MANAGEMENT OPTIONS FOR HYDROSALPINX:

SALPINGOSTOMY OR IVF

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Abstract

Hydrosalpinx refers to a dilated Fallopian tube containing clear fluid, commonly bilateral and generally accepted to be the end result of bacterial infection and resolved pyosalpinx.

Infertility can result from distal tubal obstruction and agglutination of tubal fimbriae preventing ovum entry and fertilisation in the distal Fallopian tube.

As a consequence of fluid accumulation, intraluminal pressure rises despite patency of the proximal (cornual) Fallopian tube with intermittent release of fluid to the uterine cavity. The original inflammatory process also commonly causes structural damage and loss of physiological function of the Fallopian tube.

Since the introduction and progressive improvement in outcome with IVF, use of restorative surgery as a treatment option has remained static. Because of the recognised deleterious effect of hydrosalpinx fluid on embryo implantation, to prevent fluid entering the uterine cavity, hydrosalpinges are commonly excised or proximately obstructed prior to commencing IVF.

These procedures exclude natural conception, with IVF remaining the only treatment to achieve a pregnancy.

A minority of hydrosalpinx affected women achieve intrauterine pregnancies following “salpingostomy” or” neo-salpingostomy” (surgical reopening of the distal hydrosalpinx), however the majority fail to conceive or develop tubal ectopic pregnancies.

This thesis explores how tubal function is affected by hydrosalpinx formation with the aim of determining the extent of the role, if any, of salpingostomy in the current management of hydrosalpinx.

Morphological, physical and biochemical studies were performed comparing rabbit hydrosalpinx to control oviducts and hydrosalpinx and normal Fallopian tubes in women.

Rabbit studies

Systematic evaluation of changes in morphological, physical and biochemical tissue levels were made utilising a surgically induced model of hydrosalpinx in the rabbit. The oviduct distension occurring during hydrosalpinx formation resulted in significant changes in all parameters studied. Major alterations were observed in morphology (light and surface electron microscopy), most notably epithelial deciliation, and tissue biochemistry including tissue DNA/RNA content and turnover, collagen, electrolyte, water and lipid levels.

Progessive reduction in tubal blood flow was noted with increasing intraluminal pressure.

Tubal muscular contractility, epithelial ciliary and secretory activity are critically influenced by ovarian steroids, therefore detailed examination of changes in tissue oestrogen and progesterone cytosolic and nuclear receptor levels were measured in healthy and hydrosalpinx affected oviducts.

With progressive increase in tubal distension, reduction in steroid hormone receptor levels occurred.

Reversal of all these abnormalities was achieved following microsurgical tubal reconstruction, including restoration of fertility.

Human studies

The study confirmed that in women hydrosalpinx results in similar adverse morphological and biochemical changes with variable segmental epithelial deciliation. Steroid hormone receptor levels were significantly reduced in hydrosalpinx compared to normal controls. Receptor levels measured during the proliferative, secretory phases of the menstrual cycle, menopause, early pregnancy and postpartum, reflected changes in circulating steroid hormone levels.

Fallopian tubes obtained from women with ectopic pregnancies and post-sterilisation

also showed a reduction in hormone receptor levels, in parallel with a decline in mean ciliation index (MCI).

The study confirmed that following salpingostomy, intrauterine pregnancies could be achieved when an adequate MCI was found to be present in epithelial microbiopsies taken at the time of salpingostomy procedure. Women with reduced or low MCI subsequently experienced ectopic pregnancies or failed to conceive.

Assessment of tubal morphology is laboratory dependant and requires subjecting women to a diagnostic procedure prior to carrying out salpingostomy when MCI is found to be favourable.

In an attempt to predict which Fallopian tubes are worthy of conservative surgery at a single operation, a review of the literature was undertaken to evaluate what role inspection of the tubal lumen by salpingoscopy (transabdominal) or fertiloscopy (transvaginal) plays in assessment of mucosal pathology.

The aim was to assess whether inspection of the tubal mucosa gave comparable information to MCI.

Review of salpingoscopy prediction results, suggests that women with hydrosalpinges associated with severe intraluminal adhesions, denuded or flattened mucosal folds (high abnormality grade according to set scale), had negligible chance of intrauterine pregnancy following salpingostomy. As a corollary, these pathological changes are a contraindication to restorative surgery. To perform salpingostomy an abdominal approach is preferred when correction of hydrosalpinx is considered, as opening of the distal part of the Fallopian tube, intraluminal inspection and salpingostomy procedure can best be achieved by this route.

The data also suggests that with a single endoscopic intervention, salpingoscopy followed by salpingostomy has up to a 30% chance of successful intrauterine pregnancy outcome. Women with hydrosalpinges that have mucosal folds with limited intraluminal and peritubal adhesions therefore warrant consideration of conservative

rather than destructive surgery.

Having confirmed also that mucosal assessment can be further improved by utilising microsalpingoscopy, this procedure should also be considered for assessment of the tubal mucosa. This latter procedure which involves contact epithelial inspection will determine whether post- inflammatory changes remain present at the cellular level, thus facilitating a decision of Fallopian tube conservation. Salpingostomy is particularly relevant for ovulatory women over 40 years of age who have a low chance of success with IVF treatment. The higher success rate (intrauterine pregnancy and live births) achieved by women aged 40-47 who had tubal surgery (reversal of sterilisation), in comparison to IVF treatment is consistent with this interpretation (Petrucco et al 2007).

The observation of progressive deterioration in tubal function with increasing intraluminal pressure in the rabbit model, suggests that women diagnosed with bilateral hydrosalpinx wanting future fertility could be offered salpingostomy at initial diagnosis of hydrosalpinx before fertility is considered. This endoscopic day surgery procedure may prevent deterioration in tubal function as progressive dilatation occurs with time and allow the possibility of subsequent spontaneous conception, rather than having to rely completely on artificial reproductive technology (ART) and IVF.

It is proposed that conservative surgery to achieve conception in women with hydrosalpinges should be considered when salpingoscopic assessment predicts a favourable outcome. As well as providing a chance of spontaneous conception of more than one pregnancy, this approach would reduce the enormous cost burden on society of providing repeated cycles of IVF currently funded by our national health system. Further studies and evaluation beyond this current study are required to support this proposal.

Thesis Declaration

I certify that this work contains no material presented for the award of any other degree, or diploma in any university or other tertiary institution. The thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I also certify that no part of this work will in the future be used in a submission for any other degree or diploma in any university or other tertiary institution without prior approval by The University of Adelaide.

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The following publications and conference proceedings have arisen from this work:

Petrucco, O, M. and Winston, R.M.L. (1980). Morphological and sex steroid hormone receptor study of the normal and pathological Fallopian tube. World Congress Fertility and Infertility - Madrid. Abstract 051.

Petrucco, O. (1979). Microsurgical tubal anastomosis in the rabbit following three types of sterilisation procedure. *Contraception* 20, 53-58.

Petrucco, O.M. (1981). Morphological and sex steroid receptor study of the Fallopian tube following hydrosalpinx formation*. Eighth New Zealand Congress of Obstetrics and Gynaecology, Auckland*. Abstract.

Petrucco, O.M. and Tulsi, R.S. (1981). Microsurgical reversal of the artificially induced hydrosalpinx in the rabbit – morphological, blood flow and subsequent pregnancy studies. *Eighth New Zealand Congress of Obstetrics and Gynaecology, Auckland.* Abstract.

Petrucco, O.M. and Tulsi, R.S. (1981). Microsurgical reversal of the artificially induced hydrosalpinx in the rabbit – morphological studies. *Aust N.Z J Obstet Gynaec* 21, 159-163.

Paterson, P. and **Petrucco, O.** (1987). Tubal factors and infertility. *The Infertile Couple* - 2nd Ed. Pepperell, R.J., Hudson, B., Wood., C., et al. Editors, 61-90. Churchill Livingstone.

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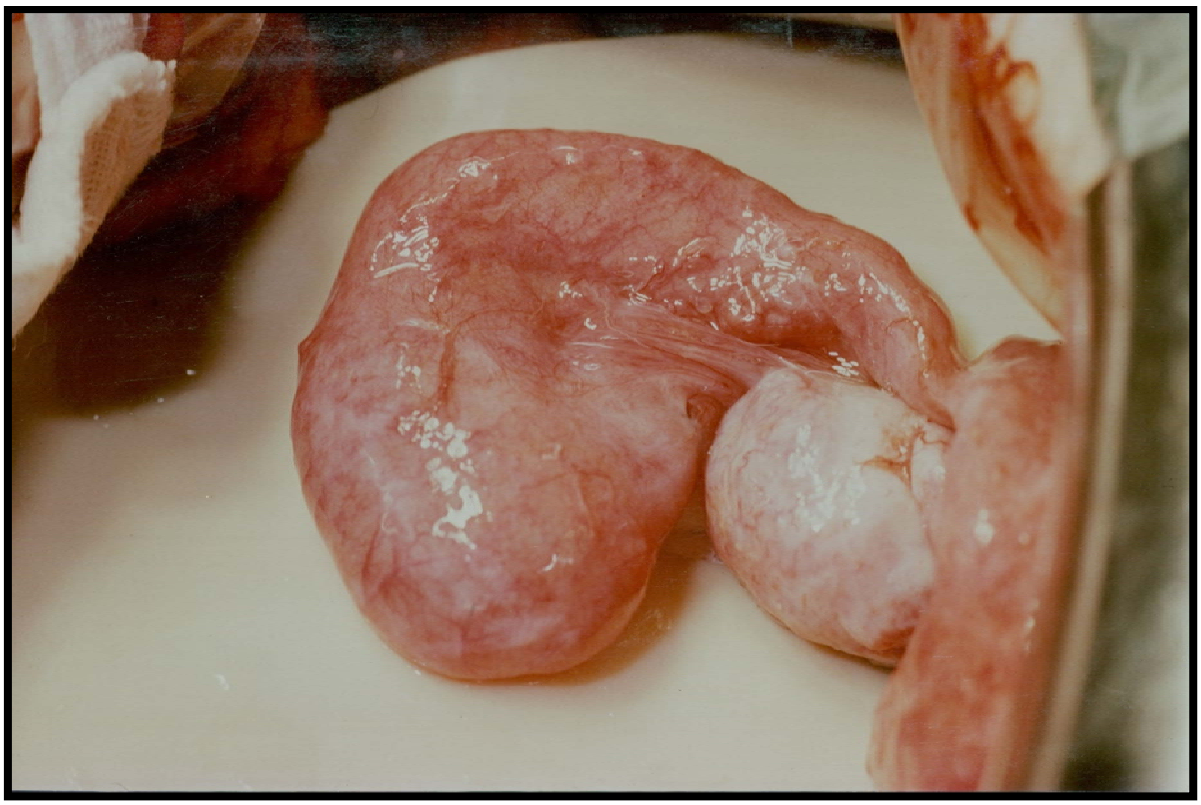
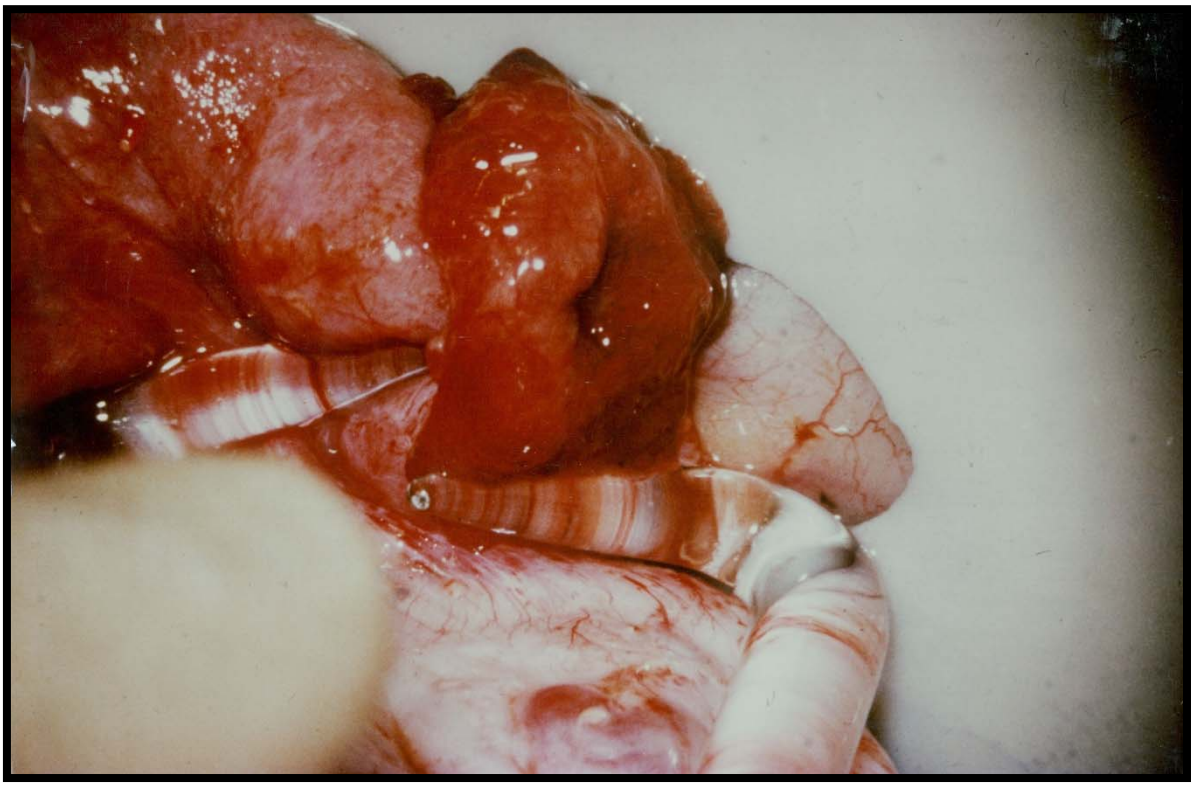
1979 - Clinicians working in the laboratories of Lord Winston at Hammersmith Hospital.

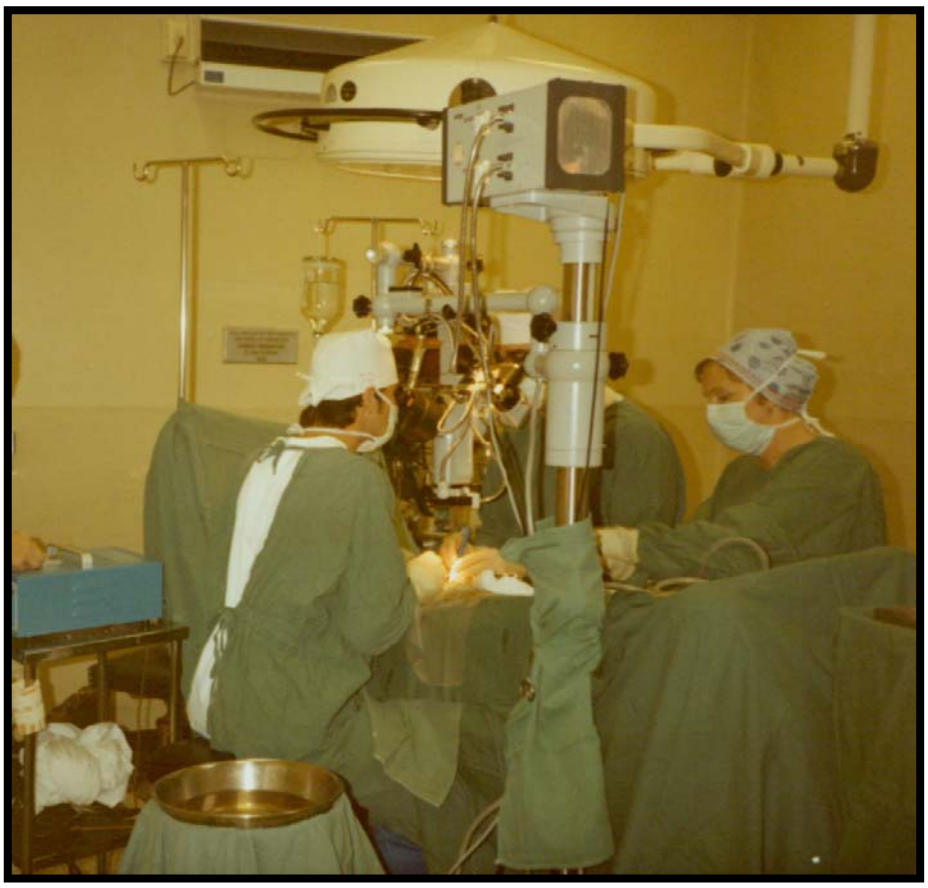
I have made use of the reviews,“The Steroid Hormone Receptors” by Klinge and Rao (2008), and “Molecular Mechanisms of Estrogen-Estrogen Receptor Signalling” by Yasar et al 2016, in addition to the PhD thesis of Lisa Akison, “The Role of Nuclear Progesterone Receptor (PGR) in Regulating Gene Expression, Morphology and Function in the Ovary and Oviduct during the Periovular Period”(The University of Adelaide, 2012). These publications have provided useful references which have been appropriately cited in my review of Steroid Hormone Receptor Physiology (Chapter 2).

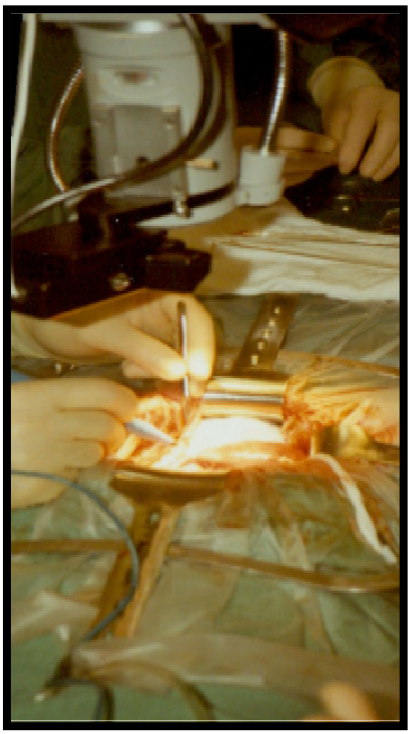




Hydrosalpinx and Microsurgical Salpingostomy

Petrucco 2020





Microsurgical Technique

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Abbreviations

AIJ: ampullary isthmic junction

ART: assisted reproductive technology

ATP: adenosine triphosphate

CBF: ciliary beat frequency

COC: cumulus oocyte complex

CSF-1: colony stimulating factor 1

ER: oestrogen receptor

E_2: oestrogen /oestradiol

GM-CSF: granulocyte macrophage – colony stimulating factor

HB-EGF: heparin binding epidermal growth factor

hCG: human chorionic gonadotrophin

hsp60: heat shock protein 60

IFNγ: interferon gamma

IFN2: interferon type II

IGF2: insulin like growth factor 2

IL-6: interleukin-6

IL-11, 13, 15, 18: interleukin, 11, 13, 15, 18

IVF: in vitro fertilisation

LH: luteinising hormone

LIF: leukemia inhibitory factor

LPS: lipopolysaccharide

MCI: mean cilia index (percentage ciliated cells in 30 fields representative of the block under examination).

M-CSF: macrophage colony stimulating factor

NO: nitric oxide

PAF: platelet activating factor

PGE: prostaglandin E

PGF2α: prostaglandin F2 alpha

P_4: progesterone

PR: progesterone receptor

SEM: surface electron microscopy

SRF: steroid receptor superfamily

TEM: transmission electron microscopy

TNFα: tumour necrosis factor alpha

TRAIL: TNF-related apoptosis inducing ligand

UTJ: uterotubal junction

VEGF: vascular endothelial growth factor

Symbols

-	not applicable
%	percentage
n	sample size
σ	standard deviation
μ	mean values

Statistical Analysis

Data is presented as Mean (μ) ± Standard Deviation (σ) unless otherwise specified. Student t-test, analysis of variance and Mann-Whitney non-parametric analysis were used for statistical analysis.

Chapter 1 Literature Review and Introduction to Thesis

1.1. Introduction

The first anatomical description of the mammalian oviduct was published in 1561 by the Italian anatomist Gabriele Fallopio. Since then experimental and clinical data has ascertained the pivotal role that the oviduct plays in reproduction.

Spontaneous pregnancy is dependent on oocyte entry, fertilization, sperm viability capacitation and transit, early development and timely delivery of the embryo to the uterine cavity, all of which occur within the Fallopian tube. Tubal muscular contraction, ciliary activity and fluid secretion, interact to achieve the sequential occurrence of these events (Jansen 1984).

One in six couples attempting conception fail to conceive (Hull et al 1985).

Fallopian tube blockage and ovarian dysfunction are the two main causes of female infertility with tubal pathology accounting for at least 30%, the majority as a consequence of salpingitis (Case and Zuspan, 1969).

Sexually transmitted diseases (STDs) adversely affect reproduction by impairment of fertilisation and intrauterine implantation, secondary to impaired tubal dysfunction and scarring following salpingitis. As STDs are frequently asymptomatic studies of incidence and prevalence are usually based on developed countries reports and analysis of data on bacterial STDs. The World Health Organisation (WHO) estimated that in 1995 there were more than 330 million new adult cases of curable STDs worldwide (Global Programme on AIDS 1995). Substantial reduction in STDs rates have been achieved in developed countries particularly for gonorrhoea and syphilis, whilst infections with chlamydia, genital herpes, human

papilloma virus (HPV), and bacterial vaginosis remain widespread. Chlamydia incidence of 5-12% are found in community and school surveys (Wasserheit 1996). The most significant tubal pathiology following salpingitis is distal tubal obstruction and formation of hydrosalpinx. The initiating inflammatory process often causes structural damage to the tubal epithelium resulting in loss of physiological function of the Fallopian tube.

In the pre-IVF area, surgical correction for hydrosalpinx (salpingostomy) achieved live births particularly following the introduction of refined microsurgical and endoscopic techniques.

With progressive improvement in IVF outcomes, the use of restorative surgery has not progressed, with fewer reproductive surgeons available to provide this service and pass on their skills to emerging infertility specialists (Gangiulo and Bhagavath, 2019).

A clinician's ability to assess tubal function following salpingitis has traditionally relied on tests of tubal patency.

Techniques to assess residual inflammation and intra-luminal pathology (salpingoscopy and microsalpingoscopy), tubal cilia (mean ciliation index on epithelial microbiopsy) and epithelial cellular response to circulating steroid hormones (measurement of steroid homone receptors), provided further insight in the assessment of tubal function and are evaluated in this thesis.

The overall goal of this thesis is to develop a greater understanding of the extent of pathological change in the Fallopian tube resulting from hydrosalpinx formation and to evaluate if corrective surgery has a role in achieving live births for women with this condition.

To better evaluate pathological changes, the following section reviews the structure and function of normal oviducts in mammals, in particular for human and rabbit.

1.2. Structure and Function of the Oviduct

The cyclical changes occurring in the reproductive tract by the action of oestrogen and progesterone similarly affect Fallopian tube morphology and function. Ovarian steroids and prostaglandins from the systemic circulation reaching highest concentration in the periovular period, influence the tubal environment and development of the early embryo by their effect on the tubal luminal epithelium, muscular and nerve function and by regulating the volume and composition of tubal fluid (Hunter 2011).

1.2.1. Morphology and structure of the mammalian oviduct

The mammalian oviduct has three anatomical and functional regions: the fimbriated terminal infundibulum, which joins the 5-8 cm ampulla and the narrowest portion the 2-3 cm isthmus joining the ampulla at the ampullary – isthmic junction (AIJ) and attaching to the uterine cornua at the utero-tubal junction, UTJ (Figure 1). Lindblom and Nordstrom (1986) identified three separate muscle layers at the utero-tubal junction comprising of a circular between two longitudinal layers.

In cross-section there are three layers (Figure 2) with the outer serosal layer composed of connective tissue and blood vessels, lymphatics and nerves surrounded by a layer of mesothelial cells.

The middle muscularis layer comprises an inner circular and outer longitudinal layer composed of smooth muscle cells. The isthmus in primates has a thick smooth muscle layer compared to the ampulla, which has more mucosal folds and less muscle in its wall.

The inner longitudinal layer becomes attenuated in the more distal part of the isthmus and is difficult to recognise past the mid-isthmus.

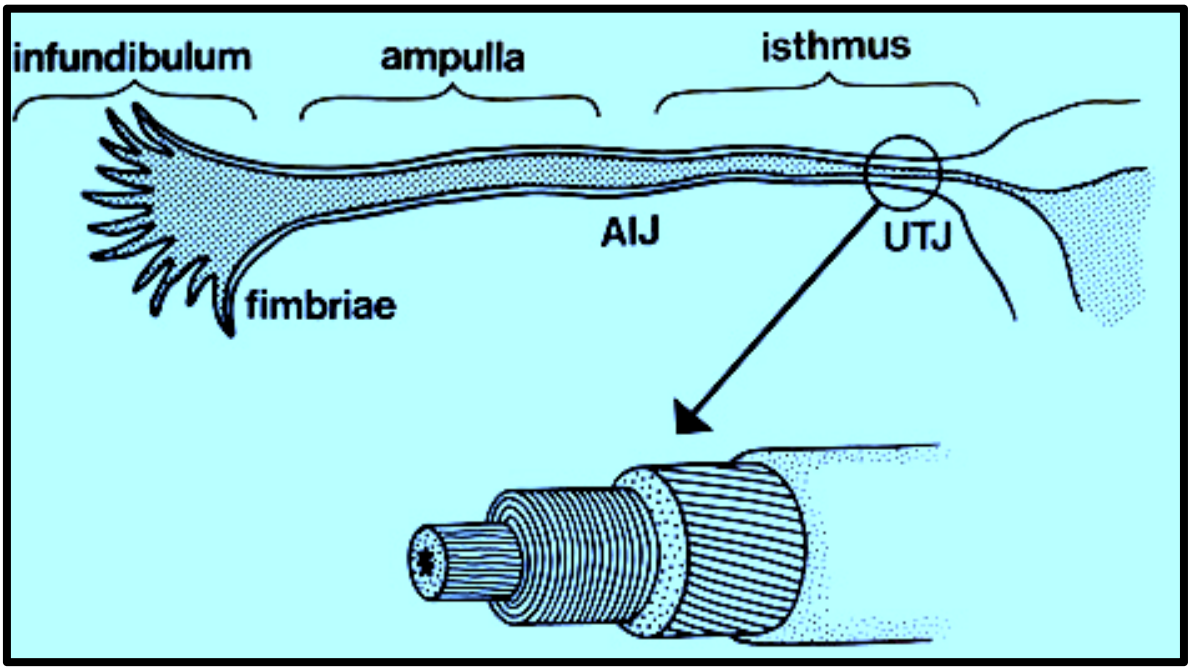

Figure 1 Anatomy uterotubal junction.

Fallopian tube anatomy at the uterotubal junction made of 3 muscle layers – outer longitudinal, intermediate circular, and innermost longitudinal layer.

(Adapted from Lindblom and Nordstrom 1986)

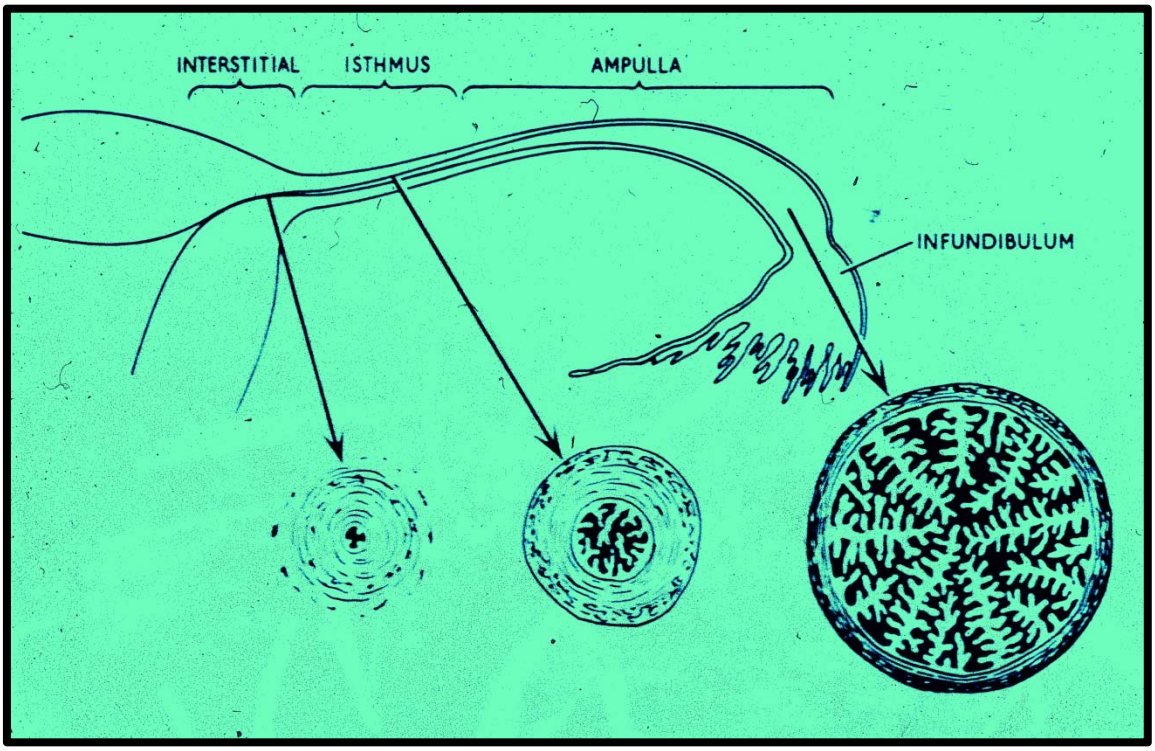

Figure 2 Differences in muscle layer thickness in three segments of oviduct.

The ampulla has a poorly defined internal longitudinal layer and a circular muscle layer which becomes progressively more prominent from the fimbriae to the ampullary-isthmic junction. This layer is interrupted by fascicles of outer longitudinal muscle fibres (Pauerstein et al 1974).

The one-centimetre wide infundibulum with fimbriae has been observed to cover the surface of the ovary aiding capture of the cumulus-oocyte complex (COC) at ovulation. It has the highest number of ciliated cells to facilitate oocyte capture and transport through the infundibulum opening to the ampulla. Fertilisation and early embryo development occur in the ampulla in the extensive highly folded mucosal layer (Figure 3).

The isthmus furthermore continues to support early embryo development and regulates embryo transport to the uterine cavity via muscular contractions (Pulkkinnen 1995).

1.2.2. Light microscopy

The inner mucosal or epithelial layer with underlying lamina propria consists primarily of two types of epithelial cells, ciliated and secretory (Figure 4). Two less common cells have been described, an intercalary or “peg” cell and a basal cell (Pauerstein and Woodruff 1967; Rendi et al 2012) (Figure 5).

The ciliated cuboid shaped cells have fine granular cytoplasm, a central nucleus. Ciliogenesis (Hagiwara et al 2000) is divided into four stages:

1. Duplication of centrioles
2. Migration of centrioles to the apical cell surface to become basal bodies
3. Elongation of cilia containing the axoneme
4. Formation of accessory structures of basal bodies.

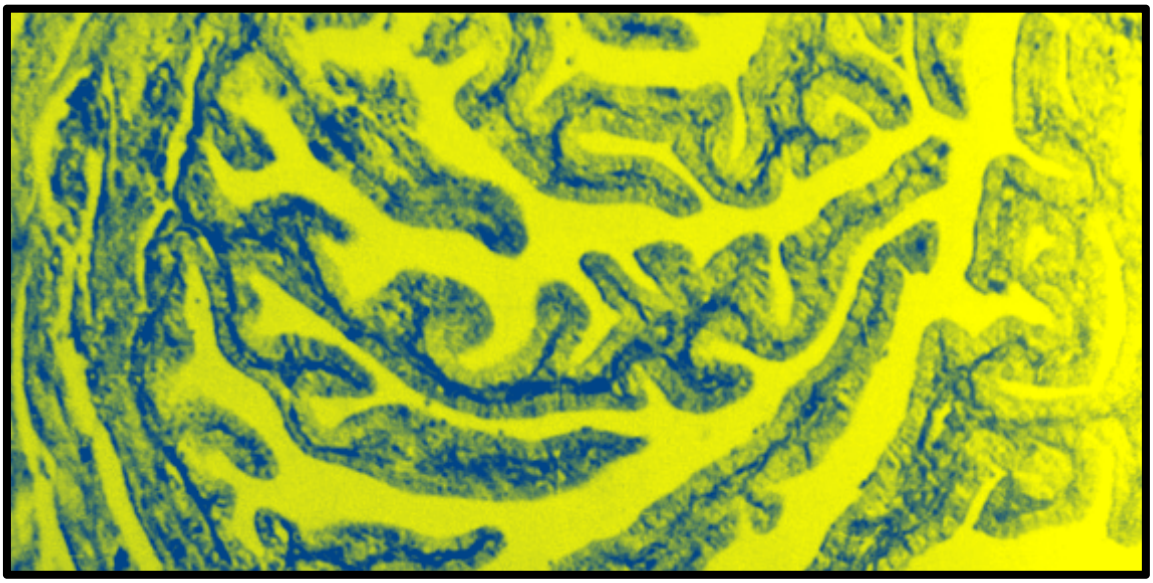

Figure 3 Oviduct ampulla with highly folded mucosal layer.

(Adapted from Lindblom 1979)

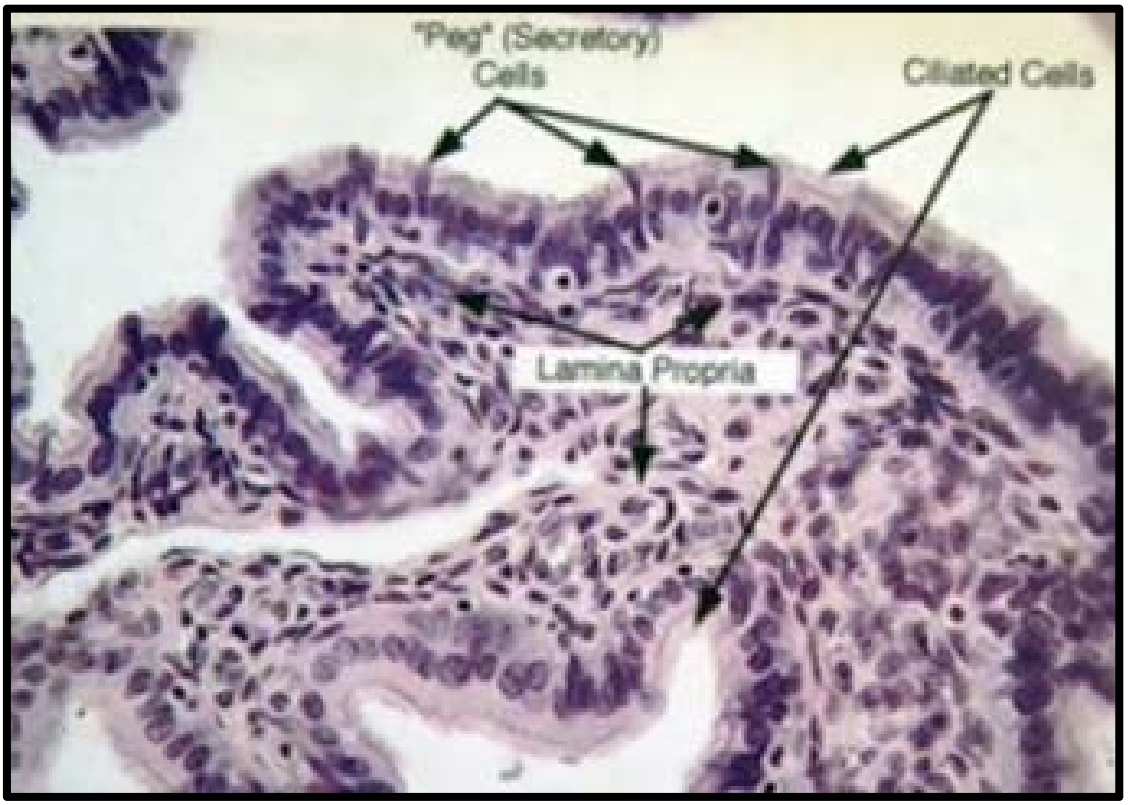

Figure 4 Fallopian tube ampullary mucosa – cellular structure.

Single layer columnar epithelial cells, both ciliated and secretory 'peg' cells with underlying loose lamina propria. A high degree of folding and less pronounced tunica muscularis is noted in this part of the Fallopian tube.

(From University of New England Histology – http://faculty.une.edu/com/abell/histo/histolab 3f.html).

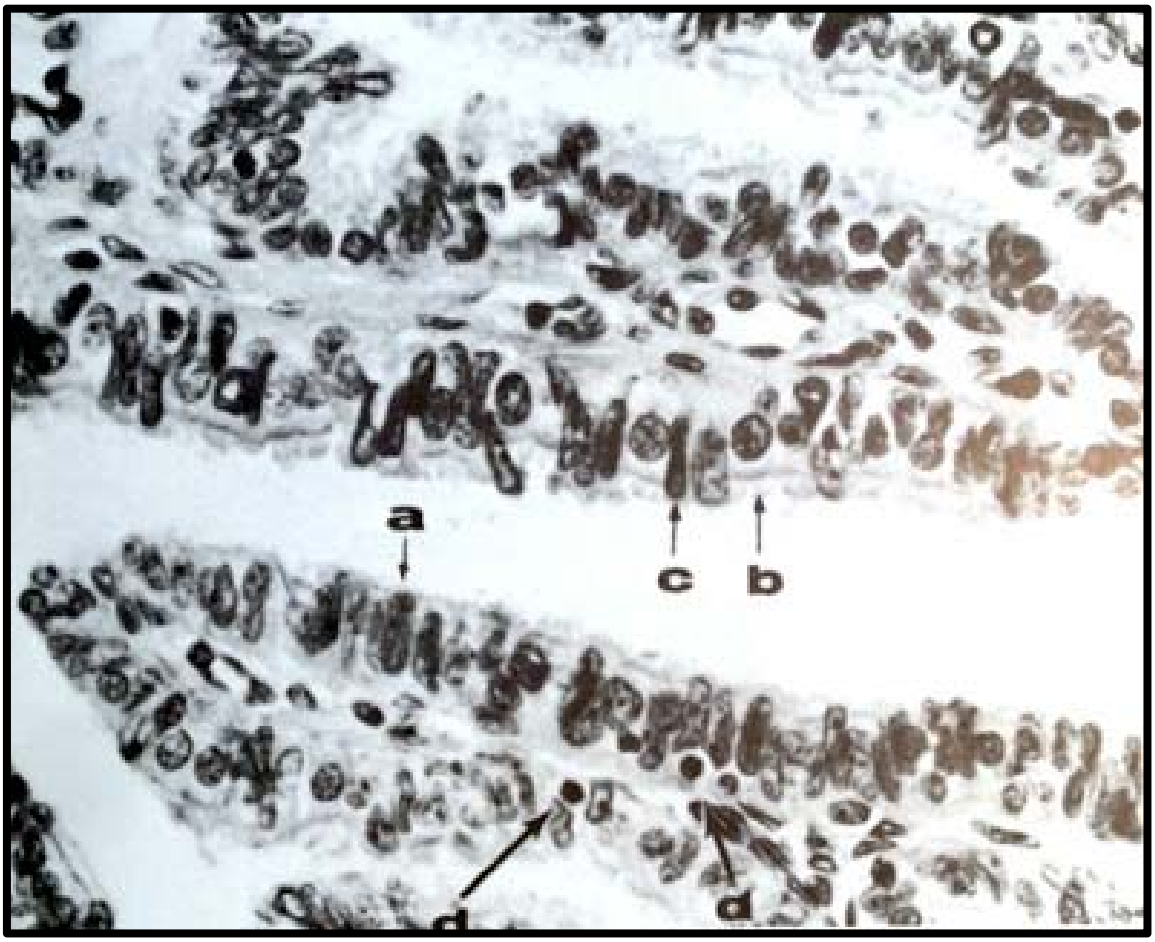

Figure 5 Cellular structure ampullary mucosa - higher magnification.

a – secretory cells
b – ciliated cells
c – intercalary or “peg” cells
d – indifferent cells

(Adapted from Pauerstein et al 1967)

Cilia are approximately 7 microns long with 2 central and 9 double lateral filaments. The secretory cells have more granular cytoplasm with nuclei in various positions in the cells.

Thinner “peg” cells are long and slender with only a small amount of cytoplasm surrounding the dark nucleus. They have been considered to be either regressed or precursor secretory cells. Basal cells may act as granulocytes and increase in number with tubal inflammation (Schulte et al 1985).

The epithelial mucosa arranged in longitudinal folds is highly developed in the thinner walled ampulla with many secondary and tertiary folds impinging on the tubal lumen. The folds are much reduced in the thicker muscular isthmus.

Ciliated cells are more frequent at the apex of mucosal folds, with secretory cells more prominent between and at the base of mucosal folds.

The epithelium is lower in the secretory phase with broader and lower ciliated cells, whilst secretory cells enlarge and bulge into the tubal lumen and often taller than ciliated cells (Figure 6).

Consensus has not been reached on the extent of morphological and distribution changes in ciliated cells during the menstrual cycle.

Novak and Everett (1928) first described an oviductal cycle in women and the influence of oestrogen (E_2) and progesterone (P_4) was documented by Christoph and Dennis (1977). Cyclical variation in human tubal morphology has been reviewed by Lyons et al (2006).

Cyclic ciliation/deciliation is not seen in the human oviduct however some renewal of ciliated cells does occur. Verhage et al (1979) described cilia changes in the fimbriae and ampulla with 12-14% loss of cilia in the luteal phase. Overall P_4 is thought to cause deciliation whilst E_2 has the opposite effect. The epithelial layer is

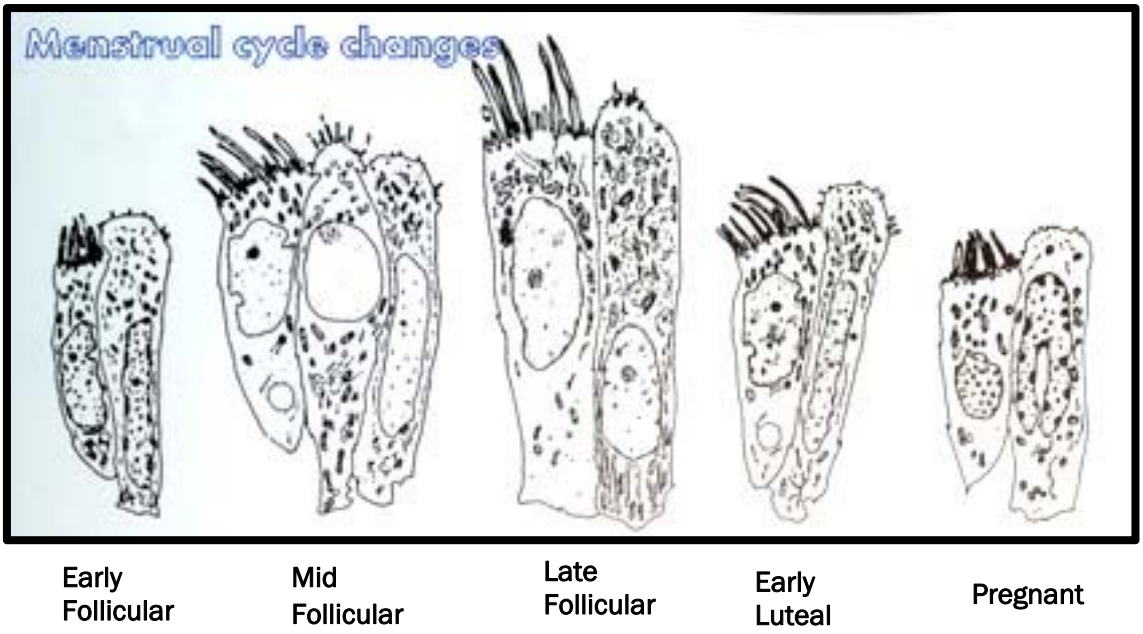

Figure 6 Fallopian tube epithelium at different stages of the menstrual cycle.

Hypertrophy and differentiation occurs in the follicular phase whilst atrophy and de-differentiation occurs during the luteal phase.
Ciliated cells are observed during the mid-follicular phase when morphological alterations also occur in the nucleus and cytoplasm (Verhage et al 1979).

(Adapted from Frederiks 1986)

made up of secretory and ciliated cells which are low in height at menstruation, increasing in size in the proliferative phase, reaching maximal height in the peri-ovular period when both cell types are of equal size.

Pauerstein and Eddy (1979) demonstrated that at ovulation secretory cells reach peak activity discharging cellular content thus reducing in height relative to ciliated cells. During the luteal phase both cell types reduce in height and partial deciliation occurs. Deciliation was demonstrated to occur in the puerperium (Seki et al 1978), with progressive reduction in ciliated cells. Nonciliated cells were in resting stage at term pregnancy with resumption of secretory activity during the puerperium.

Verhage et al (1979) and Donnez et al (1985) showed that E_2 stimulates epithelial cell hypertrophy, secretion and ciliogenesis whilst atrophy and deciliation were associated with luteal phase P_4 levels.

1.2.3. Surface electron microscopy

The tubal epithelium displays regional variation with the majority of cells being ciliated at the fimbriae with 4-6 cells forming groups (Figure 7). Non ciliated groups of 6-10 cells are found in the troughs of tubal folds with more along the crests. More extensive fields of low ciliated cells are seen in the ampulla composed of 10-20 single polyhedral cells. The ciliated cells are mostly solitary but in places occur in pairs.

1.3. Ovarian Steroid Hormone Effects on Oviduct Function

The movement of cilia, muscular contractions and oviductal fluid - all play a part in oocyte and embryo transport under the influence of E_2 and P_4.

Hafez and Blandau (1969) comprehensively reviewed the function of the

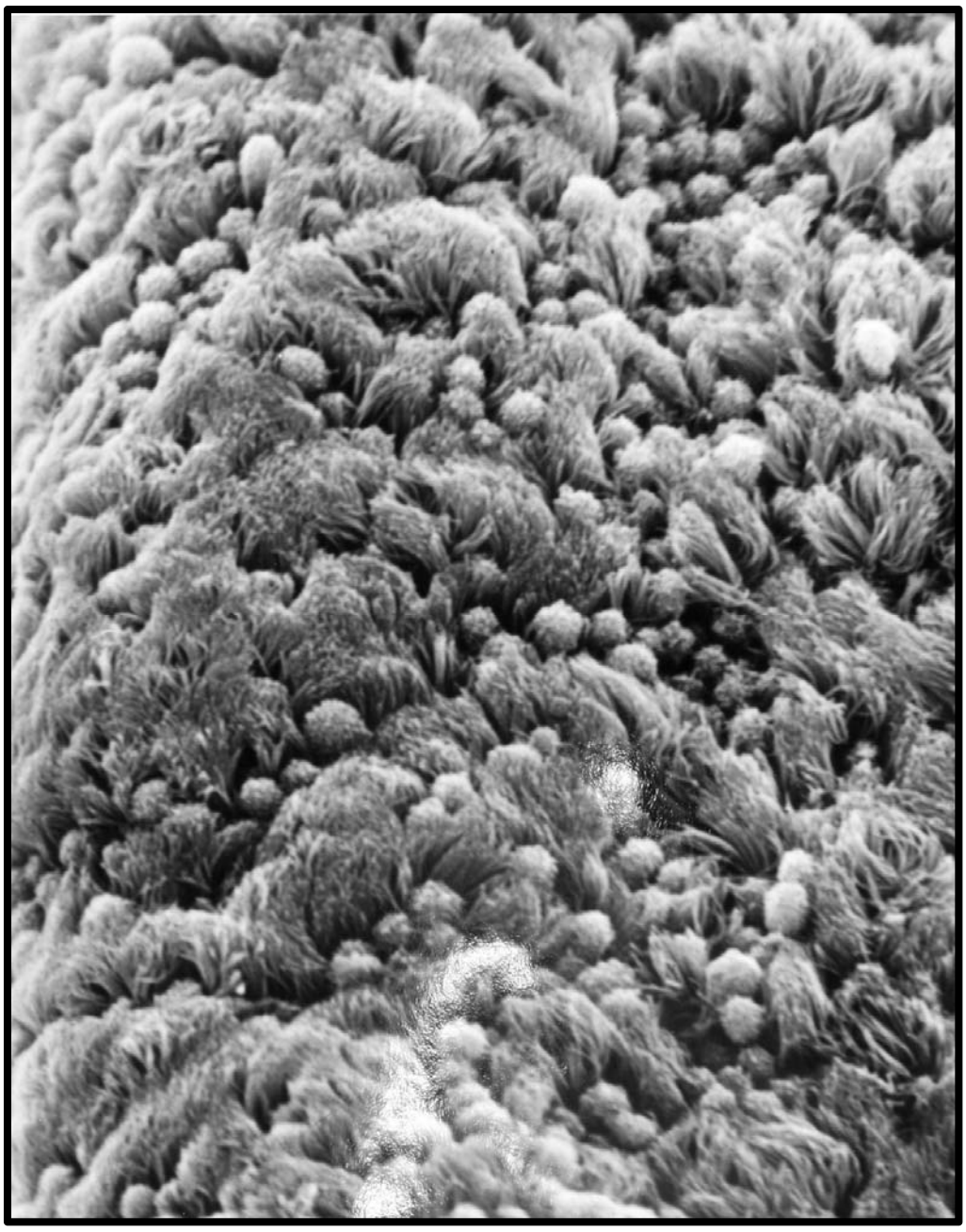

Figure 7 SEM summit ampullary fold with non-ciliated dome shaped secretory cells and clusters of ciliated cells (x7000).

mammalian oviduct highlighting the interplay between ovarian steroids and function. It has been demonstrated in several species that ovarian steroids influence growth, differentiation, fluid secretion and muscular contractility in the oviduct (Brenner and West, 1975).

The major functions of the oviduct include:

- Oocyte capture by the infundibulum
- Sperm and oocyte transport to the fertilisation site
- Storage and capacitation of sperm
- Facilitate fertilisation
- Support early embryo development
- Timely embryo transport to the uterine cavity

A review of the influence of steroid hormones on key oviduct functions now follows.

1.3.1. Rabbit ovulation, ovum and embryo transport

Endocrine and paracrine pathways play a significant role in oocyte/embryo transport, which currently have not been fully elucidated. The varying morphological features of different regions of the oviduct suggest that the cilia play a key role in oocyte transport to the fertilisation site, while muscular contractility regulates embryo transport to the uterine cavity. Ciliary beat, muscular contractions and oviduct fluid all contribute to oocyte/embryo transport coordinated by ovarian steroids, E_2 and P_4 (Lyons et al 2006). At ovulation the action of follicular fluid prostaglandins induce tubal twisting by the meso-salpinx muscle to bring the fimbria in opposition to the ovulation site on the ovarian surface (Okamura et al 1977). Prostaglandins also increase fimbria and tubo-ovarian ligament contractility thus influencing the ovum pick-up mechanism (Sterin-Speziale et al 1978).

In the human, oocyte retrieval studies from excised Fallopian tubes following the

mid cycle LH surge have indicated that the cumulus oocyte complex (COC) reaches the AIJ fertilisation site within minutes and fertilization occurring 8 hours after ovulation. The embryo remains in the ampulla for 72 hours (Croxatto et al 1978) as a result of increased muscular tone and the presence of thick mucus obliterating the isthmic lumen (Jansen 1980). Rising P_4 levels following ovulation cause disappearance of luminal mucus and relaxation of isthmic muscular tone allowing the embryo to enter the uterine cavity.

1.3.2. Oviduct ciliary activity

Oocyte and embryo transport are influenced by ciliary activity (Jansen 1984) under the control of steroid hormones (Paltieli et al 2000) and IL-6 (Papathanasiou et al 2008). Following ATP-hydrolysis the cilia anoxeme is made-up of sliding microtubules. The sliding action is converted to bending by radial spokes. At ovulation cilia strokes are oriented and synchronised to beat towards the uterus (Gaddum-Rosse et al 1973). At this time the correctly assembled oocyte cumulus matrix adheres to the fimbrial cilia completing oocyte pick up and passage through the tubal osteum (Talbot et al 2003).

