	05	12	0	888	8	8	8	8
478422	2	32	04	121089	0	4	1	0	98	1	110790	31	10	20	1	13	04	02	03	1	400	3	1	0	8
473497	1	32	05	170889	0	2	2	0	00	0	291189	41	12	24	1	14	99	99	09	3	111	1	2	0	8
473497	2	32	05	170889	0	2	2	0	00	1	280390	30	11	22	1	14	08	04	10	3	244	3	2	0	8
473497	3	32	05	170889	0	2	2	0	00	0	031090	35	12	34	0	98	98	98	98	8	888	8	8	8	8
473497	4	32	05	170889	0	2	2	0	00	0	130391	42	04	20	0	98	98	98	98	8	888	8	8	8	8
474395	1	25	04	180190	1	4	1	0	00	0	090590	31	10	20	1	13	06	01	09	3	442	2	1	1	1
474395	2	25	04	180190	2	4	1	0	00	1	200291	26	10	20	1	13	09	02	07	3	644	3	2	0	8
474395	3	25	04	180190	2	4	1	0	00	0	170791	36	11	19	1	13	11	07	14	3	111	1	2	0	8
474355	1	32	05	070989	1	1	1	5	98	1	100190	34	10	14	1	10	14	06	09	3	444	3	2	0	8
474355	2	32	05	070989	1	1	1	5	98	0	160590	43	11	20	1	14	19	07	09	0	888	8	8	8	8
474355	3	32	05	070989	1	1	1	5	98	0	080890	26	10	26	1	13	05	01	13	3	111	1	2	1	3
573227	1	28	09	110789	0	4	1	0	04	1	251089	23	07	14	0	98	98	98	98	8	888	8	8	8	8
572005	1	33	04	151189	0	4	1	0	02	1	210390	30	11	22	1	14	10	06	11	3	224	3	2	0	8
572005	2	33	04	151189	0	4	1	0	02	1	110790	29	12	26	1	15	08	03	06	0	888	8	8	8	8
572005	3	33	04	151189	0	4	1	0	02	1	311090	43	11	25	1	13	03	00	08	2	240	3	2	0	8
572005	4	33	04	151189	0	4	1	0	02	0	100491	43	10	30	1	13	09	01	10	3	111	1	2	0	8
572005	5	33	04	151189	0	4	1	0	02	0	260791	36	11	35	1	13	03	00	09	0	888	8	8	8	8
478763	1	31	03	161189	1	4	1	0	07	0	210290	27	11	22	1	14	07	04	06	2	220	2	2	0	8
478763	2	31	03	161189	1	4	1	0	07	0	270690	33	10	20	1	13	06	03	05	1	400	2	1	0	8
478763	3	31	03	161189	1	4	1	0	07	1	071190	35	12	28	1	15	05	01	08	3	344	3	1	1	1
478763	4	31	03	161189	1	4	1	0	07	1	190691	45	10	30	1	13	07	04	06	3	443	3	1	0	8
378481	1	37	06	130989	0	1	1	4	10	1	081189	38	14	56	0	98	98	98	98	8	888	8	8	8	8
378481	2	37	06	130989	0	1	1	4	10	1	250490	40	13	78	1	15	05	02	08	0	888	8	8	8	8
378481	3	37	06	130989	0	1	1	4	10	1	080890	38	12	72	1	15	03	00	06	0	888	8	8	8	8
475169	1	32	04	180190	0	2	2	0	00	0	160590	51	12	24	1	14	06	03	07	1	400	2	1	0	8
475169	2	32	04	180190	0	2	2	0	00	1	220890	25	11	26	1	14	03	02	05	0	888	8	8	8	8
475169	3	32	04	180190	0	2	2	0	00	1	100491	38	13	45	1	15	02	01	06	2	420	3	1	0	8
475942	1	35	04	220390	0	3	3	4	06	1	040790	63	09	18	0	98	98	98	98	8	888	8	8	8	8
475942	2	35	04	220390	0	3	4	4	06	1	020791	33	10	38	1	13	05	04	01	0	888	8	8	8	8
477734	1	38	04	241089	0	4	1	0	00	0	310190	31	11	28	1	14	04	01	08	3	234	2	2	1	1
477734	2	38	04	241089	1	4	1	0	00	1	250790	27	12	27	1	14	04	02	08	2	230	3	1	0	8
477734	3	38	04	241089	1	4	1	0	00	0	211190	48	12	25	1	14	05	02	12	1	300	2	2	0	8
477734	4	38	04	241089	1	4	1	0	00	1	010591	56	13	30	0	98	98	98	98	8	888	8	8	8	8
477734	5	38	04	241089	1	4	1	0	00	1	280891	36	11	38	1	13	07	02	18	0	888	8	8	8	8
572182	1	31	02	150390	0	1	1	4	04	1	110790	41	11	22	1	14	05	02	06	2	240	3	1	0	8
572182	2	31	02	150390	0	1	1	4	04	0	130291	32	10	24	0	98	98	98	98	8	888	8	8	8	8
572182	3	31	02	150390	0	1	1	4	04	0	061191	36	10	42	1	13	04	02	08	3	442	2	2	0	8
574937	1	35	08	050490	0	3	3	4	99	0	180790	29	10	19	1	13	10	05	10	3	111	1	2	1	3
090019	1	29	05	191089	0	4	1	0	07	0	170190	51	09	18	0	98	98	98	98	8	888	8	8	8	8
090019	2	29	05	191089	0	4	1	0	07	0	210391	31	10	34	1	13	05	02	03	2	340	2	1	0	8
576357	1	29	03	260790	1	4	1	0	06	0	071190	45	11	22	1	13	03	01	08	3	111	1	1	1	3
378821	1	35	03	240790	0	3	2	4	06	0	281190	52	10	20	1	13	07	04	09	3	223	2	1	0	8
378821	2	35	03	240790	0	3	2	4	06	0	190491	69	11	22	1	14	01	01	01	1	400	3	1	1	3

