CALCIUM SIGNALLING DURING HUMAN SPERM INTERACTION WITH CELLS OF THE FEMALE REPRODUCTIVE TRACT

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ABSTRACT

The female reproductive tract acts not only as a complex mediator of sperm function and selection but animal data suggests that it alters protein expression after exposure to sperm, implying two-way communication. We have used single-cell fluorescence imaging to observe $[Ca^{2+}]_i$ signalling in human female reproductive tract cells upon initial contact with sperm and in sperm during binding and release events. Parallel experiments were also performed on a model human oviductal cell line, OE E6/E7 and a control, human foreskin fibroblasts. Upon exposure to sperm, tract cells generated $[Ca^{2+}]_i$ signals through mobilisation of thapsigargin-sensitive intracellular Ca^{2+} stores. The percentage of significant $[Ca^{2+}]_i$ responses varied in different anatomical regions of the female tract. Furthermore, $[Ca^{2+}]_i$ signalling was observed upon exposure to sperm-conditioned media suggesting signalling factors may be shed or secreted by sperm. Human foreskin fibroblasts were unresponsive to sperm.

Co-culture of sperm with tract explants induced post-translational modification of sperm proteins through NO-dependant S-nitrosylation. We have also provided initial evidence for $[Ca^{2+}]_i$ alterations in sperm during binding to and detachment from oviductal explants.

We conclude that sperm can elicit $[Ca^{2+}]_i$ signals in female tract cells upon initial contact though mobilisation of intracellular Ca^{2+} stores. This may reflect events upstream of reported gene and protein expression changes. In addition, human sperm interaction with oviductal epithelium is likely to be important in modulating sperm function during migration and associated events through the female reproductive tract.

DEDICATION

This thesis is dedicated in loving memory of my dear father, John Connolly. I will always remember the jokes, the laughs and the mind-numbingly deep philosophical discussions. It is also dedicated in loving memory of my mother, Sheila Connolly, whose strength, praise and encouragement has driven me to further my education.

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ABBREVIATIONS

AC	Adenylate cyclase
ACE	Angiotensin-converting enzyme
ADM	Adrenomedullin
AIJ	Ampullary-isthmic junction
AKAP	A-kinase anchoring protein
ALH	Amplitude of lateral head displacement
APM	Apical plasma membrane
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
BSP	Bovine seminal plasma
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Cytosolic calcium concentration
$[Ca^{2+}]_o$	Extracellular calcium concentration
cAMP	Cyclic adenosine monophosphate
CASA	Computer Assisted Semen Analysis
CBF	Cilia beat frequency
CCE	Capacitative calcium entry
CICR	Calcium-induced calcium release
Cl^-	Chloride
CNG	Cyclic nucleotide-gated
COC	Cumulus oocyte complex
DAF-FM	-4-amino-5-methylamino-2', 7'-difluorescein
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSD	Dynamic sperm density
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-amino-ethylether)-N,N,N'N'-tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase

FBS	Fetal bovine serum
FPP	Fertilisation promoting peptide
FS	Fibrous sheath
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing factor
GSH	Glutathione
GSNO	S-nitrosoglutathione
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
HBSS	Hanks buffered salt solution
HCM	Human cervical mucus
HCO_3^-	Bicarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFEA	Human Fertilization and Embryology Authority
HFF	Human foreskin fibroblasts
HPV	Human papillomavirus
HSA	Human serum albumin
HSPs	Heat shock proteins
ICLC	Interstitial Cajal-like cells
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Inducible nitric oxide synthase
$InsP_3/IP_3$	Inositol 1,4,5-triphosphate
IP_3Rs	Inositol 1,4,5-triphosphate receptors
K^+	Potassium
IVF	In vitro fertilisation
LCsEBSS	Low-calcium supplemented Earle's balanced salt solution
LH	Luteinising hormone
L-NAME	NG-nitro-L-arginine-methyl ester
mAC	Membrane adenylyl cyclase
MC	Methylcellulose
mRNA	Messenger ribonucleic acid
MS	Mitochondrial sheath

Na^+	Sodium
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\cdot}$	Superoxide anion
OCM	Oviductal-conditioned media
ODFs	Outer dense fibres
OECs	Oviductal epithelial cells
OGPs	Oviduct-specific glycoproteins
PBS	Phosphate buffered saline
PDL	Poly-D-lysine
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMCA	Plasma membrane calcium ATPase
PSA	Prostate-specific antigen
ROI	Region of interest
RGD	Arginine-glycine-aspartic acid
RNE	Redundant nuclear envelope
ROCs	Receptor-operated channels
ROS	Reactive oxygen species
RYRs	Ryanodine receptors
sAC	Soluble adenylyl cyclase
sAPM	Soluble protein faction from apical plasma membrane
sEBSS	Supplemented Earle's balanced salt solution
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SH	Sulfhydryl
SMC	Smooth muscle cells
SMOCs	Second messenger-operated channels
SOCs	Store-operated channels
SOCE	Store-operated calcium entry
SOD	Superoxide dismutase

SPCA	Secretory pathway calcium ATPase
SS	Disulfide
TPBS	Triton in phosphate buffered saline
TRP	Transient receptor potential
UTJ	Utero-tubal junction
VOCs	Voltage-operated channels
WHO	World Health Organisation
ZP	Zona pellucida

CHAPTER 1

Sperm interaction with the female reproductive tract

FOREWORD TO CHAPTER ONE

This chapter summarises current understanding of the role of the female reproductive tract during sperm transport and associated interactions. Specific emphasis is placed upon the structure, expression and adaptability of the female tract environment in modulating gamete physiology and gamete modulation of the tract. The role of calcium in regulating cellular function is briefly reviewed to place the research in context.

1.1 THE HUMAN SPERM CELL

1.1.1 The structure of the human sperm cell

The sperm cell is a terminally differentiated, highly specialised cell that can be compartmentalised into two major sections: head and tail (flagellum). The head consists of the acrosome, nucleus, cytoplasm and cytoskeletal structures (Sutovsky & Manandhar, 2006).

The acrosome contains proteases and receptors involved in sperm interaction with the zona pellucida (ZP) of the oocyte, which remain inactive until initiation of acrosomal exocytosis. Inner and outer acrosomal membranes provide structural support by surrounding the dense acrosomal matrix. The outer membrane is lost during acrosomal exocytosis whilst the inner membrane remains. The equatorial region (posterior of acrosome) comprises a folded complex of perinuclear theca believed to express receptors involved in initial binding to the ovum plasma membrane (Oko & Sutovsky, 2009). The post-acrosomal sheath of the sperm is believed to express signalling proteins that may contribute to oocyte activation and initiation of zygotic development (Sutovsky *et al*., 2003).

The nucleus contains highly condensed deoxyribonucleic acid (DNA) which is generally considered transcriptionally inactive until fertilisation when the nucleus decondenses forming a pronucleus. The nucleus is surrounded by the nuclear envelope and is protected by the perinuclear theca, a rigid shell composed of proteins rich in disulfide (SS) bonds (Oko, 1995).

The flagellum contains nine symmetrically arranged microtubule doublets connected to each other by dynein arms and to a central pair by radial spokes. This 9 + 2 arrangement of microtubules forms the sperm axoneme which is highly conserved across most mammalian

species (Turner, 2006). The outer doublets are paralleled by nine outer dense fibres (ODFs) that provide flexible supporting during movement. The ultrastructure of the flagellum can be divided into four sections which are proximally to distally: connecting piece; midpiece; principal piece and end piece (Fawcett, 1975; see figure 1.1).

The connecting piece joins the head with the flagellum and extends through the entire length of the flagellum. In most animals it is comprised of nine striated columns which encase the centriole, with rodents being an exception (Sutovsky & Manandhar, 2006). The mid-piece is surrounded by the mitochondrial sheath (MS) incorporating mitochondria in a helical pattern. These spiral sheaths of mitochondria supply energy for sperm metabolism and motility. The end of the mid-piece is marked by the annulus and cessation of the MS. The principal piece is covered by two longitudinal columns of fibrous sheath (FS). The FS is unique to the principal piece and provides additional support to the ODFs. The end piece is a short terminal portion of the flagellum containing only the axonemal doublets surrounded by the plasma membrane (Turner, 2006).

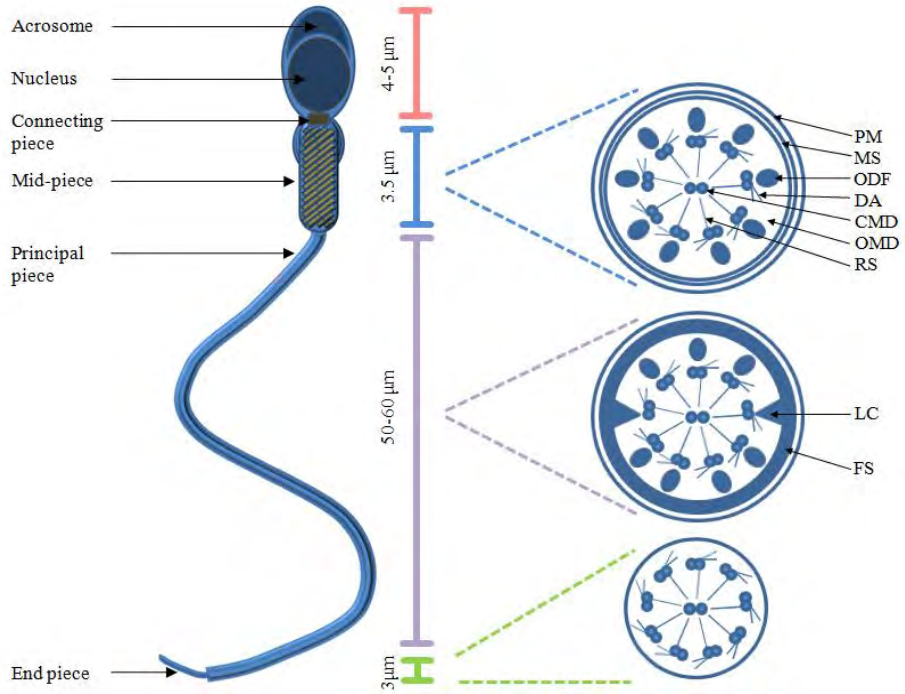


Figure 1.1. Sperm flagellum structure.

PM, Plasma membrane; **MS**, Mitochondrial sheath; **ODFs**, Outer dense fibres; **DA**, Dynein arms; **CMD**, Central microtubule doublets; **OMD**, Outer microtubule doublets; **RS**, Radial spoke; **LC**, Longitudinal columns; **FS**, Fibrous sheath. Figure has been adapted from Turner (2006).

1.1.2 Epididymal storage and transport

Sperm undergo maturation and transport within the epididymis, which in mammals can be divided into head (caput), body (corpus) and tail (cauda) regions.

Sperm transport from corpus to cauda is believed to occur as a result of spontaneous waves of contraction of the smooth muscle lining the wall of the epididymis (Harper, 1982). During epididymal transit, sperm are subjected to a sequentially changing environment where their function is modified as they undergo complex membrane remodelling and protein acquisition, reviewed by Cooper & Yeung (2006). Such properties acquired during maturation include progressive motility and the ability to fuse to and fertilise the oocyte (Bjorndahl *et al*., 2010).

In human, this process appears rapid (2-6 days) in comparison to rodents (10-13 days) (Cornwall, 2009).

The cauda region in many mammals acts as a site for sperm storage until ejaculation. A number of factors have been implicated in promoting prolonged storage including a low temperature, an androgen-dependent environment and associated local secretory products (Bjorndahl *et al*., 2010). The human epididymis in comparison to other non-human species does not present such clearly defined sections (Cooper & Yeung, 2006; Yeung *et al*., 1991). Most notably it lacks a pronounced cauda region that reduces its potential to act as a sperm reservoir (Bedford, 1994).

1.1.3 Ejaculation and seminal components

During ejaculation, sperm are transported from the distal cauda to the urethra where they become mixed with prostatic fluid and expelled. Subsequent expulsion of fluid from the seminal vesicles and bulbourethal glands form the later part the ejaculate (Bjorndahl *et al*., 2010; Mortimer, 1994). Collectively these fluids constitute seminal plasma and the suspension of sperm within seminal plasma is termed semen.

A comprehensive list of seminal plasma components has been reviewed by Owen & Katz, (2005). Each gland contributes specific factors that may support sperm functions. Seminal vesicles contribute fructose, ascorbic acid, prostaglandins as well as seminogelin I and II, the most abundant structural proteins in seminal plasma (Lilja, 1985). The prostate secretions are known to contain calcium (Ca^{2+}), zinc, magnesium, citric acid, acid phosphatase, inositol and the proteolytic enzyme prostate specific antigen (PSA). Human semen coagulates upon

ejaculation, forming a loose gel which is enzymatically degraded after approximately 30 minutes (Watt *et al*., 1986). A small contribution is made from epididymal fluid containing low levels of carnitine and inositol, and high levels of potassium (K^+). Semen also possesses a high buffering capacity compared to most other fluids in the body (Owen & Katz, 2005).

These components are incompletely mixed upon ejaculation, therefore semen is not a homogenous fluid (Bjorndahl *et al*., 2010).

1.2 REGULATION OF SPERM FUNCTION

1.2.1 Capacitation

A freshly ejaculated sperm cell must undergo a series of physiological changes to become functionally mature and capable of fertilising an ovum. This maturation process is termed „capacitation' and *in vivo*, is acquired during transport through the female reproductive tract (Austin, 1951; Chang, 1951). Events thought to play a role in inducing capacitation include sperm plasma membrane lipid rearrangements, ionic alterations of sperm membrane potential and increased tyrosine phosphorylation of sperm proteins (Salicioni *et al*., 2007).

Molecular basis of capacitation

The molecular basis of capacitation has been extensively studied however the process is still poorly understood. Recent reviews have highlighted current knowledge (Breitbart, 2003; De, 2005; de *et al*., 1997; Fraser, 2010; Salicioni *et al*., 2007; Shivaji *et al*., 2007) and are principally derived from *in vitro* studies, employing defined incubation conditions to initiate capacitation events. In the laboratory, capacitating media has been formulated in an attempt to

mimic oviductal fluid composition. This includes the addition of bicarbonate (HCO_3^-), Ca^{2+} and serum albumin at physiologically relevant concentrations (de Lamirande *et al*., 1997; Fraser, 2010). Collectively, these components modulate the induction of hyperactivated motility and receptiveness of sperm to undergo acrosomal exocytosis, necessary for acquisition of fertilising capacity (Boatman *et al*., 1991; Mariappa *et al*., 2010; Visconti & Kopf, 1998; Yanagimachi, 1994b).

Decapacitation factors

Decapacitation factors were first described by Bedford and Chang in 1962 when they observed that high speed centrifugation of seminal plasma resulted in loss of factors that regulate capacitation (Bedford & Chang, 1962; Fraser *et al*., 2003). Most decapacitation factors have been considered to originate in seminal plasma (De *et al*., 1997; Yanagimachi, 1994b). In humans, a number of small first messenger molecules present within seminal plasma have been suggested to regulate membrane adenylyl cyclase (mAC) function *in vivo* including adenosine, calcitonin and fertilisation promoting peptide (Fraser *et al*., 2003; Fraser *et al*., 2006). It is considered a prerequisite that decapacitation factors are removed from sperm for capacitation to proceed and for their intrinsic fertilising ability to be expressed (Mortimer, 1994).

1.2.2 Reactive oxygen species and Oxidative stress

Reactive oxygen species (ROS) describes a range of metabolites formed from the reduction of oxygen, producing both radical and non-radical oxygen derivates. ROS are essential for driving many biochemical pathways in sperm. However, excess production can result in oxidative stress, damage to cellular components, reduction in fertilising capability (Aitken &

Bennetts, 2006) and motility (Agarwal *et al*., 1994; Armstrong *et al*., 1999) and reduced genetic integrity (Aitken, 1999; Aitken & Clarkson, 1987).

Oxidative stress is recognised as a cause of male infertility and has been studied extensively over the last decade. Several factors have been implicated in promoting oxidative stress, particularly infections of the male and female genital tracts, presence of transition metals, poor sperm morphology and compromised antioxidative systems (Aitken & De Iuliis, 2010; Aziz *et al*., 2004; Ochsendorf, 1999; Potts & Pasqualotto, 2003; Saleh & Agarwal, 2002; Said *et al*., 2005). In addition, sperm are particularly vulnerable to oxidative stress through lipid peroxidation due to high poly-unsaturated fatty acids content of their plasma membrane (Jones *et al*., 1979) and their minimal free radical scavenging potential (Donnelly *et al*., 1999; Saleh & Agarwal, 2002). Therefore their regulation and consequent fate relies heavily on their surrounding environment.

Sperm may encounter high levels of ROS during almost every stage of their journey, from spermatogenesis and epididymal storage to transport through the female tract to the site of fertilisation. Redox regulation mechanisms within the male reproductive tract have been reviewed (Fujii *et al*., 2003) and may be of particular importance as sperm reside in the epididymis for a number of days when completing the first stages of their post-testicular maturation (Aitken & De Iuliis, 2010). Most human ejaculates are also likely to be contaminated with potential generators of ROS including immature germ cells, leukocytes and epithelial cells. These cells are derived principally from the prostate gland and the seminal vesicles (Wolff, 1995).

Defences against ROS

The antioxidative system comprises of enzymes and low molecular weight compounds such as vitamins. The system works by preventing or terminating ROS undergoing chain reactions. The reduction-oxidation (redox) system can detoxify oxidants whist also repairing oxidised molecules. It is believed that the redox system plays a key role in the regulation of signal transduction (Tanaka *et al*., 2000). ROS scavenging systems exist in both the male and female genital tracts (Fujii *et al*., 2003; Miranda-Vizuete *et al*., 2001; Sadek *et al*., 2001; Sadek *et al*., 2003; Vernet *et al*., 2004) and may play critical roles in regulating sperm functions.

Redox regulation of capacitation

At low concentrations, ROS can act as second messengers and promote capacitation events (Aitken, 1997; De and O'Flaherty 2008; Ford, 2004). ROS directly stimulate adenylyl cyclase (AC) activity (Aitken *et al*., 1998; Lewis & Aitken, 2001; Rivlin *et al*., 2004; Zhang & Zheng, 1996) and suppress tyrosine phosphatase enzymes through H_2O_2-induced oxidation of cysteine residues in the catalytic domain (Hecht & Zick, 1992). Nitric oxide (NO) is a free radical synthesised from L-arginine by nitric oxide synthase (NOS) enzymes (endothelial, neuronal and inducible types; eNOS, nNOS and iNOS) (Palmer *et al*., 1988). NO has also been implicated in promoting tyrosine phosphorylation through mechanisms involving or independent of the cyclic adenosine monophosphate (cAMP) protein kinase A (PKA) pathway (Herrero *et al*., 2003; O'Flaherty *et al*., 2006; Roy & Atreja, 2008). ROS including NO appear to play important roles in promoting capacitation.

1.2.3 Post-translational modification of sperm proteins

Despite some recent evidence for translocation of mRNAs in sperm (Gur & Breitbart, 2006; Gur & Breitbart, 2008), mature sperm are generally considered transcriptionally quiescent in comparison to somatic cells. Therefore sperm may be more reliant on post-translational modification of proteins to regulate their function. S-nitrosylation by NO is a regulated post-translational protein modification involving the covalent conversion of a thiol group from a cysteine residue to a S-nitrosothiol. This acts to control physiological cellular signalling in a similar way to phosphorylation and acetylation (Foster *et al*., 2003; Hess *et al*., 2005). S-nitrosylation has recently been implicated in targeting sperm proteins (Lefievre *et al*., 2007).

Oxidative stress can also lead to the induction of reactive carbonyl groups into specific amino acid side chains. This process termed „protein carbonylation', is thought to occur mainly in response to metal-catalysed formation of hydroxyl radicals leading to loss of protein structural integrity and function (Levine, 2002; Stadtman, 1992). Protein carbonylation is largely thought to be irreversible, „marking' proteins to be removed through proteasome-dependent degradation (Grune *et al*., 1997; Levine, 2002). Sperm are known to contain a highly active proteasome which plays a crucial role in capacitation and fertilisation events (Zimmerman & Sutovsky, 2009). Recently a number of studies have shown a correlation between protein carbonyl levels in seminal plasma and poor semen characteristics in human (Ahmad *et al*. 2009; El-Taieb *et al*. 2009; Saraniya *et al*. 2008). Further investigation is required to determine the physiological relevance of such processes and their impact on sperm functions.

1.2.4 Sperm motility

In mammals, successful fertilisation relies on the ability of ejaculated sperm to travel from the vagina to the ovulated oocyte in the oviduct. Sperm must then penetrate through the oocyte vestments in order to achieve fertilisation. Sperm motility is achieved through the specialised structure of the flagellum and is under the control of many extrinsic and intrinsic factors (Gagnon & de Lamirande, 2006). The mechanism and regulation of motility has been reviewed (Gagnon & de Lamirande, 2006; Turner, 2006).

Mechanism of flagellar propulsion

A number of ionic, metabolic and environmental factors have been identified in regulating flagellar activity and the energetics of the process have been reviewed (Ford, 2006).

Types of motility

Two forms of motility have been described in mammalian sperm characterised by differences in the amplitude of flagellar beat and trajectory pattern. Activated motility generates symmetrical, low amplitude flagellar beats resulting in relatively straight and progressive trajectory in moderately non-viscous media such as seminal plasma or saline (Katz & Vanagimachi, 1980; Suarez, 2008a; Turner, 2006). This type of motility has been suggested to aid in the initial stages of sperm transport through the female reproductive tract (Gaddum-Rosse, 1981). During transit through the female tract, motility switches to a "hyperactivated" form. This is characterised by asymmetrical, high amplitude flagellar beats and when observed in saline demonstrates a circular or star shaped trajectory (Ishijima *et al*., 2002; Yanagimachi, 1970; Yanagimachi, 1994b). However, when placed in a viscous environment, hyperactivated sperm demonstrate progressive movements (Smith *et al*., 2009b; Suarez &

Dai, 1992). This is thought to aid sperm transport through the female reproductive tract and interaction with the oocyte during fertilisation (Ho & Suarez, 2001). Hyperactivation involves complex ionic changes and although considered a marker of capacitation, it is important to note that these pathways can also occur independently (Gagnon & de Lamirande, 2006; Marquez & Suarez, 2004; Olds-Clarke, 1989).

Signalling and Regulation of motility

A number of factors appear to be involved in the regulation of sperm motility, with HCO_3^-, cAMP, Ca^{2+} and PKA thought to be central (Gagnon & de Lamirande, 2006).

HCO_3^-, cAMP and enzymatic signalling components

The concentration of HCO_3^- varies throughout both the male and female reproductive tracts. The role of HCO_3^- in stimulating motility is well documented in many mammalian species (Calson *et al*., 2007; Holt *et al*., 2002; Luconi *et al*., 2005; Wuttke *et al*., 2001) and its presence within seminal plasma plays an essential role in initiation of sperm motility (Okamura *et al*. 1985; Okamura *et al*. 1986). At the biochemical level, HCO_3^- directly activates soluble adenylyl cyclase (sAC) and raises the intracellular pH (Jaiswal & Conti, 2001; Wennemuth *et al*., 2003b) leading to an increase in cAMP production and respiratory activity (Gagnon & de Lamirande, 2006). The increasing availability of cAMP contributes towards increasing PKA activity which in turn signals through multiple pathways to regulate flagellar function (Holt & Harrison, 2002; Nolan *et al*., 2004; San Agustin & Witman, 1994). The targets of PKA phosphorylation remain largely unidentified however have been suggested to involve tyrosine kinase or kinases with targets located primarily in the flagellum (Gagnon & de Lamirande, 2006; Leclerc *et al*., 1996; Tash, 1989; Tash & Bracho, 1998;

Turner, 2003). Potential targets of tyrosine phosphorylation include the A-Kinase Anchoring Protein (AKAP) family of proteins found in the fibrous sheath of the flagellum (Luconi *et al*., 2005). The AKAP proteins are responsible for targeting PKA and other proteins to specific subcellular locations (Scott *et al*., 2000). The scaffolding of PKA to AKAP proteins has been shown to be important in initiating motility, as motility is blocked by synthetic peptides that prevent binding between AKAP proteins and the regulatory subunits of PKA (Vijayaraghavan *et al*., 1997).

Further evidence supporting cAMP-dependent PKA activity in the regulation of sperm motility has been demonstrated through the use of a PKA inhibitor, H89, and protein phosphatase inhibitors, calyculin and okadaic acid, which reduce and enhance motility characteristics, respectively (Gagnon & de Lamirande, 2006; Leclerc *et al*., 1996). In mice, targeted deletion of the PKA catalytic subunit Calpha resulted in severe motility defects (Skalhegg *et al*., 2002).

In addition to cAMP-dependent PKA activity, sperm motility may be influenced by a number of kinase signalling pathways. Extracellular signal-regulated kinase (ERK) and protein kinase C (PKC) are present within mammalian sperm including human (De Lamirande & Gagnon, 2002) and have both been associated with modulating motility (Gagnon & de Lamirande, 2006). Ras, a small GTP-binding protein found on the sperm acrosomal cap (in humans) and the flagellum (in hamster) could be involved in initiating the ERK pathway, PKC and PI3K (NagDas *et al*., 2002). Rho, another small GTP-binding protein found in bovine and murine flagellar may also play a role in driving motility (Hinsch *et al*., 1993). Ropporin, a sperm-specific binding protein of rhophilin localised in the fibrous sheath shares high homology

with the regulatory subunit type II of PKA (Fujita *et al*., 2000). Therefore, the interaction between multiple signalling pathways is likely to be important in various stages of motility acquisition.

Ca^{2+} involvement in regulating sperm motility

Ca^{2+} appears to play a complex role in regulating sperm motility (see Darszon *et al*., 2006; Publicover *et al*., 2007). There is evidence to suggest that presence of extracellular Ca^{2+} is required for HCO_3^- induced activation of sAC (Carlson *et al*., 2007) and initial cAMP generation. Ca^{2+} can stimulate cAMP production by direct activation of sAC (Jaiswal & Conti, 2003; Litvin *et al*., 2003). In turn, cAMP can stimulate further Ca^{2+} influx (Xia *et al*., 2007) suggesting that both Ca^{2+} and cAMP could function synergistically through positive feedback. Mice lacking sAC display infertility caused by severe sperm motility defects (Esposito *et al*., 2004), highlighting the importance of HCO_3^- signalling mediated through the cAMP pathway.

Ca^{2+} has been shown to be responsible for the flagellar asymmetry characteristic of hyperactivated motility (Ho & Suarez, 2001). Both cAMP and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) continue to increase during sperm capacitation conditions until a poorly defined point where there is a switch to hyperactivated motility. It is Ca^{2+} rather than cAMP that appears to act as the main second messenger triggering hyperactivation (Suarez, 2008a). The addition of Ca^{2+} to demembranated sperm has been shown to directly induce hyperactivated motility indicating that Ca^{2+} acts directly on cytoskeletal elements to promote flagellar wave asymmetry (Ho *et al*., 2002).

The importance of Ca^{2+} in hyperactivation is shown by a sperm-specific Ca^{2+} permeable ion channel, known as CatSper. CatSper channels are located on the principal piece of the flagellum and are known to be both pH-sensitive and voltage dependant. There are currently four known CatSper proteins that form the channel and all four are required for sperm to undergo hyperactivation (Carlson *et al*., 2003; Ji *et al*., 2007*;* Liu *et al*., 2007; Qi *et al*., 2007; Quill *et al*., 2003; Ren *et al*., 2001; Xia *et al*., 2007).

High concentrations of Ca^{2+} have been reported to inhibit motility (Tash & Means, 1982). This is thought to be a result of protein dephosphorylation mediated by calmodulin (CaM) and the Ca^{2+}/CaM-dependent phosphatase, calcineurin (Tash *et al*. 1988; Tash & Means, 1987).

The influence of ovarian hormones on motility

Sex hormones such as oestrogen and progesterone potentially affect sperm motility. Progesterone increases $[Ca^{2+}]_i$ and may enhance sperm motility (Kirkman-Brown *et al*., 2000; Harper *et al*., 2004). Less is known about the effects of oestrogen on motility however it has been shown to signal through rapid non-genomic cascades and increase sperm motility *in vitro* (Giretti & Simoncini, 2008; Idaomar *et al*., 1987; Idaomar *et al*., 1989). Evidence of directed motility in a chemical gradient (chemotaxis) is a controversial (in human) and active research area that is beyond the scope of this thesis. Chemotaxis has been covered in recent reviews which discuss the potential influence of ovarian hormones, particularly progesterone (Kaupp *et al*., 2008).

1.3 THE FEMALE REPRODUCTIVE TRACT

1.3.1 The human female reproductive cycle

Following menarche, the female reproductive tract undergoes remodelling in preparation for fertilisation of the oocyte. In the absence of fertilisation, hormone levels rise and fall in a characteristic pattern approximately every 28 days (Sanders & Debuse, 2003). These fluctuations and the associated changes they cause form the menstrual cycle (see figure 1.2).

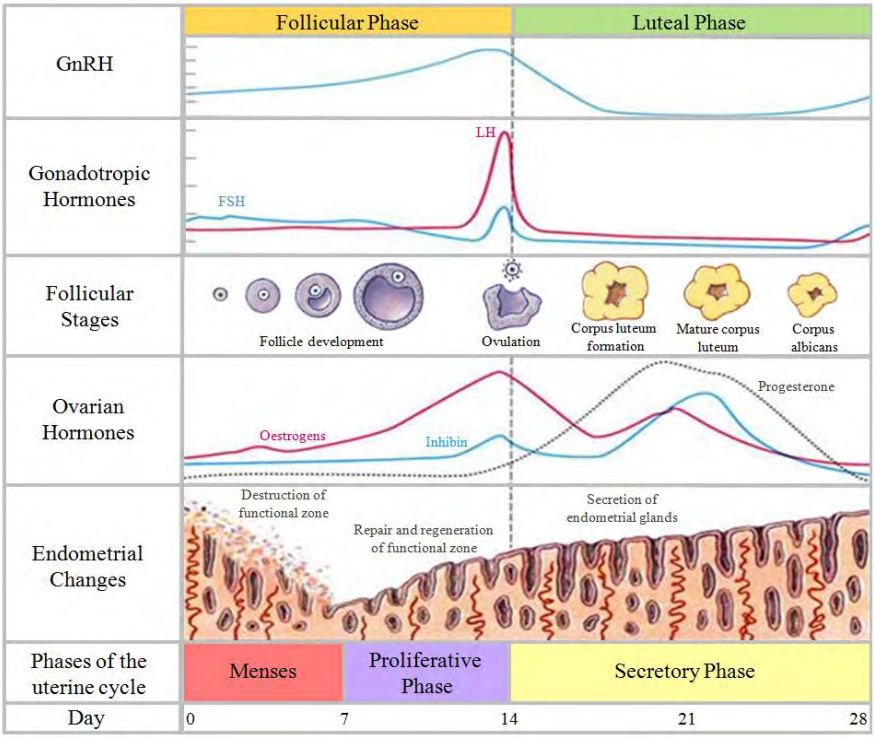


Figure 1.2. The human female reproductive cycle.

Image has been modified from Cummings (2009).

Ovarian hormones & regulation of synthesis

Ovarian hormone production is regulated by the coordination of several elements: hypothalamic control of pituitary function, ovarian follicular and luteal changes and positive

and negative feedback of ovarian hormones at the hypothalamic-pituitary axis (Rhoades & Bell, 2009). Gonadotrophin releasing hormone (GnRH) is synthesised by the hypothalamus and acts on the anterior pituitary gland to stimulate the release of gonadotrophins; luteinising hormone (LH) and follicle stimulating hormone (FSH). Gonadotrophins stimulate the ovaries to produce and secrete ovarian steroids (Sanders & Debuse, 2003). The main ovarian steroids are oestrogens, progestogens and androgens. The relative ratios of these hormones and their regulation during the ovarian cycle greatly influence the physiology of the female reproductive tract. The ovarian cycle is divided into two main stages; the follicular phase and the luteal phase, each lasting about 14 days. At around day 14, ovulation occurs (Sanders & Debuse, 2003; see figure 1.2).

1.3.2 Phases of the menstrual cycle

The follicular phase (days 1-14)

During the follicular phase, the dominant ovarian hormone is oestrogen.

Periovulatory phase (days 13 to 15)

By the end of the follicular stage, the follicle has matured into a late tertiary or „Graafian' follicle and secretes large quantities of oestrogen that feedback to the pituitary gland. The combination of high oestrogen levels (~200 pg/mL) for sufficient duration (36-48 hours) results in a surge of LH via positive feedback to the hypothalamic-pituitary axis (Johnson & Everitt, 2000). The exact mechanism behind this negative to positive switch of oestrogen is unknown (Rhoades & Bell, 2009). LH causes the Graafian follicle to rupture (ovulate) through the germinal epithelium. The oocyte is released into the peritoneal cavity and is collected into the oviducts by fimbriae. At this stage the oocyte is surrounded by ZP and

granulosa cells. The prolonged exposure to high LH levels during the surge down-regulates the ovarian LH receptors, resulting in postovulatory suppression of oestrogen synthesis (Sanders & Debuse, 2003; Rhoades & Bell, 2009).

Luteal phase (days 14-28)

During the luteal phase progesterone acts as the dominant ovarian hormone. During menstruation, oestrogen and progesterone levels are low due to regression of the corpus luteum and plasma FSH levels rise to initiate a new cycle (Adashi *et al*., 1996).

1.3.3 Variation in ovarian cycle across mammalian species

All mammalian species regulate ovarian function through the gonadotrophins, LH and FSH, similar to human. However, some species rely more heavily on genitalia-central nervous system communication to regulate ovulation and length of the luteal phase (see table 1.1) (Johnson & Everitt, 2000).

In the ovarian cycle of the cow, sow, ewe and mare, LH and FSH do not fall to negligible levels during the luteal phase as in humans. This results in significant antral expansion during the luteal phase of the previous crop of follicles. As a result the follicular phase is significantly shorter than in humans lasting 1-6 days (Johnson & Everitt, 2000). For both rat and mouse species, the cycle length depends on whether or not the female has mated. In the case of mating at the time of ovulation, the luteal phase lasts 11-12 days. However, in the absence of mating at the time of ovulation, the luteal phase is only 2-3 days long. Mechanical stimulation of the cervix by the penis at coitus has been suggested to activate sensory neurons in the cervix which feedback to the central nervous system inducing prolactin release from the

pituitary gland. In these species, prolactin is an essential part of the luteotrophic complex and without it the luteal phase is drastically shortened (Johnson & Everitt, 2000). In rabbits there is little evidence of a cycle. In the absence of mating, blood oestrogens are high and progesterone levels are low indicating a continuous follicular phase. As with the rat and mouse, mating results in mechanical stimulation of the cervix. In rabbits this induces ovulation approximately 10-12 hours post-coitus and a luteal phase of approximately 12 days (Johnson & Everitt, 2000).

Species	**Length of cycle (days)**	**Follicular phase (days)**	**Luteal phase (days)**
Human	24-32	10-14	12-15
Cow	20-21	2-3	18-19
Pig	19-21	5-6	15-17
Sheep	16-17	1-2	14-15
Horse	20-22	5-6	15-16
Rabbit	1-2	1-2	0
Rabbit* (+ infertile male)	14-15	1-2	13
Mouse/rat	4-5	2	2-3
Mouse/rat* (+ infertile male)	13-14	2	11-12

Table 1.1. Duration of ovarian cycles across mammalian species

* indicates fertile females mated with an infertile male. Data was taken from Johnson & Everitt (2000).

1.3.4 Embryology, anatomy and physiology of the female reproductive tract

The early mammalian embryo possesses both the Wolffian and Müllerian genital ducts. During sexual differentiation, in the absence of male hormones, the Müllerian ducts will develop into structures of the female reproductive tract including the oviducts, uterus, cervix and upper portion of the vagina. In the presence of male hormones, the Wolffian ducts differentiate into structures of the male reproductive tract, such as the epididymides, vas deferentia and seminal vesicles (Kobayashi & Behringer, 2003). Distinct changes in the morphology of the female reproductive tract have occurred during evolution in mammalian species (figure 1.3). This variation is largely due to the extent of fusion of the anterior ends of the Müllerian ducts. The formation of two uteri (duplex) occurs when fusion is absent or limited such as with marsupials and monotremes. The Müllerian ducts of higher primates, including humans, fuse to a greater extent, resulting in the formation of a single (simplex) uterus with a single cervix and vagina (Kobayashi & Behringer, 2003). These differences in gross anatomy along with differing sites of semen deposition should be considered when interpreting data and functions across different species.

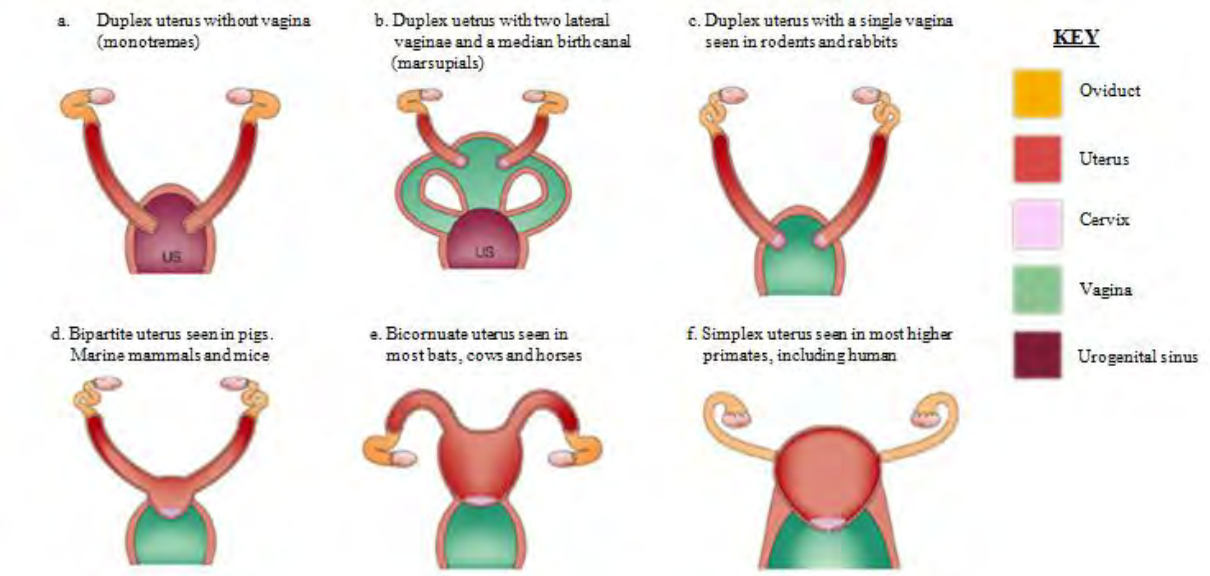


Figure 1.3. Mammalian variation of the female reproductive tract

(**a,b**) Absent or limited fusion of the Müllerian ducts presents two uteri. The urogenital sinus (US) is connected to the female reproductive tract; (**c**) the duplex uterus has a pair of cervices; (**d**) in the duplex bipartite uterus, as seen in many mammalian species, Müllerian fusion is absent resulting in the formation of paired uterine horns that can support multiple foetuses per pregnancy; (**e**) a larger portion of the uterus forms the uterine body; (**f**) Müllerian ducts fuse anteriorly resulting in a single uterine body that can support the development of a small number of foetuses per pregnancy. Figure and legend has been adapted from Feldhamer *et al*. (2003) and Kobayashi & Behringer (2003).

1.3.5 Anatomy of the human female reproductive tract

The human female reproductive tract consists of five main components: ovaries, oviducts, uterus, cervix, and vagina (see figure 1.4).

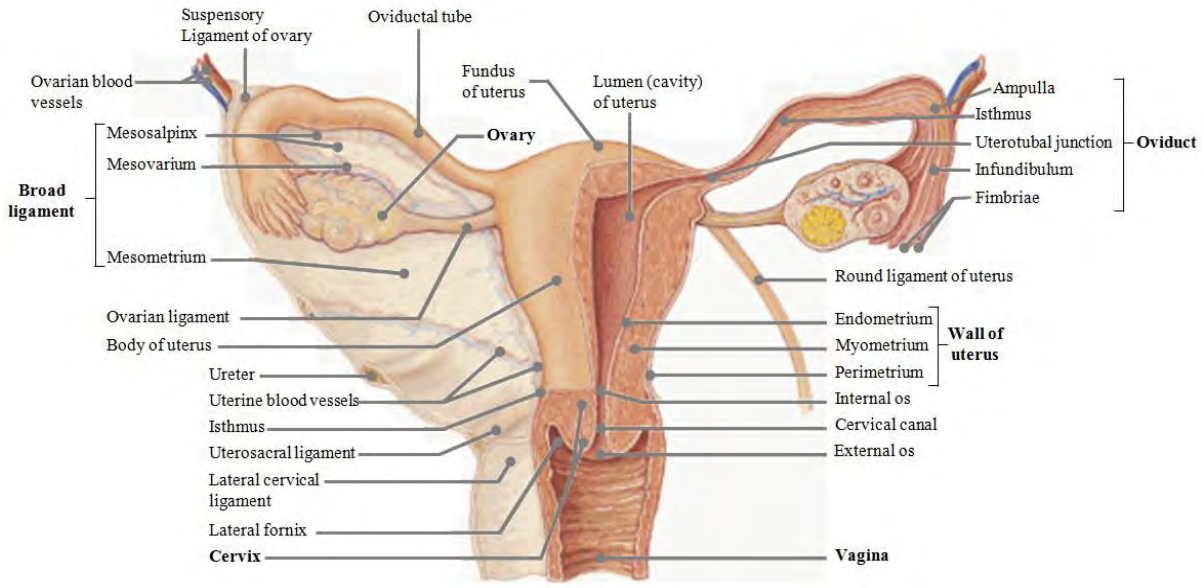


Figure 1.4. The human female reproductive tract

Image has been modified from Cummings (2001).

Vagina

The vagina is a 7-9 cm long musculomembraneous tube running from the external genitalia (vulva) to the cervix of the uterus. It is indented superiorly by the cervix forming a recess around the cervix known as the vaginal fornix (Moore *et al*., 2006). The vagina is a non-sterile area and relies on lactic acid, produced by glycogen digestion of the bacteria *Lactobacilli vaginalis*, to lower the pH to < 5 offering protection against infection (Suarez & Pacey, 2006; Ferris *et al*., 2006).

The cervix

The cervix is the narrow inferior third of the uterus which in a non-gravid adult, is approximately 2.5cm long (Moore *et al*., 2006). It consists of two main parts: the ectocervix and endocervix. The ectocervix is the area visible in the vagina (external os) consisting of non-keratinizing stratified squamous epithelium. The endocervix is the canal opening into the uterine cavity (internal os) covered by mucin-secreting, simple columnar epithelium (Adashi *et al*., 1996). Numerous glands of the endocervix produce mucus, the consistency of which is highly influenced by sex hormones (Gipson, 2001). The endocervix also contains higher numbers of immunoglobulin-containing and secreting cells than the ectocervix, oviducts, and vagina (Crowley-Nowick *et al*., 1995; Kutteh *et al*., 1988; Mestecky & Fultz, 1999) suggesting enhanced immunity. The cervix and superior structures are sterile areas. This sterility is maintained by the frequent shedding of the endometrium, thick cervical mucus and the narrow external os of the cervix (Sanders & Debuse, 2003).

Changes in cervical mucus during the ovarian cycle

The primary role of the cervix is to produce cervical mucus, a process regulated by ovarian hormones (Adashi *et al*. 1996; Katz *et al*., 1997). During the follicular phase, oestrogen stimulates secretion of clear, serous cervical mucus. During the postovulatory period, progesterone induces the formation of thick viscous cervical mucus (Rhoades & Bell, 2009).

The uterus

The uterus is a muscular organ with a trilaminar wall consisting of the perimetrium, myometrium and endometrium (Moore *et al*., 2006). The perimetrium consists of the peritoneum supported by a thin layer of connective tissue. The myometrium is a thick layer of

hormonally stimulated muscle which is richly innervated with nerves and blood vessels (Moore *et al*., 2006). The endometrium is the inner epithelial lining providing an environment in which the conceptus can be nurtured from the preimplantation stage until birth (Findlay, 1994). The endometrium can be further divided into the superficial functional layer and the deep basal layer. The superficial functional layer consists of luminal and glandular epithelial cells, with the luminal cells forming a continuum with the epithelial cells of the glands. The deep basal layer consists of stroma and is permeated by spiral arteries (Findlay, 1994). The endometrial stroma is composed mainly of fibroblasts but both regional and cyclical differences in cells exist (Blaustein, 1982). A wide variety of lymphoid cells have also been found within the endometrial stroma (Tabibzadeh *et al*., 1986).

1.3.6 Changes in the endometrium during the ovarian cycle

The endometrial lining undergoes cyclic alteration in response to changing levels of ovarian hormones, most notably in the functional layer (Sanders & Debuse, 2003). Under the influence of increasing levels of oestrogens, the stromal and epithelial layers undergo hyperplasia and hypertrophy and spiral arteries develop increasing vascularisation (Findlay, 1994). Oestrogen upregulates progesterone receptor expression and increases myometrial excitability and contractility (Mesiano & Welsh, 2007). Progesterone increases the secretory activity of the endometrium, promoting secretion of large amounts of carbohydrate-rich mucus (Findlay, 1994). The peak of secretory activity and vascularisation is seen 6-8 days after ovulation in preparation for blastocyst implantation (Adashi *et al*., 1996). The declining levels of progesterone and oestrogen result in an ischemic phase in which the functional layer of the endometrium undergoes necrotic changes and is shed during the menstrual phase (menses) (Findlay, 1994).

The Endometrial Proteome

A number of studies have characterised proteins from the endometrium and identified a number of endometrium-specific proteins such as a sialic acid binding protein (Sen *et al*., 2001), glycodelin glycoforms (Vigne *et al*., 2001) and insulin-like growth factor binding proteins (Seppala *et al*., 1992; Vigne *et al*., 2001). Proteins from samples of endometrial fluid, representing endometrial function have also been indentified (Casado-Vela *et al*., 2009). To date, a considerable amount of knowledge has been gained on phase-specific transcriptomes of human endometrium during the menstrual cycle (Van Vaerenbergh *et al*., 2010). A recent study using human endometrium in the proliferative and secretory phases identified 194 proteins including structural proteins, molecular chaperones and proteins related to metabolism and immunity. 57 of these showed differential expression in the proliferative and secretory phases (Rai *et al*., 2010). The human endometrium also shows alteration in protein expression during the window of implantation (5-9 days post-fertilisation). In particular, integrins and other attachment molecules appear to be important in mediating multiple aspects of embryo attachment and implantation (Findlay, 1994). A recent study suggested roles for annexin A2 and stathmin 1 (Dominguez *et al*., 2009). Proteomic profiling of the endometrial and oviductal surface is likely to be important in understanding the function and potential interactions between gametes and the female reproductive tract. The cilium alone comprises at least 250 distinct proteins (Marshall & Nonaka, 2006) which emphasises the challenge.

The Oviducts

The oviducts are paired, seromuscular organs connecting the ovaries to the uterus (Grudzinskas *et al*., 1994). The oviducts play a critical role in natural conception, functioning as the site of gamete interaction, transport and fertilisation. The oviductal cilia and

musculature are considered important in this respect, allowing gametes to travel in opposite directions and embryos to approach the uterus at the correct stage of development (Halbert *et al*., 1976; Mahmood *et al*., 1998). The tubes extend laterally from the body of the uterus and open into the peritoneal cavity adjacent to the ovaries. Each tube is approximately 11 cm long and are often asymmetrically arranged lying either superior or posterior to the uterus (Moore *et al*., 2006; Pauerstein & Eddy, 1979).

The oviducts have four distinguishable sections; the infundibulum, ampulla, isthmus and utero-tubal junction (UTJ) (Grudzinskas *et al*., 1994). The infundibulum is the funnel shaped distal end of the tube which opens into the peritoneal cavity. The surface area of the infundibulum is enhanced by irregular projections termed fimbriae. The infundibulum opens into the ampulla which is the widest and longest part of the tube (Sanders & Debuse, 2003). The ampulla then merges into the isthmus at the ampullary isthmic junction (AIJ) where the most significant luminal diameter change is seen (Halbert *et al*., 1988). The isthmus then opens into the uterine cavity at the UTJ (Grudzinskas *et al*., 1994).

There are distinct differences in the microanatomy and physiology of the oviductal sections (Grudzinskas *et al*., 1994). The most obvious anatomical difference relates to the degree of invagination of the epithelium, with increased size, number and complexity of epithelial folds in the infundibulum and ampulla region compared to isthmus (see figure 1.5). The isthmus region also has a highly innervated thick muscular layer in contrast to the ampulla where the muscular layer is thin and contains few nerve fibres (Helm *et al*., 1982a).

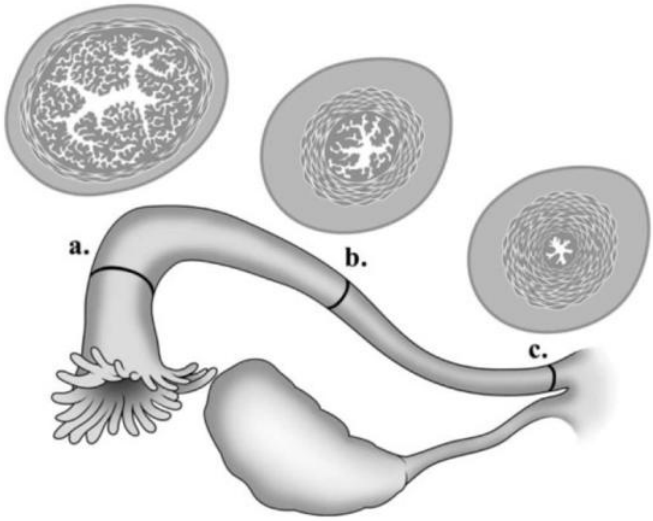


Figure 1.5. Illustration of the human oviduct

Illustration demonstrates the longitudinal folds in cross-section at (**a**) infundibulum; (**b**) ampulla; and (**c**) isthmus. Figure reproduced from Lyons *et al*. (2006).

Oviduct structure

The tubal wall consists of three layers: an internal mucosal layer (oviductal epithelium/endosalpinx), an intermediate muscular layer (myosalpinx) and an outer serosal layer.

Oviductal epithelium (endosalpinx)

The oviductal epithelium is convoluted in structure, forming folds (plicae) longitudinally around the lumen. Each fold consists of a single columnar epithelial layer on a basement membrane, rounded by a stratum of connective tissue. Beneath this lies the lamina propria containing blood and lymphatic vessels and the myosalpinx (Grudzinskas *et al*., 1994). The oviductal epithelium has a rich microvasculature allowing rapid and efficient exchange of nutrients, growth factors, gases and other communicative molecules with the intra-tubal environment (Findlay, 1994). The oviductal epithelium mainly consists of secretory cells and

columnar ciliated cells (Grudzinskas *et al*., 1994; Pauerstein & Eddy, 1979). The specific roles of ciliated and secretory epithelial cells in the oviduct remain to be fully elucidated.

Secretory cells are distributed throughout the length of the oviduct. They have a microvillar apical-surface and in contrast to ciliated cells, contain abundant amounts of rough endoplasmic reticulum, supranuclear Golgi zones and various secretory granules of differing structure (Findlay, 1994). Secretory cells have been reported to express a primary cilium considered to be a specialised cellular compartment or organelle, capable of sensory activity (Hagiwara *et al*., 2008a). The primary cilium is attached to the distal end of the basal body beneath the cell and has several striated rootlet fibres extending from the basal body forming close associations with membranous structures such as the Golgi apparatus (Hagiwara *et al*., 2008a; Singla & Reiter, 2006).

Columnar ciliated cells are most commonly found on the apices of epithelial folds (Fredericks, 1986). In species with a prolonged follicular phase such as humans, differentiation and ciliation are induced by oestrogen and attenuated by progesterone (Jansen, 1984; Verhage *et al*., 1979). Ciliated cells usually have microvilli present on their luminal surface with elongated nuclei located medially or basally (Findlay, 1994). Motile cilia are approximately 10 μM long and 0.25 μM in diameter (Satir, 1992) consisting of the 9 + 2 arrangement of microtubules (Lyons *et al*., 2006). Cilia activity is regulated by a variety of hormonal and neuronal mechanisms through Ca^{2+}-dependant hydrolysis of adenosine triphosphate (ATP) (Lyons *et al*., 2006).

The myosalpinx

Contractions of the tubal musculature may play a role in the transport of gametes (Mastroianni, 1999) but this has not yet been proven. A comprehensive structural assessment of the myosalpinx has been performed using scanning electron microscopy, revealing that the arrangement of smooth muscle cells (SMC) varies within mammalian species possibly reflecting different mating strategies (Muglia & Motta, 2001). These studies show that the myoarchitecture underlying the UTJ, isthmus and ampulla in human all demonstrate a plexiform arrangement of SMC suggesting that tubal contractility generates irregular waves of contraction capable of „stirring' movements rather than constriction and propulsion. This may serve to distribute solutes and gametes to promote successful fertilisation rather than restricting sperm transport (Muglia & Motta, 2001). A recent study identified that stromal cells termed myofibroblasts, could generate contractile movements of the mucosa (Hagiwara *et al*., 2008b). A subsequent study identified these cells as interstitial Cajal-like cells (ICLC) present in both the uterus and isthmic region of the oviduct underlying the lamina propria and musculature (Cretoiu *et al*., 2009). ICLC are also known as „gut pacemakers' that promote slow waves of depolarisation and have been found to express oestrogen receptor alpha (ER-α) and progesterone receptor A (Cretoiu *et al*., 2009). It is therefore possible that these cells contribute to tubal contractility through extracellular matrix communication with epithelial cells.

The regulation of muscular activity is influenced by adrenergic activity (Helm *et al*., 1982b), NO (Ekerhovd *et al*., 1997; Ekerhovd *et al*., 1999), oxytocin (Jankovic *et al*., 2001) , prostaglandins (Lindblom *et al*., 1979; Lindblom *et al*., 1983) and sex steroids (Helm *et al*., 1982b). Progesterone is known to exert an inhibitory action on contraction which correlates

with reduced frequency of contractions observed in the luteal phase (Lindblom *et al*., 1980; Mastroianni, 1999).

1.3.7 Changes in the oviductal epithelium during the ovarian cycle

Changes in tubal morphology and function in response to ovarian hormones are more discrete than those seen in the endometrium. Overall, the morphological and ultrastructural changes appear more pronounced in secretory cells in comparison to ciliated cells and reflect secretory activity (Fredericks, 1986). During menstruation, epithelial thickness is uniformly low, secretory cells are less active and cilia on ciliated cells appear "droopy" (Grudzinskas *et al*., 1994). During the follicular phase, increasing levels of oestrogen promote differentiation of ciliated cells most notably in the ampulla and infundibulum and cilia appear to become more erect (Patek *et al*., 1972). An increase in cell height and size has been reported for both secretory and ciliated cells reaching a maximum height of approximately 30μM, during the late follicular phase (Verhage *et al*., 1979). In secretory cells, an increase in endoplasmic reticulum activity and protein production are observed with secretory granules appearing below the cell apical membrane adjacent to the lumen. They become covered with numerous microvilli and contain apical projections at the luminal surface capable of apocrine secretion (Crow *et al*., 1994; Jansen, 1984). Secretory material accumulates and is then released into the lumen (Fredericks, 1986). There is evidence that oestrogen mediates production of oviductal-specific glycoproteins (OGPs) in secretory cells which may have important modulating affects on gametes and embryotrophic function (Adashi *et al*., 1996; Buhi *et al*., 2000). The secretory activity has been reported to be more prominent in the isthmus than in the ampulla region (Grudzinskas *et al*., 1994) with a viscous mucus observed around the time of ovulation (Jansen, 1980).

Cilia beat frequency (CBF) is reported to be highest during the periovulatory period suggesting that it is influenced by ovarian steroid hormones and prostaglandins (Critoph & Dennis, 1977; Lyons *et al*., 2002; Seibel *et al*., 1984). Although oestrogen alone does not appear to influence CBF, progesterone has been shown to reduce it by 40% (Mahmood *et al*., 1998). Furthermore, Mahmood *et al*. (1998) demonstrated that oestrogen could prevent the reduction in CBF induced by progesterone (Mahmood *et al*., 1998). Therefore the increased CBF observed during the periovulatory phase could be partly due to the relatively low ratio of progesterone to oestrogen. Prostaglandins PGE2 and F2α both stimulate CBF in the oviduct and this is believed to be regulated through prostaglandin-induced release of Ca^{2+} (Lyons *et al*., 2006; Verdugo *et al*., 1980). The physiological roles of cilia include facilitating the collection and transport of the ovum and the mixing and distribution of tubal fluid. However, the exact role of cilia activity in gamete and embryo transport remains unclear (Lyons *et al*., 2006).

In human, deciliation and atrophy of cells appears to occur during the late-luteal phase under the dominance of progesterone. Regeneration then occurs during the follicular phase of the next cycle (Adashi *et al*., 1996; Verhage *et al*., 1979).

Surface proteome of Oviductal Epithelial Cells

Oviductal epithelial cells (OECs) are in direct contact with gametes and embryos giving them the potential to mediate important events including transport and final maturation of gametes, fertilisation and embryonic development (Sostaric *et al*., 2006). Therefore, it is of interest to determine the oviductal surface proteome to discover candidates that may mediate such interactions.

Recently, progress has been made in profiling proteins on the surface plasma membrane of porcine OECs using biotin tagging (Sostaric *et al*., 2006). In this study, 40 proteins were successfully identified, many of which have been previously reported to be present in the oviduct. These include proteins associated with cell adhesion and receptor action, chaperones and a high level of heat shock proteins (HSPs). HSP and chaperone protein expression increases when cells are exposed to stress (De Maio, 1999).

The oviductal cell proteome has also been shown to change during the reproductive cycle, with distinct differences seen in the follicular and luteal phases (Seytanoglu *et al*., 2008). This change is likely to be mediated through progesterone and oestrogen receptors, which are both highly expressed in OECs (Adashi *et al*., 1996; Amso *et al*., 1994; Conneely *et al*., 2003). More recently, the human oviduct has been shown to express membrane projections termed „pinopodes', previously associated with endometrial receptivity (Makrigiannakis *et al*., 2009). Pinopodes have been associated with upregulation of integrins and alpha v beta 3, a receptor for fibronectin.

1.3.8 Oviductal fluid secretion

Oviductal (tubal) fluid composition

The composition of oviductal fluid has been extensively reviewed (Aguilar & Reyley, 2005; Leese *et al*., 2008). Oviductal fluid is rich in a wide variety of energy substrates including pyruvate, lactate, glucose and glycogen (Grudzinskas *et al*., 1994; Tay *et al*., 1997). It is also particularly high in K^+ and HCO_3^- in comparison to plasma (Leese & Gray, 1985; Leese *et al*., 2001). The ionic components as well as albumin and immunoglobulins are thought to originate from blood (Parr & Parr, 1986).

The protein concentration in oviductal fluid has been reported to range from 1.0-18.0 mg/ml in humans (David *et al*., 1973) and contain a number of OGPs (Buhi *et al*., 2000; Killian, 2004). In human, arginine, alanine and glutamate represent the highest concentrations of amino acids (Tay *et al*., 1997). In addition, oviductal fluid contains high levels of ovarian hormones, prostaglandins and growth factors (Aguilar & Reyley, 2005). These components are important in gamete and preimplantation embryo metabolism and function (Grudzinskas *et al*., 1994; Hardy *et al*., 1989).

Formation of fluid

Oviductal fluid is formed by selective transudation from blood and active secretion through the epithelial lining (Leese *et al*., 2001). Secretion is regulated through ion movements in secretory cells of the oviduct. Localised accumulation of ions in intracellular spaces is believed to create areas of high osmotic pressure (Leese *et al*., 2001). To achieve osmotic equilibrium, water follows the movement of ions leading to accumulation in the lumen (Diamond, 1971). Oestrogen stimulates oviductal fluid secretion whilst progesterone inhibits secretion (Grudzinskas *et al*., 1994). This correlates to reports that the fluid is abundant in the mid-cycle phase when gametes and/or embryos may be present.

In addition to oestrogens, a number of other factors have been shown to mediate fluid secretion including adrenergic agents (Tay *et al*., 1997), cAMP (Mahmood *et al*., 2001) and purinergic agents such as ATP (Downing *et al*., 1997; Leese *et al*., 2001;). Ca^{2+} is also known to mediate fluid secretion in a wide variety of cells types (Berridge, 1993; Villereal & Byron, 1992). It has been hypothesised that the movement of Ca^{2+} across the membrane alters the membrane potential across the cell through Ca^{2+}-dependent basolateral K^{+} channels (Dickens

et al., 1996; Leese *et al*., 2001) and associated chloride (Cl^-) flux (Reischl *et al*., 2000), leading to an increase in fluid secretion.