The adhesive attraction between the COC and cilia increases 10-40 fold at the opening of the Fallopian tube in hamsters, compared to other tubal segments. After compaction of the cumulus mass in the ostium, the adhesive strength reduces thus facilitating onward passage towards the ampulla (Lam et al 2000). Lyons et al (2002) found that in the human ciliary beat frequency (CBF) in the fimbriae was greatest in the secretory phase of the menstrual cycle following release of follicular fluid at ovulation. This fluid contains high levels of E_2, P_4 and prostaglandins (McNatty et al 1979; Seibel et al 1984) which are transported with the oocyte in the tubal infundibulum. CBF is influenced not only by changes in ovarian hormones but

also by products of oviduct cells and the embryo which have been demonstrated to occur in in vitro studies (Croxatto 2002) and likely to occur in vivo. These products include prostaglandins PGE, PGF2α (Verdugo et al 1980) and platelet activating factor (PAF). E_2 and P_4 modulate ciliated cell response to these products with P_4 decreasing and E_2 increasing CBF. Familiari et al (1996) have suggested that fibronectin, tenascin-c and laminin produced by cumulus cells in the mature COC, also play a role in promoting adhesion.

The predominant influence of ciliary action on tubal transport of oocytes and embryos has been supported by β-adrenergic suppression studies not affecting oocyte transport in the ampulla. The fact that women with immotile cilia syndrome (Kartagener's) are sub-fertile also supports this belief.

The importance of ciliary activity is further emphasised by the finding of subfertility being associated with deciliation occurring after tubal infection (Patton et al 1989). Waves of muscular activity however contribute and appear to be most important in the transport of the early embryo through the isthmus (Kolle et al 2009; Lyons et al 2006).

1.3.3. Oviduct muscular contractility

Smooth muscle contraction and relaxation influence gamete transport to the ampullary fertilisation site and transport of the early embryo to the uterine cavity (Croxatto 2002; Hunter 2011). This activity is controlled by fluctuating levels of E_2 and P_4, the adrenergic-noradrenergic system and the action of prostaglandins. Following fertilisation, the zygote pauses at the AIJ before being transferred to the uterine cavity (Halbert et al 1988). During this interval the embryo is exposed to factors in oviduct fluid to undergo further development (Lyons et al 2006). In the rabbit ongoing progress of the embryo is associated with a decrease in amplitude of

muscular contractions at the isthmus and an increase in P_4 levels (Spilman et al 1978; Weinberg and Pauerstein 1980). Oocyte transport in this species is affected by castration and administration of E_2 (Boling and Blandau, 1971).

In vitro studies by Helm et al (1989) on human tissue have demonstrated a relationship between plasma steroid levels and tubal contractility in menstrual cycles, pregnancy, menopause and oral contraceptive use (Owman et al 1996). Mild activity was noted in the peri-ovulatory period, increasing at ovulation when E_2 levels are high. This change is thought to influence the fimbriated end of the oviduct to come in contact with the ovary and aid oocyte pick up (Okamura et al 1997).

E_2 and P_4 have also been shown to affect the norepinephrine (NE) content, turnover, and activity of its synthetic enzyme and release of NE from adrenergic nerve terminals of the human uterus and oviduct (Marshall 1981).

Hormonal activity is mediated by influencing a shift in the population of alpha and beta adrenergic receptors within oviduct muscle (Hodgson and Pauerstein 1977). Alpha adrenergic receptors stimulate, whereas beta adrenergic receptors inhibit contractions (Samuelson and Sjostrand 1986). Adrenergic innervation is most pronounced in the circular layer of the isthmus (Brundin 1964) affecting constriction at the UTJ. With rising P_4 levels post ovulation, oviduct motility is inhibited, allowing passage of the embryo to the uterine cavity. Adrenergic neurone inhibition does not prevent transport or decrease fertility, (Eddy and Pauerstein 1980), suggesting that steroid hormones and tubal products play a more significant role in modulating embryo transport.

These include prostaglandins (Wanggren et al 2006; 2008), nitric oxide (NO) (Ekerhard et al 2004), prostacyclin (Arbab et al 2002), and cyclic AMP (Lindblom et al 1980).

Oxytocin (OT) and hCG have an inhibitory effect on human and porcine Fallopian tubes (Gawronska et al 1999, Jankovic et al 2001). Secreted hCG may act through hCG receptors or by up-regulation of Cox-2 expression and subsequent increase in PGE2 (Han et al 1996).

NO is involved in regulation of smooth muscle tone, platelet aggregation, cell growth, apoptosis and infection controlled immune reactions (Affanas'ev 2007). It is produced from L-Arginine by three isoforms of nitric oxide synthetase (Moncada and Higgs 1993). NO has a relaxing effect on oviduct smooth muscle. It is known to cause an increase in ciliary beat in the airway and may have a similar action on the oviduct. Oviduct musculature appears to act as a single unit, exhibiting slow conductivity, velocity of speed ranging from 1-9 mm/sec (Daniel et al 1975; Talo and Pulkkinen 1982).

Overall oviduct muscle producing oscillating movements in the isthmus slow or accelerate embryo passage through the oviduct.

It is of interest in relation to this thesis that a loss of nerve fibres has been demonstrated to occur in the Fallopian tube isthmus of women with hydrosalpinx (Zhu et al 2013). Loss of adrenergic innervations was also demonstrated to occur in the artificially induced hydrosalpinx in the rabbit (Donnez et al 1985).

The technique of live cell imaging and digital video-microscopy has provided new insights on sperm transport, fertilisation, gamete and embryo maternal interaction in near in vivo conditions (Kolle 2012).

1.3.4. Oviduct cytokines

A review by Robertson et al (2015) summarises how cytokines from the uterus and oviduct play a pivotal role in embryo development by influencing cellular events including cell survival, metabolism, division and differentiation.

A balance exists between survival agents GM-CSF, CSF1, LIF, HB-EGF and IGF2 and apoptosis-inducing factors TNFα, TRAIL and IFNγ influencing embryo implantation and development or demise.

Cytokine synthesis is regulated by steroid hormones reaching a peak during the luteal phase and at the stage of early phase embryo development.

Epithelial cells in the oviduct and endometrium secrete cytokines into the luminal cavity where the embryo develops (Hannan et al 2011). Epithelial produced colony stimulating factors (CSF1 or MCSF), leukemia inhibiting factor (LIF), transforming growth factor β, IL-11, IL-13, IL-15, IL-18, have all been progressively identified since the early 1990s.

Epithelial cells also produce chemokines and vascular endothelial growth factors (VEGFs) which regulate local leucocytes and vasculature to influence developing embryos (Hannan and Salamonsen 2007).

Apart from ovarian steroids individual cytokine genes are sequentially activated by interaction between cytokine transcription factors and steroid hormone receptors. Cytokines, growth factors, and prostaglandins derived from leucocytes, seminal fluid and the conceptus all modulate epithelial cell cytokine expression (Robertson et al 2015).

In the human, E_2 regulated cytokines (CSF1, GM-CSF, TNFα) increase during the proliferative phase with a decline occurring in the luteal phase.

It has been postulated that hydrosalpinx fluid may either contain cytokines that are embryotoxic or embryotrophic and that a distortion of their balance may negatively impact on embryo implantation rates. The concentrations of IL-8, IL-12, ILα, TNFα, TGFβ2, LIF, GM-CSF are increased in hydrosalpinx fluid, with some variation between specimens, suggesting up-regulation of these cytokines by endothelial

tissue (Strandell et al 2004). A significant increase in TNFα and IL-2 was confirmed by Bao et al (2017), in coloured as opposed to colourless hydrosalpinx fluid. Enzyme- linked immunosorbent assay (ELISA) demonstrated significant increased TNFα and IL-2 expression levels in coloured hydrosalpinx fluid compared to clear fluid, however this study also confirmed that these cytokines are not associated with embryo toxicity (Bao et al 2017). The significance of coloured fluid remains unexplained and further studies are required on this issue.

1.3.5. Oviduct fluid and secreted products

The oviduct's role in successful implantation includes aiding the survival and selection of gametes prior to fertilisation, the fertilisation process itself and the subsequent support of the embryo for its synchronous passage to the uterine cavity. These processes are affected by the secretion and movement of fluid produced by the epithelial secretory cells.

Oviduct secretory activity and volume of oviduct fluid varies quantitatively and qualitatively throughout the menstrual cycle with highest secretory rates occurring during oestrogen dominance. Oviduct fluid is abundant at mid-cycle and believed to play a role in the fertilisation process, and early cleavage. It is abundant in mucoproteins, steroid hormones, growth factors and enzymes (Aviles et al 2010). The composition of tubal fluid varies in relation to secretion by the epithelial cells and selective transudate from blood vessels (Leese 1988; Leese 2001).

A protein (present in animal species and humans) oviductal glycoprotein1 (OVGP1), is only produced by epithelial secretory cells and binds to the zona pellucida of preimplantation embryos and the acrosome region of sperm in some species (Boatman and Magnoni 1995). Because of its introduction by E_2, and presence in the oviduct in the periovular period, it is believed to be of importance for sperm

binding and reducing polyspermy thus increasing fertilisation rate (Buhi 2002; McCauley et al 2003).

Cyclic changes in oviduct fluid have been observed during the natural menstrual cycle of rhesus monkeys (Mastroianni et al 1961) and women (Lippes et al 1972) and during the estrous cycle of various species (Hamner 1973).

In the cycling ewe, Warnes (1976) found that daily oviduct secretion rates up to 1 ml/ oviduct during the luteal phase rising to over 2 mls per oviduct at oestrus, result from direct effect of ovarian hormones on cell proliferation, vascular supply to the oviduct and transudation, with oophorectomy resulting in reduced secretion whereas oestrogen administration restoring normal secretion.

E_2 has been shown to change the physical properties of oviduct secretion causing a decrease in viscosity thus influencing both ciliary activity and ovum transport (Jansen 1984).

Bovine studies have indicated that oviduct fluid influences early zygote development and early cleavage (Hunter 2011). Georgiou et al (2007) have demonstrated that oviduct epithelium alters its secretory protein profile during the pre-ovulatory interval in response to the presence of sperm or oocytes.

Overall it is apparent that oviduct and embryonic development are influenced by steroid hormones and the activity of cumulus and granulosa cells which accompany the oocyte at ovulation (Hunter 2011). P_4, locally synthesised by these cells during and following fertilisation, has been proposed to influence oviduct epithelial secretion and the developing embryo (Hunter 2011). These cells have also been proposed to influence steroid secretion from the ipsilateral ovary (Hunter et al 2005).

1.3.6. Human Fallopian tube fluid proteome in normal and hydrosalpinx tubes

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Due to the difficulty in obtaining human Fallopian tube fluid, the composition and proteome of this fluid have not been studied as extensively as the animal studies described above. A recent study has compared the proteome of lavages from healthy Fallopian tubes, at reversal of sterilisation surgery and hydrosalpinx affected tubes, by needle aspiration at laparoscopy. The study identified a total of 519 proteins (Yohannes et al 2019). An overabundant protein in the hydrosalpinx fluid was mesothelin (MSLN) a C-terminal product of the parent 69 kDA protein encoded by the MSLN gene. Other significant findings included:

- Upregulation of complement pathway proteins
- Increase of proteins related to reactive oxygen species (ROS)
- Decrease of proteins that metabolise hydrogen peroxide
- Upregulation of proteins involved in activation of immune cell trafficking, inflammation and macrophage phagocytosis
- Downregulation of proteins related to inhibition of biological processes in previous dot point

Immunoblot analysis verified the abundance of MSLN previously only associated with epithelial malignancies and CD59. This finding indicates that hydrosalpinx tubes share molecular targets with tubal and /or ovarian cancer pathology which are associated with overexpression of MSLN and binding partner mucin MUC1, MUC5B and MUC16.

Immunochemistry confirmed prevalence of MSLN staining in the mucosal epithelium of hydrosalpinx tubes.

From a diagnostic viewpoint it was of considerable interest that MSLN ELISA plasma assay confirmed a significant higher level in hydrosalpinx patients than healthy controls. The majority of patients (6 of 9) who had elevated MSLN in tubal fluid had higher level of plasma MSLN.

Two thirds of up-regulated proteins in hydrosalpinx fluid were associated with inflammation with subsets involved in neutrophil activity, binding of phagocytes and macrophage phagocytosis. Other subsets are related to complement component, including CD55 and CD59. Complement has been associated with anti-inflammatory action affecting tissue repair (Gaipl et al 2001) but is also known to be pro-inflammatory causing necrotic cell death (Koski et al 1983). It is currently unknown if the overabundance of complement protein associated with hydrosalpinx is protective or causing tubal damage (Yohannes et al 2019).

The down-regulation of detoxification enzymes involved in the ROS defence mechanism which is associated with oxidative stress may be part of the inflammatory process associated with infertility (Calagero et al 2017; Murphy et al 1998) and possibly explain the embryo-toxicity of hydrosalpinx fluid (Bedaiwy et al 2002).

Detoxification enzymes within the Fallopian tube are critical for reproduction. Induced by the presence of gametes in the oviduct, intratubal expression of SOD1, GSTP1, TXN, and PRX1 are important for fertilisation, embryo cleavage and/or embryo transit. The presence of the antioxidant enzyme SOD1 in lavages from healthy Fallopian tubes in both phases of the menstrual cycle contrasted with a significant decline of SOD1 and other redox pathway enzymes, GSTP1, TXN, and PRD6 in hydrosalpinx fluid. Down-regulation of these enzymes may result in oxidative stress affecting gamete viability, and embryo development. This may be the basis of the observed embryo toxicity and implantation failure in hydrosalpinx affected patients having IVF cycles (Bedaiwy et al 2002; Chanr et al 2004).

The deleterious effects of hydrosalpinx fluid on natural and assisted conception is thus linked to several mechanisms including:

- Presence of molecules toxic to gametes and embryos (Bao et al 2017, Zeyneloglu et al 1998)
- Abnormal utero-tubal flow cusing impaired fertilisation, endometrial receptivity and implantation (Cheng et al 2015)
- Toxic effect of hydrosalpingeal fluid on endometrium (Strandell et al 2002).

The increased risk of miscarriage and reduction in clinical pregnancy rates (Bao et al 2017, Chu et al 2015, Strandell et al 1994) has generated support for treating hydrosalpinges before commencing ART.

1.3.7. Oviduct prostaglandins and platelet activating factor

Studies in several animal species have indicated that prostaglandins (PGs) and platelet activating factor (PAF), mediate communication between embryos and the oviduct epithelium. The phospholipid ligand PAF and its receptor are expressed in human Fallopian mucosal epithelial cells have been demonstrated to influence ciliary beat frequency and smooth muscle contractility (Velasquez et al 1995; Velasquez et al 2001).

In vivo experiments have demonstrated that E-series PGs relax, whilst F-Series PGs stimulate muscular activity in humans, subhuman primates and rabbits. Ovarian steroids affect oviduct muscle response to PGs, P_4 increasing the response to PGE1, whilst decreasing the effect of PGF2α. PGF levels increase in oviduct tissue following ovulation induction in the rabbit.

The pre-ovulatory rise in E_2 is believed to stimulate PGF in oviduct tissue with peak values occurring when the oviduct isthmus is most sensitive to stimulation by PGF2α. Closure of the isthmus and prevention of premature passage of oocytes to the uterine cavity is influenced by this sequential activity of PGs.

The post-ovulatory rise in P_4 probably influences a decrease in tissue PGF, decrease the response to PGF2α and increase the response to PGF1 allowing progressive embryo movement through the isthmus to the uterus.

1.3.8. Oviduct and microorganisms

Most cases of tubal factor infertility can be attributed to untreated ascending sexually transmitted diseases which cause inflammation, cellular damage and fibrosis.

The pathogenic bacteria most commonly associated with tubal factor infertility and pelvic inflammatory disease are Chlamydia trachomatis and Neisseria gonorrhoea. Other sexually transmitted organisms including Mycoplasma genitalium, Trichomonas vaginalis and other microorganisms within the vaginal microbiome are less likely to be causative agents (Tsevat et al 2017).

Women with laparoscopic evidence of tubal damage and their husbands were shown to have significantly higher prevalence of IgG antibody to gonococcal pili in their sera, than infertile women with normal tubes. After controlling for age, the laparoscopic positive women also had significantly higher incidence of past gonorrhoea and chlamydia infection (Swasdio et al 1996).

In vitro studies on human Fallopian tube epithelium have demonstrated that Neisseria gonorrhoea endotoxin induces reduction and finally cessation of ciliary activity. Both secretory and ciliated cells are affected, however the latter are predominantly targeted with ultimate sloughing of tubal epithelium (McGee et al 1981). The bacterium generates gonococcal lipopolysaccharide and monomers of peptoglycan which cause sloughing within 12 hours of addition to Fallopian tube cultures (Cooper et al 1990). Mucosal infection is associated with increasing concentrations of TNFα, the level of which correlate with the extent of ciliated cell

loss (McGee et al 1999) and epithelial damage.

Upper genital tract infection by Chlamydia trachomatis also causes a cytotoxic effect on tubal mucosa with loss of microvilli, disruption of cell junctions and rupture of epithelial cells (Cooper et al 1990).

The 60 KDa Chlamydial heat shock protein (hsp60) has been implicated as the major antigen causing a pathogenic immune response resulting in permanent tissue scarring (Burnham and Peeling 1994). The level of antibodies to hsp60 also correlates to the presence of tubal related infertility (Toye et al 1993, Ault et al 1998). Chlamydial hsp60 causes a humoral and cell mediated immune response producing activated macrophages secreting pro-inflammatory cytokines TNFα, interlukin-1b and interferon (IFN-γ) (Ojcius et al 1998).

Chlamydial LPS and pro-inflammatory cytokines induce the production of endothelial cell adhesion molecules leading to permanent tubal damage (Kelly et al 2001). These molecules mediate the extravasation of lymphocytes promoting an inflammatory response (Butcher et al 1999).

The associated production of prostaglandins, collagen, intergrins and transforming factor B, contribute to fibrosis and scarring (Perfettini et al 2000).

Epithelial damage of varying degree particularly at the fimbriated end, is a consequence of salpingitis. With agglutination of the fimbriae and hydrosalpinx formation mucosal damage becomes more evident.

The mucosal surface may develop areas of denudation and a decrease in ciliated cells, often devoid of, or having single cilia. Early reports indicated little change in ciliated cells (Patek and Nilsson 1977; Fedele et al 1984). Later studies able to determine accurate cell counts by planimetric methodology, confirmed that deciliation varying from 6-60% was commonly seen in thin walled hydrosalpinges (Vasquez et al, 1983).

Non ciliated cells are often devoid or have decreased density of microvilli. Donnez et al (1984), have suggested that restorative surgery of hydrosalpinges is often unsuccessful because of irreversible damage to the epithelium.

Apart from deciliation the other significant repercussion of tubal inflammation is fimbrial adherence resulting in distal tubal occlusion (Patton et al 1987, 1989; Westrom and Wohler-Hanssen 1993). Following occlusion and despite patency to the uterine cavity, fluid accumulation and an increase in intraluminal pressure occur, causing further tubal damage.

According to Donnez (1984), deciliation is permanent, however the function of remaining unaffected cilia may not be compromised (Cooper et al 1990).

Tubal infertile women with positive chlamydia serology have not been shown to have changes in ciliary beat frequency unless distal tubal obstruction has occurred (Patton et al 1989).

Escherichia coli cause dose dependant deciliation in rabbits whilst secretory cells have loss of microvilli. Complete regeneration however occurs in this species by 8 weeks post infection (Laufer et al 1984).

The overgrowth of vaginal anaerobes and facultative bacteria (Bacterial Vaginosis) has been associated with pelvic inflammatory disease, particularly in the presence of other sexually transmitted diseases (Soper et al 1994) and is significantly associated with tubal factor infertility (Gaudoin et al 1999).

Microorganisms such as Mycoplasma hominis, Mobiluncus and Bacteroides ureolyticus, have also been implicated in sub-clinical pelvic inflammation and release of endotoxin which causes ciliated cell destruction or changes in ciliary beat activity.

Uteroglobulin a low molecular weight protein with anti-inflammatory properties is present in Fallopian tube epithelial cells and has been shown to have increased

expression in women with pelvic inflammatory disease and hydrosalpinges (Quintar et al 2008).

Early studies indicated that hydrosalpinx fluid was bacterial culture positive (Ng et al 2001). A more recent study using laparoscopic aseptically collected hydrosalpinx fluid instead of transvaginal collection showed no bacterial growth. The Chlamydia antigen positive rate in this fluid was > 60% (Bao et al 2017).

1.3.9. Oviduct nucleic acids

The simultaneous measurement of RNA and DNA content in tissue has facilitated assessment of cell mass and replication (Cheek 1971).

The relative RNA content as a function of total cellular activity is a constant value characteristic for specific cell lines during exponential growth. The quantity of DNA per cell is a specific constant in somatic tissue, whereas the quantity of RNA varies with the rate of protein synthesis.

The ratio of RNA/DNA has therefore been used as a useful indicator of cellular wellbeing as it provides an index of protein synthesis per cell.

Measurement of cell DNA and RNA can provide an assessment of deviation from unbalanced cell growth (Traganos et al 1982). Endogenous ovarian steroids affect RNA and protein metabolism in the oviduct and endometrium. E_2 rapidly increases the rate of protein synthesis and mean cell content of RNA at pro-oestrus in the ewe whilst P_4 was found to have no effect (Miller 1976).

Chapter 2 Steroid Hormone Receptor Physiology

2.1. Steroid Hormones and their Receptors

The steroid hormones (SHs) found in animal tissues are a group of polycyclic lipid soluble and hydrophilic biomolecules derived from cholesterol. They include adrenocortical and glucocorticoid hormones, oestrogens (estrone, estriol, E_2), androgens and P_4, which share a similar structure with sufficient differences to account for their different biological activity.

They influence growth, development and differentiation in the reproductive tract, central nervous system and adrenal system as well as many other tissues.

E_2, the most significant oestrogen, affects the development and maintenance of reproductive organs, regulation and function of the cardiovascular, musculoskeletal, and central nervous system (Gruber et al 2002; Nelson and Balun 2001). E_2 is also involved in the initiation and development of target tissue malignancies (Gruber et al 2002).

SHs circulate in blood free or bound (5%) to specific high affinity sex hormone binding globulin (SHBG). When they exceed the binding capacity of SHBG they bind to low affinity serum albumin. A dynamic equilibrium exists in the extracellular compartment between the bound and free forms. The free and albumin bound fractions are biologically the most important, able to diffuse through capillary walls and lipid plasma membranes.

SHBG binds to a specific cell membrane receptor (SHBG-R) and activates adenylate cyclase so increasing intracellular cAMP.

Hormonal activity in the cell of a target organ is dependent on the amount of free hormone gaining access to the cell. Once in the cell steroid hormones diffuse freely,

(Danzo and Joseph 1994; Joseph 1994; Porto et al 1995; Fortunati et al 1998).

Within cells binding proteins may be non–specific with low affinity and often present in high concentration or specific binding receptors exhibiting high binding capacity and the ability to alter cell function.

Before 1984, intracellular un-ligand steroids prior to hormone treatment were thought to be located predominately in the cytosol. On exposure to hormone, they were primarily detected in the nuclear fraction which led to the proposal that unoccupied receptors were in the cytosol until ligand binding resulted in translocation to the nucleus (Seiler-Tuyns et al 1986).

SHs are now known to be generally cytoplasmic or nuclear able to initiate signal transduction. In the cytosol, SHs move to the cell nucleus on activation or unligand remain in the cell nucleus waiting for the SH to enter and activate them. Uptake in the nucleus is facilitated by a nuclear localization signal found in the hinge region of the receptor. This region is covered by heat shock proteins (HSPs) which bind the receptor until the specific SH is present. SH-SHR (steroid hormone - steroid hormone receptor) binding leads to SHR conformational change, releasing the HSP so that the receptor and bound hormone enter the nucleus to act upon transcription (see detailed description in 2.1.3).

The presence of E_2 receptor (ER) and P_4 receptor (PR) in cytosol are now thought to occur following tissue homogenisation and centrifugation used in isolating the receptor proteins (Talwar et al 1964).

Recent studies support the belief that localisation and movement of ER occurs between nuclear, mitochondrial and cytoplasmic components (Monje and Boland 2002; Cammarata et al 2004; Chen et al 2004; Cammarata et al 2005; Levin 2005; Gavrilova-Jordan and Price 2007; Chen et al 2007).

Immuno-histochemical localisation studies have now shown that ER and PR

are found in the nucleus in the absence of hormonal treatment (Mangelsdorf et al 1995). In the human, 48 receptors make up the steroid receptor super-family (SRF) which includes the gonadal and adrenal steroids as well as non- steroidal ligands for thyroid hormone, Vitamin D, retinoid and fatty acids (Table 1).

SHRs are members of nuclear receptor subfamily 3 (NR3), that includes receptors for E_2 (group NR3A) and 3-ketosteroids (group NRC3C). It is also known that several G protein-coupled receptors and ion channels act as cell-surface receptors for certain SHs.In 1996, a second E_2 receptor, ERβ complementing ERα was discovered in animals and humans, forming Class 1 of the SRF (Mosselman et al 1991).

2.1.1. Molecular structure of ER and PR

Nuclear hormone receptors acting as transcription factors are distributed throughout the body and influence cellular processes (Evans 1988). E_2 effects on tissue are mediated by ERα and ERβ. Knockout (KO) animal models have been used to determine E_2 mediated signalling in E_2 target tissues. ERα is the most influential receptor in the uterus, mammary and pituitary glands, skeletal muscle, adipose tissue and bone. ERβ mediates E_2 signalling in the ovary, prostate, lung, cardiovascular, and central nervous system. Since its discovery in 1996, the action and role of ERβ in the pathophysiology of E_2 signalling remains unknown (Harris 2007).

ERα and ERβ are encoded by two distinct genes expressed at varying levels in different tissues. The ERα gene is located on chromosome 6 and its mRNA is 6322 base pairs in length encoding 595 amino acids. The ERα gene is located on chromosome 6q24-27 and ERβ on chromosome 14q22-2.4.

Table 1 Steroid hormone receptor gene superfamily - mammalian tissues.

ERα
ERβ
hAR
PR
GR
MR
VDR
TRs – hTRβ, hTRα1, hTRα2
c-erb A1
Rev-erbA alpha (Rev-Erb)
c-erbA beta
C-erbA beta-2
RARs - alpha, beta, gamma
RXRs – alpha, beta/EAR-2, H-2RIIBP, gamma
PPARs alpha, beta/delta, gamma

(Gonadal and adrenal steroids, thyroid hormones, vitamin D, retinoic acid, fatty acids, and numerous “orphan” receptors unknown or being identified).

ERα, a glycoprotein of 66 kDa, is highly specific with high affinity for E_2 with low binding capacity. ERβ is biologically unstable and heat susceptible, however the ER-E_2 complex is relatively stable.

ERβ is widely expressed as a nuclear receptor in the central nervous, cardiovascular and genito-urinary systems, bone, kidney, lung and genital tract.

The PR gene is located on chromosome 11 and its cDNA is 3014 base pairs in length and encodes 933 amino acids. PR has a molecular weight of 20-110 kDa and is highly specific with high affinity for P4 and synthesis is regulated by E_2. ERα and ERβ are subdivided into six functionally distinct domains. The structural characteristics of ERα and ERβ demonstrating differences in specific domains are shown in Figure 8 and Figure 9 (Muyan et al 2015).

The ER gene has 8 exons, encodes a protein with six domains (A-F) which are responsible for binding ER antibody, DNA binding, heat shock protein and ligand binding.

The PR gene is located on chromosome 11 and its cDNA is 3014 base pairs in length and encodes 933 amino acids. PR gene has a molecular weight of 20-110 kDa and is regulated by E_2. PR has two functional isoforms, the more potent PR-A and PR-B which are encoded by the same gene 11q-13 PR, and is regulated by two promoters A and B transcribing into two mRNAs.

2.1.2. Menstrual cycle changes of ER and PR

Influenced by ovarian steroids, expression of ER and PR differ in different phases of the menstrual cycle. E_2 in the follicular phase maintains ER and PR at a specific level reaching a peak on day 14-15. The decrease in expression post ovulation is followed by a lesser increase attributed to the supressive effect of P4 in the luteal phase (Verhage and Jaffe 1983).

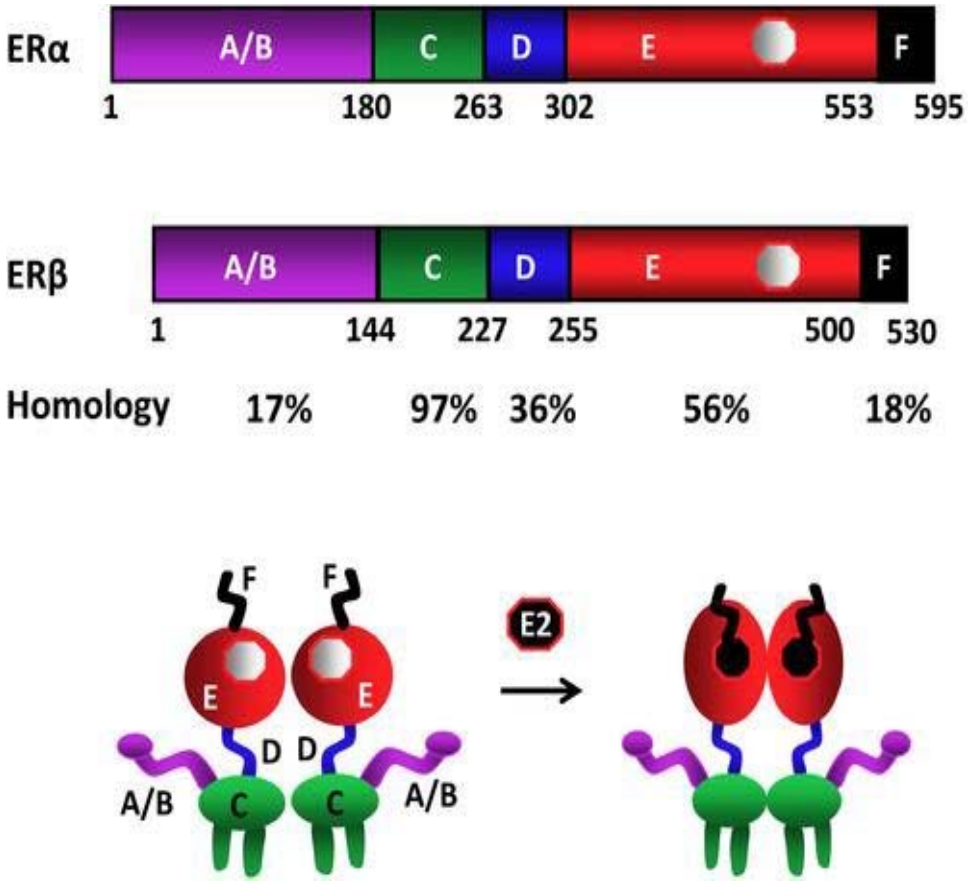


Figure 8 Schematics of ERα and ERβ structural regions.

ERα is composed of 595 amino acids, while ERβ contains 530 amino acids. The structurally distinct amino terminal A/B domains share a 17% amino-acid identity between the ERs. The near-identical central C region (97%) is the DNA-binding domain. The flexible hinge, or D, domain (36%) contains a nuclear localization signal and links the C domain to the multifunctional carboxyl terminal (E) domain, which shows 56% amino-acid homology between the ERs. The carboxyl-terminal F domain shares an 18% amino-acid identity. The ERs are dimers with or without the endogenous ligand, 17β-estradiol, the binding of which induces conformational changes in the receptors.

(Figure is modified from Muyan et al 2005, and reproduced from Yasar et al 2016)

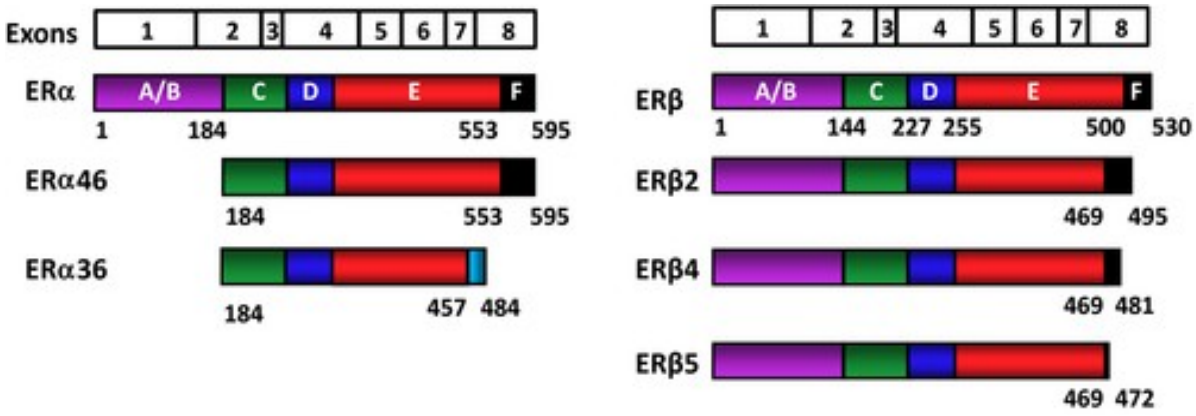


Figure 9 Schematic representation of ER isoforms.

The ERs are encoded by eight exons. The exon boundaries (lines) correspond to the regions of the ERs that are depicted with colored and labeled (A-F) structural domains. Estrogen receptor α is 595 amino acids long, whereas ERβ is composed of 530 amino acids. Estrogen receptor α46, which is generated by an alternative splicing event, lacks the amino-terminal A/B region and acts as a competitive inhibitor of ERα. Estrogen receptor α36 is generated from a promoter in the first intron of the ERα gene, together with alternative splicing events that result in a truncated protein with a unique 27 amino-acid carboxyl-terminus (light blue) that replaces the last 138 amino acids that are encoded by exons 7 and 8 of wild-type (WT)- ERα. Estrogen receptor α36 lacks both activation function (AF)1 and AF -2. Palmitoylated ERα36 localizes to the plasma membrane and cytoplasm, plays a role in the membrane-initiated 17β-oestradiol (E_2) signalling and adversely affects WT-ERα-mediated events. ERβ isoforms are formed from alternative splicing of exon 8, resulting in carboxyl-terminally truncated ERβ2, ERβ4, and ERβ5 variants with varying molecular masses. These variants cannot bind ligand and lack AF-2, but they could adversely affect E2 signaling by heterodimerizing with WT-ERα or WT -ERβ when co-synthesized.

(Reproduced from Yasar et al 2016)

2.1.3. Cellular events

Intracellular receptors modulate expression of target genes by acting as signal transducer and transcription factors. Key elements in the structure of receptor proteins induce binding with high affinity and specificity to respective ligands and cause discrete response elements within the DNA sequence of target cells to regulate gene transcription of DNA to messenger RNA.

The highly unstable un-ligated receptor either moves into the nucleus or associates with the hsp 90 complex of cytoplasmic proteins (Dittmar et al 1997). Hsp 90 a general molecular chaperone is involved in the folding of various proteins (Nover and Scharf, 1997). It also maintains the receptor in an inactive state until ligand binding occurs. (Pratt et al 2008; Pratt and Toft, 1997). Activation of adenylate cyclase which generates cAMP, also activates the protein kinase A (PKA) phosphorylation cascade.

Activation of the steroid receptor following ligand binding induces alterations in the conformation of the receptor protein allowing receptors to form homodimers and bind to specific hormone response elements (HREs) in the DNA regulatory region of the target gene. Bound to the HRE the ligand steroid hormone receptor induces a DNA bend and recruits co-activator proteins. DNA bending facilitates transcription complex binding and promotes DNA looping allowing single proteins to contact multiple DNA elements.

The hormone-receptor complex interacts with basal transcription factors to initiate the transcription complex recruiting the enzyme RNA polymerase 2 to begin transcription of the DNA sequence into mRNA.

Apart for ERα and ERβ regulating expression of target genes in the nucleus, another mechanism of E_2 action involves rapid activation of membrane associated ERs triggering a second messenger pathway over short time frames promoting genomic

events (Madak-Erdogan et al 2008). Various ovarian cells including granulosa cells and ovarian tumours, express P_4 binding proteins, PR membrane component-1 (CPGRMC1) (Peluso et al 2011).

The development of microarrays of human, mouse and rat gene and the identification of genes regulated by steroid hormone action using these assays has been a major advance in receptor physiology.

SHRs can also be studied by examining the RNA message for the receptor with radio-labeled DNA or RNA probes complementary to the SHR message. These techniques demonstrate that the gene for the steroid receptor protein is being transcribed. This methodology localising receptor messages shows good agreement with studies locating steroids by older methodology (see section 2.2).

Two methods by which steroid receptor mRNA can be studied are Northern blots and insitu hybridisation. The cloning and sequencing of SHRs have made these methods practical.

2.2. Measurement of Tissue Steroid Hormone Receptors

Assays require appropriate handling of tissue as receptors are in low number and unstable when isolated from cells.

Multiple point titration and sucrose density gradient analysis have been used in this study. Tissue homogenates are incubated with radioactive labelled high specific activity steroids in the presence or absence of corresponding excess unlabelled steroid or incubated with a fixed concentration of labelled steroid and increasing amounts of corresponding unlabelled steroid. Non- specific binding is subtracted from total binding to obtain high affinity specific binding.

Receptors and free hormones are separated by dextran coated charcoal followed by centrifugation and scintillation counting.

Scatchard (Scatchard 1949) analysis is used for calculating binding affinity (Kd) and number (binding capacity) of steroid hormone receptors. The dissociation constant for hormone binding to receptors is in the 10^{-11} to 10^{-9} M range.

Immunohistochemical methodology has been used to study ER and PR in the Fallopian tube and uterus (Amso et al 1994) at different stages of the menstrual cycle and menopause. The study confirmed increase in ER in the follicular phase to peak at mid cycle and then decline in the luteal phase.

Immunocytochemical assays with monoclonal antibodies to ER and PR protein, have been used extensively to detect receptors in normal and malignant breast tissue (King et al 1985; Berger et al 1989). Review of the literature and a series presented by Aldred et al (1990) confirmed the clinical relevance of this methodology.

It is reassuring, relevant to the receptor data presented in this thesis, that the above review confirmed concordance between immunochemical and biochemical methodology to evaluate receptor levels in tissue.

Hormone receptor definition has been facilitated by molecular biology techniques which have enabled the structure and mechanism of action of steroids and nuclear receptors to be better understood. Specific DNA, RNA and protein sequences, their size and presence previously detected by Southern, Northern, and Western blotting techniques, are currently quantitated by qPCR and next generation sequencing.

Molecular DNA cloning allows isolation of a single segment of DNA, so that following isolation and purification, structural and functional analysis can occur.

RNA extracted from hormone responsive tissue or cells enables cloning of hormone receptors to be performed. ERα and β have been studied in the rat reproductive

tract using immunohistochemistry demonstrating that protein levels of ERα and ERβ have distinct specificity for tissue and cell types similar to their respective levels of mRNA as assessed by in situ hybridization (Wang et al 2000).
The electrophoretic mobility shift assay is used to examine binding of nuclear/ steroid receptor to DNA (Kling et al 1996). Antibody addition to the steroid /nuclear receptor identifies the protein in the protein/DNA complex.
To evaluate how a particular ligand or steroid receptor affect transcription, experiments are performed on cultured mammalian cells containing or having the receptor introduced using an expression vector encoding the cDNA of the receptor with a viral promoter. These techniques quantify how different ligands and DNA sequences affect the transcriptional response of a given steroid receptor.

2.3. Progesterone and PR

Progesterone (P_4), primarily produced by the ovarian granulosa-lutein cells in response to the pre-ovulatory LH surge, is involved in oocyte maturation, sperm capacitation, fertilisation and transport of gametes and embryos (Murray et al 1995, Hunter 1998). P_4 actions are mediated by the ligand activated, nuclear transcription factor, P_4 receptor (PR) (Tsai and O'Malley 1994). Binding of P_4 to PR induces a conformational change and dimerization of the receptor which associates with specific coactivators and general transcription factors (Rowan and O'Malley 2000; Weigel 1996). The activated complex bound to P_4 elements in the promoters of target genes, results in gene transcription (McKenna and O'Malley 2002).
In mammals, the PR gene gives rise to two protein isoforms, PR-A found predominantly in the nucleus, and PR-B found in the cytoplasm of hormone free cells. The latter has been implicated in non-genomic actions of P_4 (Booiaratanakornkit et al 2008).

Unlike PR which mediates its effects via genomic mechanisms, membrane PR (mPRs) are protein cell surface receptors which alter cell signalling via modulation of intracellular signalling cascades (Thomas and Pang 2012). They are classified as mPRα, mPRβ, mPRy, mPRδ and mPRε. mPRs mediate important physiological functions in male and female reproductive tracts, immune system, neuroendocrine tissue and liver. They are involved in the neuroprotective and antigonadotropic effects of P_4 and also breast and ovarian cancer (Thomas and Pang 2012).

PR is known to control many reproductive processes including oocyte competence and successful oocyte release at ovulation (Robker and Richards 2000).

PR is predominantly found in the epithelial cells but also in smooth muscle cells. PR has also been isolated in interstitial Cajal-like cells in the human Fallopian tube (Cretoiu et al 2009).

Many PRα and PRβ promoted P_4 actions are too rapid to conform to genomic mechanisms, and it seems that P_4 can exert fast cell-surface initiated activity through activation of mPRs and their associated intracellular pathways (Thomas and Pang 2012). mPRα binds P_4 in vitro and is an intermediary in the induction of oocyte maturation and regulation of uterine functions in humans (Thomas 2008). mPRβ is a molecule implicated in cumulus-oocyte expansion by regulation of exocytosis (Qui et al 2008). mPRy is expressed in female mouse ovary and oviduct and immunohistochemical studies have demonstrated association with the apical membrane of ciliated cells in human Fallopian tube (www.uniprot.org 30-10-2015). mPRδ is a protein encoded by PAOR6 gene and mPRε by PAOR9gene.

2.4. Role of PR in Regulating Oviduct Function

The presence of PR in ciliated cells of luminal epithelium suggests a role for P_4 on ciliary activity and oocyte and embryo transport. Ciliary beat frequency (CBF) is

regulated by cyclical fluctuations in E_2 and P_4 (Nishimura et al 2010), with E_2 accelerating and P_4 decelerating activity (Mahmood et al 1998; Nakahari et al 2011).

With reference to muscular contractility, tone increases in the follicular phase and decreases in the luteal phase of the cycle. E_2 results in muscular contraction and P_4 in relaxation (Helm et al 1982; Wanggren et al 2006 and 2008). Studies inhibiting progestogen activity by RU486 seem to indicate that muscular activity induced by the prostaglandins E_2 and F2a are regulated by both progestesterone and PGR (Wanggren et al 2006 and 2008).

The role that P_4 and PR play in oviduct fluid physiology has so far been inadequately studied.

2.5. Hypotheses and Thesis Aims

For a clinician the issue of hydrosalpinx related infertility and the low live birth rate following remedial surgery remains unresolved. A hydrosalpinx that is fibrotic, surrounded by extensive adhesions to surrounding structures is clearly irreparably damaged. For hydrosalpinges of normal appearance, mildly dilated and free of adhesions, a minimal risk surgical procedure, salpingostomy, should have real prospect of restoring normal function. In light of this prospect, my concern was that IVF was fast becoming not just one, but the only solution for resolving tubal factor infertility. Reproductive endocrinology rather than surgical skill, was now playing the major role in resolving this issue, despite the acknowledged advances in surgical technique.

The role of the clinician was diminished, regulated to “egg collection” with the most critical stage of treatment now occurring in the embryology laboratory.

This study was initiated to explore the potential for steroid hormone manipulation to enhance the success rate of salpingostomy in restoring tubal function when inflammatory and other diseases had caused epithelial damage. It thus began with an investigation of steroid hormone receptors (SHR) in normal and hydrosalpinx affected oviduct tissue in the rabbit and human Fallopian tube.

Steroid hormones (SH) are known to play a fundamental role in oviduct physiology, gene expression, pathology, and function, including oocyte transport, and fertilisation. The review by Brenner (1975) and more recently by Ghazal et al (2014) have emphasised all these points. In the experimental animal, fluctuations in steroid hormone levels are reflected in changes in the concentration of tissue receptors specific to each steroid. For example, in the oviduct, E_2 domination causes an increase in E_2 / P_4 receptor ratio (ER/PR) whereas P_4 causes both receptors to decrease (Brenner and West 1975). A link between these changes with function is seen, for example, in both the cat and gilt, where E_2 has been shown to cause reciliation and secretory activity in the oviduct, whereas P_4 has the opposite effect (West and Brenner 1976; Nayak et al 1976). In the rabbit, E_2 binding to its specific receptor was demonstrated to be essential for oviduct contractility (Clemens et al 1975).

2.5.1. Main hypothesis

The main hypothesis of this thesis was to assess if surgical treatment of hydrosalpinx (salpingostomy) has a complementary role to IVF in the management of hydrosalpinx related infertility.

2.5.2. Sub-hypothesis and specific aims

The sub-hypothesis of this thesis was that measurement of ER, PR, morphology, tubal intraluminal pressure, tubal blood flow and biochemical parameters in

normal, hydrosalpinx Fallopian tubes, normal and induced hydrosalpinx rabbit oviducts, would provide an understanding of tubal pathology and suggest prognostic factors for successful remedial surgery in the human.

To support the main hypothesis, the following specific studies were performed.

Study 1

To study the pathophysiological effect of hydrosalpinx formation in women by measurement of morphological, biochemical, SHR levels. The same parameters were studied in the artificially induced hydrosalpinx in the rabbit

i) at different stages of hydrosalpinx formation

ii) following reversal of hydrosalpinx surgery

iii) following pregnancy after reversal of hydrosalpinx

Study 2

Using the rabbit hydrosalpinx model, assessment was made on how progressive oviduct distension affects the following:

i) Intraluminal pressure

ii) Oviduct blood flow

iii) Fertility following reversal surgery

Study 3

To study, by long term follow up (Adelaide Cohort Study 1980-1986) if a relationship exists between live birth rate and mucosal morphology (mean cilia index) in micro-biopsy specimens taken during remedial salpingostomy surgery.

2.6. Reviews and Thesis Presentation

Review of Fallopian tube normality, patency and function

A review of the literature was undertaken to determine if salpingoscopy and other

tubal investigation procedures may be comparable to morphology (MCI) in predicting live birth rate following salpingostomy.

Review of hydrosalpinx surgery - history, incidence, results

The aim of this review was to assess the role of tubal surgery before and following the introduction of IVF. The results of the Adelaide Cohort study are compared to results in the literature. Consensus opinions on the future of tubal surgery is also reviewed.

Thesis presentation

The thesis will be presented in the following format:

- Structure, function, morphology mammalian oviduct
- Steroid hormones and other factors affecting oviduct function
- Steroid hormone receptor physiology
- Main and sub-hypothesis, supporting studies and reviews
- Steroid hormone receptors in normal and hydrosalpinx human Fallopian tube
- Steroid hormone receptors in normal hydrosalpinx and reversed hydrosalpinx rabbit oviduct
- Rabbit fertility following reversed hydrosalpinx
- Rabbit physical studies - intraluminal pressure and oviduct blood flow
- Human Fallopian tube biochemical studies –normal and hydrosalpinx
- Rabbit oviduct biochemical studies – oestrus, hydrosalpinx and reversed hydrosalpinx
- Human Fallopian tubes morphology –menstrual cycle, pregnancy, menopause, reversal sterilisation and ectopic pregnancy
- Rabbit oviduct morphology – oestrus, hydrosalpinx and reversed hydrosalpinx
- Review of Fallopian tube normality, patency and function
- Adelaide Cohort study - surgical management hydrosalpinx

- Thesis conclusions
- Review of surgery for hydrosalpinx - history, incidence and results
- Management of hydrosalpinx infertility: findings and recommendations

Chapter 3 Steroid Receptor Measurement in Human and Rabbit Oviducts

3.1 Introduction

The review of the steroid hormone (SH) effects on oviduct function has confirmed that E_2, P_4 and their receptors are fundamental to oviduct physiology, gene expression and pathlogy. Evidence for the important role that SHs play in influencing growth, differentiation, fluid secretion and muscular contractility was first extensively reviewed by Brenner (1975) and more recently in relation to egg transport and fertilisation by Ghazal et al (2014). In the experimental animal, it has been demonstrated that in keeping with fluctuations in SHs, the concentration of specific hormone receptors similarly change. E_2 domination causes increase in ER and PR, whereas P_4 influence causes the amount of both receptors to decrease (Brenner and West 1975). In the cat and rhesus monkey, E_2 cause atrophy, reciliation and secretory activity in the oviduct, whereas P_4 has the opposite effect (West and Brenner; 1976; Nayak et al 1976). In the rabbit, E_2 binding to its specific receptor was demonstrated to be essential for oviduct contractility (Clemens et al 1975).

At the commencement of this thesis, for the human Fallopian tube there had been no reports of simultaneous measurement of both cytosolic and nuclear for either E_2 or P_4.

In 1980 this was the first study of simultaneous measurement of both cytosolic and nuclear receptors for E_2 and P_4 in human and rabbit oviducts. This study was presented at an international meeting (Petrucco and Winston 1980). Information on intracellular receptor distribution in that era was limited to the indirect evidence of Robertson et al (1975). A new initiative in relation to PR was the use of radioactive

label of the parent compound.

3.2 Methodology

Human tissue samples

Fallopian tubes were obtained from women in the premenopausal age group, undergoing hysterectomy for non malignant pelvic disease. Their menstrual history was noted and stage of menstrual cycle confirmed by measurement of serum E_2/P_4. Approval for collection of human Fallopian tubes was obtained from the University of Adelaide Ethics Commitee.

Rabbit tissue samples

New Zealand White sexually mature virgin rabbits weighing greater than 3.5 Kg. were used in animal experiments. Analysis of serum E_2 and P_4 indicated that all animals were in persistent oestrus, with no evidence of ovulation or pseudopregnancy. Tissue was trimmed of fat, mesentery and peritoneal layers using an operating microscope and kept frozen in liquid nitrogen until use within 3 months. Ethics approval for rabbit experiments was obtained from the Surgeon General in the UK, and Institute of Medical and Veterinary Science in Adelaide.
On the day of experiment, tissue was thawed, weighed and finely minced using a scalpel. All procedures were performed at 0-4^{o}C unless otherwise stated. Tissue was homogenised in 9 vol. (w/v) of buffer (0.25M sucrose, 10mM Tris-HCL, pH 7.6, 30% glycerol) using a x 10/20 homogeniser fitted with a type 10 shaft (Scientific instrument Centre, London, U.K.) with 3 bursts of 10 second duration at setting 8 with a 30 second interval between each burst. A crude nuclear pellet was obtained by centrifugation of the homogente at 800 x g for 10 min in an MSE Coolspin (Fisons, Crawley, U.K.). The pellet was resuspended to the original homogenate volume and washed three times by centrifugation and resuspension. The first supernatant obtained after centrifugation at 800 g for 10 min was further

centrifuged at 200,000 x g for one hour in an MSE Prepspin 65 (Fisons, Crawley, U.K.) to give a cytosol fraction.

Exposure of tissue to E_2

1) In vitro. Rabbit oviducts were collected, rinsed in saline to remove excess blood and incubated for 15 min in 5ml. of medium 199 (Wellcome, Beckenham, U.K.) containing unlabelled E_2 at a final concentration of 15nM and previously equilibrated at 37°C in an atmosphere of 95% O_2, 5% CO_2. Subsequently tissue was transferred to 5ml. of medium 199 without any added E_2 and incubated for a further 30 min. Preparation of tissue was as descibed above.
2) In vivo. Twenty-five micrograms of E_2 in 1 ml. of compound sodium lactate B.P. was injected intravenously 15 min. prior to laparotomy and excision of oviducts. The tissue was rinsed in homogenisation buffer and then processed for receptor analysis.

Measurement of ER

Cytosol (0.2ml., duplicate samples) was incubated with a range of concentrations (0.5-30nM) of (2, 4, 6, 7(n)-^{3}H) 17βE_2 (specific activity 85-110 Ci/mmol Radiochemical Centre, Amersham, U.K.) in the absence and presence of a 200-fold excess of diethylstilboestrol, at 4°C for 18-24 hours. Macromolecular bound radioactivity was separated from free steroid by chromatography on columns (aprox. 6x 0.6 cm) of Sephadex LH-20 (Pharmacia (G.B.) Ltd., London, UK.). Specific binding was computed as the difference in (^{3}H) E_2 binding in samples with or without competitor.