APPENDIX 4: Laboratory details of all treatment cycles included in the study

/A	/B	/C	/D	/E	/F	/G	/H	/I	/J	/JK/K	/L	/M	/N	/O	/P/Q	/R	/S	/T/U
477177	1	9999	02	02	02	06	01	0	0	1 0	030	50	3	50	1 1	100	100	4 0
477177	2	3575	00	06	00	06	00	0	0	1 6	005	70	3	45	1 1	060	080	4 1
328189	1	3500	03	24	00	10	06	0	0	1 2	060	65	3	45	2 1	400	090	3 0
470521	1	3500	00	10	00	08	07	2	0	1 0	150	60	3	60	1 1	030	090	3 1
608478	1	9999	01	07	03	07	00	3	3	1 3	100	50	3	55	1 1	050	095	3 0
476930	1	3525	01	06	00	07	04	0	0	1 2	060	70	4	40	2 1	300	090	4 1
476930	2	3692	00	05	00	05	05	0	0	1 0	200	80	4	30	1 2	200	100	4 1
572417	1	3667	03	01	00	04	04	0	0	1 3	200	80	4	30	2 1	400	100	4 1
572417	2	3700	00	03	02	05	04	0	0	1 5	110	50	3	60	1 1	250	100	4 1
573021	1	3509	04	01	00	05	00	0	0	1 4	070	80	3	70	1 1	070	085	4 1
372684	1	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
478986	1	3450	06	00	00	06	00	0	0	1 2	030	50	3	60	1 1	005	030	2 0
478986	2	3550	02	02	00	04	01	0	0	1 2	150	80	4	30	1 1	300	080	4 1
478986	3	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
478986	4	3592	03	04	01	07	06	0	0	1 2	070	15	3	55	1 1	200	100	4 1
478986	5	3800	02	05	02	09	05	0	0	1 2	150	80	4	25	1 1	250	100	4 1
479989	1	3525	08	04	00	07	04	2	0	1 0	250	80	4	10	1 1	300	100	4 0
479989	2	3517	00	15	00	15	06	0	0	1 9	087	80	4	50	1 1	050	100	4 1
479989	3	3609	09	06	04	10	00	0	0	1 6	150	70	4	60	1 1	250	095	4 2
476581	1	3550	02	01	00	03	00	0	0	1 4	070	60	3	60	2 1	500	050	3 1
476581	2	3633	00	06	02	07	06	1	0	1 3	065	70	3	25	1 1	200	080	4 1
476581	3	3600	00	04	01	05	03	0	0	1 1	080	70	4	50	1 1	150	090	4 1
476573	1	3450	02	01	00	03	00	0	0	1 6	065	70	3	35	1 1	030	030	4 0
476573	2	3492	06	06	00	08	00	2	2	1 5	030	70	4	60	1 1	080	090	4 1
570935	1	3500	03	03	00	06	04	0	0	1 8	045	70	3	30	1 1	150	090	4 1
570935	2	3675	02	03	01	06	02	0	0	1 4	060	65	4	50	1 1	100	055	4 1
570935	3	3667	00	05	00	05	00	0	0	1 7	043	55	3	50	1 1	040	070	3 1
478247	1	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
478247	2	3642	01	00	00	01	00	0	0	1 1	070	60	4	50	1 1	200	090	4 1
478247	3	9999	03	09	01	08	07	2	2	1 2	250	65	3	55	1 1	050	100	4 1
574013	1	3500	06	01	01	08	00	0	0	1 3	060	65	4	60	1 1	200	090	4 1
574013	2	9999	00	10	01	07	05	1	0	1 0	200	70	4	60	1 1	400	100	4 1
574147	1	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
574147	2	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
577717	1	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
478977	1	3475	00	06	00	06	00	0	0	1 7	200	70	3	30	1 1	250	080	3 0
478977	2	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
571714	1	3450	01	03	00	04	01	0	0	1 3	060	50	2	60	1 1	100	070	3 0
571714	2	3592	12	01	00	08	00	0	0	1 2	080	70	3	50	1 1	200	090	4 1
575340	1	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
575340	2	3600	00	02	01	03	01	0	0	1 2	090	60	4	30	1 1	200	095	4 1
575340	3	3675	02	00	01	03	01	0	0	1 2	007	60	3	50	1 1	010	000	4 2
573455	1	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
572561	1	3500	14	02	01	09	05	2	2	1 6	300	70	3	45	1 1	300	100	4 1
572561	2	3683	01	09	01	07	04	0	0	1 5	063	50	3	60	1 1	300	100	4 1
477007	1	3509	04	01	02	07	06	0	0	1 3	025	50	3	60	1 1	010	100	4 1
570381	1	3550	07	06	03	08	04	2	1	1 5	031	50	2	70	2 2	200	080	4 1
570381	2	3683	05	04	02	08	06	0	0	1 5	050	40	3	35	1 1	040	090	4 1
570381	3	3642	00	06	08	10	03	0	0	1 4	020	40	3	00	2 1	050	100	4 1
575168	1	9999	00	01	00	01	01	0	0	1 0	080	60	3	45	1 1	070	080	3 0
572164	1	3467	01	04	00	05	02	0	0	1 6	060	65	4	30	1 1	040	090	4 1
573454	1	3633	00	03	00	03	03	0	0	1 2	200	75	4	55	1 2	400	100	4 0
573454	2	3567	05	05	00	08	07	0	0	1 5	200	70	3	35	1 1	250	090	4 1
573454	3	3625	00	15	00	10	08	0	0	1 4	110	70	4	55	1 1	200	070	4 0
420501	1	9898	98	98	98	98	98	8	8	8 8	098	98	8	98	8 8	098	098	8 8
420501	2	3725	00	07	01	08	06	0	0	1 0	110	70	3	50	1 1	200	100	4 1