Regional variation in oviductal fluid

A few studies have reported regional differences in protein secretion along the oviduct perhaps reflecting functional differences (Abe, 1996; Buhi, 2002). A difference in oestrogen-specific glycoprotein secretion in ampullary and isthmic oviductal regions has been suggested in cow (Buhi *et al*., 1990; Wegner & Killian, 1992). This is important to consider as a dynamic change in the composition of oviductal fluid could influence modulation of gametes within the oviduct (Aviles *et al*., 2010). However, when this was studied in human, no differences were seen between the ampullary and isthmic regions of the oviduct (Quintero *et al*., 2005).

The Ovary and Ovarian Venous to Ovarian Artery counter-current exchange

The ovaries function as endocrine glands producing oocytes and steroid hormones in response to pituitary gonadotrophins (Sanders & Debuse, 2003). At ovulation the oocyte is released from the ovary and captured by the fimbriae of the infundibulum of the oviduct. It is then carried into the ampulla, the site of fertilisation (Rodney & Bell, 2009).

In human, the ovarian artery and vein are in close proximity (Bendz, 1977; Bendz *et al*., 1982a; Einer-Jensen & Hunter, 2000) and there is evidence for counter current exchange of progesterone and antipyrine (Bendz *et al*., 1982b). The counter-current transfer of prostaglandins and peptides has been reported in sheep (Barrett *et al*., 1971; Schramm *et al*., 1986a,b). The vessels are thought to transfer steroid hormones synthesised by the dominant

follicle directly to the uterus and oviduct at relatively high concentrations bypassing first rate metabolism through systemic circulation. Therefore, the microvasculature of the female reproductive tract may also provide a pathway for the ovary to biochemically communicate with the oviduct and uterus regulating events associated with gamete and embryo transport (Einer-Jensen & Hunter, 2005; Grudzinskas *et al*., 1994; Hunter *et al*., 1983).

1.4 SPERM TRANSPORT, MIGRATION AND INTERACTIONS WITHIN THE FEMALE REPRODUCTIVE TRACT

1.4.1 *In vivo* studies on sperm numbers and distribution within the human reproductive female tract

Knowledge of human sperm transport through and interaction with the female reproductive tract is limited, largely due to the ethical and practical difficulties of carrying out such research. Many of the early *in vivo* studies on sperm distribution in women were designed to assess sperm migration in cases of infertility or under contraceptive treatment (Adashi *et al*., 1996). Many of these studies lacked quantitative and qualitative assessment, standardisation of the time interval between insemination/coitus and sperm recovery and included infertile patients with possible pathologies likely to influence sperm migration (Williams *et al*., 1993a). For studies that did employ quantitative assessment, the methods used to recover or estimate sperm numbers were highly variable in efficiency. Nevertheless, a number of human studies were performed before the ethical restraints were introduced providing the basis of our understanding of *in vivo* sperm transport within the human female reproductive tract.

The most comprehensive *in vivo* study on sperm distribution within the human oviducts was performed by Williams *et al*. (1993a). In this study, sperm distribution within the oviducts of 10 women was quantified after insemination. A significantly larger percentage of sperm were recovered in the ovulatory ampulla in comparison to the non-ovulatory ampulla, implying that sperm access or migration into the ovulating oviduct is preferred. However, ovulation was believed to have no affect on the redistribution of sperm within each oviduct (Williams *et al*., 1993a).

Many studies have consistently reported a large decrease in numbers of sperm present in the oviduct relative to the number inseminated (Ahlgren, 1975; Mortimer & Templeton, 1982; Settlage *et al*., 1973; Williams *et al*., 1992; Williams *et al*., 1993a) however there appears to be no clear gradient between numbers recovered in the uterus and oviducts (Adashi *et al*., 1996).

Authors have reported sperm numbers within the oviducts to range from several hundred to a few thousand (Adashi *et al*., 1996; Croxatto *et al*., 1973; Williams *et al*., 1993a). In addition, sperm appear able to pass through the oviducts into the peritoneal cavity (Asch, 1976; Asch, 1978; Horne & Thibault, 1962; Stone, 1983; Templeton *et al*., 1982; Templeton & Mortimer, 1980). Ahlgren (1975) suggested that in the absence of an isthmic reservoir this may serve as a mechanism to maintain low numbers of sperm at the site of fertilisation to minimise risk of polyspermy (Adashi *et al*., 1996). These findings support the concept that in human, sperm transport to the site of fertilisation is highly controlled.

1.4.2 Sperm transport and migration through the female reproductive tract

Sperm transport within the female reproductive tract entails passage from the hostile vaginal environment through the cervical canal and uterine cavity to the oviduct, generally considered a „safe haven' for sperm. The nature and timing of this event is regulated by the female reproductive cycle and varies between species (Scott, 2000). Nevertheless, in all mammalian species only a fraction of the original sperm population deposited gains access to the site of fertilisation. It is not fully understood whether these sperm are „earmarked', „neutral' or whether the female tract itself brings about selection (Barratt & Cooke, 1991; Cohen & McNaughton, 1974; Cohen & Tyler, 1980). However, selected sperm typically demonstrate normal morphology and vigorous motility (Suarez & Pacey, 2006). Sperm transport through the female reproductive tract has been reviewed in many mammalian species (Hawk, 1983; Ikawa *et al*., 2010; Scott *et al*., 2000; Suarez, 2008b) and more focussed data on human has also been discussed (see Barratt & Cooke, 1991; Croxatto, 2002; Suarez & Pacey, 2006).

Semen deposition

The site of semen deposition varies between mammalian species (Harper, 1982; Suarez & Pacey, 2006). For instance in pigs, the vagina is bypassed and semen is deposited directly into the uterine cavity, giving sperm rapid access to the oviduct (Hunter, 1981). In human, semen is deposited in the anterior vagina after coitus and within minutes sperm swim into the cervical canal (Sobrero & Macleod, 1962). Human sperm are exposed to the acidic environment of the vagina where the pH is normally 5 or less (Suarez & Pacey, 2006). The more alkaline pH of seminal plasma may provide some protection against the acidic vaginal environment (Owen & Katz, 2005). Human semen coagulates, to form a loose gel-like structure largely consisting of structural proteins, semenogelin I and semenogelin II (Lilja,

1985; Su & Wang, 2009). It has been proposed that this coagulum may hold the sperm at the cervical os, however this is for only a limited time as the gel undergoes rapid degradation by the serine protease, prostate-specific antigen (PSA) (Watt *et al*., 1986).

Transport through the cervix

A small proportion of sperm rapidly penetrate into the mucus that fills the external os of the cervical canal (Sobrero & Macleod, 1962). Cervical mucus appears to preferentially select sperm demonstrating normal morphology and vigorous motility (Barros *et al*., 1984; Hanson & Overstreet, 1981; Katz *et al*., 1997). It has been shown that excluded sperm are more likely to have midpiece and/or tail defects impairing motility (Mortimer & Templeton, 1982).

Sperm penetration and migration through cervical mucus is influenced by ovarian hormones. Oestrogen promotes the hydration of cervical mucus, thereby reducing its viscosity (Katz *et al*., 1997) and sperm become more efficient in penetrating the mucus (Morales *et al*., 1993). Under progesterone dominance the cervical mucus becomes much less penetrable (Adashi *et al*., 1996).

It has been hypothesised that sperm may use the deep mucosal grooves of the cervix as channels to aid their migration to the uterus (Mullins & Saacke, 1989). Sperm have been found in cervical crypts which has led some authors to suggest that the cervix could potentially function as a sperm storage site (Insler *et al*., 1980). Supporting this, motile sperm have been recovered from cervical mucus 50 hours (Rubenstein *et al*., 1951), 5 days (Gould *et al*., 1984) and 7 days (Perloff & Steinberger, 1964) after insemination. However, there is no evidence to suggest that sperm stored within crypts re-emerge into the cervical canal and migrate to upper regions of the female tract (Bjorndahl *et al*., 2010).

Mucins are large glycoproteins found in cervical mucus contributing to its viscoelastic properties and microarchitecture (Carlstedt & Sheehan, 1984: Adashi *et al*., 1996). The arrangement and expression of mucins are regulated by ovarian hormones (Gipson, 2001) and during active secretory flow align forming barriers that may direct sperm migration towards the crypts while others pass through to the uterus (Suarez & Pacey, 2006; Chretien, 2003). Sperm recovered from cervical mucus up to 3 days after insemination or migration *in vitro* demonstrated incomplete capacitation when assessed by various markers however, required much shorter incubation times for its completion (Adashi *et al*., 1996; Drobnis & Overstreet, 1992). This suggests that the high shear forces experienced by sperm during migration may aid the shedding of decapacitation factors (Katz, 1991) and promote initial priming for capacitation events and hyperactivated motility (Zhu *et al*., 1992).

Shortly after insemination, an influx of leukocytic cells composed mainly of neutrophils occurs (Pandya & Cohen, 1985; Thompson *et al*., 1992). This process may be initiated by the production of inflammatory cytokines and chemokines in reproductive tract epithelia upon exposure to seminal plasma components (Sharkey *et al*., 2007). Leukocytes can invade the cervix and vagina however, few phagocytic cells have been found to contain sperm fragments. This may be explained as leukocytes specifically target sperm only if both the serological complement and complement-fixing anti-sperm antibodies are present (D'Cruz *et al*., 1992). This suggests that the main role of leukocytes is to prevent microbial invasion associated during insemination and transport (Suarez & Pacey, 2006). However, infertility can result when there are antibodies present that recognise antigens on the surface of ejaculated sperm (Menge & Naz, 1993).

Transport through the Uterus

Little is known about human sperm interaction and transport within the uterus, with most knowledge relating to the effect of uterine contractile activity on sperm transport. Interest in this area may have stemmed from early *in vivo* studies demonstrating sperm recovery from the oviducts within minutes of vaginal insemination (Settlage *et al*., 1973). Rapid transport of sperm through the uterus has also been observed in other mammalian species (Hawk, 1987; Overstreet & Cooper, 1978a) although most of these sperm appeared immotile or damaged. It has therefore been suggested that rapidly transported sperm do not contribute towards the fertile population within the oviduct (Scott, 2000). A similar process has been suggested to occur within humans (Suarez & Pacey, 2006). In the late follicular phase, the waves of human myometrial contractions intensify (Kunz *et al*., 1996; Lyons *et al*., 1991). There is also potential for myometrial contractions to be stimulated by seminal components (Robertson, 2005; Robertson, 2007) which may function to draw in sperm from the cervix (Suarez & Pacey, 2006).

Sperm survival in the uterine lumen is believed to be short (Harper, 1982). Leukocytic infiltration of the uterine cavity occurs several hours after coitus (Pandya & Cohen, 1985) when it becomes a hostile environment for sperm. In humans, sperm appear more vulnerable to phagocytosis within the uterus possibly reflecting a loss of immunological defences provided by seminal plasma constituents occurring during transport through the cervix (Dostal *et al*., 1997; Suarez & Oliphant, 1982). However, sperm have been recovered in the human uterus 24 hours post-coitus and in a number of different species, sperm manage to sequester in the uterine gland lumina (Harper, 1982).

Transport into the oviduct - Uterotubal junction (UTJ)

The uterotubal junction functions as a physiological barrier to sperm passage in most mammals (Suarez & Pacey, 2006), however in human the functional role of the UTJ is not so clear. The narrow lumen has been found to contain mucus during the periovulatory period thought to act in a similar manner to mucus within the endocervix, regulating aspects of sperm transport (Adashi *et al*., 1996; Jansen, 1980). In human, ciliary beat in the isthmus and ampulla is directed towards the uterus (Blandau & Hafez, 1969). Jansen (1980) proposed that the mucus may even protect sperm from being swept back to the uterus by ciliary beating.

In animal species, sperm transport through the UTJ requires vigorous and progressive motility. Artificially inseminated hyperactivated sperm, induced by *in vitro* capacitation, have shown incompetence in passing the UTJ (Olds-Clarke & Wivell, 1992; Shalgi *et al*., 1992). In mice, the UTJ appears to regulate sperm passage into the oviducts, closing tightly approximately 1 hour after coitus (Suarez & Pacey, 2006). Sperm lacking various molecules such as angiotensin-converting enzyme (ACE) (Hagaman *et al*., 1998; Krege *et al*., 1995), ADAM1a (Nishimura *et al*., 2004), ADAM2 (Cho *et al*., 1998), ADAM3 (Shamsadin *et al*., 1999; Yamaguchi *et al*., 2009) and calmegin (Clgn) (Ikawa *et al*., 1997) are unable to pass through the UTJ, despite being motile. In sperm, the ACE enzyme may act to release glycosylphosphatidylinositol anchored proteins associated with the sperm plasma membrane (Kondoh *et al*., 2005; Metayer *et al*., 2002). ADAM 1a, ADAM 2 and ADAM 3 are glycoproteins with EGF-like and peptidase M12B domains that have been shown to be important in sperm-oocyte fusion. Clgn is a testis-specific homolog of the ER-resident chaperone, calnexin (Ikawa *et al*., 2010). These findings suggest that in some species there may be additional factors affecting sperm migration other than sperm motility and

morphology (Suarez & Pacey, 2006). These factors may relate to the selection of sperm that are functionally mature with the ability to bind and fuse with an oocyte.

Isthmus and ampulla

After passage through the UTJ, sperm enter the tubal isthmus and many studies have focussed on interactions between sperm and the oviductal epithelium at this site.

1.4.3 Sperm binding to oviductal epithelium

There has been considerable interest regarding sperm binding to oviductal epithelium. In many species studied to date, sperm appear to bind strongly in the isthmus region of the oviduct forming a ‚reservoir' of stored sperm (Birkhead & Moller, 1993). The first description of a sperm reservoir was in the hamster tubal isthmus (Yanagimachi & Chang, 1963) and evidence has subsequently emerged for the formation of sperm reservoirs in at least 13 other eutherian mammals (Holt & Lloyd, 2010) including pigs (Hunter, 1981), cows (Hunter & Wilmut, 1984), sheep (Hunter & Nichol, 1983), rabbits (Overstreet & Cooper, 1978b) and mice (Suarez, 1987). Although there has been speculation regarding sperm storage in the isthmus region of the human oviduct, early *in vivo* studies did not support this hypothesis (Williams *et al*., 1993a). Nevertheless, pregnancies can result from intercourse up to 5 days before ovulation (Wilcox *et al*., 1995) implying that sperm are likely to be stored somewhere in the female reproductive tract to some extent (Suarez & Pacey, 2006).

Human sperm have been reported to bind intermittently to both ciliated and secretory cells of human oviductal epithelium when cultured *in vitro* (Morales *et al*., 1996; Pacey *et al*., 1995a,b). Human sperm-oviduct binding seems to be more intermittent and of lower avidity

compared to other mammalian species (Morales *et al*., 1996; Pacey *et al*., 1995a,b). In addition, the number of sperm binding to isthmus appears greater than to ampulla sections of oviductal explants (Baillie *et al*., 1997).

1.4.4 Mechanisms involved in sperm-oviduct binding and release

Sperm binding mechanisms

Sperm of non-human eutherian mammals bind to carbohydrate moieties on oviductal epithelium (Suarez, 1998; Talevi & Gualtieri, 2010). Specific mono- and oligo-saccharides have been shown to competitively inhibit sperm binding to oviductal epithelium in various species including sialic acid in hamster (Demott *et al*., 1995), galactose in horse (Dobrinski *et al*., 1996a; Lefebvre *et al*., 1995b), mannose in pig (Wagner *et al*., 2002), and fucose in cow (Lefebvre *et al*., 1997). In the human oviduct, glycoconjugates differ in expression between sialic acid residues in ciliated OECs and fucose residues in secretory OECs (Jansen, 1995). Furthermore, galactosyl residues are only found in the isthmus region (Wu *et al*., 1993). The implication of carbohydrate moieties in the binding process has currently not been identified in humans.

A number of seminal plasma derived proteins have been implicated in modulating sperm-oviductal binding. In pigs and cattle, at least two major families of seminal plasma proteins have been demonstrated to display carbohydrate and heparin binding ability, namely spermadhesins and bovine seminal plasma (BSP) proteins. In both species the binding of these proteins has been suggested to be mediated through annexin receptors expressed on the oviductal apical cell membrane (Talevi & Gualtieri, 2010). Interestingly, a BSP protein homolog (BSPH1) has been found in human (Lefebvre *et al*., 2007). In human, few studies

have investigated mechanisms involved in oviductal binding, however there is evidence for the involvement of the arginine-glycine-aspartic acid (RGD) sequence (Reeve *et al*., 2003).

Beta-defensin 126 (DEFB126), is an epididymal specific protein found to coat macaque sperm and promote sperm binding to the oviductal epithelium (Tollner *et al*., 2008). Sialic acid moieties on DEFB126 have been implicated in defence against recognition by components of the female immune system (Cohen, 1998). DEFB126 is lost or removed during capacitation in the upper region of the female tract and this removal is important for sperm-oocyte binding events (Tollner *et al*., 2004). Anti-DEF126 has been shown to partially reduce sperm binding to oviductal epithelium suggesting it is involved and that sperm adhesion is likely to rely on more than one type of molecule. A DEFB126 homolog exists in human but its role in sperm-oviduct interactions remains unknown.

Sperm release mechanisms

In many species the release of sperm after binding appears to signal the completion of sperm capacitation (Suarez, 2002; Suarez & Pacey, 2006), but the mechanisms that induce mammalian sperm release are poorly characterised (Talevi & Gualtieri, 2010). The release of sperm from epithelial binding has been hypothesised to result from a change in the oviductal milieu and is widely thought of as a periovulatory event (Hunter, 2008). Periovulatory hormones can be delivered directly to the wall of the adjacent oviduct through counter-current vascular transfer. Counter-current transfer of ovarian steroids from the ovarian vein to the ovarian artery delivers concentrations approximately 10-20 times that of the systemic circulation in the isthmus region (Einer-Jensen & Hunter, 2005). Progesterone has been implicated in promoting sperm detachment, however it is not known whether this is as a direct

effect on sperm physiology, remodelling of the oviductal epithelium or the relaxation of the isthmus musculature aiding sperm migration (Hunter, 2008).

Remodelling of the sperm plasma membrane is associated with a periovulatory influx of Ca^{2+} into bound sperm (Flesch & Gadella, 2000) and preliminary evidence has implicated Ca^{2+} mobilisation in regulating sperm release (Hunter *et al*., 1999). In addition, sperm coating proteins that may mediate binding events could be released during capacitation, a concept that agrees with observations that capacitated sperm bind less often and with lower avidity (Fazeli *et al*., 1999; Lefebvre & Suarez, 1996; Suarez, 1998).

As sperm binding appears to involve carbohydrate moieties in many mammals, some authors have suggested glycosidases present within oviductal fluid may remodel the arrangement of carbohydrate moieties on sperm membranes and therefore influence sperm binding.

In vitro, hyperactivated motility has been observed in sperm releasing from oviductal epithelium (Demott & Suarez, 1992; Pacey *et al*., 1995a; Suarez, 2008a). In human sperm it has been suggested that the switch to hyperactivated motility supplies the required force to physically disrupt sperm-oviduct attachments, aiding in the detachment of sperm (Pacey *et al*., 1995a).

ROS in the female reproductive tract

The female tract appears to have a dual function in both eliminating unsuitable sperm whilst prolonging and protecting a selected subpopulation of sperm. Lower regions of the female reproductive tract including the vagina and uterine cervix, are particularly hostile

environments for sperm. The cervix is immunologically primed and shortly after sperm exposure, leukocytes infiltrate cervical mucus and the uterus (Thompson *et al*., 1992; Williams *et al*., 1993b). Activated leukocytes can generate a 100-fold increase in ROS in comparison to non-activated leukocytes (Plante *et al*., 1994; Shekarriz *et al*., 1995). In addition, the oviduct has relatively high oxygen tension resulting in hyperoxic conditions which may enhance enzymatic activity, and generate O_2^- within cells (Guerin *et al*., 2001).

ROS appear to be important in regulating a number of significant reproductive events including steroidogenesis, follicular rupture and ovulation (Fujii *et al*., 2005). NO has been shown to regulate contractile motion in explants isolated from human oviducts (Ekerhovd *et al*., 1997) and isoforms of NOS have been identified in rat, bovine and human oviductal tissue (Rosselli *et al*., 1996). Human sperm contain multiple protein targets for NO (Lefievre *et al*., 2007) and therefore have the potential to undergo post-translational modification during their migration through the female reproductive tract (Machado-Oliveira *et al*., 2008).

Defence against ROS induced damage

In vivo, oocytes and embryos appear to be protected from oxidative stress via oxygen scavengers present in follicular and oviductal fluids (Carbone *et al*., 2003; Guerin *et al*., 2001). Many non-enzymatic compounds have been found to possess antioxidant function, with sulphur compounds such as glutathione (GSH), taurine and hypotaurine being of particular interest. OECs synthesise and secrete taurine and hypotaurine providing protection against peroxidative damage (Guerin *et al*., 1995; Guerin & Menezo, 1995). In human, a large amount of ascorbate is present in follicular fluid and may induce taurine and hypotaurine secretion into oviductal fluid at the time of ovulation (Guerin *et al*., 1995; Paszkowski &

Clarke, 1999). Hypotaurine can neutralise hydroxyl radicals effectively at low concentrations (Aruoma *et al*., 1988) and may reduce sperm lipid peroxidation (Alvarez & Storey, 1983). Pyruvate and metal chelators such as albumin and transferrin are found in human oviductal fluid and have been shown to protect sperm against ROS-induced loss of motility (Bilodeau *et al*., 2002). Albumin and transferrin are the most abundant proteins in tubal fluid and metal chelation is an important means of controlling lipid peroxidation (Guerin *et al*., 2001).

Some of the antioxidant capacity of oviductal cells has been shown to be retained in cell cultures and may improve viability of gametes or embryos during co-culture by reducing oxidative stress. For example, the OE E6/E7 immortalised oviductal cell line produces epoxide hydrolase 1, an enzyme known to reduce ROS (Cheong *et al*., 2009).

Redox regulation of sperm oviduct binding events

The potential for gametes to experience an environment both high in ROS but also greatly supported by the antioxidant system, is likely to result in complex redox events. Recently, the redox status of sperm has been suggested to play a role in regulating sperm binding to oviductal epithelium (Gualtieri *et al*., 2009; Talevi *et al*., 2007). Thiol groups, also known as sulfhydryl (SH) groups are functional groups found on cysteine residues of amino acids. Oxidation of two SH groups cause them to become linked via a disulfide (SS) bond and this reaction is rapidly reversible by reduction. Talevi *et al*. (2007) identified surface SH groups localised in the extra-acrosomal region of adhering sperm and provided evidence that SH reducing agents such as GSH could induce their release from oviductal epithelial binding (Gualtieri *et al*., 2009; Talevi *et al*., 2007). This finding suggests that ROS can rapidly and reversibly modulate SH-SS status on sperm surface proteins and that sperm binding is at least

partly dependent on SS bonds. Both SH-targeted reagents and SS reductants are able to induce sperm capacitation in bovine sperm (Talevi *et al*., 2007) and the SH-SS status has also been associated with capacitation status in human sperm (De & Gagnon, 2003). Interestingly, the SH-SS status also appears to be important in epididymal sperm transport, as SH labelling is apparent in cells of the caput epididymis but decreases towards the caudal region (Huang *et al*., 1984).

A system in which oxidative stress could promote reversible binding to oviductal epithelium would form a sophisticated mechanism to regulate sperm viability and protection from ROS-induced oxidative damage.

Selectivity of sperm binding

Some authors have suggested that mammalian sperm binding to oviductal epithelium is a selective process, in which subpopulations meeting certain criteria are preferentially bound. These criteria include intact acrosomes, (cow; Gualtieri & Talevi, 2000), (human; Pacey *et al*., 1995b), normal chromatin structure, (human; Ellington *et al*., 1998a; Ellington *et al*., 1999a), low $[Ca^{2+}]_i$, (horse; Dobrinski *et al*., 1996b), low membrane tyrosine phosphorylation, (pig; Petrunkina *et al*., 2001), (dog; Petrunkina *et al*., 2003) and uncapacitated status, (horse; Thomas *et al*., 1995a), (cow; Lefebvre & Suarez, 1996), (pig; Fazeli *et al*., 1999).

1.4.5 The role of the oviduct in regulating sperm capacitation

It appears *in vivo* that the principal contribution to capacitation is provided by the oviduct in mammals (Hunter & Rodriguez-Martinez, 2004). This conclusion has been drawn from observations that epididymal and ejaculated sperm suspended within seminal plasma

components can undergo capacitation when surgically deposited in the oviduct (Hunter *et al*., 1978; Hunter & Hall, 1974a,b). Furthermore, mammalian sperm appear to show a faster rate of capacitation if exposed sequentially to the uterine cavity and oviducts, imitating chronological events of sperm transport through the female tract. This suggests that *in vivo* capacitation has an element of synergism within different regions of the female tract (Hunter & Rodriguez-Martinez, 2004). Interestingly, insemination into the caudal isthmus yields more rapid capacitation compared to insemination into the ampulla (Hunter *et al*., 1998), implying that the secretions or cells of the isthmus region of the oviduct may be more potent in inducing capacitation (Hunter & Rodriguez-Martinez, 2004). This research has contributed to what is now a widely accepted concept that the oviduct plays a crucial role in secreting capacitation factors (Suarez, 2008b).

As previously discussed in this chapter, HCO_3^- plays an important role in promoting sperm capacitation *in vitro*. The importance of HCO_3^- *in vivo* is indicated by its relatively low concentration within the caudal epididymis in comparison to semen and upper regions of the female reproductive tract (Zhou *et al*., 2005). Concentrations of HCO_3^- have been recorded to range from 35-90 mM in oviductal fluid (David *et al*., 1973; Vishwakarma, 1962; Murdoch & White, 1986) often exceeding that routinely used to capacitate sperm *in vitro* (Visconti *et al*., 1999). Furthermore, ovulation has been reported to induce an increase in HCO_3^- secretion (Foley & Williams, 1991; Maas *et al*., 1977). This increase of HCO_3^- is likely to be a mechanism by which regulation of sperm motility is controlled by the female tract. The impact of HCO_3^- secretion in the female tract upon sperm fertilising capacity has been reviewed by Zhou *et al*. (2005).

The oviduct appears to play a complex and dynamic role in regulating sperm physiology (Holt & Fazeli, 2010). Its ability to both promote and suppress capacitation-associated events is likely to reflect the reproductive strategy of particular species. A review by Hunter & Rodriguez-Martinez (2004) proposed that the regulation of sperm capacitation by the female reproductive tract is influenced by at least two main mechanisms; the suppression of capacitation during a long pre-ovulatory interval and signalling for completion of capacitation regulated by local and systemic ovarian factors. As fully capacitated sperm are unstable and short-lived cells, suppression of capacitation until the time near ovulation would serve to prolong sperm energy resources and protect/preserve fragile surface membranes and labile acrosomal enzymes (Hunter & Rodriguez-Martinez, 2004; Rodriguez-Martinez, 2007). This strategy appears to be important in mammals with long pre-ovulatory intervals (Rodriguez-Martinez, 2007). It is interesting to note that in human, the pre-ovulatory phase is considerably longer when compared to certain mammals which this mechanism has been proposed for (Johnson & Everitt, 2000).

In mammalian species, the completion of capacitation is a periovulatory event (Hunter, 1987; Hunter & Nichol, 1986; Smith & Yanagimachi, 1989; Smith & Yanagimachi, 1990). Factors proposed to influence capacitation include follicular steroid hormones, prostaglandins and peptides transferred through the ovarian vein to ovarian artery counter-current exchange system (Hunter *et al*., 1983) and by follicular fluid/oocyte investments released at the time of ovulation (Hunter & Rodriguez-Martinez, 2004; Kolle *et al*., 2009; Yanagimachi, 1969; Yanagimachi, 1994b).

1.4.6 Studies using oviductal cultures, oviductal-conditioned media and purified proteins to assess sperm physiology

Numerous studies have demonstrated that the oviduct and its secretions can modulate several other important aspects of sperm physiology.

Influence on sperm viability and motility

A small number of human *in vivo* studies have reported sperm survival to range from 50-60 h (Rubenstein *et al*., 1951), 85 h (Ahlgren, 1975) to 16 days (Mansour *et al*., 1993). Although the latter is controversial, authors have stated that sperm remained viable for a further 9 days when co-cultured with OECs. Most *in vitro* studies have supported the notion that components of OECs maintain sperm viability and motility (e.g. Ellington *et al*., 1993a; Kawakami *et al*., 2001; Pollard *et al*., 2001; Smith & Nothnick, 1997; Yeste *et al*., 2009; Zumoffen *et al*., 2010). Human sperm-oviduct co-culture studies have suggested survival times ranging from at least 48 hours (Ellington *et al*., 1998b; Kervancioglu *et al*., 1994a; Morales *et al*., 1996) to 12 days (Akhondi *et al*., 1997). Increases in sperm motility parameters, in particular the onset of hyperactivated motility, have also been reported (Bongso *et al*., 1993; Guerin *et al*., 1991; Kervancioglu *et al*., 1994a; Kervancioglu *et al*., 2000; Pacey *et al*., 1995a) however, conflicting reports exist (Bastias *et al*., 1993).

It appears that in human, the beneficial effect of direct contact between sperm and cultured OECs extends to that of oviductal-conditioned media (OCM) (Ellington *et al*., 1999b; Quintero *et al*., 2005; Robert *et al*., 2008; Yao *et al*., 1999a; Yao *et al*., 2000; Yeung *et al*., 1994; Zhu *et al*., 1994; Zumoffen *et al*., 2010) although perhaps to a lesser extent (Kervancioglu *et al*., 1994a; Kervancioglu *et al*., 2000; Morales *et al*., 1996). The incubation

time in which sperm are exposed to cultures of OECs or OCM appears to be important, as authors have reported insignificant differences if incubation was less than 5 hours (Morales *et al*., 1996; Yao *et al*., 2000; Yeung *et al*., 1994; Zhu *et al*., 1994).

Yao *et al*. (2000) reported that the positive effect of OCM could be abolished through heat inactivation and/or trypsination, suggesting that proteins within OCM may be responsible for mediating sperm function. This agrees with other reports that soluble protein fractions derived from preparations of apical plasma membrane (sAPM) enhance motility and survival of sperm (Boilard *et al*., 2002; Dobrinski *et al*., 1997; Elliot *et al*., 2009; Fazeli *et al*., 2003; Holt *et al*., 2005; Murray & Smith, 1997).

There is limited evidence to suggest that some beneficial interactions between sperm and oviductal epithelium are not species-specific and co-culture of sperm with non-genital tract cells also enhances sperm survival (Ellington *et al*., 1998b; Kervancioglu *et al*., 1994a). However, enhanced sperm motiltiy characteristics such as hyperactivation have not been demonstrated in co-culture with non-gential tract cells (Guerin *et al*., 1991; Kervancioglu *et al*., 1994a; Lai *et al*., 1996).

How does the oviduct suppress capacitation whilst prolonging viability and motility?

The mechanisms behind suppressing capacitation whilst prolonging viability and motility are largely unknown. Aspects of capacitation such as hyperactivation and acrosomal exocytosis are known to be regulated by $[Ca^{2+}]_i$. The binding of mammalian sperm to oviductal components may stabilise the acrosome and reduce intracellular Ca^{2+} influx within bound sperm (Kawakami *et al*., 2001; Suarez, 2008b). Evidence supporting this concept includes

reports that equine sperm binding to epithelial cells and preparations of oviductal apical plasma membrane (APM) maintained lower $[Ca^{2+}]_i$ in contrast to sperm incubated with kidney APM or non-bound sperm (Dobrinski *et al*., 1996b; Dobrinski *et al*., 1997). A similar observation has been made with boar sperm in which increases in $[Ca^{2+}]_i$ and destabilisation of the plasma membrane proceeded at slower rates in cells bound to oviductal epithelium compared to unbound sperm (Petrunkina *et al*., 2001).

Zumoffen *et al*. (2010) found unidentified proteins within OCM which could exert a dose dependant decrease in both tyrosine phosphorylation of sperm proteins and level of human follicular fluid-induced acrosome reaction. As increases in tyrosine phosphorylation have been associated with capacitation, authors have suggested that proteins found within OCM could modulate events associated with sperm capacitation (Zumoffen *et al*., 2010). This supports other *in vitro* studies that have suggested that human sperm capacitation events can be suppressed by co-culture with OECs (Murray & Smith, 1997; Yao *et al*., 1999a,b).

The identification of oviductal proteins that modulate such activity is of great interest. OCM and sAPM represent a complex mixture of proteins, any of which might be active at low concentrations (Elliot *et al*., 2009). Attempts have therefore been made to identify some of these proteins specifically, along with their functions. A number of protein candidates have been implicated in modulating sperm function including HSPs, OGPs and the glycodelin glycoforms.

Heat shock proteins

Heat shock proteins (HSPs) are highly expressed on the surface of endometrium and oviductal epithelium and are candidates likely to modulate sperm viability. HSP60 and glucose regulated protein 78 (GRP78) have been detected in oviductal fluid from women in the periovulatory period and in OCM (Boilard *et al*., 2004). Both have been demonstrated to bind to sperm, increase $[Ca^{2+}]_i$ and potentiate responses to progesterone (Lachance *et al*., 2007). In a more recent study, a recombinant form of Grp78 (Rec-Grp78) was shown to decrease the number of human sperm bound to the ZP during gamete interaction (Marin-Briggiler *et al*., 2010). When Ca^{2+} from the incubation medium was replaced with strontium, rec-Grp78 enhanced sperm-ZP interaction, suggesting that it may modulate the interaction in a Ca^{2+}-dependent manner (Marin-Briggiler *et al*., 2010). Therefore HSPs have the potential to affect sperm via plasma membrane mediated cell signalling mechanisms.

Lachance *et al*. (2007) were unable to detect any viability enhancing effects of HSP60 and GRP78 on human sperm, however as their incubation period was only 4 h, effects may have been difficult to detect. Another heat shock protein, HSPA8, has been suggested to prolong the survival of sperm in the ram (Lloyd *et al*., 2009), pig and cow (Elliott *et al*., 2009). Furthermore, pre-treatment with an HSPA8 antibody reduced the ability of sAPM to prolong sperm survival (Lloyd *et al*., 2009). The conserved nature of HSPA8 between mammalian species suggests that this protein may represent a common biological mechanism for the maintenance of sperm survival in the oviduct.

Oviduct-specific glycoproteins

Oviduct-specific glycoproteins (OGPs), also known as oviductins, are a family of high molecular weight glycoproteins produced by non-ciliated secretory cells of the mammalian oviduct. They are extensively O-substituted with around 50% (dependant on species) of the molecular mass contributed to by carbohydrates (Ling *et al*., 2005). OGPs have been shown to increase the viability, motility and fertilising capacity of bovine sperm (Ling *et al*., 2005; Satoh *et al*., 1995). OGPs are believed to enzymatically modify exposed carbohydrate moieties on the surface of the ZP resulting in an increased accessibility for sperm receptors involved in binding to the ZP (O'Day-Bowman *et al*., 1996). OGPs are highly conserved across mammalian species and have been found to be secreted by human oviducts and an immortalised oviductal cell line, OE E6/E7 (Ling *et al*., 2005).

Glycodelin glycoforms in the human female reproductive tract

Another protein demonstrating the importance of glycosylation is glycodelin, known to modulate human sperm function. Glycodelin has four known glycoforms differing only in glycosylation. Each glycoform has been named after the location it was originally isolated from; glycodelin- S (seminal plasma), glycodelin-A (amniotic fluid), glycodelin-F (follicular fluid) and glycodelin-C (cumulus matrix) (Seppala *et al*., 2007; Yeung *et al*., 2006; Yeung *et al*., 2009).

Glycodelin-S

Seminal plasma contains a high concentration of glycodelin-S (Chiu *et al*., 2005) and its fast binding kinetics produce rapid effects on sperm functions. The binding of glycodelin to sperm reduces cholesterol efflux and is believed to suppress capacitation-associated changes before

sperm enter the uterine cavity. Sperm-bound glycodelin-S is shed during transport through cervical mucus as sperm begin to initiate the capacitation process (Yeung *et al*., 2009).

Glycodelin-A, F and C

Glycodelin-A is found in both human uterine and follicular fluid and reduces binding of sperm to the ZP in a dose-dependent manner (Oehninger *et al*., 1995). Fucosyltransferase-5 has been identified as a sperm plasma membrane receptor of glycodelin-A (Chiu *et al*., 2007) and glycodelin-F (Yeung *et al*., 2009), which also binds to solubilised and intact ZP (Chiu *et al*., 2007).

Glycodelin-F is highly expressed in human follicular fluid and suppresses progesterone-induced acrosome reaction (Chiu *et al*., 2003a,b). As acrosome-reacted sperm display reduced ZP binding (Yanagimachi, 1994a) and penetration of the oocyte-cumulus complex (Saling, 1989), glycodelin F has been suggested to protect sperm against premature acrosomal exocytosis (Yeung *et al*., 2009). In contrast, glycodelin-C enhances sperm–ZP binding and displaces sperm-bound glycodelin-A and glycodelin-F during interactions with the cumulus-oocyte complex (Chiu *et al*., 2007).

The nature of how these proteins interact with sperm and modulate their functions within the female tract environment is crucial when considering other aspects of reproductive physiology.

1.4.7 Sperm-induced modification of the oviductal proteome

There are some aspects of oviductal physiology and its regulation that are poorly characterised. For example, the mechanisms by which allogenic sperm are tolerated by the oviduct despite its efficient innate and adaptive immune defences (Georgiou *et al*., 2007; Wira *et al*., 2005). In addition, it not only permits the presence of sperm, but also maintains their viability before fertilisation (Georgiou *et al*., 2007). Although components derived from seminal plasma are thought to provide sperm with some protection against maternal immune defences in the lower female tract (Robertson *et al*., 2002), these „masking' components are generally believed to be absent within the oviductal environment (Georgiou *et al*., 2007). Therefore, a gamete recognition system that signals the female reproductive tract to alter its environment appears an appealing concept. Supporting this concept, the oviductal environment and the composition of oviductal fluid appears not to be solely under the influence of ovarian steroids but can additionally be modulated by the presence of gametes. Several studies have investigated the changes in gene and protein expression of female reproductive tract cells in response to gametes and embryos (Bauersachs *et al*., 2003; Das *et al*., 2006; Ellington *et al*., 1993b; Fazeli *et al*., 2004; Georgiou *et al*., 2005; Georgiou *et al*., 2007; Kapelnikov *et al*., 2008a,b; Lee *et al*., 2002; Long *et al*., 2003; Mack *et al*., 2006; Thomas *et al*., 1995b). These studies have tended to target oviductal cells, as they potentially have intimate and prolonged contact with gametes during transport and migration events.

The majority of mammalian studies investigating alterations in protein expression induced by sperm have been performed *in vitro*, using greater than the physiological number of sperm expected to reach the oviducts (Georgiou *et al*., 2007). However more recently, an elegant series of experiments by Georgiou *et al*. (2007) demonstrated that *in vivo* the composition of

porcine oviductal fluid is distinctly altered by the presence of gametes. Using isotope coded affinity tag technology to quantify relative changes in oviductal secretory proteins, 19 and 3 proteins showed a greater than 2-fold alteration by sperm and oocyte, respectively. Several candidate proteins were then focussed on namely fibrinogen, complement C3, retinol binding protein (RBP) and OGP which are all known to influence gamete maturation, viability and function (Anderson *et al*., 1993; Buhi, 2002; Buhi & Alvarez, 2003; Fazeli & Pewsey, 2008; Reyes-Moreno *et al*., 2002). Using real time-PCR and Western-blot analysis they confirmed alterations at both transcriptional and translational levels. Georgiou *et al*. (2007) concluded that the observed changes in the oviductal composition may provide a favourable environment for gametes and to prepare the oviduct for the advent of the embryo.

In bovine, the addition of sperm to cultured OECs can induce an upregulation of prostaglandin synthesis (Kodithuwakku *et al*., 2007). As prostaglandins have been shown to affect the contractile properties of the uterine myometrium and the oviductal myosalpinx, Kodithuwakku *et al*. (2007) proposed that sperm-oviduct signalling could modulate transport of gametes. Interestingly, dead or truncated sperm did not stimulate prostaglandin synthesis implying that sperm-oviduct signalling may involve mechanical stimulation. Human oviductal tissue has been shown to express prostacyclin sythases (Huang *et al*., 2002), however whether human sperm stimulate prostaglandin synthesis remains unknown.

In the drosophila model, it has been demonstrated that alterations in gene transcription occurs after mating and may be implicated in tissue remodelling mediating oviductal progression to a mature functional stage (Kapelnikov *et al*., 2008a; Kapelnikov *et al*., 2008b).

Priming effects of semen on the female reproductive tract

In many species, seminal plasma can induce the production of inflammatory cytokines and chemokines in female tract epithelia. Some authors have postulated that seminal plasma may play an important role in inducing alterations in female tract physiology which gives optimal conditions for the success of pregnancy (Robertson, 2005). Evidence for such a response in human is lacking, however reproductive tract cells cultured *in vitro* have been reported to upregulate inflammatory cytokine gene expression upon exposure to seminal plasma (Sharkey *et al*., 2007). It has also been proposed that seminal plasma constituents bound to the post-acrosomal region of human sperm may be carried to higher regions of the female tract (Robertson, 2005). Therefore, the effects of seminal plasma components on human female reproductive tract physiology may warrant further investigation.

1.4.8 Influence of endometrial and oviductal pathology on interaction

Human tissue samples for research are generally obtained from women undergoing hysterectomies so a form of pathology will inevitably be present. It is therefore important to consider these pathologies in relation to possible effects on sperm binding and interactions with cells of the female reproductive tract. Dysfunctions in sperm-oviduct binding and interactions could be a cause for infertility in some cases (Reeve *et al*., 2003; Templeton *et al*., 1998). Pathologies that are common indications for hysterectomy include leiomyomata, menorrhagia, dysmenorrhea, adenomyosis and endometriosis.

Endometriosis is a relatively common gynaecological condition, characterised by the presence of tissue resembling endometrium located outside of its normal position lining the uterus

(Heffner & Schust, 2010). Endometriosis has been implicated in contributing towards subfertility through a series of mechanisms (Gupta *et al*., 2008; Mahutte & Arici, 2002).

Reeve *et al*. (2005) reported increased binding of human sperm to OECs in cases of endometriosis in comparison to non-endometriotic derived cells and suggested that this could reduce the number of freely motile and/or detaching sperm within the oviductal lumen able to participate in fertilisation events. A previous study by Reeve *et al*. (2003) indicated the involvement of the RGD adhesion sequence and integrins in sperm-oviduct binding. As integrin expression is reported as abnormal in the endometrial epithelium in cases of endometriosis (Lessey *et al*., 1994; Ota & Tanaka, 1997; Puy *et al*., 2002), Reeve *et al*. (2005) hypothesised that an increased integrin expression in the epithelium of these women could lead to increased sperm binding and altered sperm transport.

Adenomyosis is associated with endometriosis in infertile patients and presents with lesions consisting of irregularly arranged muscle fibres (Kunz *et al*., 2000; Kunz *et al*., 2005). This alteration to the underlying myometrium can result in abnormal myometrium contractility. Therefore, adenomyotic lesions have been speculated to impaired sperm transport in infertile subjects with endometriosis (Kissler *et al*., 2007; Leyendecker *et al*., 1996).

1.5 Ca^{2+} IN CELLULAR SIGNALLING

Calcium (Ca^{2+}) is fundamental in regulating many key cellular processes, from movement of flagellar and cilia, to gene expression, differentiation and eventual cell death through apoptosis or necrosis (Berridge *et al*., 2000). The versatility of Ca^{2+} is related to its chemical

properties which enable it to complex with low affinity to many low-molecular-weight cellular components such as phosphates, amino acids, acid phospholipids and ATP (see Carafoli *et al*., 2001; Williams, 1999). For example, Ca^{2+} can bind to oxygen atoms present on certain side chains of amino acids, resulting in the formation of cross-links between segments of proteins and conformational changes (Berg *et al*., 2002). These complexes act not only to reduce the concentration of unbound „free' cytosolic Ca^{2+} ($[Ca^{2+}]_i$) whilst providing favourable energy expenditures to achieve the required changes in $[Ca^{2+}]_i$ but also exploit proteins capable of binding Ca^{2+} to transduce its signal through various pathways (Carafoli *et al*., 2001). These qualities are necessary for Ca^{2+} to function as an effective messenger.

The presence of Ca^{2+} in the cytosol is highly regulated and dependent on the dynamic relationship between its stimulatory and resting states of activity. In an unstimulated cell, the concentration of free Ca^{2+} within the cytosol is typically 100-200 nM and during agonist stimulation can increase to μM concentrations (Berridge *et al*., 2000). The majority of cytosolic Ca^{2+} binds to cytosolic „buffers' such as proteins, calretinin and parvalbumin. However, a small proportion of cytosolic Ca^{2+} binds to proteins that act as Ca^{2+} sensors or effectors and in response to binding Ca^{2+}, regulate crosstalk between signalling pathways including ion channel modulation, metabolism and gene transcription (Berridge *et al*., 2000). For example, the ubiquitous Ca^{2+} binding protein, calmodulin (CaM) is considered a major transducer of Ca^{2+} signals. Upon binding to Ca^{2+}, CaM undergoes a conformational change enabling it to bind to other non-Ca^{2+} binding proteins that in turn activate downstream targets (see Parekh, 2011). Therefore, Ca^{2+} can interact and alter the function of various proteins involved in different signal-transduction cascades and, in turn, alter cellular functions almost instantly.

1.5.1 Ca^{2+} release from intracellular stores and Ca^{2+} entry channels

An elevation in $[Ca^{2+}]_i$ can originate from two sources, either from extracellular fluid through Ca^{2+} entry channels or through the release from intracellular organelles (Berridge *et al*., 1998).

Ca^{2+} release from intracellular stores

The endoplasmic reticulum (ER) and the muscle equivalent, sarcoplasmic reticulum (SR) are the main agonist-sensitive Ca^{2+} storage compartments of eukaryotic cells (Carafoli *et al*., 2000). The release of Ca^{2+} from ER/SR stores is controlled by a number of channels with most being known about inositol-1,4,5-triphosphate receptors (IP_3Rs) and ryanodine receptors (RYRs). These Ca^{2+} channels are regulated by several Ca^{2+} mobilising second messengers and Ca^{2+} itself (Berridge *et al*., 2000).

The production of Ca^{2+} mobilising second messengers can be initiated through cell surface receptors including G-protein-linked receptors and tyrosine-kinase-coupled receptors (Berridge *et al*., 2003). The phosphoinositide cascade is an example of a signal transduction pathway initiated at the cell surface upon agonist binding to its specific receptor. During this process, several isoforms of the phosphoinositide-specific phospholipase C (PLC) are activated and catalyse the hydrolysis of phosphatidylinositol 4, 5-bisphophate (PIP_2) to the second messengers; inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Potier & Trebak, 2008; Putney, 2001). IP_3 diffuses through the cytosol and in binding to the IP_3R induces the release of Ca^{2+} from ER/SR stores. The release of Ca^{2+} into the cytosol, at low concentrations (<300 nM), can stimulate IP_3Rs and RYRs to further release Ca^{2+} known as Ca^{2+}-induced Ca^{2+}-release (CICR) however, can be inhibitory at higher concentrations

(Berridge *et al*., 2000; Marchant & Parker, 2000; Rosado *et al.,* 2004). The RYR can also be modulated by other second messengers such as cyclic-ADPribose (Marks, 1997). Both Ca^{2+} and DAG activate isoforms of protein kinase C (PKC) which are known to phosphorylate a wide range of proteins involved in signaling pathways (Fukami *et al*., 2010).

Ca^{2+} entry channels

Each cell type has a set of Ca^{2+}-permeable channels located in the plasma membrane which control the entry of external Ca^{2+} (see figure 1.6). When activated, these channels open to allow an influx of Ca^{2+}, a process driven by the large electrochemical gradient across the plasma membrane.

The mechanisms through which these channels are gated can be used to separate them into several categories. In excitable cells, Ca^{2+} entry primarily occurs through voltage-operated channels (VOCs) in the plasma membrane which open in response to a change in membrane potential to allow a rapid influx of extracellular Ca^{2+}. In non-excitable cells, where VOCs are not present, Ca^{2+} entry primarily occurs through receptor-operated channels (ROCs), second messenger operated-channels (SMOCs) and store-operated channels (SOCs) (Rosado *et al*., 2004). ROCs are particularly important in secretory and neuronal cells as they are activated by extracellular ligands such as hormones and neurotransmitters (Carafoli *et al*., 2001; Rosado *et al*., 2004). SMOCs are found in both the plasma membrane and the membrane of intracellular Ca^{2+} stores and open in response to internal second messengers such as DAG, IP_3 and cyclic nucleotides (Golovina *et al*., 2001; Parekh & Putney, 2005). The main mechanism for Ca^{2+} entry could be through activation of SOCs in response to depletion of Ca^{2+} in ER/SR stores, a process known as store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+} entry (CCE)

(Parekh & Putney, 2005; Rosado *et al*., 2004). Recent advances have implicated an important role for a Ca^{2+} store depletion sensor, Stim1 and pore-forming subunit, Orai1 during SOCE (see Parekh, 2011). Transient receptor potential (TRP) channel proteins have been presented as subunits of both ROCs and SOCs however, the exact involvement of TRP channels in Ca^{2+} entry mechanisms remains elusive (DeHaven *et al*., 2009; Putney, 2007; Potier & Trebak, 2008; Salido *et al*., 2011).

1.5.2 Cytosolic Ca^{2+} clearance mechanisms

Cytosolic Ca^{2+} has a critical influence on cell survival, with sustained elevation promoting cell death through apoptosis or necrosis. Therefore, several cytosolic Ca^{2+} clearance mechanisms exist and function to maintain intracellular Ca^{2+} homeostasis including: plasma-membrane Ca^{2+} ATPase (PMCA), Na^{+}/Ca^{2+} exchanger (NCX), sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), mitochondrial uniporter (MCU) and the more recent but less characterised, secretory-pathway ATPase (SPCA) (Berridge *et al*., 2003; Missiaen *et al*., 2007). These pumps and exchangers located in various membranes reduce $[Ca^{2+}]_i$ through extruding cytosolic Ca^{2+} extracellularly or by sequestering it into intracellular organelles such as the ER/SR, Golgi-apparatus and mitochondria (Richter & Kass, 1991).

The ATP-driven Ca^{2+} pumps (PMCA, SERCA and SPCA) have higher affinities for Ca^{2+} but relatively lower transport rates therefore respond to modest Ca^{2+} elevations and can set basal $[Ca^{2+}]_i$ (Berridge *et al*., 2003). PMCA pumps Ca^{2+} from the cytosol into the extracellular fluid and SERCA pumps cytosolic Ca^{2+} across internal membranes into ER/SR Ca^{2+} stores. SPCA appears to be important in sequestering Ca^{2+} into Golgi compartments enabling this organelle to function as a Ca^{2+} store (Berridge *et al*., 2003; Wootton *et al*., 2004).

The NCX and MCU combine a greater transport rate with a lower affinity for Ca^{2+} in comparison to ATP-driven Ca^{2+} pumps, enabling optimal function at higher (μM) concentrations of cytosolic Ca^{2+} (Berridge *et al*., 2003). The NCX is a bi-directional ion transporter that exchanges Na^{+} for Ca^{2+} through movement in opposing direction. Depending on the electrochemical driving force on the exchanger, NCX can move Ca^{2+} both into (backward mode) and out (forward mode) of the cell (Rosado *et al*., 2004). There are several types of NCX that have been described with different coupling ratios including the K^{+} independent NCX which exchanges 3 or 4 Na^{+} for each Ca^{2+} and the K^{+} dependent, NCKX which exchanges 4 Na^{+} for Ca^{2+} and K^{+}. A type of NCX has also been reported in the mitochondria (Blaustein & Lederer, 1999; Rosado *et al*., 2004).

The participation of mitochondria in recovery from Ca^{2+} stimulation is important. When $[Ca^{2+}]_i$ is high, the MCU located in the inner mitochondrial membrane transports Ca^{2+} into the mitochondria enabling it to act as a Ca^{2+} ‚buffer'. However, when Ca^{2+} within the mitochondria reaches a critical level, the mitochondrial membrane potential irreversibly collapses resulting in the activation of programmed cell death, apoptosis (Berridge *et al*., 1998; Rosado *et al*., 2004). The ability of mitochondria to both mobilise Ca^{2+} and juxtapose to the ER/SR and PM structures suggests they contribute to the spatiotemporal aspects of Ca^{2+} signalling (see Parekh, 2003).

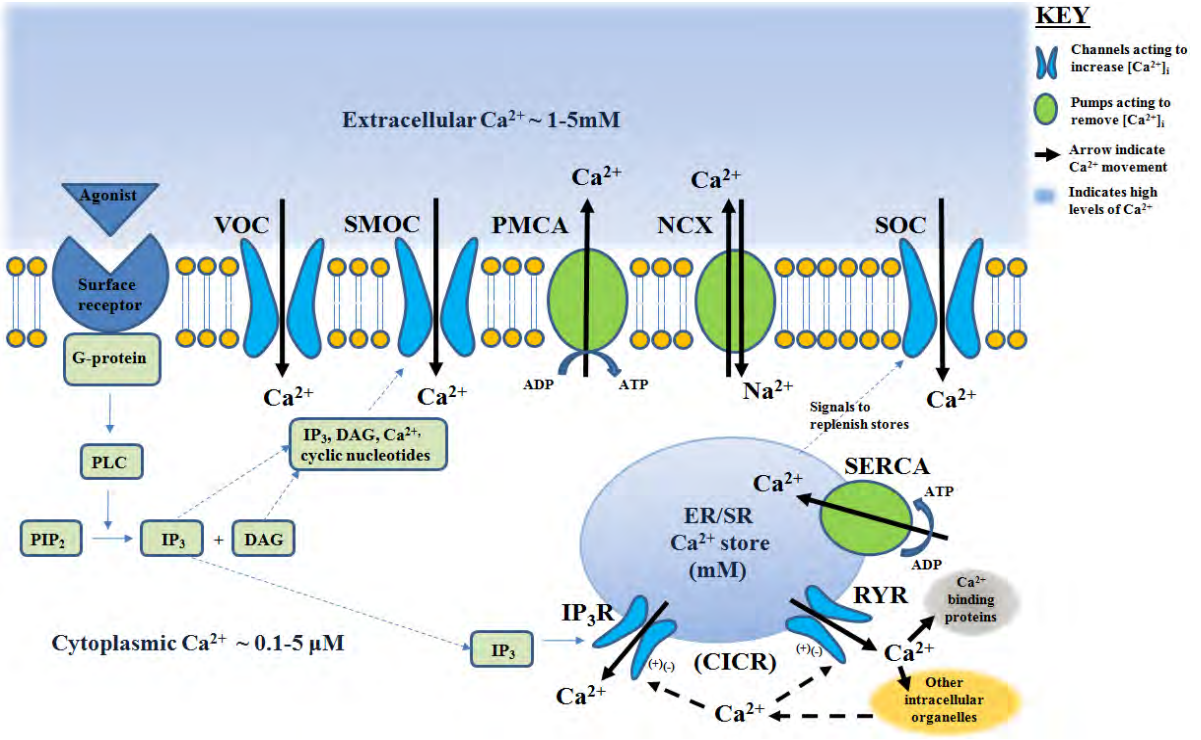


Figure 1.6. Routes of Ca^{2+} mobilisation

An elevation in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) can originate from the extracellular medium through „Ca^{2+} entry' channels, or from intracellular organelles that release Ca^{2+} upon agonist stimulation. The most common mechanism by which these two modes of Ca^{2+} mobilisation can occur is through the generation of second messengers (IP_3 and DAG) through the PI-PLC pathway in response to agonist binding. IP_3 modulates the release of Ca^{2+} through binding to the IP_3R primarily located on the internal stores (ER/SR), a process termed „Ca^{2+} store mobilisation'. Ca^{2+} released by intracellular stores can be stimulatory to both IP_3Rs and RYRs (at lower concentrations <300 nM), a process termed „Ca^{2+}-induced Ca^{2+} release' (CICR) or inhibitory (at higher concentrations >300 nM). Depletion of ER/SR Ca^{2+} stores leads to the activation of store-operated channels (SOCs) in the plasma membrane resulting in an influx of extracellular Ca^{2+}, a process termed „capacitative Ca^{2+} entry (CCE) or store operated Ca^{2+} entry (SOCE). Ca^{2+} homeostasis within the cell is maintained by the activity of various Ca^{2+} channels, pumps and cytosolic buffers. The majority of Ca^{2+} released binds to cytosolic buffers or effectors (Ca^{2+} binding proteins) that can activate various downstream signalling pathways and cellular processes. Removal of cytosolic Ca^{2+} is mainly regulated by several ATP-dependant pumps (e.g. PMCA, SERCA) and the Na^+/Ca^{2+} exchanger (NCX). The NCX and the plasma-membrane Ca^{2+}-ATPase (PMCA) extrude Ca^{2+}, whereas the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) sequesters Ca^{2+} into the ER/SR. Other intracellular organelles such as the mitochondria and the Golgi apparatus can mobilise Ca^{2+} and may participate in signalling events (Berridge *et al*., 2000; Berridge *et al*., 2003; Parekh & Putney, 2005; Rosado *et al*., 2004). **Ca^{2+}**, calcium; **PLC**, phospholipase C; **PIP_2**, phosphatidylinositol-4,5-bisphosphate; **IP_3**, inositol-1,4,5-trisphosphate; **DAG**, diacylglycerol; **PI-PLC**, phosphoinositide-phospholipase C; **ER**, endoplasmic reticulum; **IP_3R**, inositol-1,4,5-trisphosphate receptor; **CICR**, Ca^{2+}-induced Ca^{2+} release; **SERCA**, sarco/endoplasmic reticulum Ca^{2+}-ATPase; **RYR**, ryanodine receptor; **VOCs**, voltage-operated channels; **SMOCs**, second messenger-operated channels; **SOCs**, store-operated channels; **NCX**, Na^{2+}/Ca^{2+} exchanger; **PMCA**, plasma membrane Ca^{2+}ATPase; **ATP**, adenosine triphosphate ; **ADP**, adenosine diphosphate.

1.5.3 Spatial and temporal aspects of $[Ca^{2+}]_i$ signalling

The complexity and diversity of Ca^{2+} signalling is further enhanced through spatial and temporal dynamics resulting from the integrated action of channels and transporters mobilising Ca^{2+} between cellular compartments (Lytton, 2007). Spatial aspects appear to be important for fast responses of closely associated downstream effectors whilst temporal aspects, normally presented as repetitive $[Ca^{2+}]_i$ transients (oscillations) and waves, are more influential for processes such as gene transcription and proliferation (Berridge *et al*., 2003).

Ca^{2+} oscillations and waves

Oscillatory signals dispense information as frequency and amplitude (see Parekh, 2011). This nature of signalling provides a high degree of signal-to-noise discrimination and allows cells to respond to Ca^{2+} signals while avoiding sustained elevations of cytosolic Ca^{2+} that would otherwise induce cell death (Carafoli *et al*., 2001; Putney & Bird, 2008). The most commonly encountered type of $[Ca^{2+}]_i$ oscillations are baseline spikes also referred to as transients (Putney & Bird, 2008). The term baseline spikes describes a series of rapid transient increases in $[Ca^{2+}]_i$ from near resting „baseline' levels (Woods *et al*., 1986) and is believed to correlate to cycles of Ca^{2+} release and uptake by intracellular stores (Putney & Bird, 2008). Although there is no consensus on the precise mechanisms underlying baseline spikes/transients, generally they are believed to reflect positive and negative feedback through activation and modulation of the IP_3R, most likely by Ca^{2+} itself (Berridge *et al*., 2003; Carafoli *et al*., 2001; Putney & Bird, 2008). For instance, the generation of IP_3 mediates the release of Ca^{2+} via IP_3-sensitive stores, resulting in an increase in $[Ca^{2+}]_i$. The inactivation of the Ca^{2+} signal may then occur through negative feedback when high local concentrations of Ca^{2+} mediate desensitisation of the IP_3R and through Ca^{2+} reuptake by transporters into intracellular stores

(Berridge *et al*., 2003; Putney & Bird, 2008). As $[Ca^{2+}]_i$ returns to a baseline level, IP_3-sensitive channels are no longer desensitised by Ca^{2+} and can be further stimulated to release Ca^{2+}.

$[Ca^{2+}]_i$ oscillations are reported to spread through the cytoplasm as regenerative waves brought about through CICR and diffusion between clusters of IP_3Rs and RYRs on the ER/SR. As Ca^{2+} released from channel clusters can diffuse to activate neighbouring channels, this mechanism can potentiate an initial local signal to a more global Ca^{2+} wave (Berridge *et al*., 2003). In most cell types, intercellular waves of second messengers such as Ca^{2+} and IP_3 can passively diffuse into neighbouring cells through gap junctions promoting cell-to-cell signal transduction (Carafoli *et al*., 2001).