Nuclear samples (triplicate 0.2ml. of resuspension) were incubated in the presenceof radioactive E_2 (0.5-30 nm). Parallel samples contained an equivalent concentration of (^{3}H) E_2 together with 200-fold excess of diethylstilboestrol. The incubation was terminated by the additionb of 1 ml. of buffer containing 1% BSA.

Hydroxyhepatite was also included (5mg/ml) to increase the bulk of the suspension and to trap any solubilised receptor before centrifugation. The pellet was washed twice further with 1 ml of buffer containing 1% BSA only and finally with buffer alone. Radioactivity in the washed pellet was extracted into ethanol (2x 1 ml) and transferred to scintillation vials for monitoring. Binding of a saturating concentration of (^{3}H)-E_2 to nuclei was investigated at 4^{0}C, 30^{0}C, 37^{0}C for periods of 0.5-8 hours.

Measurement of PR

Radioactive P_4 (1, 2, 6, 7, (n)-^{3}H P_4 (specific activity 80-110Ci/mmol) was used for the assay of nuclear and cytosol P_4; a 50 fold excess of cortisol was included with all samples to minimise the binding of radioactive P_4 to corticosteroid binding globulin. Specific P_4 assessed on the basis of competition by a 100-fold excess of P_4 which was included in a parallel series of samples.

Cytosol (0.2ml) was incubated at 4^{0}C for 18-24 hours with (^{3}H)-P_4 (1-40nM) and chromatographed on columns of Sephadex LH-20. Nuclear samples (0.2ml of resuspension) were incubated at 4^{0}C for 18-24 hours with (^{3}H)-P_4 (1-40nM). One ml of buffer containing 1% BSA, 0.2%, Triton X-100 was added and the samples allowed to stand for 10 minutes at 4^{0}C prior to centrifugatiion. This was followed by two washes with 1ml. of buffer containing 1% BSA and finally 1ml of buffer alone before extraction of radioactivity into ethanol (2x 1ml).

Measurement of radioactivity

Cytosol fraction: Material excluded from Sephadex LH-20 was monitored for radioactivity in 3 ml. of Unisolv (Koch-Light Laboratories Ltd. Colnbrook U.K.) at an efficiency of 40% in an Intertechnique SL3000 spectrometer with automatic quench correction. Nuclear fraction: Radioactivity was measured in 10 ml. of butyl-PBD toluene (4.5 g of butyl-PBD/L toluene) at an efficiency of 40% in the spectrometer described above.

Measurement of protein and DNA

Cytosol protein was measured by the method of Lowry et al (1951), and DNA content according to the method of Burton (1956).

Mechanical induction of hydrosalpinx in the rabbit

Following adequate oviduct exposure Weck (Medium) Hemoclips were applied at the ampullary-isthmic junction and at the terminal part of the infundibulum just proximal to the fimbriated end of the oviduct. The ampullary-isthmic junction was recognised by denoting the branching artery in the mesosalpinx supplying the mid portion of the oviduct at this site.

Depending on the experiment performed hydrosalpinx was induced in either one or both oviducts, with the unoperated oviduct left as control. Care was taken to occlude only the tubal lumen with minimal interference to blood vessels in the mesentery.

Oviducts were removed 8 weeks after initial surgery via repeat flank incisions. The widest transverse diameter of the occluded oviducts was accurately measured in situ before excision.

Microsurgical reversal of hydrosalpinx in the rabbit

Microsurgery was performed utilising the principles of atraumatic dissection with microsurgical instruments, fine uni or bipolar coagulation (Bipolar-Met and Valley Lab Instruments) to ensure perfect haemostasis. Hartmann's solution was used for isotonic irrigation of tissue. The Weck clips at each site were excised with magnification provided by a Zeiss-OPMI 1 operating microscope.

Oviduct segments were then rejoined in a two-layer anastomosis of muscularis (avoiding mucosa) and serosal layers using 8/0 and 10/0 Ethicon microsutures. To facilitate anastomosis between the markedly distended ampulla and the normal size isthmic segments, the technique described by Winston (1978) was used.

At completion of the two-site anastomosis, dilute methylene blue dye was injected in the lumen of the isthmic segment proximal to the two anastomosis sites to confirm that oviduct patency had been achieved.

Ethics approval for rabbit experiments was obtained from the Surgeon General in the UK, and Institute of Medical and Veterinary Science in Adelaide.

Methodology is described in greater detail in Appendices 1 to 4 (SHR methodology; preparation of human and rabbit tissue; rabbit surgery; ER and PR assays).

3.3. Results

3.3.1 Human tissue - E_2 and P_4 dissociation constants

3.3.1.1 Normal Fallopian tube

Initially a range of radioactive E_2 was used with the specific E_2 binding reaching saturation between 5 and 10nM (^{3}H)-E_2. The non-specific binding component increased linearly and accounted for 40-50% of total E_2 binding.

E_2 binding in cytosol and nuclear fractions from human Fallopian tubes are shown in Figure 10 and Table 2. Specific E_2 binding reached saturation between 5 and 10 nM (^{3}H)-E_2. Analysis of the saturation data (Scatchard 1949) revealed a single component, high affinity E_2 binding species (Figure 10B).

The binding data for PR is shown in Figure 11. Both cytosol (n=3) and nuclear fractions (n=3) contained a P_4 binder which was saturated with (^{3}H)-P_4 at 12nM. Scatchard analysis gave a Kd of 1.6 ± 0.9 x10^{-9}M and 2.0 ± 1.1x10^{-9}M (n=3, μ ± σ), (Figure 11B and Table 2).

There was no significant difference between the proliferative and secretory phase of the menstrual cycle for cytosolic ER. There was a significant difference however. in the level of ER which was two-fold higher during the proliferative than the secretory phase of the menstrual cycle. PR in the cytosol was significantly higher in

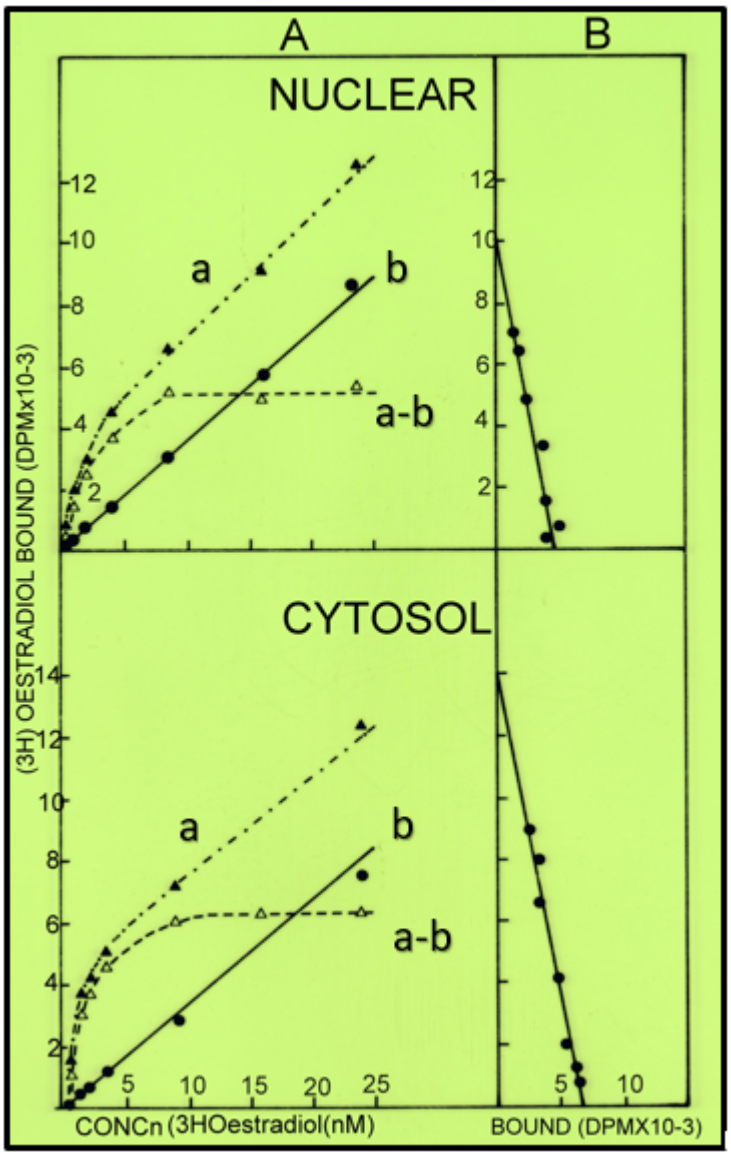


Figure 10 Binding of (^{3}H)-E_2 in nuclear and cytosol fractions of the human Fallopian tube.

A. Fallopian tube cytosol (n=5) and nuclear preparations (n=4) from separate Fallopian tubes were incubated with varying concentrations of (^{3}H)-E_2 in the presence and absence of DES (200 fold) for 18-24 hrs at 4^{0}C. Samples were removed and fractionated on Sephadex LH-20 columns (see Appendix 4). Each point on the graph is the mean of two measurements, variation <5%.
a (▲) radioactivity (high and low affinity binding)
b (●) radioactivity bound in the presence of DES (low affinity binding)
a-b (Δ) high affinity binding obtained by difference from TC (total counts) and NSB (non specific binding).

B. Scatchard plot analysis of the high affinity binding between (^{3}H)-E_2 and nuclear or cytosol ER. The linear relationship obtained from the plot ratio of bound/free radioactivity was used to calculate the dissociation constant (Kd) by the formula: slope = -(1/Kd). Constants (Kd) for nuclear and cytosol E_2 binding were respectively 1.8 ± 0.8 x10-9M (n=4) and 3.1 ± 1.5x10-9M (n=5, μ±σ), (Table 2).

Table 2 Dissociation constants (Kd) of ER and PR in human Fallopian tubes.

HUMAN FALLOPIAN TUBE DISSOCIATION CONSTANTS (Kd) of STEROID HORMONE RECEPTORS		
	μ ± σ	
ER - Nuclear	Kd = 1.8 ± 0.8 x 10^{-9}	n = 4
ER - Cytosol	Kd = 3.1 ± 1.5 x 10-9	n = 5
PR - Nuclear	Kd = 2.0 ± 1.1 x 10-9	n = 3
PR - Cytosol	Kd = 1.6 ± 0.9 x 10-9	n = 3

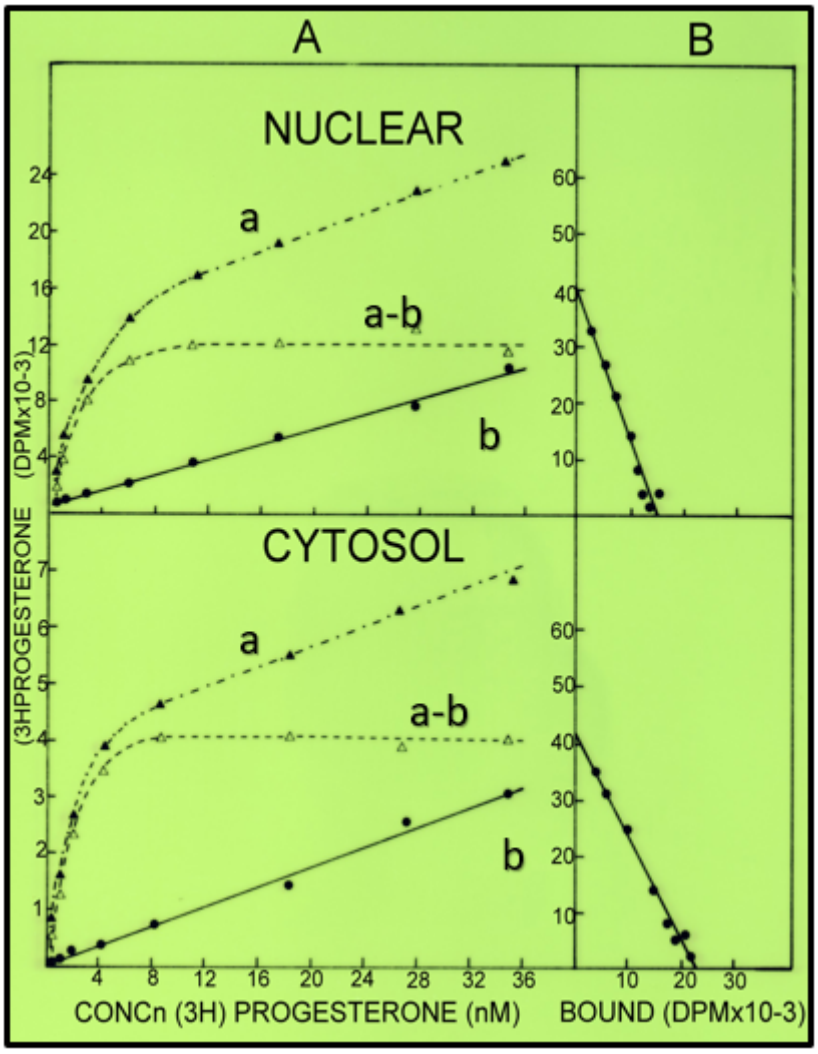


Figure 11 Binding of (^{3}H)-P_4 in nuclear and cytosol fractions of human Fallopian tube.

A. The binding of (^{3}H) P_4 in cytosol (n=3) and nuclear fractions (n=3) from separate Fallopian tubes were incubated with the indicated concentrations of (^{3}H)-P_4 in the presence and absence of competitor (100 fold unlabelled P_4) for 18-24 hr at 4^{0}C.Samples (0.2ml) were removed and fractionated on Sephadex LH-20 as described in Appendix 4. Each point is the mean of two measurements, variation < 5%.
a (▲) radioactivity (high and low affinity binding)
b (●) radioactivity bound in the presence of 200 fold competitor (low affinity binding.
a-b (Δ) high affinity binding obtained by difference TC (total counts) and NSB (non specific binding).

B. Scatchard plot analysis of the high affinity between (^{3}H)-P_4 and nuclear and cytosol PR. The linear relationship obtained from the plot ratio of bound/free radioactivity was used to calculate the dissociation constant (Kd) by the formula; slope= - (1/Kd). Kd was calculated by regression analysis to be 1.6± 0.9x 10^9 for nuclear and 2.0 ± 1.1x10^9 for cytosol PR.

the proliferative compared to the secretory phase whereas, there was no apparent difference in nuclear receptor content in the two phases of the menstrual cycle (Table 3).

In the P_4 dominant secretory phase, an inhibitory effect is observed on nuclear and cytosol E_2 binding.

The measurement of nuclear ER was studied at different incubation temperatures. Comparison of nuclear receptor measurement at 37^0C, 30^0C and 4^0C for various incubations is shown in Figure 12. At all times, measurements at 30^0C were higher than at 37^0C which was indicative of increased degradation of receptor at the higher temperature. Maximal values of nuclear receptor were obtained between one and two hours and remained constant for up to 8 hours. A similar profile was obtained at 4^0C suggesting that either exchange of (^{3}H)-E_2 with endogenous bound E_2 occurred at this temperature, or that a major proportion of the nuclear receptors were unoccupied or complexed with ligand whose affinity for receptor was lower than that for E_2.

The question of ligand exchange at 4^0C was considered. Nuclei were incubated at 4^0C for one hour with radioactive E_2 and receptor binding determined. Separate samples were further incubated after removal of radioactive E_2 with an excess of competitor DES. During a two-hour incubation period at 4^0C in the presence of competitor and after initial labelling there was no significant decrease in specific E_2 binding (Figure 13). In normal Fallopian tubes, PR in the cytosol was significantly higher in the proliferative compared to the secretory phase whereas, there was no apparent difference in nuclear receptor content in the two phases of the menstrual cycle (Table 3 and Figure 14). In the P_4 dominant secretory phase, an inhibitory effect is observed on nuclear and cytosol E_2 binding.

Table 3 Concentration of ER and PR in normal and hydrosalpinx human Fallopian tube at different stages of menstrual cycle. Confirmation by serum E_2/P_4 analysis (μ ±σ).

E_2 and P_4 binding in cytosol (fmols/mg protein) and nuclear (fmols/mg DNA) preparations of normal Fallopian tubes and hydrosalpinx at different phases of menstrual cycle (μ ± σ).

RECEPTOR	PROLIFERATIVE PHASE		SECRETORY PHASE	
	Normal (μ ± σ)	Hydrosalpinx (μ ± σ)	Normal (μ ± σ)	Hydrosalpinx (μ ± σ)
ER Nuclear (fmols/mg DNA)	3630 ± 907 n = 10	603 ± 405 n = 7 **	1665 ± 443 ϕ n = 6	429 ± 117 n = 3 *
ER Cytosol (fmols/mg protein)	31 ± 20 n = 11	14 ± 11 n = 7 +	21 ± 13 n = 6	9 ± 3 n = 4 ++
PR Nuclear (fmols/mg DNA)	3377 ± 1278 n = 5	2340 ± 1126 n = 5	3995 ± 2937 n = 5	2640 ± 1268 n = 5
PR Cytosol (fmols/mg protein)	110 ± 71 n = 9	41 ± 20 n = 5 #	46 ± 24 ϕϕ n = 4	26 ± 19 n = 4

* Significant difference from normal secretory $P < 0.005$
* Significant difference from proliferative hydrosalpinx $P < 0.04$
** Significant difference from normal proliferative $P < 0.001$
\+ Significant difference from normal proliferative $P < 0.05$
++ Significant difference from normal secretory $P < 0.05$
\# Significant difference from normal proliferative $P < 0.01$
Φ Significant difference from normal proliferative phase $P<0.02$
ΦΦ Significant difference from normal proliferative phase $P <0.05$

Student's t-test.

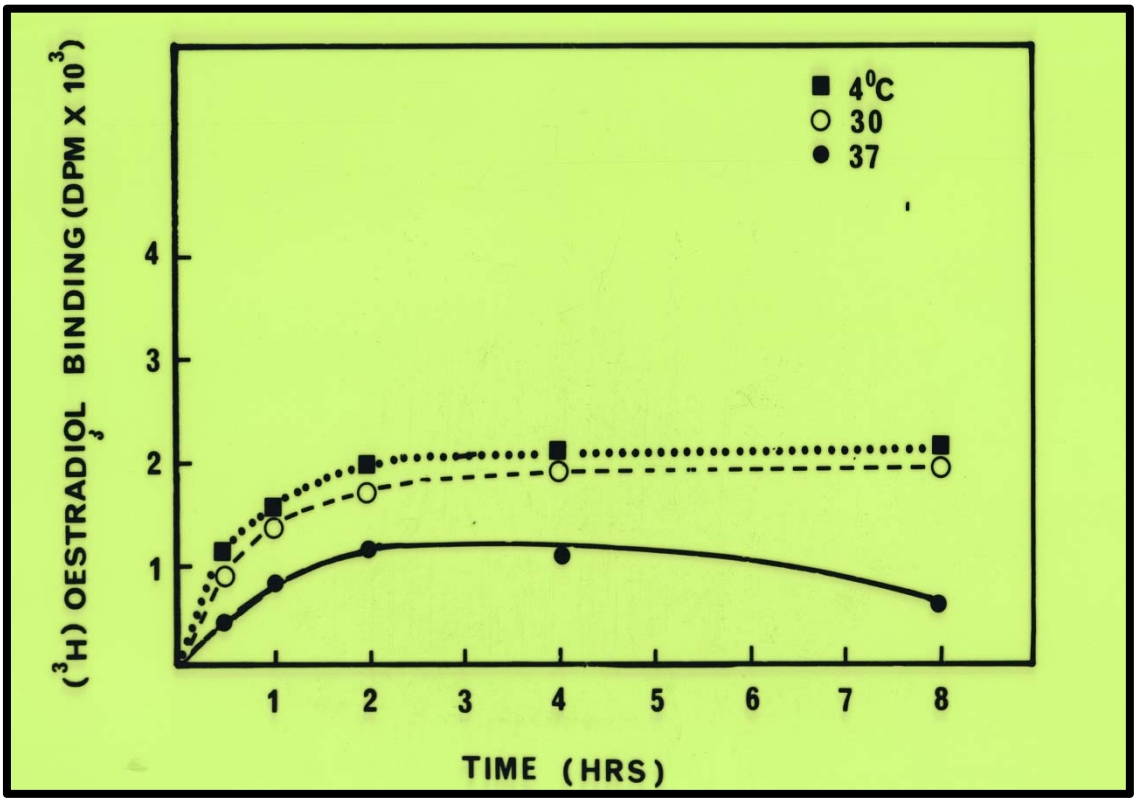


Figure 12 Time course of specific binding of (^{3}H)-E_2 in nuclear preparations at various temperatures.

At all times, E_2 measurement at 30°C were higher than at 37°C with maximal values obtained between 1 and 2 hours remaining constant for up to 8 hours. A similar profile was seen at 4°C.

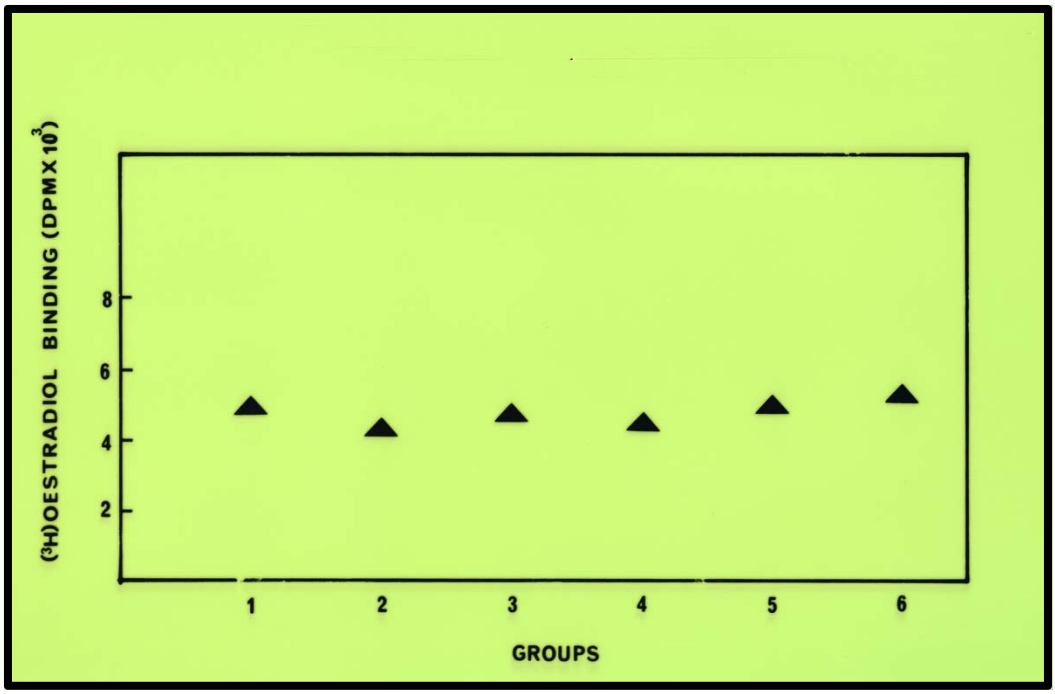


Figure 13 Nuclear incubation at 4⁰C for ER comparison measurement.

A comparison of ER measurement was made after further incubation following removal of radioactive E_2, with an excess of competitor, diethylstilbesterol (DES), at different time periods. After initial labelling during a 2 hour incubation at 4⁰C in the presence of DES, there was no significant decrease in specific binding.

Group 1	1 hr incubation – routine washes
Group 2	Buffer only wash and then as per Group 1
Group 3	4⁰C incubation with 200x DES for 15 min
Group 4	4⁰C incubation with 200x DES for 30 min
Group 5	4⁰C incubation with 200x DES for 1 hr
Group 6	4⁰C incubation with 200x DES for 2 hr

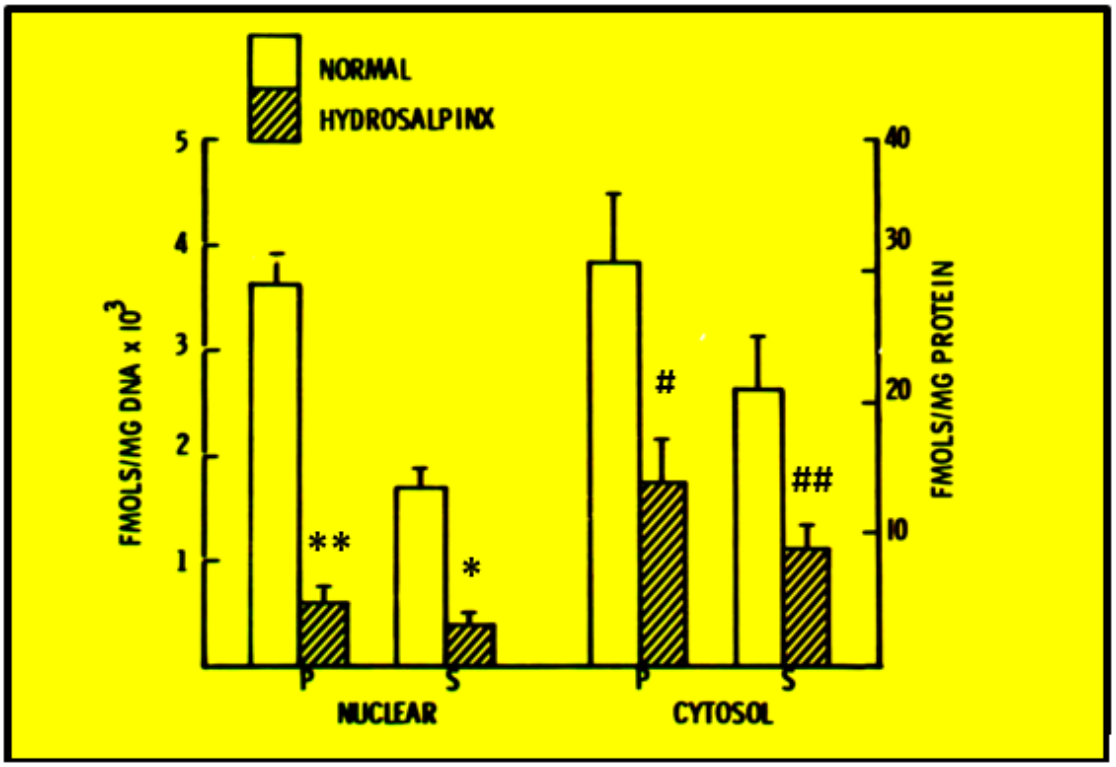


Figure 14 Nuclear and cytosol ER in normal human and hydrosalpinx affected Fallopian tubes.

* Significant difference from normal P< 0.005
** Significant difference from normal P< 0.001
\# Significant difference from normal P< 0.05
\## Significant difference from normal P< 0.05

A significant reduction is seen in both nuclear and cytosolic ER levels in hydrosalpinx compared to normal Fallopian tubes.

3.3.1.2 Hydrosalpinx

E_2 and P_4 binding in cytosol and nuclear preparations of hydrosalpinx tissue in the proliferative and secretory phase of the menstrual cycle are shown in Table 3, and Figure 14. E_2 binding in both nuclear and cytosolic fractions were significantly lower when compared with normal Fallopian tubes at both stages of the menstrual cycle. A significant reduction was also noted in PR in the cytosol of the proliferative phase of the cycle. In normal tissue there was no difference in ER cytosol but a significant increase was seen in nuclear ER (2x higher) in proliferative phase compared to secretory phase of menstrual cycle. In the PR dominant secretory phase, a significant difference was seen between proliferative and secretory phases of menstrual cycle.

A significant reduction is seen in ER cytosol and nuclear fractions in both phases of the menstrual cycle in hydrosalpinx compared to normal tissue. A significant reduction was also seen in PR cytosol in the proliferative phase of the cycle in hydrosalpinx compared to normal tissue.

3.3.2. Rabbit tissue - E_2 and P_4 dissociation constants

3.3.2.1 Oestrus tissue

11 rabbits were used for ER and PR analysis in oviduct tissue. Four animals were used for in vitro exposure to E_2 and P_4 for in vivo exposure to E_2.

The binding of (^{3}H)-P_4 in cytosol and nuclear preparations was similar to human tissue. The respective dissociation constants were 2.8 ± 0.2x10-9M (n=3) and 1.3 ± 0.2x10-9M (n=3, μ ± σ). See Table 4.

The binding of (^{3}-H)-E_2 was also similar to human tissue. Dissociation constants were 2.1 ± 2.1x10-9M (n=5) for nuclear and 1.9± 1.0x10-9M (n=5, μ ± σ) for cytosol preparations. See Table 4.

Table 4 Dissociation constants (Kd) for ER and PR in rabbit oviducts.

RABBIT OVIDUCT DISSOCIATION CONSTANTS (Kd) of STEROID HORMONE RECEPTORS		
	μ ± σ	
ER - Nuclear	Kd = 2.1 ± 2.1 x 10^{-9}	n = 5
ER - Cytosol	Kd = 1.9 ± 1.0 x 10^{-9}	n = 5
PR - Nuclear	Kd = 1.3 ± 0.2 x 10^{-9}	n = 3
PR - Cytosol	Kd = 2.8 ± 0.2 x 10^{-9}	n = 3

The effects of exposure of rabbit oviducts to E_2 in vitro and in vivo are seen in Table 5. A considerable depletion of ER cytosol was noted in both experiments. The expected increase in the ER nuclear fraction however was not apparent in vivo or in vitro despite depletion of the cytosolic ER. The procedure used in the in vitro incubation study minimises the possibility that residual steroid in the tissue subsequently interfered with the receptor assay. The similarity of depletion of cytosolic ER with both manipulations suggests that this was not an artefact related to masking of receptor by endogenous steroid.

3.3.2.2 Hydrosalpinx

Bilateral hydrosalpinx was created in 25 rabbits by application of Weck Tantalum clips to the utero-tubal junction and terminal infundibulum (Figure 15) as described in Appendix 3.

At 8 weeks following diameter measurement of hydrosalpinx (mm width at greatest diameter of hydrosalpinx) the left oviducts were excised for steroid receptor measurement, histology and surface electron microscopy (SEM) (Table 6). The right Fallopian tube had microsurgical reversal of hydrosalpinx (Figures 16 and 17) with subsequent excision at 12 weeks when the same parameters were measured. The average widest diameter of the mechanically obstructed ampulla was 10mm (8-14 mm). Clear fluid (6-10ml) was found in the distended oviducts (Figure 18).

3.3.2.3 ER and PR in increasing diameter hydrosalpinx

ER and PR binding in cytosol and nuclear fractions of varying size hydrosalpinx can be seen in Table 7.

When compared to normal oviducts from unoperated rabbits, the ER nuclear and cytosol levels were significantly lower than normal even in the smallest (less than 5 mm) hydrosalpinx. Nuclear receptor levels progressively decreased with increasing

Table 5 Concentration of nuclear and cytosol ER and PR in rabbit oviduct at oestrus and after hormonal manipulations.

RABBIT OVIDUCT				
	OESTRUS μ ± σ	PR μ ± σ	ER *in vitro*+ exposure to E_2 μ ± σ	ER *in vivo*++ exposure to E_2 μ ± σ
NUCLEAR (fmols/mg DNA)	5809 ± 569 n = 4	7339 ± 2098 n = 11	5331 ± 1791 n = 4	4167 ± 1334* n = 3
CYTOSOL (fmols/mg protein)	104 ± 24 n = 4	136 ± 26 n = 8	55 ± 17* n = 4	45 ± 8** n = 3

+ Oviducts removed and incubated with 15nM E_2 at 37^{0}C for 15 min before receptor determination.
++ Animals injected with 25ug E_2 prior to sacrifice and receptor determination.
* *P*< 0.01 compared with oestrus (Student's t-test)
** *P*< 0.001 compared with oestrus (Students t-test)

A significant decrease occurred in ER in cytosol with in vivo and in vitro exposure to E_2. A non significant reduction in of ER nuclear was seen with in vitro exposure to E_2. A similar but not significant reduction was seen in PR nuclear and cytosol preparations with invivo and in vitro exposure. The similarity of reduction of cytosol ER with both manipulations suggests that it was not an artefactrelated to masking of receptor by endogenous steroid.

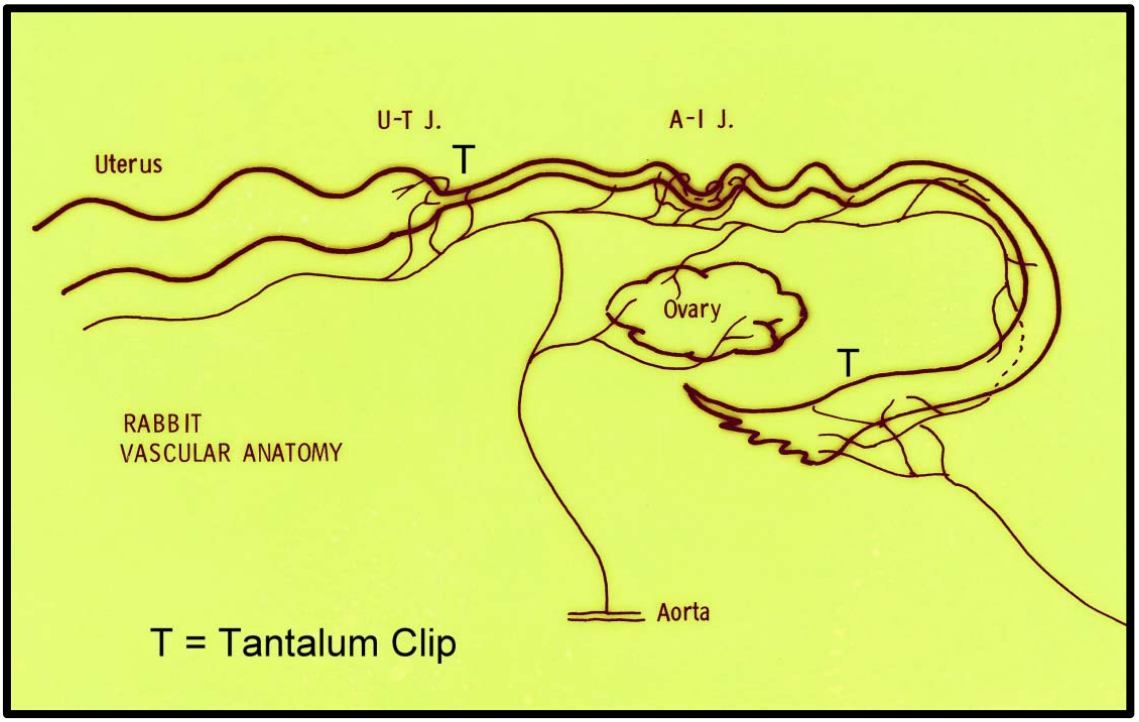


Figure 15 Surgical induction of hydrosalpinx in the rabbit.

Tantalum clip (Weck) application at ampullary-isthmic junction (AIJ) and infundibulum of oviduct. Care was taken not to occlude oviduct vasculature.

Table 6 Timeline rabbit surgery.

TIME	LEFT OVIDUCT	RIGHT OVIDUCT
0	Induction hydrosalpinx	Induction hydrosalpinx
8 weeks	Excision SR Histology / Scanning electron microscopy for MCI	Microsurgical reversal hydrosalpinx
12 - 18 weeks	-	Excision SR Histology / Scanning electron microscopy for MCI

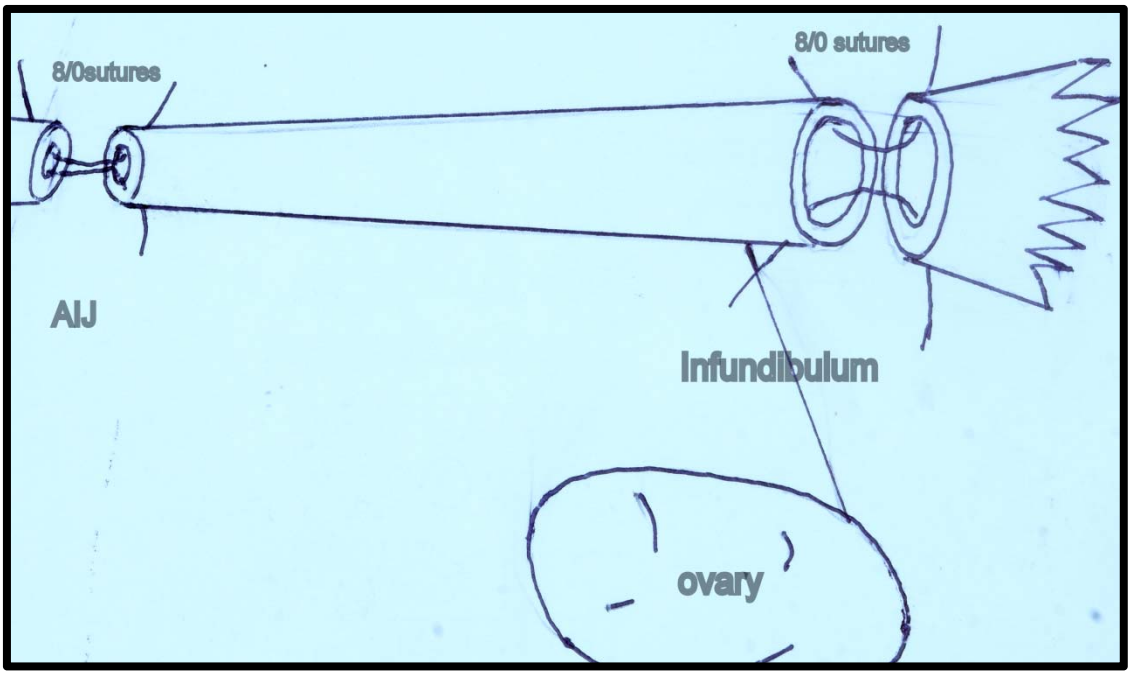


Figure 16 Procedure to reverse rabbit hydrosalpinx.

Excision of tantalum clips was followed by microsurgical anastomosis using 8/0 sutures to reverse hydrosalpinx with meticulous haemostasis.

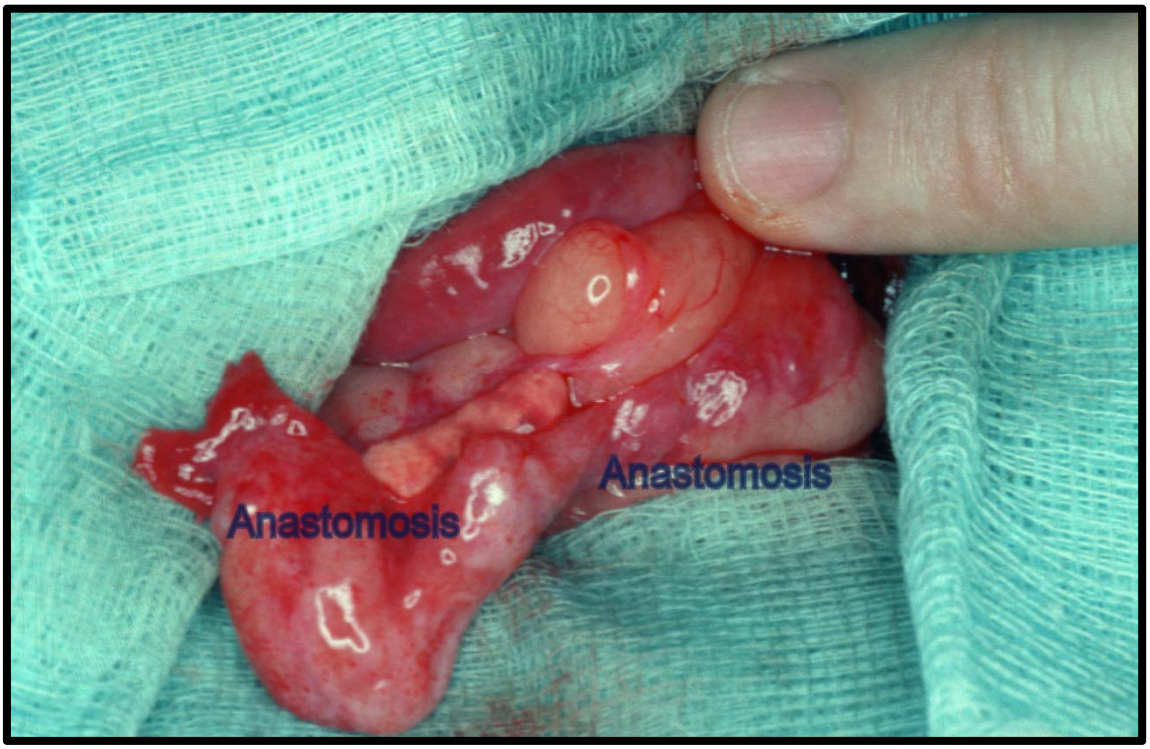


Figure 17 Rabbit oviduct following microsurgical reversal hydrosalpinx.

Microsurgical anastomosis using 8/0 nylon was performed at the ampullary-isthmic junction and infundibulum following removal of tantalum clips.

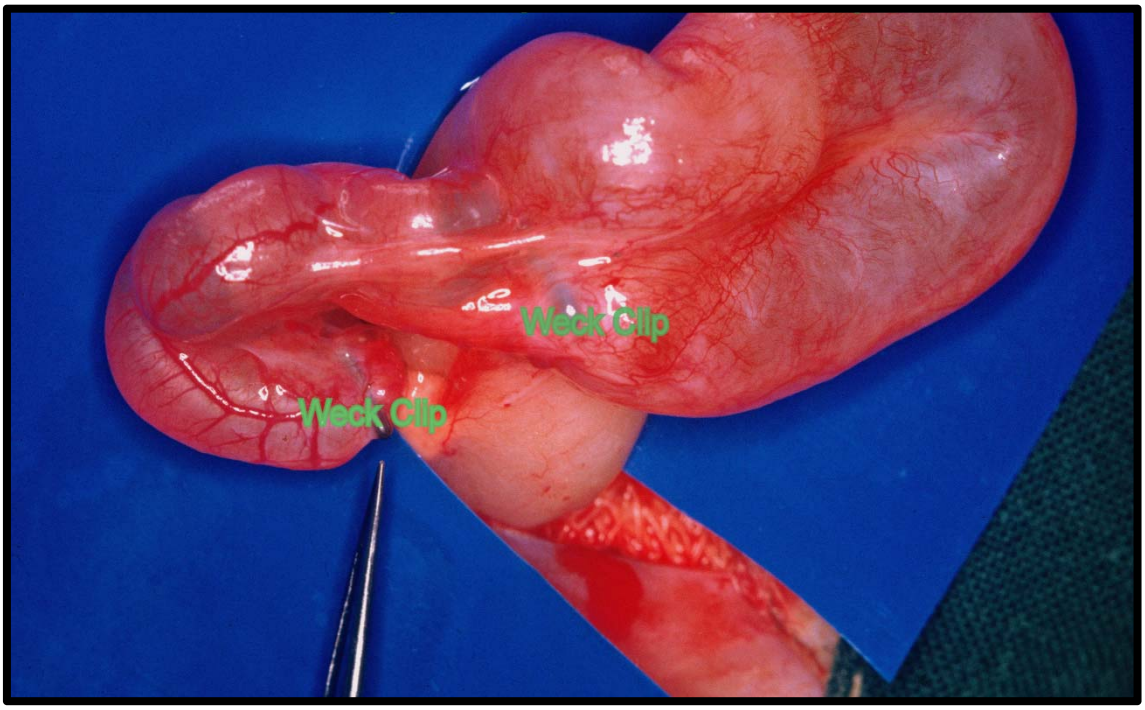


Figure 18 Rabbit hydrosalpinx containing clear fluid.

Rabbit hydrosalpinx containing clear fluid after application of tantalum clips (Weck) at the AIJ and infundibulum. The fimbriae and proximal oviduct remain normal.

Table 7 Concentration of ER and PR in increasing diameter rabbit hydrosalpinx versus control.

ER and PR in cytosol (fmols/mg protein) and nuclear (fmols/mg DNA) preparations of control (unoperated) and hydrosalpinx rabbit oviducts.

RECEPTOR	CONTROL	LEFT HYDROSALPINX		
	μ ± σ	<5MM μ ± σ	5-10MM μ ± σ	>10MM μ ± σ
ER (fmols/mg DNA)	7339 ± 2098 n = 11	5952 ± 3178 n = 10 *	3378 ± 1135 n = 12 *	1598 ± 1019 n = 3**
ER (fmols/mg protein)	136 ± 26 n = 8	90 ± 62 n = 10	58 ± 25 n = 12 **	24 ± 12 n = 3 +
PR (fmols/mg DNA)	5809 ± 569 n = 4	6844 ± 2734 n = 3	7644 ± 5569 n = 5	6057 ± 2834 n = 3
PR (fmols/mg protein)	104 ± 24 n = 4	26 ± 9 n = 7 ++	52 ± 43 n = 10+	38 ± 18 n = 3 #

* Significant difference from control $P< 0.001$
** Significant difference from control $P< 0.025$
\+ Significant difference from control $P< 0.001$
++ Significant difference from control $P< 0.01$
\# Significant difference from control $P< 0.01$
Student's t-test.

ER nuclear values were significantly lower compared to controls even in the smallest (5mm) hydrosalpinx with progressive reduction when dilatation increased. ER cytosol values were significantly reduced when the hydrosalpinx measured 5-10mm or greater.

PR nuclear values were not significantly different from controls (see text for explanation), however PR cytosol values were significantly reduced in all hydrosalpinx group.

size hydrosalpinx. PR nuclear levels were not significantly lower than normal oviducts, however cytosolic PR was significantly lower than normal oviduct. ER cytosolic receptor levels were significantly lower than normal when the hydrosalpinx measured 5-10 mm or more. PR nuclear levels were not significantly different from normal, however P_4 cytosolic receptor levels were significantly lower in each group of hydrosalpinx studied.

3.3.2.4 ER and PR in microsurgical reversed hydrosalpinx

ER and PR levels cytosolic and nuclear fractions from left established hydrosalpinx (6 weeks) and right reversed hydrosalpinx are shown in Table 8.

In the left established hydrosalpinx, ER cytosolic and nuclear receptor levels were statistically lower than normal oviducts as seen in previous study. PR cytosol did not demonstrate a reduction as seen in previous study. This result may be related to finding that in two rabbits only a minor degree of tubal dilatation was noted (2-5mm) thus explaining the small reduction in receptor levels.

In the right reversed hydrosalpinx ER cytosol levels increased to normal levels. PR nuclear levels were significantly increased above left established hydrosalpinx and returned to normal levels.

In established left hydrosalpinx ER nuclear and cytosol levels were decreased from normal as previously demonstrated, whereas PR cytosol levels were not significantly decreased from normal. In right reversed hydrosalpinx ER cytosol levels were statistically increased above left established hydrosalpinx levels and returned to normal oviduct levels. ER nuclear level failed to increase to normal levels and remained similar to established left hydrosalpinx. P_4 nuclear levels returned to normal and were significantly different to the left established hydrosalpinx.

Table 8 Concentration of ER and PR binding in rabbit left established hydrosalpinx compared to right reversed hydrosalpinx and normal oviduct.

RECEPTOR	NORMAL Unoperated μ ± σ	LEFT Established HYDROSALPINX μ ± σ	RIGHT reversed HYDROSALPINX μ ± σ
ER Cytosol	136 ± 26 n = 8	72.3 ± 26 n = 9*	97.8 ± 62 n = 8
ER Nuclear	7339±2096 n = 4	4834 ±1130 n = 9*	2942 ± 1420 n = 8*
PR Cytosol	104 ± 24 n = 8	120.3 ± 30 n = 9*	241.8 ± 71 n = 8**
PR Nuclear	5809 ± 569 n = 4	2942 ± 434 n = 9*	4834 ± 220 n = 8**

* Statistically significant from normal P< 0.025. Student's T test
** Statistically different from left hydrosalpinx P< 0.005.

In established left hydrosalpinx ER nuclear and cytosol levels were decreased from normal as previously demonstrated, whereas PR cytosol levels were not significantly decreased from normal.

In right reversed hydrosalpinx ER cytosol levels were statistically increased above left established hydrosalpinx levels and returned to normal oviduct levels. ER nuclear level failed to increase to normal levels and remained similar to established left hydrosalpinx. P_4 nuclear levels returned to normal and were significantly different to the left established hydrosalpinx.

3.4. Summary - Steroid Hormone Receptor Studies

ER nuclear values were two-fold higher in the proliferative phase of the menstrual cycle, whilst ER cytosol values remained unchanged.

PR cytosol values were also significantly higher in the proliferative phase, whilst PR nuclear values remained unchanged.

In human and rabbit tissue in both cytosol and nuclear fractions the Kd of ER and PR were similar to previously reported values for steroid dependant tissue (see Discussion). Exposure of the rabbit oviduct to E_2 in vivo and in vitro resulted in depletion of cytosol ER as expected in SH target tissue.

Human and unstimulated (oestrus) rabbit data shows depletion of steroid receptors when the oviduct is terminally obstructed and distended with fluid forming a hydrosalpinx.

Human hydrosalpinx demonstrated markedly reduced ER in cytosol and nuclear preparations compared to normal Fallopian tubes in both phases of the menstrual cycle. PR cytosol was also significantly reduced in the proliferative phase.

The reduction of receptors in women occurs despite normal circulating levels of E_2, confirming that the causative inflammatory process and subsequent terminal

obstruction causes irreparable damage to some oviducts. Although the artificially created rabbit hydrosalpinx results from mechanical obstruction rather than an inflammatory process, a similarity of steroid receptor depletion with increasing degree of tubal distension is apparent.

ER and PR studies in the rabbit confirmed that nuclear and cytosol values decreased progressively as oviduct distension occurred with the passage of time and ER values were more significantly affected than PR values.

When the hydrosalpinx was microsurgically reversed achieving restoration of oviduct patency at the uterine and fimbrial ends and reduction in intraluminal pressure, a return to normal receptor values was seen.

3.4.1. Discussion

On commencement of this study there had been no published data on the measurement of nuclear receptor by exchange assays in the human or rabbit, nor reports of the simultaneous measurements of both nuclear and cytosolic receptors for E_2 and P_4 in Fallopian tubes. Using a radioactive label for P_4 was also a first.

In this part of the study, I suceeded in partially characterising ER and PR binding in cytosolic and nuclear fractions of both human and rabbit Fallopian tubes and relate these measurements to menstrual cycle hormonal status.

A large proportion of nuclear ER was available for radioactive E_2 interaction at 4^0C. This binding was not related to dissociation of bound E_2 indicating that once translocation had occurred E_2 was no longer required for nuclear retention.

Nuclear ER significantly increased during the proliferative phase of the menstrual cycle when morphological changes are occurring in the endometrium due to increasing circulating levels of E_2. Receptor changes in the Fallopian tube suggest that it also behaves as a target tissue responsive to E_2. ER in the cytosol fraction

remained constant throughout the menstrual cycle suggesting that this receptor mediates a continuous but variable response to circulating E_2. No significant difference was seen in nuclear PR during the menstrual cycle. The observed decrease in cytosol PR in the secretory phase is consistent with the antagonistic effect of P_4 during this phase.

The study has overall confirmed that the variation in ER and PR during the menstrual cycle is secondary to changes in circulating levels of ovarian steroids. These results are in keeping with Milgrom et al (1973) who observed in the experimental animal that PR levels are regulated by both E_2 and P4, with E_2 increasing PR in cytosol, whilst P_4 had the opposite effect.

In this study, in the oestrous rabbit the Kd of ER in the cytosolic and nuclear fractions was similar to previously reported values in target tissues (King and Mainwearing 1974). PR Kd for both fractions was in agreement with that reported by Muehler et al (1976). Most previous studies utilised synthetic progestogens as ligand, whereas this study used P_4.

3.4.2. Human studies

In vitro studies using human Fallopian tube cells have demonstrated binding of E_2 (Roy et al 1972) and P_4 (Kumra et al 1974; Fuentealba et al 1975). These studies involved receptor content of the ampulla, isthmus and fimbria whereas my interest was primarily in the ampullary segment.

Several reports in the literature (Brennan and West 1975; Pollow et al 1982) confirmed ER and PR sedimentation coefficients of approximately 8s with high binding affinity for oestrogen, $Ka=10^{10}\ M^{-1}$ (Pino et al 1982; Muehler 1983) and P_4 $Ka=10^{10}\ M^{-1}$ (Punnonen and Lukola et al 1981; Pollow et al 1982) which are similar to the results of this study.