/A	/B	/C	/D	/E	/F	/G	/H	/I	/J	/JK/K	/L	/M	/N	/O	/P/Q	/R	/S	/T/U
372072	1	3500	03	14	00	11	00	0	0	2	1	060	50	3	60	1	1	030	070	3	0
372072	2	3500	02	06	01	07	00	0	0	2	0	065	70	3	30	1	1	020	100	4	0
376071	1	3475	02	03	00	05	00	0	0	2	0	050	25	2	00	1	1	030	080	2	1
376071	2	3500	04	02	00	06	00	0	0	2	0	070	80	3	20	1	1	300	090	4	0
474463	1	9999	03	01	00	04	00	0	0	1	1	007	10	2	80	1	1	010	010	3	2
474463	2	3625	00	04	01	05	00	0	0	1	1	050	30	3	65	1	1	080	080	4	0
574269	1	3525	02	01	00	03	00	0	0	1	6	005	40	3	70	1	1	010	090	4	1
325304	1	3559	03	15	00	07	04	1	0	1	3	100	80	4	15	1	1	070	080	4	1
377046	1	9999	99	99	99	07	03	0	0	2	0	030	50	2	30	1	1	030	070	3	0
377046	2	3550	04	02	00	06	06	0	0	2	0	060	50	3	20	1	1	040	090	4	1
377046	3	9999	01	01	04	04	00	0	0	2	0	060	45	4	30	1	1	040	095	4	2
377046	4	3950	01	11	00	08	04	0	0	2	0	000	00	0	00	0	0	100	070	3	0
475363	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
475363	2	3525	02	02	03	07	04	0	0	1	3	060	50	3	45	1	1	220	095	4	0
475363	3	3809	02	04	00	06	00	0	0	1	2	060	75	3	40	1	1	030	100	4	1
572195	1	3567	06	04	01	09	02	2	2	1	4	089	70	3	30	1	1	180	080	4	1
470993	1	3425	99	99	99	06	00	4	4	1	0	001	15	2	00	0	0	001	000	4	0
470993	2	3625	05	06	00	09	00	1	0	1	5	065	70	4	50	2	3	150	090	4	1
572710	1	3475	06	04	00	08	04	2	2	1	1	075	65	2	40	1	1	150	060	4	1
572710	2	3542	06	00	00	06	06	0	0	1	1	300	90	4	10	1	1	400	100	4	1
572710	3	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
573477	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
573477	2	3567	00	01	02	03	01	0	0	1	2	180	30	4	60	2	1	060	090	4	1
479857	1	3583	05	11	01	07	03	2	0	1	2	040	60	3	50	1	1	010	100	4	1
479857	2	3750	00	00	04	04	04	0	0	1	3	045	45	3	40	2	1	060	090	4	1
479857	3	3600	00	09	00	09	06	0	0	1	6	008	20	3	30	2	1	010	000	0	0
577067	1	3733	00	03	00	03	03	0	0	1	2	100	80	4	40	1	1	400	090	4	1
577067	2	3633	02	11	06	07	02	0	0	1	2	100	70	4	25	1	1	100	100	4	1
353565	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
473290	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
473290	2	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
473290	3	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
478374	1	3525	99	99	99	08	07	0	0	1	0	200	60	3	30	1	1	050	090	2	0
478374	2	3517	01	04	02	07	01	0	0	1	5	100	70	4	40	1	1	090	075	4	1
478374	3	3642	04	05	00	09	09	0	0	1	3	100	85	3	45	1	1	170	090	4	1
477872	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
477006	1	3500	08	04	00	08	05	2	1	1	2	080	70	3	40	1	1	050	080	3	1
477006	2	3717	00	08	03	07	05	0	0	1	3	030	40	3	60	1	1	020	100	4	1
477006	3	3525	99	99	99	08	00	0	0	1	0	040	25	4	50	1	1	030	090	4	0
047357	1	3525	03	02	01	05	03	1	1	1	2	060	70	3	30	2	1	080	080	3	0
575835	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
575835	2	3683	04	04	01	07	05	1	0	1	4	090	75	3	15	1	1	300	090	4	1
575835	3	3583	00	04	00	04	03	0	0	1	5	160	75	4	20	1	1	100	100	4	1
479991	1	3475	00	04	00	04	04	0	0	1	0	010	60	3	10	1	1	040	090	4	0
479991	2	3600	03	02	00	05	05	0	0	1	3	070	75	3	20	1	1	250	080	4	1
479991	3	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
479991	4	3575	00	04	02	06	03	0	0	1	4	020	70	4	50	1	1	060	100	4	1
475569	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
478762	1	3417	04	06	00	07	00	2	0	1	0	150	85	3	10	1	1	800	090	4	0
478762	2	3600	03	05	00	08	00	0	0	1	6	300	75	3	60	1	1	400	090	4	1
009127	1	3650	09	02	00	07	07	1	1	1	2	040	50	3	55	1	1	200	090	4	1
009127	2	3533	00	01	01	02	02	0	0	1	3	090	60	4	50	1	1	100	100	4	1
376487	1	3650	08	01	00	07	06	1	0	1	3	080	80	4	40	2	2	200	080	4	1
370124	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
370124	2	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
571707	1	3500	05	01	00	06	05	0	0	1	4	190	70	3	20	1	2	350	090	4	1
571707	2	3600	03	02	01	06	02	0	0	1	2	100	70	4	50	1	1	200	100	4	1