Ca^{2+} as a regulator of gene transcription

Ca^{2+} signalling is known to promote gene transcription through a number of pathways (Carafoli *et al*., 2001; Mellström & Naranjo, 2001) and there is increasing evidence to suggest that Ca^{2+} influx through Ca^{2+} entry channels is crucial for this process even when large global $[Ca^{2+}]_i$ transients arise from intracellular release (Putney & Bird, 2008). Di Capite *et al*. (2009) recently highlighted the importance of the spatial profile of Ca^{2+} signalling using the c-*fos* gene as a model system. It was demonstrated that c-*fos* induction in response to agonist-induced $[Ca^{2+}]_i$ oscillations only occurred following SOCE. The induction of c-*fos* did not occur in the absence of extracellular Ca^{2+} or in the presence of high lanthanum despite oscillations being maintained and of indistinguishable quality.

Ca^{2+} is known to activate a number of Ca^{2+}-dependant transcription factors by a large variety of mechanisms. Some of these include Ca^{2+} entering the nucleus and removing the transcription repressor known as downstream regulatory element modulator (DREAM). It can also act indirectly through the modulation of Ca^{2+}-dependant kinases (e.g. Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII) and IV (CaMKIV)) or protein phosphatases (e.g. calcineurin) that alter the phosphorylation state of various transcription factors. Alternatively, Ca^{2+} can activate gene transcription through the cAMP-signalling pathways and recruitment of Ras/mitogen-activated protein kinase (MAPK) (see Berridge *et al*., 2003; Carafoli *et al*., 2001; Mellström & Naranjo, 2001).

1.5.4 Ca^{2+} signalling in epithelial cells of the female reproductive tract

In non-excitable cells such as epithelial cells, the activation of receptors involved in Ca^{2+} mobilisation are reported to produce a series of oscillations or spikes as opposed to sustained elevations of $[Ca^{2+}]_i$. In addition, SOCE appears to be the main process of regulating Ca^{2+} influx through Ca^{2+} entry channels (Putney, 2001; Putney & Bird, 2009).

In addition to promoting alterations in gene transcription, Ca^{2+} can also influence other cellular functions relevant to epithelial cells of the female reproductive tract including secretion, absorption, volume regulation and responses to pathogens (Petersen, 1992; Zhang & O'Neil, 1999). For example, apical Cl^- channels and basolateral K^+ channels involved in directing ion movement are sensitive to increases in $[Ca^{2+}]_i$ and have been described in a number of secretory cell types (Shuttleworth, 1997). An increase in $[Ca^{2+}]_i$ can open these channels initiating secretory activity.

TRP channels are important in the regulation of Ca^{2+} influx in non-excitable cells (Song & Yuan, 2010). Several members of the TRP family of channel proteins have been found in epithelial cells of the female reproductive tract. TRP4 and polycystin-2 are two examples of TRP channels that have been located on the ciliary membrane of murine oviductal cells and are believed to function as mechanoreceptors sensing fluid movement (Andrade *et al*., 2005; Fernandes *et al*., 2008; Teilmann *et al*., 2005). Primary cilia of secretory cells are also reported to express the polycystin-1-polycystein-2 complex which functions as a Ca^{2+} entry channel (Hagiwara *et al*., 2008). This is in agreement with the notion that the bending of cilia results in increases in $[Ca^{2+}]_i$ and stimulation of CBF (Fernandes *et al*., 2008; Hagiwara *et al*., 2008; Singla & Reiter, 2006). The presence of Ca^{2+} entry channels in epithelial cells of the female tract indicates their potential role in regulating aspects of gene transcription and transport of gametes and embryos (Fernandes *et al*., 2008).

Relatively few studies have reported Ca^{2+} signalling in cells of the female reproductive tract. Dickens *et al*. (1996) reported studying a wide range of Ca^{2+} agonists in human oviductal cells and found that only ATP-induced an elevation in $[Ca^{2+}]_i$. Several other reports have also suggested that extracellular ATP can induce increases in $[Ca^{2+}]_i$ in OECs of several species including; human (Dickens *et al*., 1996; Hill *et al*., 1994), cow (Cox & Leese, 1995), mouse (Leung *et al*., 1995), hamster (Barrera *et al*., 2004) and primate (Villalon *et al*., 1995). There is evidence that ATP acts primarily through IP_3Rs and involves the activation of PKC and CaMKII in hamster oviductal cells (Barrera *et al*., 2004). Increased CBF has been associated with ATP treatment and increases in $[Ca^{2+}]_i$. Interestingly, CBF on oviductal cells has also been observed to increase after co-incubation with sperm (Chiu *et al*., 2010; Morales *et al*., 1996; Singla & Reiter, 2006).

1.5.5 Ca^{2+} signalling in sperm

Ca^{2+} plays a fundamental role in almost all important sperm functions that occur following ejaculation (Jimenez-Gonzalez *et al*., 2006). Ca^{2+} signalling in mammalian sperm has been thoroughly reviewed (see Jimenez-Gonzalez *et al*., 2006; Publicover *et al*., 2007; Wang & Gui, 2008). In contrast to somatic cells, the mature sperm cell consists of little cytoplasm and lacks many membranous organelles including the ER. Therefore, sperm primarily rely on compartmentalisation with multiple Ca^{2+} stores to gain spatio-temporal aspects of Ca^{2+} signalling (Bedu-Addo *et al*., 2008; Publicover *et al*., 2007).

The complexity of how Ca^{2+} mobilisation is regulated within the sperm cell has become more apparent in recent years with new components of the Ca^{2+} „toolkit' being continually discovered (Bedu-Addo *et al*., 2008; Publicover *et al*., 2007; see figure 1.7). In mammalian sperm, at least two Ca^{2+} stores have been identified: the acrosome, the redundant nuclear envelope (RNE) and/or calreticulin-containing vesicles associated with the cytoplasmic droplet (Bedu-Addo *et al*., 2008). IP_3Rs have been found on the outer membrane of the acrosome and release Ca^{2+} within the periacrosomal region upon agonist stimulation. The RNE and cytoplasmic droplets contain both the IP_3Rs and RYRs (Bedu-Addo *et al*., 2008; Jimenez-Gonzalez *et al*., 2006). Like somatic cells, sperm of some species studied have shown to have mitochondria capable of acting as stores or buffers for intracellular Ca^{2+} (Gunaratne & Vacquier, 2006; Wennemuth *et al*., 2003a). However, there is limited evidence regarding the involvement of mitochondria in Ca^{2+} buffering and signalling in human sperm (Jimenez-Gonzalez *et al*., 2006).

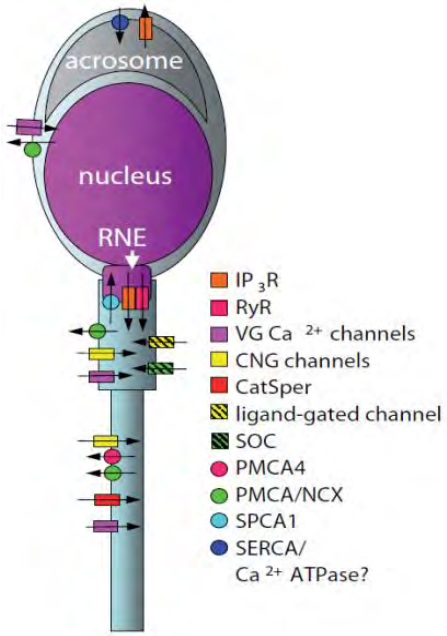


Figure 1.7. The sperm Ca^{2+} 'toolkit'

A schematic representation demonstrating the location of calcium channels (shown as rectangles) and pumps (shown as circles) within the sperm plasma and intracellular membranes. Image reproduced from Bedu-Addo *et al*. (2008).

Within the sperm cell, Ca^{2+} homeostasis may be controlled partly by a simple pump-leak balance (Breitbart, 2002; Publicover *et al*., 2007) relying on constant sequestering into stores or the extracellular environment. VOCs have been identified on the sperm plasma membrane and are believed to function similarly to those of somatic cells (Arnoult *et al*., 1996; Jimenez-Gonzalez *et al*., 2006). SOCs are also present and like those of somatic cells are believed to participate in SOCE by allowing Ca^{2+} influx in response to the depletion of internal Ca^{2+} stores (Blackmore, 1993; Jimenez-Gonzalez *et al*., 2006). The event of acrosomal exocytosis has been correlated with an initial influx of Ca^{2+} through VOCs and subsequent activation of SOCs resulting in a sustained elevation of $[Ca^{2+}]_i$ (Arnoult *et al*., 1996; Kirkman-Brown *et al*., 2003; O'Toole *et al*., 2000). It is believed that the activation of SOCs and sustained elevation of $[Ca^{2+}]_i$ is critical for acrosomal exocytosis to occur (Hirohashi & Vacquier, 2003; Kirkman-Brown *et al*., 2003).

Similarly to somatic cells, sperm employ cytosolic Ca^{2+} clearance mechanisms such as the NCX and ATP-driven Ca^{2+} pumps which extrude cytosolic Ca^{2+} either extracellularly or sequester it into intracellular Ca^{2+} stores (Jimenez-Gonzalez *et al*., 2006). However, the relative importance of each still remains to be established. There is good evidence that PMCA, SPCA and NCX function as cytosolic Ca^{2+} clearance mechanisms within mature sperm. However, the presence of SERCA remains controversial, as human sperm demonstrate little response to SERCA inhibition when using specific concentrations of SERCA inhibitor, thapsigargin (Harper *et al*., 2005; Jimenez-Gonzalez *et al*., 2006; Lawson *et al*., 2007).

The role of Ca^{2+} in regulating sperm motility

The presence of Ca^{2+} channels, with some exclusively located in the principal piece of the sperm flagellum, highlights the functional role of Ca^{2+} in regulating motility. Perhaps the most striking example of motility dysfunction resulting from a deficiency in Ca^{2+} entry has been demonstrated in mice with targeted CatSper gene deletions. CatSper channels are sperm-specific Ca^{2+} permeable ion channels and are comprised of four CatSper proteins encoded by CatSper subunit genes (CatSper 1-4). Targeted gene deletion of any one of the four CatSper subunit genes results in male infertility associated with the inability to undergo hyperactivation (Carlson *et al*., 2003; Ji *et al*., 2007*;* Liu *et al*., 2007; Qi *et al*., 2007; Quill *et al*., 2003; Ren *et al*., 2001; Xia *et al*., 2007). Reduced CatSper expression in the testis and CatSper deletions have also been associated with poor sperm motility and infertility in men (Hildebrand *et al*., 2010; Nikpoor *et al*., 2004).

In mice with targeted gene deletion of PMCA4, also expressed in the flagellum, sperm demonstrated normal activated motility, however became immotile when placed in conditions

promoting hyperactivation (Okunade *et al*., 2004; Schuh *et al*., 2004). This suggests inadequate cytosolic Ca^{2+} clearance mechanisms are detrimental to motility regulation.

More subtle abnormalities in motility regulation may be governed by other Ca^{2+} channels present in the head and flagellar as demonstrated in mice with targeted deletion of a voltage-dependent Ca^{2+} channel, $Ca_v2.3$ (α_{1E}). Sperm lacking α_{1E} demonstrated abnormal $[Ca^{2+}]_i$ transients that reduce linear projection during motility in contrast to wild-type cells (Sakata *et al*., 2002). This suggests that even subtle $[Ca^{2+}]_i$ transients can alter motility characteristics. Alterations observed in flagellar beat patterns during progesterone-induced $[Ca^{2+}]_i$ oscillations in human sperm further supports this concept (Harper *et al*., 2004). Harper *et al*. (2004) reported that progesterone-induced $[Ca^{2+}]_i$ oscillations synchronised with movements of the sperm head, driven by increased flagellar activity during the periods of high $[Ca^{2+}]_i$. These observed $[Ca^{2+}]_i$ oscillations appeared to originate from the postacrosomal and midpiece/neck regions and correlated to binding patterns of both BODIPY ryanodine and antibodies against RYR1 and RYR2 (Bedu-Addo *et al*., 2008). This led to the speculation that not only may the RNE function as a intracellular Ca^{2+} store in human sperm and contribute to $[Ca^{2+}]_i$ oscillations, but it may play a role in modulating the flagellar beat to enable penetration of oocyte vestments necessary for fertilisation (Harper *et al*., 2004).

In mammalian sperm, $[Ca^{2+}]_i$ oscillations and complex regulation of second messengers have also been associated with sperm guidance mechanisms (Harper *et al*., 2004; Publicover *et al*., 2008; Spehr *et al*., 2003; Spehr *et al*., 2004; Teves *et al*., 2006). Therefore, the regulation of Ca^{2+} signalling appears central to important events likely to be encountered by sperm during migration through the female reproductive tract.

CHAPTER 2

Preparation and culture of cells for

studying sperm-female reproductive tract interactions

2.1 INTRODUCTION

The female reproductive tract is an obstacle course that all sperm, with a chance of fertilisation, must traverse. However, it is not an inert object, the tract facilitates selecting sperm which will fertilise the oocyte and fertilisation itself (see Cohen & Tyler, 1980; Holt & Fazeli, 2010). Due to technical and ethical limitations, experimentation and observation of human sperm interaction within the female tract during *in vivo* events is almost impossible. Therefore, tissue explants and cell cultures have become the necessary research system to study human sperm-female tract interactions.

Establishing cultures of oviductal epithelial cells (OECs) has been of interest for both academic research and for clinical application in IVF treatment. For example, co-culture of OECs with embryos in many species including human, has been suggested to significantly improve implantation and pregnancy rates (Kattal *et al*., 2008). Sperm function has also been demonstrated to be positively influenced by co-culture with a number of cell types including oviductal (Chian & Sirard, 1995; Kervancioglu *et al*., 1994a; Morales *et al*., 1996; Pacey *et al*., 1995a,b), endometrial (Fusi *et al*., 1994; Guerin *et al*., 1997; Lai *et al*., 1996) and epididymal cells (Akhondi *et al*., 1997; Bongso & Trounson, 1996). Despite these reported benefits, ongoing cell cultures lose their original histology and paracrine influences, making it difficult to extrapolate observations and results, especially when considering how cells may interact *in vivo*.

Explants of human female reproductive tract have been used for a number of investigations including: sperm-oviduct interaction experiments (Baillie *et al*., 1997; Pacey *et al*., 1995a,b; Reeve *et al*., 2003); conditioning of media (Munuce *et al*., 2009; O'Day-Bowman *et al*., 1995;

Quintero *et al*., 2005; Zumoffen *et al*., 2010); muscle contractility (Wanggren *et al*., 2008); receptor expression (Bahathiq *et al*., 2002; Horne *et al*., 2009) and morphological alterations during infections and disease (Baczynska *et al*., 2007; Maisey *et al*., 2003; Reeve *et al*., 2005). The use of explants is advantageous as the original histology is maintained and sperm interaction with the surface architecture can be studied.

Establishing proliferative *in vitro* „primary' cell cultures of endometrial and oviductal epithelial cells has been previously reported (Bongso *et al*., 1988; Bongso *et al*., 1989; Kervancioglu *et al*., 1994b; Ouhibi *et al*., 1989; Takeuchi *et al*., 1991). Advantages of using primary cultures include ease of maintenance and assessment of cell viability. In addition, cells forming monolayers lend themselves more readily to high resolution imaging.

Primary epithelial cells have been shown to retain *in vivo* epithelial characteristics. The presence of prominent secretory vesicles and secretion of proteins in cell cultures suggest their secretory phenotype can be retained during culture (Gandolfi *et al*., 1989; Grudzinskas *et al*., 1994; Joshi, 1988; Joshi, 1991). This is supported by the reported presence of a secretory cell type marker, HMFG2 (Comer *et al*., 1998). Both ciliated and secretory cells have been shown to be present in primary cell cultures. However, typically there are fewer ciliated than secretory cells with many ciliated cells shedding their cilia and transforming into a non-ciliated secretory type. This suggests that oviductal cells are fundamentally secretory by nature (Ando *et al*., 2000; Comer *et al*., 1998). Deciliation of ciliated cells has been reported to be suppressed by supplementing media with oestrogens, namely oestradiol-17β (Comer *et al*., 1998; Goldberg & Friedman, 1995) which may help maintain ciliation for up to 15 days post-seeding (Comer *et al*., 1998).

Two types of secretory cells have been described: Type I and Type II (Bongso *et al*., 1989; Grudzinskas *et al*., 1994). Type I cells have large amounts of rough endoplasmic reticulum (rER), reticulate nucleoli, well developed Golgi complexes and dense elongated mitochondria of various shapes. Type II cells are less commonly seen, containing less prominent nucleoli and rER, and oval translucent mitochondria. Both types contain surface microvilli, ruffled plasma membranes, large areas of cytoplasm and several cytoplasmic processes or filopodia (Grudzinskas *et al*., 1994). Growth of cells has been reported to appear in epithelioid patterns and tightly packed with prominent nuclei. The proliferation rate has been reported to be independent of the stage of the ovarian cycle in which they were taken from, oestrogen, fibroblast growth factor, insulin and transferrin however, progesterone was reported to inhibit cell growth (Grudzinskas *et al*., 1994; Takeuchi *et al*., 1991).

Cell cultures of human OECs have been be maintained for approximately 15 passages/60 days (Grudzinskas *et al*., 1994). However, a problem commonly encountered with cell culturing is progressive changes of morphological features. Cells have often been reported to display flattened epithelioid morphology associated with a loss of cell polarity (Bongso *et al*., 1989; Dickens *et al*., 1993; Henriksen *et al*., 1990; Ouhibi *et al*., 1989; Thibodeaux *et al*., 1991). Primary cultures are also prone to fibroblast contamination. Fibroblast growth may result from contamination during preparation from tissue or through dedifferentiation of cells displaying fibroblast-like characteristics (Bongso *et al*., 1989). In previous studies, epithelial cells have been identified by morphological features such as the presence of microvilli (Ouhibi *et al*., 1989) whilst others have included cytokeratin and vimentin profiling (Ando *et al*., 2000; Comer *et al*., 1998; Henriksen *et al*., 1990).

Both explants and primary cell lines have limited proliferative and differentiated life spans, which is likely to begin immediately after removal from the natural environment. To overcome this complication, endometrial and oviductal immortalised cell lines have been developed. Immortalised oviductal cell lines have been created in a number of species including human (Ando *et al*., 2000; Lee *et al*., 2001), equine (Dobrinski *et al*., 1999), bovine (Murata *et al*., 2007), primate (Okada *et al*., 2005) and porcine (Hombach-Klonisch *et al*., 2006). Recently, an immortalised human endometrial cell line has been created (Guerin *et al*., 1997; Krikun *et al*., 2006). Immortalisation of human epithelial cells has been achieved through expression of viral oncogenes of human papillomavirus or simian virus 40. In 2001, Lee *et al*. (2001) established an immortalised human oviductal secretory cell line, OE E6/E7, which has subsequently been used in a number of studies and has been shown to retain functional characteristics of OECs (Agarwal *et al*., 2002; King *et al*., 2009; Lee *et al*., 2003; Lee *et al*., 2004; Li *et al*., 2010; Ling *et al*., 2005; Monkkonen *et al*., 2007).

Previous studies on human sperm interaction with epithelial cells have selected sperm by two main methods: density gradient (discontinuous) centrifugation (Bastias *et al*., 1993; Morales *et al*., 1996; Yeung *et al*., 1994) and direct swim-up (Pacey *et al*., 1995a,b; Reeve *et al*., 2003). Density gradients separate sperm based on cell density, typically selecting sperm with good morphology as well as removing seminal plasma components (Chen *et al*., 1995). However, centrifugation required during this method may damage sperm DNA (Twigg *et al*., 1998; Zini *et al*., 1999). The direct swim-up technique selects progressively motile sperm based on migration out of seminal plasma (Mortimer, 1994) without the need for centrifugation. *In vivo*, cervical mucus provides the main physical barrier for sperm transport, selecting highly motile sperm with good morphology (Barros *et al*., 1984; Katz *et al*., 1990).

This may therefore be the most logical way to select the correct sperm to use in experiments. Sperm penetration through cervical mucus *in vitro* has been used as a predictive test of sperm functional competence (Barratt *et al*., 1989; Eggert-Kruse *et al*., 1989). As human cervical mucus is difficult to obtain and its properties are under hormonal influence (Katz *et al*., 1997), substitutes displaying similar viscoelastic properties have been developed (Amari and Nakamura, 1973). Methylcellulose has previously been used as a cervical mucus substitute and is reported to be useful in filtering sperm based on both morphology and progressive motility (Ivic *et al*., 2002). A selection technique mimicking the physiological selection of sperm by the cervix for use in experimentation is currently unexplored but would seem a prerequisite for studying sperm oviduct interactions.

Aims

- To develop preparation techniques suitable to produce cells for both immediate and ongoing use in sperm-female tract interaction research, employing single cell imaging.
- To assess morphology and expression of epithelial/fibroblastic markers in cells during culture.
- To develop a novel technique to mimic sperm selection by the female reproductive tract for later use in experiments.

2.2 Materials and Methods

2.2.1 Materials

15 ml tubes, (Falcon). 22 x 32 glass coverslips, (Appleton woods). 35 x 10 mm tissue culture dishes, (Falcon). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), (Sigma). 50 ml syringes, (BD Biosciences). Alexa Fluor® 488 rabbit anti-goat IgG, (Invitrogen). Alexa Fluor® 594 donkey anti-mouse IgG, (Invitrogen). Anti-cytokeratin 4.62 mouse, anti-human antibody IgG, (Sigma). Anti-vimentin goat, anti-human, (Sigma). Bovine Serum Albumin (BSA), Probumin™ Fatty Acid Free (Lot: 122), (Millipore). Collagenase Type I, (Invitrogen). Disposable sterile filter unit, membrane pore size 0.2 µM, (Nalgene). Dulbecco's minimal essential medium/Hams F12 medium (1:1 v/v DMEM/F12), (Invitrogen). Dulbecco's phosphate buffered saline (PBS), (Sigma). Fetal bovine serum (FBS), (Biosera). Formaldehyde, (Sigma). Hanks buffered salt solution (HBSS) with Calcium and Magnesium (Invitrogen). Heat inactivated embryonic stem cell qualified FBS, (Invitrogen). Human Serum Albumin (HSA), (Sigma). Iscove's modified Dulbecco's medium (IMDM), (Invitrogen). Methylcellulose 4000CP (MC4000), (Sigma). Oestradiol-17β, (Sigma). Pluronic F-127, (Invitrogen). Poly-D-lysine (PDL), (BD Biosciences). Sterile filter membrane pore size 0.8/0.2 µM, (PALL life sciences). Sterile scalpel blades, (Appleton woods). Streptomycin and Penicillin, (Invitrogen). Supplemented Earls balanced salt solution (sEBSS), (Invitrogen). Syto 64 red fluorescent nucleic acid stain, (Invitrogen). Triton-X-100, (Sigma). Trypsin/EDTA, (Invitrogen).

2.2.2 Recruitment of female reproductive tract donors

Oviductal and endometrial sections were obtained with informed consent from 23 individuals undergoing hysterectomy or bilateral salpingectomy at the Birmingham Women's Hospital (Shropshire REC Reference: 06/Q2601/51). Selected patients were approached during a preoperative appointment and provided with information leaflets regarding the study and allowed at least 24 hours before providing informed consent. Written consent was taken on the day of surgery before anaesthetisation. Patients were assigned a reference code for anonymous sample identification. Stage of the menstrual cycle was determined from histology notes obtained post-pathological examination and categorised into either proliferative or secretory phase. Patients were excluded on the basis of any underlying tubal pathology. For clinical characteristics of women from whom reproductive tract was obtained, please refer to appendix II: 2.i.

2.2.3 Preparation of human female reproductive tract cells and cell lines

All explant and cell culture preparations of reproductive tract cells were performed immediately after patient surgery. Female reproductive tract tissue removed during surgery was placed into HBSS supplemented with 100 µg/ml streptomycin and 100 units/ml penicillin and 10 mM HEPES, until the operation was completed. Following this, the surgeon dissected the specimen into isthmus, ampulla and endometrial sections. These tissue sections were placed into pre-labelled individual specimen containers prior to transportation to the university laboratory. Each section of female reproductive tract tissue was clearly labelled and kept separate during all stages of transfer. This tissue was used to create explants for immediate examination and primary epithelial cell lines for later use in experiments, typically within 6 days of culture.

Explants for imaging

The uterine tubes were first washed with HBSS in a sterile petri dish and any associated tissues that were not the intended sections were discarded. Isthmic, ampullary and endometrial sections were then transferred to petri-dishes containing clean pre-warmed HBSS. The sections were then carefully slit open to expose the inner epithelial surface. Epithelium was isolated by dissecting small pieces of the epithelial surface with a pair of pointed fine scissors and watch-markers' forceps under a stereomicroscope. The resulting explants and small clusters of epithelial cells were transferred to labelled sterile centrifuge tubes containing clean pre-warmed (37°C) HBSS using a syringe and centrifuged at 300 x g for 5 min. The supernatant was discarded to reduce blood cell contamination and the pellet containing explants and epithelial cells was resuspended in HBSS (Bongso *et al*., 1989; Grudzinskas *et al*., 1994). Using a stereomicroscope, a number of small explants were then separated into separate petri-dishes so that the remaining epithelial cells could be used to create primary cultures for subsequent analysis. A small aliquot was then placed on a glass slide and analysed on an inverted microscope for epithelial ciliation and level of epithelial cell disaggregation. Pictures and time-lapse videos were taken for documentation.

Primary cell lines

After the preparation and isolation of explants outlined above, the remaining epithelial cells could be seeded onto either 22 x 32 mm glass coverslips or 35 x 10 mm tissue culture dishes for culture and use in later imaging experiments. On occasion mechanical isolation alone did not produce sufficient quantities of single or small clusters of epithelial cells and therefore enzymatic disaggregation of the epithelial layer was achieved using collagenase as previously reported by other studies (Pacey *et al*., 1995b; Takeuchi *et al*., 1991). In these cases, epithelial

cells were transferred to a 15 ml sterile polystyrene Falcon tube containing 5 ml 0.25% collagenase type I solution in HBSS pre-warmed to 37^{o}C. This tube was maintained at 37^{o}C in a water bath with gentle agitation to aid dissociation of epithelial cells. After 1 h the tube was vortexed for several seconds and left for a few minutes to allow larger fragments of connective tissue to settle. The epithelial-rich supernatant was then collected into a separate sterile polystyrene falcon tube. Fresh HBSS was then added to the remaining pellet and the process of vortexing and collection of epithelial-rich supernatant was repeated several times as previously reported by Pacey *et al*. (1995b).

Cells obtained through both methods were washed with fresh HBSS and centrifuged at 500 x g for 5min. The resulting pellet was resuspended in DMEM/F12 (1:1 v/v, w/o phenol red), supplemented with streptomycin (100 IU/ml), penicillin (100 IU/ml) and 10% FBS. A small aliquot was then examined under an inverted microscope and quantifiably assessed for cell culture seeding. Cells were then seeded at ~15000 cells coverslip and maintained at 37^{o}C, 6% CO_2.

Control cell lines

The human immortalised oviductal cell line, OE E6/E7 (donated by Lee *et al*. at Hong Kong University) were derived from ampullary oviductal tissue taken from women in the follicular phase of the menstrual cycle (Lee *et al*., 2001). Cells were cultured in DMEM/F12 (1:1 v/v, w/o phenol red) supplemented with streptomycin (100 IU/ml), penicillin (100 IU/ml) and 10% FBS at 37^{o}C, 6% CO_2. For a subset of experiments, media was supplemented with oestradiol-17β (150 pg/ml) as previously described by Comer and colleagues (Comer *et al*., 1998). Confluent growth occurred approximately every 6-7 days, at which point cells were

trypinsised and either subcultured at a typical subcultivation ratio of 1:4 or cryopreserved in liquid nitrogen until required. Human foreskin fibroblasts (HFF) were grown in IMDM supplemented with streptomycin (100 IU/ml), penicillin (100 IU/ml) and 10% FBS at 37^{o}C, 6% CO_2. Confluent growth occurred approximately every 6-7 days, at which point cells were trypinsised and either subcultivated at a 1:3 ratio or cryopreserved in liquid nitrogen until required.

2.2.4 Immunostaining for epithelial markers

All cell types were assessed for the epithelial marker cytokeratin 19 and fibroblastic marker, vimentin using anti-cytokeratin 4.62 (sigma) and anti-vimentin (Sigma), respectively.

Explants were attached to glass coverslips that had been pre-treated with 1% poly-D-lysine (PDL). Both primary and control cell lines were grown on tissue culture dishes and therefore did not require adhesion. All cell types were then treated as follows at room temperature unless otherwise stated. Cells were fixed in 4% formaldehyde for 6 mins. The formaldehyde was then rinsed off and cells were permeabilised with 0.2% Triton X-100 in PBS (TPBS) for 10 mins and subsequently washed with 0.1% TPBS for 5 mins. To reduce non-specific binding sites, cells were treated with 1% human serum albumin (HSA) in 0.1% TPBS for 1 h. The primary antibody was then added (1:50) and left in a humidified chamber overnight at 4^{o}C. For dual labelling of both anti-vimentin (Goat) and anti-cytokeratin 4.62 (mouse), primary antibodies were added simultaneously. The following day, samples were washed 3 times for 5 mins with 0.1% TPBS. The appropriate secondary antibody (1:20) in TPBS was then added and left for 1 h at room temperature. For anti-vimentin, the Alexa Fluor® 488 rabbit anti-goat IgG was used. For anti-cytokeratin, the Alexa Fluor® 594 donkey anti-mouse

IgG was used. For dual labelling both secondary antibodies added simultaneously. Note: anti-cytokeratin 4.62 and anti-vimentin antibodies were reported to have no cross-reactivity.

2.2.5 Selection, preparation and capacitation of human sperm

Sperm donors were recruited at the Birmingham Women's Hospital (HFEA Centre 0119), in accordance with the Human Embryology Authority Code of Practice. Human sperm were obtained from healthy research donors by masturbation. All donors gave informed consent (LREC 2003/239). During the semen liquefaction period, sperm were labelled with 5 µM/ml of Syto64, a red fluorescent dye, for approximately 30 mins. Sperm were then selected by a modified swim-up technique depicted in figure 2.1.

The modified swim-up technique was performed by adding 300µl of Syto64 labelled semen into several polystyrene round-bottom tubes and overlaying each tube with 0.5 ml of a viscous medium (1% MC4000 methylcellulose in sEBSS, ~ 140 centipoise) and 1 ml of sEBSS + 0.3% BSA (Fatty Acid Free), pH 7.4. After 1 h incubation at 37^{o}C, 6% CO_2 at an angle of 45^{o}, the top 0.3 ml layer of each tube, containing the selected motile cells, was collected and pooled into a 15 ml polystyrene Falcon tube. Sperm concentration was determined using a Neubauer counting chamber, in accordance with the World Health Organisation methods (World Health Organisation (WHO), 1999) and adjusted with sEBSS, to 6 million cells/ml.

On occasions when sufficient sperm were recovered after the modified swim-up, the recovered sperm population was prepared into three separate treatment samples depicted in figure 2.1. In the first treatment sample termed „non-conditioned sEBSS', sperm were

immediately removed by filtration without allowing significant incubation time. In the second treatment sample termed „sperm', sperm were incubated in capacitating sEBSS (sEBSS + 0.3% BSA (fatty acid free), 37°C, 6% CO_2) for at least 3 h without any filtration. The final treatment sample termed „sperm-conditioned sEBSS' was prepared by incubating sperm in capacitating sEBSS for at least 3 h and removing sperm by filtration immediately prior to imaging. Filtration was performed using a 50 ml syringe and a disposable sterile filter (membrane pore size 0.2 µM). On occasions when insufficient sperm were recovered, the preparation was performed as previously described, excluding the preparation of sperm-conditioned treatment.

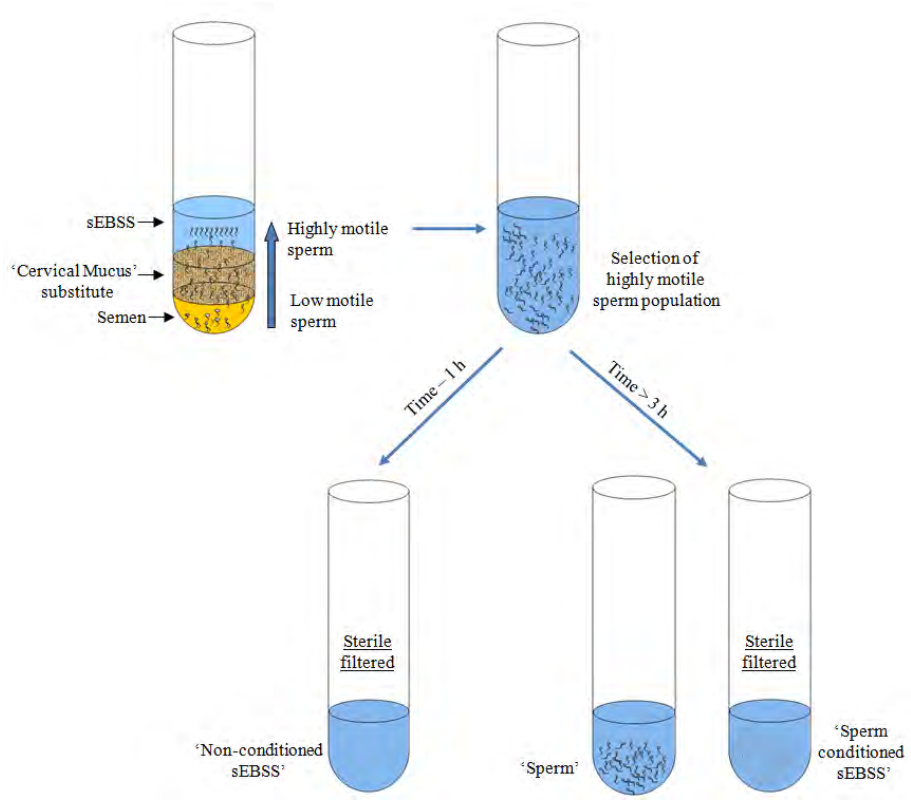


Figure 2.1. Schematic representation of the selection and preparation of sperm related treatments

Sperm were selected by their ability to migrate through a cervical mucus substitute. After selecting a highly motile population, three treatment types were prepared for use. Non-conditioned sEBSS was filtered (pore size 0.2 µM) from sperm immediately after swim-up (Time-1 h) whilst the remaining sperm population was incubated in capacitating conditions. After a minimum of 3 h, the capacitated sperm population was split into sperm-conditioned sEBSS (filtered from sperm) and sperm in sEBSS (containing sperm/unfiltered). Note: sperm-conditioned sEBSS was only performed on occasions when sufficient sperm numbers were recovered.

2.3 RESULTS

2.3.1 Preparation of female reproductive tract cells

Epithelial explants and primary cell cultures were prepared from donated surplus female reproductive tract tissue from three anatomical regions: endometrium, isthmus and ampulla. A number of different methods were tested for efficiency in separating epithelial cells from underlying structures. Explants could be obtained solely using mechanical isolation but displayed a poor yield for the generation of primary cell lines. The most successful technique for plating efficiency and retention of morphological features including ciliation, was achieved through mechanical dislodging of cells and fine dissection of epithelium after enzymatic pre-treatment with collagenase. This technique has been previously reported as successful in producing explants and primary cell lines from human oviductal cells (Henriksen *et al*., 1990; Pacey *et al*., 1995a,b).

There were considerable differences in the ease of preparation in different regions. It was more difficult to obtain epithelial cells suitable for imaging and primary culture from the isthmus region due to the glutinous properties of the tissue even after collagenase treatment. This is in contrast to ampullary derived epithelial cells that easily detached in small clusters (figure 2.2 a, b). Endometrial cells also easily detached by gentle, direct agitation after collagenase treatment, releasing as individual round cells (figure 2.2.c).

In a number of cases, epithelial cells contained active cilia clearly visible under phase-contrast microscopy. Ciliation was seen in varying degrees in all regions studied (typically ranging from highest in ampulla to lowest in endometrium). In prepared cultures, ciliation could be observed in both explants and primary cell lines (figure 2.3). Ciliated primary cell lines were

maintained for approximately 10 days with 150 pg/ml oestradiol-17β supplementation. In the absence of oestradiol-17β supplementation deciliation of primary cell lines were typically seen to occur within 6-7 days. Cilia motion in explant tissue was also observed for up to 4 days of culture. For both ciliated explants and primary cell lines cilia motion was observed to lose synchrony 24 hours post-culture.

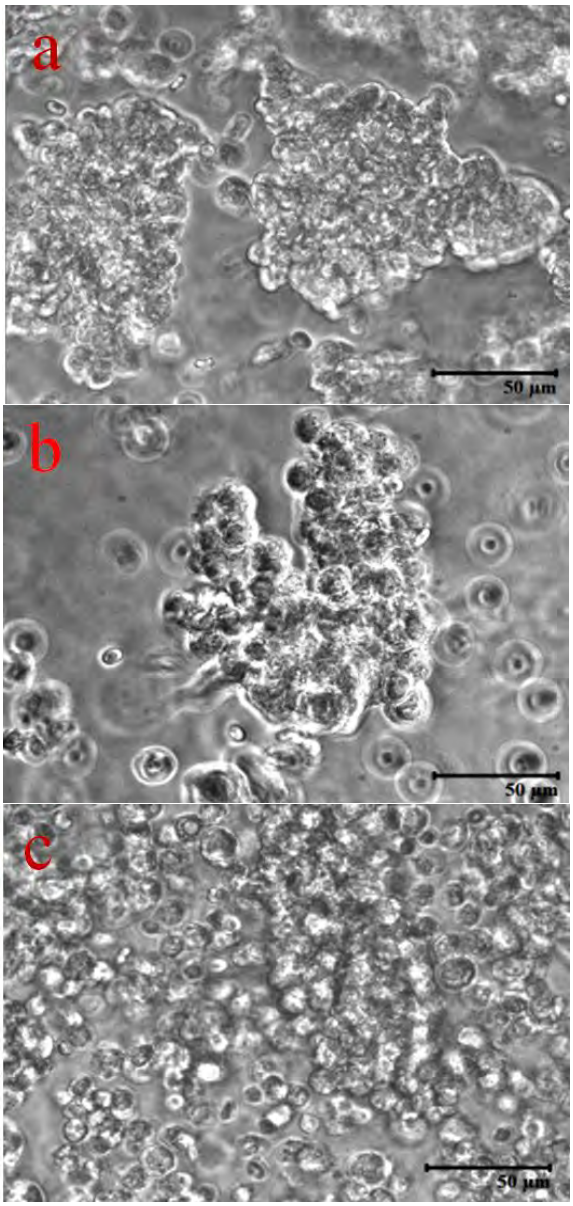


Figure 2.2. Preparation of explants and primary cell lines

Clusters of epithelial cells used for preparation of primary cell cultures: (**a**) isthmic; (**b**) ampullary; (**c**) endometrial. Epithelium has been isolated using mechanical and enzymatic treatment (collagenase).

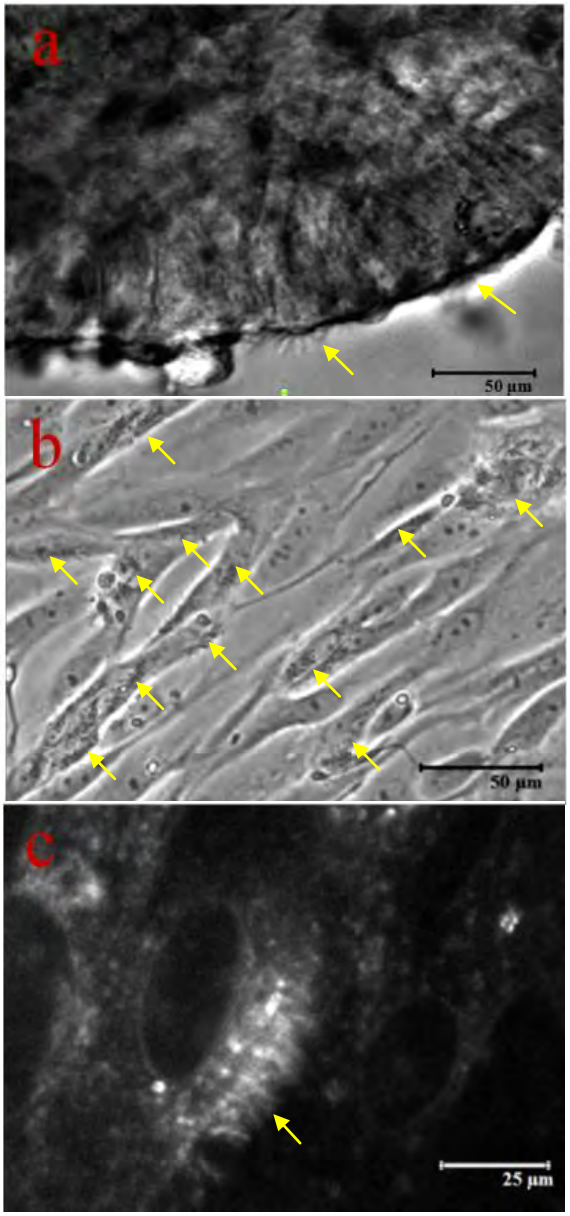


Figure 2.3. The retention of ciliated cells during cell culture studies

(**a**) An explant of endometrial tissue; (**b**) ampullary primary cells; (**c**) ampullary primary cells (taken using dark field microscopy). Arrows indicate presence of cilia.

2.3.2 Identification of single cells within explants

Explant tissue obtained during preparation was used for experimentation within 24 hours of surgical removal, often being used immediately after the surgical operation. Single cell analysis in explants was often complicated due to surface convolutions, cell size and thickness of the tissue. To aid identification of single cells, a projection of the phase and fluorescence image was created using image pro plus II software, allowing cells to be more easily identified (figure 2.4). This enhanced image was used to draw regions of interest (ROI) around single cells for subsequent data-extraction of time-fluorescence intensity values.

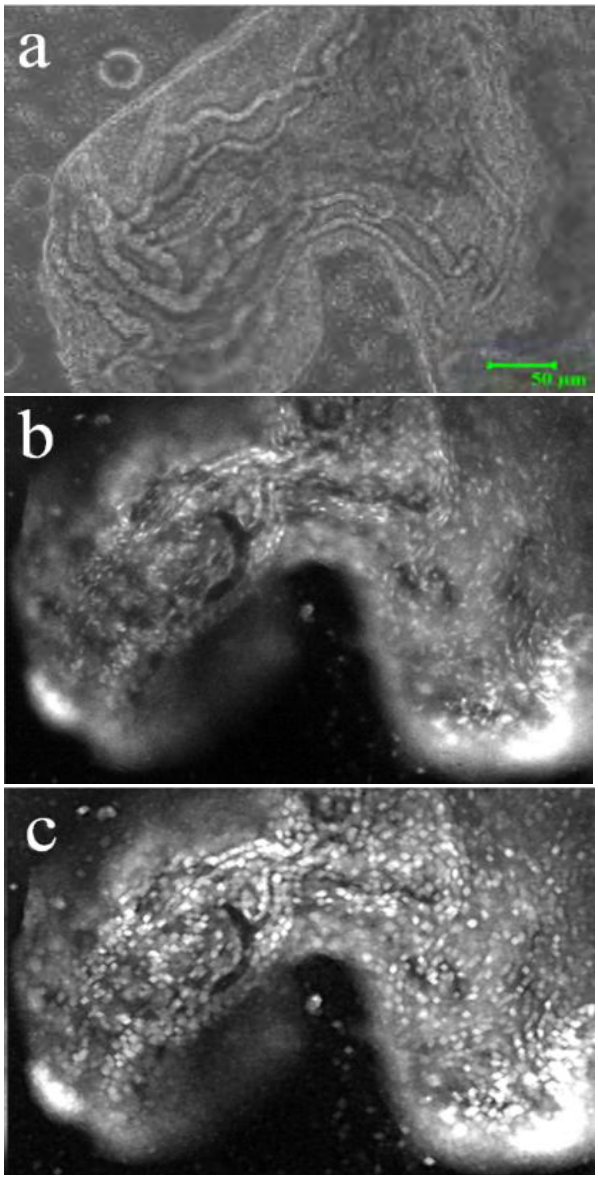


Figure 2.4. Analysing single cells in explant tissue

(**a**) Bright field image; (**b**) corresponding fluorescence image; (**c**) combined projection image used for drawing regions of interest (ROI). Explant shown is of ampulla origin.

2.3.3 Morphology and growth pattern of cells

The morphology of epithelial cultures from the oviductal regions was comparable in cell size and epithelioid growth pattern to the human immortalised oviductal cell line, OE E6/E7 (figure 2.5 a, b, c). Endometrial cultures demonstrated a more „spiral-like' epithelioid growth patterns with long thin prominent cellular projections (figure 2.5 d). This growth pattern has previously been described by Bongso and colleagues as „whorl-like', displaying remarkable similarity to that observed in this present study (Bongso *et al*., 1988). Female reproductive tract primary cell lines retained epithelioid growth patterns for several weeks however, the appearance of fibroblastic-like cells increased with time, particularly after subculturing.

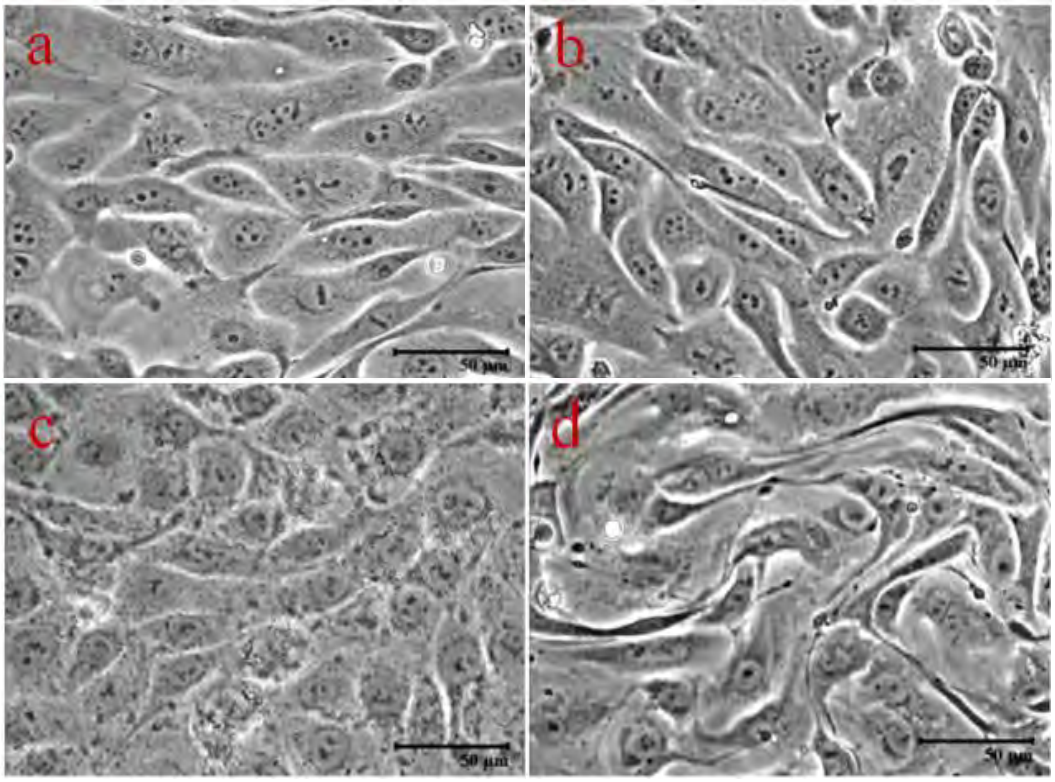


Figure 2.5. *In vitro* culture of female reproductive tract cells

Epithelioid growth patterns for: (**a**) isthmus primary cells; (**b**) ampulla primary cells; (**c**) OE E6/E7 immortalised cells; (**d**) endometrium primary cells.

2.3.4 Immunostaining for epithelial and fibroblastic cell markers

Before using cells for sperm-female tract cell interaction studies it was important to become familiar with cell morphology and expression of epithelial and fibroblastic markers. Cells of all types were assessed for expression of epithelial marker, cytokeratin 19 and fibroblast marker, vimentin, with detection via immunofluorescence in a two-step antibody labelling method. Controls for the specificity and localisation of cytokeratin and vimentin are shown in figures 2.6. Cytokeratin staining could only be observed in cells from epithelial origin and was completely absent in HFF (figure 2.7). Vimentin staining appeared to be far more intense in HFF, with patterns resembling intermediate filaments (figure 2.7c). The general pattern of vimentin staining in OE E6/E7 cells does not appear characteristic of intermediate filaments indicating non-specific binding. Dual labelling of control cell lines showed that OE E6/E7 cells stained strongly and uniformly for cytokeratin (figure 2.7 a). There was also apparent weak staining for vimentin occurring throughout the cell. Dual labelling in HFF, revealed a complete absence of cytokeratin staining with strong vimentin perinuclear and intermediate filament staining (figure 2.7 c).

A series of HFF and epithelial primary cell co-cultures were preformed to investigate whether cells of known types can be distinguished using the dual fluorescence method. This technique proved to be successful in identifying HFF in contrast to epithelial cells. Figure 2.8 a, c shows the epithelioid growth patterns of cells merging with cells of a fibroblastic appearance correlating with expression profiles of epithelial and fibroblastic markers. Dual labelling was also attempted for explant material but with limited success. Both co-localisation of cytokeratin and vimentin staining in explants was observed (shown in figure 2.8 e).

An effect of subculturing on epithelial and fibroblastic marker expression was also observed. Observations of marker expression indicated that cells that proliferated around small epithelial clusters in primary cultures (1st passage) had relatively high levels of cytokeratin expression (figure 2.9 a). The staining for cytokeratin reduced with subsequent passages (figure 2.9 c, e, g). The generalised weak staining of vimentin in epithelial cells appears consistent during passages whilst the vimentin staining of intermediate filaments appears to increase in a small percentage of cells (~2-5%) (figure 2.9 e, g). Interestingly, cells demonstrating a strong „phase halo' indicative of greater 3D nature, tended to coincide with higher levels of cytokeratin staining (figures 2.8 a, b; figures 2.9 c, d, e, f, g, h).

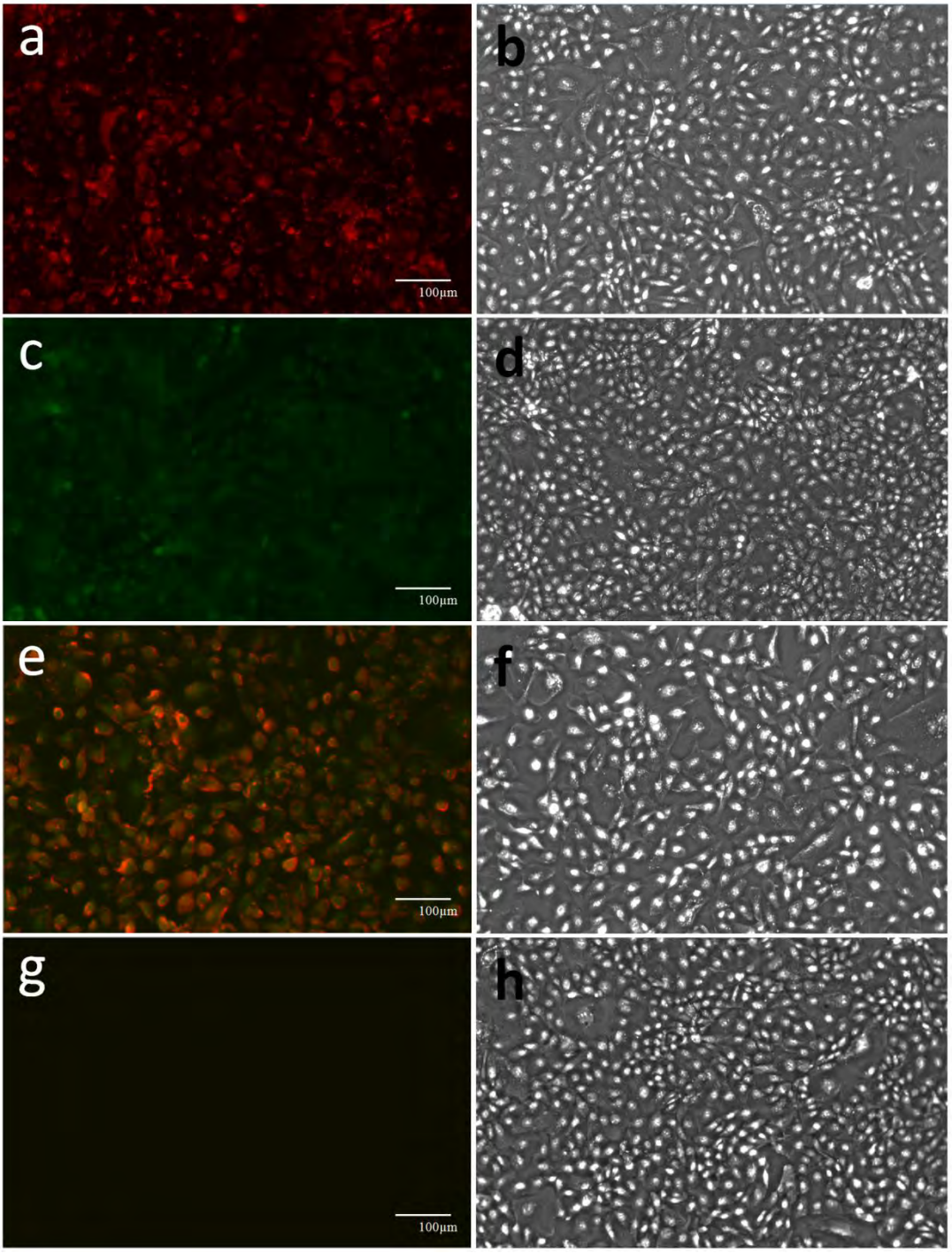


Figure 2.6. Specificity and localisation of cytokeratin and vimentin

OE E6/E7 cells labelled with: (**a**) anti-cytokeratin primary antibody only; (**c**) anti-vimentin primary antibody only; (**e**) dual labelling of primary antibodies; (**g**) absence of primary antibodies. (**b, d, f, h**) Corresponding phase-contrast images of these samples. All samples were treated with secondary antibodies specific for the primary antibodies used.

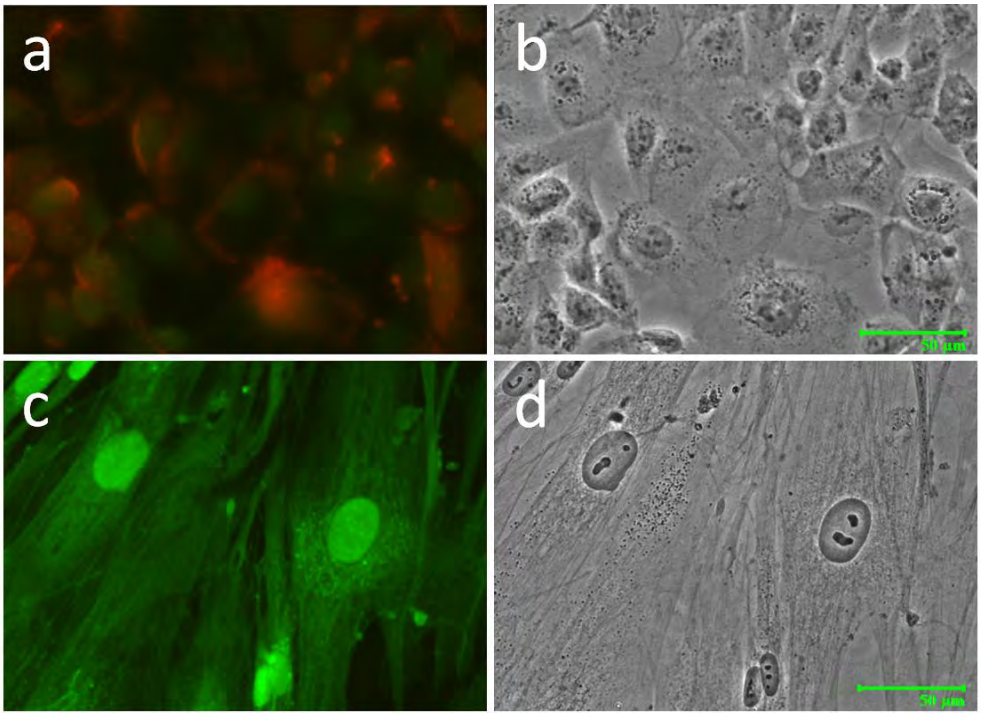


Figure 2.7. Dual fluorescence labelling for intermediate filaments in control cell lines

Control cell lines treated with intermediate filament markers for anti-cytokeratin 19 (**red**) and anti-vimentin (**green**). (**a**) Human oviductal cell line, OE OE/E7; (**c**) Human foreskin fibroblasts. (**b, d**) Corresponding phase-contrast images.

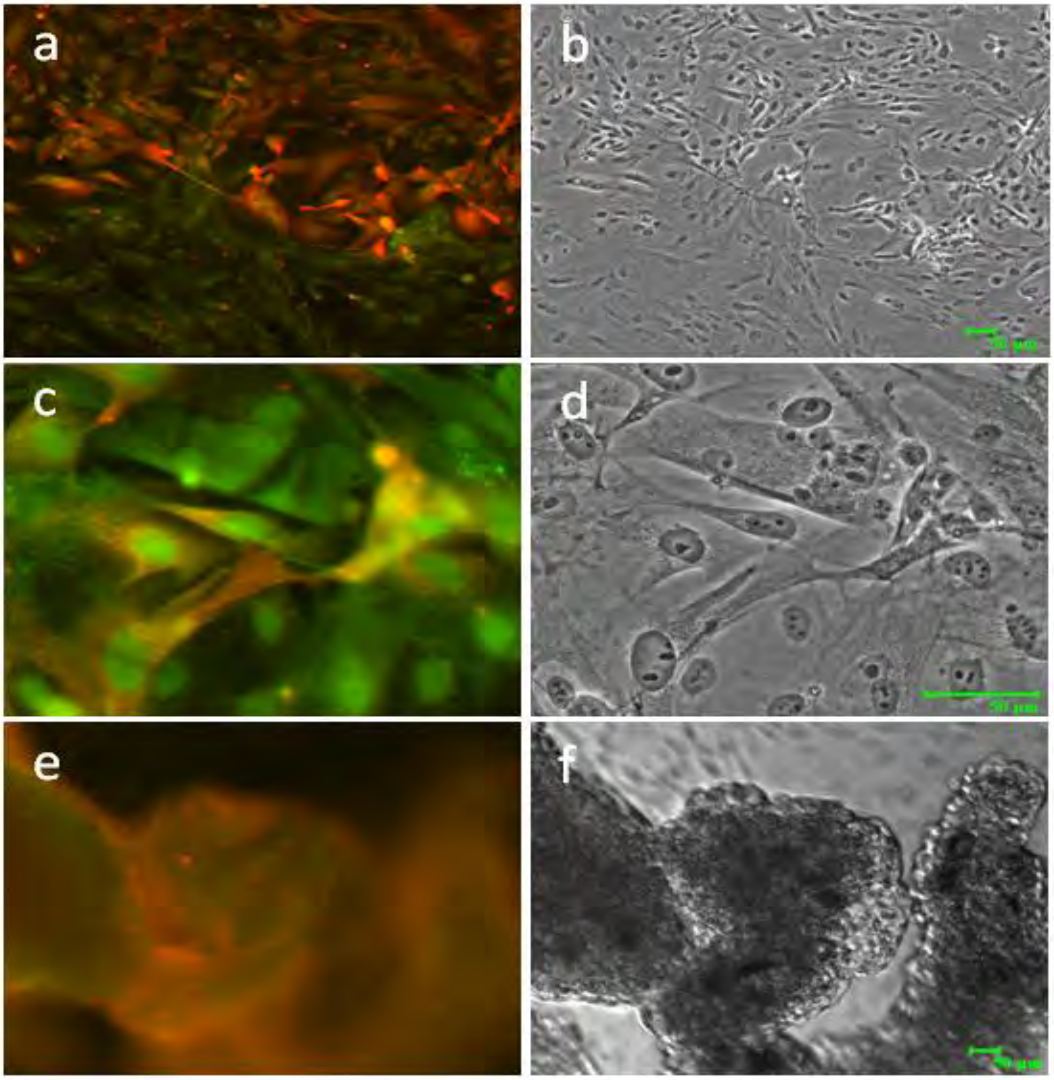


Figure 2.8. Dual fluorescence labelling for intermediate filaments in explants and mixed cell populations

(**a, c**) Co-culture of HFF and female reproductive tract primary cells; (**e**) isthmus explant tissue. (**b, d, f**) corresponding phase-contrast images. Anti-cytokeratin 19 (**red**) and anti-vimentin (**green**).