Following presentation of reduced ER and PR levels in nuclear and cytosol preparations in 1980 (Petrucco and Winston), Devoto et al (1984) and Devoto and Pino (1991), presented similar results.

Subsequent to the present study, other studies confirmed that nuclear receptor levels were highest in the late follicular phase with subsequent decline in the luteal phase (Punnonen et al 1981; 1982; Pino et al 1982). Punnonen and Lukola (1981) confirmed that cyclic changes occur during the menstrual cycle, with variations in PR in agreement with the observation of Vu Hai et al (1977) that PR is under dual hormonal control.

Verhage and Jaffe (1983) documented that ER and PR levels remain depressed during pregnancy and in the postmenopause associated with high P_4 influence during pregnancy and low E_2 levels in postmenopausal women.

The elevated levels of ER in nuclear fractions in the in the proliferative phase of the menstrual cycle, when E_2 domination is associated with highest levels of ciliogenesis, tubal fluid secretion and ciliary activity, confirm the significant physiological role that receptors play in tubal function.

3.4.3. Rabbit studies

This study finding of reduced ER and PR in the rabbit hydrosalpinx model was confirmed by Kleinstein et al (1982). Their study did not involve measurement of receptor levels subsequent to reversal of hydrosalpinx.

A strength of my study is that it was novel at the time it was conducted. A potential weakness is that the findings from the model rabbit hydrosalpinx may not accurately reflect on what happens in the obstructed human Fallopian tube. The receptor findings however are similar in the human Fallopian tube and rabbit oviduct suggesting that the findings are relevant.

3.5. Pregnancy following Reversal Hydrosalpinx in the Rabbit

Having demonstrated that ER and PR values were affected by hydrosalpinx formation, and that microsurgical reversal of the hydrosalpinx achieved a return to normal values, it remained to be resolved if fertility and pregnancies could be restored in the rabbit. A positive result would be indicative of return to normal oviduct function.

Twenty-four rabbits had hydrosalpinx induced on the right oviduct and the left oviduct left as control. Six weeks later microsurgical surgical reversal of right hydrosalpinx was performed and the animals mated 4-8 weeks later (Table 9).

The animals were sacrificed post mating to inspect for pregnancies.

Pregnancies were confirmed in 16/24 (66.6%) control oviducts and in 11/24 (46%) of the hydrosalpinx reversed oviducts (Table 10).

Post-operative terminal adhesions were found in 4 (16.6%) of the operated oviducts which were confirmed to be patent to dye, following tubal cannulation. Another 8 animals (33.3%) also had oviducts patent to dye however failed to conceive despite frequent mating with a buck of proven fertility.

The result of this part of the study shows that in the rabbit despite hydrosalpinx formation, fertility and pregnancies are possible following restoration of oviduct patency and resolution of intraluminal pressure.

A similar study confirmed pregnancies occurring in 100% control oviducts but only 6.7% of reversed hydrosalpinx oviducts, (from 16 of 30 operated oviducts) with adhesions being a complicating factor (Otubu and Winston, 1986).

Table 9 Time sequence of rabbit fertility studies.

Time 0	Left Control	Right Hydrosalpinx
6 weeks	-	Reversal Hydrosalpinx
10 weeks	Mating	Mating
14 weeks	Inspect Pregnancy	Inspect Pregnancy

Table 10 Rabbit oviduct patency and pregnancy outcome.

Comparing unoperated controls (left) and reversed hydrosalpinx oviducts (right)

Rabbits	n=24
Patent left control tube	24/24 (100%)
Patent right reversed hydrosalpinx	12/24 (50%)
Pregnancy left control oviduct	16/24 (66.6%)
Pregnancy right reversed hydrosalpinx	11/24 (46%)
Tubal adhesions	4/24 (16.6%)
Nil pregnancy	8/24 (33.3%)

Chapter 4 Rabbit Physical Studies

4.1. Aims of the Study

Results of surgical intervention when hydrosalpinges are very distended and of increased tubal thickness are associated with negligible fertility outcome (See discussion in Chapter 8).

Mucosal cellular damage secondary to pelvic inflammatory disease is likely to play the most significant role as evidenced by the observed changes in morphology and ER/PR changes previously described. Following mucosal injury and subsequent end-tubal obstruction caused by bacteria, it must also be considered that tubal dilatation and likely increase in intraluminal pressure also play a prominent role in causing cellular damage. The role of increasing intraluminal pressure with progressive tubal dilatation is of importance in deciding whether it is beneficial to relieve the pressure by performing salpingostomy to prevent progressive deterioration in tubal function with the passage of time. This decision at laparoscopic diagnosis is of importance in young women, irrespective of future fertility considerations. Salpingostomy may allow consideration of natural fertility, whereas persistence of hydrosalpinx is likely to obviate this choice and furthermore require surgical intervention before ART can be considered to achieve a pregnancy. The aim of this study was to investigate if increasing oviduct dilatation in the rabbit hydrosalpinx model is associated with an increase in intraluminal pressure and whether it may also affect oviduct blood flow.

4.2. Methodology

Unilateral occlusion of the right oviduct to create hydrosalpinx was performed on 28 animals as previously described. The left oviduct remained un-operated and kept as

control for the 15 rabbits used for intraluminal pressure measurement. The thirteen animals used for tubal blood flow assessment had unilateral or bilateral induction of hydrosalpinx with some oviducts kept as controls. Experiments were performed 8-15 weeks post-induction of hydrosalpinx for intraluminal pressure measurement and 6-8 weeks for oviduct blood flow studies.

Blood flow was measured by the method described by Janson (1975) (see Appendix 5) and blood flow calculated by the formula of Rudolf and Heymann (1967).

Intraluminal pressure measurement

A Statham P23 AC transducer kept at the same level as the experimental animal was connected to a recording device (Grass polygraph model 5D) via a 21gauge needle inserted in the surgically induced hydrosalpinx (Figure 19).

Manometry tubing was filled with fluid before calibration and pressure measurement (mm HG). An example of recording is seen in Figure 20. The right oviduct was occluded 8 to 15 weeks before intraluminal pressure measurement and the left was used as the control.

E_2 (Estradurin 0.2cc) was administered intramuscularly daily to ensure physiological functioning of the oviducts.

At the completion of the experiment, the control and hydrosalpinx oviducts were excised and wet weight measured. Pressure measurement was technically not possible in four animals and wet weight not recorded in three.

4.3. Results

The diameter of hydrosalpinx varied from 4-10mm and the mean intraluminal pressure was 10 mm. Wet weight increased 3 times in hydrosalpinx oviducts compared to normal controls (Table 11). Figure 21 shows a statistically significant

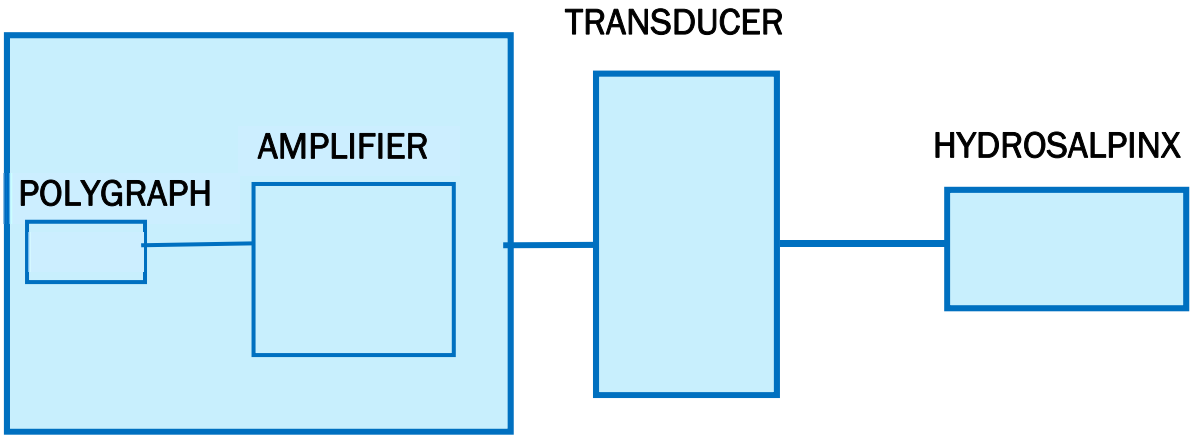


Figure 19 Intraluminal pressure measurement.

Polygraph and amplifier connected to transducer and tubing to hydrosalpinx. Instruments all at same horizontal level as experimental animal. Manometry tubing filled with fluid before calibration and pressure measurement.

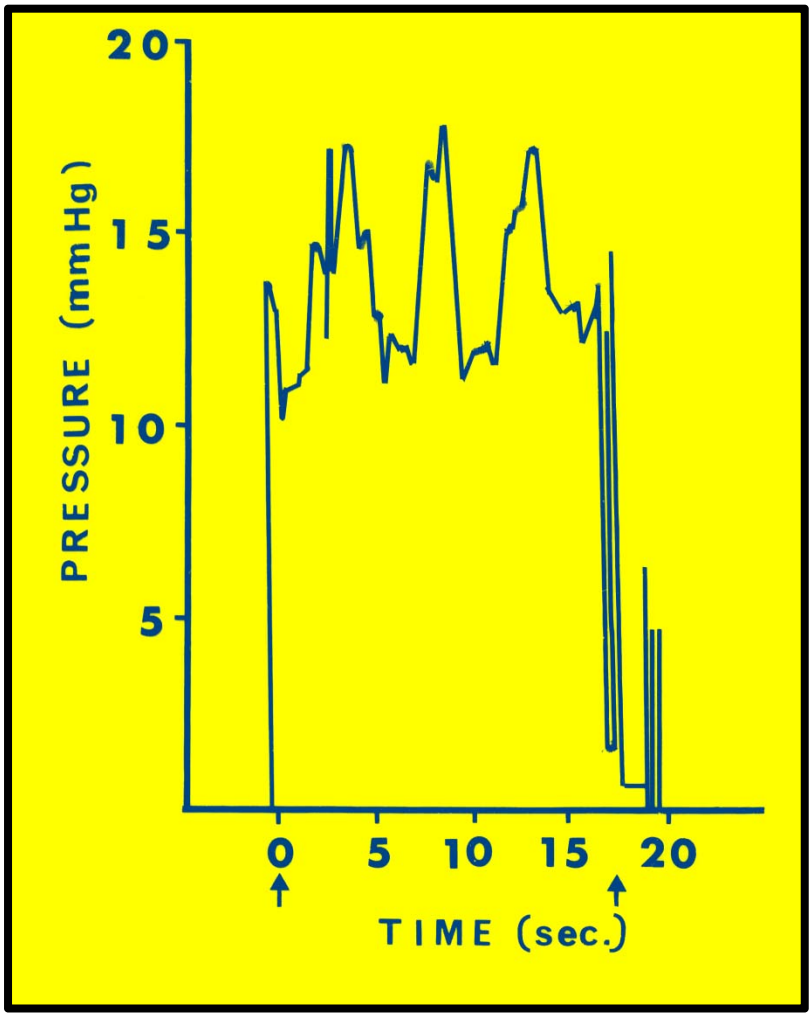


Figure 20 Recording of intraluminal pressure measurement.

Measurement using 21 gauge needle inserted in hydrosalpinx and connected to recording device and Statham transducer.

Table 11 Rabbit hydrosalpinx diameter, wet weight and intraluminal pressure.

No.	Weeks	Size (mm)	Wet weight Hydrosalpinx (gms)	Wet weight Normal (gms)	Pressure (mm)	E_2 (days)
1	13.5	8	0.658	0.288	15	7
2	8	4	0.474	0.276	-	7
3	8	10	1.278	0.362	-	7
4	8	10	0.915	0.277	-	7
5	8	18	1.154	0.278	-	7
6	8	6	-	-	18	4
7	8	18	-	-	17	4
8	8	7	-	-	11	4
9	8	15	2.087	0.495	21	4
10	8	8	0.663	0.362	-	10
11	15	10	1.196	0.362	14	10
12	15	10	0.753	0.255	20	10
13	15	6	1.12	0.279	15	10
14	14	7.5	0.638	0.337	7.5	10
15	14	10	0.763	0.388	17.5	10
		n = 15 μ = 10	n=12 μ =0.96 σ ± 0.73	n = 12 μ = 0.36 σ ± 0.4	n = 10 μ = 15.6	

A significant increase is seen in wet weight and intraluminal pressure with increasing diameter of hydrosalpinx, compared to normal oviduct.

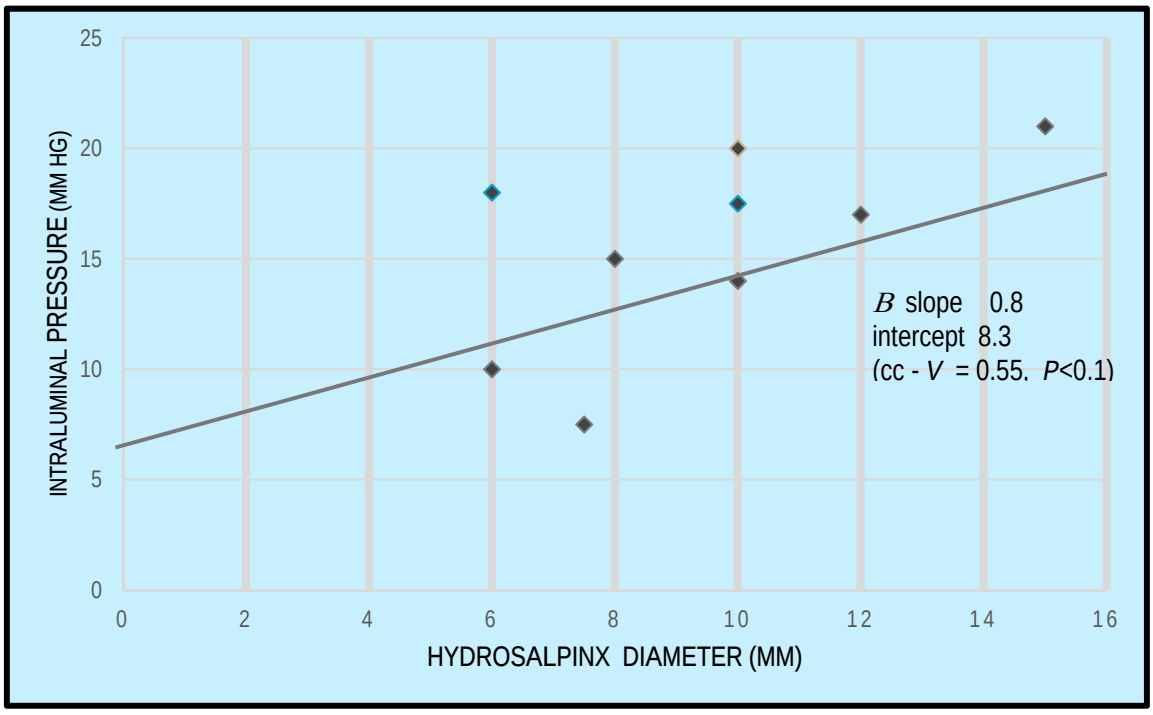


Figure 21 Intraluminal pressure versus diameter hydrosalpinx.

Significant increase occurs in intraluminal pressure as hydrosalpinx diameter increases.

relationship between wet weight, intraluminal pressure and diameter hydrosalpinx (cc – 0.55, p< 0.1).

4.4. Oviduct Blood Flow

Six of the rabbits had hCG administered either 7 hours (x3) or 18 hours (x3), before blood flow estimations to assess the effect of ovulation on ovarian and oviduct blood flow.

4.4.1. Results

The results of oestrus and hCG treated animals were similar and therefore combined. In two rabbits reference values indicated that microsphere distribution was not acceptable and these results were omitted from final assessment. Oviduct blood flow (mls /100gm tissue/min) was significantly higher in control compared to hydrosalpinx affected oviducts (Table 12). Hydrosalpinx blood flow was median level 31.8 mls /100gm tissue/min (95% CI 70-95) whilst control value was 75.3mls/100gm tissue/min (95% CI 0-75). A significant difference was observed in combined as well as paired oviducts, (Wilcoxon test P < 0.028).

Oviduct distension by hydrosalpinx formation has a pronounced effect on blood flow. Following administration of hCG, ovarian blood flow was significantly higher (p=0.0038, Mann-Whitney non parametric analysis) 18 hours post hCG, (p=0.0067), 7 hours post hCG.

A significant difference was also found in hydrosalpinx compared to control at 18 hours post hCG (p=0.0339). Only 2 data points were available at 7 hours post hCG so difference from control could not be assessed.

The higher oviduct and ovarian blood flow following hCG administration confirmed that a peri-ovulatory regulatory relationship exists between gonadal steroids and adnexal blood flow.

Table 12 Rabbit oviduct blood flow in control and hydrosalpinx (combined and paired oviducts).

OVIDUCT BLOOD FLOW (mls /100gm tissue / min)	
COMBINED OVIDUCTS	
CONTROL n = 11 μ = 122 σ ± 32	HYDROSALPINX n = 11 μ = 27* σ ± 17
PAIRED OVIDUCTS	
CONTROL n = 6 μ = 75.3 σ ± 26	HYDROSALPINX n = 6 μ = 31.8** σ ± 13

* Student t-test $P < 0.01$
** Wilcoxon test $P < 0.028$

Significant difference in blood flow between hydrosalpinx and combined and paired control oviducts.

A strength of this study is that it was novel when performed and not repeated in the literature. A weakness is that the findings in the rabbit hydrosalpinx model may not be representative of what happens in the human Fallopian tube following hydrosalpinx formation.

4.5 Summary of Physical Studies

The observed increase in intraluminal pressure in the rabbit hydrosalpinx model was directly proportional to the diameter and therefore volume of fluid within the oviduct. With increasing tubal distention a complementary increase in weight was noted. As discussed in the next chapter, this was associated with an increase in water and collagen content.

Oviduct blood flow was significantly reduced in the hydrosalpinx compared to normal oviducts. The observed reduction in blood flow per gram of tissue is likely to be a contributory factor to altered cellular activity and wellbeing.

Utilising the same rabbit hydrosalpinx model, Jones and Verco (1983) studied methyl methacrylate corrosion vascular casts to examine vascular changes by scanning electron microscopy. They found a major change in the interplical microvascular architecture with fewer capillaries present and absence of large ampullary plical veins. The subserosal veins were more distended. This study suggested a higher venous pressure and locally decreased fluid absorption capacity causing fluid accumulation in the hydrosalpinx.

The results of these studies are complementary to the blood flow results and confirm that vascular changes play a significant part in the pathological changes observed in tubal function.

Chapter 5 Biochemical Studies

5.1. Aims of the Study

The purpose of this study was to investigate whether the DNA unit could be used to detect changes that may occur in cell mass and cell replication in hydrosalpix compared to control Fallopian tubes and rabbit oviducts.

The deoxyribonucleic acid (DNA) is constant per cell and in polypoid tissue the DNA content and cytoplasm increase proportionally so that cell water to cell protein is a constant in mammalian tissue (Cheek et al 1977). The relative RNA content as a function of total cellular nucleic acid content is also a constant value characteristic for particular cell lines during growth under optimal conditions so that changes in cellular growth rate are reflected in cellular DNA/RNA content thus providing information on deviation from normal cell growth (Troganoff et al 1982).

It is also known that alterations in serum E_2 effects mean cell content of RNA, cell protein and RNA synthesis (Miller 1976).

The aim of this study was to measure nucleic acid ratios and water content to assess whether unbalanced cell growth occurs in hydrosalpinx compared to control Fallopian tubes and rabbit oviducts.

ER/PR levels per cell protein and DNA levels, and mean cilia index were also measured to compare hydrosalpinx to control tissue.

5.2. Results

DNA, RNA and RNA/DNA ratios were measured in 8 control and 25 hydrosalpinx human Fallopian tubes. Methods used for all biochemical studies are detailed in Appendix 6.

5.2.1. Nucleic acids content - human tissue

A slight reduction in DNA, RNA and RNA/DNA values was noted in hydrosalpinx Fallopian tubes which did not reach significance from controls (Table 13).

5.2.2. Nucleic acids versus mean cilia index - human tissue

SEM studies were only possible in 16 of 20 hydrosalpinx affected Fallopian tubes. Four hydrosalpinges had normal ciliated cell counts and 16 had markedly reduced ciliated cell counts when compared to controls.

The RNA and DNA values (mg/gm fresh tissue) were significantly reduced in the hydrosalpinx specimens with reduced ciliated cell counts compared to those with normal cilia counts and normal controls (Table 13).

The reduced DNA levels were similar to those previously found in postmenopausal and postnatal Fallopian tubes known to have reduced cellular activity.

In human Fallopian tubes, no difference was found in DNA, RNA and RNA /DNA ratio between normal and hydrosalpinx Fallopian tubes. However, a significant difference was noted when hydrosalpinx with low cilia count was compared with hydrosalpinx with normal cilia (Table 14). See Chapter 6 for MCI methodology.

RNA counts per mg of tissue were reduced in the low cilia group indicating a reduction in RNA turnover and less active cells. Compared with results of normal Fallopian tubes and hydrosalpinx with normal MCI, the hydrosalpix with low MCI had a significant reduction in RNA and DNA values.

Results for postnatal and postmenopausal patients who are known to have low ciliated cell counts similarly had significantly reduced DNA values.

5.2.3. Nucleic acid content - rabbit oviducts

In the rabbit, a significant difference was found in DNA, RNA, and RNA /DNA ratio in

Table 13 Human Fallopian tube and hydrosalpinx nucleic acids (per gram fresh tissue).

	FALLOPIAN TUBES – HUMAN				
	NORMAL μ ± σ	n	HYDROSALPINX μ ± σ	n	*P* VALUE
DNA (mg/gm fresh tissue)	3.66 ± 1.10	8	3.22 ± 0.98	25	NS
RNA (mg/gm fresh tissue)	1.03 ± 0.47	8	0.815 ± 0.4	25	NS
RNA/DNA	0.29 ± 0.1	8	0.27 ± 0.13	25	NS

NS = not significant

No significant difference seen in DNA, RNA, RNA/DNA between hydrosalpinx and normal Fallopian tubes.

Table 14 Human Fallopian tube nucleic acids comparison normal to hydrosalphinx (low and normal cilia), postmenopausal and postnatal Fallopian tubes.

	n	RNA μ ± σ	DNA μ ± σ	RNA / DNA μ ± σ
NORMAL	8	1.03 ± 0.47	3.66 ± 1.10	0.29 ± 0.11
HYDROSALPINX (NORMAL CILIA)	4	1.10 ± 0.39	4.80 ± 0.17	0.23 ± 0.08
HYDROSALPINX (LOW CILIA)	16	0.62 ± 0.27*	2.79 ± 0.68*	0.27 ± 0.14
POSTMENOPAUSAL	3	0.70 ± 0.17	2.68 ± 0.80*	0.26 ± 0.02
POSTNATAL	6	0.81 ± 0.41	2.29 ± 0.56*	0.39 ± 0.21

Student's t-Test

* Indicates $P < 0.05$ for hydrosalpinx (normal cilia) compared to hydrosalpinx (low cilia)
$P<0.05$ for RNA and DNA.

A significant difference was found between RNA and DNA values in hydrosalpinx with low mean cilia count and normal Fallopian tubes.

Compared to normal Fallopian tubes, a significant difference was also found in DNA content in postmenopausal and postnatal specimens compared to normal Fallopian tubes.

hydrosalpinx compared to normal control oviducts (Table 15).

RNA and DNA synthesis results are seen in Table 16.

RNA counts per mg of tissue of hydrosalpinx were reduced indicating a reduction in RNA turnover and less active cells. RNA counts per mg RNA again confirmed less active cells and a decrease in cellular turnover in hydrosalpinx tissue.

DNA counts per gram tissue were also reduced indicating a reduction in cell replication per gram of tissue. The DNA count per mg DNA was significantly increased indicating increased cell replication in the cells present in that tissue.

5.3. Water, Electrolytes, Lipid and Collagen Studies

The methods used to measure water, lipid, collagen and electrolytes (sodium, potassium, chloride) are detailed in Appendix 6.

5.3.1. Human tissue results

Compared to normal tissue, hydrosalpinx Fallopian tubes had a significant increase in water and collagen content (Table 17).

Sodium content was the only electrolyte significantly increased.

5.3.2. Rabbit tissue results

A significant increase was seen in hydrosalpinx collagen content and the electrolytes Na, K, Cl (Table 18).

5.4. Steroid Receptors per Cell Protein and DNA

ER and PR were measured in 15 normal and 28 human hydrosalpinx affected oviducts.

No significant difference was noted in receptor/mg protein in tissue or receptor /mg DNA (Table 19).

Table 15 Rabbit nucleic acids – normal and hydrosalpinx oviducts (per gram fresh tissue).

	OVIDUCTS – RABBIT				
	NORMAL μ ± σ	n	HYDROSALPINX μ ± σ	n	*P* VALUE
DNA (mg/gm fresh tissue)	2.60 ± 0.78	8	1.14 ± 0.35*	8	<0.01
RNA (mg/gm fresh tissue)	1.37 ± 0.78	8	0.89 ± 0.20*	8	<0.01
RNA/DNA	0.530 ± 0.11	8	0.83 ± 0.24*	8	<0.002
TOTAL WEIGHT (gms)	0.358 ± 0.077		0.896 ± 0.319*		<0.01

Student's t-test

* Significant difference between normal oviduct and hydrosalpinx.

Table 16 Rabbit oviducts – RNA and DNA synthesis

OVIDUCTS - RABBIT			
	NORMAL n = 8 μ ± σ	HYDROSALPINX n = 25 μ ± σ	
RNA (cpm/gm tissue)	5000 ± 3202	2301 ± 1747*	*P*<0.01
RNA (cpm/mgm RNA)	3236 ± 1177	1948 ± 1198 *	*P*<0.01
DNA (cpm/gm tissue)	11243 ± 10353	5936 ± 6326*	*P*<0.01
DNA (cpm/mgm DNA)	3701 ± 2765	5559 ± 6145	NS

* Significant difference p<0.01 between normal oviduct and hydrosalpinx.

Table 17 Human Fallopian tubes - lipid, collagen, electrolytes and water (per gram fresh tissue).

	FALLOPIAN TUBES – HUMAN				
	NORMAL μ ± σ	n	HYDROSALPINX μ ± σ	n	*P* VALUE
WATER	82.2 ± 2.7	7	85.0 ± 1.6*	24	0.001<*P* <0.005
LIPID (gm/100gms)	0.21 ± 0.19	7	0.34 ± 0.7	24	NS
COLLAGEN (gm/110gms fresh frozen, dried tissue)	32.5 ± 6.6	8	38.2 ± 6.7*	27	0.02 <*P* <0.05
CHLORIDE (mEq/110gms fresh frozen, dried tissue)	40.5 ± 8.8	6	46.9 ± 8.9	23	NS
SODIUM (mEq/110gms fresh frozen, dried tissue)	41.6 ± 8.4	6	52.7 ± 9.6*	23	0.01 <*P* <0.02
POTASSIUM (mEq/110gms fresh frozen, dried tissue)	21.5 ± 5.6	6	19.7 ± 3.7	23	NS

NS = not significant
Student's t-test

* Significant difference between normal Fallopian tube and hydrosalpinx.

Table 18 Rabbit oviducts – lipid, collagen, water and electrolytes.

	OVIDUCTS – RABBIT				
	NORMAL μ ± σ	n	HYDROSALPINX μ ± σ	n	*P* VALUE
WATER (gm/100gms)	79.7 ± 3.2	7	82.4 ± 3.0	7	NS
LIPID (gm/100gms)	4.6 ± 3.1	7	4.3 ± 2.8	7	NS
COLLAGEN (gm/110gms fresh frozen, dried tissue)	19.9 ± 4.2	7	26.8 ± 1.6*	7	<0.01
CHLORIDE (mEq/110gms fresh frozen, dried tissue)	26.3 ± 1.7	7	41.5 ± 8.4*	7	<0.01
SODIUM (mEq/110gms fresh frozen, dried tissue)	37.0 ± 1.3		60.0 ± 3.5*		<0.002
POTASSIUM (mEq/110gms fresh frozen, dried tissue)	33.0 ± 2.9		12.3 ± 1.4*		<0.002

NS = not significant
Student's t-test

* Significant difference between normal oviduct and hydrosalpinx.

Table 19 Concentration of ER and PR per tissue protein and DNA in human Fallopian tubes

	FALLOPIAN TUBES – HUMAN				
	NORMAL μ ± σ	n	HYDROSALPINX μ ± σ	n	*P* VALUE
ER / PROTEIN (fmols receptor/mg protein)	54.3 ± 30.3	15	53.4 ± 25.0	28	NS
PR / PROTEIN (fmols receptor/mg protein)	71.7 ± 42.5	15	50.4 ± 38.8	28	NS
ER / DNA (fmols receptor/mg DNA)	2012 ± 2336		1758 ± 1439	28	NS
PR / DNA (fmols receptor/mg DNA)	1699 ± 661	15	1900 ± 1397	28	NS
K / DNA	1.2 ± 0.5	5	1.0 ± 0.4	21	NS

NS = not significant
Student's t-test

No significant difference was found in SHR / protein or SHR / DNA.

5.5. Summary - Biochemical Studies

Nucleic acid studies in hydrosalpinges with reduced MCI indicative of increased cellular damage were significantly different from controls.

In the rabbit. RNA and DNA synthesis and turnover were significantly reduced in hydrosalpinx tissue. Cells were less active with a reduction in cell replication, protein synthesis and cell growth.

The increase in water and collagen content is in keeping with the observed increase in weight, tubal thickness and tubal dilatation.

In the human Fallopian tubes, the reduction of DNA in hydrosalpinx tissue was similar to that seen in the relatively inactive postmenopausal and postnatal specimens.

In the human and rabbit hydrosalpinx tissue, the higher content of water, collagen and electrolytes was indicative of altered cell function.

In rabbit hydrosalpinx, the wet weight was observed to have increased three-fold in hydrosalpinx tissue in keeping with the observed increase in water content.

The increase in water and collagen content is in keeping with the observed increase in weight, tubal thicknes and tubal dilatation.

Chapter 6 Oviduct Morphology Studies

6.1. Aim of the Study

The purpose of this study was to examine the morphological changes occurring in the epithelium of human Fallopian tubes and rabbit oviducts following hydrosalpinx formation. These changes are compared to control rabbit oviducts and healthy Fallopian tubes at different stages of the menstrual cycle, pregnancy, reversal of sterilisation, ectopic gestation and menopause. This study enabled assessment from normal of pathological conditions affecting Fallopian tubes and hydrosalpinx formation in the rabbit model.

6.2. Methodology for Tubal Morphology Studies

Light microscopy (Appendix 8)

Stained tissue sections for examination by light microscopy were produced by a step-wise proces beginning with fixation of tissue in buffered formalin (10%), followed by dehydration and clearing of tissue before inpregnation with wax. Thin sections are attached to a glass slide for staining with haematoxylin and eosin.

Surface electron microscopy (SEM) tissue preparation and mean cilia index- MCI (Appendix 7)

Tissue biopsies were fixed in 3% glutaraldehyde (TAAS) and 2% formaldehyde in cocodylate buffer (0.05M, pH 7.2, 395 Mosm), dehydrated in alcohol, critical point dried (Denton D502) and coated with 60:40 gold- palladium. Specimens were mounted on stubs using “Silver Dag” (Electrodag 915- high conductivity point) A Siemen’s Autoscan was used for viewing, mainly at 20 kV.Counting of ciliated cells was accomplished by a technique described by Tulsi and Dreosti (1981) at 2000 x magnification using a Perspex graticule divided into 36 squares (3600um) and

finding the average number of ciliated cells in 30 fields representative of the block under examination. The median of ciliated cells in all field becomes the mean cilia index (MCI).

Transmission electron microscopy (TEM) tissue preparation (Appendix 7)

Tissue biopsies were fixed in 3% glutaraldehyde fixative (TAAS) and washed 3 times in cocodylate buffer as above and fixed for 1 hour in 1% Oso4. Following dehydration in alcohol tissues were subjected to 50/50 absolute alcohol/spurs resin and then pure spurs resin overnight at 63^0C.

Tissue blocks were trimmed into trapezoid shape and tissue cut with glass knife into 0.5 micron thickness sections. On grids, tissue was stained with uranyl acetate and distilled water. The lead stain was made with one pellet NaOH in 50mls distilled water/0.25g lead citrate. After wash in distilled water and drying, scanning was performed using transmission electron microscope.

Normal Fallopian tubes

Thirty five women at different stages of menstrual aged 28-42 were undergoing hysterectomy with removal of Fallopian tubes for non-malignant gynaecological conditions. Menstrual history and serum E_2/P_4 confirmed their menstrual cycle status. Ampullary, isthmic and fimbrial specimens (proliferative and secretory phase cycle) were processed as described below.

Normal pregnancy

Fourteen women having sterilisation procedures related to pregnancy, agreed to have ampullary microbiopsies taken during their procedure. Four women were 4 days postpartum after vaginal delivery, nine patients were undergoing Caeserean section and one woman was having termination of pregnancy at 10 weeks gestation.

Hydrosalpinx

Twenty-seven infertile women at different stages of the menstrual cycle, aged between 27-37 (Median age 29), with bilateral hydrosalpinx were undergoing laparotomy for microsurgical salpingostomy. Complete terminal tubal blockage was diagnosed preoperatively at diagnostic laparoscopy or by hysterosalpingogram. Forty nine ampulla, isthmus, and fimbrial microbiopsy specimens were taken at salpingostomy using an operating microscope for magnification (10-40) of the mucosal surface.

Reversal of sterilisation

The study involved thirty one women aged 25-36 years peviously sterilised by application of Fallope rings undergoing mini-laparotomy and microsurgical reversal of sterilisation (5-10 years post procedure). Microbiopsy specimens were taken under magnification from the proximal occluded tubal segment, and processed as described above.

Ectopic pregnancy

Eleven women having ectopic pregnancies (6-10 weeks gestation) had microbiopsies taken during laparoscopic surgery. Ectopic pregnancies were all sited in the ampulla of the Fallopian tube and biopsies were taken adjacent to the ectopic site in proximity to the fimbriated end of the tube.

Menopause

Three women with at least 12 months amenorrhoea and within 5 years of last menstrual period, having hysterectomy with removal of Fallopian tubes were also included in the study.

Rabbit oviduct specimens

Microbiopsy specimens from control and experimental animals were processed with same methodology described for human Fallopian tubes.

6.3. Rabbit Hydrosalpinx Results

Ten animals were used in this study. The rabbit hydrosalpinx model was used as previously described (see also Appendix 3). Care was taken to occlude only the oviduct lumen with tantalum clips without interference to the blood vessels in the mesosalpinx. Excision of the left hydrosalpinx was performed 6 weeks later and subjected to light and surface electron microscopy (SEM). Prior to excision, each oviduct was measured at its widest segment. In all cases almost equal dilatation was observed on each side with the average widest diameter of the mechanically obstructed ampulla being 10mm (range 8-14mm).

6.3.1. Light microscopy

Loss of longitudinal folds and a flattened epithelium and de-differentiation of cells are the major changes observed (Figures 22, 23).

At higher magnification there was evidence of hyalinization (scarring) of sub-epithelial connective tissue. No inflammatory changes were observed.

6.3.2 Surface electron microscopy

Marked deciliation was seen in all oviducts. The MCI was 53% (95% confidence interval 0-75). At low magnification disorganization of surface architecture was obvious with loss of secondary folds and considerable separation and flattening of the remaining longitudinal folds (Figure 24).

The majority of cells were of secretory type and had early or late microvillus formation on their surfaces indicating different degrees of secretory activity (Figures 25, 26 and 27). The remaining ciliated cells displayed normal cilia (Figure 28).

Mucosal polyps were frequently seen, the largest measuring 400mu in height and

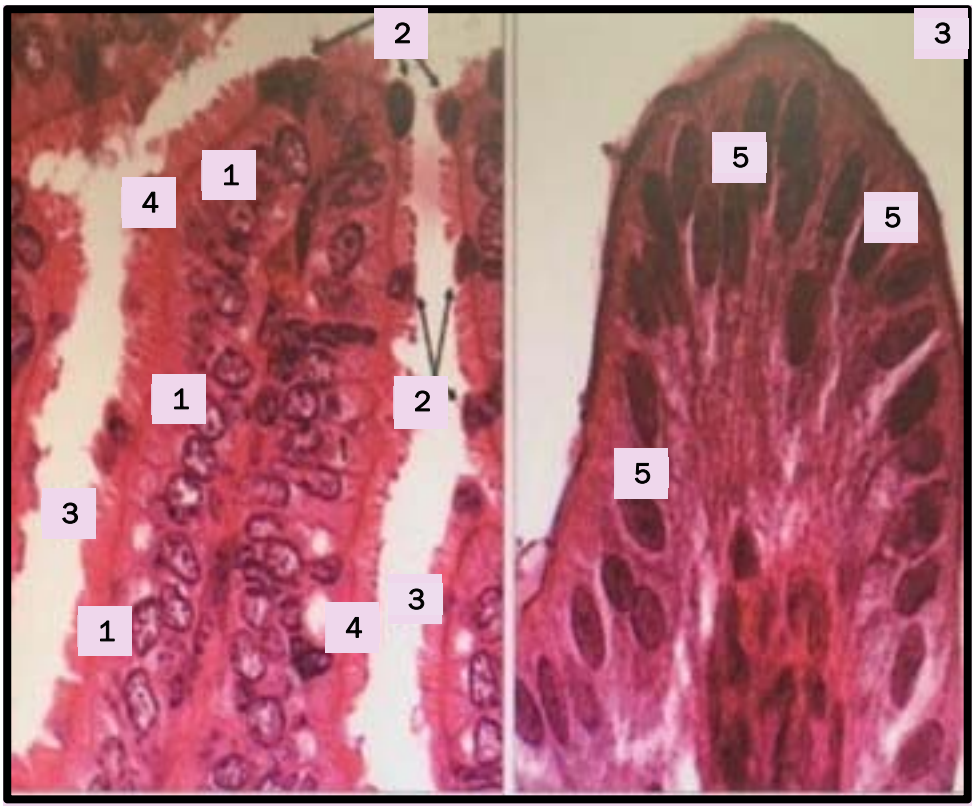
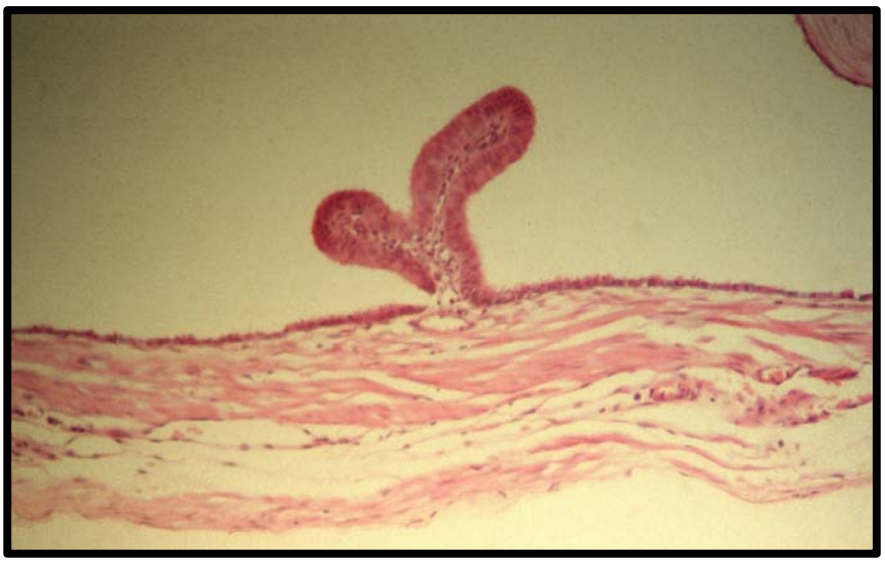


Figure 22 Light microscopy of oestrus rabbit normal oviduct and hydrosalpinx (H&E x150).
Left panel shows normal oviduct and right panel shows hydrosalpinx.
1= Ciliated epithelial cells
2= Secretory cells
3= Lumen of oviduct
4= Cilia
5= Non ciliated cells



Bottom panel shows loss of cilia and scarring in sub-epithelial layers. Mucosal polyp not normally seen in normal tissue.

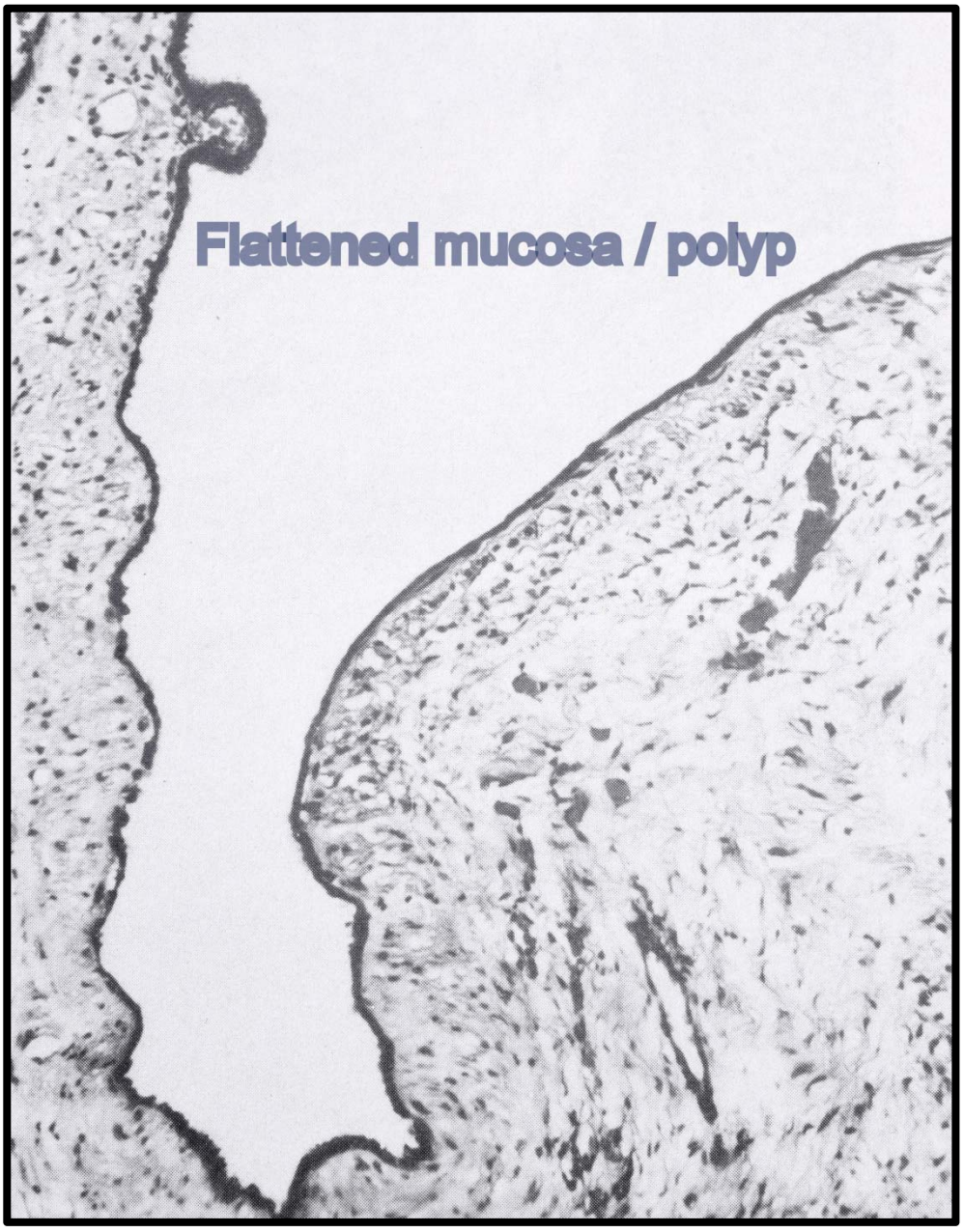


Figure 23 Rabbit hydrosalpinx - flattened mucosa (H&E x160).

Light micrograph of a region comparable to Figure 24 showing loss of longitudinal folds (only one shown in this micrograph) and flattening of epithelial layer, with evidence of scarring in sub-epithelial layers.

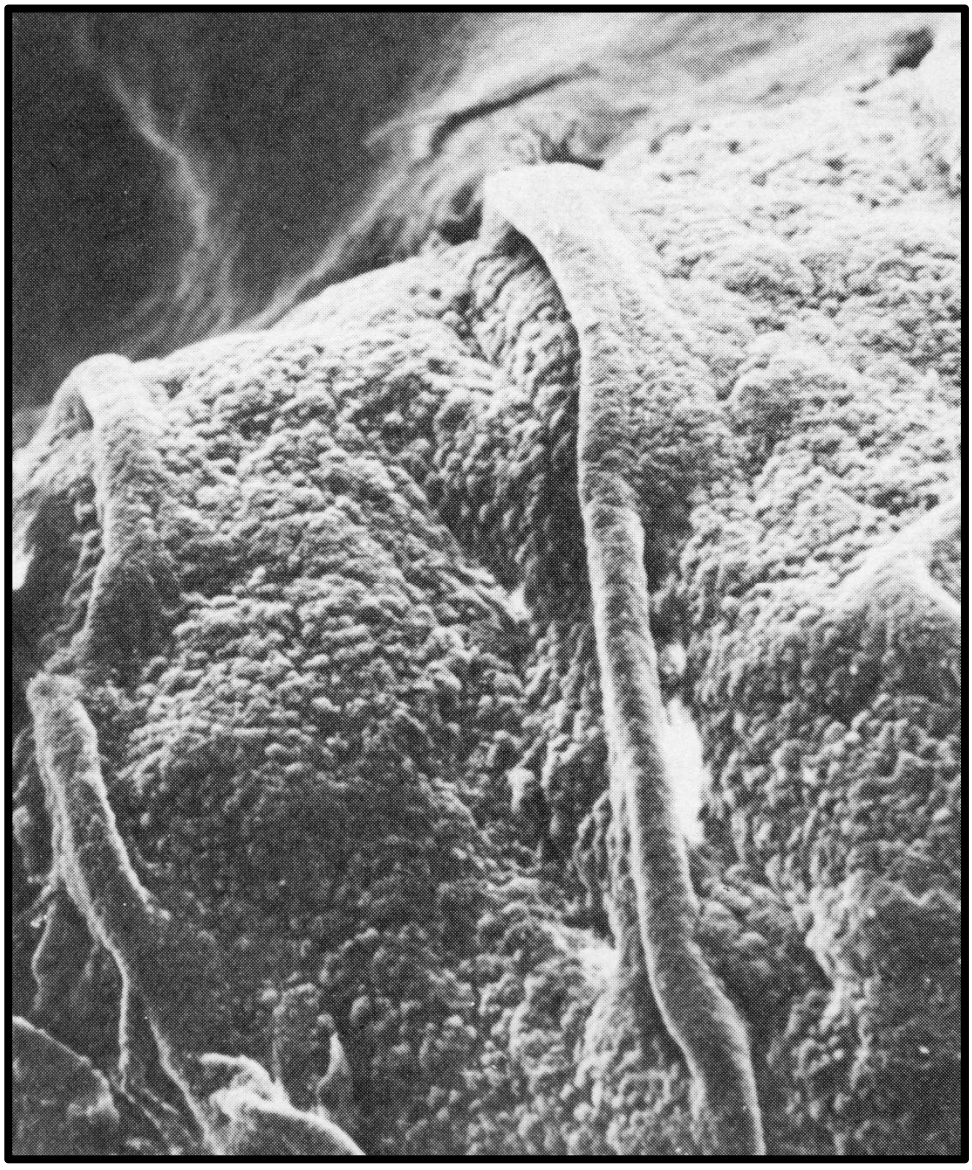


Figure 24 SEM rabbit hydrosalpinx at six weeks following induction of hydrosalpinx (x100).

Scanning electron micrograph of the infundibulum of the oviduct 6 weeks after mechanical occlusion showing loss of secondary folds and separation (the 2 folds in the picture are approximately 500 micrometres apart) and flattening of the longitudinal folds.

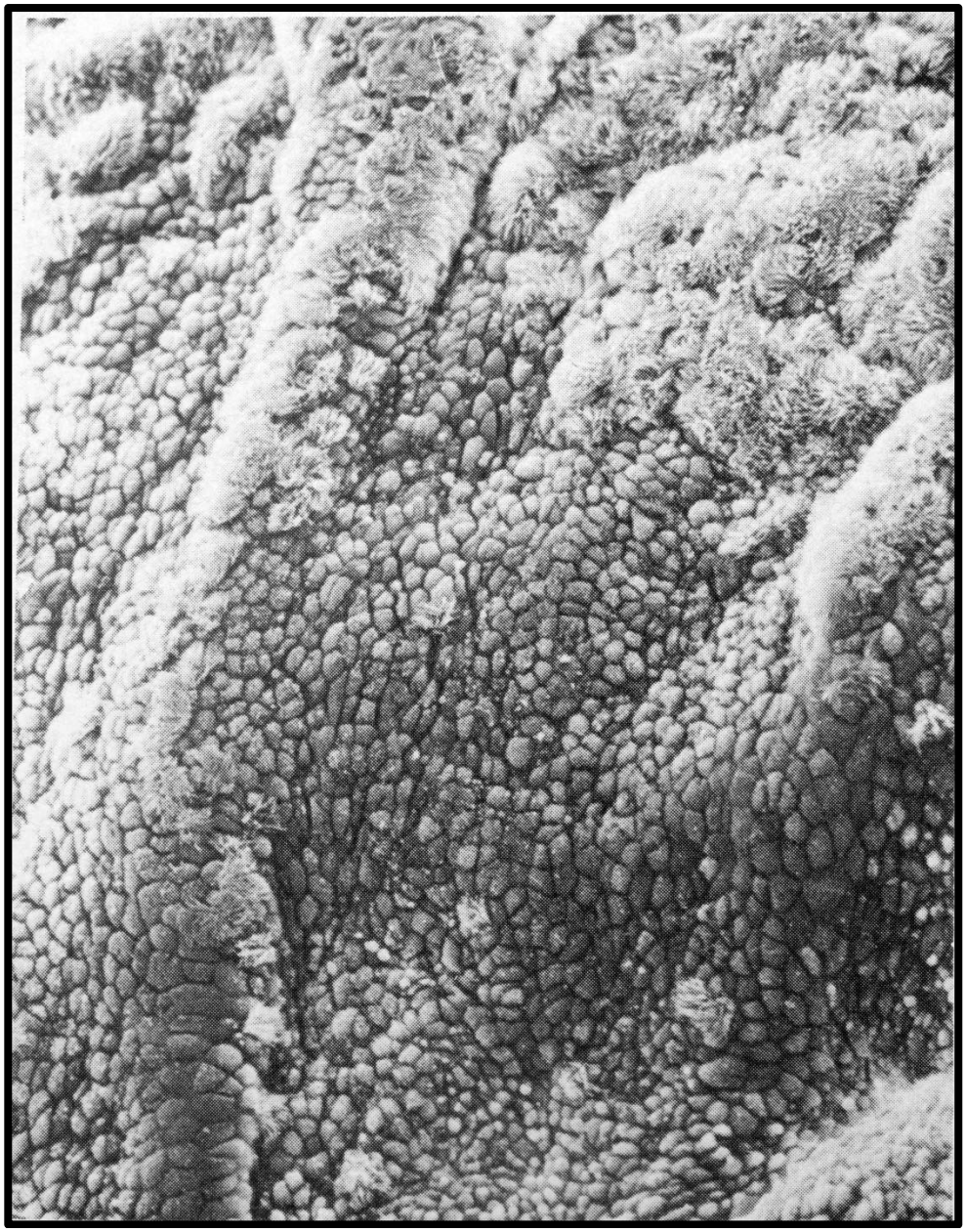


Figure 25 SEM rabbit hydrosalpinx epithelial fold and adjacent area seen in Figure 24 (x360).

An epithelial fold, at higher magnification, adjacent to area seen in Figure 24. Loss of cilia is observed with ciliated cells at early stage of secretory activity.