/A	/B	/C	/D	/E	/F	/G	/H	/I	/J	/JK	/K	/L	/M	/N	/O	/P	/Q	/R	/S	/T	/U
479702	1	3692	04	05	00	07	05	2	1	1	2	080	75	4	40	1	1	100	100	4	1
479702	2	3650	02	10	00	09	07	0	0	1	0	200	75	4	35	1	1	400	090	4	1
475941	1	3759	07	01	00	07	06	1	0	1	2	070	90	4	15	2	1	300	100	4	1
475941	2	3609	02	03	01	06	04	0	0	1	5	170	60	4	25	1	1	400	100	4	1
574277	1	3759	03	02	00	05	00	0	0	1	2	070	50	3	40	1	1	080	070	3	1
574277	2	3533	03	05	00	08	01	0	0	1	2	080	55	4	30	1	1	020	070	3	0
574311	1	3633	01	02	00	03	00	0	0	1	2	060	40	3	50	1	1	100	070	3	1
574311	2	9999	99	99	99	07	00	0	0	1	0	010	10	3	60	1	1	001	000	0	2
477310	1	3550	05	01	00	06	03	0	0	1	4	035	60	3	40	1	1	070	090	4	1
477310	2	3609	02	05	00	07	05	0	0	1	5	120	50	3	35	1	1	400	080	4	1
278210	1	3533	00	11	01	06	00	3	0	1	4	120	55	3	65	1	1	400	095	3	0
278210	2	3567	06	07	02	07	00	2	0	1	5	060	40	4	65	2	1	120	090	4	1
574081	1	3500	15	03	00	08	00	0	0	2	0	060	75	3	20	1	1	100	090	4	1
574081	2	3683	03	05	00	08	05	1	1	2	0	070	60	3	25	1	1	300	080	4	0
574081	3	3650	03	09	04	07	05	0	0	2	0	000	00	0	00	0	0	050	060	4	1
474462	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
474462	2	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
473658	1	3609	00	03	01	04	03	0	0	1	2	050	75	3	50	1	1	050	100	4	1
473658	2	3650	00	04	00	04	00	0	0	1	6	060	50	4	45	1	1	050	090	4	1
575032	1	3559	01	03	00	04	02	0	0	1	2	060	50	4	60	1	1	300	100	4	1
575032	2	3625	02	06	01	07	03	0	0	1	2	060	50	3	60	2	1	200	095	4	1
575032	3	3500	00	05	01	06	04	0	0	1	0	028	60	3	30	1	1	030	090	4	1
575032	4	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
475836	1	3617	12	03	00	08	04	0	0	2	0	070	60	4	25	1	1	100	090	4	1
475836	2	3567	03	05	01	07	00	0	0	2	0	100	70	4	25	1	1	040	090	4	1
475836	3	3600	00	05	01	06	00	0	0	2	0	050	30	4	30	2	1	020	050	4	1
474546	1	3467	00	19	00	12	03	0	0	1	0	070	75	3	25	1	1	800	090	3	0
474546	2	3515	03	06	00	09	05	0	0	1	6	020	50	3	65	1	1	080	095	3	0