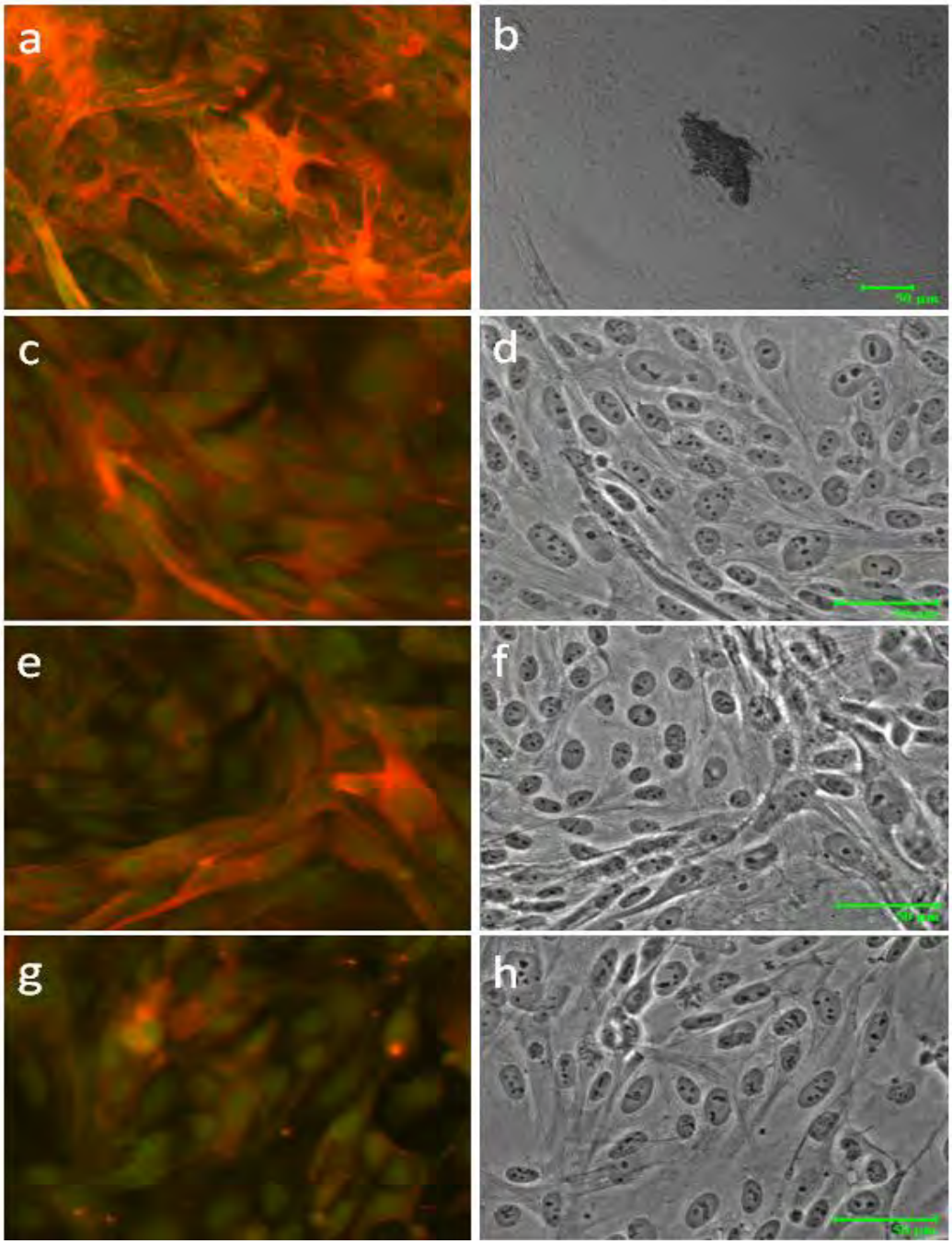


Figure 2.9. The effect of subculture on epithelial and fibroblastic marker expression

(**a**, **c**, **e**, **g**) Dual immunofluorescence labelling of cytokeratin (**red**) and vimentin (**green**) in: (**a**) primary cultures; (**c**) 1st passage cultures; (**e**) 2nd passage cultures; (**g**) 3rd passage cultures. (**b, d, f, h**) Corresponding phase-contrast images. Cells were derived from ampulla regions of the female reproductive tract tissue.

2.3.5 Selection of sperm for use in sperm-female tract interaction experiments

Human sperm were selected on their ability to migrate through a viscous layer of 1% methylcellulose into sEBSS. This method was based on a development of the direct swim-up preparation technique (Mortimer, 1994). The addition of Syto64 (5 μM) to semen during the liquefaction stage allowed Syto64 labelled sperm to be collected after swim-up without the need for further washing steps, as seminal plasma was retained underneath the layer of methylcellulose. Preliminary results carried out in our laboratory suggest that the modified methylcellulose swim-up technique selects sperm populations with lower DNA damage (data not shown) and high motility. In this study, a subset of preparations was performed to assess sperm recovery via the modified methylcellulose preparation. The concentration of sperm was also assessed before and after performing the selection method and indicated that sperm recovery was 10.5 ± 1.9% (mean ± SEM, n = 7). Computer aided semen analysis (CASA) indicated that sperm prepared by this method displayed a mean progressive motility of 72.0 ± 3.8% (mean ± SEM, n = 7) compared to 33.3 ± 4.5% observed in seminal plasma (mean ± SEM, n = 7).

2.4 DISCUSSION

Several methods have been reported for isolation and culture of epithelial cells including enzymatic pre-treatment with trypsin-EDTA (Ouhibi *et al*., 1989; Sattar *et al*., 1999) or collagenase (Pacey *et al*., 1995a,b; Takeuchi *et al*., 1991) and in combination (Jung-Testas *et al*., 1986) to aid mechanical separation (Grudzinskas *et al*., 1994). In this study, mechanical isolation without enzymatic pre-treatment was suitable for the preparation of small explants of epithelial cells for single cell imaging. This process was rapidly completed only hours after surgery and therefore is ideal for studying *ex vivo* sperm-female tract interactions. Epithelial cells derived from the isthmus region were more difficult to obtain for fresh explant imaging and primary culturing due to glutinous properties. This may be due to the increased thickness of the lamina propria and smooth muscle in this area, which may have decreased enzymatic digestion efficiency with collagenase and/or mechanical manipulation. Nevertheless, epithelial cells were obtained from all regions and cultured to generate primary cell lines for use in later experiments.

In this study, endometrial and oviductal epithelial cells were observed to grow in an epithelioid growth pattern, as previously reported by other studies (Bongso *et al*., 1988; Bongso *et al*., 1989; Comer *et al*., 1998; Henriksen *et al*., 1990). Both secretory and ciliated cells were observed in cell cultures. These cell types have been reported to be functionally different with secretory cells producing oviductal specific glycoproteins (OGPs) (Rapisarda *et al*., 1993) and ciliated cells playing a role in gamete transport and formation of sperm reservoirs (Suarez *et al*., 1991a). In cultures originating from ciliated explants, ciliated cells appeared in patchy clusters accounting for approximately 5% of total cell population. The patchiness of ciliated cells in primary cultures is likely to have originated from small clusters

of epithelial cells seeded during the initial preparation procedure. This is because ciliated cells observed within primary cultures were sparse and not seen to proliferate. This agrees with the concept that ciliated cells are terminally differentiated and non-proliferating cells similar to ciliated cells of the upper respiratory tract, as suggested by Comer *et al*. (1998). In addition, the relative proportion of ciliated to secretory cells may have been influenced by reduced plating efficiency due to active cilia beating physically preventing attachment to the surfaces, as previously reported by Henriksen *et al*. (1990). Cilia observed on primary cells were prominent, well developed and appeared to move synchronously. After approximately 24 hours of culture, cilia movements lost synchronicity with deciliation occurring approximately 6-7 days later.

OECs express both oestrogen and progesterone receptors (Amso *et al*., 1994). There is conflicting evidence for (Donnez *et al*., 1983; Donnez *et al*., 1985; Eddy *et al*., 1978; Verhage & Brenner, 1976) and against (Ouhibi *et al*., 1989; Thomas *et al*., 1995c) steroid hormones effecting morphology of cells in culture. In a subset of cultures, supplementation of media with oestradiol-17β extended the observation of active cilia on cells by approximately 2-3 days however, no induction of ciliated morphology was observed. This is in agreement with other studies (Goldberg & Friedman, 1995) suggesting that the process of dedifferentiation of ciliated cells *in vitro* can be delayed by oestradiol-17β treatment but cannot promote the differentiation of secretory into ciliated phenotypes. Comer *et al*. (1998) reported the induction of a ciliated phenotype in secretory cells by oestradiol-17β measured through increases in cilia marker expression, $LhS28^{+}$ however, no quantification of active cilia was documented (Comer *et al*., 1998).

In human, secretory cells undergo cyclical maturation and regression during the menstrual cycle (Crow *et al*., 1994; Grudzinskas *et al*., 1994; Verhage *et al*., 1979). Oestrogen has been suggested to regulate secretion of OGPs, some of which are up-regulated around the time of ovulation (Gandolfi, 1995). In the present study secretory cells were dominant, displaying prominent nuclei with surface microvilli. No visual differences could be identified with oestradiol-17β supplementation in cultures.

Fibroblastic contamination has been frequently reported to occur during primary cell culture (Bongso *et al*., 1989; Grudzinskas *et al*., 1994; Henriksen *et al*., 1990). In this study fibroblast-like cells were seen to occur particularly after subculture appearing long, flat and spindle-shaped with several long cytoplasmic processes similar to the description reported by Bongso *et al*. (1989). Phase-contrast microscopy has been widely used to assess cells as either epithelial or fibroblast-like. This alone may not be accurate enough especially for an inexperienced researcher. A more accurate method to determine cell type is to observe staining characteristics of intermediate filament proteins, namely cytokeratin (for epithelial cells) and vimentin (for mesenchymal cells) (Grudzinskas *et al*., 1994). Intermediate filaments represent a group of cytoskeletal components typically 10 nm in diameter located in the cytoplasm of virtually all mammalian cells (Pieper *et al*., 1992). Intermediate filaments are expressed in a developmental and tissue specific fashion (Pieper *et al*., 1992). Cytokeratins are part of the intermediate filament family, consisting of about 20 related polypeptides with each tissue having a specific expression of these cytokeratin types. Expression of cytokeratin 19 has been used in previous studies to characterise human OECs (Comer *et al*., 1998; Lee *et al*., 2001; Moll *et al*., 1983). Vimentin is an intermediate filament present in both normal and pathological tissue of mesenchymal derivation including fibroblasts, endothelial cells and

lymphoid tissue (Gupta *et al*., 2006). Vimentin expression in epithelial cultures has also been reported to be an *in vitro* sign of dedifferentiation (Giese & Traub, 1986; Pieper *et al*., 1992; Siebert & Fukuda, 1985). As both anti-cytokeratin and anti-vimentin antibodies have little or no reported cross reactivity with each other or other members of the intermediate filament family, it was advantageous to incorporate dual labelling for accurate assessment of samples. A similar method of dual labelling has previously been reported by Henrikson *et al*. (1990) as a three-step procedure using cytokeratin antigen, PKK1 and anti-vimentin.

In this study, dual immunostaining of both vimentin and cytokeratin intermediate filaments was achieved in a two-step procedure allowing the identification of epithelial and fibroblast-like cells. Almost all epithelial cells displayed some degree of cytokeratin 19 labelling. In addition, the use of anti-vimentin in epithelial cells produced a slight labelling pattern across the whole cell. This explains why co-localisation of vimentin (green) with cytokeratin (red) results in an „orange' colour even when preformed in the epithelial control cell line (figure 2.7 a). However, it has been documented that vimentin expression can occur during culture of epithelial cell lines and/or be induced by serum (Pieper *et al*., 1992; Rittling & Baserga, 1987). The co-expression of vimentin and cytokeratin has been reported in only a few cells of aged cultures of human OECs (Henriksen *et al*., 1990). Despite this, the strong contrast to staining patterns observed in HFF in which staining appeared to resemble intermediate filament patterns implies that the staining is non-specific in epithelial cells (figures 2.7 a, 2.7 c). There were no obvious differences observed between regions of the female tract or between patients.

Dual labelling of fresh explant tissue demonstrated a similar labelling pattern to that observed in primary cultures. Cytokeratin labelling can be seen particularly around the edges of explant tissue (figure 2.8 e), however there appears to be a stronger staining for vimentin. This may have been due to technical problems related to the thickness of explant tissue and retention of vimentin primary antibodies, or could indicate underlying connective tissue of mesenchymal origin. It may also be simply due to non-specific binding of vimentin. Despite the possibility of non-specific binding of anti-vimentin in this study, this method appeared to be successful in distinguishing cell types. This was demonstrated by co-culturing cells of known epithelial and mesenchymal origin at opposing ends of the culture dish. The two cell types could be distinguished by expression profiles (figure 2.8 a).

There was a significant reduction in the intensity of cytokeratin 19 labelling observed after subsequent passaging (figure 2.9) however, cytokeratin 19 expression could still be observed in the majority of cells even after the 2nd passage. Vimentin staining of intermediate filaments was typically seen after subculture in a small percentage of cells and increased with subsequent passages. Subculture was performed typically every 6-7 days. Therefore in this study we report the observation of fibroblast-like cells typically 15 days after initial culture. The identification of vimentin-positive cells has also been reported in cultures of 10-14 days (Henriksen *et al*., 1990). Some studies have reported a loss in cytokeratin during prolonged cultures of epithelial cells (Henriksen *et al*., 1990) with levels of cytokeratin-positive cells being 38% after 42 days of culture (Akhondi *et al*., 1997). Others have reported cultures of OECs to have predominantly retained cytokeratin expression (Baillie *et al*., 1997) however, noted a marked change in cell morphology, reduced ciliation and an appearance of fibroblast-like cells after 15-20 days.

An observation of phase-contrast halos was associated with the cytokeratin labelling patterns in primary cultures. Phase-contrast halos appeared in most cases to be associated with a more intense labelling of cytokeratin (figure 2.9). A phase-contrast halo indicates the refraction of light off a cell boundary and may represent cells of an increased height. Epithelial cells which appeared flattened, often associated with ongoing cell culture and non-polarised cell characteristics, displayed a more even, less intense labelling pattern for cytokeratin. It would be interesting to investigate whether this labelling intensity was correlated with cell polarity. Polarised cells resemble *in situ* morphology and may be particularly important to consider for ongoing cell cultures. Maintenance of polarity in epithelial cell culture can be encouraged by using air-liquid interface collagen inserts and may be beneficial in maintaining epithelial-associated characteristics during extended culturing (Clark *et al*., 1995; de Jong *et al*., 1994; Hanamure *et al*., 1994; Kervancioglu *et al*., 1994b; Levanon *et al*., 2010). This type of culturing system was trialled in the present study but abandoned as they were not suitable for imaging.

An important aspect when studying sperm-female tract interaction is the selection of sperm displaying characteristics of functional competence. In addition, sperm must be effectively isolated from seminal plasma. Not only do seminal plasma components modify sperm behaviour but the presence of cytokines and prostaglandins may promote inflammatory pathways and cellular toxicity in female tract cells (Maegawa *et al*., 2002; Politch *et al*., 2007; Robertson, 2007; Scott *et al*., 2009). In the present study, the standard swim-up technique was modified by incorporating an additional layer of viscous medium between semen and the collection media. Sperm penetration into 1% MC 4000 has been demonstrated to be comparable to that of human cervical mucus (HCM) (Ivic *et al*., 2002). The viscosity of HCM

varies during the menstrual cycle and there is a considerable difference in values reported in the literature due to measurement methods (Karni *et al*., 1971; Wolf *et al*., 1977). Wolf *et al*. (1977) found the average viscosity of HCM to range from 180-520 centipoise (cp) being lowest two days before ovulation. This reflects the influence of oestrogen on HCM hydration also reported to be highest periovulatory (Katz *et al*., 1997). In our laboratory, measurements of 1% MC4000 using the Wolf *et al*. (1977) method revealed an approximate viscosity of 140 cp, approximately 200 times the viscosity of water (at 37^{o}C). Although this is lower than values for HCM reported by Wolf *et al*. (1977), the relative increase in viscosity in comparison to current selection techniques is likely to better reflect physiology. Increased viscosity affects flagellar bending patterns and trajectory (Smith *et al*., 2009b). Viscoelastic properties of fluid may select sperm and this is currently not replicated in current selection techniques.

Preliminary results suggest that this modified swim-up technique may be useful in selecting sperm with lower levels of DNA damage whilst displaying characteristics associated with functional competence. Sperm motility has been correlated to sperm chromatin structure (Giwercman *et al*., 2003) and it is widely accepted that the female reproductive tract plays a crucial role in selection of sperm through motility and morphology characteristics (Holt & Van Look, 2004; Satake *et al*., 2006). More recently, in animal models it has been demonstrated that sperm with chromatin damage may have reduced ability to bind and interact with oviductal epithelium (Ardon *et al*., 2008) and interact with oocytes (Hourcade *et al*., 2010). Therefore, selection of sperm with low DNA damage would be advantageous to model sperm-female tract interactions.

In conclusion, this study has demonstrated that donated female reproductive tract tissue can be used for preparing fresh native explants suitable for immediate use in studying sperm-female tract interactions at the single cell level. Additional preparation techniques using enzymatic pre-treatment with collagenase to aid separation of epithelial cells from underlying connective tissue can also be performed to generate primary cell cultures. This method is simple and inexpensive, producing primary cell cultures displaying epithelial characteristics similar to those of the immortalised OECs in terms of appearance and growth patterns. The present work also agrees with previous studies reporting that epithelial cells from human oviducts can be grown in culture for several weeks retaining the expression of epithelial specific marker, cytokeratin. However, morphology and ciliation dedifferentiation is seen to occur approximately 7 days after culture and particularly after subculture. For these reasons an effort will be made to use primary cells within 6 days of culture without passaging to maintain epithelial characteristics for investigating sperm-epithelial interactions. Finally a novel method for preparing and selecting sperm has been introduced in an effort to mimic physiological events encountered during sperm transport through the female reproductive tract.

CHAPTER 3

Sperm-induced $[Ca^{2+}]_i$ signalling in female reproductive tract cells

3.1 INTRODUCTION

The female reproductive tract is not merely a conduit but is also secretory in nature producing a complex milieu of proteins, some of which have been demonstrated to play roles in both enhancing and suppressing the fertilising potential of sperm (Buhi *et al*., 2000; Grudzinskas *et al*., 1994; Yeung *et al*., 2009). Recently, it has been demonstrated that sperm may communicate with cells of the female tract controlling aspects of protein secretion. The ability of sperm to modulate their environment has been described in a variety of species including: altered gene expression profiles in mouse (Fazeli *et al*., 2004), turkey (Long *et al*., 2003) and fruit fly (*Drosophila*) (Mack *et al*., 2006); alterations in the oviductal secretory proteome in cow (Ellington *et al*., 1993b) and pig (Georgiou *et al*., 2005; Georgiou *et al*., 2007); and more recently, reproductive tract tissue remodelling in *Drosophila* (Kapelnikov *et al*., 2008a). Some authors have suggested that sperm-induced signalling may even have implications in regulating their own transport, through enhancing oviductal contractions (Kodithuwakku *et al*., 2007).

In human, the interactions of sperm and the female reproductive tract remain poorly characterised and relation of animal data can prove difficult due to fundamental differences in anatomy and reproductive diversity (reviewed in chapter one). What is known is that human sperm form intimate associations with oviductal epithelium (Baillie *et al*., 1997; Pacey *et al*., 1995a,b; Reeve *et al*., 2003) whilst being sensitive to modulation by oviductal secretions (Marin-Briggiler *et al*., 2010; Smith *et al*., 1998; Zumoffen *et al*., 2010). A previous study has reported that the concentration of cytosolic calcium ($[Ca^{2+}]_i$) in cultured equine oviductal epithelial cells (OECs) is altered in the presence of sperm (Ellington *et al*., 1993c). This could be upstream of other observed changes and warrants further investigation.

$[Ca^{2+}]_i$ signalling is known to induce activation of signal transduction cascades which can alter cellular functions almost instantly and is often implicated in cellular communication. Increases in $[Ca^{2+}]_i$ have been implicated in gene regulation (Di Capite *et al*., 2009), ion and fluid secretion (Petersen, 1992; Shuttleworth, 1997), absorption, volume regulation and immune response to pathogens (Zhang & O'Neil, 1999). In mammalian ciliated cells, elevations in $[Ca^{2+}]_i$ are also associated with an increase in cilia beat frequency (CBF) (Salathe, 2007). An increase in CBF persists even when $[Ca^{2+}]_i$ return towards or reach baseline levels, indicating that even transient increases in $[Ca^{2+}]_i$ may have ongoing effects on cell physiology.

There are few studies that focus on $[Ca^{2+}]_i$ signalling in cells of the female reproductive tract. Dickens *et al*. (1996) studied a wide range of Ca^{2+} agonists in human OECs and found that only adenosine-5'-triphosphate (ATP) induced $[Ca^{2+}]_i$ signals. This is in agreement with several other reports suggesting that extracellular ATP can induce increases in $[Ca^{2+}]_i$ in OECs of several species including; human (Dickens *et al*., 1996; Hill *et al*., 1994), cow (Cox & Leese, 1995), mouse (Leung *et al*., 1995), hamster (Barrera *et al*., 2004) and primates (Villalon *et al*., 1995). In other cell types it has been reported that the bending of primary cilia can induce $[Ca^{2+}]_i$ signals (Singla & Reiter, 2006). As secretory OECs have been reported to express primary cilia (Hagiwara *et al*., 2008a), a potential role may exist for sperm-induced mechanical stimulation. Interestingly, increased CBF of OECs has been observed after co-incubation of sperm (Chiu *et al*., 2010; Morales *et al*., 1996; Singla & Reiter, 2006). Demonstration of sperm-induced $[Ca^{2+}]_i$ signals in cells of the human female reproductive tract would provide further evidence for a mechanism through which sperm-cell communication could lead to a change in cellular function.

Aim

- To assess the occurrence of $[Ca^{2+}]_i$ signalling in human female reproductive tract cells on exposure to sperm at the single cell level.

3.2 MATERIALS AND METHODS

3.2.1 Materials

12 mm round coverslips, (Warner instruments). 22 x 32 glass coverslips, (Appleton woods). 35 x 10 mm tissue culture dishes, (Falcon). 5ml syringes, (BD Biosciences). Adenosine-5'-triphosphate (ATP), (Sigma). Bovine Serum Albumin (BSA), Probumin™ Fatty Acid Free (Lot: 122), (Millipore). Calcium green-1 AM, (Invitrogen). Collagenase Type I, (Invitrogen). Hanks buffered salt solution (HBSS) with Calcium and Magnesium, (Invitrogen). Imaging chamber series 20, (Warner instruments). IVF culture oil, (Cook® Medical). Methylcellulose 4000CP (MC4000), (Sigma). Pluronic F-127, (Invitrogen). Poly-D-lysine (PDL), (BD Biosciences). Silicone grease, (Warner instruments). Sterile scalpel blades, (Appleton woods). Supplemented Earls balanced salt solution (sEBSS), (Invitrogen). Syto 64 red fluorescent nucleic acid stain, (Invitrogen).

3.2.2 Recruitment of female reproductive tract donors

Refer to section 2.2.2.

3.2.3 Preparation of human female reproductive tract cells and cell lines

Refer to section 2.2.3.

3.2.4 Labelling female reproductive tract cells for $[Ca^{2+}]_i$ imaging

Female reproductive tract cells and cell lines were labelled with 7.6 µM Calcium Green-1 AM in sEBSS for 1 h at 37^{o}C, 6% CO_2. Cells were then washed for 10 mins 3 times with fresh sEBSS supplemented with 10 mM HEPES. Cells were kept in the dark at all stages. During

replicates, an effort was made to keep the length between labelling and use in experiments equal.

3.2.5 Selection, preparation and capacitation of human sperm

Refer to section 2.2.5.

3.2.6 Setting up imaging apparatus

Two methods were used to image single cell $[Ca^{2+}]_i$ signalling in cells upon exposure to sperm and related treatments: Perfusion and injection (figure 3.1).

Perfusion chambers (series 20, Warner instruments) were assembled as depicted in figure 3.1a. The glass coverslip base on which cells had been grown on or in the case of explants, adhered to by pre-coating with 1% poly-D-lysine (PDL, 10 mg/ml) was sealed to the chamber using a small amount of silicone grease peripherally. Experimental treatments were added to a 5 ml disposable syringe and perfused into the chamber using a peristaltic pump. To minimise mixing of sequential treatments, each treatment was added to the syringe when the previous treatment contents were visibly low. The syringe and tubing were replaced after each experiment. The perfusion rate was measured at ~ 0.7 ml/min. The overflow was removed by vacuum suction from the outlet pool. The chamber shape was designed to allow laminar flow and fast exchange of environments, ideal for assessing treatment effects on cells whilst allowing the removal of any pre-existing treatment.

The injection method was carried out for experiments in culture dishes and is depicted in figure 3.1 b. Cells were either grown on or in the case of explants, adhered to the surface of

35 x 10 mm tissue culture dishes by pre-treatment with 1% PDL. Cells were then overlaid with 1.5 ml of sEBSS and 1 ml of IVF culture oil to prevent water evaporation during the experimental time course. Treatments were then injected using a 1ml sterile wide-bore tipped pipette near the field of view during the experiment. Treatment concentrations were calculated with consideration of the total volume after addition. The culture dish injection method was simple in design and used to introduce sperm near female tract cells with minimum turbulence.

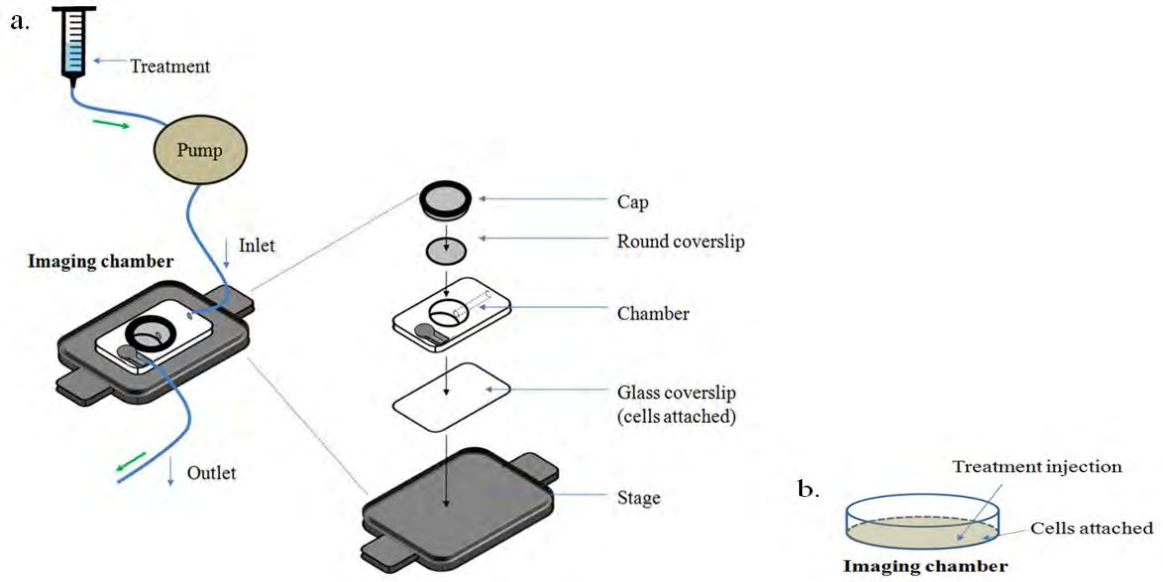


Figure 3.1. Preparation of imaging chambers for sperm-female tract cell interactions

(**a**) Perfusion and (**b**) injection methods.

3.2.7 Imaging and co-localisation of cells

Cells were imaged using a Nikon TE2000 inverted fluorescence microscope with Cairn Optosource and Apple Mac with Improvision Openlab software. Two sets of images were captured using alternating spectral wavelengths of 488/535 nm and 575/610 nm (excitation/emission) correlating to the spectral characteristics of Calcium green-1 AM and

Syto64, respectively (see appendix III: 3.i). Videos were co-localised for observation during data analysis.

3.2.8 Imaging data processing

Data was processed offline using Image pro plus II software (MediaCybernetics). An outline was drawn around each individual cell, considering as many as possible. Each cell was directly observed to ensure cellular viability, indicated by retention of $[Ca^{2+}]_i$ indicator (fluorescence). The average fluorescence intensity within the selected region of interest (ROI) was acquired for each individual cell. Raw intensity values were then imported into Microsoft Excel and normalised to pre-stimulus values with the following equation:

$$\mathbf{R = [(F - F_{rest}) / F_{rest}] \times 100}$$

Where ***R*** is normalised fluorescence intensity; ***F*** is fluorescence intensity at a time point; and $\boldsymbol{F_{rest}}$ is the mean of approximately 10 determinations of ***F*** acquired during the control period. This allowed individual cells to be observed from time-fluorescence intensity plots.

Note: normalised fluorescence intensity values (*R*) for individual cells were compiled to generate an average of normalised cell fluorescence for each time point (R_{tot}). The total series of R_{tot} values were then plotted to give the mean normalised cell fluorescence intensity for that experiment.

3.2.9 Imaging data and statistical analysis

Microsoft Excel Logic was employed to calculate the mean, variance, standard deviation, 95% confidence interval, median and maximum response values for each cell during test regions. An initial trial and error testing of various statistical approaches failed to identify every peak within an expected peak range and a new method termed „thresholding' was used

based upon a further development of the analytical methods employed by Kirkman-Brown *et al*. (2000 & 2003). Thresholding involved comparing treatment period values to the control period mean and standard deviation (Std.Dev). Time points were selected as indicated in fig 3.2. For a treatment to be reported as inducing a ‚significant' increase in $[Ca^{2+}]_i$, at least 2 values within the selected treatment period must be greater than the mean of the control period plus twice the standard deviation and be at least a 5% increase in relative fluorescence intensity:

$$\mathbf{X_r > ((con + 2 \times Std.Dev) \,\&\, (5\ Finc))}$$

Where $\boldsymbol{X_r}$ is the treatment period values; ***con*** is the mean value for the control period; ***Std.Dev*** is standard deviation of the control period; and ***Finc*** is a relative increase in normalised fluorescence (%).

This method of analysis was verified manually and confirmed to be effective in selecting fluorescence peaks occurring within a treatment time period whilst accounting for confounding factors such as experimental noise. Maximum peak heights were reported for significant responses using Excel Logic. As many experiments demonstrated a small but constant decline in base levels of fluorescence, the median value for the control period was subtracted from the maximum peak height. Maximum peak heights from each significantly responding cell were averaged for each treatment and recorded for each experiment.

3.2.10 Data grouping used for statistical means testing

SPSS (version 14.0) was used to test for normal distribution of data sets and subsequently used to perform the appropriate statistical test of means. A combination of both non-

parametric and parametric testing was used for data presented. The statistical approach taken has been stated in the figure legends and statistical significance was set at $P < 0.05$. The P values recorded are presented in appendix tables (appendix III). The percentages of significantly responding cells from each experiment in a series of repeats were pooled to calculate the mean percentage of cells responding to the treatment (stated in the text as mean ± SEM). Summary data tables correlating to figures are provided, indicating number of replicates, number of cells analysed and mean ± SEM values.

Data was grouped (post-analysis) retrospectively for comparison of the responsiveness of different regions of the female reproductive tract to sperm. In addition, the stage of the menstrual cycle and supplementation of oestrogen was also examined. An effort was made to perform experimental replicates on all three regions comparatively for each individual patient sample. However, only very small sections of tissue were received for each tract region and as a result some experimental series failed to analyse all three regions (isthmus, ampulla and endometrium) comparatively for each individual patient.

3.2.11 Experimental plan for studying sperm-induced $[Ca^{2+}]_i$ signals

The primary aim of experiments was to assess the ability of sperm ($500x10^3$) to induce $[Ca^{2+}]_i$ signalling within human female reproductive tract cells and a non-reproductive tract cell line. The preparation of non-conditioned and sperm-conditioned sEBSS is outlined in section 2.2.5. Briefly, non-conditioned sEBSS was filtered (pore size 0.2 µM) from sperm immediately after swim-up (time-1 h) whilst the remaining sperm population was incubated in capacitating conditions. After > 3 h, the capacitated sperm population was split into sperm-conditioned sEBSS (filtered from sperm) and sEBSS containing sperm (unfiltered). This treatment

preparation required equal splitting of the recovered sperm population. As a result, sperm-conditioned sEBSS was only obtained when sufficient sperm numbers were recovered after the methylcellulose swim-up method and in consideration of numbers of experimental replicates to perform. Adenosine-5'-triphosphate (ATP) at a concentration of 100 μM was also used as a positive control for eliciting a $[Ca^{2+}]_i$ response in all cell types.

The exact experimental plan varied depending on whether sufficient sperm numbers were recovered to permit use of sperm-conditioned sEBSS. However, the essential method remained consistent. For perfusion experiments, cells were initially perfused with sEBSS for at least 2 minutes before subsequently exposing cells to treatments. For perfusion experiments, an initial control period during the start of the experiment during the perfusion of sEBSS was used to assess rates of peaks induced by non-conditioned sEBSS (figure 3.2 a). After cells were exposed to each treatment, cells were perfused with sEBSS (washed) before subsequent treatment. For injection experiments, an injection of non-conditioned sEBSS was performed before the addition of sperm (figure 3.2 b).

For both methods, individual cell responses were assessed for significance by comparing normalised fluorescence values (Δ fluorescence (%)) from the expected response period (50 s) to a prior control period (50 s). Each individual cell was assessed for significant responses (for determination of significance responses, see section 3.2.9) and sorted using Excel logic. Excel logic was written to separate cells into categories indicating which of the various treatments elicited significant responses. This allowed linkage analysis to be performed (presented in Chapter 4). Numbers of cells responding to each treatment then contributed to a percentage of responsive cells within that experiment population. Each percentage of cell

responders for various treatments within experiments was averaged and used for statistical analysis.

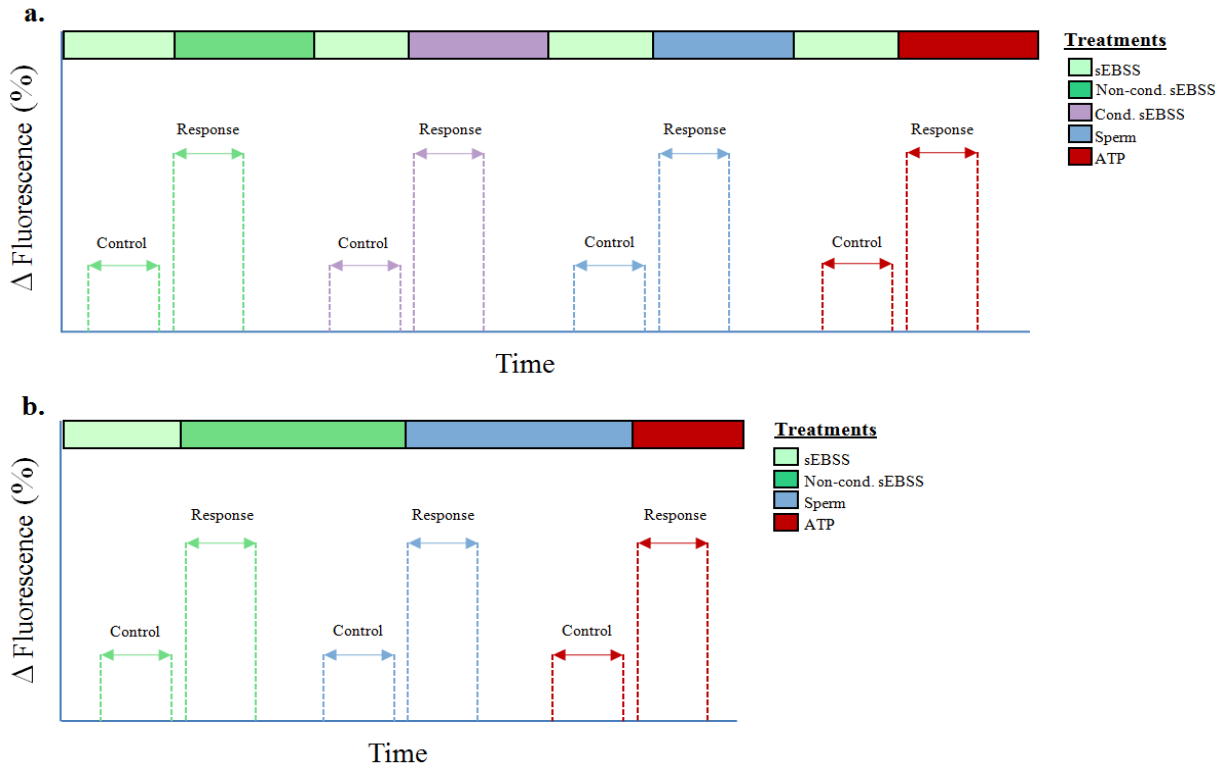


Figure 3.2. Experimental design for assessing cell responses to treatments

To determine changes in relative $[Ca^{2+}]_i$ in tract cells, the normalised fluorescence values (Δ fluorescence (%)) during treatment response periods were compared to control periods prior to treatment additions. All time periods (represented by double headed arrows) were assessed for an equivalent period of time (50 s). The percentage of significant responses to non-conditioned sEBSS treatment was used as the internal control and compared to all other sequential treatments in statistical tests. (**a**) Shows the experimental design for assessing treatment responses in perfusion experiments; (**b**) shows the experimental design for assessing treatment responses in injection experiments.

3.3 RESULTS

3.3.1 A novel approach to study sperm-female tract interactions

Single cell $[Ca^{2+}]_i$ imaging was carried out to investigate whether human sperm or products secreted during sperm capacitation generate a response in human female reproductive tract cells. The nature and timing of such an interaction in human remains unknown and therefore experiments were planned to capture the moment in which sperm first approach the female tract cells. This required introducing sperm to an imaging chamber in which female tract cells were attached, without causing artefacts due to delivery methods.

For initial experiments carried out, sperm were introduced to other cell types via perfusion into an enclosed imaging chamber (figure 3.1). Capturing images of alternating wavelengths specific to two differing dyes allowed real-time visualisation of sperm arrival in the focal field (figure 3.3; figure 3.4). Once sperm were observed, perfusion was stopped to allow cells to interact. This timing was also replicated for control periods and other treatments. Initial experiments revealed that a subpopulation of cells were responsive to sperm and sperm-conditioned sEBSS, resulting in a rapid increase in $[Ca^{2+}]_i$ upon exposure. Using Excel Logic, cells were sorted into categories based on treatment responses and viewed to validate that true peaks were being correctly identified by logic (figure 3.5). This perfusion method revealed that cell responses appeared to occur rapidly to both sperm-conditioned sEBSS and sperm, however, the proportion of latent responses occurring during the non-perfusion period was less for sperm-conditioned sEBSS in comparison to sperm treatment (figures 3.5 a, 3.6 a). The observed difference in responses related to perfusion led to more detailed investigation of the effect of perfusion activity with the timing of cell responses to treatments. Individual cell analysis revealed that some cell responses seemed to correspond with activity of the perfusion

system ~5-10% (figure 3.6.b). This relationship only existed after exposure to sperm-conditioned sEBSS or sperm, with such responses being absent during control perfusion activity.

As the frequency of sperm-conditioned sEBSS-induced $[Ca^{2+}]_i$ responses were lower during periods of non-perfusion, experiments were performed to investigate the effect of sEBSS and sperm when applied during constant perfusion (figure 3.7). Results demonstrated that continual perfusion of sperm-conditioned sEBSS resulted in an increased response frequency, comparable to that seen with sperm treatment. Generally, cell responses to sperm persisted during the sEBSS wash period but at a lower frequency, however this was not quantified (figure 3.7.a). Observations of co-localised videos revealed that during experiments of this type, fewer sperm were able to interact with female tract cells, probably due to shear force of continuous perfusion. Despite this, cells demonstrated responses to both sperm-conditioned sEBSS and sperm indicating that direct-binding of sperm was not solely responsible for eliciting $[Ca^{2+}]_i$ responses in the female reproductive tract cells.

To minimise the possibility of artefacts resulting from perfusion, a simple injection method was developed to introduce sperm near female reproductive tract cells with minimal turbulence (figure 3.8). An injection of non-conditioned sEBSS was used as an internal control. Using this approach, co-localised videos revealed that the introduction of sperm resulted in a rapid response in female tract cells similar to that observed using the perfusion method whilst allowing time to study subsequent binding and interaction behaviour.

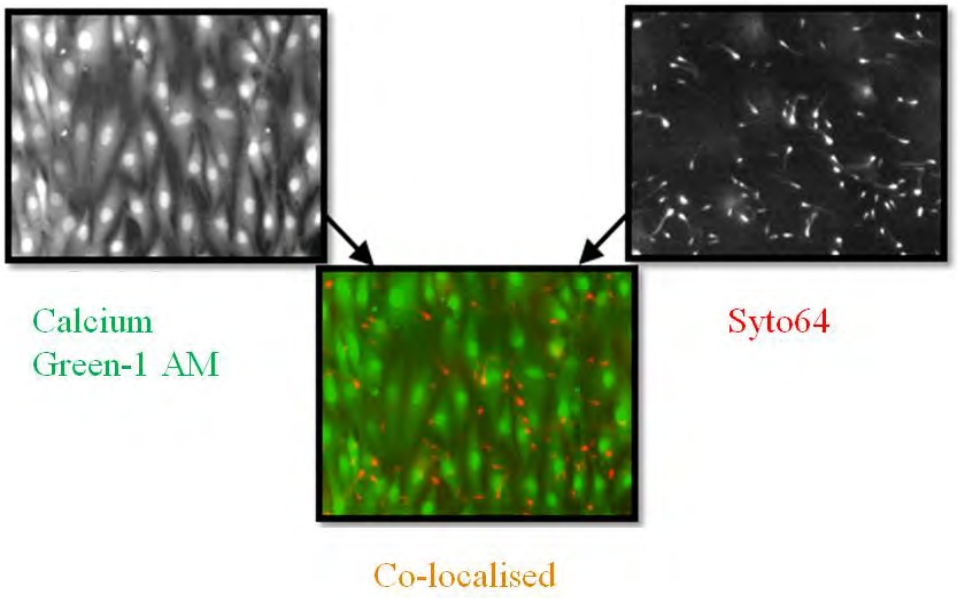


Figure 3.3. Co-localisation of cells

Dual labelling enables visualisation of sperm position relative to female reproductive tract cells and $[Ca^{2+}]_i$. Sperm and female tract cells are labelled with Syto64 (**red**) and Calcium green-1 (**green**), respectively.

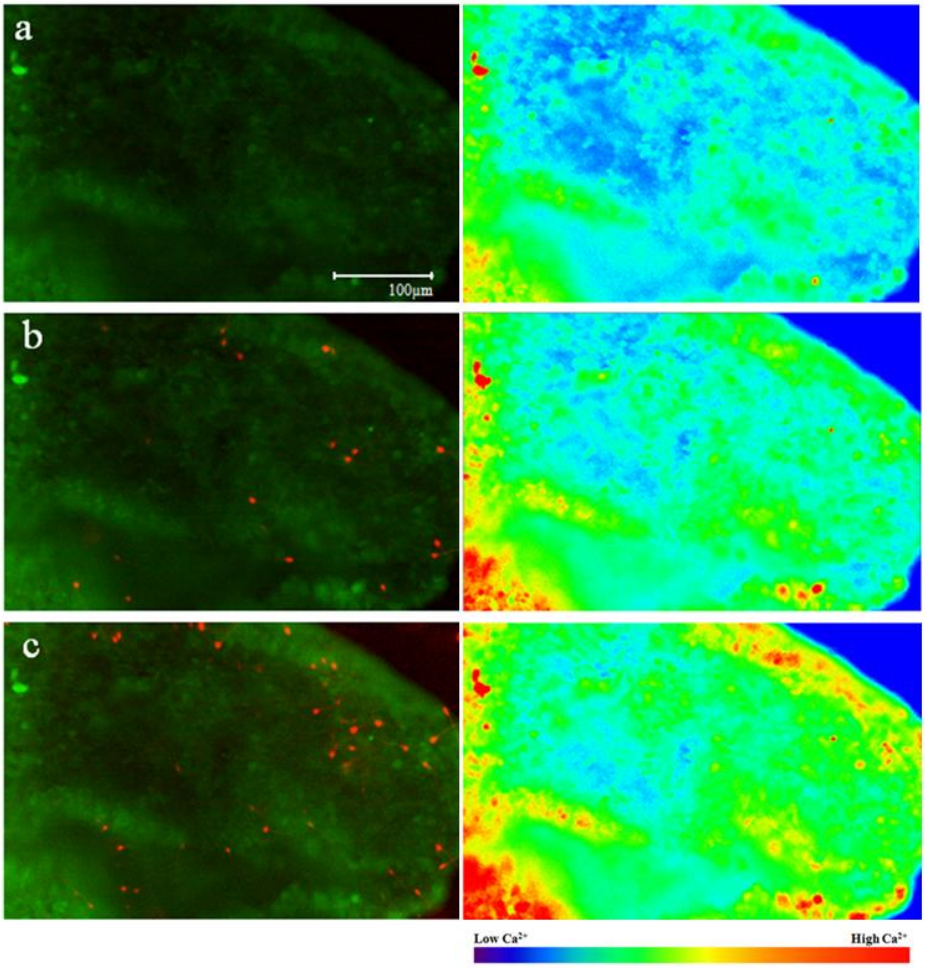


Figure 3.4. Visualising changes in $[Ca^{2+}]_i$ in response to sperm using co-localisation and pseudo-colouring

Panels demonstrate co-localisation of sperm and female tract cells (left) and corresponding pseudocolour images (right) for an ampulla explant: (**a**) before the addition of sperm; (**b**) after; and (**c**) after ATP (100µM) treatment. Warm colours show high $[Ca^{2+}]_i$.

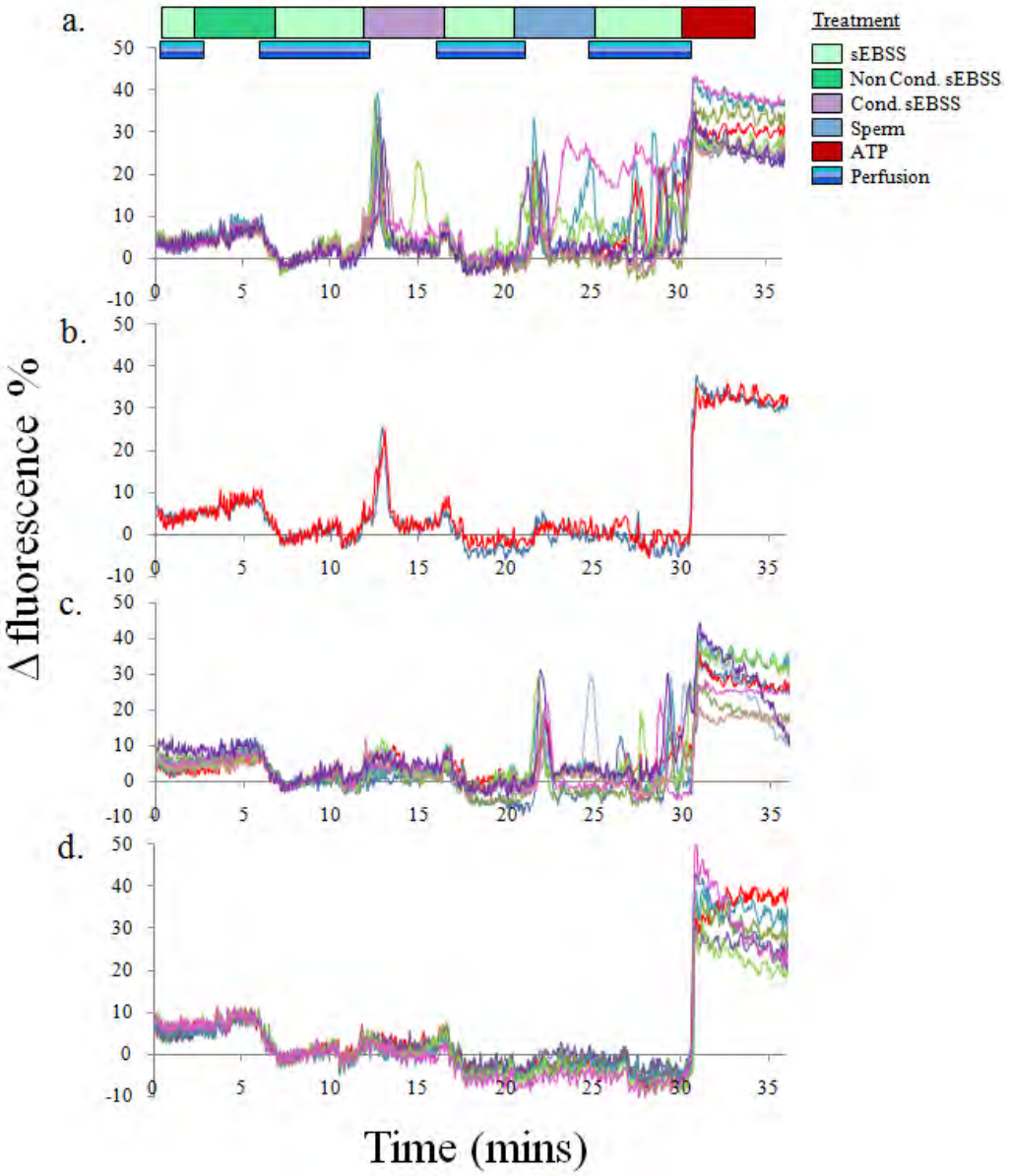


Figure 3.5. Sperm and sperm-conditioned sEBSS elicit $[Ca^{2+}]_i$ responses in OECs

Representative traces of an individual experiment performed on OE E6/E7 cells. Cell responses were separated by logical analysis into the following responsive groups: (**a**) both sperm-conditioned sEBSS and sperm; (**b**) sperm-conditioned sEBSS only; (**c**) sperm only; (**d**) ATP only. The treatment bar indicates time periods in which cells were exposed to the various treatments. The perfusion bar indicates time periods in which perfusion was active. ATP (100 μM) was used at the end of the experiment as a positive control for eliciting a $[Ca^{2+}]_i$ response.

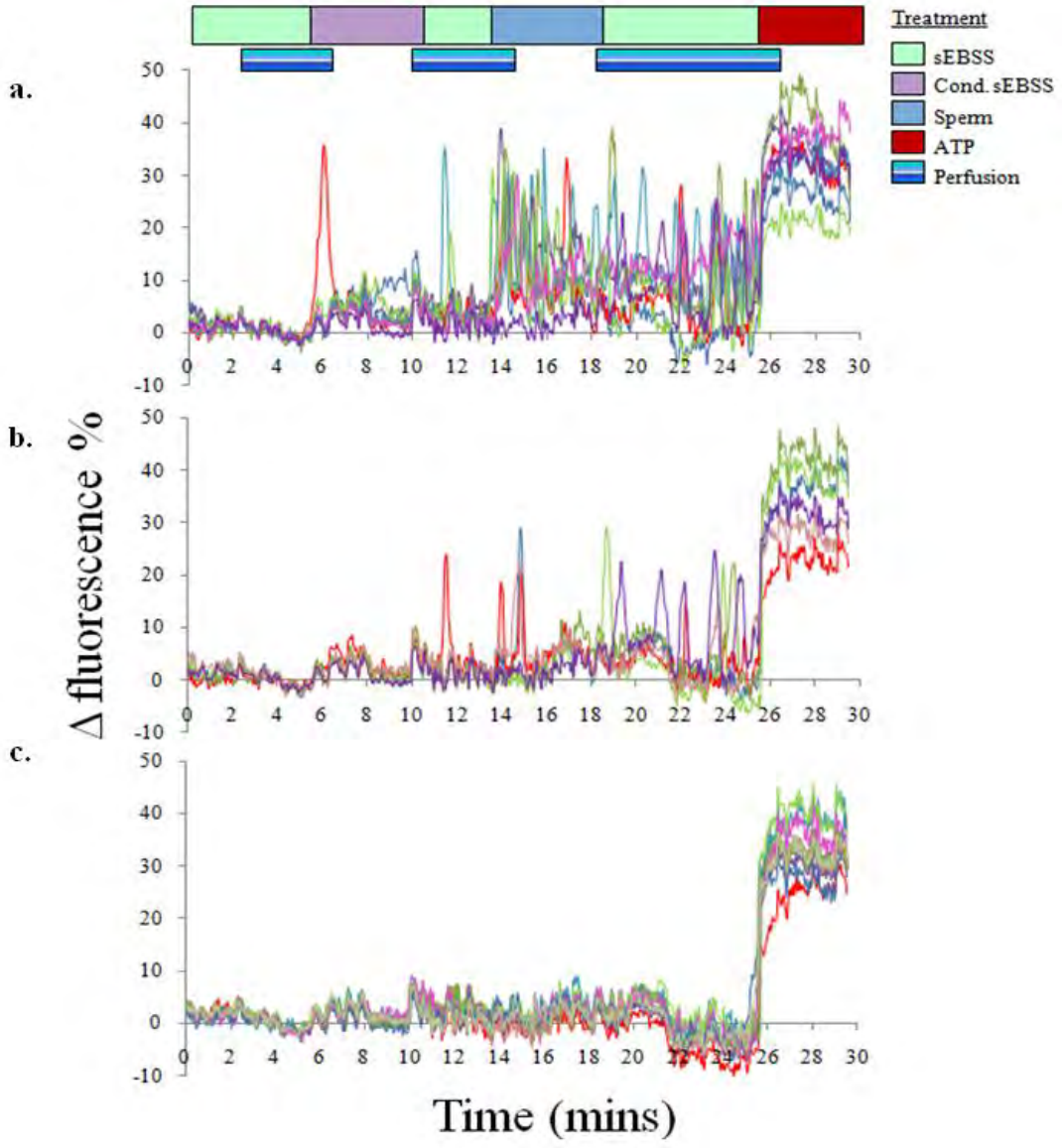


Figure 3.6. The effect of perfusion activity on cell responses to sperm and sperm-conditioned sEBSS

Representative traces of an individual experiment performed on OE E6/E7 cells using the perfusion method. Shown are representative traces of the following: (**a**) responsive cells; (**b**) cells in which responses occurred in synchrony with perfusion activity; (**c**) only ATP responsive cells. The treatment bar indicates time periods in which cells were exposed to treatments. The perfusion bar indicates time periods in which perfusion was active. ATP (100µM) was used at the end of the experiment as a positive control for eliciting a $[Ca^{2+}]_i$ response.

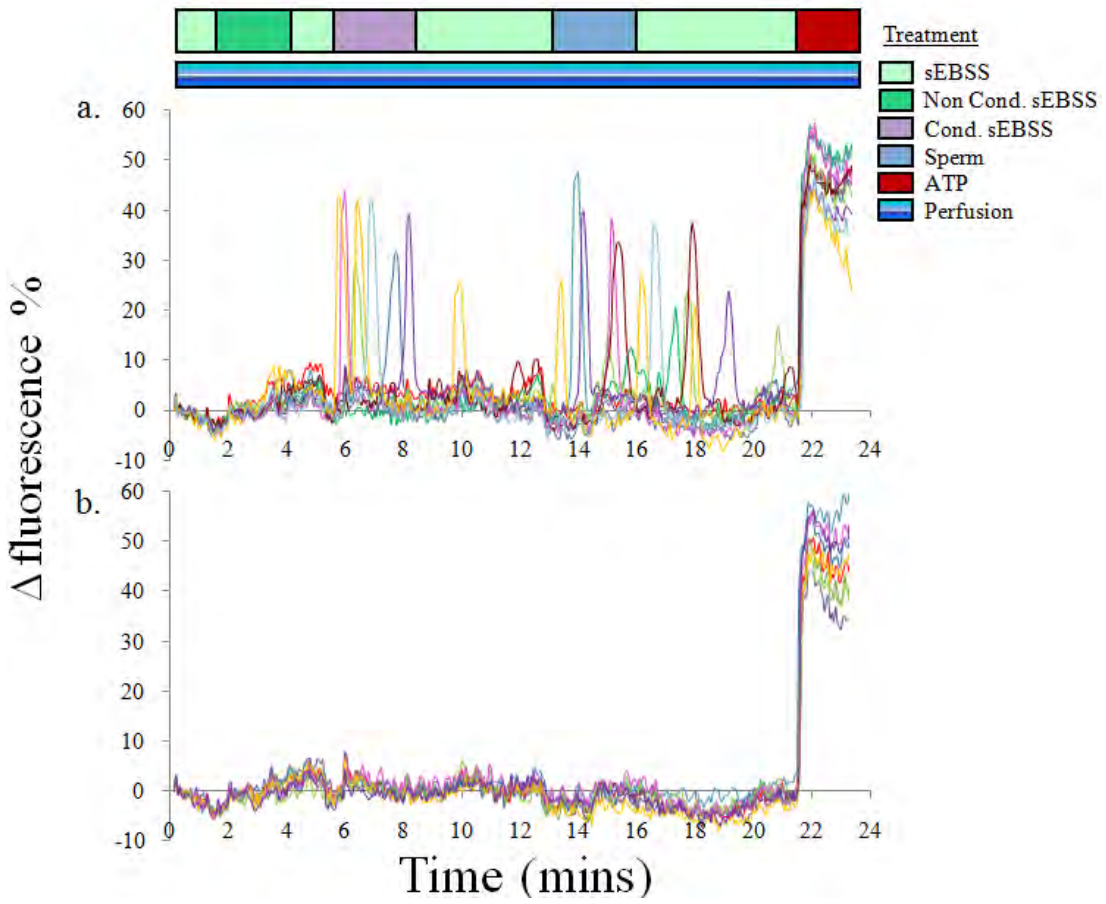


Figure 3.7. The effect of using constant perfusion on cell responses to sperm and sperm-conditioned sEBSS

Representative traces of an individual experiment performed on OE E6/E7 cells using a constant perfusion method. Shown are subgroups of the following: (**a**) responsive cells; (**b**) only ATP responsive cells. The treatment bar indicates time periods cells were exposed to the various treatments. The perfusion bar indicates the time periods in which perfusion was active. ATP (100 μM) was used at the end of the experiment as a positive control for eliciting a $[Ca^{2+}]_i$ response.

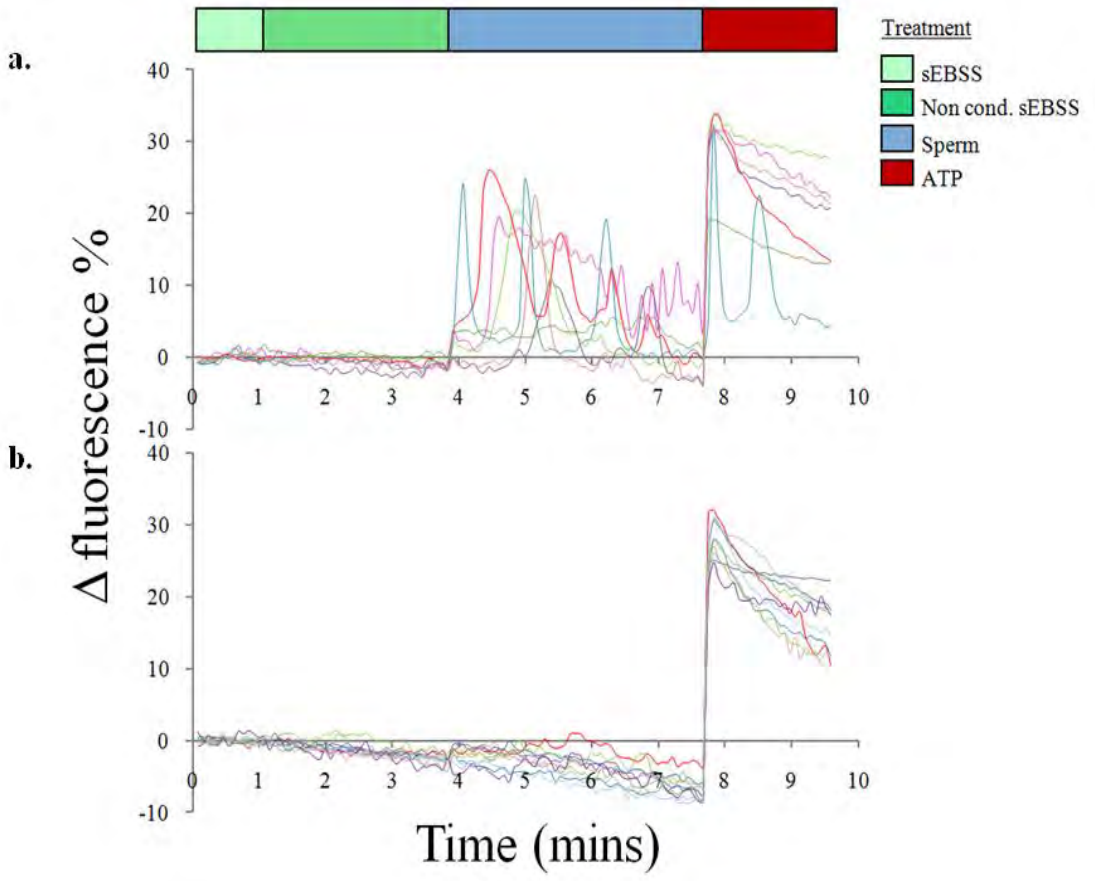


Figure 3.8. Studying sperm-induced $[Ca^{2+}]_i$ signalling in cells using a simple injection method

Representative traces of an individual experiment performed on primary isthmus cells using the injection method for introducing sperm to female reproductive tract cells. Shown are subgroups of the following: (**a**) responsive cells; (**b**) only ATP responsive cells. The treatment bar indicates time periods in which cells were exposed to the various treatments. ATP (100 μM) was used at the end of the experiment as a positive control for eliciting a $[Ca^{2+}]_i$ response.