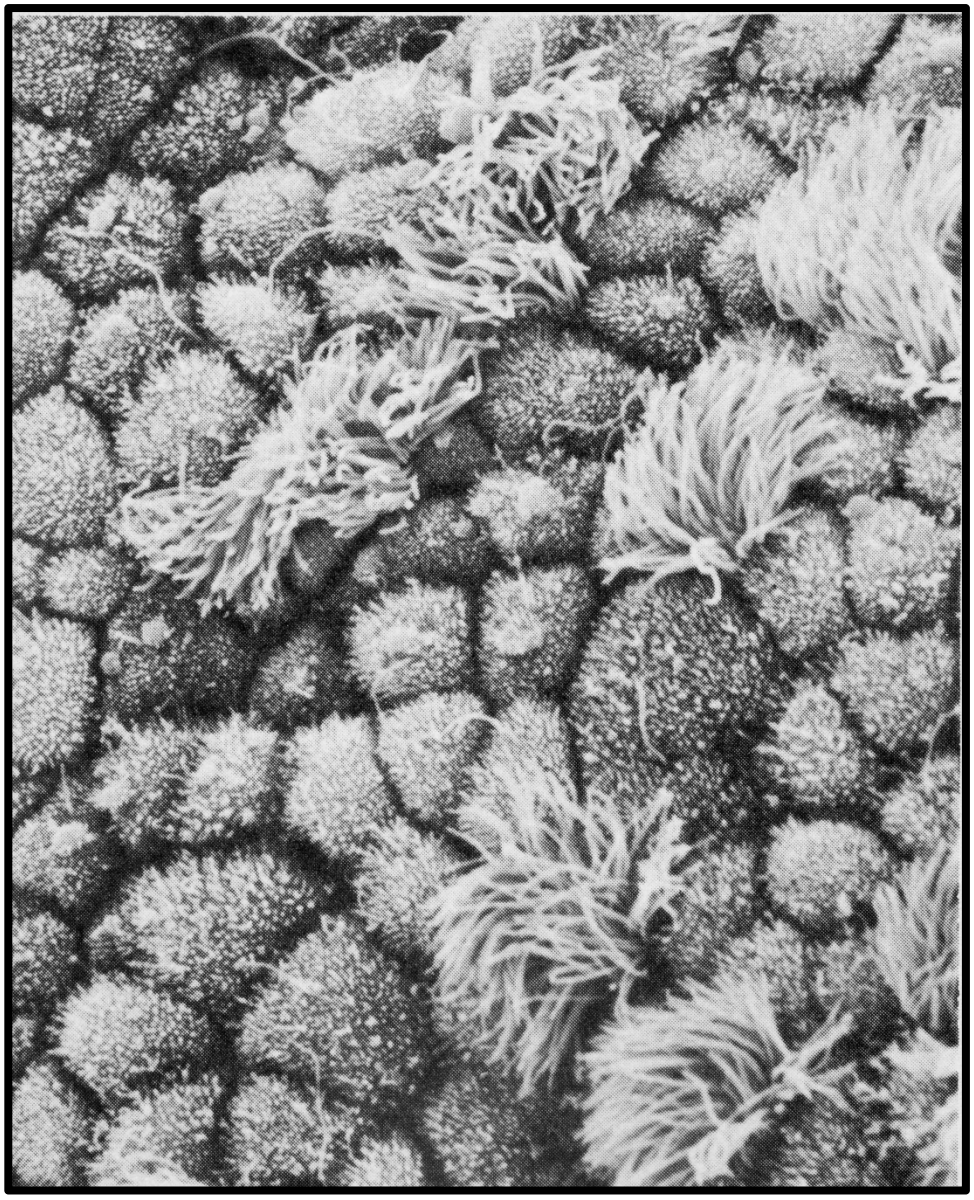


Figure 26 SEM rabbit hydrosalpinx higher magnification (x2000).

Higher magnification of an area adjacent to Figure 25 showing several ciliated cells surrounded by a large number of secretory cells in an early stage of secretory activity.

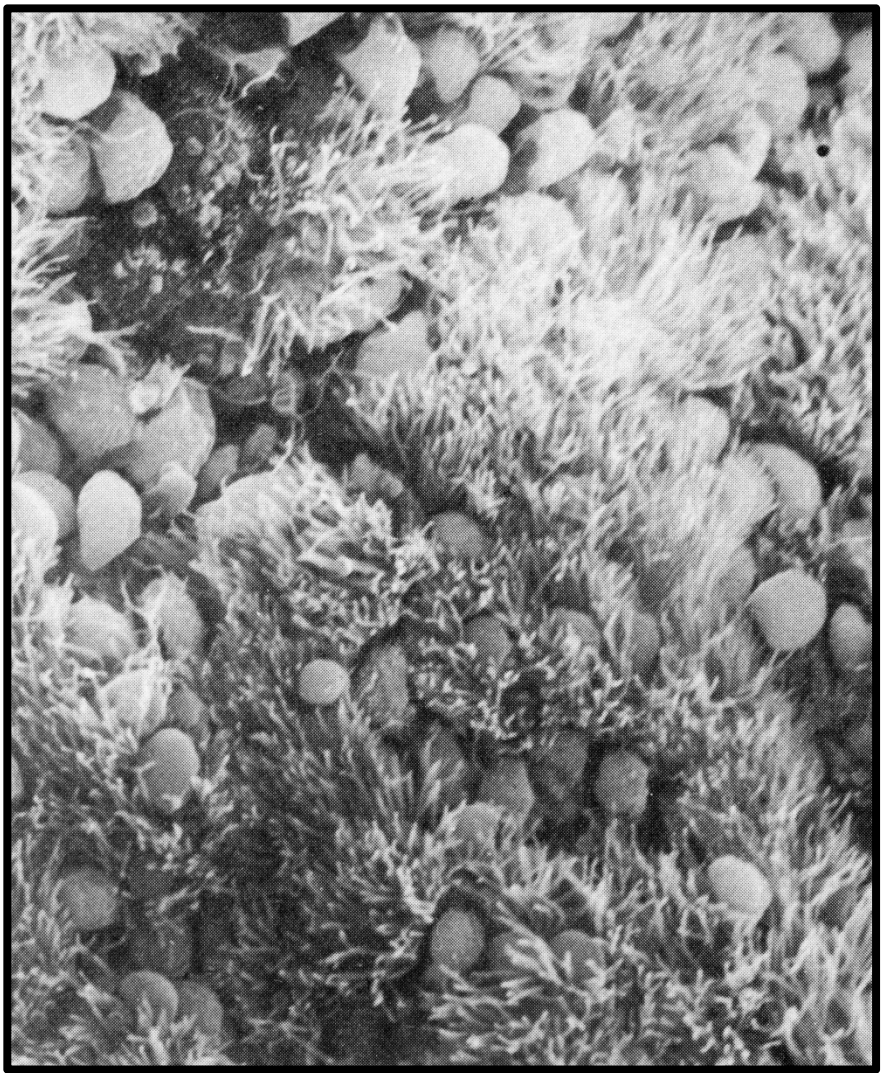


Figure 27 SEM rabbit hydrosalpinx showing ciliated and secretory cells (x2000).

Note that in this example most of the secretory cells are in an advanced stage of secretory activity.

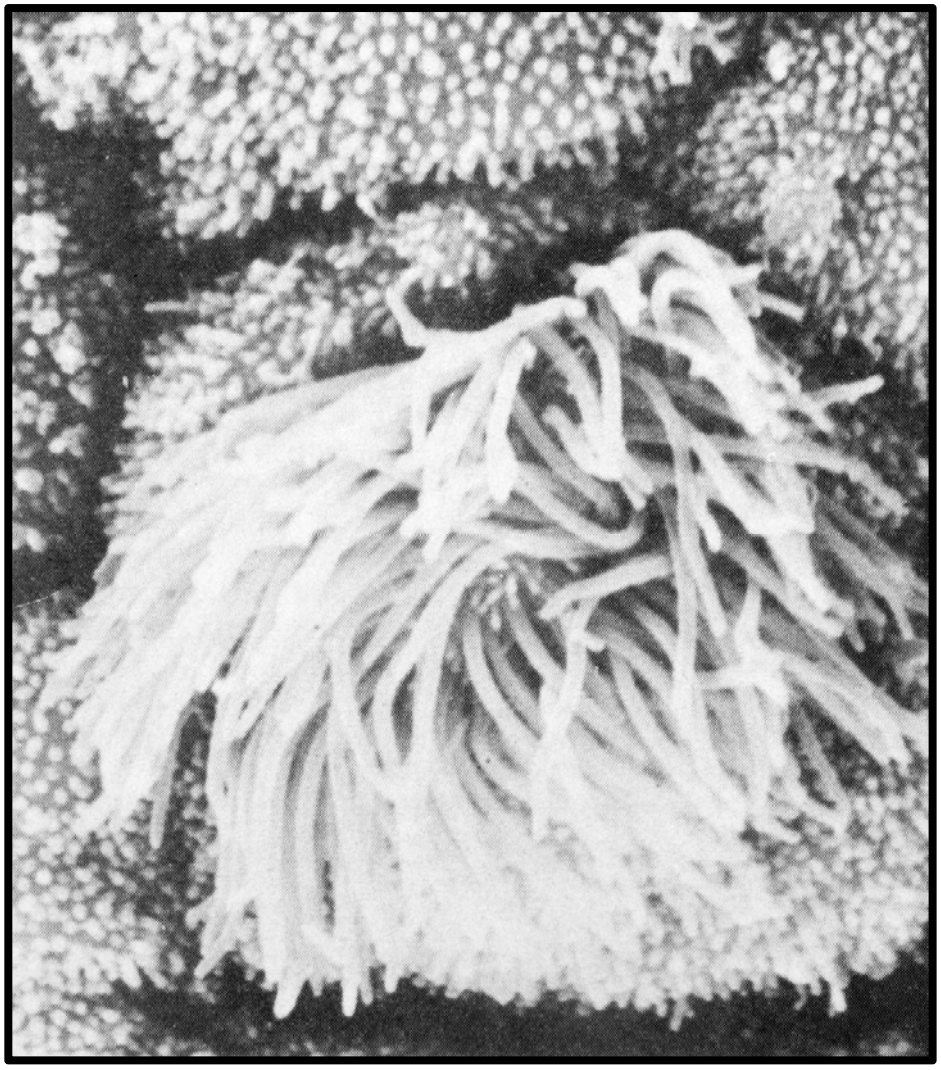


Figure 28 SEM rabbit hydrosalpinx ciliated secretory cells with normal appearance microvilli (x6600).

Higher magnification of an area adjacent to Figure 25. Note the essentially normal appearance of microvilli and cilia.

200mu in width (Figure 29). Cell populations on these polyps resembled adjacent areas of mucosa and there was usually a similar degree of deciliation as found on mucosa surfaces. The ciliation index of another 10 rabbits used in the hormone receptor study gave similar results (84% versus 35%) for control and hydrosalpinx oviducts.

6.4. Rabbit Reversed Hydrosalpinx

The right oviduct, six weeks following induction of hydrosalpinx, was subjected to microsurgical anastomosis of the AIJ and infundibulum, with removal of tantalum clips as previously described (see Appendix 6). Using magnification, a dilute methylene blue dye was injected into the isthmic part of the tube proximal to the two anastomoses to establish that oviduct patency had been achieved. A laparotomy was again carried out 6 weeks later and the right oviduct excised and subjected to light and surface electron microscopy.

6.4.1. Light microscopy

Epithelial flattening and separation of folds were no longer apparent and MCI had returned to normal (as seen in Fig 30).

6.4.2. Surface electron microscopy

Almost complete reversal to normal mucosal architecture and morphology were observed. Although some areas of deciliation persisted the number of ciliated cells had greatly increased (median value 90%; 95% confidence interval 70-95). This value was significantly different to the ciliation index of the control oviduct ($p=0.002$; Mann-Whitney non-parametric analysis).

Longitudinal folds were more prominent with secretory cells difficult to distinguish

Figure 29 SEM rabbit hydrosalpinx mucosal polyp and fold showing deciliation (x1000).

A mucosal polyp partly associated with a low fold showing deciliation. This particular polyp is 85 micrometres in its greatest diameter and 160 micrometres in height.

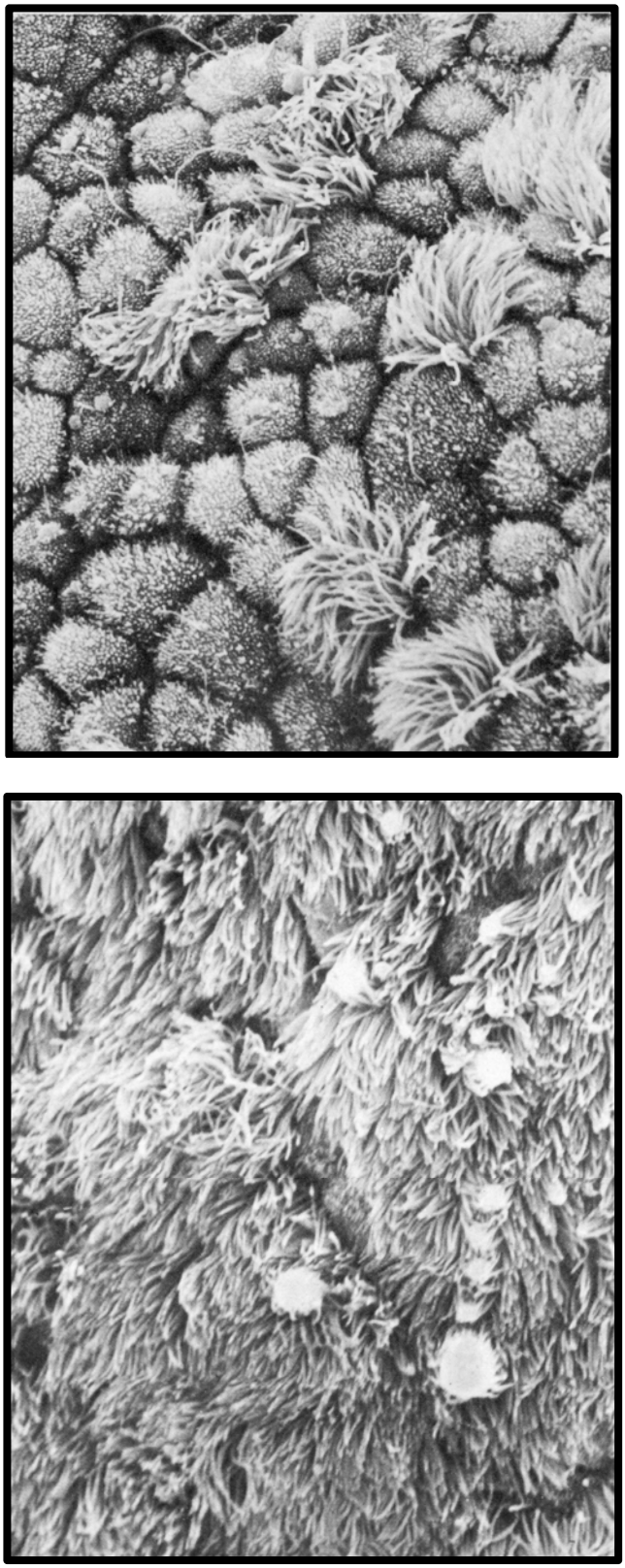


Figure 30 SEM reversal rabbit hydrosalpinx – return to normal ciliation (x2000).

Top panel as in Figure 26 SEM rabbit at maximal hydrosalpinx formation. Bottom panel magnification (X2000).

because of cilia regeneration in most areas. Figure 29 demonstrates deciliation of the control oviduct at maximal hydrosalpinx formation, and Figure 30, the return to normal cilia in the reversed oviduct in the same animal.

The cilia index of another 10 rabbits used in the hormone receptor study gave similar results, (35% versus 84% for control oviducts).

Return to normal MCI was seen following oviduct reconstruction.

6.5. Human Fallopian Tube Results

6.5.1. Normal tissue

The MCI results for ampulla isthmus and fimbriae are seen in Table 20. There was no significant difference in median counts between the 3 segments with the fimbria having the highest counts. Fimbriae counts ranged from 83-98%, ampulla 52-78% and isthmus 37-47%.

The ampulla, isthmus and fimbriae TEM and SEM examples are shown in Figures 31, 32, 33 and 34. No polyps or clumping of ciliated cells were seen in normal oviducts.

Mean cilia index for pregnant and postnatal patients were significantly reduced (Figure 35).

Ampulla specimens from 3 menopausal patients not on hormone replacement therapy, had median counts which varied from 60-68% and did not differ from menstruating women.

Mucosal folds had shallower depths with flat surfaced with non ciliated cells having sparse microvilli (Figure 36).

Epithelial atrophy was seen with flat columnar cells with reduced microvilli. Healthy cells were also apparent in other specimens (Figure 37).

Table 20 Human Fallopian tube mean cilia index for different anatomical segments.

Site	n	Mean ciliation index
ISTHMUS	4	41% (± 4-8)
AMPULLA	13	63% (± 11-15)
FIMBRIA	9	92% (± 6-9)

Specimens were from premenopausal subjects with regular menstrual cycles.
No significant difference in MCI between sites.
$P<0.5$.

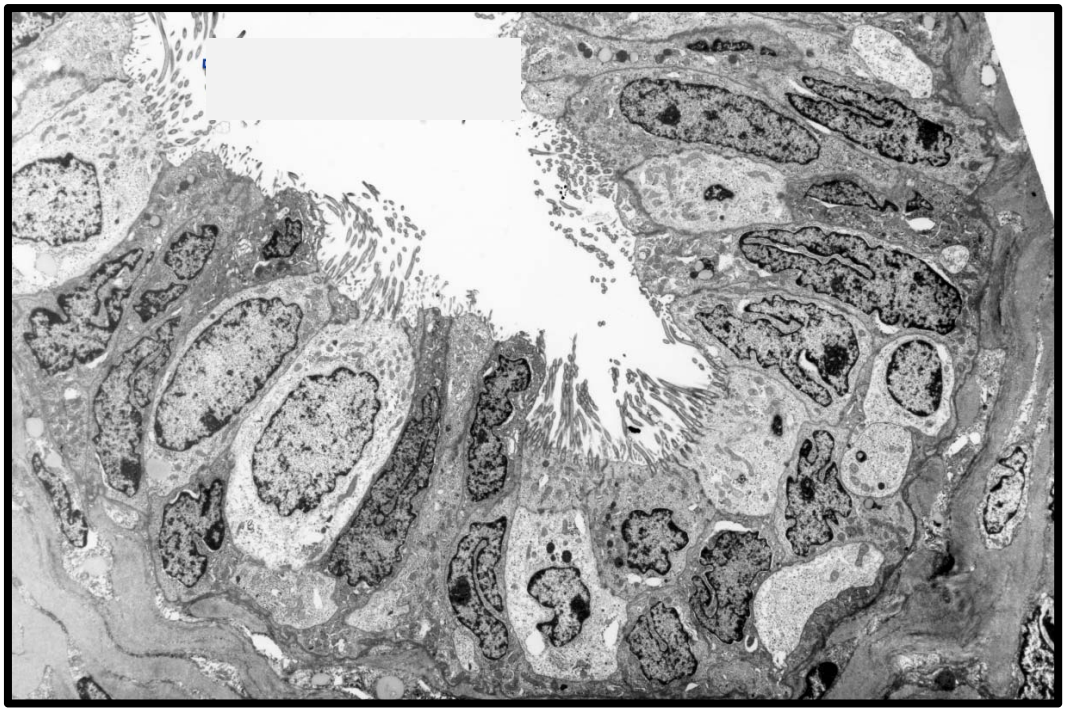


Figure 31 TEM human Fallopian tube ampulla - secretory phase (x25000).

Normal tissue with normal cellular appearance and cilia.

Figure 32 SEM human Fallopian tube ampulla - secretory phase menstrual cycle (x1000).

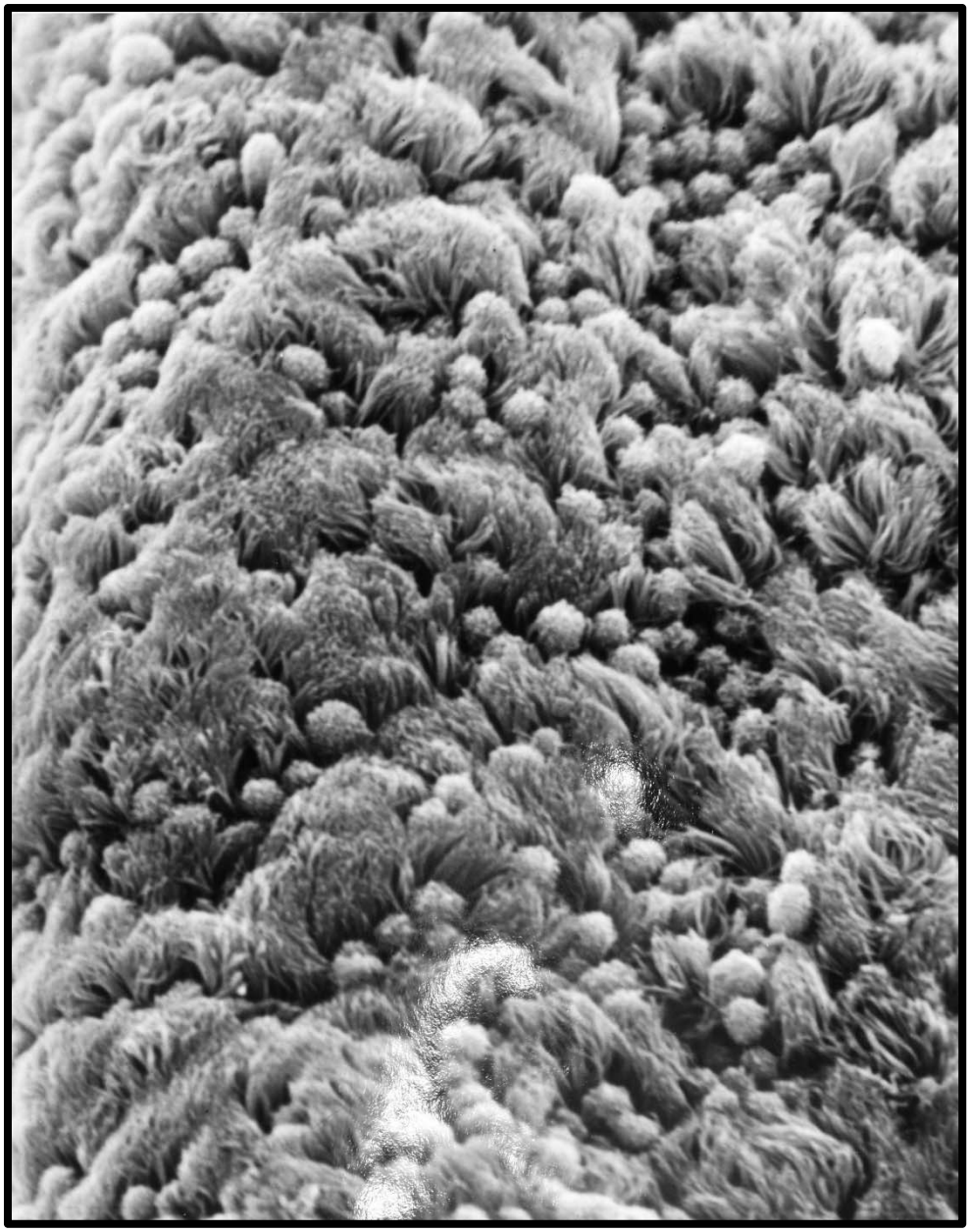


Figure 33 SEM human Fallopian tube ampulla – peri-ovular phase cycle (x1000).

At ovulation having reached peak activity, secretory cells are lower than ciliated cells. Secretory cells are in an advanced stage of secretory activity.

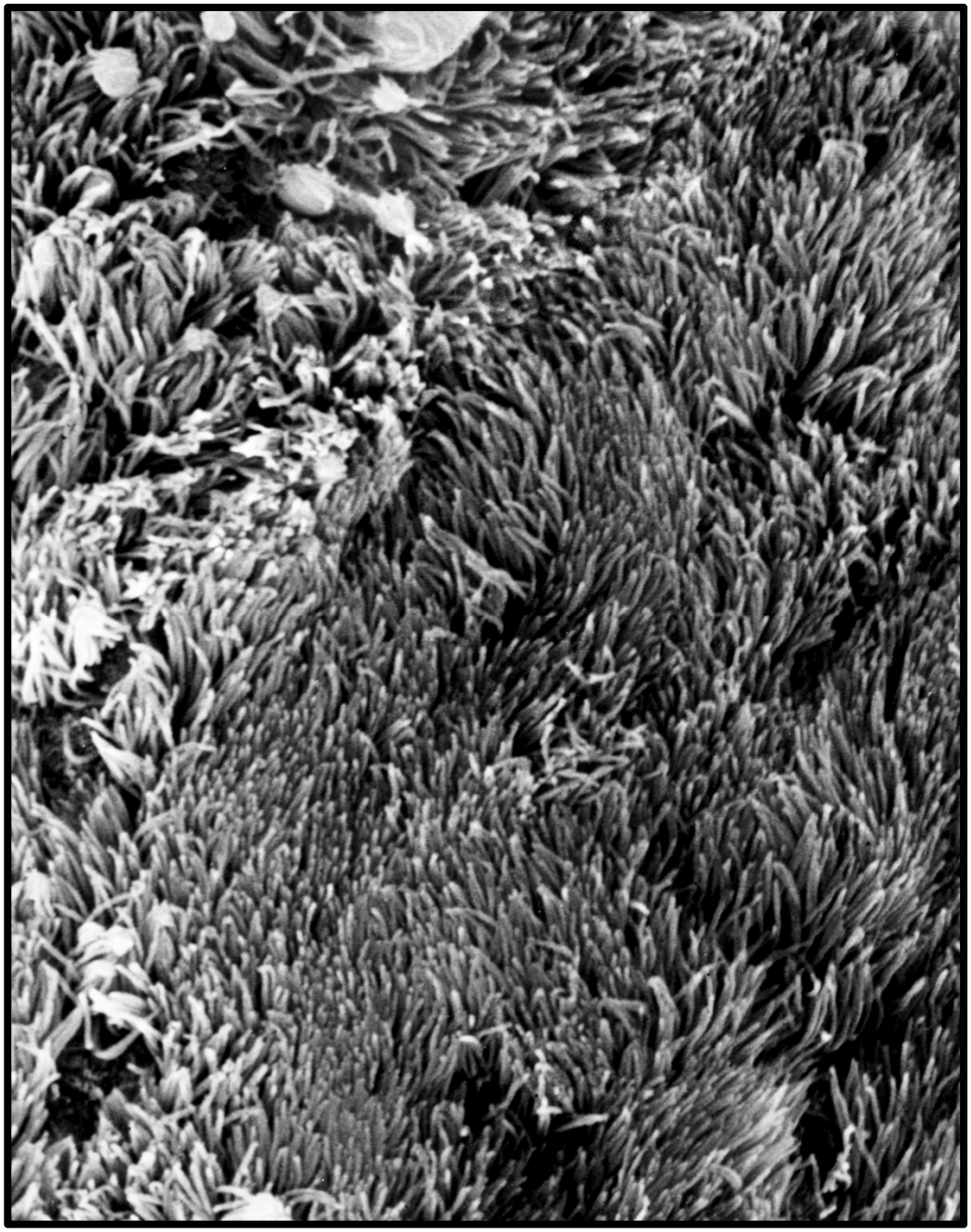


Figure 34 SEM human Fallopian tube - fimbriated portion (x1000).

The predominant cell type in this segment of the Fallopian tube are ciliated cells.

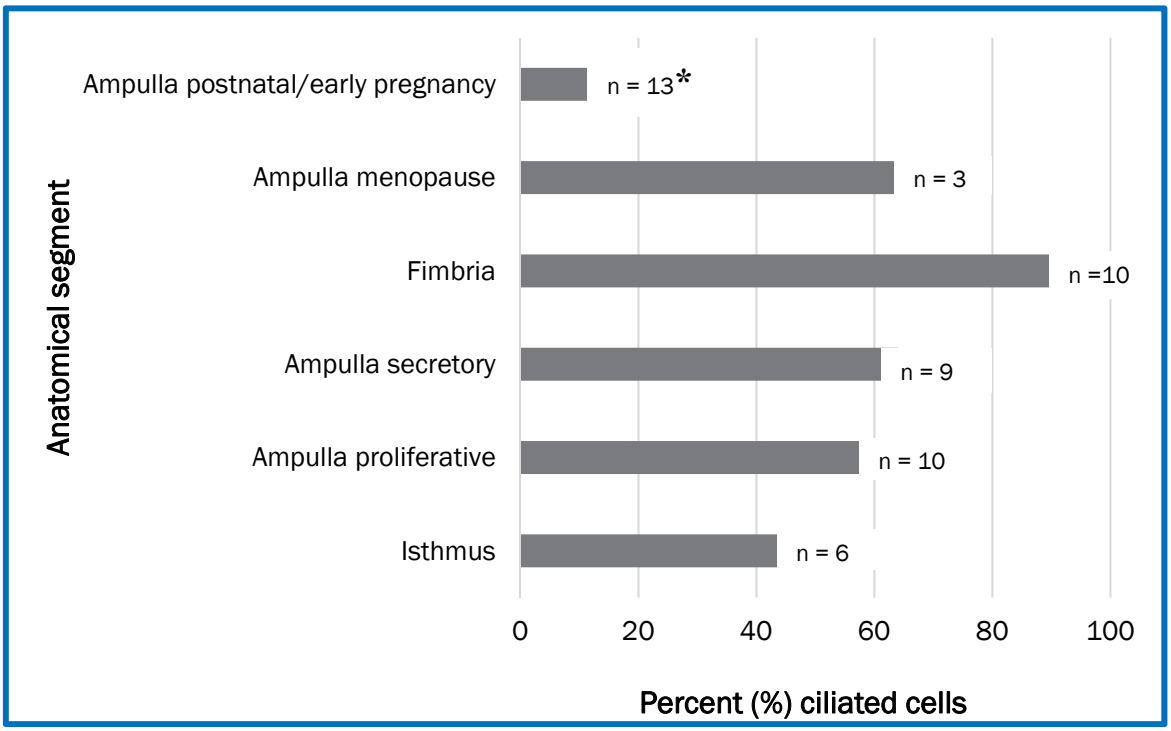


Figure 35 Mean cilia index of Fallopian tube – proliferative/secretory phase cycle, menopause and post natal.

Different stages shown include proliferative and secretory phase of the menstrual cycle, menopause and postnatal. The highest mean cilia index is seen in the fimbria and lowest in the isthmus. Very low values are seen in menopausal and pregnant patients.

Student's t-test: significant difference $p<0.5$ between pregnancy samples* and all other Fallopian tube segments.

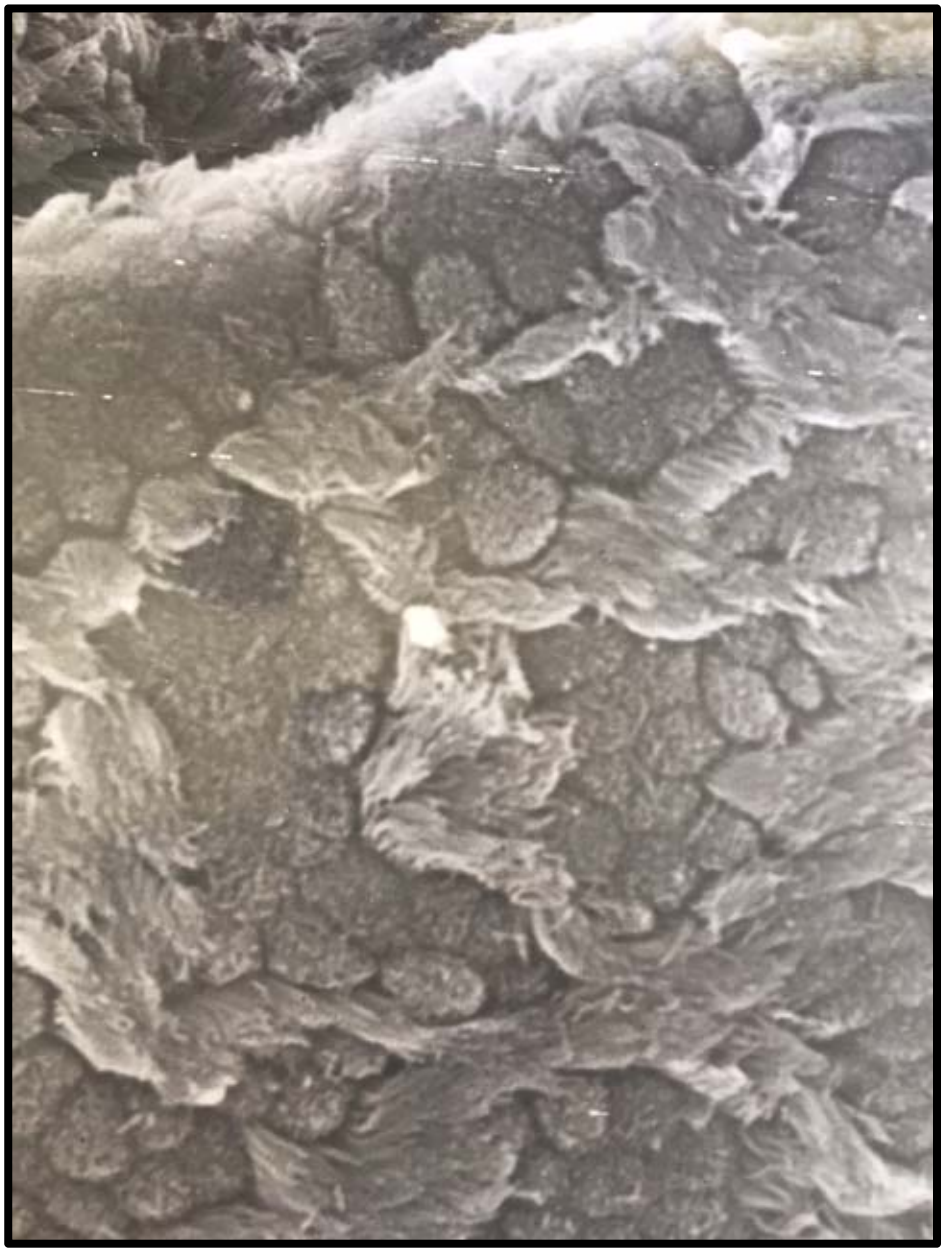


Figure 36 SEM human Fallopian tube ampulla – menopause (x1000).

Epithelial atrophy is evident by presence of flat columnar cells without microvilli.

Figure 37 SEM human Fallopian tube – menopause at higher magnification (x1000).

Healthy cilia in reduced numbers at higher magnification.

6.5.2. Pathological tissue

6.5.2.1. Hydrosalpinx

The changes occurring in the mucosa of Fallopian tubes affected by hydrosalpinx formation are progressive as seen in Stages 1 to 3 (Figure 38). Mild changes to mucosal folds are seen at first with subsequent loss of secondary and tertiary folds and finally progressing to flattening and atrophy of all folds and severe deciliation. Normal cellular ratios are maintained at first with normal surface microvilli changing to cupuloid, pleomorphic and finally flat hexagonal cells. The end result in some specimens is progressive desquamation, atrophy and cell necrosis. Surviving ciliated cells have fewer cilia and secretory cell microvilli are lost or blunted.

Except for the changes observed with the passage of time in the surgically induced hydrosalpinx model in rabbits, there is no evidence in the literature to indicate the time over which changes occur in the human hydrosalpinx.

All these changes were observed in the 49 ampulla, isthmus and fimbria microbiopsy specimens taken at different stages of the menstrual cycle.

All specimens demonstrated some degree of deciliation compared to normal Fallopian tubes with MCI being at least 10% below normal. Counts varied from 10-30% (Figure 39).

Epithelial folds were usually present but large flattened areas were also commonly seen (Figure 40 and Figure 41).

Polyp formation was present in 3 of the 49 specimens (Figure 42). In 13 specimens severe clumping of cilia was observed (Figure 43). Secretory cells were at different stages of development.

Transmission microscopy of hydrosalpinx epithelium is seen in Figure 44. Flattening of epithelial cells with reduced ciliary distribution and degenerating secretory cells are clearly identified. The MCI of the 49 specimens is shown in Figure 39.

HYDROSALPINX FORMATION

STAGE 1

1. Increased vascularity
2. Increased secretory activity
3. Cell types normal ratio
4. Cells normal morphology
5. Fold pattern conserved

STAGE 2

1. Loss of secondary and tertiary folds
2. Deciliation starting at fold bases
3. Secretory cells cupoloid; pleomorphic
4. Excess secretion still common
5. Secretory cell microvilli normal

STAGE 3

1. Progressive flattening /atrophy all folds
2. Progressive severe deciliation
3. Fewer cilia on some ciliated cells
4. Secretory cells flat, hexagonal, atrophic
5. Microvili lost or blunted
6. Progressive desquamation /cell necrosis

Figure 38 Features of stages in hydrospalinx formation.

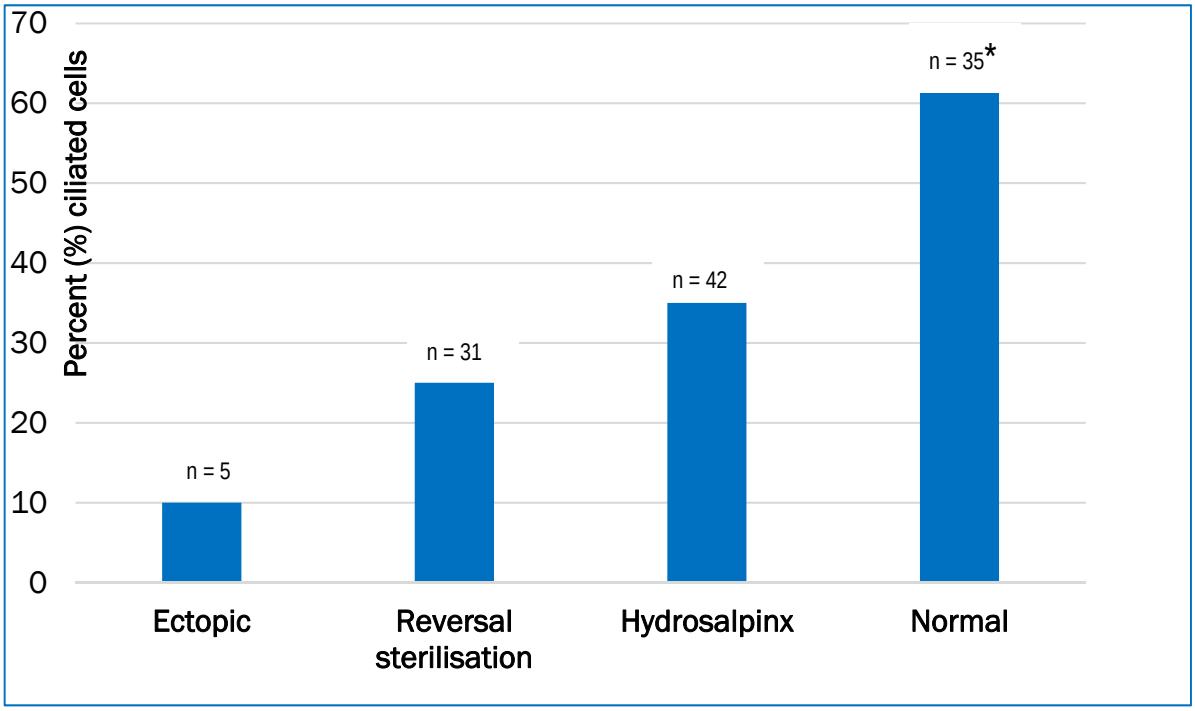


Figure 39 Mean cilia index of pathological Fallopian tubes.

Mean cilia index (MCI) was reduced in hydrosalpinx, reversal sterilisation and ectopic pregnancy specimens.
Student's t-test: Significant difference $p<0.5$ between normal Fallopian tube* and pathological specimens (ectopic, reversal sterilisation, hydrosalpinx)

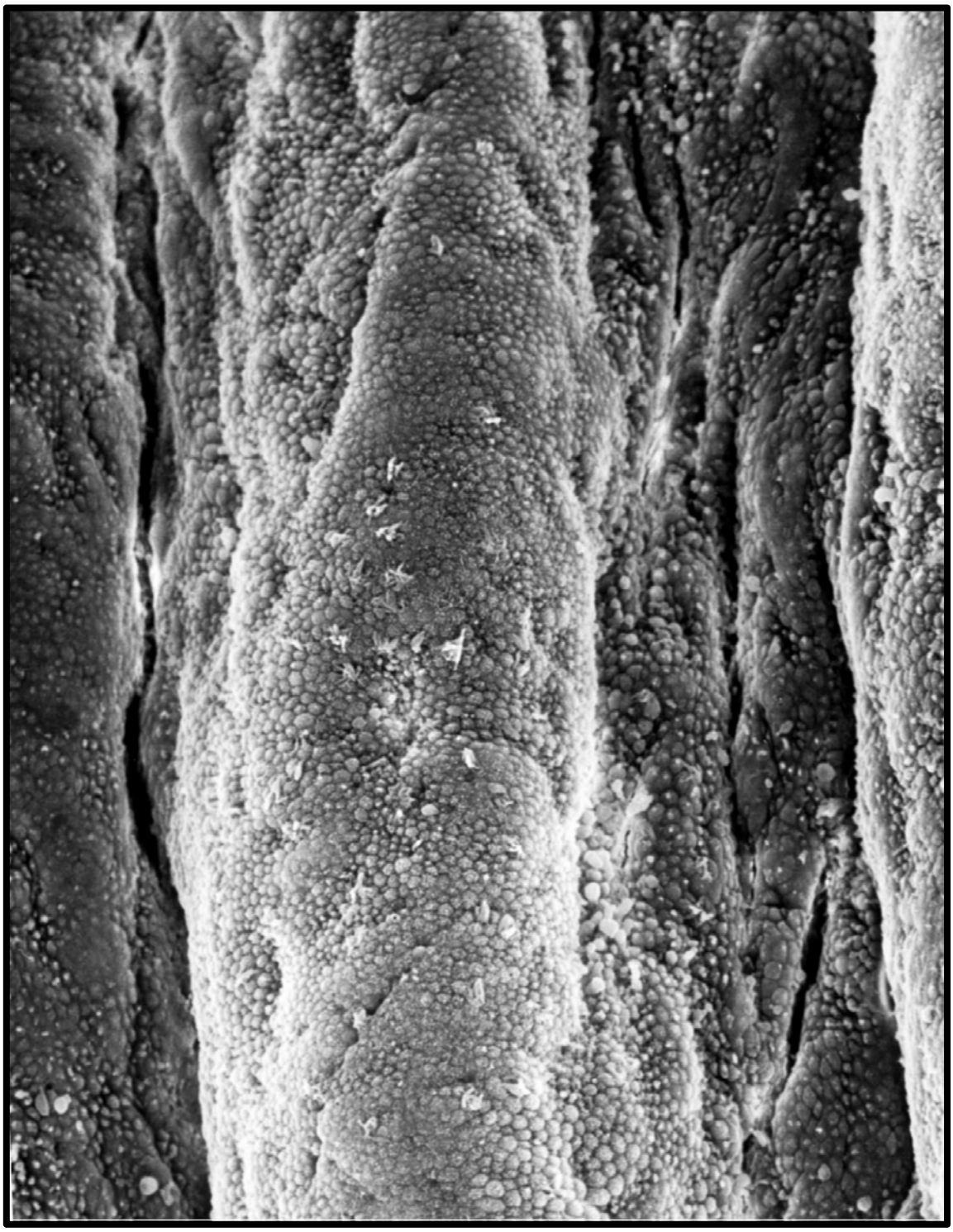


Figure 40 SEM human hydrosalpinx – marked deciliation (x 360).

Tubal lumen showing tubal fold and marked deciliation.

Figure 41 SEM human hydrosalpinx – flattened mucosa (x360)

Large flattened areas of mucosa are seen with extensive deciliation.

Figure 42 SEM human hydrosalpinx – polyp formation (x3000).

Fallopian tube epithelium showing polyp formation.

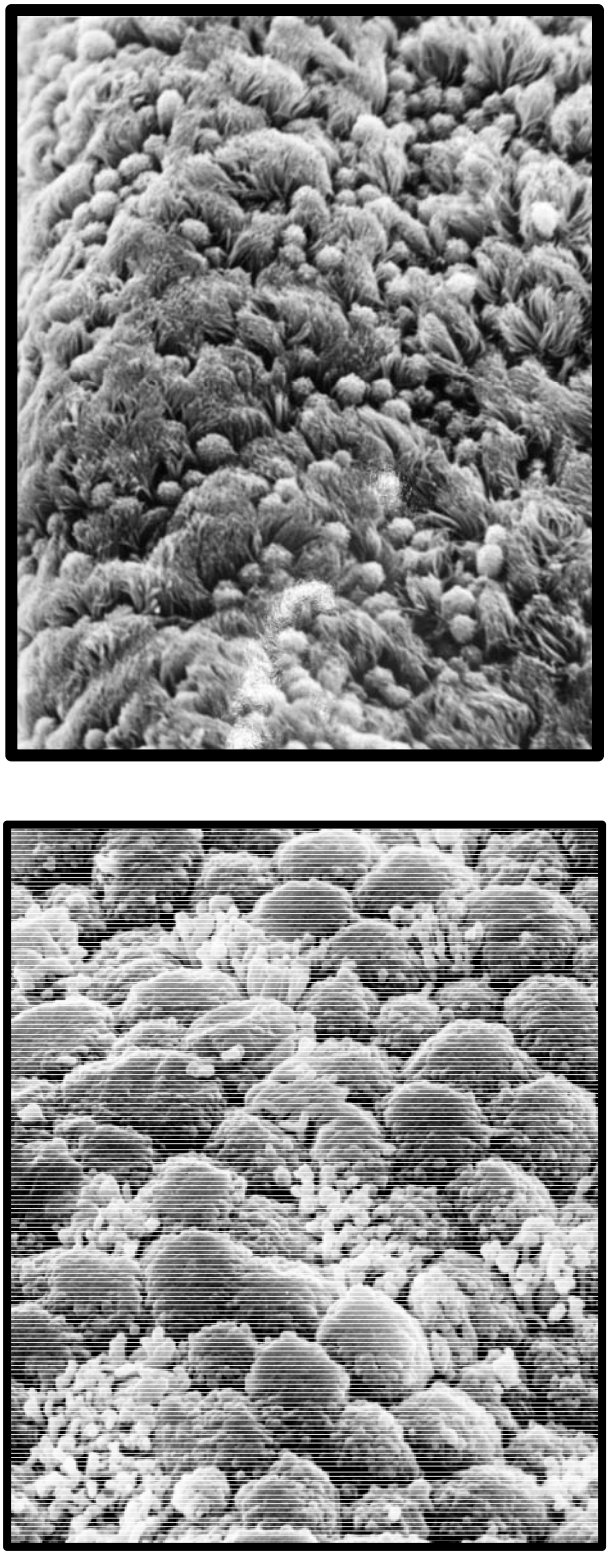


Figure 43 SEM human normal Fallopian tube and hydrosalpinx.

Top panel: Human Fallopian tube ampulla - periovular phase cycle. Secretory cells have reached peak secretory activity and are lower than ciliated cells. (x1000).
Bottom panel: Fallopian tube epithelium showing clumping cells and abnormal microvilli (x2500).

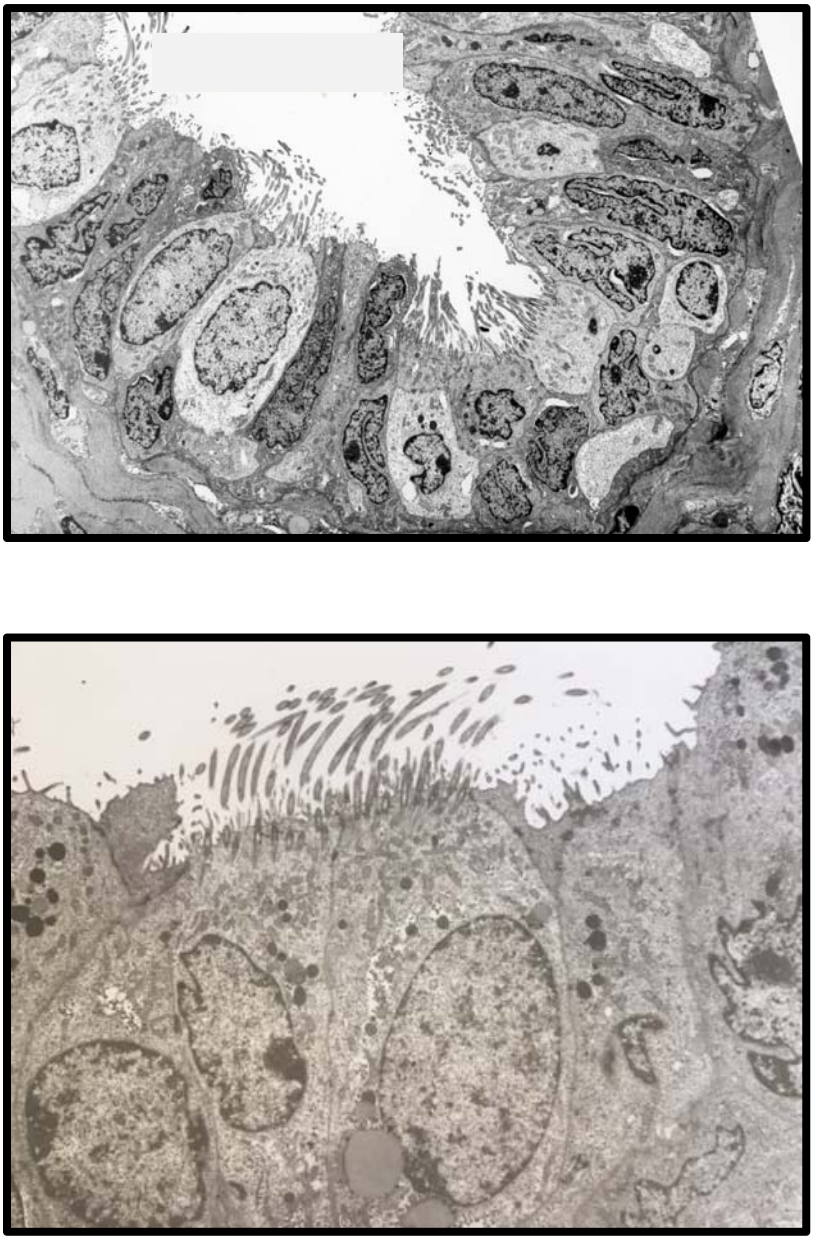


Figure 44 TEM human normal Fallopian tube and hydrosalpinx - secretory phase cycle.

Top panel: Normal Fallopian tube with normal cilia and cellular structure (x7000).
Bottom panel: Hydrosalpinx showing degenerating secretory cells, large intracellular vacuoles and also swollen nuclei with sparse chromatin, (X7500).

6.5.2.2. Sterilisation reversal

Sterilisation was performed by application of a Fallope ring as seen in Figure 45. 31 specimens were taken from the proximal occluded tubal segment following sterilisation performed varying number of years previously.

Ciliated counts varied from 10-30% with median 25 (± 10.6%). See Figure 39.

SEM revealed that luminal folds were shallower with mostly longitudinal folds seen in the majority of specimens. Six of the 31 specimens had no mucosal folds and a further six only had segmented folds.

Polyp formation was seen in 13 specimens with single and multiple polyps in clusters occasionally seen.

Clumping of ciliated cells was seen in 23 specimens. Secretory cells displayed various stages of development.

6.5.2.3. Ectopic pregnancy

Multiple specimens were taken from 11 patients having surgery for ectopic gestation, at 6 to 10 weeks of pregnancy. They were all sited in the ampulla of the Fallopian tubes. Biopsies were taken adjacent (within 5mm) to the ectopic site in proximity to the fimbriae. Mucosal folds were identified in all but one specimen with marked deciliaton being displayed (Figure 46).

Ciliated cell counts varied from 3-20% (Figure 39) and appeared uniform when multiple biopsies were taken from the same oviduct. Cilia stunting was observed with swollen distal portions and polyp formation was seen in 3 specimens.

The secretory cells in all specimens appeared to be in mature stage of development with secretion being apparent.