474546	3	3633	00	07	02	07	04	0	0	1	5	100	80	4	40	1	1	300	100	4	1
477578	1	3483	02	04	00	06	05	0	0	1	0	120	65	3	70	1	1	250	085	4	1
477578	2	3559	07	01	00	07	03	0	0	1	2	060	70	3	60	1	1	150	100	4	1
479926	1	3650	02	03	00	05	04	0	0	1	2	080	85	4	75	1	1	120	100	4	1
574301	1	3633	05	01	00	06	00	0	0	1	2	005	70	3	60	1	1	040	090	4	1
476211	1	3425	00	20	00	13	13	0	0	1	5	095	75	3	20	1	1	800	090	4	0
476211	2	3467	00	07	01	08	02	0	0	1	4	250	90	4	20	2	1	600	085	4	0
570757	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
576942	1	3609	04	13	02	08	00	2	0	1	4	075	55	3	60	1	1	020	100	3	1
576942	2	3550	00	14	11	10	06	0	0	1	4	045	60	4	35	1	1	120	090	4	0
377863	1	9999	02	06	03	11	00	0	0	1	0	020	80	3	40	1	1	050	070	2	0
377863	2	3433	07	04	00	07	00	0	0	1	2	050	70	3	50	1	1	080	095	4	1
577288	1	3517	00	04	00	04	04	0	0	1	2	070	70	3	45	1	2	400	095	4	0
577288	2	3775	00	02	01	03	03	0	0	1	6	150	60	3	15	1	2	300	095	4	1
577288	3	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
377188	1	3442	01	02	00	03	00	0	0	1	0	007	10	2	60	1	1	005	000	0	0
377188	2	9999	00	00	00	00	00	0	0	1	0	035	65	3	50	1	1	020	090	4	2
377188	3	3550	00	01	00	01	01	0	0	1	2	015	30	3	65	1	1	100	080	4	1
377188	4	3642	00	01	00	01	00	0	0	1	5	090	60	4	40	1	1	070	100	4	1
220276	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
220276	2	3550	04	01	01	06	02	0	0	1	2	150	90	4	20	1	2	400	100	4	1
575917	1	3475	02	01	00	03	00	0	0	1	0	039	60	3	40	1	1	030	080	4	1
575917	2	3533	02	03	00	05	04	0	0	1	4	055	60	4	20	1	1	200	090	4	1
378488	1	3550	07	11	06	17	00	5	4	1	5	100	50	3	50	1	1	030	095	3	0
571826	1	3567	00	01	02	03	00	0	0	1	0	060	50	3	30	1	1	100	095	4	0
571826	2	3559	00	02	03	05	03	0	0	1	2	100	50	3	40	1	1	100	090	4	0
571826	3	3583	00	03	05	08	00	0	0	1	2	100	75	3	30	1	1	400	095	4	1
476133	1	3542	12	05	02	09	08	2	2	1	3	065	55	4	40	1	1	300	090	4	1
476133	2	3609	00	09	01	07	03	0	0	1	2	100	80	4	30	1	1	300	100	4	1