3.3.2 Cell type responses to sperm

Having established sperm-induced $[Ca^{2+}]_i$ signalling in OE E6/E7 cells, several different cell types were used in parallel to assess whether sperm-induced $[Ca^{2+}]_i$ signalling can be examined in human female reproductive tract explants and primary cell lines. In addition, a human non-reproductive tract cell line, HFF, was also used for comparison. In total 16,097 cells were individually assessed for significant increases in $[Ca^{2+}]_i$ in response to sperm. This included explants (29 experiments), primary cell lines (53 experiments), OE E6/E7 cells (28 experiments) and HFF (11 experiments). A combination of both perfusion and injection methods were used and details of the data separated by these two methods can be seen in appendix III: 3.ii.

A summary graph of the mean % of cell responses to non-conditioned sEBSS, sperm and ATP are shown in figure 3.9. The introduction of sperm to cells of all regions of female reproductive tract examined elicited a significant increase in $[Ca^{2+}]_i$ signalling in comparison to the control, non-conditioned sEBSS. In contrast, this effect was not observed in HFF. Explants and primary cell lines demonstrated a mean % population response to sperm treatment of 18.0 ± 2.0 and 18.3 ± 1.9%, respectively. Primary cells had a greater but insignificant increase in responsiveness to sperm-conditioned sEBSS in comparison to explants, 13.5 ± 3.6% and 8.2 ± 1.5%, respectively. OE E6/E7 cells were significantly more responsive to sperm than other cell types examined ($P<0.05$). For all cell types there appeared to be a general increase in the % of cells responding to sperm treatment compared to sperm-conditioned sEBSS, although this was only significant in the OE E6/E7 cell line ($P<0.05$; figure 3.9; table 3.1).

When analysing explants it was difficult to detect changes in $[Ca^{2+}]_i$, as thickness and architecture of the epithelial surface meant that not all cells could be clearly visualised. ATP was useful in identifying cells that demonstrated a detectable change in $[Ca^{2+}]_i$ (figure 3.4). ATP was found to be a potent inducer of $[Ca^{2+}]_i$ response in all cell types studied and was used at the end of the experiment as a method for „calibrating' sperm-induced $[Ca^{2+}]_i$ responses. The average % of cells that responded to ATP ranged from 80.2 - 93.5% (figure 3.9; table 3.1). Both primary cell lines and HFF were observed to be slightly less responsive to ATP. Almost all responses to sperm occurred in cells that were responsive to ATP (data not shown). As a result we speculate that an inability to detect a significant response to ATP may indicate an inability to detect changes in Ca^{2+} in those cells when exposed to sperm.

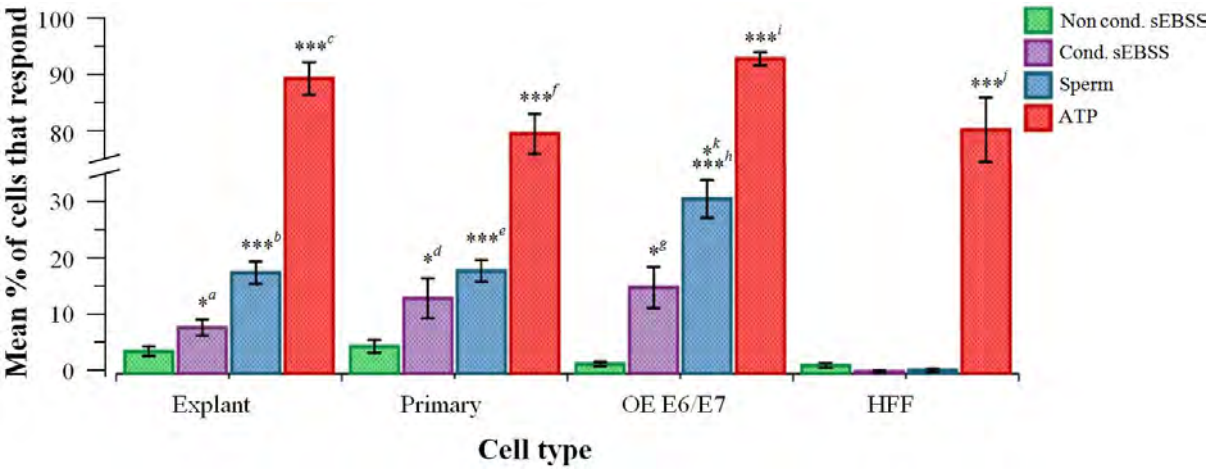


Figure 3.9. Mean % of cell populations that initiate a $[Ca^{2+}]_i$ response to differing treatments separated by cell type

Data was classified by logical analysis of significant responses. Female reproductive tract explant tissue, primary cell line and model cell line, OE E6/E7 cells were exposed sequentially to non-conditioned sEBSS, sperm-conditioned sEBSS, sEBSS containing sperm ($500x10^3$) and ATP (100µM). Human foreskin fibroblasts (HFF) were also studied as a non-reproductive tract cell type. All treatments were prepared in sEBSS + 0.3% BSA (fatty acid free), 10 mM HEPES. Error bars represent SEM. Asterisks denote statistical significance *** (P<0.001) and * (P<0.05) performed by a Wilcoxon (paired) test. Significant responses were demonstrated for explant tissue [a, b, c], when compared to explant responses to non-conditioned treatment. Significant responses were also demonstrated for primary cells [d, e, f], when compared to primary cell responses to non-conditioned sEBSS treatment. Significant responses were demonstrated for OE E6/E7 cells [g, h, i], when compared to OE E6/E7 cell responses to non-conditioned sEBSS treatment. A significant difference in response was only found between sperm and sperm-conditioned sEBSS in OE E6/E7 cells, [k]. Statistical P values are listed in appendix III: 3.iii.

	Explant				Primary cell line			
	Non-cond. sEBSS	Cond. sEBSS	Sperm	ATP	Non-cond. sEBSS	Cond. sEBSS	Sperm	ATP
No. of replicates (*n*)	29	5	29	17	53	12	53	30
No. of cells analysed	4460	1270	4460	2948	6187	1441	6187	3965
Mean % response ± SEM	4.0 ± 0.9	8.2 ± 1.5	18.0 ± 2.0	90 ± 2.9	4.9 ± 1.1	13.5 ± 3.6	18.3 ± 1.9	80.2 ± 3.6

	OE E6/E7				HFF			
	Non Cond. sEBSS	Cond. sEBSS	Sperm	ATP	Non Cond. sEBSS	Cond. sEBSS	Sperm	ATP
No. of replicates (*n*)	28	9	28	20	11	7	11	11
No. of cells analysed	4294	1248	4294	3380	1156	722	1156	1156
Mean % response ± SEM	1.7 ± 0.4	15.4 ± 3.6	31.2 ± 3.3	93.5 ± 1.1	1.5 ± 0.5	0.4 ± 0.2	0.6 ± 0.3	80.8 ± 5.7

Table 3.1. Summary data used for figure 3.9

3.3.3 Regional variation in responsiveness to sperm

Cell type responsiveness to sperm was further examined by separating data by anatomical region from which cells were derived (figure 3.10; table 3.2). This revealed a significantly greater % of responsiveness to sperm in explant tissue and primary cells derived from the isthmus region of the oviducts ($P<0.05$). For explants and primary cells, the mean % of isthmic cells that responded to sperm was 26.4 ± 2.7% and 24.2 ± 3.2%, respectively. OE E6/E7 cells (ampulla in origin) displayed a similarly level of responsiveness, 31.2 ± 3.3%. However, both ampullary derived explants and primary cells were significantly less responsive, 14.6 ± 2.4% and 12.5 ± 2.1%, respectively ($P<0.05$). Endometrial explants and primary cells responsiveness was comparable to ampullary cells, 15.0 ± 3.5% and 15.8 ± 3.5%, respectively. HFF were found to be non-responsive to sperm in comparison to controls with a mean cell population responsiveness of 0.6 ± 0.3% and 1.5 ± 0.5%, respectively. This was statistically lower than any other female reproductive tract cell type examined ($P<0.001$).

Data was re-examined by expressing the mean % of cells that respond to sperm within ATP responsive populations (appendix III: 3.v). This alternative way of expressing the data did not alter statistical findings but, there were subtle increases in mean % cell responses.

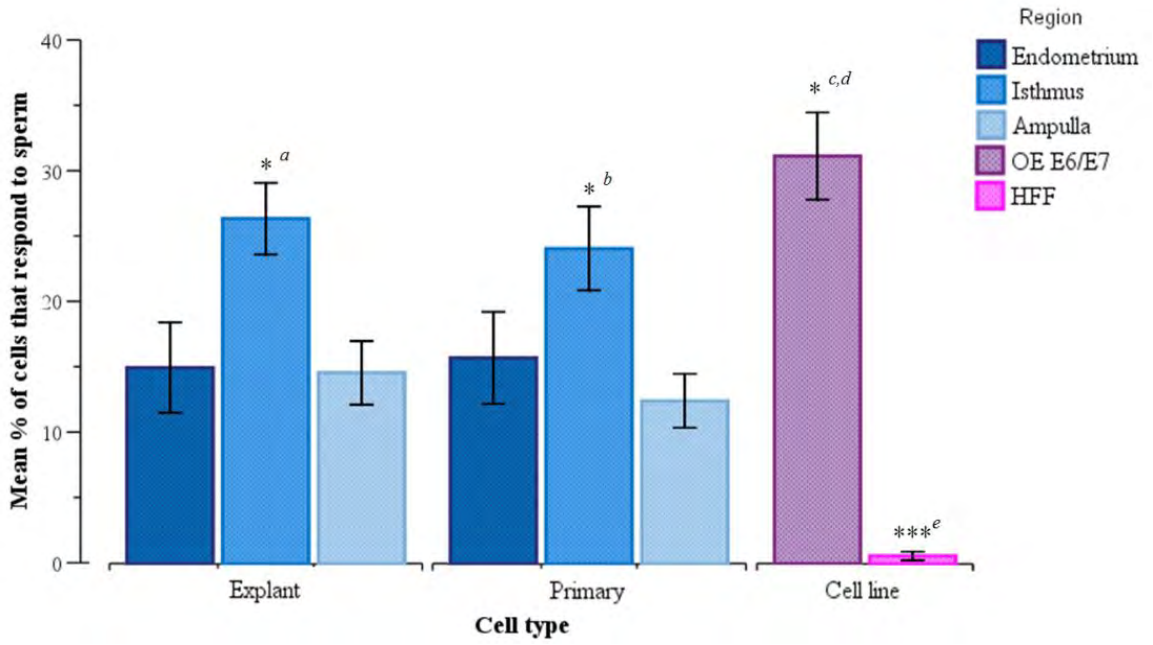


Figure 3.10. Mean % of cell populations that initiate a $[Ca^{2+}]_i$ response to sperm separated by cell type and region

Data classified by logical analysis of significant responses. Both explant tissue and primary cell lines derived from human female reproductive tract donors were separated into the three anatomical regions: endometrial, isthmic and ampullary epithelium. Parallel experiments were also performed in a model oviductal cell line, OE E6/E7 and non-reproductive tract cell line, human foreskin fibroblasts (HFF). Cells were exposed to sperm in sEBSS ($500x10^3$). Error bars represent SEM. Regions within both explant and primary cell types were compared for significance using Kruskal-Wallis test and Dunn's test. Cell lines were compared using Mann-Whitney U test. Asterisks denote statistical significance *** ($P<0.001$) and * ($P<0.05$). Isthmic cells for both explant and primary cell types showed a significant increase [a,b] in responsiveness in comparison to endometrial and ampullary derived cells within cell type. OE E6/E7 cells were compared to both explant [c] and primary [d] ampullary cells. HFF were compared to all cell type regions and the OE E6/E7 cell line [e]. Statistical P values are listed in appendix III: 3.iv.

	Explant			**Primary cell line**			**Cell line**	
	Endo.	Isthmus	Ampulla	Endo.	Isthmus	Ampulla	OE E6 E7	HFF
No. of replicates (*n*)	12	8	9	16	22	15	28	11
No. of cells analysed	1398	952	2110	1825	2357	2005	4294	1156
Mean % response ± SEM	15.0 ± 3.5	26.4 ± 2.7	14.6 ± 2.4	15.8 ± 3.5	24.2 ± 3.2	12.5 ± 2.1	31.2 ± 3.3	0.6 ± 0.3
Mean Δ Fluo % ± SEM	12.8 ± 2.0	6.9 ± 0.8	10.0 ± 1.9	15.5 ± 2.2	12.9 ± 1.1	9.1 ± 1.0	17.6 ± 0.9	8.9 ± 1.8

Table 3.2. Summary of data used for figure 3.10

3.3.4. Responsiveness to sperm separated by stage of the female menstrual cycle and oestrogen supplementation

Data was also sorted and separated according to estimated stage of the menstrual cycle, based on histology of the endometrium (figure 3.11 a, b; table 3.3). Samples in which the cycle stage was unclear or unremarked were excluded from analysis. In addition, values recorded for experimental replicates between patients were averaged to avoid skewing of the data as numbers of replicates from individual patients were often unequal. Replicate numbers were low (table 3.3) but there was a tendency for female tract tissue displaying proliferative phase histology to have a higher mean % of cells that respond to sperm than female tract cells derived during the secretory phase (figure 3.11 a, b). A statistical difference in responsiveness between proliferative and secretory derived tissue was only observed for primary cells (figure 3.11 a; $P = 0.028$).

In a subset of experiments, OE E6/E7 cells were cultured in media supplemented with 150 pg/ml of oestradiol-17β (figure 3.11.c; table 3.3). This level of supplementation has been reported to be effective in retaining and sometimes inducing a ciliated phenotype in OECs (Comer *et al*., 1998). Results indicate that a slightly higher mean % of cells respond to sperm after receiving oestrogen supplementation in comparison to controls but this was not significant, 31.2 ± 7.3% and 27.5 ± 6.4%, respectively ($P = 0.69$).

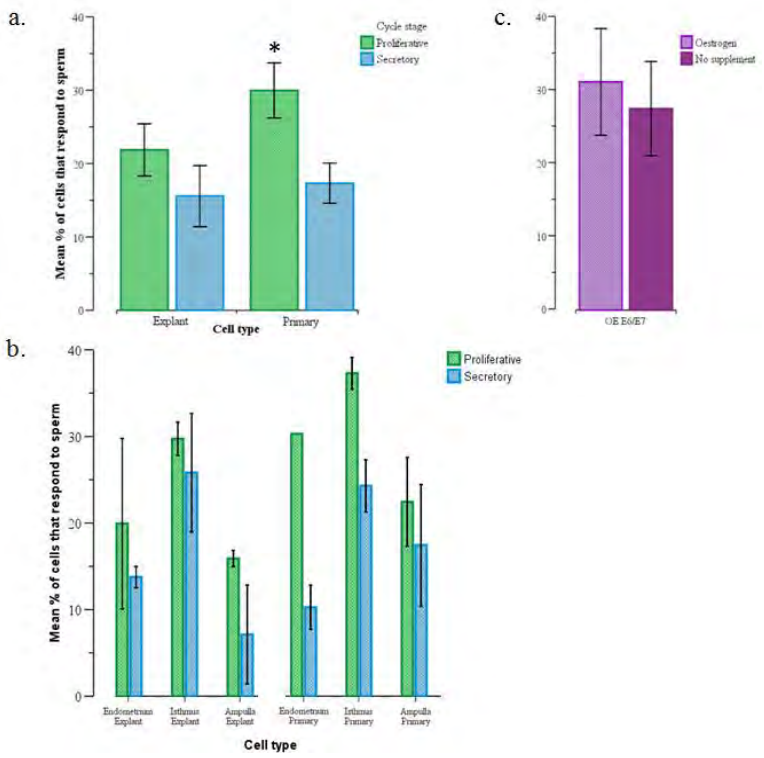


Figure 3.11. Mean % of cells that respond to sperm separated by stage of the female menstrual cycle and oestrogen supplementation

Data was classified by logical analysis of significant responses. Cell type responsiveness to sperm (500 x 10^3) was assessed by stage of menstrual cycle (estimated by endometrial histology) and in OE E6/E7 cells, supplementation with oestrogen (oestradiol-17β, 150 pg/ml). (**a**) Cell type responsiveness to sperm (region grouped); (**b**) cell type and regional responsiveness to sperm; (**c**) OE E6/E7 cells cultured with or without supplementation of oestradiol-17β. All experimental replicates for patient were averaged to minimise any potential bios. Error bars represent SEM. An asterisk denotes statistic significance ($P<0.05$) determined by Mann-Whitney U test. A significant difference in responsiveness was only found when comparing responses from proliferative to secretory derived cells in primary cell lines. Statistical P values are listed in appendix III: 3.vi.

	Explant					
	Endometrium		**Isthmus**		**Ampulla**	
	Proliferative	Secretory	Proliferative	Secretory	Proliferative	Secretory
No. of replicates (*n*)	3	2	3	2	3	2
No. of cells analysed	974	424	689	263	1115	574
Mean % response ± SEM	19.9 ± 9.8	13.8 ± 1.2	29.7 ± 1.9	25.8 ± 6.8	15.9 ± 0.9	7.1 ± 5.7

	Primary cell line					
	Endometrium		**Isthmus**		**Ampulla**	
	Proliferative	Secretory	Proliferative	Secretory	Proliferative	Secretory
No. of replicates (*n*)	1	4	2	4	2	2
No. of cells analysed	493	1291	849	1249	771	992
Mean % response ± SEM	30.3 ± n/a	10.3 ± 2.5	37.3 ± 1.8	24.3± 3.0	22.4 ± 5.1	17.4 ± 7.0

	OE E6/E7	
	Oest. Suppl	No supple.
No. of replicates (*n*)	5	5
No. of cells analysed	900	3394
Mean % response ± SEM	31.2 ± 7.3	27.5 ± 6.4

Table 3.3. Summary data for figure 3.11

3.3.5. Propagation of $[Ca^{2+}]_i$ in cells responding to sperm-induced signalling

$[Ca^{2+}]_i$ signals were occasionally observed to propagate in sequence through neighbouring cells after one cell initially responded to sperm. In contrast, increased $[Ca^{2+}]_i$ in response to ATP (100μM) were observed to occur in synchrony (figure 3.12).

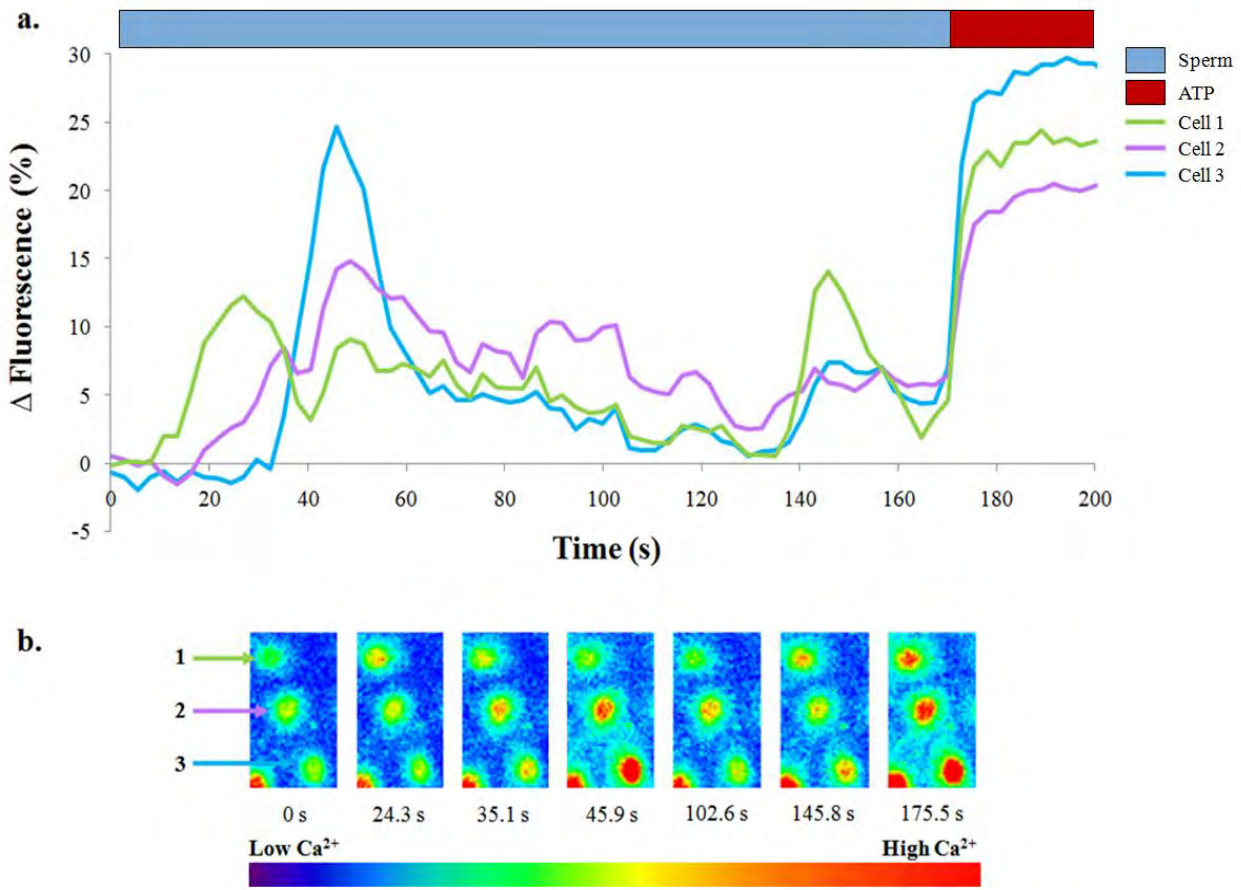


Figure 3.12. A representation of propagating $[Ca^{2+}]_i$ signals in cells responding to sperm-induced signalling

Figure (**a**) shows the fluorescence traces of three cells shortly after the addition of sperm and during addition of $[Ca^{2+}]_i$ agonist, ATP (100µM). Cells 1 to 3 demonstrate the initiation of transient increases in $[Ca^{2+}]_i$ in sequential order occurring at ~ 8, 17 and 32 s after exposure to sperm, respectively. The observed kinetics of the three responses to sperm varies in duration and peak amplitudes. Cells 1-3 demonstrate synchronous increases in response to ATP occurring at ~ 172 s; (**b**) pseudo-colour images of Cells 1 to 3 at various time points corresponding to the traces presented in (a). Note: Cells are identified by numbers on the far left panel and arrow colours correspond with fluorescence trace colours.

3.4 DISCUSSION

Recent progress in studying maternal communication with gametes has been made using high-throughput genomic and proteomic tools (Fazeli, 2008; Fazeli & Pewsey, 2008; Georgiou *et al*., 2007). Emerging evidence supports the notion that the female reproductive tract is „sensitive' to the arrival of sperm and modulates its environment to support the possibility of fertilisation (Georgiou *et al*., 2007). Despite this, the mechanisms by which tract cells recognise sperm remain poorly characterised. In this study we have demonstrated $[Ca^{2+}]_i$ signalling as a mechanism of cellular communication between sperm and female tract cells in human. Furthermore, we have found regional variation in responsiveness to sperm indicating that certain areas of the female reproductive tract may be more „sensitive' to detecting sperm.

Novel techniques for studying interaction

A novel approach has been developed for studying cell interactions using a dual fluorescence labelling technique in which both cell types can be selectively observed. This is achieved as the Calcium green-1 and Syto64 excitation and emission spectra have minimal overlap (see appendix III: 3.i). This approach allowed the co-localisation of both cell types whilst being able to monitor $[Ca^{2+}]_i$ signalling within female tract cells (figure 3.3; figure 3.4). In addition, sperm used in this study were selected by a novel modified methylcellulose swim-up technique in an attempt to replicate physiological selection of highly motile and morphologically superior sperm (section 2.3.5).

Initial experiments performed in the human oviductal cell line, OE E6/E7 revealed that both sperm-conditioned sEBSS and sperm can elicit Ca^{2+} mobilisation (figures 3.5; 3.6; 3.7, 3.8). Co-localised videos were important in making the observation that some cells responded

before intimate contact suggesting that sperm related secretions/products may be partly responsible for eliciting responses in tract cells. This is in agreement with fact that sperm-conditioned sEBSS was capable of inducing responses albeit to a lesser extent (figure 3.9; table 3.1).

Two different experimental approaches were used to study this interaction incorporating perfusion and injection methods for application of sperm suspensions or sperm-conditioned sEBSS. The perfusion technique allowed rapid exchange of environmental conditions ideal for assessing the effects of multiple treatments. However, a disadvantage of the perfusion method was less stable baseline fluorescence in cells particularly during on/off activity observed in figure 3.5. Dye leakage in intact cells can occur due to organic anion transporters and differs between cell types (Bird *et al*., 2008). The switching on of perfusion generally resulted in a fall in the base line fluorescence, probably reflecting removal of the $[Ca^{2+}]_i$ indicator. Baseline fluorescence of cells appeared more stable when perfusion was constant and during injection experiments (figures 3.7; 3.8).

Closer examination of cell responses in perfusion experiments also revealed a small percentage of cells (~ 5-10%) which appeared to respond in synchrony with perfusion activity after exposure to sperm and sperm-conditioned sEBSS (figure 3.6.b). This may have been related to female tract cells adjusting to a change in shear force. Cell responses to sperm-conditioned sEBSS appeared to largely occur synchronously with a reduced frequency when perfusion was switched off (figure 3.5; 3.6). The frequency of responses to sperm-conditioned sEBSS treatment appeared to increase during constant perfusion (figure 3.7.a). For sperm-induced responses, there were also cells that responded synchronously, comparable to sperm-

conditioned sEBSS treatment; however there appeared to be a greater occurrence of later peaks suggestive of an on-going stimulus for OE E6/E7. A simple injection method was found to be effective in studying sperm-induced $[Ca^{2+}]_i$ signalling. The injection of sperm resulted in a range of responses occurring similarly to those observed in perfusion experiments (figure 3.8). Amongst these response types included oscillating and sustained $[Ca^{2+}]_i$ responses.

With all of the above taken into account the following conclusions can be made: 1) both sperm-conditioned sEBSS and sperm in sEBSS can induce rapid $[Ca^{2+}]_i$ signalling in female tract cells; 2) Ca^{2+} mobilisation in female tract cells did not require intimate prolonged contact with sperm as responses were observed during constant perfusion experiments; 3) treatment with sperm appeared to elicit a higher number of $[Ca^{2+}]_i$ responses than observed with sperm-conditioned sEBSS; 4) $[Ca^{2+}]_i$ responses observed in the vast majority of cells are unlikely to be due to shear force caused by perfusion, as similar responses were observed using a slow injection technique.

Initial investigation of cell responses suggested that the timing of peaks varied with the majority occurring within the first 50 s of exposure to sperm. However, there were peaks occurring after this time particularly when treated with sperm. This may have resulted from subsequent binding and/or „tickling' of the cell's surface by sperm as it was seen less in pre-treatment with sperm-conditioned sEBSS. It is known that the bending of primary cilia can induce $[Ca^{2+}]_i$ signals and this in turn, may initiate increased CBF (Lorenzo *et al*., 2008; Masyuk *et al*., 2006; Satir & Christensen, 2007; Singla & Reiter, 2006). Increased CBF on OECs has been noted after the introduction of sperm (Chiu *et al*., 2010; Morales *et al*., 1996).

Such a change is interesting as it could influence transport of gametes and embryos through the oviduct.

Cell type responses to sperm

To our knowledge this is the first report of single cell $[Ca^{2+}]_i$ imaging using female reproductive tract explants. Imaging explants was technically challenging in part due to the thickness and complexity of the epithelial surface. In addition, explants were often difficult to attach to imaging chambers and as a result it was challenging to introduce sperm without focal drift. Nevertheless, subgroups of explant experiments were successful and revealed significant detectable $[Ca^{2+}]_i$ responses upon exposure to sperm and sperm-conditioned sEBSS.

Non-conditioned sEBSS treatment was used for each experiment as an internal control. Levels of cell responses to non-conditioned treatment were used to determine whether sperm treatments were significantly different and not an artefact of the method. The fact that all cell types including the human oviductal cell line, OE E6/E7 were significantly more responsive to sperm ($P<0.001$) and sperm-conditioned sEBSS ($P<0.05$) was convincing. This result was further strengthened by the relatively low responsiveness of HFF (figure 3.9; table 3.1).

Our results show that both reproductive explant tissue and primary cell lines responded at similar levels to sperm (figure 3.9; table 3.1). This finding could perhaps be explained by considering that the vast majority of primary cell experiments were performed within 6 days of isolation and culture. In addition, responses in explants may have been affected by reduced focal clarity lowering the ability to detect changes in $[Ca^{2+}]_i$.

OE E6/E7 cells appeared to be significantly more responsive to sperm and ATP treatments in comparison to other cell types (figure 3.9; table 3.1). It was noted that on many occasions primary cell lines in particular appeared unstable during experiments. Unstable basal Ca^{2+} levels may have reflected mechanotransductive mediated generation of Ca^{2+} signals, as primary cilia have been reported to act as a transducer of extracellular stimuli and shear flow (Masyuk *et al*., 2006; Satir & Christensen, 2007). It is also arguable that both explant tissue and primary cell lines are more unstable due to inevitable dedifferentiation that occurs once cells are removed from their physiological environment. This may have affected cell stability during experimentation resulting in less stable control periods and therefore greater standard deviations. These factors would have contributed towards lower detection of significant responses as treatment peak values were compared against control means and standard derivations. This may also explain why both explants and primary cell lines had higher levels of responses to non-conditioned sEBSS in contrast to OE E6/E7 cells (figure 3.9; table 3.1). The OE E6/E7 immortalised cell line is reported to be stable and to not require differentiating factors whilst retaining characteristics specific to OECs (Ling *et al*., 2005; Xu *et al*., 2001). It is also possible that differences between these cell types may be attributed to other factors such as OE E6/E7 cells being derived from the ampullary region of oviducts taken from a premenopausal woman during the proliferative phase of the menstrual cycle (Lee *et al*., 2001). Patients recruited during this study were not ruled out by age or menopausal status (see appendix II: 2.i). Nevertheless, all female reproductive tract cell types were significantly more responsive to sperm ($P<0.001$; paired) and sperm-conditioned sEBSS ($P<0.05$; paired).

To our knowledge only one previous study has reported the effect of sperm on Ca^{2+} mobilisation in OECs. Ellington *et al*. (1993c) reported $[Ca^{2+}]_i$ alterations in cultured equine

OECs (38 cells) measured before and 15 minutes after the addition of 2.5 x10^6 stallion sperm. 37 out of 38 cells demonstrated an increase in fluorescence intensity (correlating to an increase in $[Ca^{2+}]_i$). In contrast, this study focussed on rapid $[Ca^{2+}]_i$ signalling events occurring within ~50 s after the addition of sperm. It is possible that we have underestimated the total number of responsive cells as some would have undergone delayed responses.

The components responsible for inducing Ca^{2+} responses are currently unidentified. Potential candidates may include membrane fragments such as surface proteins shed during capacitation and/or metabolic products. Other possible targets may include the binding of integrins to arginine-glycine-aspartic acid (RGD) sequence containing ligands. This concept has been previously discussed by Reeve *et al*., (2003). RGD containing ligands are known to be capable of inducing rapid Ca^{2+} signalling (Iwao & Fujimura, 1996; Schwartz & Ginsberg, 2002). Sperm express both RGD-binding integrins and RGD containing intracellular matrix proteins (e.g. fibronectin (Fn) and vitronectin (Vtn)) on their surface membranes (Glander *et al*., 1998). Recently it has been demonstrated that Fn is expressed apically on human oviductal epithelium (Makrigiannakis *et al*., 2009). Vtn has also been found localised to the mucosal folds of the isthmic region of the oviducts (Schultka *et al*., 1993). Furthermore, Vtn was only observed in oviducts obtained from women of a younger age. However, it is expected that the RGD sequence containing proteins are likely to be located on the surface of sperm as only very low levels of RGD sequence containing oligopeptides have been reported to bind to sperm. Therefore, the location and identification of molecular candidates involved in signalling events remains uncertain but warrants further investigation.

Regional variation in responsiveness

Both explant and primary cell data sets were separated by anatomical origin; endometrial, isthmic and ampullary epithelium. Isthmic derived cells of both explant and primary cell types were found to be significantly ($P<0.05$) more responsive in comparison to other corresponding regions within cell types (figure 3.10; table 3.2). This is not the first study to suggest that the isthmus may differ in its response to sperm in comparison to other anatomical regions of the female reproductive tract. Recently, human oviductal explants and OE E6/E7 cells have been shown to upregulate adrenomedullin (ADM) expression after co-culture with sperm (Li *et al*., 2010). ADM has been implicated in stimulating cilia motility and was seen to be highest in the isthmic region after co-culture.

In most species studied to date, preferential and prolonged contact between sperm-oviductal epithelium occurs in the isthmus region (Hunter, 2005; Hunter & Leglise, 1971; Hunter & Nichol, 1983; Jansen, 1980; Lefebvre *et al*., 1995a; Suarez *et al*., 1991a). The isthmus region may function as a sperm storage reservoir until further stimulus around the time of ovulation promotes sperm release and progression to the ampulla region for fertilisation (Holt & Lloyd, 2010; Hunter, 2005; Talevi & Gualtieri, 2010). In human, identification of a sperm reservoir has been unsuccessful, however *in vitro* it has been demonstrated that higher numbers of sperm bind to isthmic compared to ampullary derived OECs (Baillie *et al*., 1997; Reeve *et al*., 2003). Interestingly, beads coupled with RGD sequence containing oligopeptides have been reported to preferentially bind to the isthmus in comparison to the ampulla region in human (Reeve *et al*., 2003). An increase in RGD-integrin interactions in the isthmus region, in theory, could contribute towards an increased responsiveness to sperm as observed in this study.

Recently, the presence of sperm within the female tract has been demonstrated to alter the expression of at least 20 proteins including fibrinogen, complement C3 (C3) and oviductal glycoprotein (Georgiou *et al*., 2007). Both fibrinogen and C3 have been implicated in modulating the immune response to pathogens and may play a role in clearance of sperm (Anderson *et al*., 1993; Lee *et al*., 2004). In light of this evidence it would be tempting to hypothesise that sperm may induce signals particularly to isthmic cells during transport that may induce changes modifying the oviductal environment. These alterations of the female tract environment may be important in regulating the motility, survival and clearance of sperm.

Gap junction mediated cell communication

The mean % of cell responders to sperm varied considerably, not only between patient samples but also between experimental replicates on the OE E6/E7 cell line performed in parallel with the same sperm sample. The reason for such variation remains unknown. In viewing cell responses, it was often observed that active cells seemed to trigger adjacent cells in a manner resembling gap junction signalling (figure 3.12). Cell confluency may have therefore impacted on cell-to-cell communication resulting in variation in percentages of cell responders. Gap junction mediated cell communication has previously been implicated in sperm-oviduct interactions with cultured equine OECs (Ellington *et al*., 1993c). Cell communication through gap junctions provides a mechanism in which cells may respond synchronously to external stimuli, without each cell being directly exposed to the primary stimulus (Ellington *et al*., 1993c; Schultz, 1985). By this concept, responses in only a subpopulation of 'receptive' female reproductive tract cells may be sufficient for propagation of a sperm-induced signal. Increased levels of secondary messengers such as Ca^{2+} have also

been found to enhance the permeability of gap junctions (Arellano *et al*., 1988). Therefore, gap junction signalling may play a role in propagating Ca^{2+} waves in response to sperm-induced signalling and hence aid communication between cells (Cotrina *et al*., 1998).

The data presented in this study demonstrate that sperm and products secreted by sperm can induce Ca^{2+} signalling in cells of the female reproductive tract. Initial findings suggest isthmic derived cells may be of increased receptivity to sperm-induced signalling. This initial communication between sperm and the female tract may be a mechanism contributing towards the observed sperm-induced modification of the female reproductive tract environment reported in other mammalian species.

CHAPTER 4

Characterisation of $[Ca^{2+}]_i$ responses in female reproductive tract cells

4.1 INTRODUCTION

The previous chapter focused on studying the regionalised responsiveness of female reproductive tract cells and cultures to human sperm. Having established that upon exposure to sperm, tract cells demonstrate cytosolic calcium concentration ($[Ca^{2+}]_i$) fluctuations, the mechanisms underlying this signalling were targeted for characterisation.

An elevation in $[Ca^{2+}]_i$ can originate by mobilising Ca^{2+} from extracellular fluid through Ca^{2+} entry channels and/or through release from intracellular organelles (reviewed in chapter one). The release of Ca^{2+} from intracellular organelles is commonly mediated by a number of channels with most being known about inositol-1,4,5-triphosphate receptors (IP_3Rs) and ryanodine receptors (RYRs) located primarily on endoplasmic/sarcoplasmic reticulum (ER/SR) (Carafoli *et al*., 2000; Putney, 2005). These Ca^{2+} channels are regulated by several Ca^{2+} mobilising second messengers including inositol-1,4,5-triphosphate (IP_3) and Ca^{2+} itself (Berridge *et al*., 2000).

In most non-excitable cells such as epithelial cells, the activation of IP_3Rs and RYRs is reported to produce transient increases or oscillations in $[Ca^{2+}]_i$ (Putney & Bird, 2009). Such events are believed to reflect both positive and negative feedback through activation and desensitisation of these receptors, most likely by Ca^{2+} itself (Berridge *et al*., 2003; Carafoli *et al*., 2001; Putney & Bird, 2008). For instance, IP_3 can be generated via the phosphoinositide cascade in response to an extracellular stimulus such as the binding of an agonist to its specific receptor on a cell's surface (Potier & Trebak, 2008; Putney, 2001). IP_3 diffuses through the cytosol and on binding to the IP_3R mediates the release of Ca^{2+} from IP_3-sensitive stores (e.g. ER/SR). The release of Ca^{2+} into the cytosol, at low concentrations, stimulates

IP_3Rs and RYRs to further release Ca^{2+}, a process known as Ca^{2+}-induced Ca^{2+}-release (CICR). CICR and Ca^{2+} diffusion between neighbouring channel clusters may function as a mechanism to potentiate an initial local signal resulting in a more global Ca^{2+} wave (Berridge *et al*., 2003). In most cell types, intercellular waves of second messengers such as Ca^{2+} and IP_3 can also passively diffuse into neighbouring cells through gap junctions which may result in cell-to-cell signal transduction (Berridge *et al*., 2000; Carafoli *et al*., 2001). However, at higher concentrations Ca^{2+} reduces CICR by mediating the desensitisation of the IP_3R (Berridge *et al*., 2000; Berridge *et al*., 2003; Marchant & Parker, 2000; Putney, 2007; Rosado *et al.,* 2004). A number of pumps and exchangers located in various membranes also reduce $[Ca^{2+}]_i$ through extruding cytosolic Ca^{2+} extracellularly or by sequestering it into intracellular organelles such as the ER/SR, Golgi-apparatus and mitochondria (Richter & Kass, 1991). As $[Ca^{2+}]_i$ returns to baseline levels, IP_3-sensitive channels are no longer desensitised by Ca^{2+} and can be further stimulated to release Ca^{2+} into the cytosol.

The release of Ca^{2+} from ER/SR depletes the „reservoir' of stored Ca^{2+}, an event that is detected and subsequently results in the activation of store-operated channels (SOCs). SOCs mediate an influx of Ca^{2+}, a process known as store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+} entry (CCE) (Parekh & Putney, 2005; Rosado *et al*., 2004). This mechanism appears to be important for regulating the activity of certain functional proteins and transcription factors (Berridge *et al*., 2000). For example, alterations in gene expression following Ca^{2+} mobilisation has been reported to occur after Ca^{2+} entry accompanied a transient $[Ca^{2+}]_i$ signal (Di Capite *et al*., 2009).

Sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps are located on the ER in epithelial cells and play an essential role in sequestering Ca^{2+} into intracellular stores. Thapsigargin is a specific inhibitor of SERCA pumps (Treiman *et al*., 1998) and has been used as a pharmacological tool for depleting Ca^{2+} stores and activating SOCE (Takemura *et al*., 1989). Many studies have used thapsigargin and low extracellular Ca^{2+} ($[Ca^{2+}]_o$) conditions to determine the involvement of extracellular/intracellular Ca^{2+} sources in response to agonists.

Relatively little is known about $[Ca^{2+}]_i$ signalling events in female reproductive tract cells. Transient receptor potential (TRP) channel proteins have been described as subunits of SOCs and appear to be important in the regulation of Ca^{2+} influx in non-excitable cells. Several members of the TRP family of channels have been found in epithelial cells of the female reproductive tract. TRPV4 and polycystin-2 are two examples of TRP channels that have been located on the ciliary membrane of murine oviductal cells and are believed to function as mechanoreceptors sensing fluid movement (Andrade *et al*., 2005; Fernandes *et al*., 2008; Teilmann *et al*., 2005). Primary cilia of secretory cells are also reported to express the polycystin-1-polycystein-2 complex which may function as a Ca^{2+} entry channel (Hagiwara *et al*., 2008). These channels indicate an importance of Ca^{2+} signalling not only in potentially regulating aspects of gene transcription but also for transport of gametes and embryos (Fernandes *et al*., 2008).

Aims

- The aim of this chapter is to further characterise $[Ca^{2+}]_i$ signals in cells of the female reproductive tract which occur on exposure to sperm, including:
    - $[Ca^{2+}]_i$ peak kinetics.
    - The role of intracellular Ca^{2+} stores in sperm-induced Ca^{2+} mobilisation.

4.2 Materials and Methods

4.2.1 Materials

12 mm Round coverslips, (Warner instruments). 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), (Sigma). Adenosine-5'-triphosphate (ATP), (Sigma). Calcium green-1 AM, (Invitrogen). Ethylenediaminetetraacetic acid (EDTA), (Sigma). Imaging chamber series 20, (Warner instruments). Low Ca^{2+} sEBSS (LCsEBSS), (see appendix I for full recipe), (MP Biomedicals). Pluronic F-127, (Invitrogen). Silicone grease, (Warner instruments). Sodium lactate, (Sigma). Sodium pyruvate, (Sigma). Supplemented Earls balanced salt solution (sEBSS), (Invitrogen). Syto 64 red fluorescent nucleic acid stain, (Invitrogen). Thapsigargin, (Sigma).

4.2.2 Recruitment of female reproductive tract donors

Refer to section 2.2.2.

4.2.3 Preparation of human female reproductive tract cells and cell lines

Refer to section 2.2.3.

4.2.4 Labelling female reproductive tract cells for $[Ca^{2+}]_i$ imaging

Refer to section to 3.2.4. For experiments performed in LCsEBSS, Maxchelator software (version 1.3/http://www.stanford.edu/~cpatton/CaEGTA-TS.htm) was used to calculate the concentration of EGTA required to reduce unbound Ca^{2+} to approximately 150 nM.

4.2.5 Selection, preparation and capacitation of human sperm

Refer to section 2.2.5. For experiments performed in LCsEBSS, sperm were treated with EGTA to reduce extracellular Ca^{2+} to approximately 150 nM. EGTA was added dropwise to sperm samples approximately 10 mins before use in experiments. In a subset of experiments, sperm were prepared by the methylcellulose modified swim-up technique as outlined in section 2.2.5 using LCsEBSS in replacement of sEBSS.

4.2.6 Experimental design

Refer to section 3.2.11. Only the perfusion method was used for experiments investigating Ca^{2+} store mobilisation.

4.2.7 Imaging data processing

Refer to section 3.2.8.

4.2.8 Imaging data and statistical analysis

Data analysis used „thresholding' logic as outlined in section 3.2.9.

4.2.9 Characterisation of $[Ca^{2+}]_i$ responses

Cell $[Ca^{2+}]_i$ responses evoked by exposure to sperm, as initially demonstrated in chapter three were examined for an additional occurrence of a 2^o peak. The 2^o peak assessment period was ~ 75 s and taken ~1.5 mins after exposure to sperm. A schematic representation of the sorting of cell response types is depicted in figure 4.1. Responses were characterised into one of four groups: 1) cells that display both significant 1^o and 2^o peaks indicating that a cell has rapidly responded to treatment with a prolonged signalling event. This is most likely due to cells

oscillating or having a sustained increase in $[Ca^{2+}]_i$; 2) the occurrence of 1° peaks without subsequent 2° peaks indicating a cell has responded transiently without prolonged $[Ca^{2+}]_i$ signalling; 3) the occurrence of 2° peaks without 1° peaks indicating a slow or delayed response to treatment; 4) without the detection of 1° or 2° peaks.

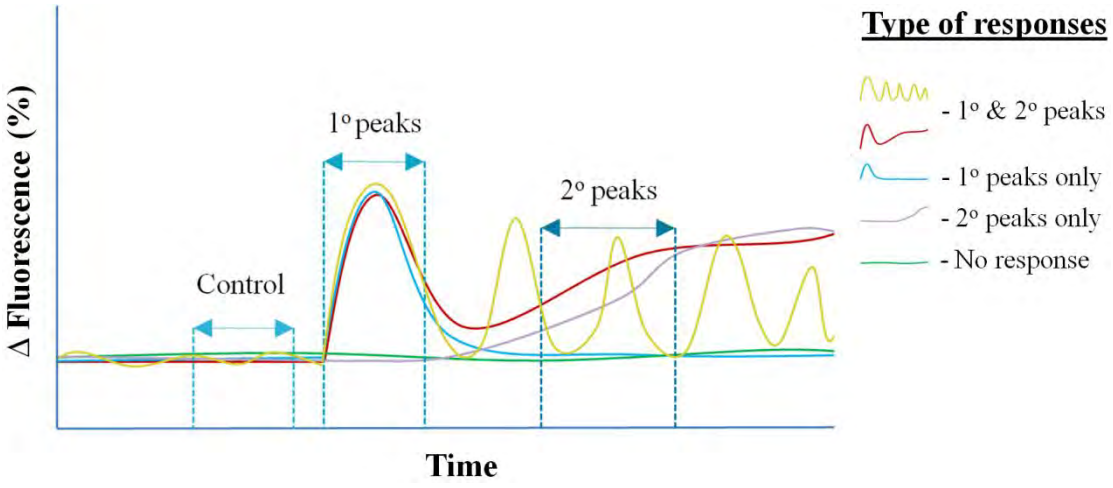


Figure 4.1. Schematic demonstrating logical sorting and characterisation of cell responses

4.2.10 Data grouping

Refer to section 3.2.10. Statistical P values are listed in appendix IV.

4.3 RESULTS

4.3.1 Characterisation of cell $[Ca^{2+}]_i$ responses to sperm, sperm-conditioned sEBSS and ATP

Figure 4.2 shows a representative example of logical sorting through characterisation of 1^o and 2^o peaks, performed for a single experiment on an isthmus primary cell line. The averaged fluorescence (R_{tot}) plots demonstrate that the logic was effective in separating different response characteristics. Figure 4.3 shows the relative % of categorised cell responses separated by cell type and regions. The occurrence of responses characterised as containing both 1^o and 2^o peaks was generally higher for explants when compared to regions of primary cell lines. Statistical significance was found for both isthmic and ampullary derived cells ($P<0.05$) but not endometrial cells ($P = 0.171$). Cells derived from the isthmus appeared to display a greater % of responses consisting of both 1^o and 2^o peaks although this was not significant when compared to other regions.

For analysis of the relationship between occurrence of sperm-induced 1^o and 2^o peaks, data from all experiments in which responses were characterised were combined for each region and cell type to generate total numbers for each of the four categories of response: 1^o & 2^o responsive; 1^o response only; 2^o responsive only and none response (table 4.1). Deviation from the null hypothesis (i.e. random association between the two types of responses) was tested using a chi-squared (χ^2) test. All regions across explants, primary cultures and OE E6/E7 cells demonstrated a significant association between the occurrences of these two peaks. This indicates that a 2^o peak is more likely to occur in a cell after occurrence of a 1^o peak (table 4.1; χ^2 $P<0.001$).

The relationship between the 1^o and 2^o peaks was also checked in response to sperm-conditioned sEBSS treatment. Data from all experiments were combined for each cell type without regional separation (table 4.2) due to low experimental numbers. Similarly to sperm-induced responses, sperm-conditioned sEBSS-induced responses showed a positive relationship between the occurrence of 1^o and 2^o peaks (table 4.2; χ^2 $P<0.001$).

Finally the responses to sperm and sperm-conditioned sEBSS were examined for linkage. Data from all experiments in which cells were exposed sequentially to sperm-conditioned sEBSS and sperm were combined for each cell type (table 4.3). The percentage of both sperm-conditioned and sperm responsive cells varied considerably between experiments but there was a significant association between cells responding to sperm-conditioned and sperm treatments suggesting that responses were not random (table 4.3; χ^2 $P<0.001$).

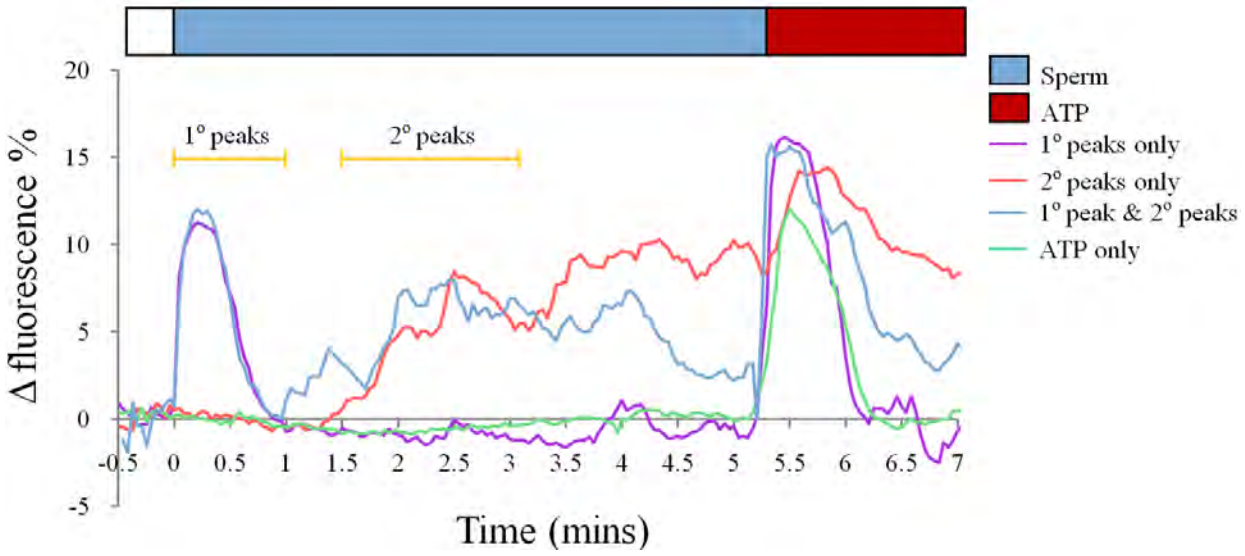


Figure 4.2. Characterisation of female tract cell $[Ca^{2+}]_i$ responses to sperm and ATP

Cell $[Ca^{2+}]_i$ responses were assessed using logical analysis. Significant responses were further defined as having an initial primary „fast' response (peaks occurring within 1 minute of exposure to treatment), a secondary „late' response (peaks occurring after 1.5 min of treatment exposure) or both types of response indicating either a „fast and sustained' or „fast and oscillating' response to treatment. Above is an example of logical sorting of cell responses from an experiment using primary isthmic cells. Representative traces of the mean response (R_{tot}) to sperm for each response type: 1° peak only (14 cells); 2° peaks only (17 cells); 1° & 2° peaks (15 cells) and ATP only (62 cells). The experiment was performed using the injection method.

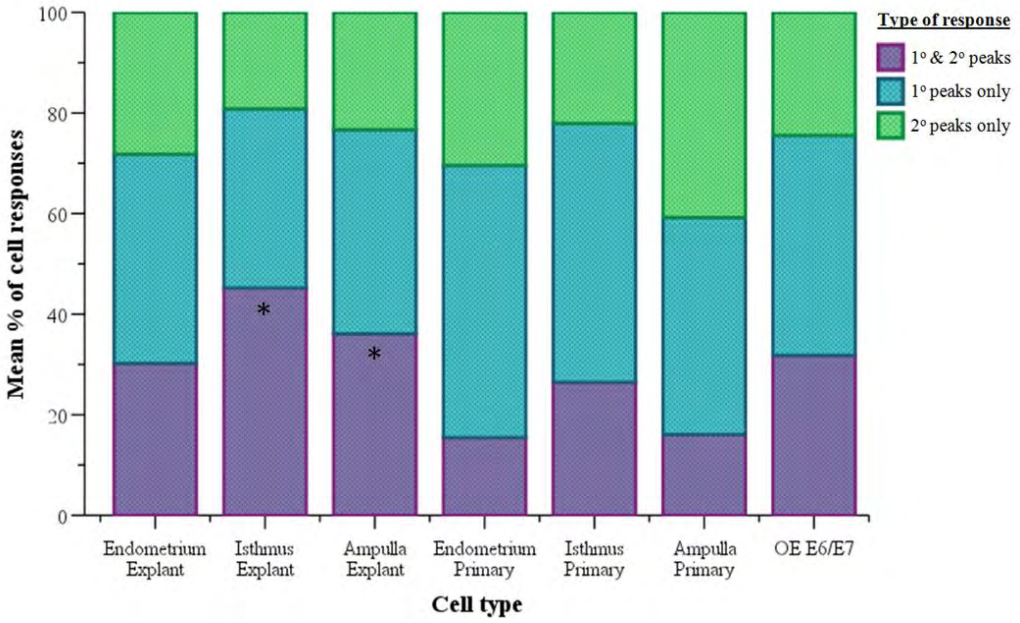


Figure 4.3. Mean % of categorised cell responses to sperm separated by cell type and region

Characterisation of the type of cell response was performed using logical analysis in Excel. Statistical analysis was performed to assess differences in the occurrence of both 1^{o} and 2^{o} peaks for both cell types and regions. An asterisk denotes statistical significance of ($P<0.05$). Regions within both explants and primary cell types were compared for significance using one-way ANOVA. There were no statistical differences observed across explant regions when compared to corresponding regions within primary cell cultures using a *t-test* (independent). A significant increase in the occurrence of both 1^{o} and 2^{o} peaks was found for isthmic and ampullary explants in comparison to corresponding regions within primary cell cultures. No statistical difference was observed when comparing OE E6/E7 cells to ampullary regions across cell types using *t-test* (independent). For further details on P values, see appendix IV: 4.i.

	No. of cells (% of total)					
Cell type	**1^o**	**1^o & 2^o**	**2^o**	**No response**	**Total**	**χ^2**
Endometrium explant	107 (3.4)	76 (6.6)	168 (14.7)	793 (69.3)	1144	< 0.001
Isthmus explant	81 (9.5)	138 (16.2)	56 (6.6)	575 (67.3)	850	< 0.001
Ampulla explant	214 (10.6)	90 (4.5)	76 (3.8)	1642 (81.2)	2022	< 0.001
Endometrium primary	214 (12.0)	68 (3.8)	99 (5.5)	1403 (78.2)	1784	< 0.001
Isthmus primary	252 (15.3)	158 (9.6)	132 (8.0)	1100 (67.0)	1642	< 0.001
Ampulla primary	150 (8.2)	72 (3.9)	170 (9.3)	1443 (78.6)	1835	< 0.001
OE E6/E7	237 (11.1)	219 (10.3)	147 (6.9)	1524 (71.7)	2127	< 0.001
Total cells	1255	821	848	8480	11404	
Mean % ± SEM	10.9 ± 0.9	7.9 ± 1.7	7.8 ± 1.3	73.4 ± 2.2		

Table 4.1. The relationship between primary (1^o) and secondary (2^o) peaks occurring during sperm-induced $[Ca^{2+}]_i$ signalling

Summary data demonstrating the relationship between primary (1^o) and secondary (2^o) peaks occurring during sperm-induced $[Ca^{2+}]_i$ signalling. Number of cells with percentage of all cells in that category in parentheses is shown. Columns 2-5 show responses sorted by category; **1^o** (primary peaks only); **2^o** (secondary peaks only); **1^o & 2^o** (both primary and secondary peaks). Column 5 "total" gives details of total cells analysed for each cell type. Column 6 shows P values from a Chi-squared test (χ^2). χ^2 P values indicated a positive relationship between primary and secondary peaks in cell responses.

	No. of cells (% of total)					
Cell type	**1^o**	**1^o & 2^o**	**2^o**	**No response**	**Total**	**χ^2**
Explant	178 (15.0)	53 (4.5)	33 (2.8)	920 (77.7)	1184	< 0.001
Primary	133 (14.2)	23 (2.5)	47 (5.0)	733 (78.3)	936	< 0.001
OE E6/E7	146 (13.2)	62 (5.6)	42 (3.8)	854 (77.4)	1104	< 0.001
Total cells	457.0	138.0	122.0	2507.0	3224	
Mean % ± SEM	14.2 ± 0.5	4.2 ± 0.9	3.9 ± 0.6	77.8 ± 0.3		

Table 4.2. The relationship between primary (1^o) and secondary (2^o) peaks occurring during sperm-conditioned sEBSS-induced $[Ca^{2+}]_i$ signalling

Summary data demonstrating the relationship between primary (1^o) and secondary (2^o) peaks occurring during sperm-conditioned sEBSS-induced $[Ca^{2+}]_i$ signalling. Number of cells with percentage of all cells in that category in parentheses is shown. Columns 2-5 show responses sorted by category; **1^o** (primary peaks only); **2^o** (secondary peaks only); **1^o & 2^o** (both primary and secondary peaks). Column 5 "total" give details of total cells analysed for each cell type. Column 6 gives p values from a Chi-squared test (χ^2). χ^2 P values indicates a positive relationship between primary and secondary peaks in cell responses.

	No. of cells (% of total)					
Cell type	**S**	**S & C**	**C**	**No response**	**Total**	χ^2
Explant	188 (14.5)	104 (8.0)	143 (11.1)	858 (66.4)	1293	< 0.001
Primary	163 (10.4)	52 (3.3)	144 (9.2)	1212 (77.1)	1571	< 0.001
OE E6/E7	183 (14.7)	98 (7.9)	112 (9.0)	855 (68.5)	1248	< 0.001
Total cells	534.0	254.0	399.0	2925.0	4112	
Mean % ± SEM	13.2 ± 1.4	6.4 ± 1.5	9.7 ± 0.7	70.7 ± 3.3		

Table 4.3. The relationship between cell responses to sperm-conditioned sEBSS and sperm

Summary data demonstrating the relationship between cell responses to sperm-conditioned sEBSS and sperm. Number of cells with percentage of all cells in that category in parentheses is shown. Columns 2-5 show responses sorted by category; **S** (sperm responsive cells); **C** (sperm-conditioned sEBSS responsive cells); **S & C** (cells responsive to both sperm and sperm-conditioned sEBSS). Column 5 "total" provides details of total cells analysed for each cell type. Column 6 gives P values from a Chi-squared test (χ^2). χ^2 P values indicates a positive relationship between cell responsiveness to sperm-conditioned sEBSS and sperm.

4.3.2 Peak kinetics

To observe the kinetics of tract cell responses to sperm, 1^{o} peaks were aligned by identifying the initiation of the rising phase. There was considerable variation in peak amplitudes even within individual experiments (figure 4.4). The mean % change in normalised fluorescence of 1^{o} peaks induced by sperm, sperm-conditioned sEBSS and ATP are presented in figure.4.5; table 4.4. Results demonstrated that both sperm-conditioned sEBSS and sperm-induced $[Ca^{2+}]_i$ responses were similar in respect to % change in normalised fluorescence whilst in comparison, ATP produced a significantly greater rise in fluorescence for all cell types studied ($P<0.05$). OE E6/E7 cells appeared to produce a significantly greater % change in fluorescence for all treatments when compared to corresponding treatments in both female reproductive tract explants and primary cells (sperm-conditioned, $P<0.05$; sperm $P<0.05$, ATP $P<0.001$).