6.6. Discussion

Morphology of the mammalian oviduct was reviewed in Chapter 1, describing tubal

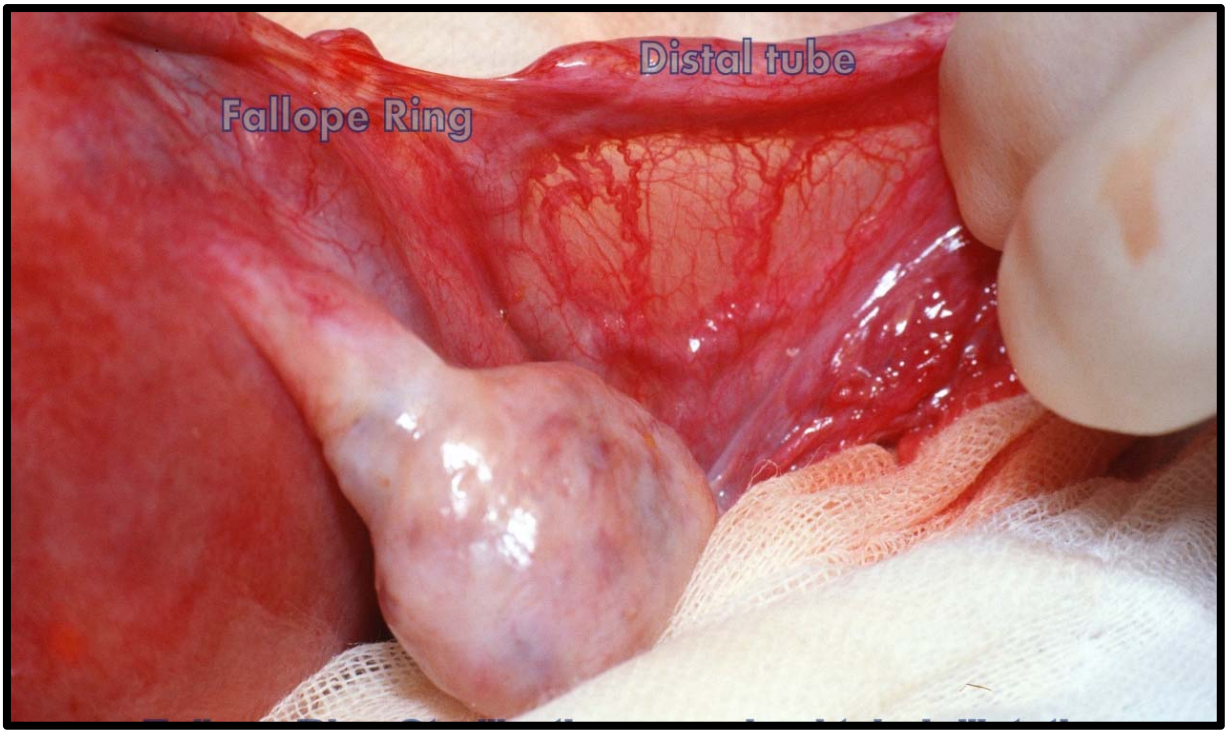


Figure 45 Fallope ring sterilisation – proximal tubal dilatation.

Fallope ring seen buried in adhesions showing 1-2cms disruption of the Fallopian tube and closed proximal and ampullary segments of the uterus. The proximal isthamic part of the tube is dilated. Normal ovary and mesenteric vascular architecture.

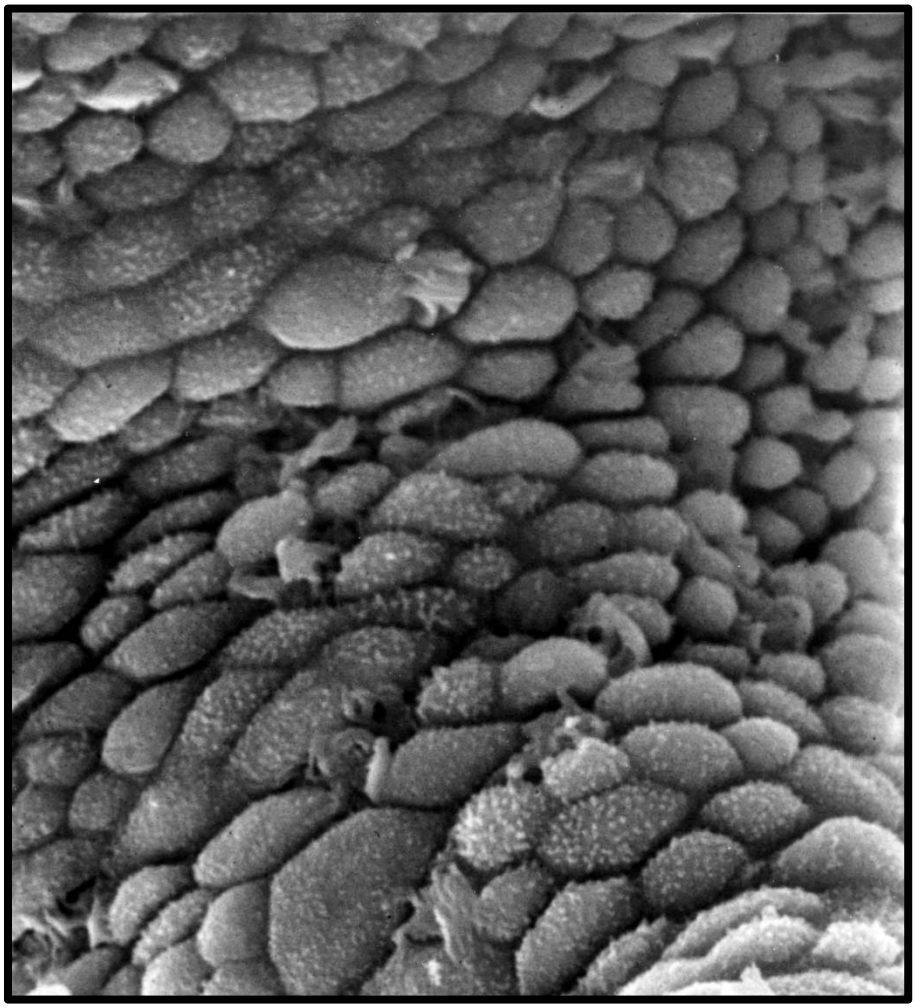


Figure 46 SEM human Fallopian tube – ectopic pregnancy

Ampulla adjacent to ectopic pregnancy demonstrating marked deciliation.

epithelial changes during the ovarian cycle, pregnancy, and the puerperium under the influence of steroid hormones. The result of current study was in agreement with previous published data.

The rabbit hydrosalpinx model produces deciliation without involvement of an inflammatory process. The degree of deciliation found in this study was also demonstrated by Vasquez et al (1981), Vemer et al (1984), Formelli et al (1985) and Donnez et al (1986), who also found almost complete deciliation in the same rabbit model. Most severe changes were seen after double clipping as used by all studies described above. Clipping at the fimbrial end only resulted in hydrosalpinx formation in only one of 34 oviducts as opposed to 67 of 99 oviducts that were double clipped (Vemer et al 1984). This finding probably explains the variance in results found by Halbert and Patton (1981) as only the fimbrial end was occluded in their study. Double clipping produces a closed loop hydrosalpinx which causes increased intraluminal pressure and reduced oviduct blood flow as described in Chapter 4.

It is reasonable to deduce that these physical changes are responsible for the observed deciliation as the reversal of hydrosalpinx study confirmed a high degree of reciliation, and subsequent fertility. The study of Gauwerky et al (1994), also demonstrated deciliation with loss of microvilli, reduced secretory activity and de-dedifferentiation of epithelial cells with maximal changes occurring from 4 weeks onward from initial surgery. Deciliation was fully reversible within 8 weeks of reversal of hydrosalpinx. Their study also found that deciliation was uneven with fields and islets beig affected. The finding of capillaries under ciliated patches but not under flattened deciliated areas suggested that vascularization changes may also be responsible for the morphological alterations. The microvascular architecture studies of Verco et al (1983) in the rabbit model are supportive of this

view. The time interval to resumption of fertility following restoration of patency in the rabbit hydrosalpinx model was addressed by Karbowski et al (1988). Two weeks after correction no pregnancies occurred despite patency rate of 63%. Pregnancies were observed from 4 weeks onwards, concomitant with partial or complete recovery of morphological changes.

Early studies (Patek and Nilsson (1977, Woodruff and Pauerstein, 1969) of epithelial ultrastructure in Fallopian tubes following salpingitis indicated no apparent change in ciliation. The findings in this thesis are similar to those reported by Vasquez et al (1983). They found cell height of thin walled hydrosalpinges to be significantly different to controls, mucosal surface ciliation varying from 5.8-59.9% with thin-walled hydrosalpinges having fewer ciliated cells than thick-walled hydrosalpinges. Deciliation had persisted in a few patients in microbiopsies taken more than 12 months following unsuccessful salpingostomy. Poor clinical outcome was reported following reversal of sterilisation after long term occlusion of the Fallopian tubes (Vasquez et al 1980) in women with demonstrable loss of ciliated cells. These findings are suggestive that long term occlusion of Fallopian tubes either by hydrosalpinx formation or sterilisation may result in irreparable damage to tubal mucosa. The SEM study of Li and Chen (1993) confirmed loss of cilia and microvilli in the 1 cm. of proximal blocked Fallopian tube after sterilisation, and suggested that this lenght of Fallopian tube should be excised before anastomosis is performed to reverse sterilisation.

The results presented in this thesis on reversal of sterilisation tubal microbiopsies are complementary to the above studies, with the added finding of frequent polyp formation.

The SEM results for ectopic pregnancy described in the thesis are in agreement with the findings of Vasquez et al (1983), who found that the percentage of ciliated

surface was always lower than in other segments of the same tube. Their study also determined that the degree of deciliation was more than seen in normal pregnant women at an equivalent gestational age. It is plausible to suggest that ampullary deciliation may play a role in affecting tubal transport and facilitate tubal implantation of the embryo.

Chapter 7 Review - Assessment of Fallopian Tube Normality, Patency and Function

The complete investigation of couples presenting with infertility includes assessment of patency of the Fallopian tubes to confirm that fertilisation can take place in the ampulla.

The gold standard for some decades has been laparoscopy with chromo-pertubation (passage methylene blue dye), (Kodaman et al 2004). Hysteroscopy is usually performed at the same time to exclude congenital or acquired abnormalities of the uterine cavity.

Alternative tests have been promoted, particularly by non-operative ART specialists, to eliminate the need for regional or general anaesthesia, reduce surgical costs and surgical risk.

7.1. Hysterosalpingography (HSG)

This is a useful radiological procedure to detect congenital anomalies, leiomyomas, synechiae, salpingitis isthmica nodosa, hydrosalpinx and tubal patency.

It fails to provide adequate assessment of peritubal adhesions, ovarian morphology and endometriosis.

It suffers the drawbacks of often being painful, may be associated with vasovagal reactions and rarely allergic reactions to iodinated contrast media. When performed on patients that have had pelvic inflammatory disease, may precipitate an acute flare up, unless prophylactic antibiotics have been commenced before the procedure. A retrospective analysis of post HSG incidence of pelvic infection was estimated to occur in 3.1% of patients having this procedure (Stumf and March 1980).

Major factors for recurrence of pelvic infection were:

- Previous pelvic inflammatory disease.
- Infertility history
- Past pelvic surgery for infection
- Adnexal tenderness during the procedure
- Presence of adnexal mass.

A higher incidence of allergic reactions and anaphylaxis associated with oil based contrast media, have led to water soluble based media being predominantly used. One of the reason for resurgence of HSG usage is related to the findings of a Cochrane review meta-analysis, which described a higher conception rate following HSG when oil soluble contrast media was used with an odd ratio of 1.9 (95% c.i. 1.6-2.29). It was suggested that this finding may be associated with a demonstrated reduction in peritoneal macrophage function (Luttjeboer et al 2007; Boyer et al 1986), improved endometrial receptivity and possible effect on pelvic endometriosis.

Despite the fact that laparoscopy does not have perfect sensitivity, specificity, or positive and negative predictive values for detection of tubal pathology or patency, comparisons between HSG and laparoscopy show discrepancies up to 45% (Volpi et al 1996).

A meta-analysis involving 20 articles and 4100 patients (Swart et al 1995) concluded that whilst laparoscopy was not the ideal standard for tubal patency, HSG had a sensitivity of only 65% and specificity of 85% compared to laparoscopy.

7.2. Ultrasonography and Sonohysterography (SIS)

Intrauterine saline infusion whilst performing pelvic ultrasound improves detection of intrauterine cavity defects. The ability to perform SIS as an outpatient procedure without anaesthesia has attracted clinicians to utilise this technique.

Comparing SIS to HSG and hysteroscopy has shown statistical equivalence for evaluation of intrauterine pathology (Deichert et al 1987).

For tubal assessment transvaginal ultrasound is useful for detection of hydrosalpinges with sensitivity of 86% (Sokalska et al 2009). These procedures whilst able to evaluate ovarian and uterine cavity abnormalities have limitations with respect to image interpretation and ultrasonographer experience. Furthermore, ultrasonography and SIS require supportive studies for tubal patency and oviduct pathology.

7.3. Hysterosalpingo-Contrast Sonography (HYCOSY)

Combining transabdominal ultrasound and infusion of Hyscon in the uterine cavity (Dextran70 (32% w/v in dextrose) (Watrelot and Dreyfus 2000; Mohiyiddeen et al 2015; Papaioannou et al 2007), tubal patency can be confirmed by identifying fluid in the pelvic culdesac indicating patency of at least one Fallopian tube. The introduction of hyperechoic contrast media has allowed tubal patency assessment by visualisation of intratubal flow of echogenic contrast with ultrasound and demonstration of flow to the distal end of the tube and over the adjacent ovary.

A learning curve of 50-100 procedures is necessary to achieve visualisation in up to 95% of cases (Hamilton et al 1998).

The HYCOSY procedure has a 10% false occlusion rate and 7% false patency rate when compared to laparoscopy (Holz et al 1997).

Campbell et al (1994) demonstrated concordance of 86.3% with laparoscopy and 83.2% with HSG.

Review of the literature supports the view that HYCOSY is a reliable and useful screening procedure for infertility investigation.

7.4. Laparoscopy and Chromopertubation

The advantages of this procedure include:

- Complete evaluation abdominal and pelvic cavities for pathology
- Simultaneous treatment of endometriosis and pelvic adhesions
- Evaluation and management of oviduct pathology including hydrosalpinx and peritubal adhesions.

Surgical complication rates of 5.7% per 1000 laparoscopy procedures have been reported in large studies (Jansen et al 1997). These include haemorrhage from vascular injuries and intestinal injuries.

Diagnostic procedures have less frequent complications (2.7per 1000), compared to operative procedures (17.9 per 1000) (Jansen et al 1997).

Retrospective reviews involving more than 1.5 million women revealed an overall complication rate of 0.2-10.3% with 20-25% being unrecognised during the procedure.

In the presence of a positive history, laboratory or positive non-invasive tests suggesting oviduct related pathology, laparoscopy remains the procedure of choice for definitive diagnosis and possible operative treatment of pelvic pathology causing infertility.

7.5. Transvaginal Hydrolaparoscopy (THL)

The original transvaginal technique for inspection of the pelvis was culdoscopy, which was widely used in the 1960s and 1970s. It was superceded by laparoscopy, which offered many advantages. Watrelot et al (1997) and Brosens, Campo and Gordts (1998) were among the first to propose and report the development of the

standardised procedure “transvaginal hydrolaparoscopy” or THL. Initially a modified reusable laparoscope was used with subsequent introduction by Watrelot of a disposable instrument, which could also be used for hysteroscopy, labelling it a “fertiloscope” and the procedure fertiloscopy (Watrelot and Dreyfus 2000). This procedure combines the passage of a fine telescope via the posterior fornix of the vagina and simultaneous perfusion of the pelvic cavity with warm Ringer’s lactate solution. This achieves flotation and visualisation of the adnexa and posterior aspect of the uterus and pelvis.

Introduction of the telescope into the tubal opening enables salpingoscopy to be performed. The tubal mucosa can usually be inspected from the infundibulum to the ampullary-isthmic junction. Passage of dye through the cervix, progressing through the Fallopian tube and spill from the fimbriated end can be directly visualised.

Hysteroscopy and laparoscopy can be performed at the same time in an ambulatory setting, using local analgesia in conscious patients or general anaesthesia depending on patient preference. The technique, diagnostic accuracy, safety and patient tolerance were evaluated by Gordts et al (2002).

Concerns regarding risk of bowel injury and sepsis have prevented the widespread use of this technique in Australia. A multinational survey evaluating the incidence of bowel injury involving 3667 THL procedures reported 24 bowel injuries (0.65%), (Gordts et al 2001). The authors confirmed that the injuries were recognised during the procedure and were managed conservatively without apparent consequences. The risk of bowel injury increases in the presence of a retroverted uterus or Pouch of Douglas endometriosis.

THL has limitations compared to laparoscopy as the procedure provides only a view of the posterior but not the anterior aspect of the uterus. Inability to manipulate the

adnexa means that not all adnexal pathology may be seen.

Operative procedures such as ovarian capsule drilling, treatment of superficial endometriosis, lysis of tubo-ovarian adhesions and endometrioma have all been reported (Gordts et al 2000; Fernandez et al 2004).

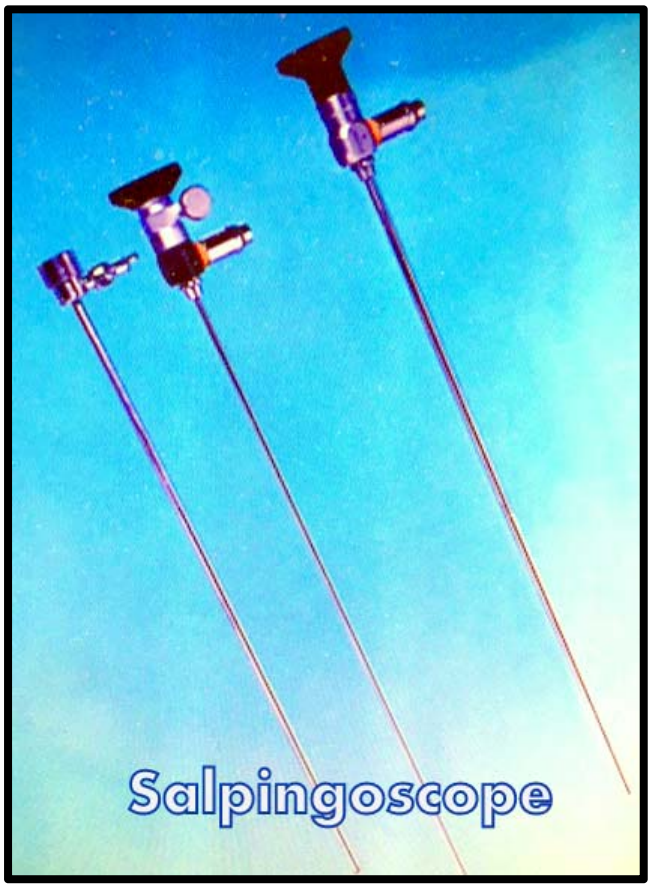
These authors have suggested that outpatient THL combined with hysteroscopy and tubal dye studies should be considered as an alternative to HSG as a first line investigative tubal patency procedure.

7.6. Salpingoscopy

Salpingoscopy involves the introduction of a telescope into the Fallopian tube from the fimbriated end.

Henry Suchet et al (1981) were the first to use a 4 mm hysteroscope inserted into the Fallopian tube at laparotomy. Cornier (1985) used a 3.5 mm flexible bronchoscope introduced via an operating laparoscope. Brosens (1988) described “tubal endoscopy” using a 2.6mm rigid “salpingoscope” and outer 5 mm protective sheath and obturator introduced via the operating channel of a laparoscope (Figure 47). Watrelot designed a multi-function instrument he called a “fertiloscope” with viewing, flushing and biopsy channels (Figure 48).

The terminal part of the tube is grasped with fine atraumatic forceps to introduce the 5mm sheath and telescope through the tubal osteum (Figure 48). Ringer’s lactate infusion via the outer sheath enables distension of the tubal lumen. Atraumatic grasping forceps are used to clamp the tube around the sheath at the level of the infundibulum. The obturator is replaced by the telescope to allow direct vision of the distended ampulla. The mucosal folds can be inspected whilst floating in the perfused fluid. When adhesions are encountered anchoring the tube and ovary, fine scissor dissection must be performed to mobilise the tube and ovary



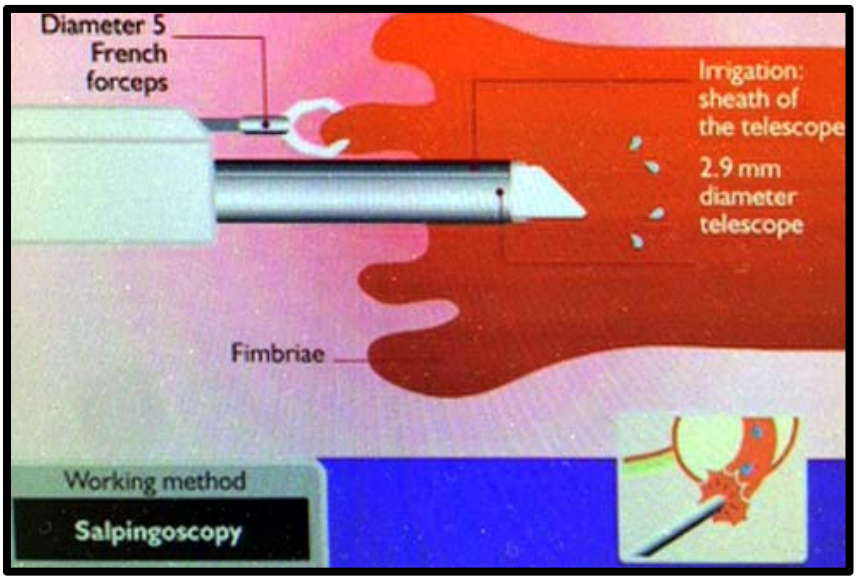


Figure 47 Salpingoscope and salpingoscopy technique.

Gentle use of grasping forceps at the fimbria allows introduction of the 2.9mm telescope for visualisation of the Fallopian tube lumen.

(Adapted from Watrelot 2002)

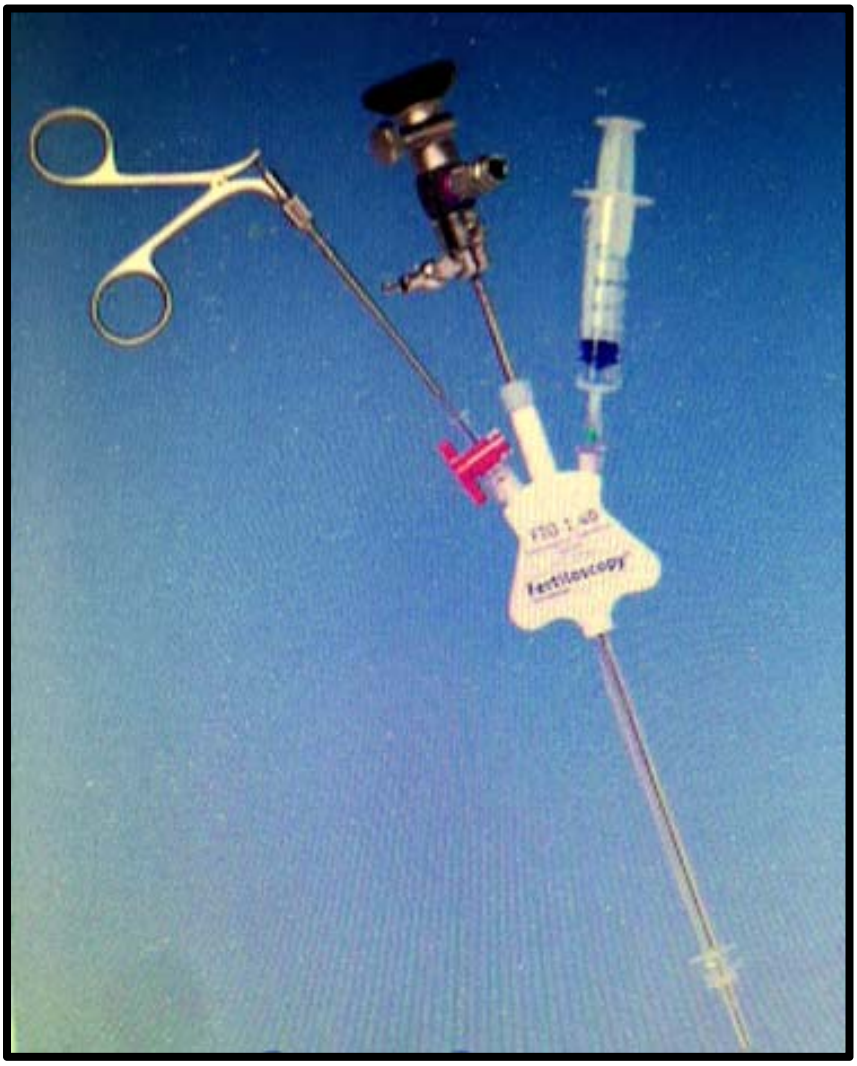


Figure 48 Watrelot multi-function salpingoscope

(Adapted from Watrelot 2002)

before the end of the tube can be manipulated and entered.

Presence of a hydrosalpinx requires a small incision to be made at the terminal part of the tube to allow introduction of the sheath and telescope.

I have used a modification of the above technique, introducing a 2mm hysteroscope and sheath via a lateral second entry port in the abdomen. This method requires a second camera system to enable laparoscopy and salpingoscopy to be performed simultaneously.

7.6.1. Normal salpingoscopy

The ampullary mucosa has 4-5 major longitudinal folds with several accessory folds arising on each side. Between the major folds are 3-5 minor folds (Figure 49). The major folds have a delicate structure with fine vascular network. The normal Fallopian tube is very distensible and with separation of the folds the tubal wall appears thin and transparent.

7.6.2. Salpingoscopy - mucosal abnormalities

Focal or extensive filmy or vascularised adhesions occur between ampullary folds (Figure 50).

Tubal fibrosis which is usually generalised may result in complete destruction of the mucosa with fibrotic fold remnants occurring in otherwise patent tubes (Figure 51).

Hydrosalpinx formation may be associated with flattening and separation of major mucosal folds and absence of adhesions. In this situation the tubes are usually thin walled. More severe mucosal destruction and fibrosis often results in the formation of a thick walled hydrosalpinx.

Using a simple classification system (Table 21), evaluation of the extent of tubal inflammatory damage and likely impact on fertility can be assessed.

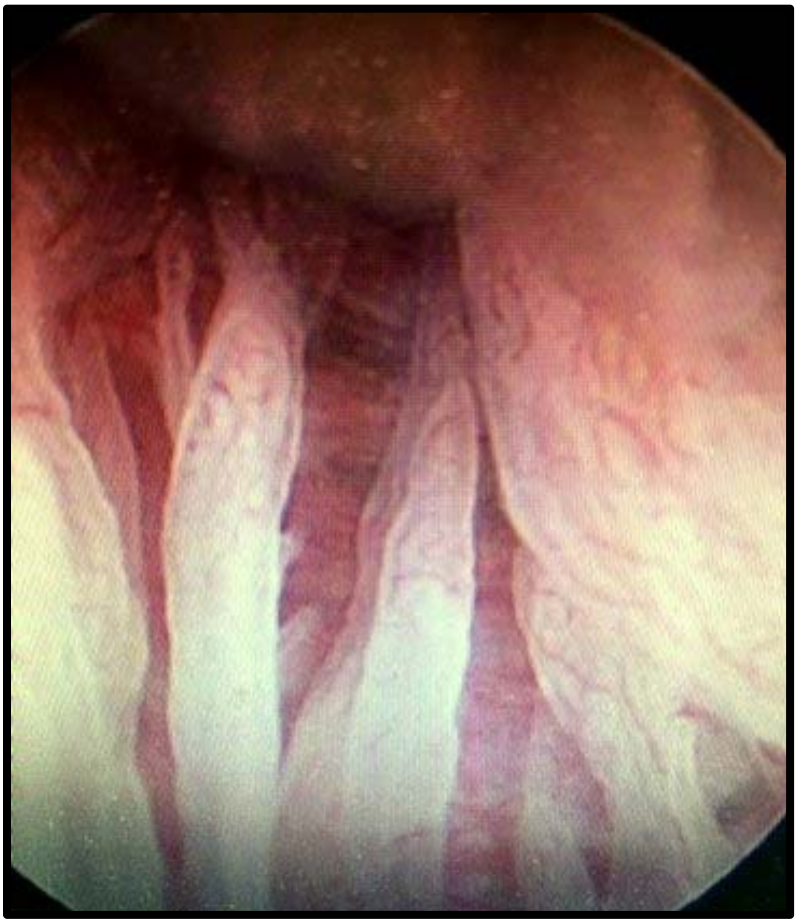


Figure 49 Salpingoscopy - normal ampullary mucosa.

Inspection of the tubal lumen shows intact major and minor ampullary folds with normal ampullary mucosa (Class 1).

(Adapted from Marconi 1998)

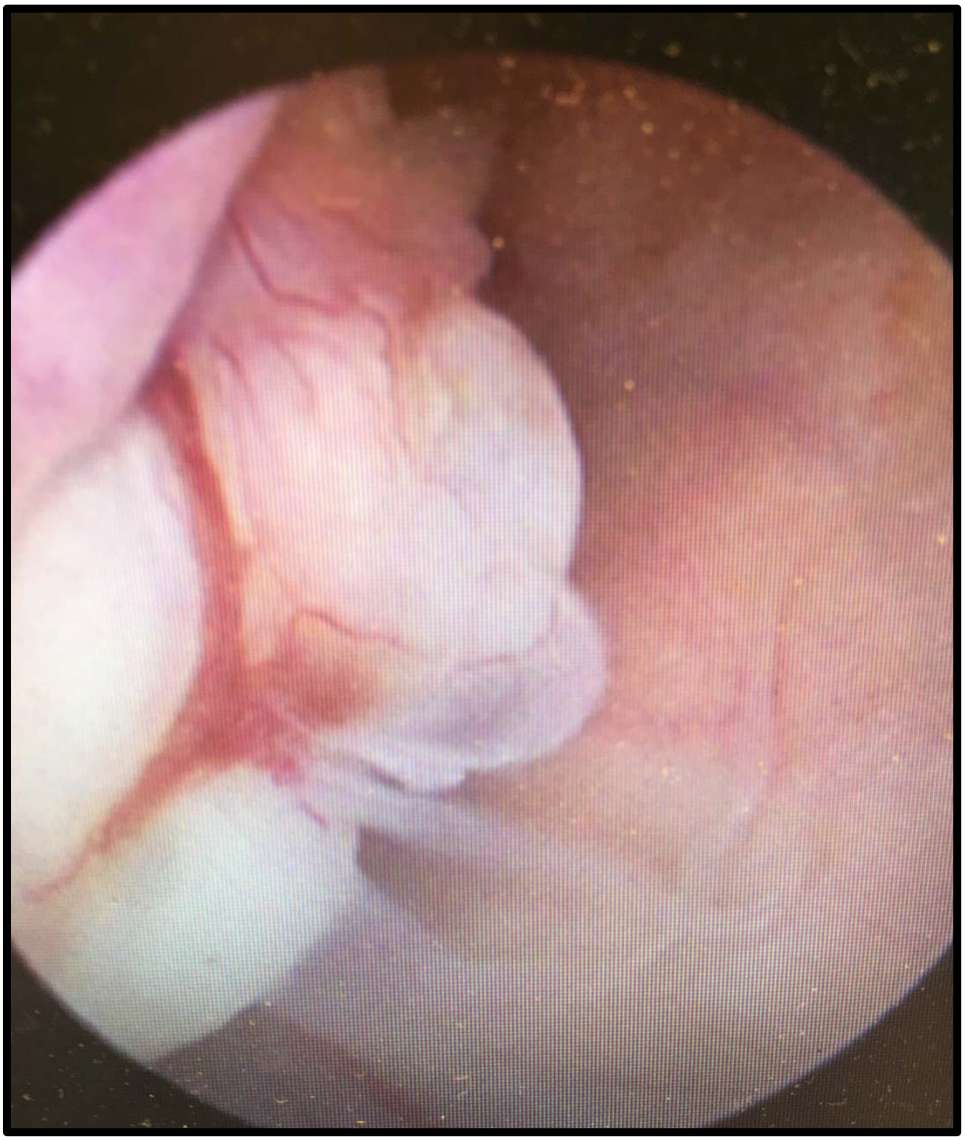


Figure 50 Salpingoscopy – loss of mucosal folds and adhesions.

Tubal lumen with evidence of cystic and extensive vascularised adhesions (Class 4). Tubal epthelium denuded of normal mucosa.

(Adapted from Brosens 1998)

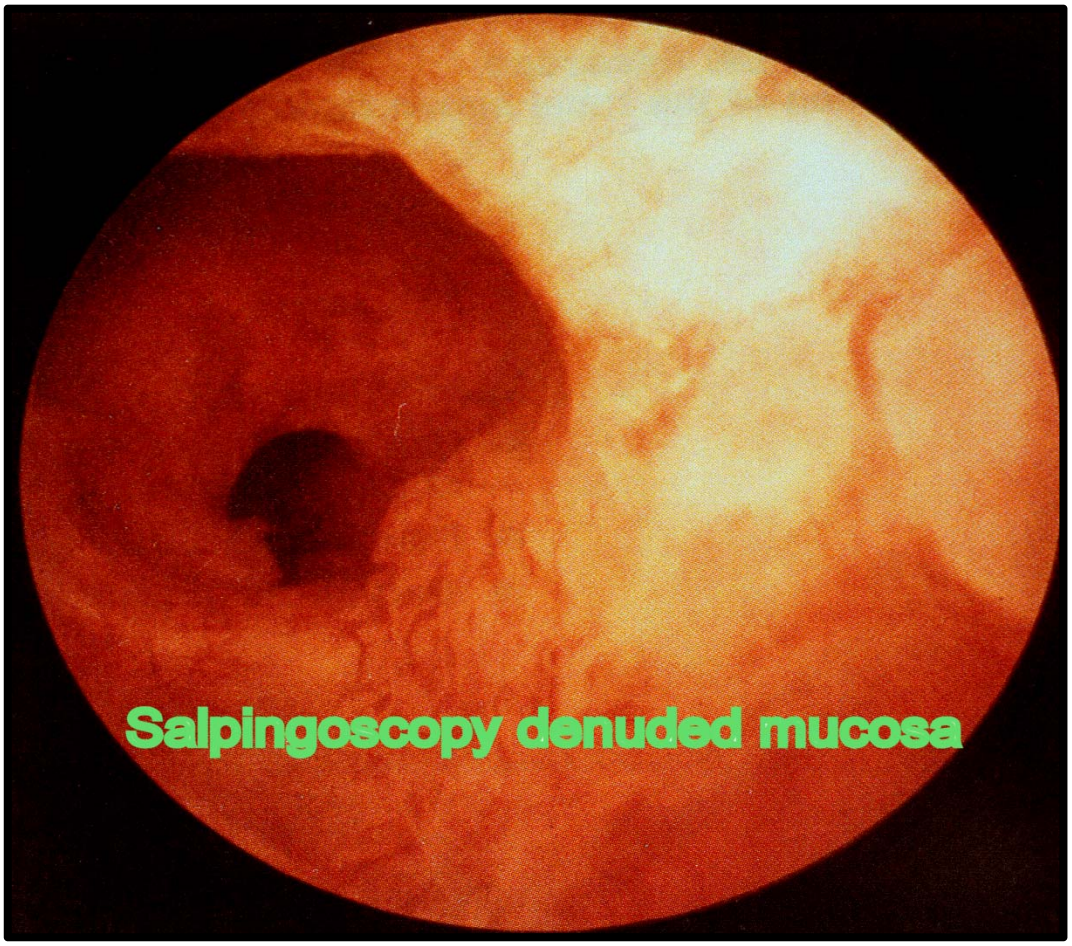


Figure 51 Salpingoscopy - denuded mucosa.

Inspection of tubal lumen showing complete disruption of tubal architecture. There is loss of major and minor folds as well as ciliated epithelial surface cells (Class 5).

(Adapted from Brosens 1998)

Table 21 Salpingoscopy - classification of ampulla mucosal lesions.

Class 1	Normal mucosal pattern
Class 2	Mucosal folds preserved but separated by a greater distance and with a variable degree of flattening
Class 3	Mucosal folds preserved with presence of focal lesions such as agglutination of and/or adhesions between folds
Class 4	Presence of extensive adhesions and agglutination of folds, structure of lumen or disseminated flat areas
Class 5	Complete loss of mucosal fold pattern

(Adapted from Brosens 1988)

7.7. Microsalpingoscopy

A more precise evaluation of the tubal epithelium is achieved by using the Hamou II telescope (Storz, Germany) which on contact allows up to 180 times cellular magnification. Following passage of methylene blue dye, which becomes incorporated in mucosal cells enables detection of stained nuclei and differentiate intermediate or inflammatory cells (mastocytes) in the middle of tubal folds. The number of dye stained nuclei allows classification into 4 stages. Stage 1 with no nuclei dye stained, ascending to Stage 4, where a pathological number of cells are dye stained (Marconi and Quintana; 1998) (Figure 52 and Figure 53).

Evaluation of 500 infertile women with no obvious pathology, salpingoscopy was possible in 85% of cases with transvaginal hydrolaparoscopy. When no pathology was detected on pelvic inspection only 8.2% had abnormal salpingoscopy but 37% had abnormal microsalpingoscopy, confirming the usefulness of this added technique (Watrelot et al 2000).

A further transvaginal hydrolaparoscopy study found that salpingoscopy was only feasible in 41% of 130 infertile patients. A correlation however was found between salpingoscopic and previous laparoscopic findings (Susuki et al 2005).

Salpingoscopy performed on 50 infertile patients with no evidence of pelvic pathology at laparoscopy, confimed that 8% had major pathology, with 34% having evidence of severe mucosal damage (Stages 3-4) with microsalpingoscopy (Verhoven and De Wilde 2002).

7.8. Salpingoscopy versus Imaging and Morphology

Comparison of salpingoscopy to other assessment methods has been addressed by several authors.

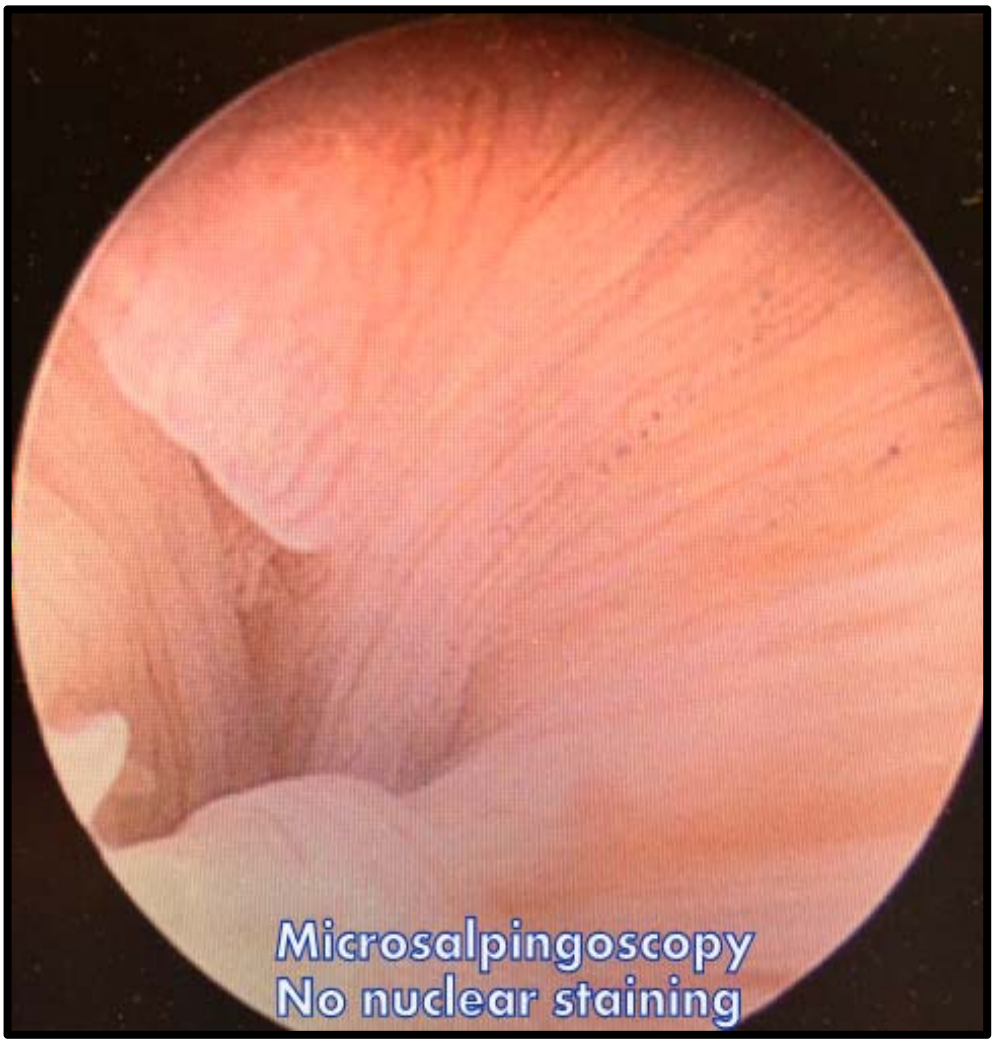


Figure 52 Microsalpingoscopy – no nuclear staining.

Methylene blue dye has been used to demonstrate incorporation by inflammatory cells. No nuclear staining is visible indicating normality of epithelial cells (Class 1).
An in vivo method for evaluating vitality of tubal epithelium.

(Marconi and Quintana, 1998)

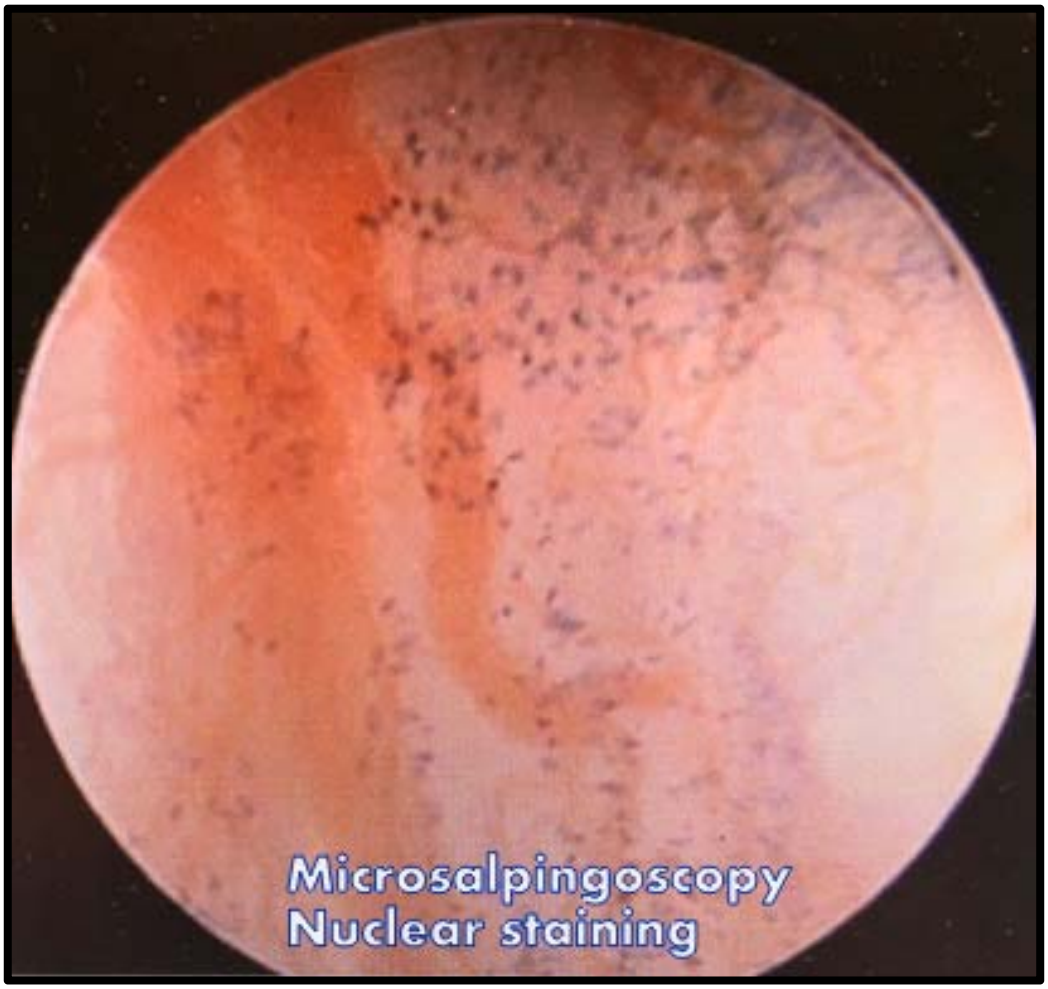


Figure 53 Microsalpingoscopy – nuclear staining.

Methylene blue dyeing of cellular nuclei at salpingoscopy. Considerable nuclear staining indicative of ongoing inflammatory changes in epithelial cells (Class 4).

(Marconi and Quintana 1998)

Puttemans et al (1987) found salpingoscopy superior to HSG in evaluating mucosal lesions. Marana et al (1995) found a significant correlation between salpingoscopy grade and pelvic pathology (American Fertility Society Classification of adnexal tubal occlusion and adnexal adhesions) and term pregnancy rate following corrective surgery.

A small study (32 Fallopian tubes) compared salpingoscopy findings to light microscopic and electron microscopic evaluation (SMS). Salpingoscopic observations were consistent with histologic findings when endotubal disease was severe. Moderate pathologic changes documented by light and TEM were frequently not diagnosed with salpingoscopy. Overall a significant correlation (correlation coefficient=0.71) was found between the salpingoscopic and histologic scores (Hershlag et al 1991).

A prospective study of 91 infertile women (Canadian Task Force classification 1-2), to evaluate correlation between laparoscopic and salpingoscopic findings confirmed that laparoscopy alone was not sufficient to predict normality of Fallopian tubes. Concomitant salpingoscopy significantly increased accuracy of predicting fertility outcome (Marchina al 2001).

7.9. Salpingoscopy and Subsequent Fertility

De Bruyne et al (1989) carried out salpingoscopy in 22 patients with bilateral hydrosalpinx who were considered suitable for restorative surgery. Lumen distension and flattening of the major and minor folds was observed in all tubes, and mucosal adhesions were absent in 17 patients. Ten of these patients conceived after microsurgical salpingostomy and had intrauterine pregnancies. Of the 5 patients with evidence of adhesions between mucosal folds, no intrauterine pregnancy occurred in this group, and one ectopic pregnancy occurred in a patient

with focal adhesions. This study was one of the first to suggest that the prognosis for restorative surgery can be significantly improved by selection of patients by pre-operative salpingoscopy.

Antony et al (1996) found a correlation between endometritis and tubal mucosal lesions. Women investigated for infertility by laparoscopy had 25.8 % incidence of mucosal abnormalities when the Fallopian tubes were considered normal externally. Fallopian tubes considered abnormal at laparoscopy were found to have mucosal abnormalities in 86.5%.

Heylen et al (1995) studying 158 consecutive patients (232 Fallopian tubes), carried out salpingoscopy at laparoscopy and using the Brosens table assessed the relationship between abnormality classification and subsequent cumulative pregnancy rates.

- Patients with a normal salpingoscopy (Class 1 and 2) had a 71% rate
- Patients in intermediate group (Class 3 and 4) had 34% rate
- Patients with severe mucosal pathology (Class 5) had a 0% rate.

Puttemans et al (1998) summarised a decade of experience with salpingoscopy with the following statement " With salpingoscopy and using a simple classification system a trained endoscopist can evaluate the sequelae of tubal inflammatory disease and their impact on fertility nearly as efficiently as with mucosal microbiopsies and they can direct their patients accordingly towards reconstructive surgery or medically assisted reproduction".

Brosens (1996) provided an elegant summary of salpingoscopy for diagnosis and predictive value for subsequent fertility.

My own experience with salpingoscopy commenced at the completion of this study and I am therefore unable to provide sufficient data to compare mean cilia index and salpingoscopy results.

7.10 Falloposcopy

Kerin et al (1992) were the first to successfully visualise the intramural and isthmic segment of the Fallopian tube endoscopically. A 0.5mm micro-endoscope or falloposcope was introduced via the uterine cavity. Tubal cannulation was achieved by using either a coaxial catheter system at hysteroscopy, or a linear everting balloon catheter system for non-hysteroscopy introduction.

Perforation or partial dissection of the proximal tube occurs in 3-10% of attempts which does not seem to have any serious sequelae. Both methods require a learning curve of 50-100 cases.

Once cannulated the tubal lumen is usually inspected whilst retracting the protecting catheter and falloposcope from the fimbriated end of the tube. A “white-ant effect” on contact with the tubal epithelium, soiled lenses and insufficient distension of the tubal lumen by irrigating fluid can impair visualisation, with an overall failure rate of 7-10% (Rimbach et al 2001).

Proximal tubal obstruction prevents sufficient distension whilst over-distension, perforation and “curling” of the catheter can occur in hydrosalpinges.

Ampullary vascularised folds are easiest to inspect and flattening or agglutination of folds, web-like adhesions and polyps can be identified as post-inflammatory changes. Tubal obstruction or dilatation can be assessed in the isthmic and intramural part of the tube not accessible at salpingoscopy. Tubal wall endometriosis, fibrosis and inflammation cannot be detected by falloposcopy.

A classification and scoring system proposed by Kerin et al (1992), allowed a cumulative severity score to be calculated for the entire tube.

Falloposcopy remains the only method for visualisation of the Fallopian tube from the utero-tubal to the ampullary-isthmic junction and is complementary to salpingoscopy for inspection of the distal tube.

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Having identified obstructions, adhesions or debris within the lumen, this procedure allows removal to treat infertility. Limitations of falloposcopy include:

- Falloposcope fragility
- Entry difficulties
- Light reflexion
- Adhesions limiting passage.

A large multi-centre study (Rimbach et al 2001) indicated that in only 2 of 3 cases, complete examination could be performed with tubal perforation occurring in 4% of procedures.

Interest in falloposcopy has decreased since this study and the instrument is no longer manufactured or available for sale.

7.11 Summary

To confirm patency of the Fallopian tubes, laparoscopy and passage of dye has been the gold standard for some decades. Combined with hysteroscopy to exclude congenital and aquired uterine cavity abnormalities, it provides almost complete assessment of the reproductive organs except for intraluminal Fallopian tube evaluation. Several studies have confirmed that normal external appearance does not exclude intraluminal pathology. Alternative tests have been promoted by non operative ART specialists to eliminate the need for regional or general anaesthesia, so reducing surgical risk and cost. Despite its drawbacks of being a painful procedure, associated with vaso-vagal reactions and flare up of pelvic inflammatory disease, failure to provide accurate assessment of ovarian pathology, peri-tubal adhesions and endometriosis, HSG has had a resurgence because a meta-analysis study (odds ratio 1.9) linked oil-based media used for HSG and increased post HSG conception rates. Review of large population studies have however confirmed that

HSG has a sensitivityof 65% and specificity of 85% for tubal patency compared to laparoscopy. Pelvic ultrasonography and SIS can be performed as an outpatient procedure and have shown statistical equivalence for intrauterine pathology, and a sensitivity of 80% for detection of hydrosalpinges. Other limitations include the lack of expert image interpretation, ultrasonographer experience and the need for supportive studies for tubal patency and pathology. HYCOSY allows detection of patency of at least one Fallopian tube by the presence of infused Hyscon in the Pouch of Douglas. It has a 10% false occlusion and 7% false patency rate compared to laparoscopy and concordance of 86% with laparoscopy and 83% with HSG for tubal patency.

THL involves inspection of the pelvic cavity and visualisation of the adnexa and posterior aspect of the uterus. It enables the introduction of the telescope through the tubal ostium to perform salpingoscopy, observe passage of methylene blue dye and exclude intratubal pathology. Ovarian drilling for poly-cystic ovarian syndrome division of adhesions and cautery of mild endometriosis can be performed. Despite the documented low risk of bowel injury and infection, this technique has not been accepted for routine use in Australia as it has in Europe and the UK. Salpingoscopy performed at laparoscopy or THL allows intraluminal inspection of mucosal folds, presence of adhesions or loss of mucosa which cannot be excluded at laparoscopy. Hydrosalpinges can be inspected internally following small incision at the occluded end. At laparoscopy this requires the introduction of a second telescope and camera system, whereas THL can achieve the same result with one telescope. The presence or absence of of mucosal folds, adhesions and flattening of the mucosa will determine whether conservative or excision surgery should be considered. Contact microsalpingoscopy and methylene blue staining of cell nuclei allows classification of four stages of residual inflammation. Scoring by salpingoscopy and

microsalpingoscopy will determine severity score, extent of tubal pathology and inpact on fertility following remedial surgery. Salpingoscopy has been shown to be superior to HSG to evaluate mucosal damage. A correlation has been confirmed between salpingoscopy grading of tubal pathology and prediction of live births following salpingostomy.