/A	/B	/C	/D	/E	/F	/G	/H	/I	/J	/JK	/K	/L	/M	/N	/O	/P	/Q	/R	/S	/T	/U
574064	1	3709	00	05	02	07	02	0	0	1	2	200	80	4	25	1	1	200	090	4	1
574064	2	3533	00	04	00	04	04	0	0	1	3	150	70	4	50	1	1	200	095	4	1
574137	1	3642	01	02	00	03	00	0	0	1	3	046	75	2	70	1	1	050	080	4	1
574137	2	3959	00	08	00	08	00	0	0	1	2	016	10	2	40	2	1	010	050	2	1
571627	1	3533	06	00	00	06	04	0	0	1	7	075	60	3	30	1	3	200	080	4	1
571627	2	3600	00	02	00	02	00	0	0	1	2	080	60	3	50	1	1	100	100	4	1
571627	3	3692	00	06	01	07	00	0	0	1	3	150	75	3	45	1	1	100	085	4	1
570933	1	3742	00	04	00	04	04	0	0	1	2	080	65	4	55	1	3	100	090	4	1
570933	2	3600	00	03	00	03	00	0	0	1	6	038	60	3	30	1	1	009	000	0	0
572543	1	3550	05	03	00	07	07	1	1	1	6	065	80	3	15	1	2	050	090	4	1
572543	2	3600	02	04	00	06	02	0	0	1	2	020	40	3	30	1	1	020	090	4	1
572543	3	3709	01	06	01	08	08	0	0	1	4	050	65	3	50	1	1	030	100	4	1
572543	4	3800	00	10	00	07	04	0	0	1	3	020	30	3	35	1	1	020	075	4	0
478422	1	3583	09	03	00	08	00	2	0	1	4	100	80	4	25	2	1	300	100	4	0
478422	2	3492	02	01	00	03	01	0	0	1	3	055	50	3	40	2	1	090	090	4	1
473497	1	3509	99	99	99	09	09	0	0	1	0	250	80	3	30	1	1	999	095	4	0
473497	2	3467	07	03	00	08	08	0	0	1	5	150	90	4	10	1	2	350	100	4	1
473497	3	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
473497	4	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
474395	1	3517	02	06	01	07	05	2	0	1	0	200	95	4	05	1	1	400	090	4	0
474395	2	3575	00	07	00	07	06	0	0	1	2	150	75	4	40	1	1	250	100	4	1
474395	3	3659	00	09	05	08	05	2	1	1	2	200	60	4	25	1	1	150	070	4	1
474355	1	3525	03	06	00	09	08	0	0	2	0	000	00	0	00	0	0	000	000	0	0
474355	2	3483	02	07	00	08	01	0	0	2	0	000	00	0	00	0	0	050	060	3	0
474355	3	3650	08	05	00	08	04	0	0	2	0	060	70	3	15	1	1	200	090	4	1
573227	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
572005	1	3533	07	04	00	08	03	1	1	1	2	070	50	3	10	1	1	200	100	4	2
572005	2	3483	02	03	01	06	00	0	0	1	3	030	60	3	25	1	1	070	080	4	2