The kinetics of 1^{o} peaks for sperm and ATP-induced $[Ca^{2+}]_i$ signalling were compared between explants and primary cell types in two representative experiments (figure 4.6). Typically, maximum amplitudes were reached after 10-15 s with a total duration of approximately 50 s in both explant and primary cells (figure 4.6 a). Peak amplitudes in response to sperm in explants were generally smaller than in primary cell lines (figure 4.6 b). ATP-induced responses reached maximum peak amplitudes 5-10 s after addition in both explant and primary cell types (figure 4.6 c). In explant tissue, ATP-induced $[Ca^{2+}]_i$ responses were typically smaller in peak amplitudes demonstrating a transient increase in $[Ca^{2+}]_i$ followed by recovery to near baseline fluorescence (figure 4.6 c, d). In comparison, primary cells ATP responses often induced a sustained elevation of $[Ca^{2+}]_i$, as shown in figure 4.6 c, however in a minority of cases transient increases were observed. Kinetics of sperm-induced

1^{o} peaks were compared to sperm-conditioned-induced 1^{o} peaks in a selected experiment on primary isthmic cells (figure 4.7). The peak kinetics of the two types of responses were almost indistinguishable.

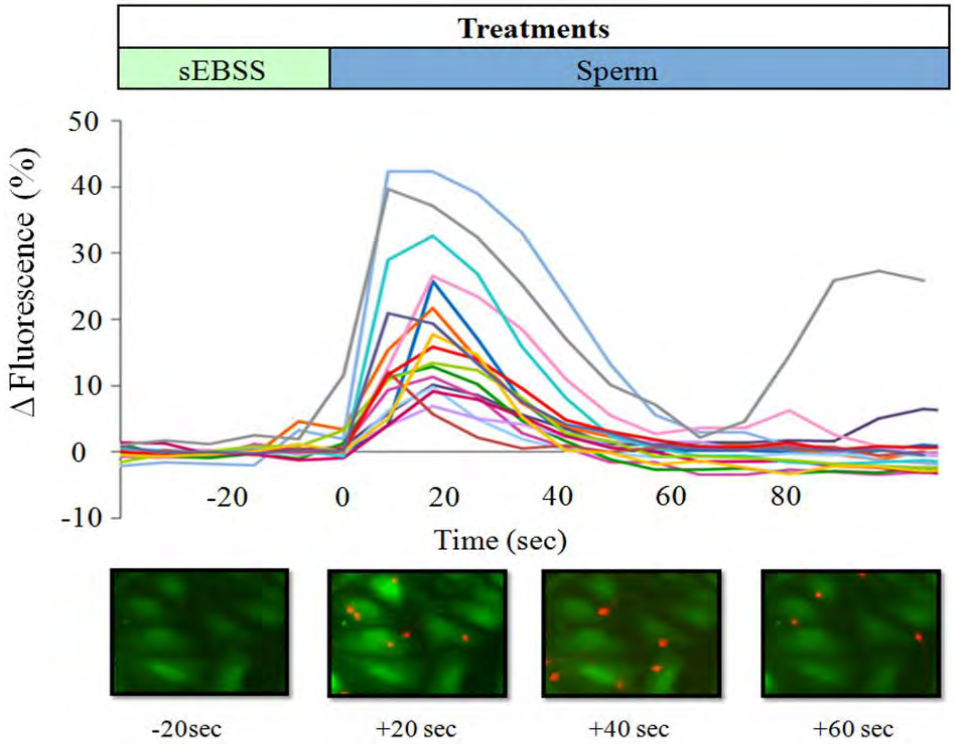


Figure 4.4. Sperm-induced $[Ca^{2+}]_i$ responses in a subset of isthmic primary cells

Time-normalised fluorescence ($[Ca^{2+}]_i$) plots from seventeen individual cells. Insert shows corresponding co-localisation images in a subset of the cell population as $[Ca^{2+}]_i$ rises during interaction.

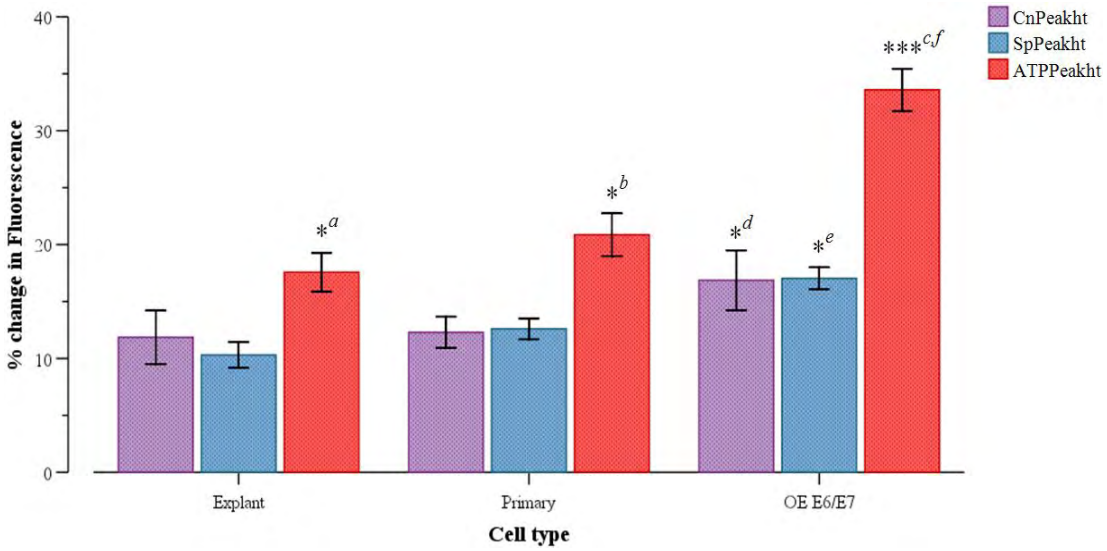


Figure 4.5. Mean increase in fluorescence ($[Ca^{2+}]_i$) induced by sperm, sperm-conditioned sEBSS and ATP separated by cell type

Bar chart shows the mean % increase in normalised fluorescence measured for 1° peaks induced by treatments. Error bars represent SEM. Asterisks denote statistical significance *** (P<0.001) and * (P<0.05) obtained from a (independent) *t-test*. A significant increase in fluorescence % was demonstrated for ATP (100 µM) responses when compared to either sperm-condition sEBSS or sperm for all cell types studied [a, b, c]. OE E6/E7 cells demonstrated a significantly larger increase in mean fluorescence % values when each treatment type was compared to corresponding treatments within explant and primary cell types [d, e, f]. For further details on P values, see appendix IV: 4.ii.

	Explant			Primary cell line			OE E6/E7		
	Cond. sEBSS	Sperm	ATP	Cond. sEBSS	Sperm	ATP	Cond. sEBSS	Sperm	ATP
No. of replicates (*n*)	5	29	17	12	53	30	9	28	20
No. of cells analysed	234	790	2311	196	1068	3035	210	1358	3133
Mean Δ Fluo % ± SEM	11.9 ± 2.3	10.3 ± 1.1	17.4 ± 1.6	12.3 ± 1.4	12.6 ± 0.9	20.9 ± 1.9	16.9 ± 2.6	17.6 ± 0.9	33.5 ± 2.2

Table 4.4. Summary data used for figure 4.5

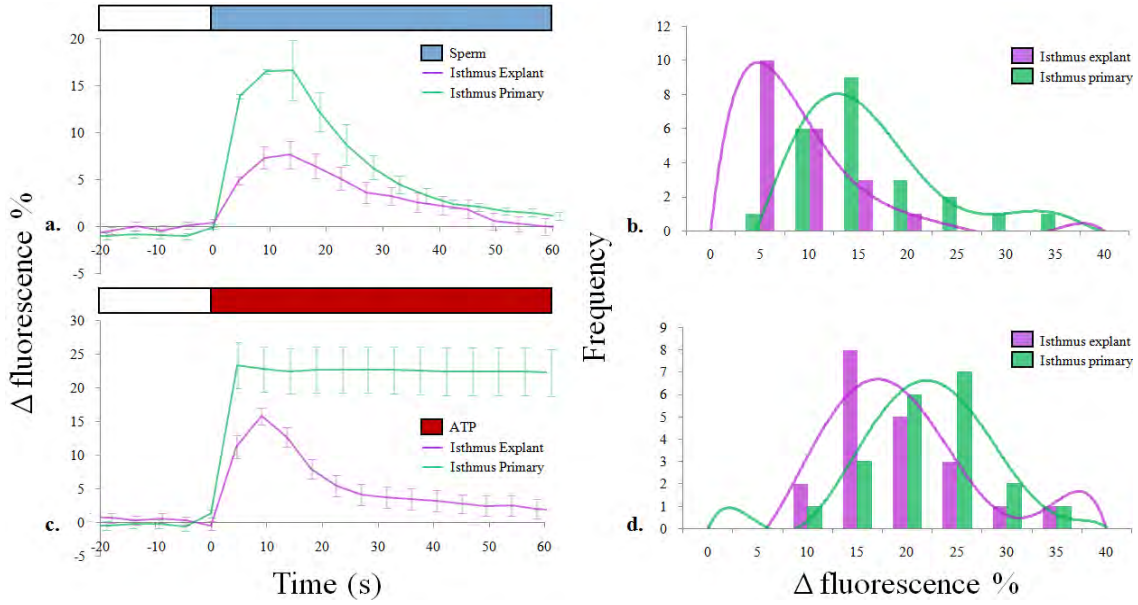


Figure 4.6. Comparison of $[Ca^{2+}]_i$ peak kinetics induced by sperm and ATP

(**a**) Representative traces of the mean response (R_{tot}) for sperm (500 x 10^3) induced 1^o peaks (20 cells) in isthmic-derived cells. Responses for both sperm and ATP were obtained from one experiment for both an isthmic explant and primary cells performed in sEBSS. Peaks were aligned to examine the mean peak height and duration; (**b**) summary amplitude distribution for corresponding sperm-induced 1^o peaks (20 cells) with trend line (polynomial); (**c**) representative traces of the mean response (R_{tot}) for ATP (100μM) induced 1^o peaks (20 cells) in cells; (**d**) summary amplitude distribution for corresponding ATP-induced 1^o peaks (20 cells) with trend line (polynomial). All experiments were performed using the injection method.

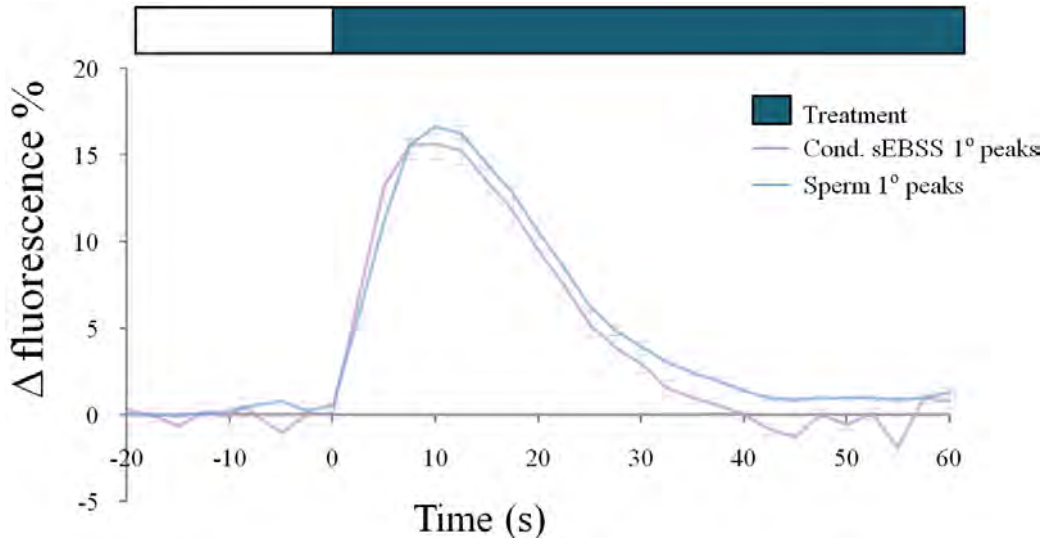


Figure 4.7. Comparison of $[Ca^{2+}]_i$ peak kinetics induced by sperm and sperm-conditioned sEBSS

Representative traces of the mean response (R_{tot}) for sperm (500 x 10^3) induced 1° peaks (6 cells) and sperm-conditioned sEBSS induced 1° peaks (6 cells) in isthmic-derived cells. Responses for both sperm and sperm-conditioned sEBSS were obtained from one experiment using the perfusion method. Peaks were aligned to examine the mean peak height and duration.

4.3.3 Ca^{2+} stores involvement in sperm-induced $[Ca^{2+}]_i$ signalling

The change from high to low extracellular Ca^{2+} ($[Ca^{2+}]_o$) was performed during experimental recording to monitor its effects on Ca^{2+} mobilisation. 32.2 ± 6.9% of cells demonstrated a significant elevation in $[Ca^{2+}]_i$ as a result of switching from sEBSS to LCsEBSS (see appendix IV: 4.iii). After 1.7 minutes, the % of cells that maintained a significantly elevated $[Ca^{2+}]_i$ had fallen to 1.7 ± 0.5%. This was not significantly different to control rates of spontaneous responses, suggesting that cell Ca^{2+} homeostasis was re-established before starting subsequent treatments.

In low $[Ca^{2+}]_o$ conditions (LCsEBSS), sperm stimulated a significant increase in $[Ca^{2+}]_i$ in 38.0 ± 8.4% of OE E6/E7 cells which was not significantly different to that observed for cells bathed in sEBSS, 29.9 ± 10.5% (figure 4.8; table 4.5, P = 1.98). Pre-treatment of OE E6/E7 cells with 100 nM thapsigargin in low $[Ca^{2+}]_o$ significantly reduced the mean % of cells responding to sperm to 0.8 ± 0.5% (figure 4.8; table 4.5, $P<0.05$).

Figure 4.9 shows a representative experiment performed to assess the effect of thapsigargin on sperm-induced $[Ca^{2+}]_i$ signalling in low $[Ca^{2+}]_o$ conditions. The addition of thapsigargin resulted in an increase in normalised fluorescence % reaching a mean peak amplitude of 17.7 ± 3.0%, within 20-30 s. The peak durations were broader than those resulting from sperm-induced Ca^{2+} responses. Thapsigargin was then washed off with LCsEBSS resulting in $[Ca^{2+}]_i$ returning to baseline levels within 1.25 mins, before subsequent exposure to sperm. The addition of sperm did not elicit further elevations in $[Ca^{2+}]_i$. Subsequent reintroduction of high $[Ca^{2+}]_o$ conditions (sEBSS), resulted in a rapid increase in $[Ca^{2+}]_i$ and high amplitude

responses which were sustained throughout the rest of the experiment. Subsequent ATP treatment had little effect on $[Ca^{2+}]_i$.

Figure 4.10 shows a representative experiment in which sperm induced $[Ca^{2+}]_i$ responses in low-$[Ca^{2+}]_o$ conditions. In these conditions, sperm produced a transient peak that resembled 1^o peaks observed in high $[Ca^{2+}]_o$ conditions however, in contrast there appeared to be a distinct reduction in 2^o peaks, with most cells returning to near baseline $[Ca^{2+}]_{i,}$ (figure 4.10 b). When high $[Ca^{2+}]_o$ (sEBSS) was reintroduced to OE E6/E7 cells after exposure to sperm, there was an observed increase in sustained and/or oscillating responses (figure 4.11 b), suggesting a role for extracellular Ca^{2+} in sustaining $[Ca^{2+}]_i$ signalling. The mean % of OE E6/E7 cells that respond to reintroduction of high $[Ca^{2+}]_o$, with or without prior sperm-induced Ca^{2+} mobilisation was 20.9 ± 6.2% and 3.0 ± 3.4%, respectively (figure 4.12; table 4.6). The mean % fluorescence increase recorded for cells that responded to sEBSS reintroduction having been previously exposed to sperm was 15.5 ± 1.0%, this is considerably lower than that recorded after sEBSS in cells pre-treated with thapsigargin in LCsEBSS, 34.2 ± 4.0% (table 4.6; appendix IV: 4.v, respectively).

The peak kinetics for sperm and ATP-induced 1^o peaks in sEBSS and LCsEBSS were examined in more detail in a single experiment. In terms of peak durations both sperm-induced responses in sEBSS and LCsEBSS were similar, reaching maximum peak after ~ 10-15 s lasting for ~ 50 s (figure 4.13 a). Peak amplitudes were also similar (figure 4.13 b). It should be noted that when grouped the data suggested the maximum peak amplitudes are slightly greater for responses occurring in LCsEBSS when compared to sEBSS being 20.7 ± 1.% and 15.4 ± 1.%1, respectively (table 4.5). ATP was found to be as effective in eliciting

significant Ca^{2+} responses in OE E6/E7 cells bathed in LCsEBSS, resulting in a rapid and sustained increase in $[Ca^{2+}]_i$ reaching maximum amplitude within 5-10 s as previously observed in sEBSS conditions (figure 4.13 c, d). However, the duration of $[Ca^{2+}]_i$ elevation was transient in LCsEBSS in contrast to sustained in sEBSS conditions as shown in figure 4.13 c. Responses to ATP in LCsEBSS were dramatically reduced with pre-treatment of 100 nM thapsigargin from 93 ± 1.5% to 4.6 ± 1.4% ($P<0.001$; see appendix IV:4.vi).

The effect of preparing sperm in LCsEBSS on sperm-induced $[Ca^{2+}]_i$ signalling was also examined. Unfortunately, due to a limited sperm sample from recovery, only one experiment was performed. Sperm prepared in LCsEBSS induced a higher than expected level of cell responses being 72.1% in comparison to standard in sEBSS, 29.9 ± 10.5% (figure 4.14; table 4.7). The resulting sperm-conditioned LCsEBSS resulting from this preparation was sufficient to use in three experimental repeats. Sperm-conditioned LCsEBSS produced a mean % response in cell populations of only 5.5 ± 3.3%. This level is lower than that previously observed for OE E6/E7 responses to sperm-conditioned sEBSS prepared in sEBSS, 15.4 ± 3.6% (chapter 3, figure 3.9; table 3.1).

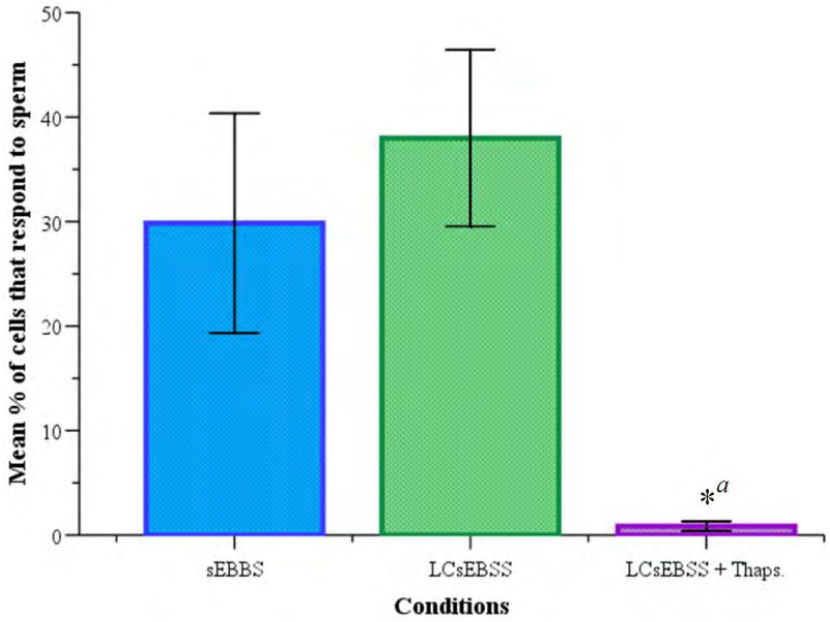


Figure 4.8. The effects of LCsEBSS and thapsigargin on the sperm-induced $[Ca^{2+}]_i$ signalling

Data was classified by logical analysis of significant responses. Sperm-induced $[Ca^{2+}]_i$ signalling in OE E6/E7 cells was assessed in either sEBSS, LCsEBSS or LCsEBSS after pre-treatment with thapsigargin (100 nM). Experiments were performed in parallel and cells were exposed to sperm ($500x10^3$) via the constant perfusion method. Error bars represent SEM. Asterisk denotes statistical significance ($P<0.05$) calculated by a Mann-Whitney U (independent) test. The % of cells responding to sperm in LCsEBSS significantly decreased when pre-treated with thapsigargin *a*. For further details on P values, see appendix IV: 4.iv.

	sEBSS	LCsEBSS	LCsEBSS + Thaps.
No. of replicates (*n*)	5	6	7
No. of cells analysed	860	1208	1196
Mean % response ± SEM	29.9 ± 10.5	38.0 ± 8.4	0.8 ± 0.5
Mean Δ Fluo % ± SEM	15.4 ± 1.1	20.7 ± 1.5	17.7 ± 3.0

Table 4.5. Summary data used for figure 4.8

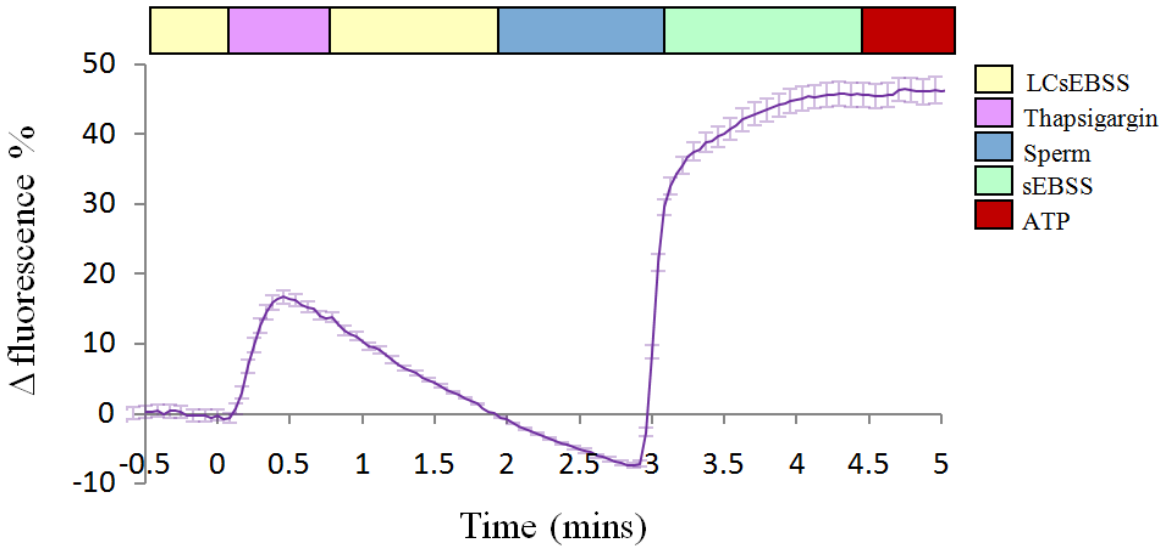


Figure 4.9. The effect of thapsigargin on sperm-induced $[Ca^{2+}]_i$ signalling

The representative trace shows the mean response (R_{tot}) for a single experiment (101 cells, OE E6/E7 cell line). Treatment bar indicates different conditions exposed to OE E6/E7 cells including the pre-treatment of thapsigargin (100 nM) before exposure to sperm (500 x 10^3). Time 0 corresponds with the introduction of thapsigargin. Experiments were performed in parallel using the constant perfusion method. Error bars represent SEM and are displayed for every time point.

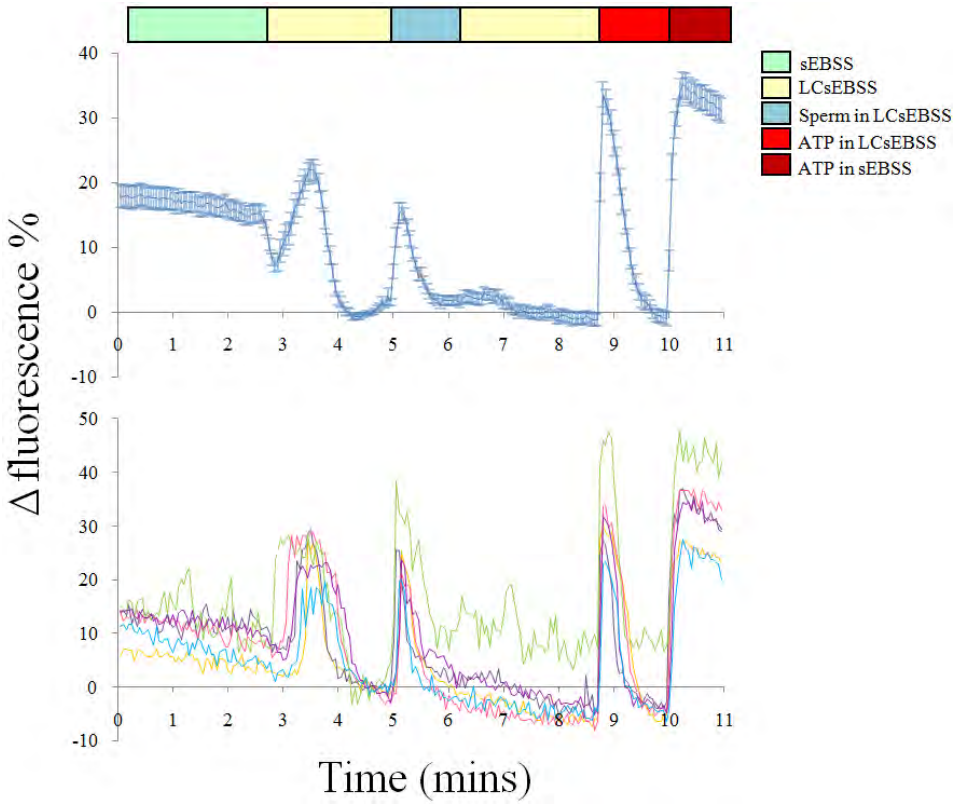


Figure 4.10. Sperm-induced $[Ca^{2+}]_i$ signalling in LCsEBSS conditions

(**a**) Shows the mean fluorescence (R_{tot}) plots for a single experiment (47 cells, OE E6/E7 cell line). Treatment bar indicates different conditions exposed to OE E6/E7 cells including the introduction of LCsEBSS before exposure to sperm in LCsEBSS conditions (500 x 10^3). sEBSS was reintroduced after exposure to sperm and cells were subsequently treated with ATP; (**b**) shows 6 representative traces of the cell population. Experiments were performed in parallel using the constant perfusion method. Error bars represent SEM and are displayed for every time point.

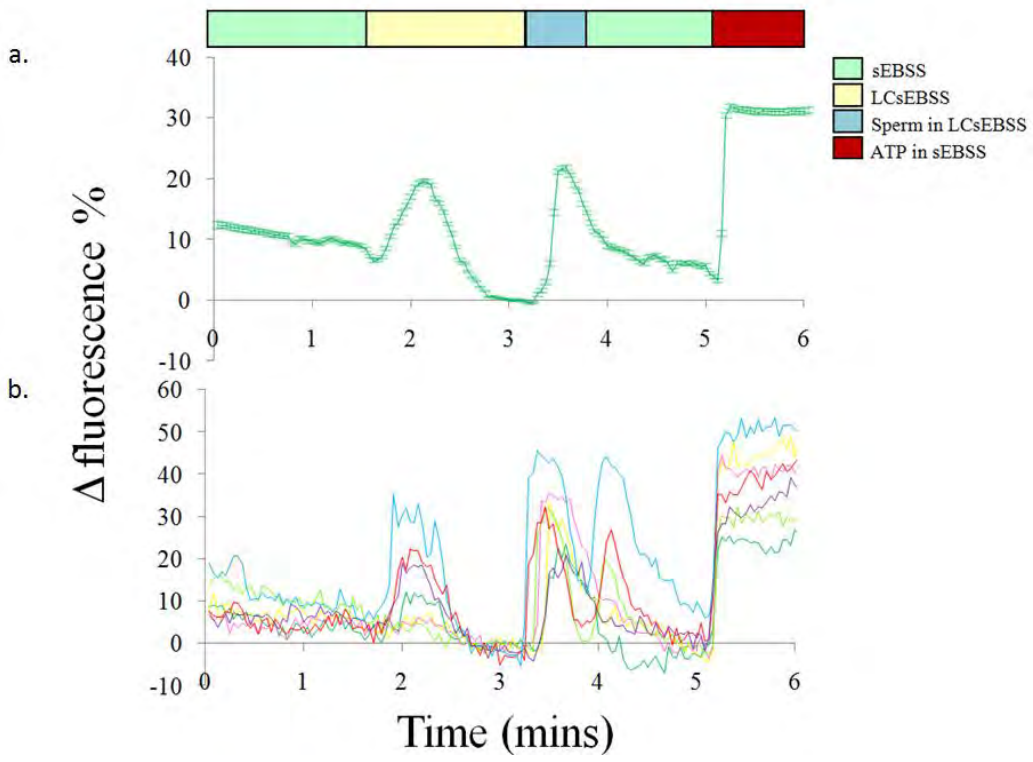


Figure 4.11. Sperm-induced $[Ca^{2+}]_i$ signalling in LCsEBSS and the effect of reintroducing sEBSS

(**a**) Shows the mean fluorescence (R_{tot}) plots for a single experiment (347 cells, OE E6/E7 cell line). Treatment bar indicates different conditions exposed to OE E6/E7 cells including the introduction of LCsEBSS before exposure to sperm in LCsEBSS conditions (500 x 10^3). sEBSS was reintroduced after exposure to sperm and cells were subsequently treated with ATP; (**b**) shows 7 representative traces of the cell population. Experiments were performed in parallel using the constant perfusion method. Error bars represent SEM and are displayed for every time point.

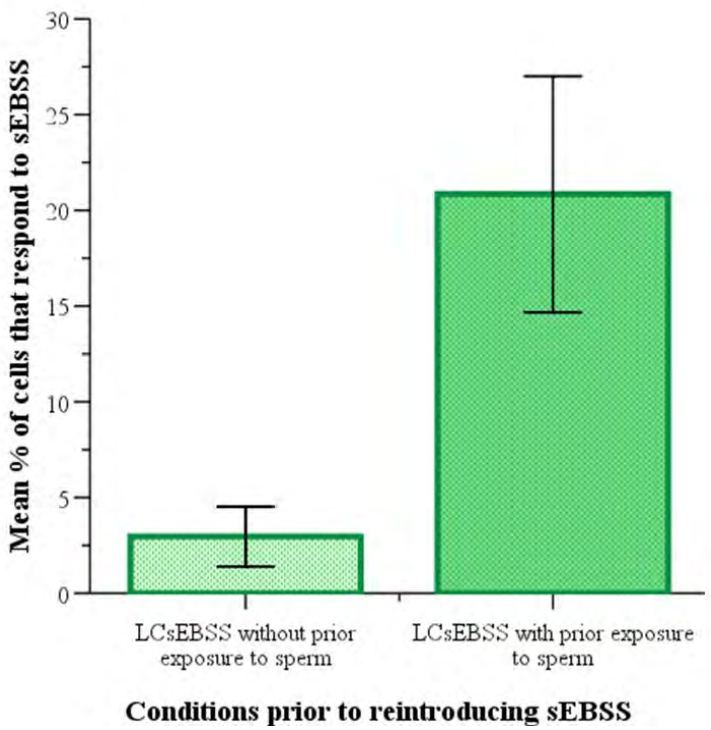


Figure 4.12. The effect of prior sperm exposure on the mean % of cells that respond to the reintroduction sEBSS

Data was classified by logical analysis of significant responses. sEBSS-induced $[Ca^{2+}]_i$ signalling in OE E6/E7 cells was assessed in either sEBSS or LCsEBSS before and after exposure to sperm. Experiments were performed in parallel and cells were exposed to sperm ($500x10^3$) via the constant perfusion method.

	LCsEBSS w/o prior exp. to sperm	LCsEBSS with prior exp. to sperm
No. of replicates (*n*)	2	3
No. of cells analysed	655	935
Mean % response ± SEM	3.0 ± 1.6	20.9 ± 6.2
Mean Δ Fluo % ± SEM	15.7 ± 3.4	15.5 ± 1.0

Table 4.6. Summary data for figure 4.12

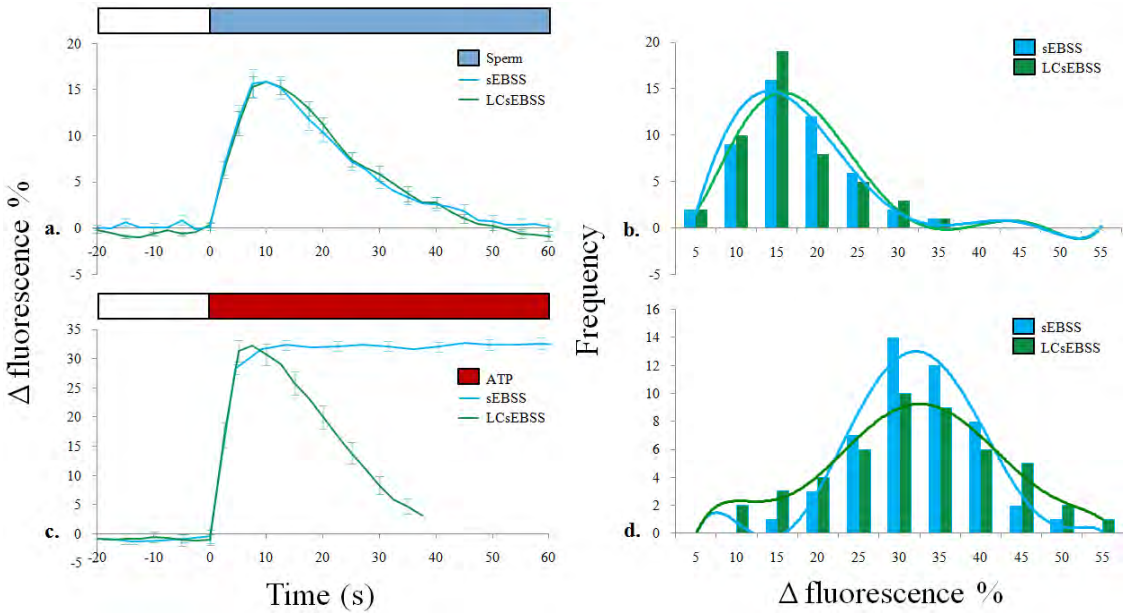


Figure 4.13. Comparison of $[Ca^{2+}]_i$ peak kinetics induced by sperm and ATP in sEBSS vs. LCsEBSS conditions

Responses were obtained from two separate experiments performed in sEBSS and LCsEBSS conditions. Peaks were aligned to examine the mean peak height and duration. (**a**) Representative traces of the mean response (R_{tot}) for sperm (500 x 10^3) induced 1^o peaks (48 cells) in OE E6/E7 cells; (**b**) summary amplitude distribution for corresponding sperm-induced 1^o peaks (48 cells) with trend line (polynomial); (**c**) representative traces of the mean response (R_{tot}) for ATP (100µM) induced 1^o peaks (48 cells) in OE E6/E7 cells; (**d**) summary amplitude distribution for corresponding ATP-induced 1^o peaks (48 cells) with trend line (polynomial). Experiments were performed in parallel and using the constant perfusion method.

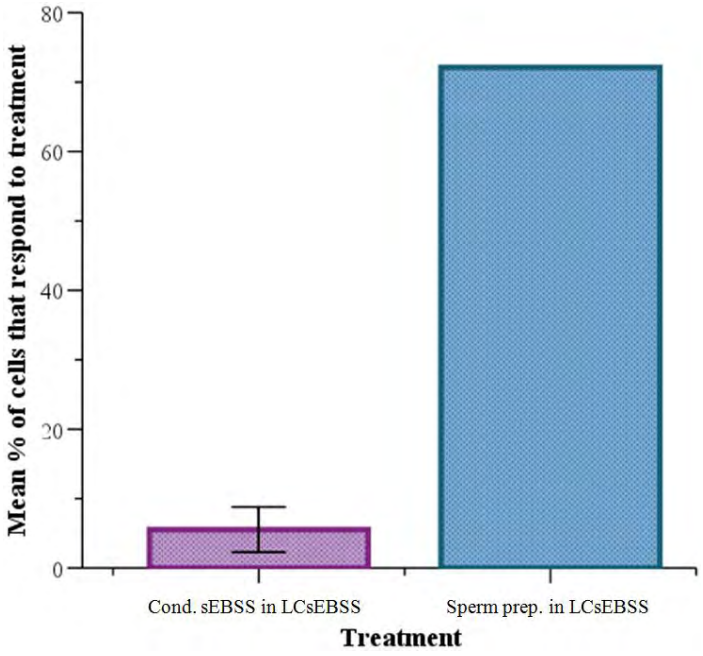


Figure 4.14. The mean % of cells that respond to sperm-conditioned LCsEBSS and sperm when prepared in LCsEBSS conditions

Sperm-induced $[Ca^{2+}]_i$ signalling in OE E6/E7 cells was assessed after being prepared in LCsEBSS. Data was classified by logical analysis of significant responses. Experiments were performed using the constant perfusion method.

	Cond. sEBSS in LCsEBSS	Sperm prep. in LCsEBSS
No. of replicates (*n*)	3	1
No. of cells analysed	719	796
Mean % response ± SEM	5.5 ± 3.3	72.1 ± n/a
Mean Δ Fluo % ± SEM	13.9 ± 2.2	18.4 ± n/a

Table 4.7. Summary data used for figure 4.14

4.4 DISCUSSION

Characterisation of responses

A novel attempt was made to characterise $[Ca^{2+}]_i$ responses by the occurrence of 1^o „fast' and 2^o „late' peaks. Cells displaying both types of peaks were identified as either oscillating or sustained $[Ca^{2+}]_i$ type responses, both indicating prolonged signalling events. There was some evidence to suggest that prolonged signalling events to sperm occurred more frequently in explants when compared to corresponding primary cells (figure 4.3). This suggests subtle differences in the regulation of $[Ca^{2+}]_i$ signalling within explants in response to sperm, however further work is required to confirm this finding.

A relationship was demonstrated for 1^o and 2^o peaks in both sperm-conditioned and sperm-induced $[Ca^{2+}]_i$ responses ($P<0.001$; table 4.1; table 4.2). This suggests that the occurrence of a 2^o peak was more likely to occur after the occurrence of a 1^o peak, possibly indicating that primary Ca^{2+} responses trigger secondary pathways for Ca^{2+} entry as suggested by other studies (Kirkman-Brown *et al*., 2000). A positive relationship between cells responding to both sperm-conditioned and sperm treatments was detected for all types of female reproductive tract cells ($P<0.001$; table 4.3). This suggests that cells responding to sperm-conditioned sEBSS were more likely to respond to sperm treatments.

Peak kinetics

Peak heights were recorded for all significant treatment responses. These values indicate relative $[Ca^{2+}]_i$, as fluorescence is correlated to Ca^{2+} binding to Calcium green-1. It is important to note that an increase in fluorescence is not directly proportional to $[Ca^{2+}]_i$ and requires calibration to determine true values. Nevertheless, normalised fluorescence is useful

to indicate relative changes in $[Ca^{2+}]_i$ that can be used to compare parallel conditions. Mean $[Ca^{2+}]_i$ increases induced by sperm-conditioned and sperm treatments were similar, whilst ATP-induced Ca^{2+} peaks were on average greater in size across all cell types (figure 4.5; table 4.2). This suggests that ATP may induce greater increases in $[Ca^{2+}]_i$ when compared to sperm and sperm-conditioned sEBSS.

The duration of sperm-induced 1^o peaks were typically ~ 40-50 s in all cell types studied (figures; 4.4, 4.6 a, 4.13 a). There was considerable variability in duration of ATP-induced peaks between cell types and external Ca^{2+} conditions (figures 4.6 c, 4.10 a, 4.13 c). Frequently, only transient ATP peaks were observed in explant tissue whilst both transient and sustained responses were observed in primary cells. In OE E6/E7 cells, ATP treatment in sEBSS conditions consistently resulted in large sustained responses.

Sperm induced Ca^{2+} store mobilisation

A series of experiments was performed to investigate the mechanism underlying sperm-induced Ca^{2+} mobilisation within female reproductive tract cells. These experiments were performed exclusively in OE E6/E7 cells to reduce experimental variation between patient samples. These experiments addressed the role of intracellular Ca^{2+} stores by comparing responses of cells exposed to sperm in sEBSS containing low extracellular Ca^{2+} ($[Ca^{2+}]_o$), (LCsEBSS, $[Ca^{2+}]_o$ = ~ 150 nM) with or without intracellular store depletion with those performed in sEBSS with high $[Ca^{2+}]_o$, (sEBSS, $[Ca^{2+}]_o$ = ~ 5 mM).

Treatment of OE E6/E7 cells with sperm (500 x 10^3) resulted in a clear rise in $[Ca^{2+}]_i$ in a subpopulation of responsive cells. The ability of sperm to elicit Ca^{2+} responses in OE E6/E7

cells was not significantly altered by the reduction of extracellular unbound Ca^{2+} to approximately 150 nM (figure 4.8; table 4.5). This shows that the effect of sperm-induced signalling reflects intracellular Ca^{2+} store mobilisation. This was further supported by pre-treatment of cells with thapsigargin (100 nM) which effectively abolished sperm-induced $[Ca^{2+}]_i$ responses (figure 4.8; table 4.5). In somatic cells, thapsigargin is known to be a non-competitive irreversible SERCA inhibitor (Rogers *et al*., 1995). Sperm however, have been reported to be insensitive to thapsigargin at the concentrations used in this study (Harper *et al*., 2005). Therefore, it can be assumed that the effects of thapsigargin on OE E6/E6 cells response to sperm are not likely to be due to effects on sperm physiology.

The increase in $[Ca^{2+}]_i$ observed in OE E6/E6 cells in response to thapsigargin treatment is consistent with the inhibition of SERCA ability to sequester $[Ca^{2+}]_i$ back into intracellular ER stores, as reported in previous studies (Ghosh *et al*., 1991; Inesi *et al*., 2005). In sEBSS conditions, thapsigargin-induced increases in $[Ca^{2+}]_i$, were transient, returning towards baseline levels approximately 1.25 minutes after initial exposure. As the actions of thapsigargin are irreversible, the removal of raised cytosolic Ca^{2+} is likely to have been through alternative plasma membrane Ca^{2+} pumps and exchangers such as plasma membrane ATPase (PMCA), the Na^+/Ca^{2+} exchanger (NCX) and uptake by the mitochondrial uniporter (MCU) (Berridge *et al*., 2003) resulting in the depletion of both stored and cytosolic free Ca^{2+}. This explains the observations that neither subsequent exposure to sperm or ATP in LCsEBSS were capable of generating $[Ca^{2+}]_i$ elevations and also demonstrated that LCsEBSS provided insufficient extracellular Ca^{2+} to maintain a Ca^{2+} influx as seen in sEBSS conditions.

Depletion of intracellular Ca^{2+} stores is associated with activating SOCs and SOCE (Parekh & Putney, 2005; Rosado *et al*., 2004). SOCs mediate the influx of extracellular Ca^{2+}, remaining open until Ca^{2+} stores are replenished (Putney, 1997; Putney, 2001; Putney, 2005). This mechanism explains the rapid $[Ca^{2+}]_i$ elevations observed in OE E6/E7 cells during the reintroduction of extracellular Ca^{2+} (sEBSS) after store depletion resulting from thapsigargin and/or sperm-induced $[Ca^{2+}]_i$ signalling in LCsEBSS conditions (figures 4.9; 4.11, respectively). Ca^{2+} signalling of this nature has been implicated in altering gene expression profiles (Di Capite *et al*., 2009).

The mean fluorescence increase for cells that responded to reintroduction of sEBSS after exposure to sperm in LCsEBSS was lower than that in cells pre-treated with thapsigargin in LCsEBSS conditions, 15.5 ± 1.0% and 34.2 ± 4.0%, respectively (table 4.6; appendix IV: 4.v). Furthermore, the mean % fluorescence increase induced by sperm and sEBSS reintroduction after exposure to sperm in LCsEBSS conditions were similar, 20.7 ± 1.5% and 15.5 ± 1.0%, respectively (table 4.5; table 4.6). This suggests that sperm do not completely empty Ca^{2+} stores and demonstrates that the influx of extracellular Ca^{2+} is highly regulated by Ca^{2+} store refilling requirements. In agreement, the reintroduction of sEBSS did not have significant effects on Ca^{2+} mobilisation in cells without prior exposure to sperm (figure 4.12; table 4.6).

In this experimental series, ATP (100 μM) induced significant $[Ca^{2+}]_i$ responses in OE E6/E7 cell populations when performed in either sEBSS or LCsEBSS, 95.5 ± 1.1% and 93.0 ± 1.5%, respectively (see appendix IV: 4.vi). This is in agreement with previous reports on cultured oviductal epithelial cells (OECs) (Barrera *et al*., 2004; Cox & Leese, 1995; Dickens *et al*.,

1996; Tiemann *et al*., 1996). ATP has been shown to act through specific P2Y purinoreceptors, leading to IP_3 production via the phosphoinositide cascade, which in turn mediates intracellular Ca^{2+} store release (Janssen *et al*., 2009). In addition, ATP has been reported to activate plasma membrane IP_3Rs triggering a Ca^{2+} influx in ciliated OECs (Barrera *et al*., 2007). In this study, thapsigargin was effective in reducing significant ATP-induced responses to 4.6 ± 1.4% in LCsEBSS conditions (see appendix IV: 4.vi), supporting the notion that ATP exerts its effect, at least partially, through store activated pathways.

Further investigation of sperm-induced 1^o peaks indicated that the kinetics were comparable in sEBSS and LCsEBSS (figure 4.13 a, b). The mean $[Ca^{2+}]_i$ elevation that occurred under low $[Ca^{2+}]_o$ conditions on average was slightly higher than that occurring under normal Ca^{2+} conditions (table 4.5). This may result from fluorescence values being normalised to lower baseline levels in LCsEBSS. Only calibrated results would allow accurate assessment of micromolar $[Ca^{2+}]_i$ actually occurring. ATP peaks appeared more rapid then sperm peaks, reaching maximum amplitude typically within 5-10 s (figure 4.13 c). An ATP response in OE E6/E7 cells consistently resulted in a fast rise followed by a sustained elevation of $[Ca^{2+}]_i$ when performed in sEBSS conditions. This was contrary to OE E6/E7 cell responses to ATP when performed in LCsEBSS in which cells responded transiently (figure 4.13 c). This difference is again likely to reflect the activity of plasma membrane channels in removing cytosolic Ca^{2+} combined with the insufficient $[Ca^{2+}]_o$ in LCsEBSS for subsequent Ca^{2+} influx regulated by SOCs.

Treatment of OE E6/E7 cells with sperm-conditioned LCsEBSS resulted in a lower mean % of significant responses, 5.5 ± 3.3% (figure 4.14; table 4.7) in comparison to sperm-

conditioned sEBSS 15.4 ± 3.6 (chapter 3, figure 3.9; table 3.1). Conversely, sperm prepared in LCsEBSS produced a larger than expected mean % of cells demonstrating significant responses, 72.1% (figure 14; table 4.7). However, replicates could not be performed due to limited sperm recovery during the selection process, as LCsEBSS reduced sperm migration during preparation. The lower than average cell responses resulting from sperm-conditioned LCsEBSS may reflect the altered conditions in which sperm were incubated in. Ca^{2+} is important in capacitation related events and its absence may have affected sperm metabolism, secretion or shedding of membrane proteins (Bedu-Addo *et al*., 2005). Further work is required to confirm this finding.

As mentioned in the previous chapter, the components associated with sperm responsible for inducing Ca^{2+} responses are currently unidentified. Integrins have been implicated (Reeve *et al*., 2003) as they are known to induce rapid Ca^{2+} signalling and be present on sperm (Glander *et al*., 1998; Iwao & Fujimura, 1996; Schwartz & Ginsberg, 2002). Furthermore, the elevation of $[Ca^{2+}]_i$ via integrins has been reported to involve IP_3-evoked Ca^{2+} release from sarcoplasmic/endoplasmic reticulum and subsequent extracellular Ca^{2+} influx in other cell types (Kwon *et al*., 2000). Further research is required to determine whether integrins are involved in sperm-induced $[Ca^{2+}]_i$ signalling in female reproductive tract cells.

In summary, the data presented in this chapter suggest that $[Ca^{2+}]_i$ responses to sperm in cells of the female reproductive tract may vary in kinetics depending on region and cell type. $[Ca^{2+}]_i$ response kinetics to sperm-conditioned sEBSS and sperm were similar suggesting that they act via the same signalling mechanism. In addition, cells that responded to sperm were more likely to respond to sperm-conditioned sEBSS. Sperm-induced $[Ca^{2+}]_i$ responses can

result in prolonged signalling in a subpopulation of cells, indicated by the presence of oscillations and sustained $[Ca^{2+}]_i$ signalling. Prolonged signalling events may vary subtly in explant cells in comparison to primary cells. Finally, sperm-induced $[Ca^{2+}]_i$ signalling appears to result through mobilisation of intracellular Ca^{2+} stores which may in turn, trigger activation of SOCs to replenish Ca^{2+} stores. Repeated activation of this pathway could be a mechanism for the observed sustained or oscillatory Ca^{2+} signalling seen in a subpopulation of responsive cells.

CHAPTER 5

Observations and signalling in sperm during interaction with cells of the female reproductive tract

5.1 INTRODUCTION

Sperm storage has been demonstrated across many species (Holt & Lloyd, 2010; Overstreet & Cooper, 1979; Rodriguez-Martinez *et al*., 2005; Smith & Yanagimachi, 1990). It is thought to regulate capacitation-associated events whilst maintaining sperm viability until ovulation-associated signals induce detachment, allowing low numbers of sperm to progress towards the ampulla for fertilisation (Smith & Yanagimachi, 1991; Suarez & Pacey, 2006). The biochemical nature concerning the regulation of sperm detachment remains poorly characterised, partly due to the technical difficultly of studying such events *in vivo* (Hunter *et al*., 1999; Taitzoglou *et al*., 2007).

In human, several studies have contributed towards our understanding of sperm behaviour using *in vitro* cultures of oviductal epithelial cells (OECs). Pacey *et al*. (1995a,b) employed a simplistic co-culture system in which human sperm were shown to bind randomly via the head whilst displaying active flagellar beating. In the absence of paracrine influences, bound sperm detached transiently displaying alterations in flagellar beat patterns associated with hyperactivated motility (Pacey *et al*., 1995a). Interestingly, this pattern of behaviour has been observed to aid sperm migration along the oviductal epithelial surface in other species (Demott & Suarez, 1992; Lefebvre & Suarez, 1996; Smith & Yanagimachi, 1991). Therefore, a „switch' to hyperactivated motility may supply the force required for detachment from oviductal cellular contacts (Pacey *et al*., 1995a).

Calcium (Ca^{2+}) is important in regulating motility, capacitation and hyperactivation (Carlson *et al*., 2003; Ho & Suarez, 2003). An increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) has been associated with the onset of hyperactivated motility (Marquez & Suarez, 2007),

characterised by increased amplitude of flagellar bending and lateral head movements (reviewed by Suarez, 2008a). Some authors have reported free swimming sperm (unbound) to have consistently higher $[Ca^{2+}]_i$ than sperm bound to monolayers of cultured OECs (Dobrinski *et al*., 1996b; Petrunkina *et al*., 2001). These observations have lead to the consensus that sperm with low $[Ca^{2+}]_i$ preferentially bind to OECs and that the low $[Ca^{2+}]_i$ maintained through binding may play a role in delaying capacitation events and preserving sperm viability. To our knowledge, there are currently no reports demonstrating the regulation of $[Ca^{2+}]_i$ during real-time observations of binding to and detachment from oviductal epithelium.

Ovulation signals and timing of initial activation of sperm release varies between species and there is uncertainty over what may induce release of sperm bound to epithelium. Hunter *et al*. (1983) proposed that sperm could be exposed to high levels of progesterone through the counter-current mechanism whereby the ovarian vein runs alongside the ovarian artery, supplying the wall of the isthmus. Progesterone has been identified as a Ca^{2+} agonist and is a major component of follicular fluid. Hunter *et al*. (1999) showed that when sperm residing in the isthmus of bovine oviducts were exposed to both follicular fluid and a Ca^{2+} ionophore, a high rate of polyspermic fertilised oocytes were obtained with high numbers of sperm associated with the zona. This implied that an elevation in $[Ca^{2+}]_i$ acting on either sperm or OECs may induce sperm to release. There is also convincing data suggesting that sperm detachment is induced by the presence of the cumulus-oocyte complex (COC) within the oviduct (Kolle *et al*., 2009), however the underlying molecular factors are unknown.

Female reproductive tract cells produce other soluble messengers likely to influence sperm activity and responses to physiological cues. Reactive oxygen species (ROS) such as nitric oxide (NO) play an importance role in sperm (Herrero & Gagnon, 2001) and oviductal physiology (Lapointe *et al*., 2006; Rosselli *et al*., 1998). NO is uncharged allowing diffusion directly through biological membranes, with a reported reactivity radius of 100-200 μm from synthesis (Lancaster, 1997). This allows NO to act as an effective intracellular and extracellular biological messenger. Recently, NO donors have been shown to alter sperm $[Ca^{2+}]_i$ kinetics to progesterone (Machado-Oliveira *et al*., 2008) and this may in part be regulated by S-nitrosylation of proteins involved in Ca^{2+} mobilisation (Lefievre *et al*., 2007; Stoyanovsky *et al*., 1997; Xu *et al*., 1998). As sperm are generally considered transcriptionally inactive, regulation of sperm activity by post-translational modifications during their interactions with cells of the female reproductive tract may be of crucial importance (Lefievre *et al*., 2009).

Aims

- Make basic observations on binding and behaviour of sperm interacting with cells of the female reproductive tract.
- Characterise the variation in sperm binding duration with primary cell cultures derived from endometrial, isthmic and ampullary anatomical regions.
- Assess $[Ca^{2+}]_i$ in individual sperm during initial stages of binding and detachment from oviductal explants.
- Assess the effect of progesterone treatment on Ca^{2+} mobilisation and behaviour whilst either bound or in the absence of oviductal explants.
- Assess NO production in explant tissue and NOS isoform expression within primary OECs.
- Investigate S-nitrosylation of sperm surface proteins after co-culture with oviductal explants.

5.2 Materials and Methods

5.2.1 Materials

4,5-diaminofluorescein, DAF-FM, (Invitrogen). Anti-Biotin, Monoclonal Mouse IgG antibody, (Jackson ImmunoResearch Laboratories). Bovine Serum Albumin (BSA), Probumin™ Fatty Acid Free (Lot: 122), (Millipore). Calcium green-1 AM, (Invitrogen). Collagenase Type I, (Invitrogen). Dimethyl sulfoxide (DMSO), (Sigma). Dulbecco's minimal essential medium/Hams F12 medium (1:1 v/v DMEM/F12), (Invitrogen). Dulbecco's phosphate buffered saline (PBS), (Invitrogen). EZ-Link Biotin-N-[6-(biotinamido)hexyl]-3_-(2_-pyridyldithio)propionamide (EZLink Biotin-HPDP), (Perbio Science). Formaldehyde, (Sigma). Hanks buffered salt solution (HBSS) with Calcium and Magnesium (Invitrogen). L-NG-Nitroarginine methyl ester (L-NAME), (Sigma). Nitrocellulose membrane, (GE Healthcare), Oestrodiol-17β, (Sigma). Pluronic F-127, (Invitrogen). Poly-D-lysine (PDL), (BD Biosciences). Progesterone, (Sigma). Protease inhibitor cocktail tablets, (Roche Diagnostics). Rabbit polyclonal anti-eNOS, nNOS, iNOS and donkey anti-rabbit Texas Red, (Santa Cruz Biotechnologies). Supplemented Earls balanced salt solution (sEBSS), (Invitrogen). SYTOX, (Molecular Probes). Triton X-100, (Sigma).

5.2.2 Recruitment of patients and sperm donors

Refer to section 2.2.2 and 2.2.5.

5.2.3 Preparation of human female reproductive tract cells and cell lines

Refer to section 2.2.3.

5.2.4 Selection, preparation and capacitation of human sperm

For the assay of sperm protein S-nitrosylation and visualisation of S-nitrosoproteins (section 5.2.10) sperm were obtained by direct swim-up into sEBSS (pH 7.3-7.4) with 0.3% BSA and adjusted to 6 million cells/ml (Kirkman-Brown *et al*., 2000). Sperm were allowed to capacitate at 37°C, 6% CO_2 for 5-6 h. For all other experiments, sperm were selected and prepared by the modified methylcellulose swim-up technique as outlined in section 2.2.5 with the modification of labelling sperm with 7.6 µM of Calcium green-1, AM in replacement of the red nuclear dye, Syto64.

5.2.5 General observations and interaction experiments

Observations of sperm interaction with epithelial and fibroblastic cells were performed at various stages through culture ranging from within hours of obtaining tissue, to several days after sub-culturing. Before adding sperm, the explant tissue was thoroughly rinsed with fresh sEBSS to reduce contamination with erythrocytes. Loose dissociated epithelial cells were removed by gentle washing. For cells that had been cultured in culture media, the change to sEBSS was performed at least 1 h before the addition of sperm and general observations.

Sperm were added at a concentration of 2 x 10^6 in sEBSS + 0.3% BSA and were observed at the initial moments of interaction and after 1 h of addition. The interaction of sperm with epithelial cells was video recorded which allowed playback to make more detailed observation. At least 20 videos of sperm interacting with explants, primary cultures and cell lines were recorded.

For a subset of interaction experiments, a high acquisition camera (~ 300 Hz) was used to capture cilia motion in both explants and primary (ampullary) cell lines and movements of sperm flagellum during binding. On several occasions after observations were noted, sperm binding avidity was assessed by subjecting sperm interactions to either gentle (2 ml/min) or strong (6.5 ml/min) washing conditions of sEBSS, administered using a perfusion system (see figure 3.1). Washing was performed whilst directly observing its effects on binding behaviour.

5.2.6 Characterisation of numbers and binding durations of sperm to primary OECs

A series of experimental replicates was performed using 4 day old primary OECs derived from a single patient. All 12 replicates were performed consecutively, alternating between cell lines originating from different anatomical regions of the female tract. Sperm were prepared from a single donor using the methylcellulose swim-up technique, as described in section 2.2.5. 2 x 10^6 sperm were added during video recording allowing binding and interactions to be assessed at the initial point of exposure/contact. Images were taken every 5 s for a total duration of 375 s after the addition of sperm.

Analysis of video data was performed using Image Pro Plus II to view and playback video data. Clear acetate sheets were used to mark and record sperm binding durations. A sperm was considered to be bound or interacting when static for ≥ 10 s. Binding numbers and durations were then entered on to Microsoft Excel and sperm binding durations were grouped into 5 categories selected to best represent the data. These groups were as follows: 10-15 s; 20-45 s; 50-75 s; 80-125 s; and >125 s. Any sperm binding within the last 125 s that remained

bound until the end of video capture were excluded as the binding duration would not be known. Exclusion of sperm within this category was minimal (<1%) and is unlikely to affect the overall pattern of data.

5.2.7 Tracing sperm flagellar and head activity during interactions

Video data was viewed extensively using Image Pro plus II. Sperm were selected for analyses on the basis that they were not disrupted by other sperm binding and interactions. Images were captured every 4 s. Sperm selected were traced for 5 sequential frame sequences (equating to 20 s) for each series, producing multiple image traces for each sperm. Traces were then superimposed to give a representation of flagellar and head location during that time period.

5.2.8 Detection of NO production in oviductal explants

Human ampullary explants were washed with sEBSS and incubated in dark conditions at 37°C, 6% CO_2 with 5 µM 4,5-diaminofluorescein (DAF)-FM diacetate for 30 minutes. Excess DAF-FM was removed by three washes in sEBSS and the explants were transferred to microscope slides under a cover slip supported on spots of vacuum grease to gently compress it. The slides were examined using a Nikon inverted fluorescence microscope (488 nm excitation / 540 nm emission).

5.2.9 Immunofluorescent staining for NOS isoforms in primary OECs

Ampullary explants were washed in HBSS before being incubated with 0.25% type I collagenase in DPBS for 1 h at 37°C with gentle agitation. The supernatant was collected and pelleted by centrifugation at 500 ***g*** for 5 minutes. The resulting cell suspension was then

plated onto slides in DMEM/F12 supplemented with 150 pg/mL oestradiol-17β and left to adhere. Cells were cultured at 37°C, 6% CO_2 for 2 days before use.