Chapter 8 Salpingostomy Study–Adelaide Cohort (1980–1986)

8.1. Aims of the Study

The purpose of this study was to correlate morphology of Fallopian tube mucosa (MCI) to live birth rate following salpingostomy procedure in women with infertility caused by bilateral hydrosalpinx.

Although all conceptions including miscarriages were recorded only ectopic gestations and live births are included in the results as the focus of the study was to evaluate live births and ectopic pregnancies.

8.2. Methodology

8.2.1 Patient Selection

One hundred and thirty one infertile women scheduled for bilateral salpingostomy procedure between 1978 and 1987, who consented to provide details of post-operative clinical outcome by postal questionnaire or phone consultation were included in the study. Follow up was for at least 3 and up to ten years post operatively. Except for the ectopic pregnancy group follow-up was discontinued once a live birth had been achieved; subsequent live births have not been recorded. Fertility outcome of women who chose to have IVF following failed salpingostomy have also not been included in this study.

The study received ethics approval from University of Adelaide Ethics Committee and all phone consultations at six monthly intervals, were carried out by the author of this thesis.

Hydrosalpinx formation was diagnosed preoperatively either by HSG or diagnostic

laparoscopy. At laparoscopy sub-diaphragmatic adhesions between the liver and diaphragm (Fitz Hugh Curtis Syndrome), secondary to Chlamydia infection were occasionaly seen (Figure 54).

The main focus of this study was to assess MCI results relative to surgery outcome and SHR studies were not performed.

Women with gross pelvic pathology and a “frozen” pelvis causing complete distortion of normal anatomy by adhesions binding the uterus, Fallopian tubes, ovaries, bowel and bladder were excluded from the study as this degree of pathology was considered a contraindication to fertility surgery (Figure 55).

Women in premenopausal age group with thin walled hydrosalpinges of equal or less than 2 cms dilatation and minimal adhesions to surrounding structures and absence of endometriosis, were primarily-selected as they were considered to most likely benefit from remedial surgery (Figures 56 and 57).

Women with congenital Fallopian tube abnormalities were also excluded as hydrosalpinx formation in these cases results from lack of development of the fimbriated end of the Fallopian tube and not post infection end-tubal obstruction (Figure 58).

8.2.2. Surgical Techniques

8.2.2.1. Microsurgical salpingostomy

Laparotomy access was used for the majority of women (110) and the procedure is illustrated in Figure 59. The terminal part of the tube is visualised with 4-20x magnification using an operating microscope and incisions made along scar lines (Figure 60) with microelectrodes so that the fimbria remnants can evert creating a new stoma using 8/0 nylon microsutures to maintain eversion. Requirements for microsurgical tuboplasty and principles of microsurgcal technique used for this

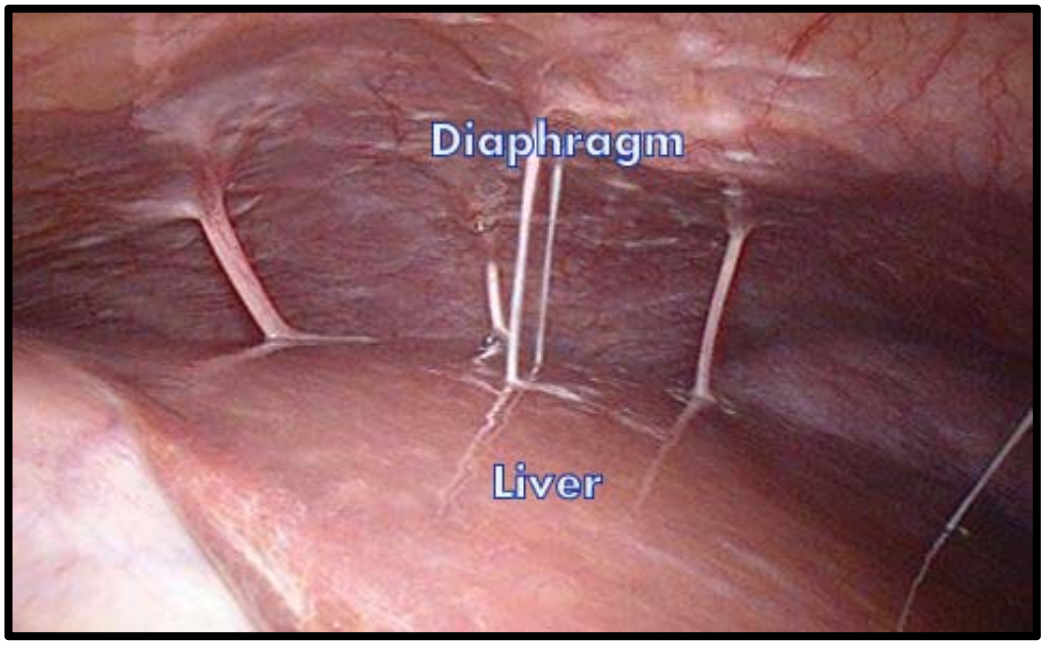

Figure 54 Fitz Hugh Curtis Syndrome.

Thick fibrous adhesions are seen at laparoscopy connecting the dome of the liver to the sub-diaphragmatic peritoneum.

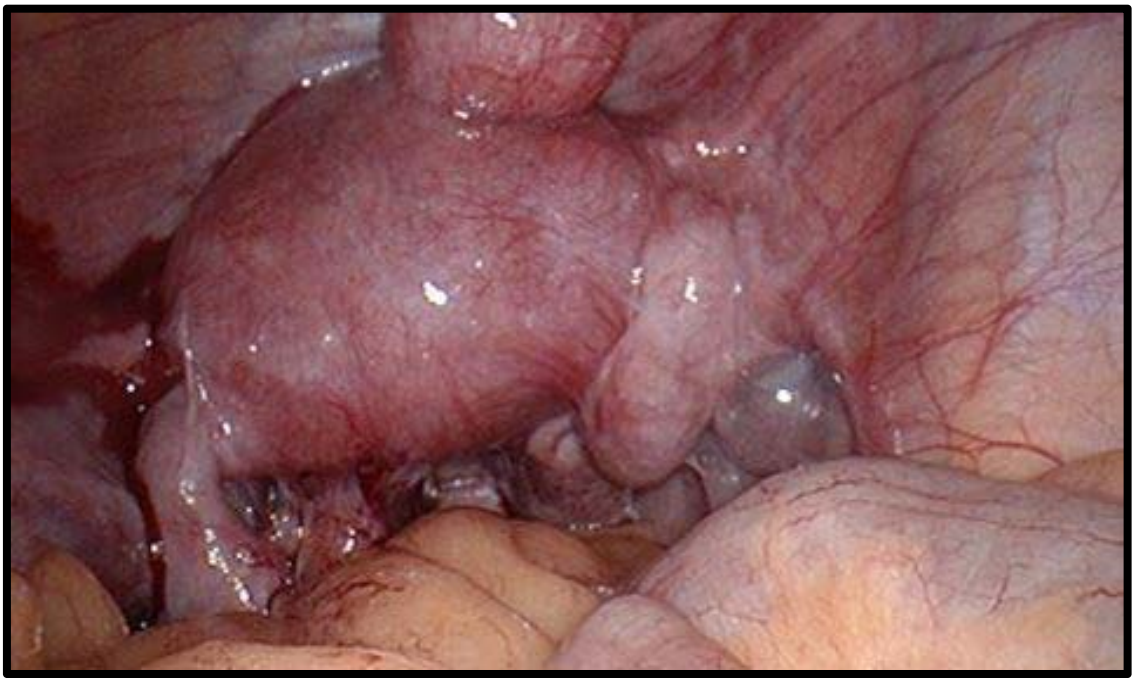

Figure 55 Gross post-inflammatory (frozen) pelvic pathology

The thickened Fallopian tubes are adherent and buried by vascular adhesions to the uterus, ovaries, descending colon and parametrium. A myoma is seen on the anterior aspect of the uterus which is also adherent to the bladder anteriorly.

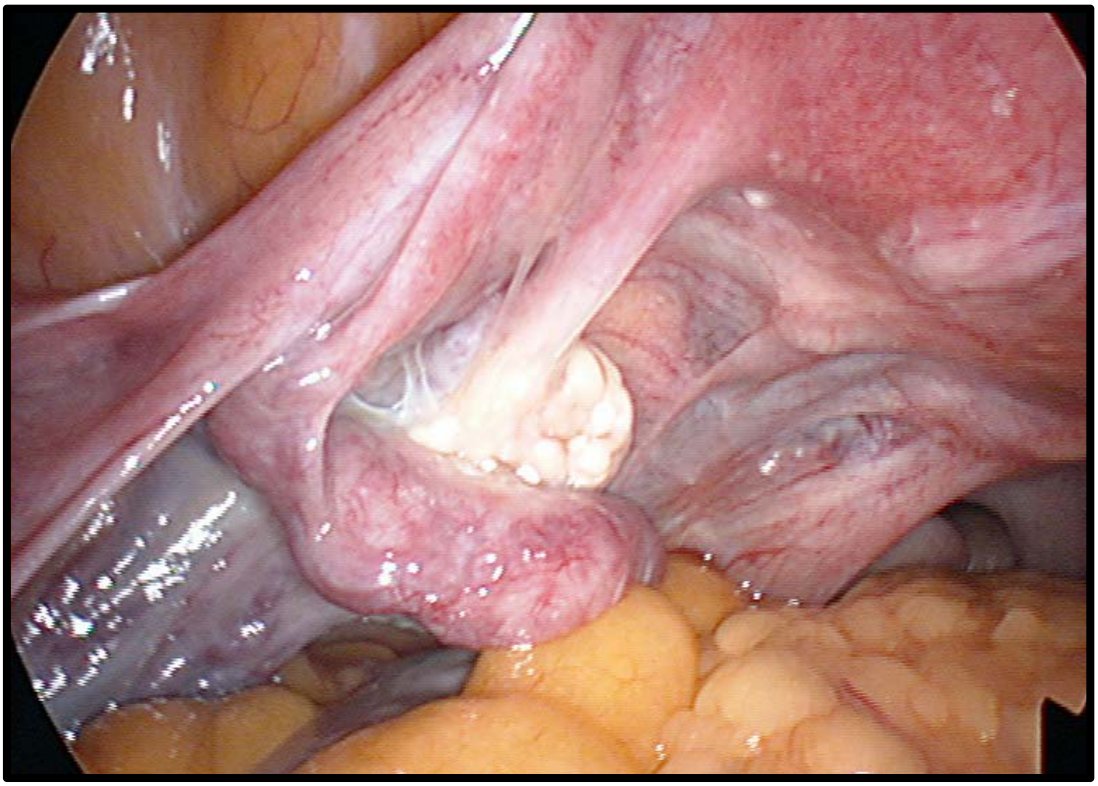

Figure 56 Hydrosalpinx with mild adhesions between ovary and Fallopian tube.

Thin walled hydrosalping overlying and adherent to the ovary by fine adhesions.

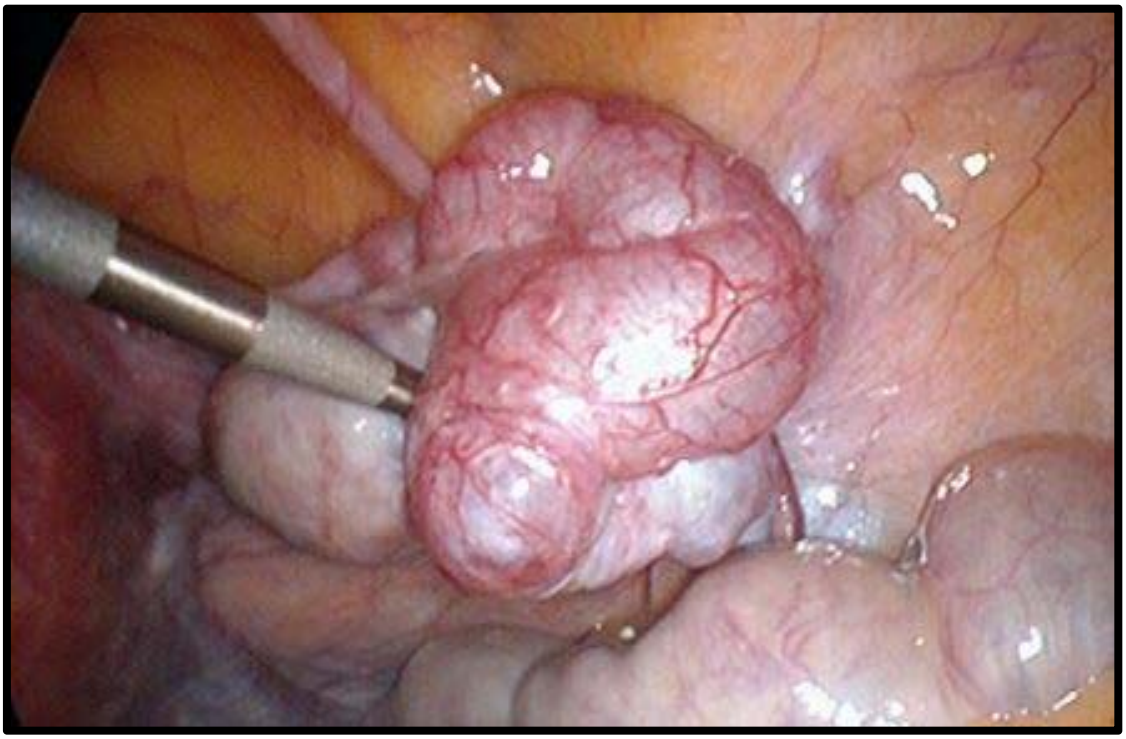

Figure 57 Thin walled hydrosalpinx less than 2 cm diameter.

The Fallopian tube has remained thin walled, is filled with clear fluid and terminally obstructed. Fimbria are not seen and the tube is free of peritubal adhesions.

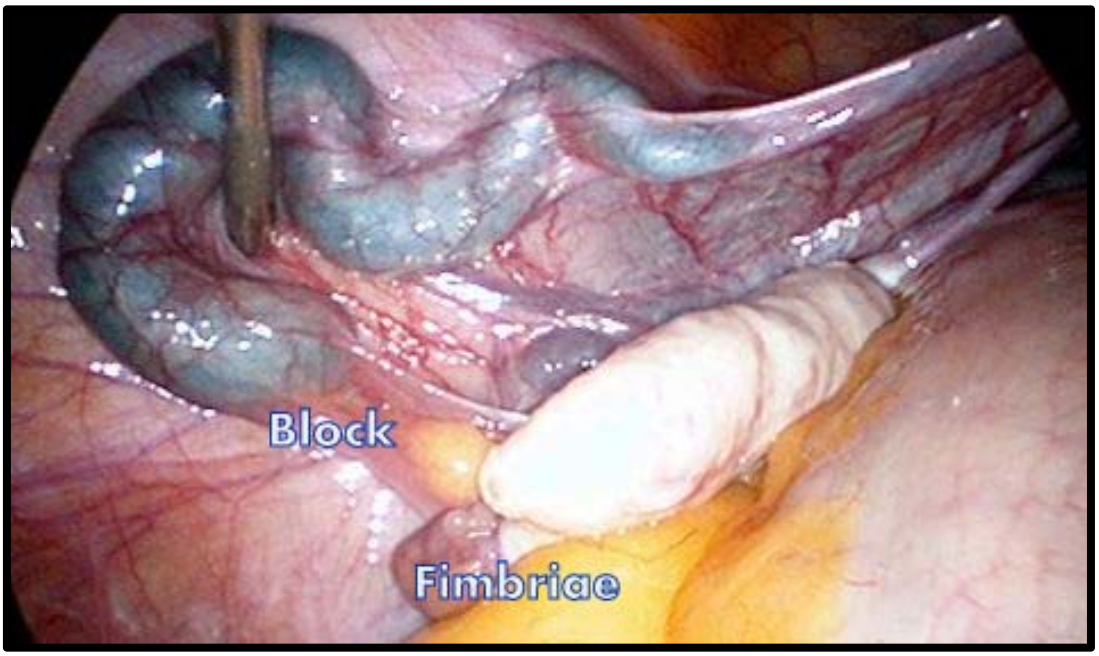

Figure 58 Congenital failure of development of terminal infundibulum.

The Fallopian tube is distended with methylene blue with apparent block in the ampullary segments. Vestigal fimbria development is seen attached to the ovary.

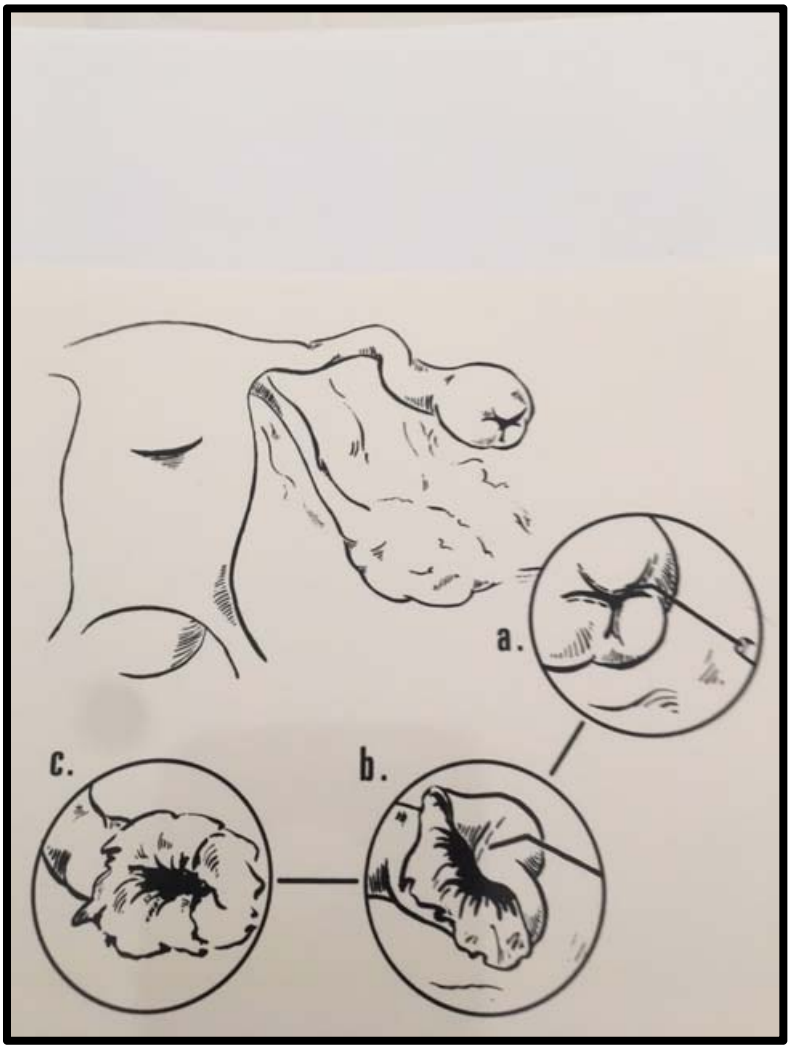

Figure 59 Salpingosotomy procedure.

a. Microdissection along scar lines using fine needle electrocautery
b. Eversion to free fimbriated end
c. Completion of procedure by fixation edges with microsutures to maintain patency of Fallopian tube.

(Adapted from Paterson and Petrucco 1987)

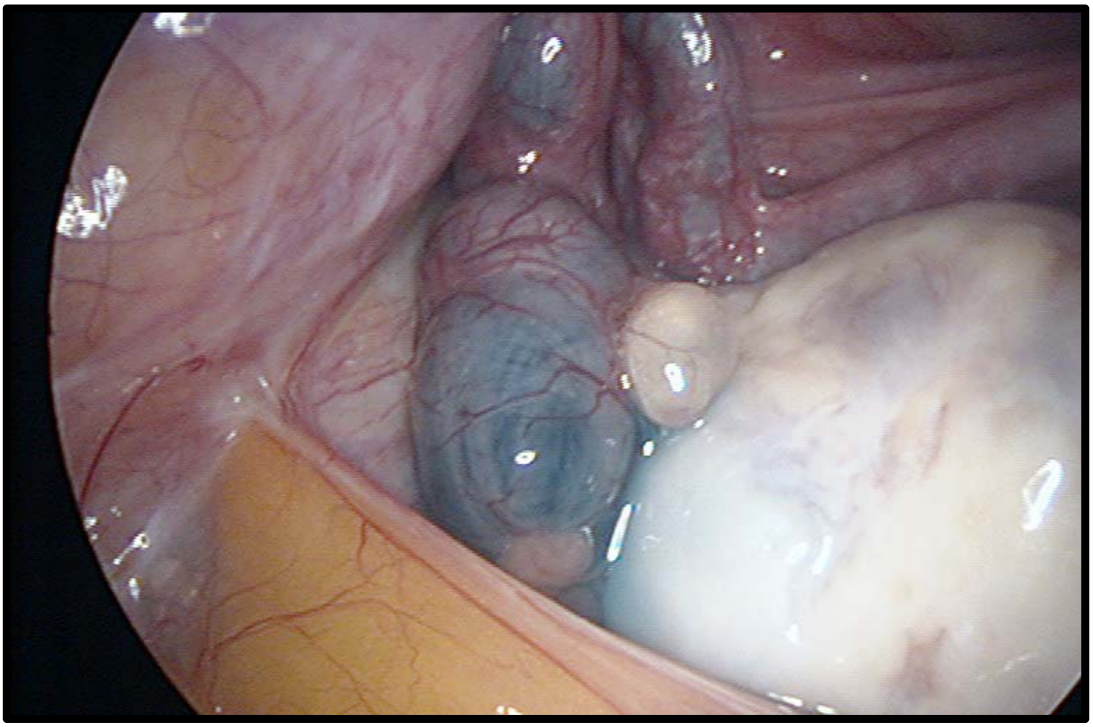

Figure 60 Hydrosalpinx – terminal scar lines.

The Fallopian tube distended with methylene blue dye is adjacent to the ovary. The occulded end displays scar lines with no evidence of fimbria. There are no adhesions between the tube and ovary with only a small benign cyst on the surface of the ovary.

study are shown in Table 22.

The fimbriated end of a Fallopian tube inspected at caesarean section following successful salpingostomy is seen in Figure 61.

8.2.2.2. Laparoscopic salpingostomy

For the remaining 21 women, three ports were used at laparoscopy, one to view and two to operate on the hydrosalpinges. Low level mono and bipolar electrosurgery, argon and CO_2 lasers were employed for division of adhesions and opening the hydrosalpinx. Following eversion of the fimbriated end, the posterior aspect of the new osteum was held in place by welding the serosol surfaces with the same energy source used for incision (laser or electrocautery) or with 7/0 non-absorbable sutures. The end result of tubal eversion to create a new stoma is seen in Figure 62.

8.2.2.3. Microsurgical mucosal biopsy

Microbiopsies (3-5mm) long were taken with fine scissors from mucosal folds when present, otherwise from flattened areas around the new tubal stoma and immediately fixed in buffered formalin and gluteraldehyde for light microscopy, SEM and MCI evaluation as described in Chapter 6. To minimise bias in collection of microbiopsies magnification (10-25x) was used to select sites that accurately represented the mucosa being inspected. Specimens could not be taken from all women in the study group as technical back-up was not always available during operating sessions. MCI assessment was possible in 47% of the group.

8.3. Results - Adelaide Cohort Study (1980-1986)

Table 23 indicates clinical outcome and incidence of mucosal microbiopsy sampling for the study group. The live birth rate for the the subgroup having salpingostomy with MCI measurement was 18.3%.

Table 22 Requirements for microsurgical tuboplasty.

REQUIREMENTS FOR TUBOPLASTY

1. Experience and patience
2. Minimal tissue/peritoneal trauma
3. Fine instruments
4. Needle point bipolar cautery
5. Fine non-reactive nylon sutures for peritoneal closure
6. Constant saline irrigation
7. Adequate surgical exposure
8. Magnification – operating microscope

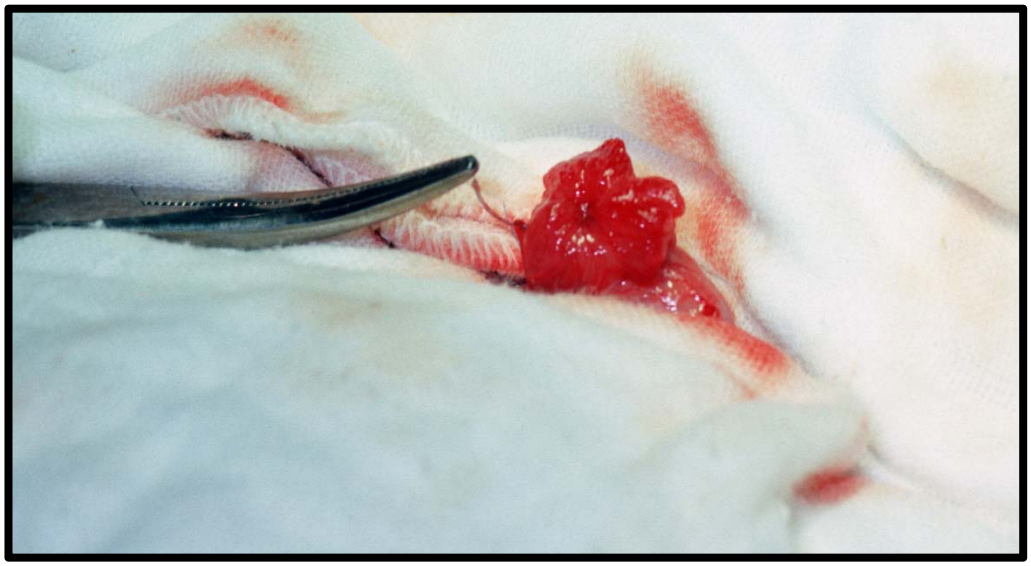

Figure 61 Appearance of terminal segment Fallopian tube at Caesarean section.

Salpingostomy performed for hydrosalpinx followed by successful pregnancy and live birth.

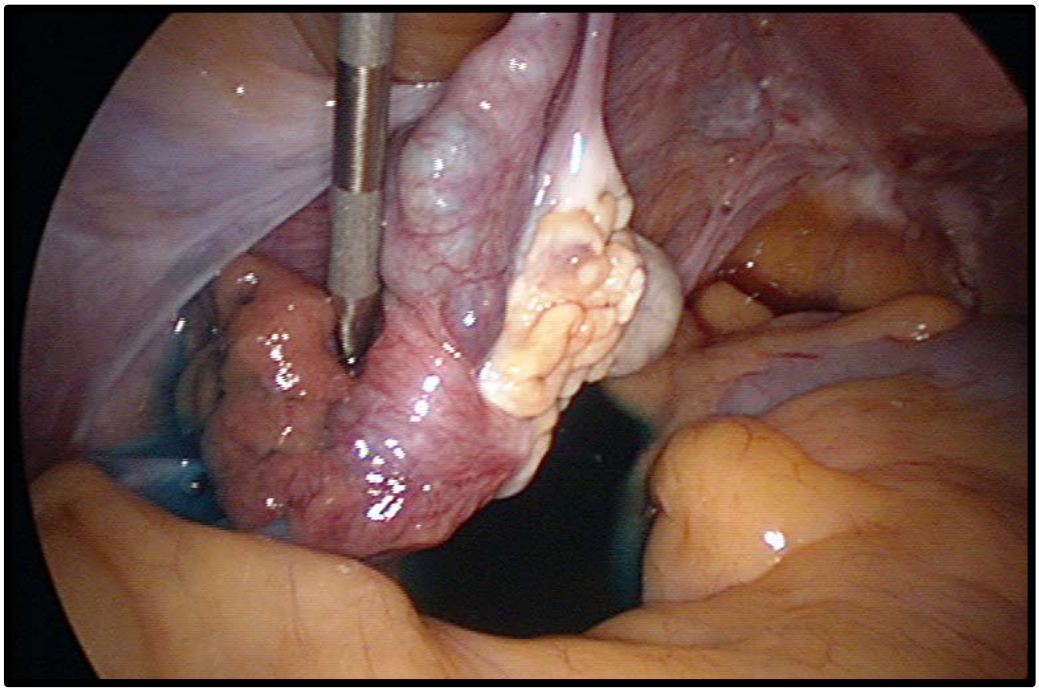

Figure 62 Salpingostomy procedure - eversion of terminal hydrosalpinx to create a new osteum.

Free passage of dye seen in culdesac indicating tubal patency. New osteum covered with normal appearing mucosal lining.

Table 23 Salpingostomy with microbiopsy results (1980-1986) – Clinical outcome and incidence mucosal microbiopsy.

n	IINCIDENCE MUCOSAL MICROBIOPSY	CLINICAL OUTCOME
102	39 (38.2%)	Failed surgery (77.8%)
24	18 (75%)	Live birth (18.3%)
5	5 (100%)	Ectopic pregnancy (3.8%)
Total 131	**62 (47.3%)**	**-**

From a study population of 131 individuals, a total of 62 (47.3%) had fimbria mucosal micro-biopsy at surgical procedure (salpingostomy).
Of the 18 /24 (75%) women with micro-biopsies, 18.3% had successful live births following salpingsotomy, and from 39 /102 (38.2%) of women with micro-biopsies, 77.8% failed to conceived.
All 5 women who experienced ectopic pregnancy following salpingostomy (3.8%) had mucosal micro-biopsy.

Clinical outcome relative to MCI is seen in Table 24. The MCI was significantly higher in the live births group compared to failure to conceive and ectopic pregnancy categories.

SEM of ampullary mucosal biopsies of patients who had live births, ectopic pregnancies or failed to conceive are seen in Figures 63, 64 and 65.

8.4. Discussion

The MCI and pregnancy results following salpingostomy in this study indicate that live birth outcome was most likely to occur when the tubal mucosa and cilia were not severely compromised by hydrosalpinx formation.

The main focus of this study was to assess MCI results relative to salpingostomy outcome and SHR studies were not performed. Inclusion of parallel steroid hormone receptors in the study studies would have been of interest however excision of sufficient tissue to perform the assays would have been detrimental to successful surgical outcome.

The criteria used for patient selection also limit the value of the study but were chosen to provide women optimal chance of successful outcome. The results of the prospective study by Vasquez et al (1995) which demonstrated that significantly more live births occurred following surgery on thin walled hydrosalpinges less than 1 cm in size, compared to moderate (1-2 cm) or large (> 2 cm) hydrosalpinges are in agreement with this choice. Donnez et al (1985, 1986) and Singhal et al (1991) also considered ampullary dilatation and tubal wall thickness to be of paramount significance, finding that thick wall hydrosalpinges and tubal dilatation greater than 2.5 cm were associated with very low pregnancy rates. A further limitation of this study is the lack of assessment of successful outcome relative to survival analysis. The probability of conception in a defined time period ideally should include survival

Table 24 Salpingostomy by laparoscopy and laparotomy – clinical outcome versus MCI.

CLINICAL OUTCOME	MUCOSAL MICROBIOPSY	MEAN CILIA INDEX μ ± σ
Failure to conceive	39	36.5 ± 2.4*
Live birth	18	50.5 ± 4.0*
Ectopic pregnancy	5	10 ± 3.8*

* All MCI results were of significant difference $P < 0.05$ (one-way ANOVA).

A significant difference was found in mean cilia index for women who had live births from those that failed to conceive or had ectopic pregnancies.

Figure 63 SEM of ampulla – successful live birth after salpingostomy.

Ampulla in secretory phase cycle from a patient who had a successful live birth following salpingostomy. Cell population and appearance is similar to normal tissue in an unoperated patient.

Figure 64 SEM of ampulla - ectopic pregnancy following salpingostomy.

Ampulla from a patient who had an ectopic pregnancy following salpingostomy. Marked deciliation is evident with normal appearance of secretory cells. In 5 ectopic pregnancy specimens, the MCI varied from 3-20%.

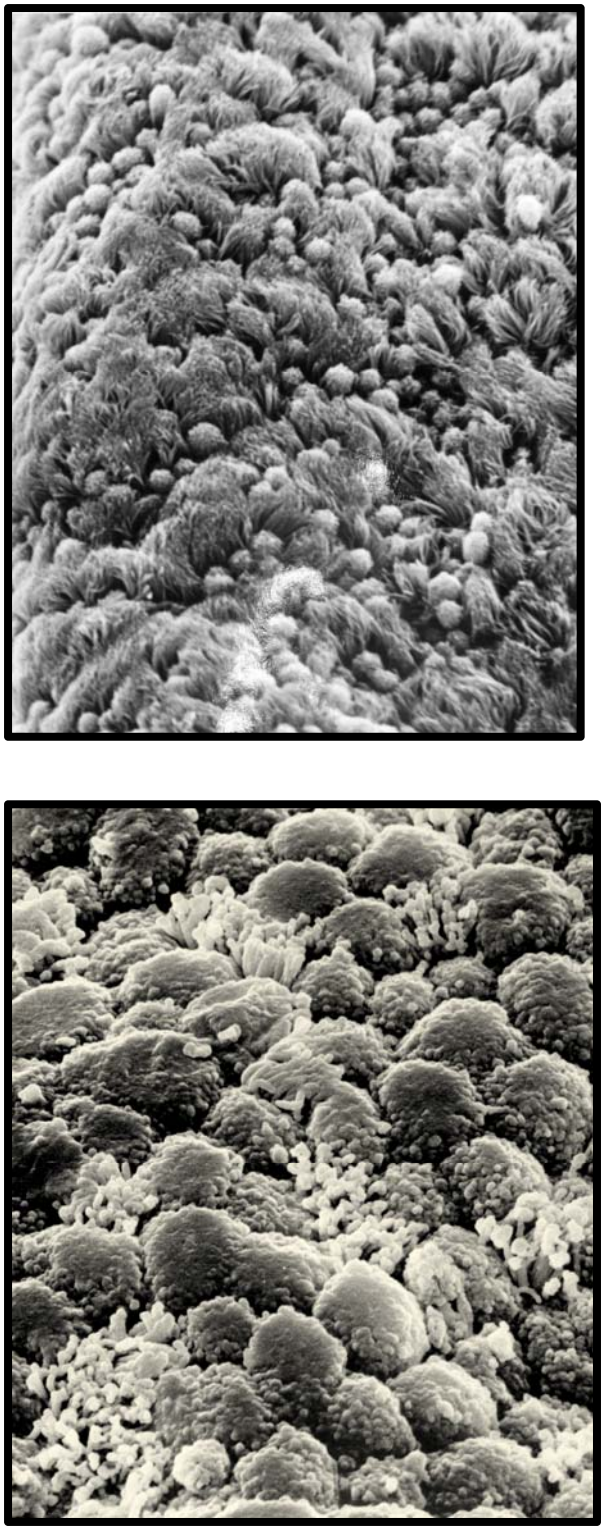

Figure 65 SEM of ampulla - normal and failure to conceive after salpingostomy

Top Panel: normal summit ampullary fold with non-ciliated dome shaped secretory cells and clusters of ciliated cells (x7000).
Bottom Panel: Ampulla in proliferative phase of the menstrual cycle from a patient who failed to conceive following salpingostomy. Severe clumping of cilia is observed, with secretory cells at different stages of development (x7000).

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analysis to present unbiased representation of data (Gillett 1997). This Adelaide study can be criticised for not including miscarriage, live births following ectopic gestation, women having more than one live birth or live births following IVF after failed salpingostomy.

The clinical results, SHR, morphological and biochemical studies on the human Fallopian tube presented in separate studies in this thesis, however have confirmed that hydrosalpinx formation results in severe cellular and particularly epithelial abnormalities causing in some cases irreparable damage and failure to conceive. The pathological changes however are not universal, some Fallopian tubes being less affected than others and the challenge remains to be able to carry out tests that will determine the extent of mucosal damage before deciding to carry out salpingostomy.

Salpingoscopy was introduced in Adelaide at the end of the clinical study, therefore I am unable to relate MCI results to salpingoscopy scores. Following literature review of salpingoscopy and microsalpingoscopy, it seems reasonable to use these investigations to assess intraluminal pathology and mucosal deciliation. Salpingoscopy and microsalpingoscopy should be a prerequisite for assessment of luminal and particularly mucosal damage. Pre-operative discussion with patients regarding proposed surgery should include a statement that salpingostomy would only be performed if salpingoscopic findings exclude severe pathology or evidence of persistent inflammatory process.

Morphological assessment (by MCI) necessitates a time interval and the performance of two endoscopy procedures. The first for evaluation and performing fimbria micro-biopsy and the second to perform salpingostomy. The review of salpingoscopy data indicates that this investigation may be of equivalent value to morphology to decide if salpingostomy should be considered with reasonable

expectation of successful outcome. Since its introduction in the 1980s, salpingoscopy has greatly improved the assessment of tubal factor infertility. Salpingoscopy and micro- salpingoscopy demonstrate anatomical distortion, adhesions between mucosal folds, complete destruction of mucosal folds and the presence or absence of inflammatory cells in the mucosa.

These techniques are complementary to mucosal morphological studies, but are not interval time dependent for diagnosis. It has enabled clinicians to consider reconstructive surgery at diagnostic laparoscopy, when intraluminal pathology has been excluded.

When it was considered that the Fallopian tubes are not suitable for salpingostomy, closure of the proximal segment with Filshie clips or excision (salpingectomy) was performed ensuring that the ovarian vascular pedicle was not damaged ensuring maintenance of normal blood supply to the ovary for subsequent ART (Figure 66).

The results of this study are similar to other large published series performed by different surgical techniques either by laparotomy or laparoscopy (Table 25).

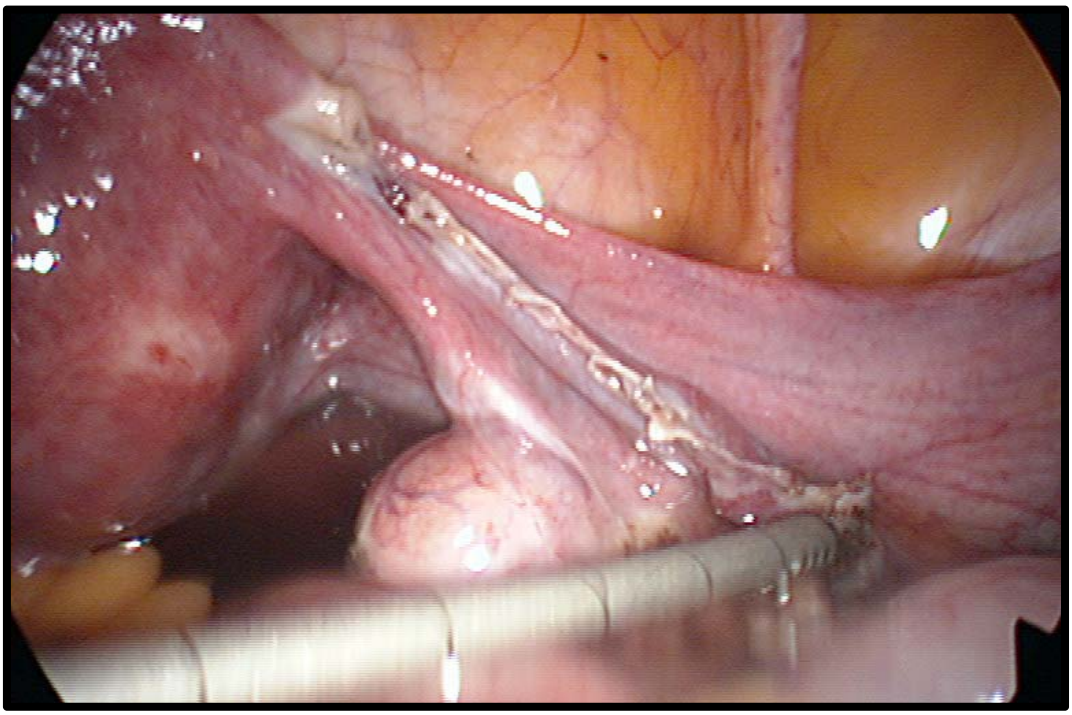

Figure 66 Salpingectomy with conservation ovarian vascular pedicle.

The Fallopian tube has been excised at the utero-tubal junction and infundibulum, with preservation of the blood supply in the mesosalpinx and ovarian attachment to the uterus and broad ligament.

Table 25 Salpingostomy results by laparoscopy and laparotomy.

OUTCOMES FOLLOWING SALPINGOSTOMY BY LAPAROTOMY				
Authors	No. patients	Follow up (years)	Intrauterine pregnancy (%)	Ectopic pregnancy (%)
Swolin, 1975	33	>8	39.0	24.0
Rock et al, 1978	87	>4	22.0	6.0
Gomel, 1978	41	>1	29.0	12.0
DeCherney & Kase, 1981	54	2	26.0	17.0
Kelly and Roberts, 1983	28	1	7.0	4.5
Mage and Bruhat, 1983	68	>1	27.0	9.0
Tulandi and Vilos, 1985	67	2	26.0	4.3
Donnez and Casanas-Roux, 1986	83	1-6	31.0	7.0
Jacobs et al, 1988	71	3	41.0	11.0
Schlaff et al, 1990	64	>1	20.3	7.8
Singhal et al, 1991	97	1	33.0	6.0
Winston and Margara, 1991	323	>10	33.0	10.0
TOTAL	**1016**		**27.9**	**9.9**
OUTCOMES FOLLOWING LAPAROSCOPIC SALPINGOSTOMY				
Authors	**No. patients**	**Follow up (years)**	**Intrauterine pregnancy (%)**	**Ectopic pregnancy (%)**
Gomel, 1978	9	1	44.4	0
Daniell and Herbert, 1984	21	>1	19.0	5.0
Dubuisson et al, 1990	34	>1	29.4	2.9
Canis et al, 1991	87	>3	33.3	6.9151
TOTAL	**151**		**31.5**	**3.7**

Each author appears in references.

(Reproduced from Tulandi 1995)

Chapter 9 Thesis Conclusions

9.1. Thesis Overview

To assess management options for hydrosalpinx related infertility the Main Study proposes a role for restorative surgery (salpingostomy) to achieve natural fertility, accepting that IVF is an alternative option.

To support this proposal a literature review was first undertaken to substantiate current understanding of the physiological processes involved in oviduct function in healthy Fallopian tubes, so that a comparison can then be made with Fallopian tubes affected by hydrosalpinx.

This included a review of:

- Anatomical structure and morphology
- Ciliary activity
- Muscular contractility
- Cytokines
- Fluid and secreted proteins
- Prostaglandins
- Platelet activating factor
- Microorganisms
- Nucleic acids
- Steroid hormones and their receptors.

This review formed the basis for sub-hypothesis studies to be performed to assess how hydrosalpinx affects Fallopian tube function.

9.2. Results - Human Studies (Study 1)

9.2.1. Steroid hormone receptors

This part of the study confirms that variation in ER and PR during the menstrual cycle is secondary to changes in plasma E_2 and P_4.

Charecterisation of ER cytosol and nuclear binding in hydrosalpinx tissue showed significantly lower levels than controls in both phases of the menstrual cycle. A significant reduction was also seen in the PR cytosol level in the proliferative phase. This reduction in steroid hormone receptors with normal levels of plasma steroid hormones suggests that the pathological changes causing hydrosalpinx formation has resulted in reduced cell number and that some of the mucosal cells affected by intraluminal pressure have been irreparably damaged and are not responding to circulating steroid hormones.

At the commencement of this study I considered the possibility of inducing E_2 dependant ciliogenesis in hydrosalpinx tissue by intraluminal or systemic administration of E_2. This was not proceeded with as it was considered that serum E_2 during the menstrual cycle is of sufficient quantity to allow ciliogenesis to occur in normal or damaged epithelium. Rabbits also have adequate serum E_2 levels in oestrous state. It was of interest to find that human tubal epithelial cultures in vitro supplemented with E_2 demonstrated initial prevention of deciliation, however once deciliation commenced high levels of E_2 failed to limit the process of deciliation or induce ciliogenesis (Goldberg ans Friedman 1995).

The rabbit experiment which demonstrated depletion of cytosol and nuclear ER in vitro and invivo with exposure to E_2 also suggests that hormonal manipulation may not be useful for remedial theraphy.

9.2.2. Biochemical studies

Nucleic acids levels (per gram fresh tissue) demonstrated that DNA, RNA and

RNA/DNA ratios were significantly decreased (compared to controls) in the hydrosalpinx with reduced morphological MCI levels.

Hydrosalpinx tissue demonstrates a significant increase in water, sodium and collagen content.

No significant difference is found in ER and PR levels per mg tissue protein or ER and PR levels per mg DNA.

The significant decrease in DNA and RNA synthesis and turnover is indicative of reduced cells activity with altered metabolism in hydrosalpinx tissue. A similar reduction is also found in the relatively inactive menopausal and postnatal specimens. The increased content of water, electrolytes and collagen also suggests altered cell function. Similar to the other sub-hyphothesis studies the biochemical change are relevant but not universal to all hydrosalpinx specimens, therefore supporting the main hypothesis

9.2.3. Morphology studies

Microbiopsies taken during salpingostomy from patients with hydrosalpinx demonstrated a variety of pathological changes ranging from normal to complete loss of secondary and tertiary mucosal folds and varying levels of deciliation. Cellular ratios also varied from normal with healthy surface microvilli to cells becoming cupuloid, pleomorphic and finally flat hexagonal cells. All specimens demonstrated some degree of deciliation. MCI was at least 10% below normal and polyp formation was seen in 3/49 specimens with severe clumping of cilia on polyps and flattened areas.

TEM confirmed flattening of epithelial cells with reduced cilia and degenerating secretory cells at different stages of development.

Specimens from the proximal occluded segment of the Fallopian tube from sterilisation reversal surgery showed polyp formation with shallower and mostly

longitudinal folds and clumping of ciliated cells.

Biopsies taken from sites adjacent to ectopic pregnancies in the ampulary segment of the Fallopian tube had the lowest MCI (3-20%) as demonstrated in other pregnancy specimens.

The results of this study support the main hypothesis as it demonstrates that morphological changes are variable with some hydrosalpinges having normal mucosa and therefore amenable to restorative surgery.

9.2.4. Adelaide cohort salpingostomy study (Study 3)

Study 3 presents a relationship between live birth, ectopic pregnancies and surgical failure rates following salpingostomy relative to MCI of Fallopian tube mucosa.

Low MCI was found in women who failed to conceive or had ectopic pregnancies, whilst MCI was near normal in women who had live births. This study is supportive of the main hypothesis as it indicates that successful oucome is posible when Fallopian tubes with healthy mucosa are selected for restorative surgery.

9.3. Results - Rabbit Studies (Study 2)

9.3.1. Rabbit hydrosalpinx

9.3.1.1. ER and PR hydrosalpinx

ER nuclear was significantly lower in hydrosalpinx compared to control and progressively decreased in value as the diameter of hydrosalpinx increased.

ER cytosol were significantly lower when the hydrosalpinx was ≥ 5-10mm. PR nuclear was unaltered from control however PR cytosol was significantly lower in all size hydrosalpinx.

9.3.1.2. ER and PR reversed hydrosalpinx

ER binding in cytosol and nuclear preparations from surgically corrected

hydrosalpinx showed an increasein ER cytosol whereas ER nuclear level was unchanged. PR nuclear and cytosol returned to control levels.

9.3.1.3. Reversed hydrosalpinx fertility

Following microsurgical repair of hydrosalpinx and mating 4-8 weeks later, resulted in pregnancies occurring in 16/24 (66.6%) unoperated control and 11/24 (46%) of the reversed hydrosalpinx animals. Fertility and pregnancy in the rabbit model was possible following restoration of oviduct patency and resolution of intraluminal pressure.

9.3.2. Biochemical studies

9.3.2.1. Nucleic acids studies

A significant reduction was found in DNA, RNA, and RNA/DNA rati in hydrosalpinx compared to controls. RNA (per mg tissue) and RNA (per mg RNA) confirmed that hydrosalpinx had a decreased turnover of RNA and less active cells. DNA (per mg DNA) was significantly higher indicating icreased replication of active cells. The reduced DNA/RNA was similar to that seen in Fallopian tubes from postnatal and menopausal women.

9.3.2.2. Water, electrolytes, lipid and collagen studies

A significant increase was seen in hydrosalpinx collagen and electrolytes Na, K and Cl, compared to control.

9.3.2.3. Summary - Biochemical studies

Rabbit oviduct nucleic acids suggested altered cell metabolism in hydrosalpinx compared to control.RNA and DNA synthesis and turnover were significantly reduced in less active cells with a reduction in cell replication, protein synthesis and cell growth. Hydrosalpinx had higher content of cell water, collagen and electrolytes

also indicative of altered cell function. Wet weight was increased three-fold in hydrosalpinx commensurate with the observed increase in water content.

9.3.3. Physical studies

9.3.3.1. Hydrosalpinx intraluminal pressure

Mean intraluminal pressure was 10mm Hg, in hydrosalpinx varying from 4-10mm diameter.

9.3.3.2. Blood flow

Oviduct blood flow was significantly lower in hydrosalpinx compared to control. Oviduct distension had a pronounced effect on blood flow. Higher oviduct and ovarian blood flow was demonstrated following hCG administration confirming that a periovular regulatory relationship exists between steroid hormones and adnexal blood flow. These results indicate that vascular changes play a significant role in the pathological changes occurring in hydrosalpinx.

9.3.4. Morphology studies

Light microscopy and SEM confirmed loss of longitudinal folds, flattened epithelium and hyalynisaton of subepithelial layers without inflammatory changes in hydrosalpinx. Marked deciliation was seen in all hydrosalpinx with median MCI of 53% although some cells retained normal cilia. The majority cells were secretory with early or late microvillus formation indicating varying degree of secretory activity. Following microsurgical reversal of hydrosalpinx light microscopy demonstrated lack of separation and flattening of epithelial folds. Although some areas of deciliation persisted, MCI increased to median value of 90%. This was significantly different to the unreversed hydrosalpinx level. SEM indicated almost

complete return to normal mucosal architecture with more prominent longitudinal folds. Secretory cells were more difficult to distinguish because of cilia regeneration in most areas.

9.3.4.1 Reversed hydrosalpinx fertility

Following microsurgical repair of hydrosalpinx and mating 4-8 weeks later, resulted in pregnancies occurring in 16/24 (66.6%) unoperated control and 11/24 (46%) of the reversed hydrosalpinx animals. Fertility and pregnancy in the rabbit model was possible following restoration of oviduct patency and resolution of intraluminal pressure.

The rabbit model supported the findings in human Fallopian tube and highlighted the significant consequenses of of increased intraluminal pressure as dilatation increases.

Chapter 10 Review - Surgery for Hydrosalpinx - History, Incidence and Results

10.1. Historical Background

Reproductive surgery in the 1960-1970 time period was performed via a laparotomy incision with disappointing results because of postoperative adhesion formation (Di Zerega and Holtz 1982). During this period the incidence of sexually transmitted disese increased resulting in a doubling of infertility and ectopic pregnancy rates (Westrom et al 1981). The recognition that reduction of surgical trauma and postoperative inflammation were of crucial importance led to the introduction of microsurgical approach (Swolin 1967, Winston 1980). The concensus by experienced microsurgeons that patient selection was as important as surgical technique was another important determinant of successful outcome (Winston 1982; Beyth and Bercovici 1982). The next important step was the introduction of laparoscopy and video laparoscopy using high definition cameras and monitors. Tubal surgery could now be performed accurately using microsurgical principles, (Petrucco 2000), with day care surgery rather than prolonged hospital stay. Fertility interventions (salpingolysis, salpingostomy, reversal sterilisation, ovarian cystectomy and endometriosis surgery) became possible as laparoscopic surgery expanded.