572005	3	3609	02	06	00	08	02	0	0	1	2	080	60	4	35	1	1	100	080	4	2
572005	4	3609	00	05	05	08	08	0	0	1	3	027	30	3	20	1	1	180	090	4	1
572005	5	3659	00	08	01	08	02	0	0	1	2	018	50	2	55	1	1	020	090	3	0
478763	1	3450	01	05	00	06	02	0	0	1	2	100	80	4	20	2	1	060	090	4	0
478763	2	3492	01	03	01	05	01	0	0	1	4	250	65	3	50	1	1	350	100	4	1
478763	3	3550	03	03	02	08	06	0	0	1	2	090	80	4	25	2	1	300	100	4	1
478763	4	3525	00	04	02	06	04	0	0	1	5	100	50	3	20	1	1	120	080	4	1
378481	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
378481	2	3550	05	02	01	07	00	1	1	1	9	075	50	3	40	1	1	010	070	3	2
378481	3	3600	03	01	02	06	00	0	0	1	7	060	65	3	30	1	1	100	090	4	0
475169	1	3517	00	05	02	06	02	1	0	1	3	100	75	3	70	1	1	600	090	3	0
475169	2	3509	04	01	00	05	01	0	0	1	2	090	80	4	45	1	1	100	090	4	1
475169	3	3592	02	04	00	06	02	0	0	1	2	090	65	4	55	1	1	100	090	4	1
475942	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
475942	2	3575	00	00	01	01	00	0	0	1	3	050	45	2	50	1	1	050	050	2	2
477734	1	3475	03	05	00	08	05	0	0	1	2	300	80	4	20	1	1	050	020	2	0
477734	2	3375	04	04	00	08	02	0	0	1	6	060	50	4	20	2	1	100	070	4	1
477734	3	3633	05	06	01	08	01	0	0	1	2	080	60	3	20	1	1	030	040	3	2
477734	4	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
477734	5	3609	02	16	00	14	01	0	0	1	3	250	55	3	50	1	1	009	025	2	1
572182	1	3500	04	02	00	06	02	0	0	1	0	045	30	3	60	2	1	060	090	4	1
572182	2	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
572182	3	3833	00	08	00	08	06	0	0	1	5	120	40	3	25	1	1	010	080	3	0
574937	1	3550	03	06	01	07	03	2	1	1	3	050	50	4	55	1	2	250	080	4	1
090019	1	9898	98	98	98	98	98	8	8	8	8	098	98	8	98	8	8	098	098	8	8
090019	2	3459	01	01	01	03	02	0	0	1	3	190	80	4	20	1	2	300	100	4	1
576357	1	3600	02	05	01	07	05	1	0	1	2	100	80	4	30	1	1	200	100	4	1
378821	1	3517	00	07	02	07	03	1	1	1	3	010	60	4	70	1	1	110	095	4	1
378821	2	3567	01	00	00	01	01	0	0	1	3	080	85	4	25	1	1	030	090	4	0