Cells were fixed with 4% formaldehyde for 6 minutes at room temperature and then permeabilised using 0.2% Triton X- 100 for 15 minutes. Cells were washed after with 0.1% (v/v) Triton X-100 in PBS. Slides were treated with 1% (w/v) BSA and 5% (v/v) goat serum in PBS (30 minutes, 37°C, 6 % CO_2 in air) then incubated with rabbit polyclonal anti-eNOS, -nNOS or -iNOS (1:50 dilution in 1% (w/v) BSA in PBS, 37°C, 6 % CO_2 in air, 60 minutes). Slides were then washed with PBS and treated with the secondary antibody [donkey anti-rabbit Texas Red or FITC, 1:200 dilution in 1% (w/v) BSA in PBS] for 60 minutes at 37°C, 6% CO_2. Finally, the samples were examined using a Nikon inverted fluorescence microscope (575 nm excitation / 610 nm emission).

5.2.10 Visualisation of sperm surface S-nitrosoproteins

S-nitrosylation status of human sperm surface proteins was assessed using the biotin switch assay as described in a previous by Lefievre *et al*. (2007). To visualise S-nitrosoproteins in sperm exposed to female reproductive tract-synthesised NO, sperm (50 million cells/ml) were incubated with fresh human oviductal and endometrial explants (fragments ~ 3 mm^3) in 50µl DMEM/F12 medium supplemented with 150 pg/ml oestradiol-17β at 37°C in 5% O_2/6% CO_2 balance N_2 for 2 h. Sperm were then retrieved and fixed on slides using 4% formaldehyde and S-nitrosoproteins were detected using a method adapted from Yang & Loscalzo, (2005), as described previously by Lefievre *et al*. (2007). This method involves blocking thiols with a thiol-reactive agent, methyl methanethiosulfonate (MMTS) followed by the reduction of S-

nitrosothiols with ascorbate and labelling with fluorescently tagged methanethiosulfonate (MTSEA).

5.2.11 $[Ca^{2+}]_i$ imaging and velocity tracking of sperm

Experiments investigating sperm $[Ca^{2+}]_i$ during binding to and detachment from female reproductive tract cells were performed using explants obtained with 24 h of surgery. Explants were prepared as outlined in section 2.2.3 and placed in culture dishes containing 1.5 ml of sEBSS, pre-warmed to 37°C. Culture dishes were then overlaid with 1 ml of IVF-culture oil. Sperm (pre-labelled with Calcium green-1) were added to oviductal explants at a concentration of 6 x 10^6 / ml and allowed to interact for 1 h before fluorescence imaging. Images were captured at 4 s intervals.

Analysis of video data was performed using Image Pro plus II. Sperm were selected for analysis on the basis that their fluorescence plots were not disrupted by other sperm binding and interactions, or by excessive rotation and flipping. A fixed sized region of interest (ROI) was drawn around the midpiece/neck of the sperm to measure changes in fluorescence intensities over time (see appendix V: 5.i). Image Pro Plus II software supported ROI tracking of midpiece/neck movements of a single sperm thus enabling motile sperm to be assessed. For selected examples, the velocity of sperm motility could also be assessed using ROI tracking. Velocity was calculated using the following equation, $\mathbf{v} = \mathbf{d}/\mathbf{t}_2\text{-}\mathbf{t}_1$, where **v** is velocity, **d** is distance (μm) and **t** is time (s).

Fluorescence plots for individual sperm were then separated into three groups relating to general behaviour characterisation, namely: bound sperm; binding sperm; and

detaching/releasing sperm. The „bound' group consisted of fluorescence plots of sperm that were bound during the entire video and demonstrated little or no movement. The 'binding' group consisted of fluorescence plots of sperm that demonstrated free swimming and binding during video capture and remained bound throughout the duration of recording. Finally, the „detaching/releasing' group consisted of fluorescence plots of sperm that were either bound at the start or bind and subsequently detach from oviductal epithelium during video acquisition. Fluorescence plots were individually aligned so that time 0 marked the initiation of the associated event.

5.2.12 The addition of progesterone to sperm in the presence or absence of oviductal explants

Sperm and oviductal explants were prepared as outlined in section 5.2.4 and 2.2.3, respectively. Experiments were performed in culture dishes containing 1.5 ml of sEBSS overlaid with 1 ml of IVF-culture oil. Images were obtained every 4 s. Sperm were added at a concentration of 6 x 10^6 / ml and exposed to 3.2 µM progesterone by gentle injection into the culture dish, adjacent to the chosen imaging focal field.

Progesterone-induced $[Ca^{2+}]_i$ responses within sperm whilst bound or in close proximity to oviductal explants

Sperm were added to culture dishes containing oviductal explants and allowed to interact for approximately 1 h before imaging. Before imaging, a focal field was chosen which allowed visualisation of both the explant and the imaging chamber surface. ROI tracking (as described above) was used to obtain fluorescence intensity plots for individual sperm responses to progesterone. Fixed ROI tracking was required due to subtle movements of sperm throughout

the duration of the video. Sperm were considered in close proximity to oviductal explants if within 20 µm.

Progesterone-induced $[Ca^{2+}]_i$ responses within sperm in the absence of oviductal explants.

Sperm were added to culture dishes pre-coated with 1% poly-D-lysine (PDL) and left for approximately 1 h before imaging. Fluorescence intensity plots for individual sperm cell responses to progesterone were obtained using a fixed static ROI.

5.2.13 Image data processing

Fluorescence values were normalised as described in section 3.2.8. Individual cell responses were compiled for all experimental replicates and averaged to generate R_{tot} plots.

5.3 RESULTS

5.3.1 Sperm behaviour with cells of the female reproductive tract

Basic observations were made of sperm behaviour/interactions with explants and primary cultures derived from different anatomical regions of the female reproductive tract. In addition, sperm interactions with the OE E6/E7 cell line and human foreskin fibroblasts (HFF) were also observed as a control.

The effects of female tract architecture on free-swimming sperm

Crypts & Ciliated explants

Free swimming sperm were seen to become entrapped in crypts within the architecture of the epithelial surface of explant tissue. Within the crypts, sperm clustered and displayed highly active flagellar beating. Frequently, sperm were observed to make contact with each other (referred to here after as sperm-sperm contact) and display erratic back and forward movements.

Sperm migration appeared to be altered when placed on highly ciliated explants in comparison to non-ciliated explants. Generally, less sperm were observed to traverse across ciliated explants with the majority of sperm being located within crypts displaying characteristics as described above. Sperm interactions with cilia were easier to observe using ciliated primary cell cultures, in which cilia were in sparse clusters. Sperm approaching cilia slowed upon contact, often displaying a directional change to swim around the periphery of cilia clusters. Sperm movement over cilia appeared to sometimes increase their velocity, although the relatively small area of cilia clusters in which sperm traversed through made it difficult to quantify (data not shown). Occasionally, sperm appeared to intermittently

associate with cilia, rapidly moving back and forth with cilia motion. It was noted that cilia beat frequencies appeared to increase after exposure to sperm although this observation was not quantified. It should be noted that the exact z-plane of sperm verses cilia was not discernible in this data.

Sperm binding interactions with epithelial and fibroblastic cells

Sperm did not bind to HFF, erythrocytes or cellular debris. Upon addition to these cell types, sperm were observed to freely swim without any observable interactions with the exception of contact with clearly defined boundaries.

In contrast, sperm bound to explants of all regions examined, with a tendency to bind in focal areas. This resulted in sperm accumulating in clusters throughout the duration of experiments. Sperm within clusters were not bound to each other and could release from binding independently. Sperm bound were often transiently contacted by other binding sperm, which was frequently associated with detachment either during contact or shortly after.

Sperm were observed to bind through non-specific regions of the head. Many, sperm would appear to pivot and roll whilst remaining in contact with the epithelial surface. Occasionally, sperm were observed to bind via the apical tip of the acrosome, but this was only observed around the edges of explant tissue and rarely during interactions with primary cultures.

Avidity of sperm binding to epithelial and fibroblastic cells

Evidence supporting the low binding avidity of sperm to HFF was further demonstrated by being easily removed during gentle washing conditions (~ 2 ml/min) with sEBSS. Gentle

washing was insufficient to remove sperm bound to female reproductive tract explants of all regions examined. This was also observed for primary cell lines and OE E6/E7 cells but to a lesser extent. In primary cell lines, it was clear that sperm bound with a higher avidity to isthmic compared to ampullary and endometrial derived cells as sperm attachments were more resistant to vigorous washing conditions (~ 6.5 ml/min) using sEBSS. This perfusion rate was sufficient to remove all loose cellular debris and free swimming sperm. Although, this perfusion rate partially removed sperm bound to endometrial and ampullary cells, some remained bound indicating substantial strength in attachment. During gentle perfusion of sEBSS over sperm and female tract cells, sperm mainly orientated to oppose the direction of flow whether they were bound or free. Sperm that were loosely attached were often observed to progress against the flow by what appeared as transient binding to female tract cells.

The location of sperm addition had a great impact on overall sperm numbers binding to explants. The addition of sperm directly above the explant of interest resulted in a large number of sperm settling on the epithelial surface. When sperm were placed next to but not directly above explants, only very few sperm progressed onto central points of the explant, remaining largely bound around the periphery. The few sperm progressing to more central points were observed to display swimming patterns indicative of hyperactivated motility.

This apparent variability in dynamic sperm density was observed to a much lesser extent during sperm addition to confluent primary cell lines. For this reason, sperm binding behaviour was characterised for a series of experiments on confluent primary cell lines derived from endometrium, isthmus and ampulla regions. In these experiments, 2 x 10^6sperm were added during live video recording. Videos were examined in detail for characterisation

of interactions for a period of 375 s over a surface area of 0.1 mm^2. The number of sperm present over any given area was calculated to be approximately 200 per 0.1 mm^2. Figure 5.1 a shows the number of sperm that bound over the duration of 375 s after initial exposure. An average of 49 ± 7, 114 ± 10 and 70 ± 28 sperm bound to endometrial, isthmic and ampullary cells, respectively (mean ± SEM, $n = 4$).

The duration of sperm binding/interactions were further analysed by categorising them into binding duration groups ranging from 10-125 s to >125 s as shown in figure 5.1 b. Interestingly, 60%, 51% and 59% of sperm that bound to endometrial, isthmic and ampullary cells, respectively, bound for only 10-15 s. Only a small fraction of the bound sperm population, 4.6% and 3.5% remained bound for longer than 125 s for endometrial and isthmic cells, respectively. Sperm were not observed in any experimental replicate to bind for longer than 125 s to ampulla-derived primary OECs. The average durations for which sperm bound to endometrial, isthmic and ampullary cells were 15.5 ± 6 s, 46.7 ± 6 s and 29.1 ± 12 s, respectively.

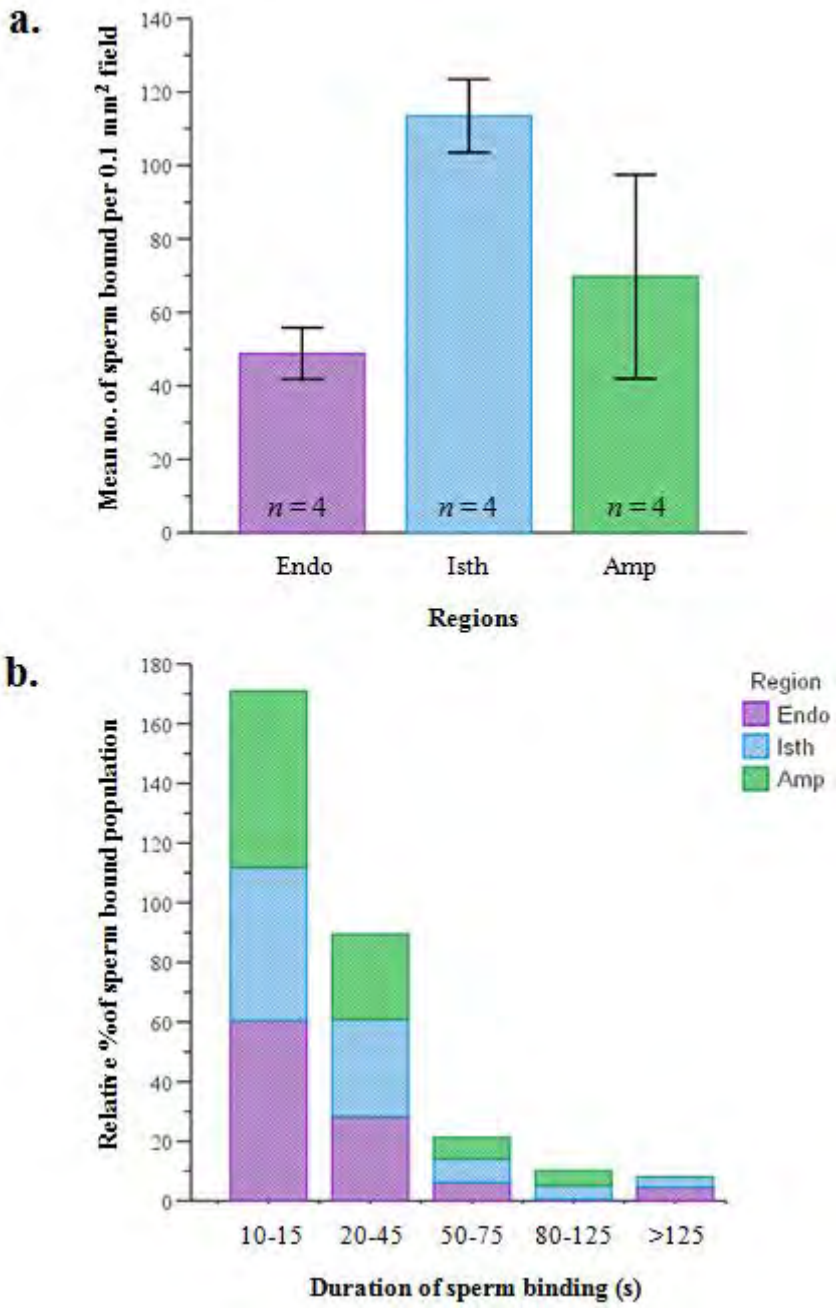


Figure 5.1. Characterisation of the number and duration of sperm binding to primary OECs

(**a**) The mean number of sperm which bound per 0.1 mm^2 field of primary isthmic, ampullary and endometrial epithelial cells. A total of four replicates for each female tract region were performed in alternating order, using the same sperm sample on primary cell cultures originating from the same patient, culture age and confluency. 2 x 10^6 sperm were added and assessed for binding for the first 360 s of interaction. Sperm were considered bound if static for >10 s. Error bars represent SEM; (**b**) binding was then further characterised by assessing the duration of sperm binding before detachment. Sperm were classified into five time duration groups ranging from 10-125s. Any sperm bound for longer than 125 s were grouped in the > 125 s category. The average durations for which sperm bound to endometrial, isthmic and ampullary cells were 15.5 ± 6 s, 46.7 ± 6 s and 29.1. ± 12 s, respectively. Values represent the percentage of sperm that bound for that duration (s) category in respect to the total number bound and are separated by anatomical origin (isthmic, ampullary and endometrial).

5.3.2 Monitoring $[Ca^{2+}]_i$ during sperm binding and detachment

Labelling sperm with a fluorescent probe had the dual advantage of aiding visualisation under fluorescent conditions in comparison to phase-contrast (demonstrated in figure 5.2) and allowing monitoring of $[Ca^{2+}]_i$. This method facilitated more accurate observations of sperm behaviour and numbers on explant tissue.

Fluorescence values (relating to $[Ca^{2+}]_i$) were assessed for sperm binding, bound to and detaching from oviductal epithelium. Average normalised fluorescence plots (R_{tot}) indicated a decline in $[Ca^{2+}]_i$ occurring gradually after initial binding (mean decrease of 7.0% after 290 s; figure 5.3 b). Sperm that were bound before imaging and showed no signs of release demonstrated a more stable R_{tot} plot (mean decrease of 3.3% after 290 s; figure 5.4 b). In contrast, R_{tot} plots in sperm observed to detach from epithelial binding showed a gradual increase in fluorescence (mean increase of 8.2% occurring over 290 s; figure 5.5 b) prior to detaching with a small spike correlating with the point of release (mean increase of 12.7% at time 0; figure 5.5 b).

Sperm were occasionally seen to detach and reattach on explants, primary cultures and immortalised cell lines. A representative trace for fluorescence ($[Ca^{2+}]_i$) and velocity of a transiently binding sperm during release, reattachment and subsequent release is shown in figure 5.6. Results suggest that the increase in $[Ca^{2+}]_i$ associated with detachment occurs before sperm movement as indicated by velocity (red) and fluorescence (green) traces. Interestingly, the sperm shown in figure 5.6 was observed to have come into contact with another sperm (sperm-sperm contact) at ~ 22 s shortly before detachment. This event was commonly seen, where the movement of sperm adjacent to each other or directly in contact

could induce an increase in $[Ca^{2+}]_i$ in both cells (figure 5.6, 5.7), whilst often being associated with subsequent detachment or movement of one or both cells involved. This transient binding was observed more frequently with sperm interacting with primary and OE E6/E7 cell lines.

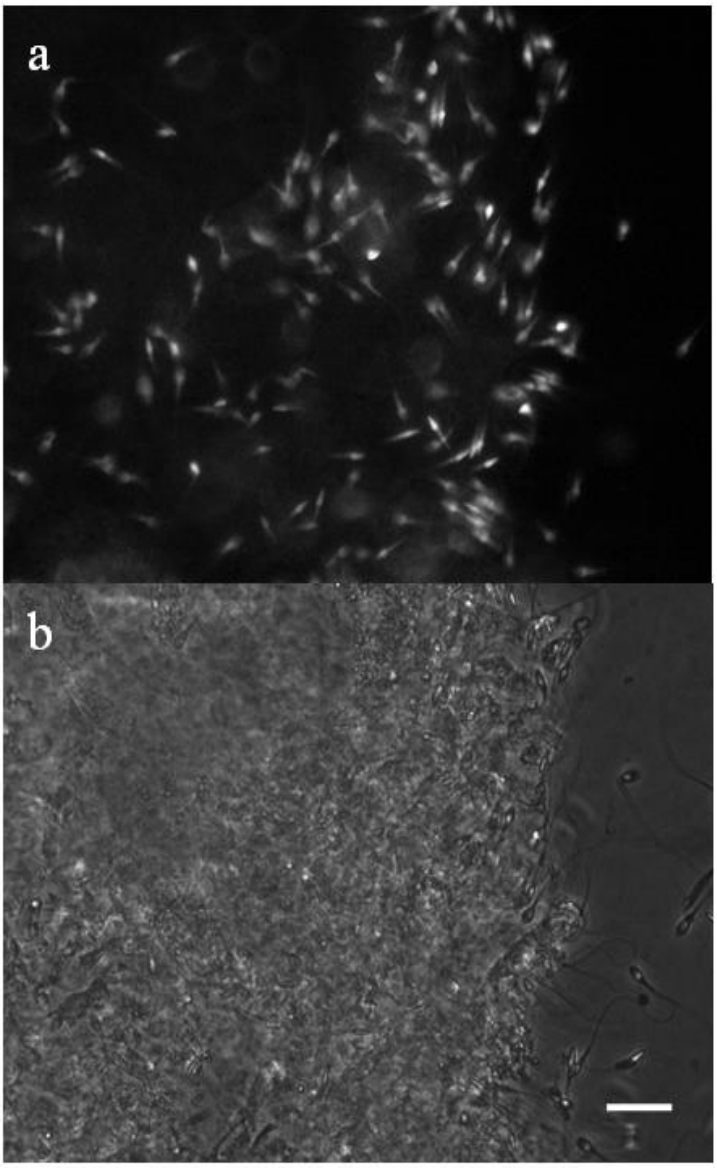


Figure 5.2. Monitoring sperm $[Ca^{2+}]_i$ and binding behaviour on oviductal explants

(**a**) Sperm labelled with Calcium-green-1, allowed monitoring of $[Ca^{2+}]_i$ whilst improving the visualisation of sperm on oviductal explants (isthmus explant shown). Note: choosing a focal field capturing an edge of an explant allows distinction between how sperm behave on plastic or glass verses cells; (**b**) corresponding phase-contrast image. Pictures were taken 5 s apart. Scale bar: 20 µm.

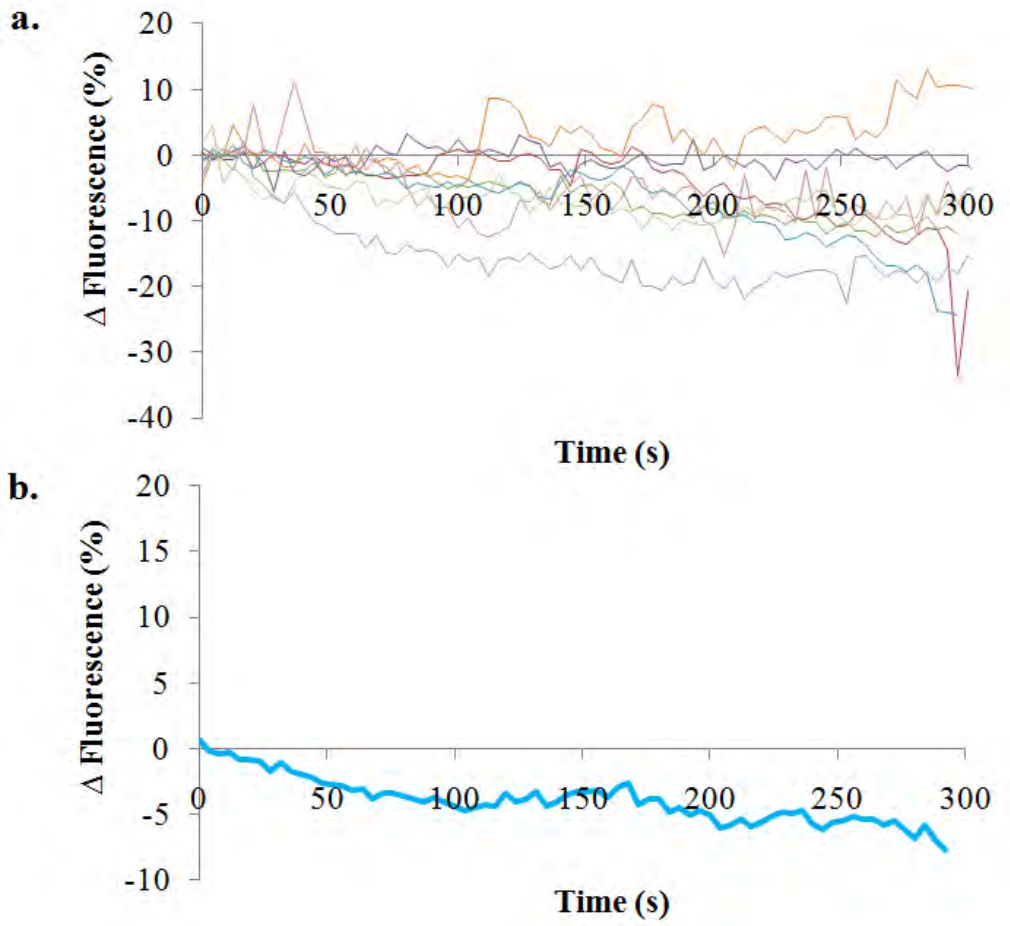


Figure 5.3. $[Ca^{2+}]_i$ in sperm immediately after binding to oviductal explants

Sperm were selected on the basis that they were captured at the initiation of binding (time 0 s) and subsequently remained bound throughout analysis. (**a**) 8 superimposed individual sperm fluorescence traces from 4 experiments (chosen to demonstrate the variability in $[Ca^{2+}]_i$ during this behaviour characterisation); (**b**) mean response (R_{tot}) for all 53 cells in 4 experiments.

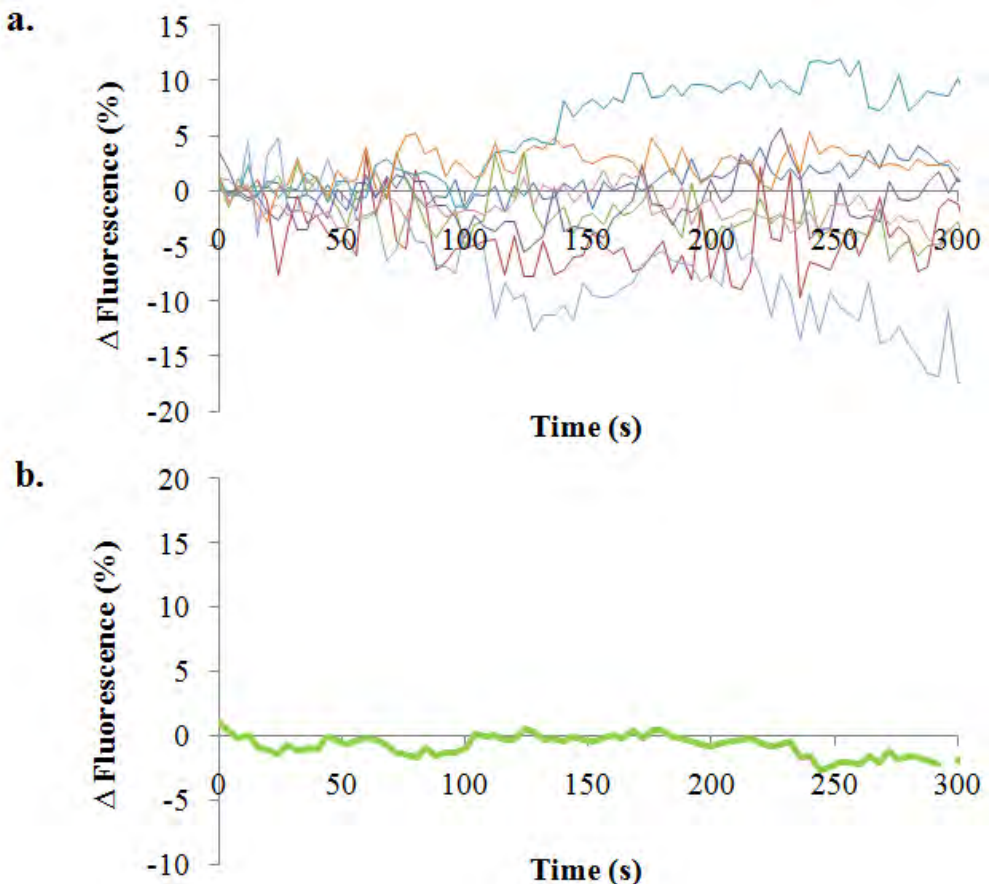


Figure 5.4. $[Ca^{2+}]_i$ in sperm bound to oviductal explants

Sperm were selected on the basis that they were bound at the start of recording and remain tightly bound throughout the analysis. (**a**) 8 superimposed individual sperm fluorescence traces from 3 experiments (chosen to demonstrate the variability in $[Ca^{2+}]_i$ during this behaviour characterisation); (**b**) mean response (R_{tot}) for all 50 cells in 3 experiments.

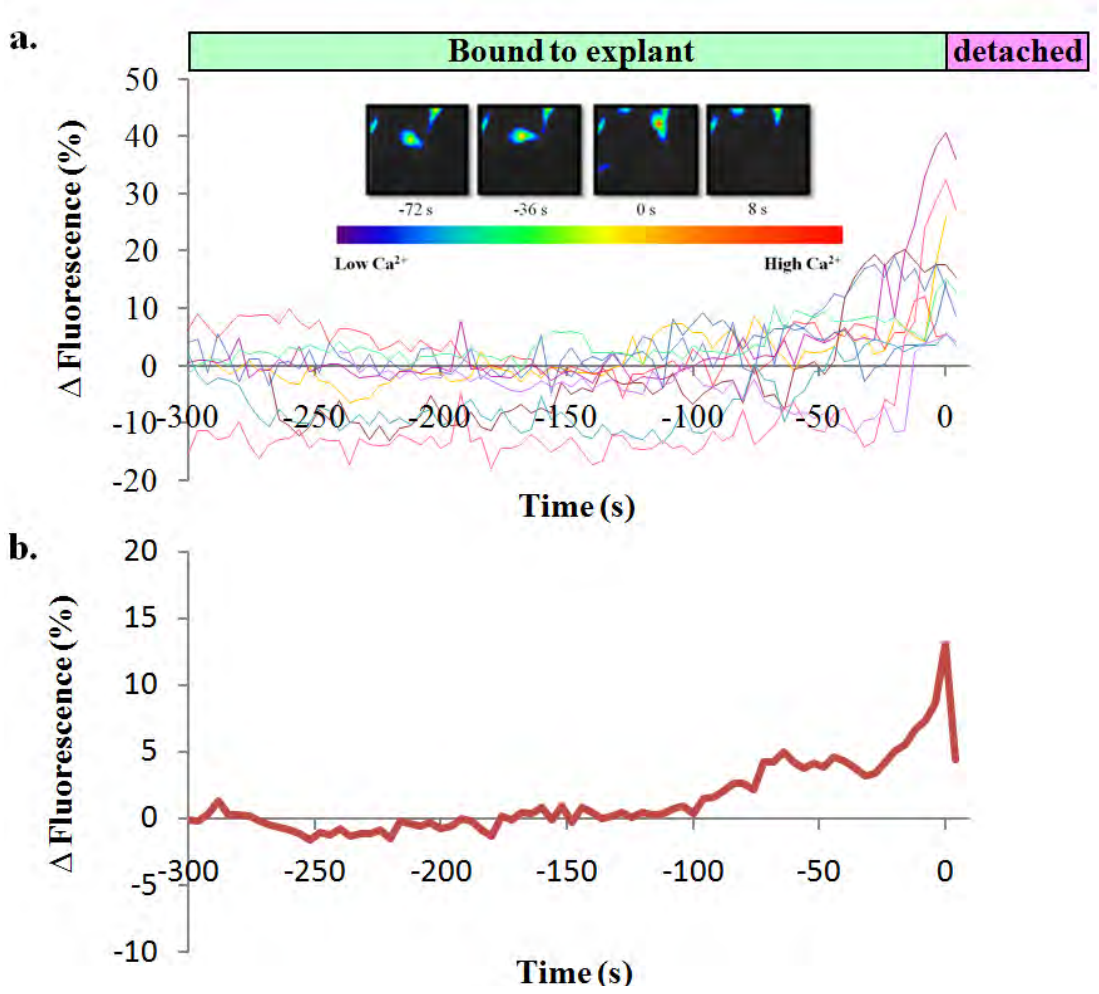


Figure 5.5. $[Ca^{2+}]_i$ in sperm detaching from oviductal explants

Sperm were selected on the basis that they were bound at the start of recording and detached during the analysis (time 0 s). Data shown was accumulated from sperm detaching from both isthmic and ampullary explants. (**a**) 10 superimposed individual sperm fluorescence traces from 5 experiments (chosen to demonstrate the variability in $[Ca^{2+}]_i$ during this behaviour characterisation). Insert shows pseudocolour images of a sperm during detachment; (**b**) mean response (R_{tot}) for all 99 cells in *5* experiments.

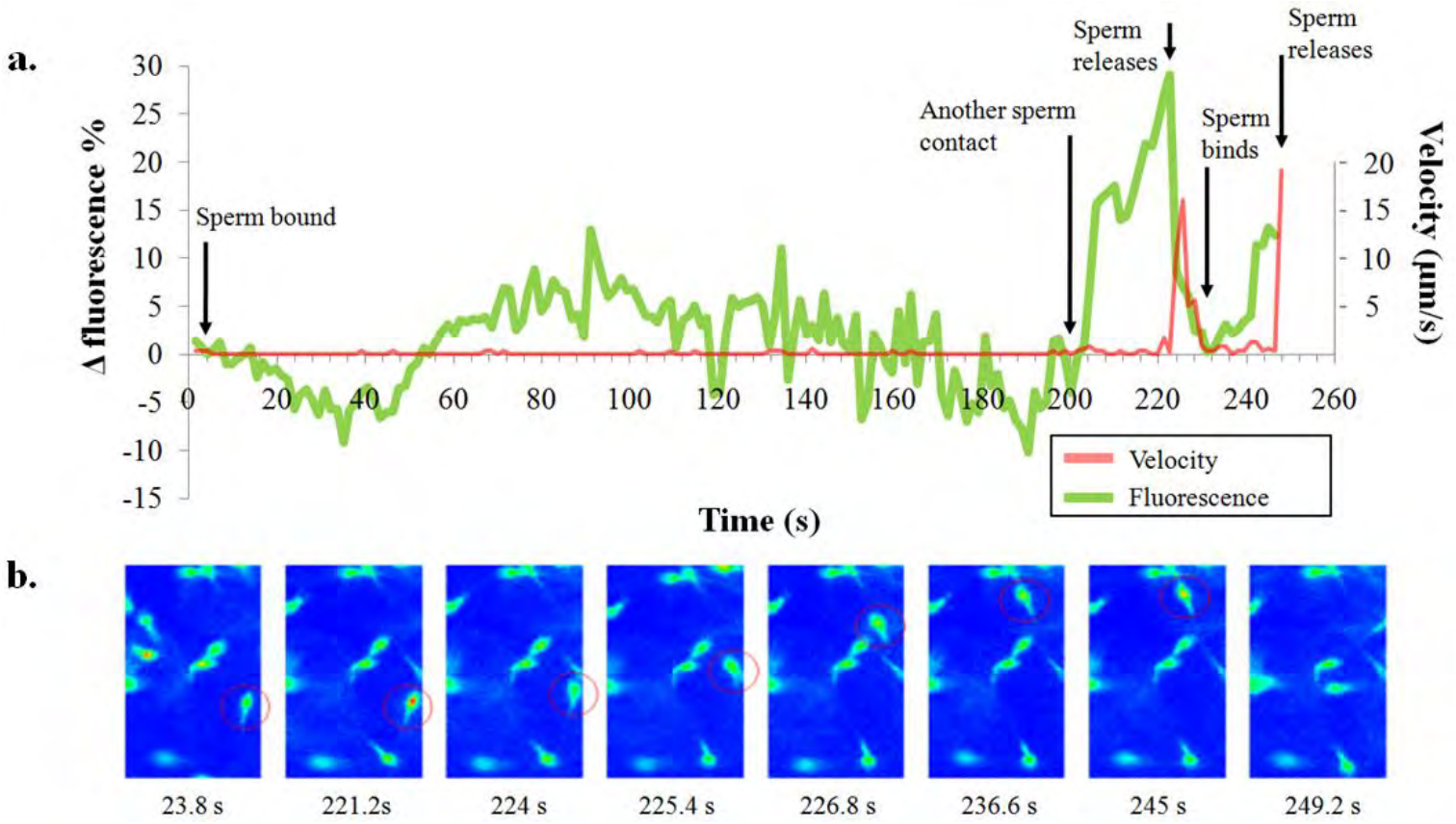


Figure 5.6. $[Ca^{2+}]_i$ and velocity tracking of a single sperm during transient binding to an isthmus derived oviductal explants

(**a**) Shows the fluorescence intensity (correlating to relative $[Ca^{2+}]_i$) and velocity of a single sperm during transient binding to an isthmus derived explant. A fixed ROI was drawn around the midpiece/neck of the sperm and was tracked using Image Pro Plus II® software for approximately 250 s. The sperm of interest was present at the start of recording and appeared bound with little movement for ~ 200 s. At ~ 200 s another sperm makes contact resulting in an increase in $[Ca^{2+}]_i$. At ~ 220 s the sperm releases and migrates before subsequently binding again at ~ 230 s. This transient binding lasted for ~ 17 s before detaching again at ~ 248 s; (**b**) pseudocolour image series of the tracked sperm (indicated by the red circle) during transient binding patterns (warm colours represent high $[Ca^{2+}]_i$). Numbers show time in (s).

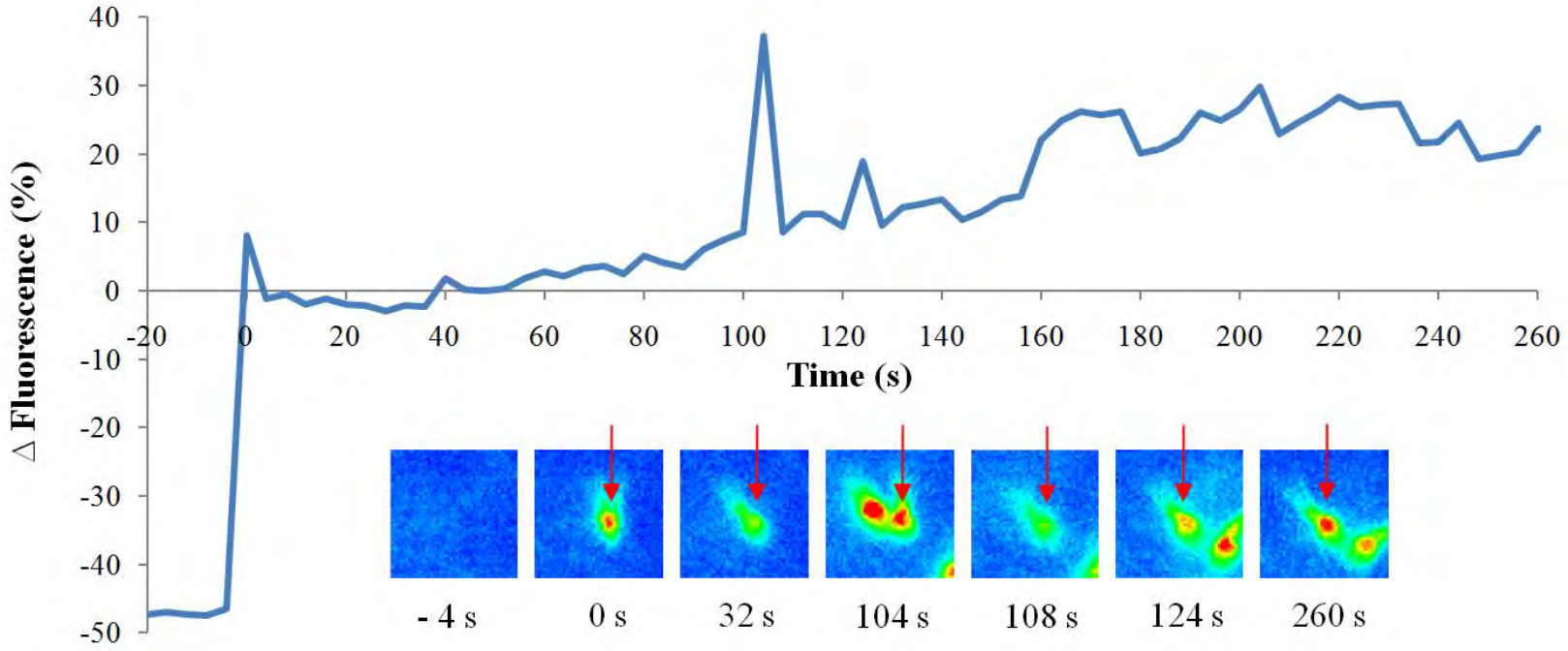


Figure 5.7. Sperm-sperm contact elicits an increase in $[Ca^{2+}]_i$

A representative trace of fluorescence intensity (correlating to relative $[Ca^{2+}]_i$) within sperm in response to contact with another sperm. The sperm of interest binds to an isthmus explant at time 0 s. At ~100 s another sperm binds adjacently for ~ 8 s before detaching at 108 s. At ~120 s an adjacently bound sperm moves into closer proximity. Insert shows a pseudocolour image series correlating to the above fluorescence trace (warm colours represent high $[Ca^{2+}]_i$). Red arrows indicate the sperm of interest. Numbers show time in (s).

5.3.3 Sperm flagellar activity during binding to oviductal explants

Detailed observations of sperm interactions on explants revealed subtle movements of sperm during apparent binding. Some sperm appeared to go through cyclical changes in tail bending movements. This cycle consisted of vigorous tail beating with intermittent pauses in which demonstrating an increase flagellum bend angle and lateral movement of the sperm head. This type of flagellum activity appeared to be correlated with $[Ca^{2+}]_i$ as shown in figure 5.8 a-c. The duration of the increased flagellum bend angles was observed to vary dramatically. Figure 5.8 b demonstrates an increase in flagellum bend angle for ~ 100 s, correlating to an observed rise in fluorescence shown in figure 5.8 a. This association could be mimicked by the addition of 3.2 µM progesterone. Using a high speed acquisition camera (300 Hz), sperm were observed to rapidly alternate between static and motile flagellar activity within only a few hundred milliseconds (ms) (data not shown). On occasions this type of behaviour was associated with sperm detaching however, this pattern of movement was not always sufficient to release bound sperm.

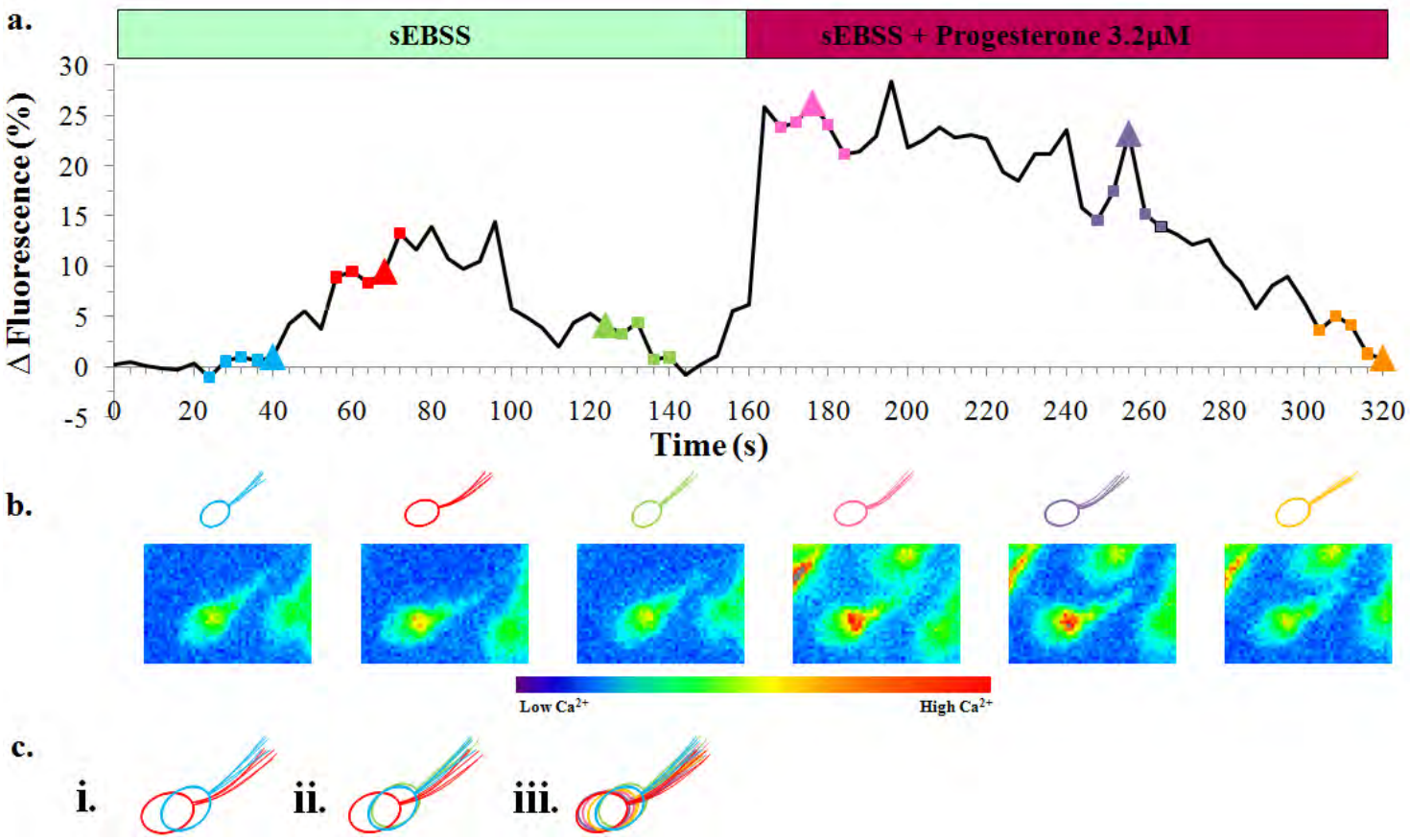


Figure 5.8. $[Ca^{2+}]_i$ in sperm during subtle movements whilst bound to oviductal explants

(**a**) Graph shows a representative normalised fluorescence trace of a single sperm undergoing cyclical changes in midpiece bend angle and movements whilst bound to an oviductal explant (isthmus). The coloured bar above the graph indicates when progesterone is added; (**b**) pseudo-coloured images of the sperm correspond to time points indicated by Δ symbols on graph (a). Above each pseudo-coloured image is an outline trace of the sperm's position for 5 sequential time points indicated by □ symbols on graph (a). Note: all image traces were taken at equal time distances and for the same duration of 20 s. Colour of traces correlates to coloured markers shown on the fluorescence trace in graph (a); (**c**) outline traces presented in (b) superimposed to demonstrate the overall bend angles of the midpiece and head movement corresponding with time points on the graph (indicated by matching colours).

5.3.4 Progesterone-induced $[Ca^{2+}]_i$ responses within sperm in the presence or absence of oviductal explants

Progesterone, a known Ca^{2+} agonist in human sperm, was used to further investigate the role of Ca^{2+} signalling during sperm interaction with oviductal explants. Figure 5.9.a compares R_{tot} plots of sperm responses to progesterone (3.2 µM) when bound to (green trace) or within a 20 µm perimeter of oviductal explants (blue trace) and for sperm attached to a glass surface pre-coated with PDL (red trace) in the absence of oviductal explants. Individual representative traces for sperm responses whilst bound to PDL (figure 5.9 b) or oviductal explants (figure 5.9 c) are shown to demonstrate peak kinetics. Generally, sperm responses whilst bound to PDL demonstrated a transient increase in fluorescence (mean increase of 23.7% at 20 s; figure 5.9 a) that gradually returns toward baseline levels (mean increase of 2.5% at 400 s; figure 5.9 a) after a duration of 400 s. Sperm bound to or within 20 µm of oviductal explants demonstrated responses of similar peak amplitudes in response to progesterone (mean increase of 20.3% and 24.8%, respectively at 20s; figure 5.9 a) however, had a sustained/prolonged elevation in $[Ca^{2+}]_i$ (mean increase of 18.1% and 15.7%, respectively, at 400 s; figure 5.9 a).

The addition of progesterone appeared to have a complex role in sperm detachment. The timing of release for a subpopulation of sperm coincided with responses to progesterone addition. However, there appeared to an overall increase in numbers of sperm binding to oviductal explants after exposure of cells to progesterone (data not quantified).

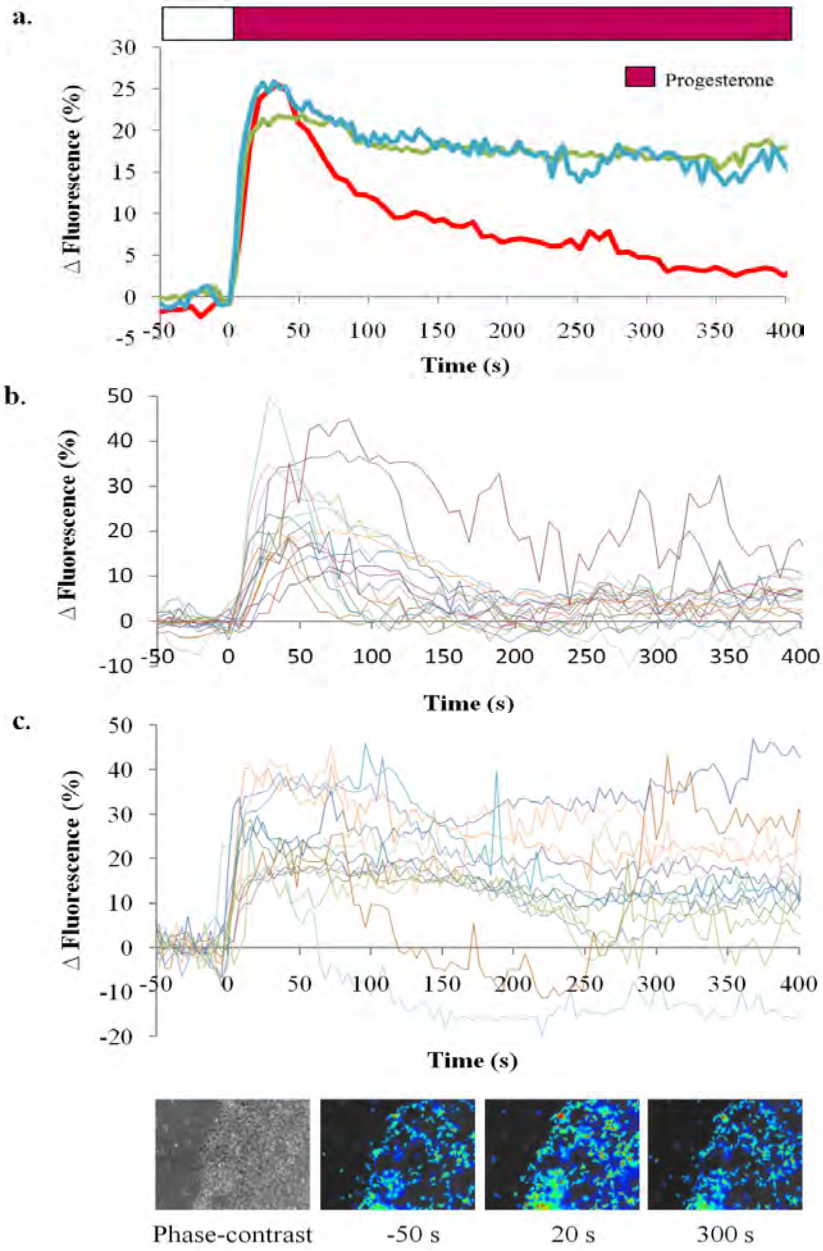


Figure 5.9. Progesterone-induced $[Ca^{2+}]_i$ responses within sperm in the presence or absence of oviductal explants

(a) Mean response (R_{tot}) plots for sperm treated with progesterone whilst bound to explants (n = 290; 5 experiments), bound to PDL coated glass near explants (n = 14; 3 experiments) or bound to PDL coated glass in the absence of oviductal explants (n = 1394; 17 experiments). Time 0 represents the initiation of Ca^{2+} response to progesterone; (**b**) 17 superimposed, single cell $[Ca^{2+}]_i$ responses to progesterone whilst bound to PDL coated glass. Taken from 17 experiments and chosen to demonstrate the variability in $[Ca^{2+}]_i$ responses; (**c**) 14 superimposed, single cell $[Ca^{2+}]_i$ responses to progesterone whilst bound to oviductal explants (both ampulla and isthmus derived). Taken from 5 experiments and chosen to demonstrate the variability in $[Ca^{2+}]_i$ responses; (**Image panel**) pseudocolour image series taken from one experiment to demonstrate a typical global increase observed in sperm $[Ca^{2+}]_i$ in response to progesterone whilst bound to oviductal explants (isthmic derived). From left to right: a phase-contrast image; before progesterone addition (-50 s); shortly after progesterone addition (20 s); and a later image taken after progesterone addition (300 s). Warm colours represent relatively high $[Ca^{2+}]_i$ and numbers show time in (s).

5.3.5 Human oviductal explants produce NO and retain expression of NOS in primary cultures

The production of NO by female reproductive tract explants was demonstrated by labelling with a nitric oxide probe, DAF-FM (figure 5.10). Female reproductive tract explants showed strong labelling with DAF-FM which was attenuated but not completely abolished by 1 mM of L-NAME, a NOS inhibitor (data not shown). All three NOS isoforms were detected through immunocytochemistry in primary OECs as shown in figure 5.11, indicating that NO synthesis could still occur.

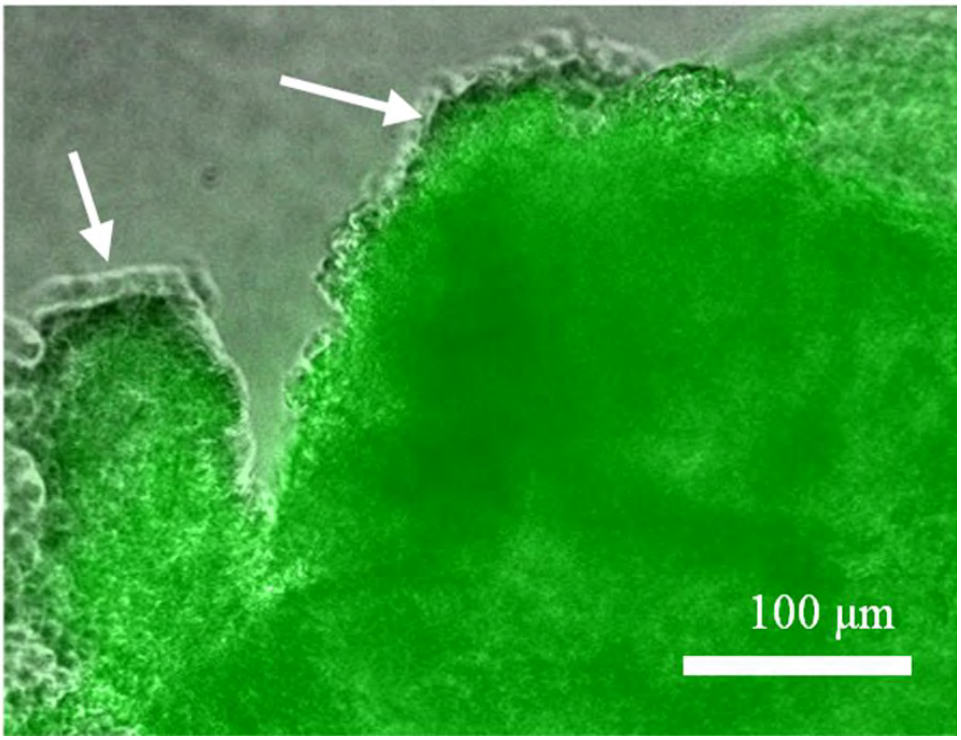


Figure 5.10. Human oviductal explants labelled with DAF

Phase image of an oviductal explant (ampullary region), overlaid with fluorescent image from DAF-FM staining. NO production is occurring throughout the explant. Arrows show beating cilia that are visible where they extend beyond the edge of the explant. Scale bars: 100 µm.

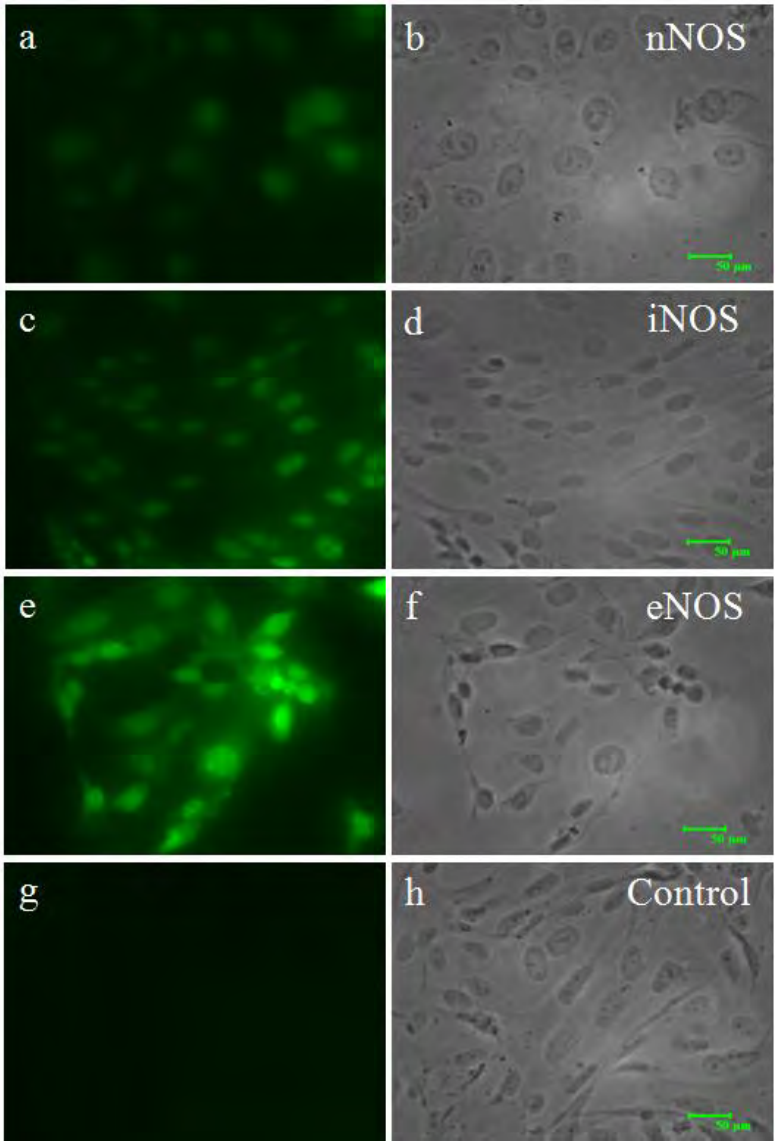


Figure 5.11. Immunofluorescent staining for NOS isoforms in primary OECs

Staining of human primary OECs derived from explants of human oviduct (ampullary region) for: (**a**) nNOS; (**c**) iNOS; (**e**) eNOS; (**g**) no staining was detectable in control incubations in which the primary antibodies were omitted. (**b**,**d**,**f,h**) Corresponding phase-contrast images of these samples. Images were taken using the same equipment settings. Scale bars: 50 µm.

5.3.6 Incubation of sperm with explants of human female reproductive tract induces protein S-nitrosylation

To determine whether NO production by female reproductive tract is sufficient to induce modification events in sperm, sperm were incubated with tract explants for 2 h and assessed for evidence of protein S-nitrosylation.

Sperm were retrieved from these incubations and processed for labelling of S-nitrosothiols. This showed levels of labelling equivalent in intensity and distribution to that induced by parallel incubation with 100 μM S-nitroso-glutathione (GSNO) and slightly greater than that seen with 100 μM spermine NONOate, shown in figure 5.12. Sperm incubated with oviductal explants of female tract showed higher levels of S-nitrosylated proteins (fluorescence labelling with MTSEA) than those incubated with endometrium. The immunocytochemistry of S-nitrosylated proteins was performed by Dr. L. Lefièvre.

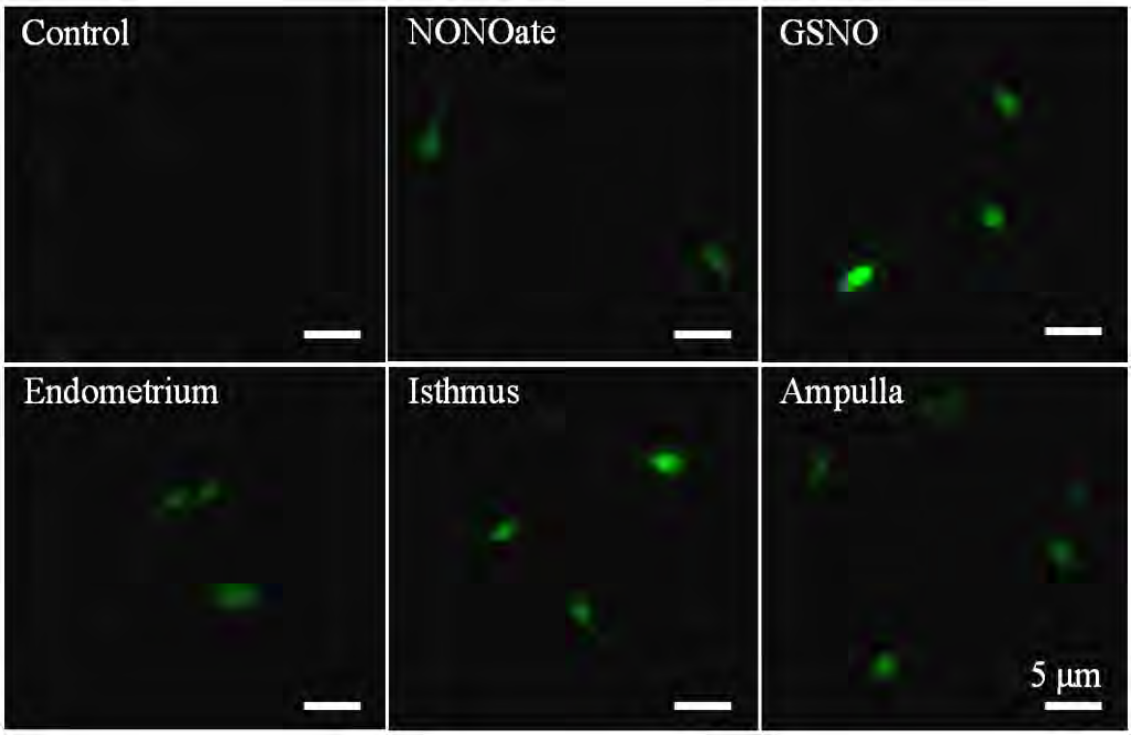


Figure 5.12. NO production by female reproductive tract explants induces S-nitrosylation in human sperm

S-nitrosylated proteins were identified using fluorescently tagged methanethiosulfonate (MTSEA), as described in the text. Negligible levels of labelling were present in controls. Treatment with 100 µM spermine NONOate or GSNO caused clear labelling, particularly at the back of the sperm head. Incubation of sperm with explants derived from endometrial or oviductal regions (ampulla and isthmus) induced levels of S-nitrosylation at least as much as those seen with NONOate. Scale bars: 5 µm.