In the early 1980s, with the introduction of IVF, women with tubal factor infertility were now able to choose tubal surgery or IVF. Partly because tubal surgery had become a subspecialty field requiring supervised training and surgical cost, a major swing towards IVF took place. Many women prefer IVF because of immediacy of result, involvement in minor surgical procedure and reduced incidence of ectopic

pregnancy. Despite the increased usage of IVF however some women continue to choose surgery because of risks associated with IVF (ovarian hyperstimulation, multiple pregnancy), the need to have multiple cycles to conceive as age increases and for religious reasons. Unless performed in public hospitals in Australia, surgery may be expensive but it has the benefit that it does allow repeated attempts at conception and the possibility of more than one spontaneous pregnancy.

10.2 Classification

It is difficult to counsel women regarding surgery versus IVF because published results include a heterogeneous group of patients and the unclear definition of the type of end tubal surgery (salpingostomy or fimbriolysis) performed. A system for grading severity of tubal disease has also not been universally accepted with a new classification being recently introduced (Zou et al 2014). Many classifications exist for tubal scoring, each with a number of limitations. These include the pelvic adhesion scoring in the revised American Fertility Society (AFS-r) (1997) which only evaluates pelvic adhesions, the Hulka (1978) and Rutherford and Jenkins (2002) classifications, which are considered too general. Hydrosalpinges are also heterogeneous because of differing intra and extraluminal pathological change.

10.3 Incidence

The prevalence of tubal surgery in the USA has remained static with 3.2% ever using salpingostomy surgery and 3.1% ever using IVF (Chandra et al 2014). Australian Institute of Health and Welfare figures for public hospital based salpingoscopy (laparoscopy and laparotomy) amounted to 1911 between 2000 and 2017. During those years, the incidence declined from 261 in 2000 to 95 in 2017. I am not aware of the incidence of these operations in private hospitals. During the same time period 234,508 oocyte recovery and 410,480 embryo

transfers were performed. The Assisted Reproductive Technology Australia and New Zealand 2015 report included 1725 (33.5%) of 45945 IVF treatment cycles that were performed for female factor infertility. 1725 (66.0%) of these were for “tubal disease only”. The live delivery rate per initiated non freeze all cycles for this group was 18%.

In an Australasian survey of the management of hydrosalpinx in women due to undergo IVF, involving thirty three clinics, reported that 94% of 74 specialists offered surgical treatment (Johnson and Norris 2004). The majority performed salpingectomy, 14% proximal tubal occlusion, 7% ultrasound guided needle aspiration of hydrosalpinx fluid at oocyte collection and 24% salpingostomy. A followup study has not been done to date.

10.4 Ectopic and Intrauterine Pregnancy

The 2-5% tubal pregnancy rate associated with IVF is higher, than the 1-2% rate occurring with spontaneous pregnancy (Strandell et al 1999; Farquar 2005). Women who have IVF for tubal factor infertility are at higher risk of ectopic pregnancy than to those who have IVF for male factor reasons (Strandell et al 1999; Weigert et al 2009). The ectopic pregnancy rate reported in the Adelaide Cohort study (3.8%) is similar to the IVF rate however it relates to a selected group of patients. Review of published salpingostomy series, seldom include ectopic pregnancy rates. Kelly and Roberts (1983) reported a 7% ectopic rate and larger series including a review of 241 cases (Winston 1981), reported a 17.5% intrauterine and 9.5% ectopic pregnancy rate. Gomel (1978) reported a 22% intrauterine and 10% ectopic pregnancy rate in a poorly controlled case series. Swolin (1975) in his series reported 24% intrauterine and 18% ectopic pregnancy rate. The conclusion from these case series was that an approximate 20%

intrauterine and 10-20% ectopic pregnancy rate could be achieved by operating on unselected cases.

The most important factor in assessment of salpingostomy results however is the lack of any randomised controlled trial comparing tubal surgery and IVF (Chua et al 2017). The conclusion of this Cochrane Review was that "The effectiveness of tubal surgery relative to expectant management and IVF in terms of live birth rate for women with tubal infertility remains unknown". The review included all classes of tubal surgery however the statement similarly applies to salpingostomy.

The update Cochrane Database System Review on "Surgical treatment for tubal disease in women due to undergo IVF" (Melo et al 2020) again states "there is no consensus on how best to manage these women with tubal surgery mainly aiming to remove the deleterious effect of hydrosalpingeal fluid on embryo development and receptivity".

Salpingectomy, tubal occlusion, aspiration of hydrosalpingeal fluid and salpingostomy were assessed for effectiveness to prevent hydrosalpingeal fluid from entering the uterine cavity.

Randomised controlled trials comparing surgical treatment versus no surgical treatment and head to head surgical interventions, live birth rates (LBR), surgical complications and clinical outcomes (muiltiple and ectopic pregnancy, miscarriage oocyte retrieval and embryos obtained) were assessed. Eleven parallel-design trials involving 1386 participants were considered to have limitations due to lack of blinding, wide confidence intervals and sample size.

Salpingectomy an invasive irreversible and possibly technically difficult (Dryer 2016) was considered to possibly increase clinical pregnancy rate (CPR), versus no surgery (RR 2.02, 95% CI 1.44-2.82). Comparing the CPR rate of 19% without surgery, the CPR for salpingectomy was thought to vary between 27% and 52%.

There was moderate quality evidence that salpingectomy prior to ART probably increases CPR compared to no surgery.

Proximal tubal occlusion currently only performed by laparoscopic application of isthmic tubal clips or cauterisation, as hysteroscopic application of the Essure device has been discontinued due to safety concerns. This procedure had a CPR of aproximately 21% to 74% although the evidence was of low quality.

No study reported live birth rate for ultrasound guided transvaginal aspiration of hydrosalpingeal fluid versus no surgery and it was uncertain whether there was an increase in CPR compared to no tubal surgery. On a positive note this procedure is less invasive than salpingectomy and able to be performed in an outpatient setting, however has a recurrence rate of up to 30% (Hammadieh 2008).

Salpingostomy to allow drainage of hydrosalpingeal fluid was considered to have a low surgical complication rate, live birth rate by natural conception of 25% with a 10% risk of ectopic pregnancy, Chu et al (2015), this study is further discussed below.

No randomised studies were able to be included in this review to assess salpingostomy versus no surgery or studies where salpingostomy was preceded by salpingoscopic assessment of intraluminal pathology thus selecting suitable cases for the procedure.

The Cochrane Review authors concluded that “further high-quality trials are requiered to determine the impact of tubal surgery on IVF and pregnancy outcome of females with hydrosalpinges, particularly for live birth rate, surgical complications and to investigate the relative efficacy and safety of different surgical modalities in the treatment of hydrosalpinges prior to ART.

The studies and reviews presented in this thesis indicate that such trials should include women who undergo salpingoscopic selected salpingostomy versus other treatment modalities.

The largest case series (Ponomarev 2009), reported an intrauterine pregnancy rate of 72.8% for terminal salpingoneostomy and salpingo-ovariolysis in a conference abstract, making it difficult to compare with other studies.

Tran (2010) with a 30 year experience of open microsurgery for tubal disease included 153 patients having salpingostomy resulting in a 55% live birth and 6,7% ectopic pregnancy rate. The study was retrospective, included women with fimbriae phymosis and involved resection of the terminal end of the tube, making this study also difficult to evaluate.

The study of Chanelles et al (2011), unlike other series included results for surgery followed by IVF. It involved 81 women who had at least one hydrosalpinx, thus differing from other series which usually involve bilateral hydrosalpinx. Fallopian tubes were evaluated by an institutional score, enabling a decision to be made to either perform salpingostomy or salpingectomy. 35 women (50 hydrosalpinges, thus some with unilateral disease) who had salpingostomy 30.4% conceived spontaneously. Unsuccessful women proceeded to IVF achieving a cumulative 63.3% pregnancy rate.

Results of microsurgical salpingostomy from a pooled series of 333 cases operated on by seven experienced microsurgeons resulted in an intrauterine pregnancy rate of 17% and ectopic rate of 5.7% (Winston 1981). Winston and Margara (1991) reviewed the results of 388 women having microsurgical salpingostomy procedures who had examplary follow up (10 years), and grading of tubal pathology (Grade 1-4). Term pregnancy rate of 39% (Stage 1), 20% (Stage 2) was achieved for 155 women, with pregnancies mostly occuring whithin 12 months of intervention. The

ectopic pregnancy rate was 12.9% for this group. Conclusion from this study was that microsurgical salpingostomy is not an obsolete procedure and should be considered for women with limited tubal disease. Bontis and Theodoridis (2007) suggest that following salpingoscopic evaluation confirming Stage 1 or 2 hydrosalpinx, laparoscopic salpingostomy should be performed. They allowed 18 months trial of conception before further intervention.

A recent review and meta-analysis of salpingostomy (Chu et al 2015), mentioned above included 22 observational studies from 1972 to 2014, which scored well on the Newcastle Ottawa quality assessment scale. It involved 2810 women attempting conception with surgery performed by experienced surgeons. Strict inclusion criteria were used however the studies were clinically heterogeneous in relation to patient characteristics, surgical technique and duration of follow up following salpingostomy. Cumulative clinical pregnancy rates were 8.7%, 20%, 25.5% at 6, 12, and 24 months respectively. The pooled birth rate (10 studies and 1469 patients) was 25%, and ectopic pregnancy rate (19 studies, 2662 patients) was 10%. Based on these results the authors suggested that prior to commencing IVF, salpingostomy should be considered, rather than tubal occlusion or salpingectomy for women with hydrosalpinges. The included studies were clinically heterogeneous and not all involved preoperative tubal assessment, with the suggestion by the authors that further prospective high-quality studies should be performed to identify the sub population that would benefit from remedial surgery.

The systematic review of Volodarsky-Perel et al (2019) addressed treatment of hydrosalpinx in relation to IVF outcome. The theme of this Canadian review was management of hydrosalpinx to optimise embryo implantation at IVF. The role of reconstructive surgery was considered to be limited, and Fallopian tube damage best evaluated at surgery. The rate of pregnancy was low with rigid, thick Fallopian

tubes without mucosal folds and up to 50% with minimal damage (Tulandi and Marzal 2012). The study of Millingos et al (2000), indicated that of 61 women having laparoscopic neosalpingoscopy the cumulative pregnancy rate (CPR) was 13.6% for patients with only distal tubal disease and 23% with mild degree of tubal disease and periadnexal adhesions. The CPR rose to 20.5 and 29% at 24 months for the same categories respectively. An overall conception rate of 41.7% (18% live births), 2.5-16.5% rate of ectopic pregnancy and mean conception time of 17.7 months was quoted for the study reported by Taylor et al (2001) involving unilateral and bilateral operations. The review concludes that "regardless of the surgical approach the results of salpingostomy remain poor".

The views of USA gynaecologists, is reflected in the American Society of Reproductive Medicine (ASRM) supported Fertility and Sterility journal, which recently published in Views and Reviews section a series of articles adressing "Reproductive Surgery: glimpses into the past and thoughts for the future (Parts 1 & 2)". One article in this series by Goldberg et al (2019), discussing attitudes for management of hydrosalpinx state "another surgical procedure that has all but been abandoned is neosalpingostomy", however quote that data from 5 non controlled studies indicate that two thirds of good prognosis patients had intrauterine pregnancies and low ectopic pregnancy rates (Boer-Meisel et al 1986; Donnez and Casanas-Roux 1986; Oh 1996; Rock et al 1978; Schlaff et al 1990). The overall conclusion was that "appropriately selected patients can benefit from a cost effective minimally invasive outpatient procedure to avoid the need for IVF. Many patients would prefer this if given the option". The article in the series by Raff and DeCherney (2019), addressing "Reproductive surgery and invitro fertilization: the future reevaluated" state "Currently the majority of reproductive endocrinologists address hydrosalpinges with surgical removal", and recognise that

a delicate balance exists between the increasing use of ART and the loss of surgical skills that comes with fewer opportunities for surgical intervention. This view was supported by the review of Gangiulo and Bhagavath (2019), who addressed "Reproductive surgey: decreasing skills and advancing technology - an existential conundrum". They concluded that surgical innovations have created more opportunities for both reproductive endocrinologists and general gynaecologists to engage in reproductive surgery with patients reaping the benefits.

The findings presented in this study positively support the above statement which is in agreement with the main theme in this thesis that restorative surgery has a complementary role to IVF in the management of hydrosalpinx related infertility. The live birth rate in women in the Adelaide Cohort study with normal MCI (18.3%), is comparable to the live birth rate following a cycle of IVF (Fitzgerald et al, 2017).

The review by Koninckx et al (2018) also supports this view commenting "The comparison of IVF/ART versus reproductive surgery is the wrong debate as the cumulative pregnancy rate of reproductive surgery and IVF are additive. The large majority of women with infertility should have a diagnostic laparoscopy during which reproductive surgery can be performed if needed. Having excellent reproductive surgery readily available to patients, similar to the availability of IVF would increase cumulative pregnancy rate in women with infertility and dicrease the overall cost"

This opinion was also reached by Gebeh and Metwally (2017), who concluded that "surgical intervention is necessary in most cases of distal tubal disesase with hydrosalpinges and selected cases should have salpingostomy".

In the invited lecture to the Royal College of Obstetricians and Gynaecologists (RCOG) "Surgical Management of Distal Tubal Disease" on 13 September 2016, Li commented that his group achieved a live birth rate of 29% (28/97women) for salpingostomy surgery (Singhal et al (2016). He emphasised the advantages for

selective salpingostomy - salpingectomy policy following salpingoscopic assessment to grade severity of endotubal pathology. Women with mild tubal disease would have the chance of spontaneous conception as well as increased chance of conception if it was subsequently necessary to proceed to IVF. He commented that women who conceived after surgery had a more satisfactory clinical experience, and that salpingostomy was potentially more cost effective.

The Practice Committee of the American Society for Reproductive Medicine (2015) reviewing the role of tubal surgery in the era of assisted reproductive technology have indicated that the advantages of tubal surgery are:

- Minimally invasive surgical procedure
- Avoidance of IVF complications
- Chance of monthly natural conception
- Ability to have more than one pregnancy.

While the disadvantages are

- Possible complications (haemorrhage, infection, organ damage, anaesthetic risk
- Longer more painful postoperative recovery and increased risk of DVT
- Higher risk ectopic pregnancy than IVF.

The suggested main advantages of IVF, are higher per cycle success rate and less surgically invasive procedure.

The Committee further recommended that age, prior fertility, ovarian reserve, number of children desired, extent of tubal pathology, presence of other infertility factors, patient preference, religious beliefs and experience of the surgeon should all be considered.

To optimise chance of sucess and reduce surgical risk reproductive surgeons experienced in laparoscopic and microsurgical technique should perform restorative surgery ideally in centres where both ART and surgery are supported by collaborating multidisciplinary teams. Emotional support and counseling are a priority. This concept has proven to be of value in the management of severe cases of endometriosis with the development of centres of excellence able to achieve quality improvement for such women (Ebert et al 2009).

Emotional adjustment to IVF and infertility has been extensively reviewed emphasising the psychological and emotional support that must be provided during surgical or ART treatment (Verhaak et al 2007). Treatment-induced stress is related to possible failure, with unsuccessful treatment raising levels of negative emotions which dissappear when treatment is successful. Counseling should be provided for male partners as well to reduce the sense of helplessness of men and encourage greater involvement and provision of emotional support for their partner. Giving hope to couples is just as important during critical stage of IVF treatment (between embryo transfer and pregnancy test) as during surgical intervention (Gabnay-Nagy et al 2020).

Chapter 11 Management of Hydrosalpinx Infertility: Findings and Recommendations

11.1. Findings

The findings of this thesis are that

- The pathological changes in Fallopian tube morphology and function following hydrosalpinx formation are variable.
- Deleterious changes are time dependant and likely due to progressive increase in intraluminal pressure as evidenced in the rabbit hydrosalpinx model.
- Salpingoscopy and microsalpingoscopy are useful techniques to assess tubal endothelial damage and persistence of inflammation thus enabling a rational decision to be made to either excise irreparably damaged tubes prior to commencing IVF/ART or to proceed to salpingostomy from a “one key-hole” laparoscopic intervention.
- The presence of loss of cilia, cellular inflammation, and intraluminal or severe peritubal adhesions are contraindications to salpingostomy.

11.2. Recommendations

Clinicians and service providers should consider the benefits of day surgery resolution of hydrosalpinx related infertility before proceeding to IVF/ART. These benefits include:

- Emotional and psychological improvement associated with the knowledge that spontaneous pregnancy may be possible (Li 2016)
- Likely partner acceptance/preference

- More accessible treatment in public hospitals for uninsured patients without medical cover.
- Financial savings when compared to long term IVF treatment.

These recommendations are particularly relevant to ovulatory women over 40 years of age who have a low take-home baby rate with IVF, but have been shown in a reversal of sterilisation study, to have an improved outcome following tubal surgery (Petrucco et al 2007).

11.3. Guidelines

- Women with Fallopian tubes that have been proven to have minor and not severe epithelial damage, can be considered for day surgery endoscopic salpingostomy. In pre-operative counselling the higher incidence of ectopic pregnancy than associated with IVF should be discussed.
- Due to the time dependant deterioration in tubal function, salpingostomy for bilateral hydrosalpinx should be considered for young women at diagnostic laparoscopy, even before fertility is considered.
- Preoperative counseling to perform salpingostomy should be considered when there exists high suspicion of hydrosalpinx. The higher incidence of ectopic pregnancy than associated with IVF should be discussed.
- Women should be involved in the decision to perform surgery and give consent for either salpingostomy or salpingectomy depending on intraluminal salpingoscopy findings
- A large prospective randomised controlled trial comparing IVF treatment to salpingostomy should be performed in suitably selected (salpingoscopy assessed) women with adequate follow up before proceeding to IVF. The study would require international cooperation by selected specialised ART

units able to provide IVF and tubal laparoscopic and/or microsurgery intervention. In principle the design of the study would ensure that surgical cases would proceed to IVF if unsuccessful after an agreed interval of time (12 months preferably). A second arm would include women who would proceed with IVF following surgery whilst also attempting spontaneous conception.

- The no-surgery IVF group would undergo an agreed number of IVF attempts with fresh and frozen embryo transfers according to age range selected. Depending on the above factors and agreement on accepted difference in outcome for the two arms, I estimate that 400-1400 women would need to be enrolled in the trial.

Recent opinion on debating the relevance of surgery in infertility practice in Australia however, only considered patients with endometriosis, with no mention of hydrosalpinx or other Fallopian tube disease correctable by surgery (Yazdahl 2017; Abbott 2017).

In the current clinical climate in Australia it would seem that a randomised trial would be poorly supported.

APPENDICES

Appendix 1 Steroid Hormone Receptor Methodology

Materials:

- Chemicals (A.R. Grade) were obtained from British Drug Houses (BDH) Ltd. Other companies supplied the following:
- Oestrogen 17b, diethylstilbestrol, dithiothreitol and calf thymus DNA (type1) from Sigma Chemical Company Ltd., Kingston, Surrey, UK.
- Sephadex LH-20 from Pharmacia (GB) Ltd., Hounslow, Middlesex, UK.
- Butyl-PBD (5-(4-biphenyl)-2) 4-t-butylphenyl)-1-oxa-3,4 diazole from Fisons Ltd., Loughborough, Leicester UK. Biosolv BBS-3 from Bechman-RIIC Ltd., High Wycombe, Bucks., UK.
- B.S.A., BDH Chemicals, Poole, UK.
- Hydroxylapatite, Bio-Rad Laboratory, UK.
- Weck Medium Hemoclips, Edward Weck and Co., USA.
- Radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks., UK.
- Fluothane (Halothane) was supplied by ICI Ltd., (Pharmaceutical Division), Alderly Park, Macclesfield, Cheshire, UK.
- Anaesthetic Gases were from B.O.C. (Medical Gases), Wembley, Middlesex, UK.
- Polypropylene tubes (LP3) and stoppers were obtained from Luckams Ltd., Burgess Hill, Sussex, UK.
- Dextran – coated charcoal tablets, Steranti Research Ltd., Herts., UK.
- PPO, POPOP Scintillation chemicals, Koch-Light Laboratories, UK.

Apparatus:

- A type x10/20 Homogeniser (Intern Lab. App. GmbH) supplied by the Scientific Instrument Centre, London, UK.
- Centrifugation was performed on a range of MSE centrifuges (Fisons, Manor Royal, Crawley, Sussex, UK) Centrifuges used were the MSE Coolspinand MSE Mistral 4L.
- Ultracentrifugation was carried out in a MSE Prepspin and Beckman L5-65B with a SW50.1 Rotor (Beckman Instruments, California, USA).
- Radioactivity was determined using an Intertechnique PG4000 Liquid Scintillation Counter, Intertechnique Ltd., Middlesex, UK. Activity was expressed in dpm1s determined by external standard ratio counting coupled to quench correction by computation from pre- programmed quench curves. An Isocap/300 Liquid scintillation Counter, Nuclear, Chicago, USA, was used in the latter part of the study.

Measurement of Radioactivity:

A scintillation fluid of 0.45% butyl- PBD in toluene (Scintillation grade) was used. Radioactivity extracted into ethanol was counted directly in this fluid (1-2 ml ethanol per 10 ml scintillation fluid). Aqueous samples were monitored for radioactivity using this fluid containing 10% BBS-3 (from 0.5-1.5 ml aqueous sample per 10 ml scintillation fluid).

Appendix 2 Experimental Animals and Preparation of Human and Rabbit Tissue

Experimental Animals:

New Zealand White (Foxfield Rabbits Petersham, UK, and Institute of Medical and Veterinary Science, Adelaide, South Australia) strain sexually mature, virgin female rabbits weighing greater than 3.5 kg were used. The animals were individually caged and kept separate from male and pregnant female rabbits. All animals were kept on a 12 hour dark / 12 hour light cycle, with food and water and libitum. Animals were kept for at least one week to acclimatise before experiments were commenced. To ensure that animals were in constant oestrus peripheral oestrogen levels were measured to confirm oestrus phase and no P4 activity.

Anaesthesia and Postoperative Care:

Premedication prior to shaving of fur was instituted by an intramuscular injection of Ketalar (Ketamine Hydrochloride, Parke- Davis, UK and Australia) in appropriate dosage per kilogram. Anaesthesia was maintained using Innovar-Vet (Fentanyl androperidol, Ethnor, UK and Australia), supplemented with gas, oxygen and Fluothane via a Boyle's machine and Fluotec vaporiser.
Penicillin was administered postoperatively and appropriate care was given to all animals.

Preparation of Tissue:

- Rabbit

After induction of anaesthesia, flank incisions in the lower abdomen were utilised to expose the right and left oviducts. Following application of haemostats to supplying blood vessels, the oviducts were excised free. Euthanasia was then carried out by the injection of Lethabarb (Pentobarbitone sodium, Arnolds, Boronia Victoria). The oviducts were kept at 4^0C and trimmed free of mesenteric fat and peritoneal layers using low magnification (OPMI 1 operating microscope). The tissue was then immediately frozen in liquid nitrogen and kept in this medium for use within 3 months. Preliminary experiments indicated that within this time period there was no deterioration in hormone receptor values in the tissue.
In continuing experiments only the required oviduct was excised and the animals were allowed to recover after ensuring that haemostasis was achieved.

- Human tissue

Fallopian tubes were obtained from patients undergoing surgery for non- malignant pelvic disease following prior informed consent. Only that part of the Fallopian tube from the ampullary-isthmic junction to the fimbrial end was used.
Hydrosalpinges were removed from infertile patients when corrective surgery was not possible.
To confirm stage of menstrual cycle E_2 and P_4 were measure in peripheral blood collected at the time of surgical excision.
Dissection of peritoneal coat and mesenteric layers was carried out at 4^0C (Petrie dish supported on ice) before snap freezing in liquid nitrogen.

Appendix 3 Rabbit Surgery

Mechanical Induction of Hydrosalpinx in the Rabbit:

Following adequate oviduct exposure Weck (Medium) Hemoclips were applied at the ampullary-isthmic junction and at the terminal part of the infundibulum just proximal to the fimbriated end of the oviduct. The ampullary-isthmic junction was recognised by denoting the branching artery in the mesosalpinx supplying the mid portion of the oviduct at this site.
Depending on the experiment performed hydrosalpinx was induced in either one or both oviducts, with the unoperated oviduct left as control. Care was taken to occlude only the tubal lumen with minimal interference to blood vessels in the mesentery.
Oviducts were removed 8 weeks after initial surgery via repeat flank incisions. The widest transverse diameter of the occluded oviducts was accurately measured in situ before excision.

Microsurgical Reversal of Hydrosalpinx in the Rabbit:

Microsurgery was performed utilising the principles of atraumatic dissection with microsurgical instruments, fine uni or bipolar coagulation, (Bipolar-Met and Valley Lab Instruments) to ensure perfect haemostasis. Hartmann's solution was used for isotonic irrigation of tissue. The Weck clips at each site were excised with magnification provided by a Zeiss-OPMI 1 operating microscope.
Oviduct segments were then rejoined in a two layer anastomosis of muscularis (avoiding mucosa) and serosal layers using 8/0 and 10/0 Eticon microsutures. To facilitate anastomosis between the markedly distended ampulla and the normal size isthmic segments, the technique described by Winston (1978) was used.
At completion of the two site anastomosis dilute methylene blue dye was injected in the lumen of the isthmic segment proximal to the two anastomosis sites to confirm that oviduct patency had been achieved.

Appendix 4 Steroid Hormone Receptor Assays

ER Assay:

Homogenisation

Tissue was thawed, weighed and finely minced on an ice cold watch glass and place in a pre- cooled vessel. All procedures were performed at 0-4^{0}C.
Tissue was homogenised in nine volumes (W/V) of buffer (0.25M sucrose, 10nM Tris-HCL, ph 7.6, 0.025M KCL, 30% glycerol) utilising x10-20 homogeniser with 3 bursts of 10 second duration at setting 8 with 30 second interval between each burst. The vessel was constantly cooled in an ice water bath.

Subcellular Fractionation (see Figure 9)

A crude nuclear myofibrillar pellet was obtained by centrifugation at 2000g for 10 minutes in an MSE-Coolspin refrigerated centrifuge (Fisons Crowley Kent). The pellet was then washed 4x in the original volume of sucrose buffer, each time being re-centrifuged at 1200g for 10 minutes. The first supernatant obtained after centrifugation at 800g was further centrifuged at 200,000g for 1 hour at 4^{0}C in a MSE Prepspin 65 (Fisons Crawley Kent) to give a cytosol fraction and microsomal pellet.
After the final wash the pellet was resuspended in the original volume of sucrose buffer by vortexing and gentle aspiration through a pasteur pipette. This suspension was kept at 4^{0}C before use.

DNA Estimation

DNA in the crude nuclear myofibrillar pellet was measured using the Burton (1956) method. The crude nuclei from 0.5 ml of the suspension was pelleted by centrifugation at 1200g. This pellet was washed twice with 0.2 PCA at 4^{0}C. The pellet was then hydrolysed with 0.5M PCA for 60 minutes at 8^{0}C in a shaking water bath before cooling at 4^{0}C and re-centrifugation to isolate the hydrolysate.

- Reagents
  - 1.5% (W/V) diphenylamine in glacial acetic acid containing 1.5% (V/V) sulphuric acid. This solution was stored in the dark.
  - Aqueous acetaldehyde (16.2 mg/ml).
  - 1 ml of aqueous acetaldehyde was added to each 20 ml of diphenylamine solution prior to use.
  - Stock calf thymus DNA (400ug/ml in 5mM NaOH) kept at 4^{0}C.
  - Each volume of stock and 1.0M PCA were mixed and heated at 70^{0}C for 15 minutes. From this working standard of 200ug/ml DNA a range of standard solutions (0-200ug) was prepared in 0.5M PCA.

- Procedure

One volume of the hydrolysate (0.5ml) was mixed with 2 volumes of the diphenylamine solution (1ml) and incubated at 30^{0}C in a shaking water bath. The standard DNA solutions (calf thymus) were similarly treated. Absorbency was measured at 600nM and a standard curve prepared for the known DNA concentrations. The concentration of DNA in the experimental samples was derived by reference to this standard curve.

Protein Estimation

The protein concentration of some of the cytosol preparations was measured using the Lowry et al (1951) technique. Cytosol was diluted 50 fold in distilled water prior to use.

- Reagents
  - A reference protein solution (Versatol) was prepared at 1.44mg/ml in distilled water and kept at -20^{0}C until required when it was diluted to give a standard solution from 0-144ug/ml.
  - Copper tartrate/carbonate solution (200ml of 2% sodium carbonate containing 4 ml of 1% sodium tartrate and 0.4ml 5% copper sulfate).
  - Folin's reagent diluted 1 in 3 in distilled water prior to use.

- Procedure

Each 1 ml of protein solution (standard or known) 5 ml of copper tartrate/carbonate solution was added and the whole allowed to stand for 15 minutes at bench temperature. Then 0.5ml of dilute Folin's reagent was added, the solution mixed and left for 15 minutes at room temperature before reading the absorbance at 720nM. A standard curve was prepared for the known protein standards and the protein concentration of the unknowns derived from that.

ER Nuclear Assay:

Procedure

Nuclear samples (triplicate 0.2ml of suspension) were incubated in the presence of (2,4,6,7 (n)-^{3}H)17B E_2 (specific activity 85-110 Ci/mmol Radiochemical Centre, Amersham UK) at 37^{0}C for 1 hour; parallel samples contained an equivalent concentration of (^{3}H)-E_2 with a 200-fold excess of diethylstilboestrol (DES) (Amersham et al 1972), to replace the endogenous E_2 bound to the nuclear receptors. All incubations were carried out in a stoppered 2 ml polystyrene tube.
In preliminary experiments to determine the dissociation constant for the nuclear ER–E_2 interaction a range of (^{3}H)-E_2 concentrations from 0.5 to 30nM were employed, each being with or without 200 fold excess of DES.
For routine determinations of nuclear ER levels a saturating concentration of 15nM (^{3}H)-E_2 was used. Measurement of nuclear receptor at 37^{0}C gave a value for total nuclear receptors (ie. occupied and unoccupied).
The incubation was terminated by the addition of 1 ml of ice cold buffer containing 1% BSA (Vu Hai and Milgrom 1978) and the tubes placed on ice.
Hydroxylapatite was also included (5mg/ml) to increase the bulk of the suspension and to trap any solubilised receptor (Pavlik and Coulson 1976) before centrifugation for 10 minutes at 4^{0}C.
The pellet was washed twice more with 1 ml of buffer containing 1% BSA only and finally with 1 ml of buffer alone. During each washing step the resuspended nuclear preparation was left to stand for 15 minutes at 4^{0}C before centrifugation. After each centrifugation the supernatant was removed with a pasteur pipette to minimise loss of of the pellet. The (^{3}H)-E_2 in the final pellet was extracted twice with 1 ml of absolute alcohol. The ethanol was left in contact with the nuclear preparation for 15 minutes in each extraction. The ethanol extract was mixed with 10mls of scintillation fluid, 4.5ml of butyl-PBD/litre toluene and radioactivity

determined in an Intertechnique SC 3000 spectrometer with automatic quench correction at an efficiency of 40%.

ER Cytosol Assay:

Procedure

Cytosol (0.2ml duplicate samples) was incubated with (2,4,6,7 (n)- ^3H) 17B oestrogen (specific activity 85-110 Ci/mmol Radiochemical Centre Amersham UK) in the presence or absence of 200-fold excess DES for 18 to 24 hours in polystyrene tubes. A range (0.5 to 30 nM) of (^3H)-E_2 concentrations with or without DES was used in preliminary experiments to determine the dissociation constant for the cytosol ER-E_2 interaction.
For routine determination of cytosol receptor levels, a saturating concentration of 15nM (^3H)-E_2 was used. Incubation of cytosol with labelled E_2 at 4^0C measures unoccupied receptors which normally constitute greater than 90% of total receptors. Endogenous E_2 interferes little with cytosol assay by this method (White et al 1978).

Sephadex LH-20 Chromatography

Macromolecular bound radioactivity was separated from free steroid by exclusion chromatography on sephadex LH-20 (Pharmacia GB Ltd., UK) chromatography columns (Ginsburg et al 1974). Sephadex LH-20 was swollen with 10 volumes (w/v) of TED buffer (1.21g Tris, 0.504g EDTA, 0.1543g Dithiothreitol ph7.6) at 4^0C for 24 hours prior to use.
The" fines" were removed before use with excess buffer and the slurry degassed under vacuum. The slurry was then poured into borosilicate-glass pasteur pipettes (0.6 cm width) which had been plugged with glass wool at their necks. The pipettes were filled to a 6 cm high column and placed in a purpose built perspex tank and surrounded by granulated ice to keep the columns at 4^0C. The columns were washed with 2ml TED buffer at 4^0C before chromatography of cytosol. Flow through each column was stopped by capillarity when the buffer level had reached the top of the surface of the Sephadex gel.
A 0.2ml aliquot of cytosol pre-labelled with (^3H)-E_2 was applied to the top of the column and allowed to enter the gel bed. Separation of (^3H)-E_2 bound to macromolecular species from that which was free was initiated by addition of 0.5ml TED buffer to the column. Macromolecular bound steroid was excluded from the gel bed whilst free steroid entered the gel bed. The flow of free steroid through the column was retarded relative to bound steroid.
Preliminary experiments with the addition of 10ul of a 1% solution of blue dextran to the cytosol had shown that 0.5 ml of buffer was sufficient to move the macromolecular bound steroid down the column to just above the glass wool plug (Myatt 1980). The haemoglobin in cytosol was similarly found to serve as a marker to check the column flow was regular. A further increment (usually 1 ml) of buffer was then added to the column to elute the macromolecular bound steroid. The void volume was collected directly into scintillation vials containing 3mls of scintillation fluid prepared for counting aqueous samples (UNISOLV). The radioactivity in each sample was then determined in the instruments previously described. The difference I radioactive counts between samples incubated with or without DES gave a measurement of specific ERs.

Dextran-Coated Charcoal Analysis

Due to the lack of availability of the 0.6 cm pipettes used for the sephadex chromatography assay, use was made of dextran coated charcoal for the latter part of this study in Australia. Results of simultaneous assays using both techniques indicated similarity of results so that values obtained in each part of the study could be similarly interpreted.

The principle used in this method relies on the fact that unbound radio-inert ligand is absorbed by the charged dextran-coated charcoal and can thus be separated from the bound hormone which remains in the supernatant following centrifugation.

A dextran coated charcoal suspension was prepared by utilising 1 tablet of dextran coated charcoal (250mg activated charcoal and 25 mgs dextran) I 50 ml of TED buffer, thus achieving a final suspension of 0.5% activated charcoal and 0.05% dextran T70.

Cytosol (0.2ml duplicate samples) was incubated with (2,4,6,7 (n)-^{3}H) 17B E_2 in the absence of DES as previously described for 18-24 hours. The charcoal suspension (0.5ml) is added to each assay tube, vortex mixed and after 15 minutes at 4^{0}C centrifuged at 2500 rpm for 10 minutes. The supernatant is decanted into vials and scintillation fluid (UNISOLV or DCSII) added and radioactivity counted. The difference in radioactive counts between samples incubated with or without DES gave a measurement of specific oestrogen receptors.

PR Assay:

Homogenisation and Subcellular Fractionation

These studies were carried out as previously described for the ER assay.

PR Nuclear Assay:

Procedure

Triplicate 0.2ml samples of the crude nuclear myofibrillar suspension were incubated at 4^{0}C for 18-24 hours with (1,2,6,7(n)-^{3}H)-P_4 (specific activity 80-110 Ci/mmol, Radioactive Centre, Amersham, UK). Parallel samples contained an equivalent concentration of (^{3}H)-P_4 with a 100-fold excess of unlabelled P_4. A 50-fold excess of cortisol was included with all samples to minimise the binding of radioactive P_4 to corticosteroid binding globulin.

In preliminary experiments, as described for the E_2 nuclear assays, I determined that for routine determination of nuclear P_4 receptor levels, a saturating concentration of 15nM (^{3}H)-P_4 was used.

The incubation was terminated by the addition of 1ml ice cold buffer containing 1% BSA and samples allowed to stand for 10 minutes at 4^{0}C prior to centrifugation at 2500g for 10 minutes. A further 2 washes with 1ml buffer containing 1% BSA were followed by a final wash with 1 ml of buffer alone.

During each washing step the resuspended nuclear preparation was left to stand for 15 minutes at 4^{0}C before centrifugation. After each centrifugation the buffer wash was removed with a pasteur pipette to minimise losses from the pellet. The (^{3}H)-P_4 in the final pellet ws extracted twice with 1 ml of buffer alone. The ethanol was left in contact with the nuclear preparation for 15 minutes in each extraction. The ethanol extract was mixed with 10 mls scintillation fluid (4.5g of butyl-PBD/L toluene) and radioactivity measured as previously described.

Non-Specific Binding in the Exchange Assay

Preliminary experiments revealed that a high level of nonspecific binding of (^{3}H)-P_4 was occurring with the technique described above. Because of this following exchange at 5^{0}C, 0.2% Triton x-100 was added to the terminating buffer containing 1% BSA. This step was then followed by 2 washes with 1% BSA buffer and finally by buffer alone. Thereafter the Triton X-100 was included in all routine assays.

PR Cytosol Assay:

Procedure

Cytosol (0.2ml duplicate samples) was incubated with 1,2,6,7 (n)^{3}H-P_4 (specific activity 80-110 Ci/mmol, Radioactive Centre, Amersham, UK) in the absence or presence of 100-fold excess of unlabelled P4 for 18-24 hours at 4^{0}C in polystyrene tubes. A range of (^{3}H) P_4 concentrations was used in preliminary experiments to determine the dissociation constant for the cytosol PR interaction. For routine assays of cytosol receptors saturating concentration of 15nM (^{3}H)-P_4 was used. A 50 fold excess of cortisol was included with all samples to minimise binding of radioactive P_4 to corticosteroid binding globulin.

Sephadex LH-20 Chromatography

A similar procedure described above for the ER cytosol assay was used to measure PR in the cytosol, using columns of Sephadex LH-20.

Dextran Coated Charcoal Analysis

The procedure described for ER cytosol assay was similarly used for P4 cytosol receptor measurement. Comparable results to Sephadex chromatography was achieved.

Exposure of Tissue to E_2:

In vitro: Rabbit oviducts were surgically collected, rinsed in saline to reduce excess blood, and incubated for 15 minutes in 5ml of medium 199 (Welcome, Beckenham, UK), previously equilibrated at 37^{0}C in an atmosphere of 96% O_2, 5% CO_2, which contained unlabelled E_2 at a final concentratuion of 15nm/L. Subsequently, tissue was transferred to 5ml of medium 199 without any added E_2 and incubated for a further 30 minutes. Preparation of tissue for receptor measurement as described above.

In vivo: Twenty mcgm of E_2 in 1ml of compound sodium lactate B.P. was injected intravenously prior to excision of oviducts. Blood samples were collected prior to E_2 administration and at excision of oviducts for E_2 assay. The tissue was rinsed in homogenisation buffer and then prepared as described above.

Appendix 5 Measurement of Oviduct Blood Flow

Rabbits:
13 rabbits were used. A hydrosalpinx had been induced in the left, right or both oviducts.
6 of the animals were treated with HCG (human chorionic gonadotrophin) 7or 18 hours prior to use.

Method:
The method was similar to that described by Janson (1978) for ovarian blood flow. Following induction of anaesthesia P.E. catheters (O.D. 1.0mm, I.D. 0.8mm) were inserted in the

- Right femoral artery for monitoring of blood pressure (Stathem P23 AC transducer connected to a Grass Model 5D Polygraph).
- Left femoral artery for withdrawing a reference blood sample using a Harvard infusion/withdrawal Pump.
- Left ventricle via the left femoral artery.

The position of the cannula was checked by observing the pattern of blood pressure recording.
Microspheres (3M Company, St. Paul, Minnesota, U.S.A.) with diameter of 15.2u (+/- 0.7u) were labelled with strontium-85. A suspension (0.5ml-(1-1.5x10^6) microspheres in 20% dextran was infused into the left ventricle over a period of 30 seconds.
A reference blood sample was withdrawn at a constant rate (1.7mls/minute) 15 seconds prior to, during and after infusion of microspheres.
Following euthanasia with sodium pentobarbitone the oviducts were dissected free and the number of microspheres lodged in the oviduct determined by counting the activity in a Backman Biogamma counter.
The blood flow was calculated according to the formula (Rudolf and Heymann 1967):

$$Q_{organ} = \frac{Q_{ref} \times N_{org} \times 100}{N_{ref} \times W}$$

Q organ = Blood flow mls/100g tissue/min
Q ref = Withdrawal Rate of reference sample
N ref = Number of spheres in reference sample
Norg = Number of spheres in the organ
W = Weight of organ

Appendix 6 Biochemical Studies

All biochemical studies were performed in the laboratories of Prof. Donald Cheek (deceased) at The Queen Victoria Hospital (now The Women's and Children's Hospital) in 1982.

Materials and Methods:

Fallopian tubes of women with or without hydrosalpinx were collected as previously described.
The oviducts of rabbits with or without induced hydrosalpinx (8 weeks duration) were processed as previously described.
It was not possible to measure sodium and potassium on all samples because of the amount of tissue required. Six normal rabbits provided 3 measurements of sodium and potassium (paired oviducts) whilst 2 measurements of potassium and sodium were carried out on hydrosalpinx specimens.
Biochemical methods were those described by Cheek and Hill (1971) with minor modification.

DNA and RNA Extraction:

Frozen tissue was cut into fine pieces with scissors and placed in a pre -weighed chilled tube. Five volumes of cold 0.2M perchloric acid was added and the mixture homogenised using Ystral X10/20 homogeniser for 3 bursts of 30 second duration ay maximal speed. The homogeniser was then rinsed with a further five volumes of 0.2M perchloric acid and the two volumes combined by a brief agitation with a vortex mixer. The homogenate was allowed to stand for 30 minutes at 4^0C then centrifuged at 3^0C for 10 minutes at 1000g.

The supernatant was resuspended in 20 volumes of 1M perchloric acid. The tubes were capped securely and the RNA extracted by constant rotation for 18 hours at 4^0C. After centrifuging at 1000G for 10 minutes the supernatant was removed and kept for RNA measurement. The residue was washed with 20 volumes of cold perchloric acid, the supernatant was discarded and the residue resuspended in 10 volumes of cold 0.5m perchloric acid and used for DNA measurement.

- RNA Reagents
  - Ferric chloride-HCl (Stock)
  - 10 gms anhydrous ferric chloride (BDH) dissolved in 100mls conc. HCl. 0.5 ml stock is diluted to 100mls with conc. HCl (Univar, Ajax Chemicals) just before use.
  - Orcinol Reagent - 0.4 gms orcinol (Sigma Chemicals) dissolved in 10 mls of absolute alcohol (Ajax Chemicals) just before use.
  - RNA Standard - (200ug/ml). 20 gms calf liver RNA (Sigma Chemicals) dissolved in 100mls of 1M perchloric acid. Working standards of 10, 25, 100, 200ug/ml are prepared from this.

- RNA Method
  1ml supernatant standard or 1M perchloric acid was added to a 10 ml tube. 20 mls fresh Ferric chloride-HCl and 0.12 mls orcinol reagent were then added. Tubes were mixed, capped and placed in a water bath for 20 minutes. After allowing to cool O.D. of samples are read at 660mu on spectrophotometer (Sequoia-Turner, Model 340).

- DNA Reagents
  - DNA Standard-(1mg/ml). 50mg calf thymus DNA (Sigma Chemicals) dissolved in 50mls 0.005M sodium hydroxide. Working standards of 50, 100, 200, 300, 400, 500 ugmDNA/ml were prepared by adding the required volume of stock to 2mls of 1m perchloric acid and adjusting the volume to 4mls with distilled water.
  - 4mls of 0.5 perchloric acid is used as a blank.
  - Diphenylamine Reagent- (prepared just before use). 1.5gms of diphenylalamine (Sigma Chemicals) dissolved in 100 mls glacial acetic acid (Univar, Ajax Chemicals).

- DNA Method

Blank standards and samples were kept at 70^{0}C in a water bath for 30 minutes. Samples were spun for 10 minutes at 1000G. 1ml of blank standard or sample supernatant was added to a 10ml tube and 2mls of diphenylamine reagent added. Tubes were mixed, capped and placed in boiling water for 10 minutes. After allowing to cool, O.D. of samples are read at 600mu on a spectrophotometer.

Preparation Fat Free Dried Tissue:

Method

Oviducts were weighed in a pre-weighed tissue bottle and dried for 2 days in an oven (Thermoline) maintained at 100^{0}C (with lid inverted). Bottles were cooled and weighed to determine the weight of the dry sample and hence the water content. Samples are then immersed in Petroleum Spirit (May & Baker BPT 40^0-60^{0}C) and allowed to stand for 3-4 hours. The solvent was decanted and specimens washed with more petroleum spirit. Residual petroleum spirit is evaporated off and the samples dried in the 100^{0}C oven for one hour. After cooling the samples are weighed to determine the weight of fat free, dried tissue and hence lipid content. The fat free, dried powder is ground to a fine powder with a mortar and pestle and then dried in the oven for another hour before storing in an air-tight tube at a temperature below 0^{0}C.

Collagen Estimation:

- Reagents
  - Potassium borate (pH8.7). 6.18 gms of boric acid (Analar BDH) and 22.5 gms KCl (Sigma Chemicals) were added to 70mls cold water. The pH was adjusted to 8.7with 5M KOH, volume was made up to 100mls with distilled water and stored at 4^{0}C.
  - Alanine Solution. 10 gms Alanine (Sigma Chemicals) was dissolved in 90mls of distilled water and adjusted to pH 8.7 with 5M KOH, and the volume adjusted to 100mls with distilled water and stored at 4^{0}C.
  - Sodium Thiosulphate (Pronalys, May& Baker) 3.6 mls distilled water. This solution was stable at room temperature under a layer of toluene.
  - Ehrlich's Reagent - 20 gms of p-dimethylamino benzaldehyde (Sigma Chemicals) was dissolved in 33mls of absolute alcohol. 4.56 mls of conc.

sulphuric acid was slowly added to another beaker containing 34 mls of absolute alcohol. After cooling this is slowly added to the first solution with thorough mixing and the solution stored at 4^0C.

- 1% Phenolphthalein (May & Baker) in ethanol.
- Humin precipitant-20 gms of an anion exchange resin (BIORAD AG-1-X8 200-400 mesh, chloride form) and 10gms of NORITA charcoal (Sigma Chemicals) are mixed thoroughly in a beaker. The mix is washed 5 times with 6MHCl and dried with suction, by washing four times with ethanol ether (1:2).
- Chloramine–T (Prepared fresh before use)-2.8 gms of Chloramine T (GOR, BDH) is dissolved in 50mls Ethylene Glycol Monomethyl Ether (Sigma Chemicals).
- Hydroxyproline (Sigma Chemicals) Standard (1mg/ml). Working standards of 10, 20, 30, 40, 50g/ml are prepared by diluting the stock in distilled water.

Tissue Hydrolysis of Collagen Method:

10mg of fat free dried tissue was weighed directly into a pyrex tube to which were added 2mls distilled water and 2mls conc. HCl. The tubes were capped tightly with Teflon cushioned screw caps and a tight seal ensured by covering the seal with autoclave tape. The tubes were heated for 18 hours in an oven maintained at 118^0C. The hydrolysate was cooled to room temperature and diluted to a volume of 8mls with distilled water. 0.5 gms of humin precipitant was added and the tubes thoroughly mixed and centrifuged at 1000G. The supernatant was used for hydroxyproline determination.

Hydroxyproline Estimation:

0.2 mls sample or standard or distilled water (blank) were pipetted into 15mls tubes containing 0.8mls of distilled water and 5ul of phenylpthalein solution. The contents were titrated to a faint pink colour with 5M KOH and diluted to 2mls with distilled water before saturating with KCl. 0.5mls of borate buffer and 0.25mls alanine solution was added. After vortexing 0.25 mls of chloramine-T solution was added and the tubes again vortexed before left to stand for 20 minutes at room temperature. 1.5 mls of sodium thiosulphate solution was added and the tubes vortexed. If necessary samples were again saturated with KCl. 2.5mls of toluene was added and the tubes vortexed for 30 seconds. The organic phase was decanted and discarded. The tubes were tightly capped, heated in a boiling water bath for 30 minutes and placed immediately in an ice bath to cool to room temperature. The pyrrole thus formed is extracted into 2.5 mls of toluene by mixing for 30 seconds. 2mls of the toluene extract were transferred to separate tubes and 0.8mls of Ehlich's reagent added with stirring. After 30 minutes, O.D. is measured at 560mu using a spectrophotometer. The colour was found to be stable for at least an hour.

Water and Electrolyte Content:

Water

Water content was measured by the difference in dry tissue weight (following dessication).

Electrolyte Measurements

Chloride Estimation

- **Method**

50gms of fat free, dried tissue was weighed directly into a small platinum crucible. 0.2mls of 0.2 M KOH and 5 mls of glass distilled water were added and the mixture evaporated to dryness in an oven maintained at 100^0C. The crucible was placed in a muffle furnace and the contents ashed by increasing the temperature to 500^0C and maintaining that temperature for 90 minutes.
The ash was dissolved in 0.1mls of 2M sulphuric acid and the extract transferred to a 5 ml volumetric flask with several washes of glass distilled water before making up to volume.
2mls of water (blank), standard or unknown solution were added to 0.1 mls of perborate solution, capped, mixed and allowed to stand for 18 hours at room temperature.
200ul gelatin indicator and 0.5mls of Nitric acetic reagent were added. The mixture is titrated, using the low setting on the Chloridometer (Buchler-Cotlove Chloridometer Buchler Instruments Inc Fort Lee, NJ, USA).

- **Reagents**:
  - 0.2 M KOH
  - 2M sulphuric acid
  - Perborate solution (Fresh each day) - 0.3 gms NaBo3 (H_2O) (Ajax Chemicals) was dissolved in 5mls of 0.5M HNO3 to which was added 5 mls of 6M NaOH.
  - Gelatin Indicator - 600mg gelatin (Ajax Chemicals), 10mgs Thymol Blue (water soluble), 10 mg Thymol Crystals (Ajax Chemicals), were all dissolved in distilled water. This solution was aliquoted into 5 lots and new lot used each day. Before use the tube was immersed in hot water to liquefy the gelatin.
  - Nitric acid reagent - 1.3M HNO_3 in 50% acetic acid.
  - Chloride Standard - (5ug Eq/ml) - 292mgs NaCl dissolved in 0.04M sulphuric acid.

Sodium and Potassium

200mgs of fat free, dried tissue was ashed in a small crucible at 620^0C for 90 minutes. The ash was dissolved in 5mls of 0.1M HCl.
After filtering through a Millipore filter (MILLEX-PF) the sodium and potassium levels were measured by atomic absorption.

Appendix 7 Surface Electron and Transmission Electron Microscopy Tissue Preparation

Method:

Tissue biopsies were fixed in 3% glutaraldehyde (TAAS) and 2% formaldehyde in cocodylate buffer (0.05M, pH 7.2, 395 Mosm), dehydrated in alcohol, critical point dried (Denton D502) and coated with 60:40 gold-palladium. Specimens were mounted on stubs using "Silver Dag" (Electrodag 915- high conductivity point)

A Siemen's Autoscan was used for viewing, mainly at 20 kV.
Counting of ciliated cells was accomplished by a technique described by Tulsi and Dreosti (1981) at 2000 x magnification using a Perspex graticule divided into 36 squares (3600um) and finding the average number of ciliated cells in 30 fields representative of the block under examination.
Part of the biopsy was also processed for light microscopy study by fixation in buffered formalin.

Transmission Electron Microscopy Tissue Preparation:

Tissue biopsies were fixed in 3% glutaraldehyde fixative (TAAS) and washed 3 times in cocodylate buffer as above and fixed for 1 hour in 1% Oso4. Following dehydration in alcohol tissues were subjected to 50/50 absolute alcohol/spurs resin and then pure spurs resin overnight at 63^0C.
Tissue blocks were trimmed into trapezoid shape and tissue cut with glass knife into 0.5micron thickness sections.
On grids tissue was stained with uranyl acetate and distilled water. The lead stain was made with one pellet NaOH in 50mls distilled water/0.25g lead citrate. After wash in distilled water and drying, scanning was performed using transmission electron microscope.

Appendix 8 Light Microscopy Stained Tissue Section Preparation

Method:

Production of stained tissue sections for examination by light microscopy followed a step-wise process beginning with fixation of tissue in buffered formalin (10%) followed by dehydration and clearing of tissue before impregnation with wax. Thin sections are attached to a glass slide for staining with hematoxylin and eosin.

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