APPENDIX 5: LIST OF PRESENTATIONS AT MAJOR MEETINGS AND PUBLICATIONS RESULTING FROM THESE STUDIES

Presentations:

1989 Silver Jubilee British Congress of Obstetrics and Gynaecology, London, England
"Analysis of cyclical changes of human oviductal proteins"

1989 Annual Meeting of the British Fertility Society, The London Hospital, London, England
"Patterns of ovarian response in Assisted Reproduction. Can they be altered?"

1991 7th World Congress on In Vitro Fertilization and Assisted Procreation, Paris, France
"A randomized controlled trial of in vitro fertilisation and uterine embryo transfer (IVF-UET) vs tubal embryo transfer (IVF-TET) in the management of idiopathic and male infertility"

1991 1st International Meeting of the British Fertility Society, London, England

(i) "Does tubal embryo transfer improve the pregnancy rate? A randomized controlled trial of IVF-uterine ET versus IVF-tubal ET in the management of unexplained and male factor infertiltiy"

(ii) "Is ultrasound guided vaginal egg collection safe? Evaluation of the immediate and late effects as assessed by laparoscopy"

(iii) "Comparative immunohistochemical study of oestrogen and progesterone receptors in the fallopian tube and endometrium in different hormonal circumstances"

1992 The Fallopian tube: Advances in diagnosis and surgical treatment, The Royal London Hospital, London, England

(i) "Comparative ultrastructural studies of the endometrium and Fallopian tube at different stages of the menstrual cycle"

(ii) "Cyclical changes of oestrogen and progesterone receptors in the Fallopian tube and the endometrium under different hormonal circumstances. A comparative immunohistochemical study"

(iii) "Assessment of the influence of Fallopian tube embryo transfer on pregnancy and implantation rates in women undergoing assisted reproduction treatment"

(iv) "Evaluation of the immediate and late effects of repeated vaginal egg collections on pelvic organs as assessed by laparoscopy or laparotomy"

1993 The British Fertility Society, Oxford, England

"Factors influencing the outcome of tubal embryo transfers. Results of a randomized controlled trial comparing tubal and uterine embryo transfers"

Publications:

Amso N, Shaw RW. 1992 Clinical trials in assisted reproduction (letter). Hum Reprod 7:580-581.

Amso NN, Shaw RW. 1993 A critical appraisal of Assisted Reproduction techniques. Hum Reprod 8:168-174.

Amso N, Curtis P, Preutthipan S, Keith E, Bernard A, Shaw RW. 1991 A randomized controlled trial of in vitro fertilisation and uterine embryo transfer vs tubal embryo transfer in the management of idiopathic and male infertility. In: "Proceedings of the 7th World Congress on In Vitro Fertilization and Assisted Procreation (30.6.1991-3.7.1991)". Hum Reprod 6 (suppl 1):128-129.

Amso NN, Crow J, Shaw RW. 1994a Comparative immunohistochemical study of oestrogen and progesterone receptors in the Fallopian tube and the uterus at different stages of the menstrual cycle and the menopause. Hum Reprod 9:1027-1037.

Amso NN, Crow J, Lewin J, Shaw RW. 1994b A comparative morphological and ultrastructural study of endometrial gland and Fallopian tube epithelia at different stages of the menstrual cycle and the menopause. Hum Reprod 9:2234-2241.

Crow J, Amso NN, Lewin J, Shaw RW. 1994 Morphology and ultrastructure of Fallopian tube epithelium at different stages of the menstrual cycle and menopause. Hum Reprod 9:2224-2233.

Curtis P, Amso N, Parnaby RM, Kibbler C, Shaw RW. 1992 Successful twin delivery despite proven intraperitoneal chlamydial infection at implantation. Case report. *Br J Obstet Gynaecol,* 99:263-264.

Jackson AE, Curtis P, Amso N, Shaw RW. 1992 Exposure to LHRH agonists in early pregnancy following the commencement of mid-luteal buserelin for IVF stimulation. Hum Reprod 7:1222-1224.