5.4 DISCUSSION

The purpose of these experiments was to characterise signalling and behaviour in human sperm during interactions with cells of the female reproductive tract.

Basic qualitative observations

Previous studies have reported that the thickness of human oviductal explants reduces illumination transmission, resulting in poor contrast between sperm and the epithelial surface. As a consequence, 'live' observations have been made mainly around the edges of explants, where illumination was brighter, or on collagenase-dissociated tissue (Pacey *et al*., 1995a). In this study, we found that sperm could be observed on undissociated explant tissue when using optimal contrast set-up. Using the $[Ca^{2+}]_i$ indicator, Calcium green-1, we were able to visualise sperm in central regions of explants and mucosal folds whilst also monitoring $[Ca^{2+}]_i$.

Free swimming sperm appeared to accumulate in regions of explants resembling crypt-like structures (epithelial folds) or swim around the periphery of the explant tissue. It was difficult to determine whether these entrapped sperm were interacting with the epithelium or were held there due to tendencies of sperm to accumulate at boundaries (Rothschild, 1962; Smith *et al*., 2009a). In other mammalian species, sperm have been reported to be found in pockets formed by mucosal folds (Suarez, 2008b).

Observations of sperm swimming over small clusters of ciliated cells in primary OECs indicated that cilia may impede sperm progression, alter swimming trajectories or „waft' sperm in a given direction. Cilia generated currents have been reported to move sperm quickly

past ciliated cells (Tollner *et al*., 2008) and in addition cilia beat frequency has been reported to increase after exposure to human sperm (Chiu *et al*., 2010; Li *et al*., 2010; Morales *et al*., 1996). However, in our experiments, the cilia in primary cultures were too sparse and also had lost synchronicity of the metachronal wave. Therefore, reliable conclusions could not be made.

Pacey *et al*. (1995a) reported transient binding to human oviductal explants, lasting on average 33.6 and 20.6 s for isthmic or ampullary derived cells, respectively. Our observations using explants indicated that although a subpopulation of sperm were observed to demonstrate this type of behaviour the vast majority of „binding' sperm remained bound for greater periods of time. However, similar average binding durations were observed for primary cultures being 15.5 ± 6 s , 46.7 ± 6 s and 29.1. ± 12 s for endometrial, isthmic and ampullary cells, respectively. It is feasible that this difference may be due to our use of undissociated explant tissue in which the mucosal surface had not been enzymatically treated. This suggests that sperm may form more intimate associations with explants than previously described. Sperm binding durations were vastly transient in primary cell cultures, which is in agreement with previous human *in vitro* studies (Baillie *et al*., 1997; Morales *et al*., 1996; Yeung *et al*., 1994).

At times sperm binding resembled „Velcro' or „chewing-gum'-like interactions where they were observed to move and rotate with or without progressive movements. Sperm appeared able to re-orientate to oppose flow direction during washing conditions with some loosely bound sperm observed to progress against directional flow by intermittent binding to oviductal epithelium. The physiological implication of this is unknown, however sperm

rheotaxis has previously been observed by Rothschild, (1962) and it is possible that this type of behaviour could aid sperm migration through the female reproductive tract *in vivo*.

Quantitative observation

The dynamic sperm density is a term used here to describe the estimated number of sperm swimming above or across female reproductive tract cells at any given time. Attempts were made to quantify sperm binding to explants during the initial point at which cells were mixed however, we found that numbers observed to bind or interact were confounded by the architecture of the epithelial surface and position of sperm injection. To reduce variation associated with dynamic sperm density and surface architecture, we used confluent monolayers of primary cells (4 day cultures) derived from endometrial, isthmic and ampullary regions of the female reproductive tract (figure 5.1). Our observations indicate that sperm interacted with cells from all regions of the female reproductive tract, with higher numbers of binding to isthmic derived cells (figure 5.1 a). This is in agreement with previous studies on human (Baillie *et al*., 1997) and other mammalian species (Lefebvre *et al*., 1995a; Suarez *et al*., 1991a; Thomas *et al*., 1994).

The number of sperm observed to bind to primary OECs in this study was higher than previously described by Baillie *et al*. (1997). However, in this study, the interest was in sperm binding over the course of 320 s and is therefore not comparable to the method used by Baillie *et al*. (1997), which included washing, fixation and assessment after 1 hour (Baillie *et al*. 1997). Nevertheless, the average ratio of sperm bound per field of isthmic:ampullary and isthmic:endometrial cells for this experimental series was approximately 1:0.6 and 1:0.4,

respectively. This is in close agreement with previous reports by Baillie *et al*. (1997) of 1:0.7 and 1:0.4, respectively.

Sperm binding to primary OECs was more transient, with the majority of sperm binding for less than 15 s (figure 5.1 b). This is in agreement with previous reports of reduced binding avidity of sperm with cultured cells (Baillie *et al*., 1997; Morales *et al*., 1996; Yeung *et al*., 1994). The proportion of bound sperm in each time category up to and including 75 s was similar across all regions examined. The observation that no sperm bound to ampullary cells for > 125 s indicates that derivation and culture of ampullary cells creates a cell line which is less effective in binding sperm for long durations. However, the small numbers of sperm binding observed during the greater time categories in these experiments could be due to random chance. Pacey *et al*. (1995a) suggested binding durations between regions to be similar however, the mean duration of binding was lower for ampullary derived tissue.

Sperm $[Ca^{2+}]_i$ during binding and detachment from oviductal explants

In non-human species, sperm bound to OECs have consistently been reported to maintain lower $[Ca^{2+}]_i$ in comparison to free swimming sperm (Dobrinski *et al*., 1996b; Kawakami *et al*., 2001; Petrunkina *et al*., 2001). Petrunkina *et al*. (2001) reported the kinetics of such temporal changes to be rapid having already occurred within the first sampling point (3 mins after co-incubation). Authors suggested that sperm with lower $[Ca^{2+}]_i$ may preferentially bind. In this study, single cell fluorescence plots of sperm at the initial point of binding revealed a general decrease in cell fluorescence plots (figure 5.3 a) with a mean fluorescence (R_{tot}) plot indicating a gradual decline in $[Ca^{2+}]_i$ occurring rapidly from the onset of binding (figure 5.3 b). Although the data presented here does not disprove that sperm with relatively low $[Ca^{2+}]_i$

may preferentially bind, to our knowledge this provides the first evidence that upon binding there is a reduction in $[Ca^{2+}]_i$.

Single cell fluorescence plots of sperm bound (before and throughout the duration of recording) revealed that $[Ca^{2+}]_i$ was generally stable (figure 5.4 a). The mean fluorescence (R_{tot}) plot of all bound cells indicated a reduction in $[Ca^{2+}]_{i,}$ albeit smaller than that observed in the initial phase of binding (figure 5.4 b), suggesting that $[Ca^{2+}]_i$ then reached a plateau.

Mechanisms involved in lowing $[Ca^{2+}]_i$ during sperm binding to oviductal explants remain elusive, but it has been speculated that oviductal apical membrane proteins may stabilise the plasma membrane of sperm and in turn reduce Ca^{2+} influx associated with capacitation events (Suarez, 2008b). In contrast to bound sperm, sperm observed to detach from oviductal epithelium generally demonstrated an increase in $[Ca^{2+}]_i$ prior to release (figures 5.5 a, b). The mean fluorescence (R_{tot}) plot of all detaching sperm indicated a gradual increase in $[Ca^{2+}]_i$ occurring over ~100 s prior to detachment. A number of previous studies have reported higher $[Ca^{2+}]_i$ in free swimming sperm assumed to have detached or not bound to OECs (Boilard *et al*., 2002; Dobrinski *et al*., 1996b; Petrunkina *et al*., 2001). In addition, bull sperm treated with heparin were observed to detach from binding, an event associated with a gradual increase in $[Ca^{2+}]_i$ occurring over a time period of ~160 s (Gualtieri *et al*., 2005).

Sperm behaviour whilst bound to oviductal explants

Sperm were observed to undergo cyclical alterations in which the midpiece of the flagellum increased in bend angle with moments of no flagellar activity. Interestingly, similar behaviour has been described by Pacey *et al*. (1995b). In this study, sperm were observed to release on

occasions during this type of behaviour. Parallel observations of fluorescence traces indicated increased $[Ca^{2+}]_i$ was associated with increased flagellar bending and lateral head movements (figure 5.8 a-c). Application of progesterone elevated sperm $[Ca^{2+}]_i$ in the neck/midpiece and also increased flagellar bending. This has also been noted in our work on sperm bound to coverslips and may indicate hyperactived motility (Bedu-Addo *et al*., 2007; Bedu-Addo *et al*., 2008; Harper *et al*., 2004).

Increased $[Ca^{2+}]_i$ in the flagellum is associated with the onset of hyperactivation and has been reported to involve both voltage sensitive Ca^{2+} channels (CatSpers) and ryanodine receptors (RYRs), located in the principal piece and redundant nuclear envelope, respectively (Suarez, 2008a). CatSper channels appear to be highly sensitive to a rise in intracellular pH, suggesting that alkalisation occurring during sperm capacitation may activate CatSpers to increase $[Ca^{2+}]_i$ and induce hyperactivation (Kirichok *et al*., 2006; Qi, 2007). The increase in the amplitude of flagellar bend is indicative of hyperactived motility and has been suggested to assist sperm in detaching from oviductal attachments (Demott & Suarez, 1992; Lefebvre & Suarez, 1996; Pacey *et al*., 1995a; Smith & Yanagimachi, 1991). As hyperactivation is reversible (Suarez, 1987) it may serve as a useful mechanism for sperm to progress through the oviduct *in vivo* (Suarez, 2008b), via repeated dynamic attachment and detachment.

Examining sperm displaying transient binding patterns (figure 5.6) represents a good model to study $[Ca^{2+}]_i$ behaviour, as individual sperm are alternating between detaching, free swimming and binding. Results demonstrated that short bursts of movement may be related to increases in $[Ca^{2+}]_i$. Sperm-sperm contact resulted in increases in $[Ca^{2+}]_i$ typically in both sperm cells (figure 5.7) and may have influenced detachment of sperm from binding to

oviductal epithelium. Sperm were also noted to make forward and backward movements when swimming together in folds, as though competing in a race, although there was no net forward progression.

Progesterone-induced $[Ca^{2+}]_i$ responses within sperm in the presence or absence of oviductal explants

Progesterone is perhaps the most studied physiological Ca^{2+} agonist in sperm physiology and has been reported to be a weak inducer of hyperactivation (Calogero *et al*., 2000; Jaiswal *et al*., 1999; Uhler *et al*., 1992; Yang *et al*., 1994). We used progesterone to study its effects on populations of sperm whilst bound to oviductal explants. In control experiments, the addition of progesterone to sperm bound to PDL demonstrated transient responses typically of 90-150s in duration and were similar to established data (Kirkman-Brown *et al*., 2000). Application of progesterone to sperm bound to oviductal explants resulted in a rapid elevation in $[Ca^{2+}]_i$ with peak amplitudes similar to controls (23.7 and 20.3 %, respectively; figure 5.9 a). However, peak durations (from start of rise to inflexion at start of falling phase) showed that sperm on explant tissue demonstrated prolonged responses in comparison to controls (figure 5.9 b, c). To investigate whether this effect was due to intimate sperm-epithelial associations, sperm bound within 20 μm of an explant were also analysed. In this group numbers of cells analysed were low (n=14) as not many sperm bound to the surface were in view for analysis. Despite this, responses to progesterone in these cells resembled those observed for sperm bound to explants (figure 5.9 a).

These results suggest that oviductal explants may produce diffusible factors that alter the kinetics of responses to progesterone. Sperm $[Ca^{2+}]_i$ response kinetics to progesterone have

been reported to be altered in the presence of NO donors (Machado-Oliveira *et al*., 2008). Machado-Oliveira *et al*. (2008) reported pre-treatment and continuous exposure of sperm to NONOate, a NO donor, significantly increased progesterone-induced $[Ca^{2+}]_i$ transient durations of > 150 s from 42 to 92 %. Progesterone is hypothesised to mobilise Ca^{2+} stores in the neck/midpiece of human sperm through activation of RyRs (Harper *et al*., 2004). RyR2 has previously been shown to be a target for S-nitrosylation in human sperm supporting this concept (Lefievre *et al*., 2007; Machado-Oliveira *et al*., 2008; Stoyanovsky *et al*., 1997; Xu *et al*., 1998).

Although the addition of progesterone to sperm bound to oviductal epithelium clearly demonstrated elevations in $[Ca^{2+}]_i$ there was no evidence that progesterone provoked sperm detachment. Gualtieri *et al*. (2005) made similar observations that sperm detachment could not be induced by Ca^{2+} agonists. Collectively, this suggests that $[Ca^{2+}]_i$ increases may induce alteration in flagellar activity, however other factors are also important in regulating detachment. These may be related to capacitation-associated remodelling of plasma membranes which agrees with the concept that capacitation status could be the main regulating factor of sperm-oviductal binding. However, the repeated binding of sperm reveals that any such system is highly dynamic and reversible in humans.

Human female reproductive tract explants produce NO, induce S-nitrosylation of sperm surface proteins and retain expression of NOS in primary cultures

The findings of this study indicate that the human female reproductive tract is a potential source of NO exposure to sperm. Explants labelled with DAF-FM indicated that NO was being produced (figure 5.10) and was further supported by the detection of all three NOS

isoforms in ampullary OECs (figure 5.11). This finding is consistent with previous reports supporting the role of NO production in female tract cells (Ekerhovd *et al*., 1999; Lapointe *et al*., 2006; Rosselli *et al*., 1996).

Co-incubation of sperm with tract explants appeared to be at least as effective in inducing S-nitrosylation in comparison to spermine NONOate (figure 5.12). This finding suggests that sperm could experience post-translation protein modifications via S-nitrosylation whilst in the female reproductive tract. A wide range of sperm proteins have been reported to be S-nitrosylated (Lefievre *et al*., 2007). In addition, S-nitrosylation has been shown to be reversible by thiol reducing agents (Machado-Oliveira *et al*., 2008; Stoyanovsky *et al*., 1997). Evidence for thiol reducing agents inducing sperm detachment from binding to OECs has been shown in a bovine model (Gualtieri *et al*., 2009; Talevi *et al*., 2007). Thiol reducing agents exist within the oviductal environment and therefore *in vivo* it is likely that this potential „switch' plays a complex role in modulation of sperm functions and interactions with OECs.

In summary, the observations made in this study show that sperm bound to and interacted with all regions of the female reproductive tract to varying extents, with isthmic-derived cells binding more sperm on average. Sperm binding to explants appeared stronger in comparison to primary and immortalised oviductal cell lines, indicating that cells may lose expression of factors involved in sperm binding during extended culture despite immortalisation. We provide initial evidence that in human sperm, binding to oviductal epithelium may lower or stabilise $[Ca^{2+}]_i$. Sperm observed to detach from oviductal epithelium, demonstrated a mean increase in $[Ca^{2+}]_i$ that may regulate flagellum activity. Sperm detachment could not be

induced by progesterone, implying there are more complex factors regulating this event. Finally, NO produced from female reproductive tract explants may play a role in modulating sperm function through protein S-nitrosylation.

CHAPTER 6

General Discussion

In this study, the use of single-cell calcium (Ca^{2+}) imaging has allowed detailed analysis of signals evoked during sperm interaction with epithelial cells of human female reproductive tract. The use of long wavelength dyes meant that absolute levels of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) could not be quantified. However, this is compensated for by the stability of signals from the cells and the ability to record for long time periods without marked effects of photo-damage. The data presented support the occurrence of communication between sperm and female reproductive tract cells through generation of $[Ca^{2+}]_i$ signals. Furthermore, we believe that increases in $[Ca^{2+}]_i$ are primarily regulated through mobilisation of intracellular Ca^{2+} stores, leading to the activation of store-operated Ca^{2+} channels.

The results suggest that sperm or their secretory products generate these signals in several regions of the female reproductive tract, which they encounter during migration toward the site of fertilisation *in vivo*. Significantly greater responsiveness was observed in isthmic-derived oviductal epithelial cells (OECs) in comparison to other regions of the female tract. This implies that isthmic cells may have a higher expression of receptors for sperm signalling factors, a concept that is consistent with observations that in most species, including human, sperm appear to bind in higher numbers to isthmic-derived cells. In addition, the isthmus is believed to be a site of sperm storage (at least in non-human mammals) and where sperm capacitation is completed.

Future work must aim to discover the agonists and receptors involved and candidates worth exploring are sperm integrins. The RGD integrin recognition sequence is up-regulated on isthmic cells and has been hypothesised to be involved in integrin-ligand associated intracellular signalling (Reeve *et al*., 2003).

The observation that sperm bind to and induce signalling within endometrial cells is interesting, as relatively little is currently known about how sperm interact with the endometrium. Human *in vivo* studies suggest that few sperm can be recovered from the uterine cavity after uterine flushing (Williams *et al*., 1993b). Rapid transport of sperm through the endometrial cavity has been hypothesised to result from myometrial contractions (Suarez & Pacey, 2006). A recent study has suggested that sperm may induce oviductal contractions through up-regulation of prostaglandin production and secretion (Kodithuwakku *et al*., 2007). Currently, it is not known whether human sperm up-regulate such factors or if oviductal contractility influences sperm transport. This would therefore be an interesting concept to further investigate.

Activity of sperm 'bound' to OECs

Sperm that appeared bound to OECs demonstrated a range of motility characteristics that related to changes in $[Ca^{2+}]_i$. Mean $[Ca^{2+}]_i$ in sperm was observed to decline after initial binding to oviductal explants whilst fluctuations in $[Ca^{2+}]_i$ in adhered sperm appeared to relate to changes in flagellar bending. Sperm responsive to progesterone demonstrated transient elevations of $[Ca^{2+}]_i$ correlating to flagellum bending which is consistent with previous observations (Harper *et al*., 2004; Machado-Oliveira *et al*., 2008). This is the first report to describe direct correlation between alterations in $[Ca^{2+}]_i$ and flagellar bending in a physiological setting (i.e. bound to oviductal epithelium).

Various groups have looked at how molecules and physiological stimuli affect sperm binding to the female tract in different species during ovulation (e.g. Hunter and Kolle work). In our

experiments we were unable to induce sperm to release from binding to oviductal explants, implying that an immediate increase in $[Ca^{2+}]_i$ or flagellum motility is not sufficient alone.

Sperm incubated with female tract explants have been demonstrated here to nitrosylate which had previously been suggested to occur within the cumulus (Machado-Oliveira *et al*., 2008). The presence of nitric oxide, as a switchable signal that may be induced by sperm, is a new and interesting addition to mechanisms underlying regulation in transit lower down the female reproductive tract.

Collectively, this data demonstrates that interaction of sperm with epithelial surfaces of the female tract is not a passive or „inert' process. Interaction causes responses in both sperm and female tract cells. The involvement of $[Ca^{2+}]_i$ in these processes has not previously been shown with this detail in human. Our experiments suggested that when sperm were incubated under non-capacitating conditions, the resulting sperm-conditioned media was less effective in inducing oviductal $[Ca^{2+}]_i$ signals. Therefore, this supports the concept that factors metabolised or shed during sperm capacitation may have a direct effect on surrounding cells. The proposed model for Ca^{2+} signalling during sperm interaction with epithelial cells of the female reproductive tract is shown in figure 6.1.

It should be noted that although sperm were prepared using a „physiological-like' mechanism, it was not truly physiological. It is possible that responses to cells which have been exposed to cervical mucus *in vivo* and not recognised or phagocytosed by leucocytes would be quite different. However, the preparation is likely to replicate the effects encountered during artificial insemination and responses that would occur in female tract cells. In addition, bound

sperm which induce a signal may be those that the female reproductive tract removes. For example, Ca^{2+} signals generated in lung epithelial cells can be a means of leukocyte recruitment (Chun & Prince, 2009). This may also fit with the observation that sperm induce an upregulation of C3 (Georgiou *et al*., 2007) which has been associated with increased immune clearance of sperm.

We hypothesis that changes in protein expression observed in the female reproductive tract upon sperm exposure (e.g. Fazeli and colleagues) occur downstream of the interactions and rapid signalling observed in this study. The $[Ca^{2+}]_i$ signals within female tract cells may therefore be a precursor for an immediate sperm modulating effect (e.g. nitric oxide synthase activation), as well as affecting later secretory events that potentially influence embryo development and implantation.

In conclusion, the data presented raises many more exciting questions than it has answered, but provides a new research front in understanding and examining fertility *in vivo* and potential for developing further diagnostics *in vitro*.

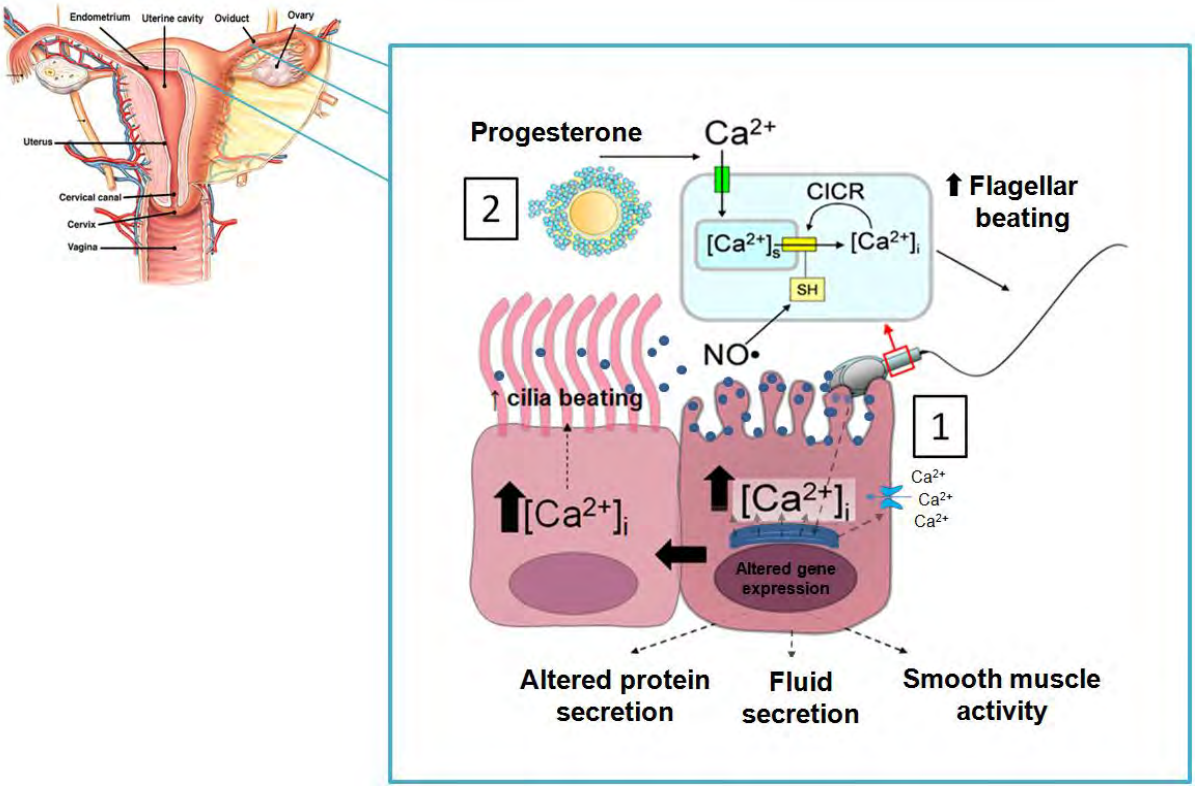


Figure 6.1. Ca^{2+} signalling events during sperm-female tract cell interactions

1) Sperm-female tract cell interactions can result in an increase in $[Ca^{2+}]_i$ within female reproductive tract cells through mobilisation of intracellular Ca^{2+} stores. The depletion of stored intracellular Ca^{2+} triggers store operated Ca^{2+} channels resulting in an influx of Ca^{2+} through the plasma membrane. Ca^{2+} signals can propagate to neighbouring cells and may serve as a mechanism to initiate changes in the tract environment.

2) Sperm binding to a female reproductive tract cell is likely to involve binding of apical membrane proteins implicated in modulating sperm functions. Increases in $[Ca^{2+}]_i$ are associated with increased flagellar activity and may provide the necessary force to induce detachment from binding. External factors such as progesterone and nitric oxide are likely to have synergistic roles in modulating $[Ca^{2+}]_i$ signalling during sperm-female tract cell interactions.

APPENDICES

APPENDIX I: MEDIA

Supplemented Earle's Balanced Salt Solution (sEBSS)

Sodium Dihyd. Phosphate	0.122 g/l (1.0 mM)
Potassium Chloride	0.4 g/l (5.4 mM)
Magnesium Sulphate.7H2O	0.2g/l (0.81 mM)
Dextrose Anhydrous	1.0 g/l (5.6 mM)
Sodium Pyruvate	0.3g/l (2.7 mM)
DL-Lactic Acid, Sodium	4.68 g/l (41.8 mM)
Calcium Chloride.2H2O	0.73 g/l (5.0 mM)
Sodium Bicarbonate	2.2 g/l (26.2 mM)
Sodium Chloride	5.0 g/l (85.6 mM)
HEPES	2.38 g/l (10.0 mM)

sEBSS recipe was based upon Supplemented Earle's Balanced Salt Solution w/o Phenol Red recipe. Sodium chloride was added until osmolarity was 285-295 mOsm. Media osmolarity was checked using an Advanced Micro Osmometer (Vitech Scientific Ltd, West Sussex, UK) which has been pre-calibrated using a 50 mOsm/Kg H_2O and a 850 mOsm/Kg H_2O calibration standards. sEBSS pH was adjusted to 7.3-7.4 with 1M HCl and 1M NaOH and subsequently stored as 100 ml volumes in glass beakers at 4°C until use. 0.3% Bovine serum albumin (BSA, Probumin Fatty acid free) was added on experimental day.

Supplemented Earle's Balanced Salt Solution-Ethylene glycol-bis (β-amino-ethylether)-N,N,N'N'-tetraacetic acid (LCsEBSS)

Sodium Dihyd. Phosphate	0.122 g/l (1.0 mM)
Potassium Chloride	0.4 g/l (5.4 mM)
Magnesium Sulphate.7H2O	0.2g/l (0.81 mM)
Dextrose Anhydrous	1.0 g/l (5.6 mM)
Sodium Pyruvate	0.3g/l (2.7 mM)
DL-Lactic Acid, Sodium	4.68 g/l (41.8 mM)
Calcium Chloride.2H2O	0.73 g/l (5.0 mM)
Sodium Bicarbonate	2.2 g/l (26.2 mM)
Sodium Chloride	5.0 g/l (85.6 mM)
HEPES	2.38 g/l (10.0 mM)
EGTA	3.43g/l (9.000 mM)

Preparation as for standard sEBSS. 0.3% BSA (fatty acid free) was added on experimental day.

APPENDIX II: CHAPTER 2

Appendix 2.i

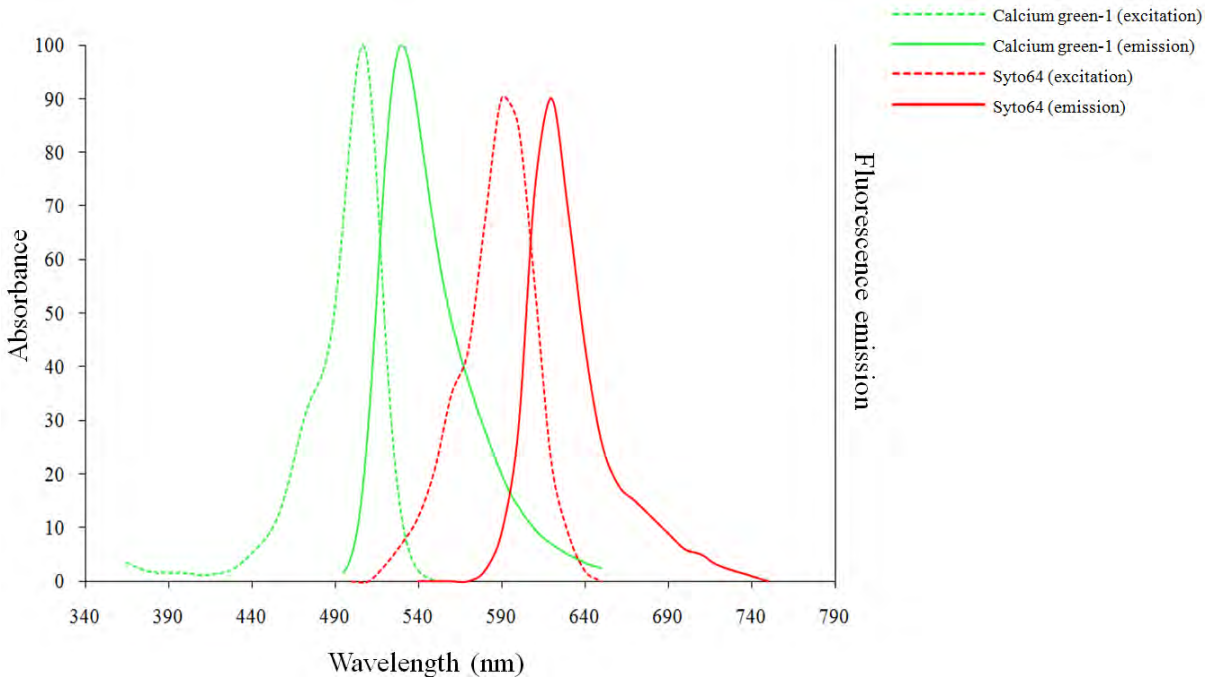
Specimens	Age	Endometrial histology	Pathology diagnosis	Proven fertility
A	49	Secretory	Dysmenorrhea	Yes
B	47	Secretory	Leiomyomata	Yes
C	39	Proliferative	Menorrhagia, dysmenorrhea	Yes
D	50	Proliferative	Leiomyomata, adenomyosis	No
E	44	Proliferative	Leiomyomata, adenomyosis	Yes
F	48	Not known	Leiomyomata, adenomyosis	Yes
G	45	Late secretory	Leiomyomata	Not known
H	42	Early secretory	Adenomyosis	Yes
I	39	Secretory	Leiomyomata	Yes
J	42	Not known	Leiomyomata, adenomyosis	Yes
K	49	Late secretory	Menorrhagia, leiomyomata	Yes
L	56	Secretory	Leiomyomata	Yes
M	47	Late secretory	Menorrhagia	No
N	43	Progestational effect (a)	Adenomyosis	Yes
O	44	Proliferative	Adenomyosis, leiomyomata	Yes
P	49	Progestational effect (a)	Leiomyomata, menorrhagia	Not known
Q	43	Proliferative phase	Menorrhagia, leiomyomata	Yes
R	49	Not known	Not known	Not known
S	45	Secretory	Adenomyosis	Yes
T	52	Proliferative	Leiomyomata, adenomyosis	Not known
U	52	Proliferative	Adenomyosis	Yes
V	40	Proliferative	Dysmenorrhea	Yes
W	46	Post-menstrual	Pelvic organ prolapse	Yes

Clinical characteristics of women from whom reproductive tract tissue were obtained

Individual specimens were assigned a letter code. Phase of menstrual cycle was determined by histological dating of the endometrium. Histology demonstrating evidence of decidualised stroma characteristic of women taking exogenous progestins is denoted with (**a**).

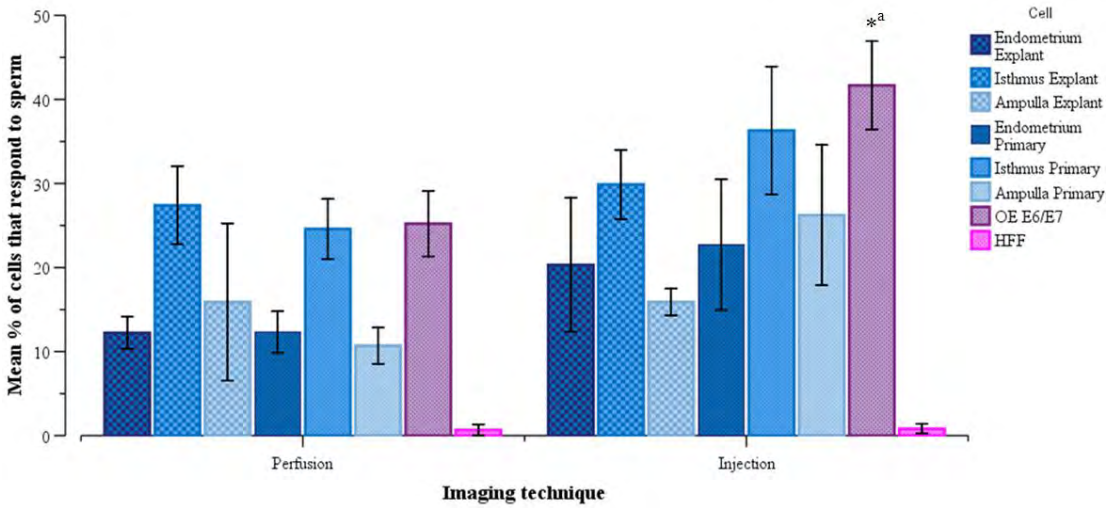
APPENDIX III: CHAPTER 3

Appendix 3.i



Excitation and emission spectra for Calcium green-1 AM and Syto64

Appendix 3.ii



Mean (%) of cell populations that initiate a significant calcium response to sperm separated by imaging technique

Data was classified by logical analysis of significant response. Female reproductive tract explant tissue, primary cultures and model cell lines, OE E6/E7 and HFF were exposed to media containing sperm ($500x10^3$). Sperm were introduced to cells by either the injection or perfusion method. Error bars represent SEM. Asterisk denotes statistical significance ($P<0.05$) performed by a Mann-Whitney U (independent) test comparing each cell type and regions responsiveness to sperm when performed by two different imaging techniques. A significant difference was found for OE E6/E7 cells, [a].

	Explant			Primary cell line			Cell line	
	Endometrium	Isthmus	Ampulla	Endometrium	Isthmus	Ampulla	OE E6 E7	HFF
Perfusion								
No. of replicates (*n*)	7	3	3	9	15	8	15	4
Mean % response ± SEM	12.2 ± 1.9	27.4 ± 4.7	15.9 ± 9.4	12.3 ± 2.5	24.6 ± 3.6	10.7 ± 2.2	25.2 ± 3.9	0.7 ± 0.7
Mean Δ Fluo % ± SEM	14.2 ± 3.2	7.4 ± 1.7	7.4 ± 1.7	19.4 ± 3.4	12.4 ± 1.5	9.2 ± 1.7	17.6 ± 1.5	8.9 ± 3.6
Injection								
No. of replicates (*n*)	5	5	6	7	7	7	13	7
Mean % response ± SEM	20.3 ± 8.0	29.9 ± 4.1	15.9 ± 1.6	22.7 ± 7.8	36.3 ± 7.6	26.2 ± 8.4	41.7 ± 5.2	0.82 ± 0.6
Mean Δ Fluo % ± SEM	10.9 ± 1.8	6.1 ± 1.0	11.4 ± 2.9	10.6 ± 1.5	13.8 ± 1.3	8.9 ± 1.1	17.6 ± 0.9	8.8 ± 2.6

Summary data used for 3.ii

Perfusion vs. Injection	P value
Endo explant	0.755
Isth explant	0.571
Amp explant	0.714
Endo primary	0.351
Isth primary	0.142
Amp primary	0.232
OE E6/E7	0.037
HFF	0.927

P values from Mann-Whitney U statistical test relating to 3.ii

Appendix 3.iii

	Cell type	N number	P value
Cond. vs. Control	Explant	5	0.043
	Primary	12	0.002
	OE E6/E7	9	0.015
	HFF	7	0.080
Sperm vs. Control	Explant	29	0.000
	Primary	53	0.000
	OE E6/E7	28	0.000
	HFF	11	0.028
ATP vs. Control	Explant	17	0.001
	Primary	30	0.000
	OE E6/E7	20	0.000
	HFF	11	0.003

	Cell type	N number	P value
Cond. vs. Sperm	Explant	5	0.08
	Primary	12	0.424
	OE E6/E7	9	0.008
	HFF	7	0.715

P values from Wilcoxon statistical test relating to Figure 3.9

Appendix 3.iv

	Endo	Isth	Amp
Explant endo (n =12)		0.0069	2.2008
Explant isth (n = 8)	0.0069		0.0317
Explant amp (n = 9)	2.2008	0.0317	
Primary endo (n = 16)		0.0689	2.2833
Primary isth. (n = 22)	0.0689		0.0316
Primary amp (n = 15)	2.2833	0.0316	

P values from Kruskal Wallis - Dunns statistical test relating to Figure 3.10

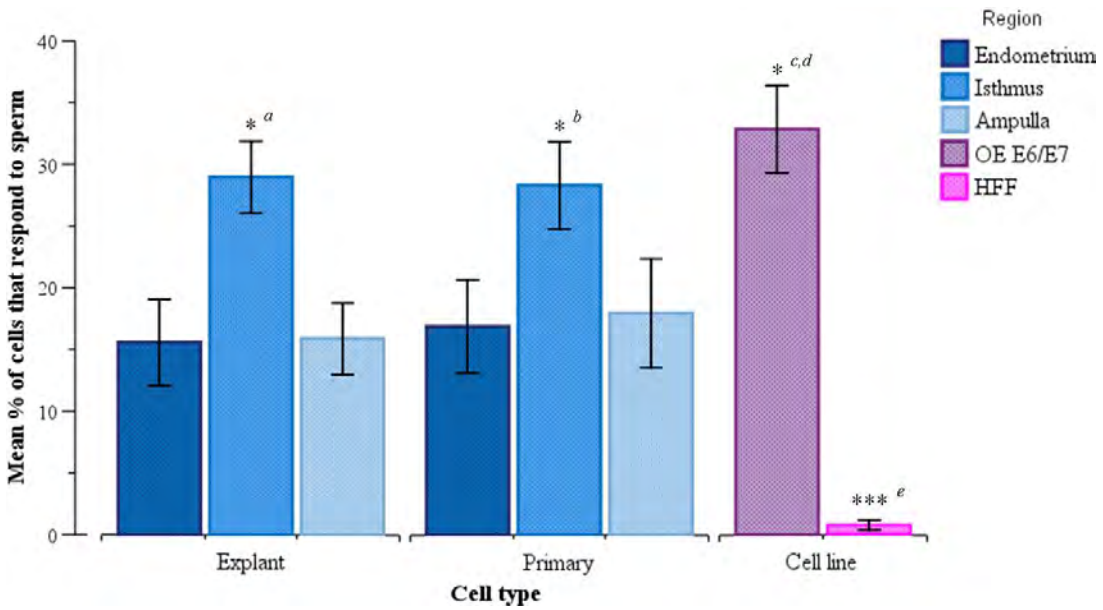
	Endo explant	Isth explant	Amp explant	Endo primary	Isth primary	Amp primary	OE E6/E7	HFF
Endo explant		0.007	0.508	0.982	0.031	0.648	0.002	0.000
Isth explant	0.007		0.001	0.013	0.298	0.003	0.723	0.000
Amp explant	0.508	0.001		0.978	0.147	0.379	0.006	0.000
Endo primary	0.982	0.013	0.978		0.039	0.984	0.002	0.000
Isth primary	0.031	0.298	0.147	0.039		0.005	0.154	0.000
Amp primary	0.648	0.003	0.379	0.984	0.005		0.002	0.000
OE E6/E7	0.002	0.723	0.006	0.002	0.154	0.000		0.000
HFF	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

P values from Mann Whitney U statistical test relating to Figure 3.10

	Sperm treatment							
Internal control (non-cond. treatment)	**Endo explant**	**Isth explant**	**Amp explant**	**Endo primary**	**Isth primary**	**Amp primary**	**OE E6/E7**	**HFF**
Endo explant	0.002 (n=12)							
Isth explant		0.018 (n=8)						
Amp explant			0.012 (n=9)					
Endo primary				0.027 (n=16)				
Isth primary					0.000 (n=22)			
Amp primary						0.001 (n=15)		
OE E6/E7							0.000 (n=28)	
HFF								0.028 (n=11)

P values from Wilcoxon statistical test relating to Figure 3.10

Appendix 3.v



Mean (%) of ATP responsive cell populations that initiate a significant calcium response to sperm separated by cell type and region

Data was classified by logical analysis of significant responses. Only cells that demonstrated a significant response to ATP (100 µM) were used for analysis. Both explant tissue and primary cell lines derived from human female reproductive tract donors were separated into the three anatomical regions: endometrial, isthmic and ampullary epithelium. Parallel experiments were also performed in a model oviductal cell line, OE E6/E7 and non-reproductive tract cell line, human foreskin fibroblasts (HFF). Cells were exposed to sperm in sEBSS ($500x10^3$). Error bars represent SEM. Regions within both explant and primary cell types were compared for significance using Kruskal-Wallis test and Dunn's test. Cell lines were compared using Mann-Whitney U. Asterisks denote statistical significance *** ($P<0.001$) and * ($P<0.05$). Isthmic cells for both explant and primary cell types showed a significant increase [a,b] in responsiveness in comparison to endometrial and ampullary derived cells within cell types. OE E6/E7 cells were compared to both explant [c] and primary [d] ampullary cells. A significant difference in responsiveness was found when HFF were compared to all cell type regions and the OE E6/E7 cell line [e].

	Explant			Primary cell line			Cell line	
	Endo.	Isthmus	Ampulla	Endo.	Isthmus	Ampulla	OE E6 E7	HFF
No. of replicates (*n*)	12	8	9	16	22	15	28	11
No. of cells analysed	1309	880	1815	1690	1840	1729	4047	1158
Mean % response ± SEM	15.7 ± 3.6	29.0 ± 2.9	15.9 ± 2.9	16.9 ± 3.8	28.3 ± 3.5	18 ± 4.4	32.9 ± 3.5	0.8 ± 0.4
Mean Δ Fluo % ± SEM	12.8 ± 2.0	6.9 ± 0.8	10.0 ± 2.1	15.5 ± 2.3	12.9 ± 1.1	9.1 ± 1.0	17.6 ± 0.9	8.9 ± 1.8

Summary data used for 3.v

	Endo	Isth	Amp
Explant endo (n =12)		0.0079	1.9553
Explant isth (n = 8)	0.0079		0.0472
Explant amp (n = 9)	1.9553	0.0472	
Primary endo (n = 16)		0.0329	2.7847
Primary isth (n = 22)	0.0329		0.0492
Primary amp (n = 15)	2.7847	0.0492	

P values from Kruskal Wallis - Dunns statistical test relating to 3.v

	Endo explant	Isth explant	Amp explant	Endo primary	Isth primary	Amp primary	OE E6/E7	HFF
Endo explant		0.007	0.464	0.945	0.012	0.905	0.001	0.000
Isth explant	0.007		0.002	0.019	0.662	0.028	1.000	0.000
Amp explant	0.464	0.002		0.978	0.052	0.640	0.010	0.000
Endo primary	0.345	0.019	0.978		0.016	0.800	0.003	0.000
Isth primary	0.012	0.662	0.052	0.016		0.001	0.379	0.000
Amp primary	0.905	0.028	0.0.64	0.800	0.010		0.004	0.000
OE E6/E7	0.001	0.000	0.001	0.003	0.379	0.004		0.000
HFF	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

P values from Mann Whitney U statistical test relating to 3.v

Internal control (non-cond. treatment)

	Sperm treatment							
	Endo explant	**Isth explant**	**Amp explant**	**Endo primary**	**Isth primary**	**Amp primary**	**OE E6/E7**	**HFF**
Endo explant	0.002(n=12)							
Isth explant		0.018(n=2)						
Amp explant			0.012 (n=9)					
Endo primary				0.036(n=16)				
Isth primary					0.000(n=22)			
Amp primary						0.001(n=15)		
OE E6/E7							0.000(n=28)	
HFF								0.028(n=11)

P values from Wilcoxon statistical test relating to 3.v

Appendix 3.vi

Stage of cycle	P value
Explant pro. vs. sec.	0.328
Primary pro. vs. sec.	0.028
OE E6/E7 oest. vs. no oest	0.690

P values from Mann-Whitney U statistical test relating to Figure 3.11 a, c

Cell type/stage	P value
Explant	
Endo pro. vs. Endo sec.	0.800
Isth pro. vs. Isth sec.	0.800
Amp pro. vs. Amp sec.	0.200
Primary	
Endo pro. vs. Endo sec.	0.400
Isth pro. vs. Isth sec.	0.133
Amp pro. vs. Amp sec.	0.667

P values from Mann-Whitney U statistical test relating to Figure 3.11 b

APPENDIX IV: CHAPTER 4

Appendix 4.i

Cell type / 1° & 2° peaks	Cell type	P value
Endometrial explant	Isthmus explant	0.6050
	Ampulla explant	0.9012
Isthmus explant	Endometrial explant	0.6050
	Ampulla explant	0.8342
Ampulla explant	Endometrial explant	0.9012
	Isthmus explant	0.8342
Endometrial primary	Isthmus primary	0.0924
	Ampulla primary	0.9930
Isthmus primary	Endometrial primary	0.0924
	Ampulla primary	0.1377
Ampulla primary	Endometrial primary	0.9930
	Isthmus primary	0.1377

One way ANOVA + Tukey statistical test relating to Figure 4.3

Cell type / 1° & 2° peaks	P value
Endometrial primary vs. Endometrial explant	0.171
Isthmus primary vs. Isthmus explant	0.033
Ampulla primary vs. Ampulla explant	0.011
Ampulla explant vs. OE E6/E7	0.706
Ampulla primary vs. OE E6/E7	0.054

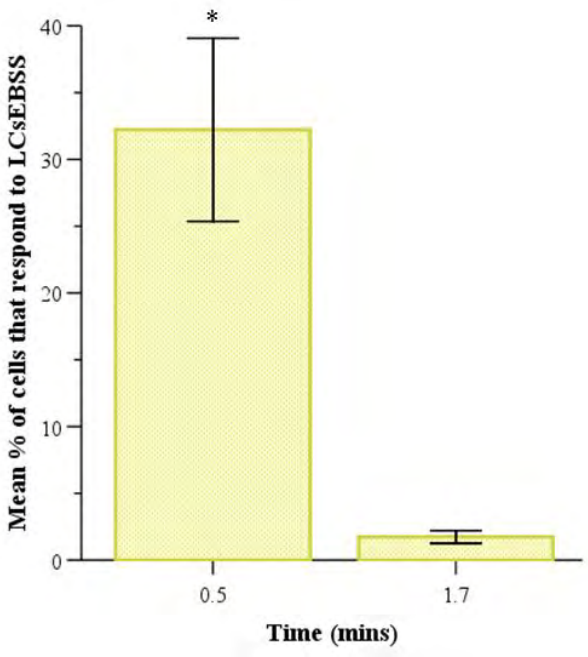
P-values from Independent t-test relating to Figure 4.3

Appendix 4.ii

	Explant cond	Explant sperm	Explant ATP	Prim Cond.	Prim sperm	Prim ATP	OE E6/E7 cond.	OE E6/E7 sperm	OE E6/E7 ATP
Explant cond. (n=6)		0.568	0.032	0.876	0.794	0.046	0.206	0.016	0.000
Explant sperm (n=29)	0.568		0.001	0.444	0.126	0.000	0.012	0.000	0.000
Explant ATP (n=17)	0.032	0.001		0.086	0.011	0.223	0.846	0.918	0.000
Prim cond. (n=6)	0.876	0.444	0.086		0.913	0.006	0.207	0.016	0.000
Prim sperm (n=53)	0.794	0.126	0.011	0.913		0.000	0.088	0.000	0.000
Prim ATP (n=30)	0.046	0.000	0.223	0.006	0.000		0.29	0.134	0.000
OE E6/E7 cond. (n=9)	0.206	0.012	0.846	0.207	0.088	0.29		0.734	0.000
OE E6/E7 sperm (n=28)	0.016	0.000	0.918	0.016	0.000	0.134	0.734		0.000
OE E6/E7 ATP (n=20)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

P values form Independent t-test relating to Figure 4.5

Appendix 4.iii



Mean (%) of cell populations that initiate a significant calcium response to the introduction of LCsEBSS media

Data was classified by logical analysis of significant responses. OE E6/E7 cells were continuously perfused with sEBSS prior to switching to LCsEBSS for experiments investigating calcium store mobilisation. The graph shows the mean % of cells that responded to the introduction of LCsEBSS was assessed at 0.5 min and 1.7 min after initial exposure. Error bars represent SEM. Asterisk denote statistical significance ($P<0.05$) performed by a wilcoxon (paired) test. A significant increase in cells with elevated fluorescence was observed after 0.5 mins of LCsEBSS addition. There was no statistical difference in % of cells with elevated levels of fluorescence at 1.7 mins after the introduction of LCsEBSS when compared to controls.

	LCsEBSS (0.5 min)	LCsEBSS (1.7 min)
No. of replicates (*n*)	13	13
No. of cells analysed	2393	2393
Mean % response ± SEM	32.2 ± 6.9	1.7 ± 0.5
Mean Δ Fluo % ± SEM	17.4 ± 0.6	10.8 ± 1.7

Summary of data used for 4.iii

Treatment	P Value
LCsEBSS	0.002
LCsEBSS after 1.7 min	0.866

P values from Wilcoxon statistical test relating to 4.iii

Appendix 4.iv

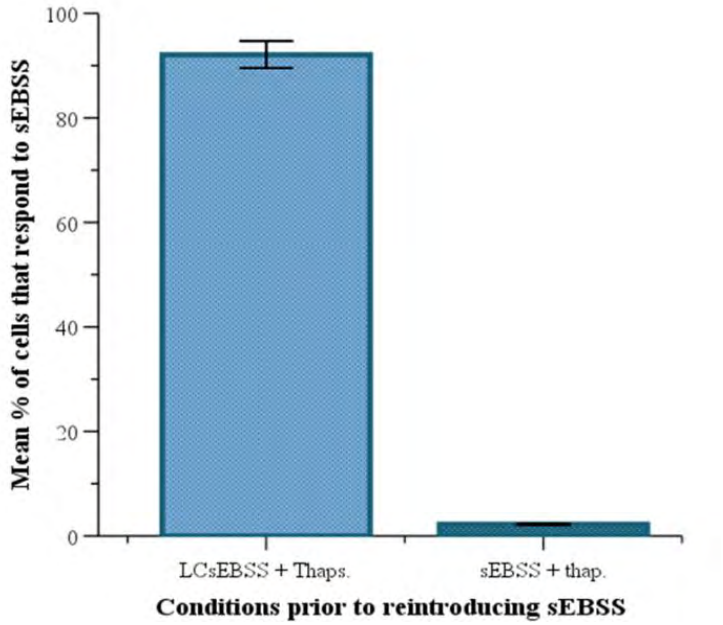
Treatment	P value
sEBSS vs. LCsEBSS	1.9834
sEBSS vs. LCeEBSS + Thaps.	0.0250
LCsEBSS vs. LCsEBSS + Thaps.	0.0056

P values from Kruskal Wallis + Dunns statistical test relating to Figure 4.8

Treatment	P value
sEBSS vs. control	0.043
LCsEBSS vs. control	0.043
LCsEBSS + Thaps. vs. control	0.593

P values from Wilcoxon statistical test relating to Figure 4.8

Appendix 4.v



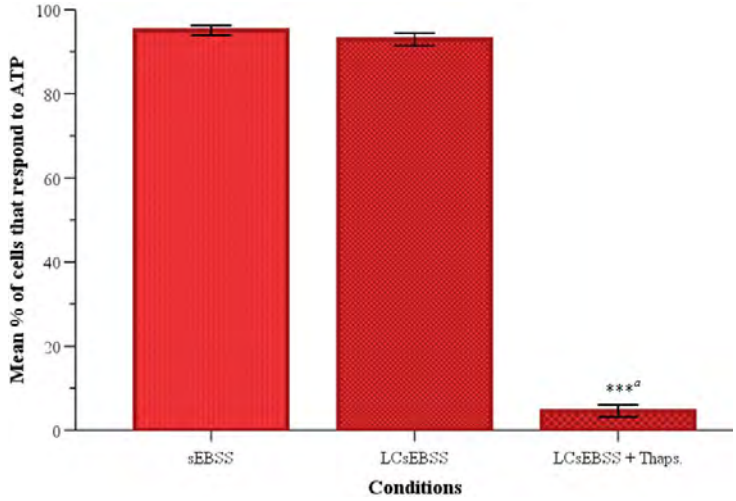
The effects of the reintroduction of sEBSS on the mean % of cells that respond after pre-treatment with thapsigargin in either sEBSS or LCsEBSS

Data was classified by logical analysis of significant responses. sEBSS induced calcium signalling in OE E6/E7 cells were assessed in either sEBSS or LCsEBSS after pre-treatment with thapsigargin (100 nM). Experiments were performed in parallel via the constant perfusion method.

	LCsEBSS + Thaps.	sEBSS + Thaps.
No. of replicates (*n*)	6	2
No. of cells analysed	801	436
Mean % response ± SEM	92.1 ± 2.6	2.2 ± 0.2
Mean Δ Fluo % ± SEM	34.2 ± 4.0	10.7 ± 1.3

Summary data used for 4.v

Appendix 4.vi



The effects of LCsEBSS and thapsigargin on the % of cells responding to ATP

Data was classified by logical analysis of significant responses. ATP induced calcium signalling in OE E6/E7 cells were assessed in either sEBSS, LCsEBSS or LCsEBSS after pre-treatment with thapsigargin (100 nM). Experiments were performed in parallel and cells were exposed to ATP (100 µM) via the constant perfusion method. Error bars represent SEM. Asterisks denote statistical significance (P<0.001) performed by a Mann-Whitney U (independent) test. The % of cells responding to ATP in LCsEBSS significantly decreased when pre-treated with thapsigargin [a].

	sEBSS	LCsEBSS	LCsEBSS + Thaps.
No. of replicates (*n*)	10	16	8
No. of cells analysed	1129	3928	1196
Mean % response ± SEM	95.1 ± 1.2	93.0 ± 1.5	4.6 ± 1.4
Mean Δ Fluo % ± SEM	37.6 ± 3.8	31.2 ± 1.5	21.8 ± 2.5

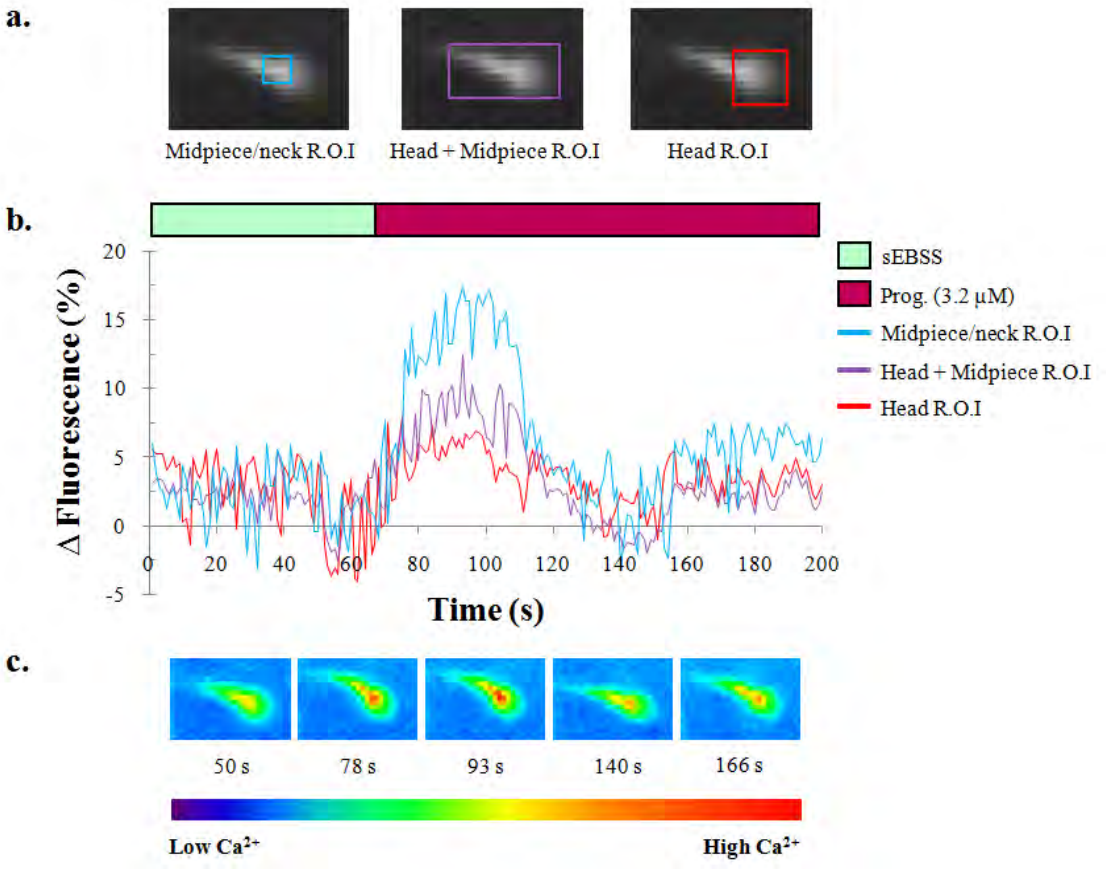
Summary data used for 4.vi

Treatments	P value
sEBSS vs. LCsEBSS	1.8385618
sEBSS vs. LCsEBSS + Thaps.	0.0003352
LCsEBSS vs. LCsEBSS + Thaps.	0.0005072

P values from Mann Whitney U statistical test relating to 4.vi

APPENDIX V: CHAPTER 5

Appendix 5.i



Detecting calcium changes in sperm during interaction with explants of female reproductive tract

Three regions of interest (ROI) were applied to measure fluorescent intensity changes induced by progesterone (3.2 µM) in single sperm whilst bound to an explant (isthmus region). (**a**) Shows the location and size of the three ROI used, depicted by blue, lilac and red boxes. The area within the ROI is used to measure changes in fluorescence intensity and indicates relative $[Ca^{2+}]_i$ in sperm; (**b**) shows fluorescence intensity plots corresponding to the three different ROI depicted above in figure (a); (**c**) Pseudocolour image series of a single $[Ca^{2+}]_i$ oscillation correlating to (a) and (b) (warm colours represent high $[Ca^{2+}]_i$). Numbers show time in (s).

APPENDIX VI: PUBLICATIONS AND PRESENTATIONS

Publications

Nash K, Lefievre L, Peralta-Arias R, Morris J, Morales-Garcia A, **Connolly T**, Costello S, Kirkman-Brown JC, Publicover SJ. 2010. Techniques for imaging Ca2+ signaling in human sperm. *J Vis Exp*., Jun 16;(40). pii: 1996. doi: 10.3791/1996.

Machado-Oliveira G, Lefièvre L; Ford C; Herrero M B; Barratt C; **Connolly TJ**; Nash K; Morales-Garcia A; Kirkman-Brown J; Publicover S. 2008. Mobilisation of Ca^{2+} stores and flagellar regulation in human sperm by S-nitrosylation: a role for NO synthesised in the female reproductive tract. *Development*.; 135(22):3677-86.

Ogunbayo, O.A., Lai, P.F., **Connolly, T.J**., & Michelangeli, F. 2008. Tetrabromobisphenol A (TBBPA), induces cell death in TM4 Sertoli cells by modulating Ca2+ transport proteins and causing dysregulation of Ca2+ homeostasis. *Toxicol.In Vitro*, 22, (4) 943-952.

Conference Posters and abstracts

Fertility 2011 (7th Biennial Conference Meeting), Dublin (2011). SIGNAL INITIATION BY SPERM-FEMALE TRACT INTERACTION. **T.J.Connolly**, S.J, Publicover and J.C, Kirkman-Brown.

Maternal interaction with Gametes and Embryo 2nd General Meeting (COST-GEMINI), Italy (2009). CALCIUM SIGNALLING DURING SPERM-FEMALE TRACT INTERACTION**. T.J.Connolly**, D.J, Smith, S.J, Publicover and J.C, Kirkman-Brown.

Physiological Society Annual Meeting, Dublin (2009). CALCIUM SIGNALLING DURING SPERM-FEMALE TRACT INTERACTION. **T.J.Connolly**, D.J, Smith, S.J, Publicover and J.C, Kirkman-Brown.

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Aitken, R.J. & Clarkson, J.S. 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J.Reprod.Fertil.*, 81, (2) 459-469.

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