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CatSper

Characterisation in Human Spermatozoa and Clinical Significance

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CatSper: Characterization in Human Spermatozoa and Clinical Significance

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Declaration

I, Hannah Lauren Williams, declare that I am the sole author of this thesis. The work was carried out by myself and all of the references were consulted by myself. None of the work contained within this thesis has been accepted for a previous higher degree.

Signature of Candidate

Hannah Lauren Williams

Statement by Supervisors

I certify that Hannah Lauren Williams has fulfilled the conditions of ordinance 39 and of the relevant regulations, such that she is qualified to submit this thesis in application for the higher degree of Doctor of Philosophy.

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Professor Christopher L.R. Barratt

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Summary

Before a mature spermatozoon is able to fertilise the oocyte, it must first traverse the female reproductive tract, through obstacles such as highly viscous mucus. As the sperm is thought to be transcriptionally and translationally inactive, it must rely on cues from its external environment in order to alter its function. Membrane-localised ion channels are fundamental in this process, where activation/inhibition of the channel results in modification of intracellular ion concentrations, resulting in change in behaviour of the sperm, in particular sperm motility. The sperm-specific calcium-permeable cation channel CatSper is necessary for initiation of hyperactive motility in murine spermatozoa, and was previously identified to be disrupted in sperm from an infertile patient. However, little is known about the incidence of CatSper defects in the IVF (*in-vitro* fertilisation) patient population.

In the present study two groups of patients, 1) IVF patients and 2) IVF or ICSI (intra-cytoplasmic sperm injection) patients who had experienced a cycle of low/failed fertilisation were screened for defects in calcium regulation and particularly CatSper function. In a novel finding, one patient from each of these groups were found to have normal semen analysis, yet sperm were found to have a complete absence of calcium response to progesterone, thus indicating CatSper dysfunction.

This study also investigated the effect of cryopreservation on human sperm function, with particular reference to calcium regulation. Cryopreservation was able to significantly diminish the calcium response to progesterone, basal hyperactivation and penetration into viscous media. The assay identified a subset of donors who were particularly susceptible to cryodamage, which may be of significant clinical relevance, due to the common use of cryopreservation of semen in ART. The present study also

describes the novel use of single cell calcium imaging as a sensitive screening tool for calcium defects in healthy volunteer donors, IVF and ICSI patients, thus enabling study of sperm samples of low concentration, particularly relevant for male infertility patients exhibiting oligozoospermia.

List of abbreviations

4-AP – 4-aminopyridine

ACU – assisted conception unit

ALH – amplitude of lateral head displacement

ANOVA – analysis of variance

AR – acrosome reaction

ART – assisted reproductive technology

ATP – adenosine triphosphate

BCF – beat cross frequency

BSA – bovine serum albumin

Ca^{2+} - calcium ion

cAMP – cyclic adenosine monophosphate

CASA – computer-assisted sperm analysis

CatSper – cation channel of sperm

CC – cumulus cells

CICR – calcium-induced calcium release

CO_2 – carbon dioxide (gas)

DFI – DNA fragmentation index

DGC – density gradient centrifugation

DMSO - dimethyl sulfoxide

DNA – deoxyribonucleic acid

EGTA – ethylene glycol tetraacetic acid

GP – general practitioner

HCO^{3-} - bicarbonate ion

HEPES – 4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid

HFEA – Human Fertilisation and Embryology Authority

HOS test – hypoosmotic swelling test

HSA – human serum albumin

HTF – synthetic human tubal fluid

ICSI – intra-cytoplasmic sperm injection

IUI – intra-uterine insemination

IVF – *in vitro* fertilisation

LIN - linearity

MAR test – mixed antiglobulin reaction test

MII – metaphase II

NP – non-progressive (motility)

PB – polar body

PDE – phosphodiesterase

PKA – protein kinase A

PLCζ – phospholipase C zeta

PN – pronuclei

PR – progressive (motility)

RNE – redundant nuclear envelope

ROI – region of interest

ROS – reactive oxygen species

SA – semen analysis

sAC – soluble adenylate cyclase

SEM – standard error of the mean

SD – standard deviation

SPS – serum protein substitute

STF – synthetic tubal fluid

STR – straightness

TFF – total fertilisation failure

TMA – trimethylamine hydrochloride

VAP – average path velocity

VCL – curvilinear velocity

VOCC - voltage-operated Ca^{2+} channels

VSL – straight line velocity

WHO – World Health Organization

ZP – zona pellucida

Chapter 1

General Introduction

1.1 Infertility

1.1.1 Subfertility, infertility and the use of assisted reproductive technologies

Clinical infertility is defined by the World Health Organization (WHO) as a disease of the reproductive system, characterised by the failure of a couple to conceive after 12 months of regular unprotected intercourse (Zegers-Hochschild et al., 2009). The WHO (World Health Organization, 1992) estimates that around 10-15% of couples trying to conceive will experience infertility or subfertility and will require assisted conception: infertility may be as a result of female, male, or a mixture of female and male or of unexplained origin (Department of Health, 2009). Infertility is a significant global health issue, with an estimated 72.4 million people affected by infertility worldwide (Boivin et al., 2007). The development of assisted reproductive technologies (ART) as treatments for infertile patients led to the birth of the world's first 'test tube baby' Louise Brown in 1978 via the *in vitro* fertilisation of a single oocyte extracted laparoscopically (Steptoe and Edwards, 1978). This is now a mainstream treatment. It was announced in 2012 that over 5 million babies have now been born by assisted conception worldwide (Ferraretti et al., 2012) and the number of ART cycles is increasing by 3.9% each year (HFEA, 2014).

ART consists of multiple *in-vitro* techniques to assist achievement of a pregnancy, including intra-uterine insemination (IUI) for mild infertility, *in vitro* fertilisation (IVF) for moderate infertility, or intra-cytoplasmic sperm injection (ICSI) for severe infertility, as well as gamete/embryo cryopreservation and frozen embryo transfer (Zegers-Hochschild et al., 2009). In the UK, over 60,000 cycles of IVF and ICSI are performed annually, at a proportion of 47.4% versus 52.6% respectively (HFEA, 2014). Although the introduction of assisted reproductive techniques has enabled

pregnancies and live births for many otherwise infertile couples, the success rate of live birth per cycle remains low at 26.5% for women of all ages (HFEA, 2014). Furthermore, the likelihood of pregnancy is reduced following multiple unsuccessful IVF cycles, with a particularly sharp decrease in pregnancy rates after the third cycle of IVF where the first two cycles have failed to produce a pregnancy or live birth (Martin-Johnston et al., 2009).

1.1.2 Fertilisation failure in ART

It is estimated that around 5-20% of all IVF cycles will result in total fertilisation failure (Johnson et al., 2013), with a fertilisation failure recurrence rate in subsequent IVF cycles of 30-67%. Total fertilisation failure in ICSI is lower, estimated to be around 1-3%, with low fertilisation in up to 14% of cases, with factors including inability of the oocyte to become activated by the sperm (Flaherty et al., 1995, Saunders et al., 2002, Esfandiari et al., 2005).

The occurrence of failed fertilisation is often unpredictable and with no obvious identifiable male or female deficiency. However, it has been estimated that low sperm binding is the largest cause of failed fertilisation in IVF, where <5 of the partner's sperm are found to be tightly bound to the oocyte. In this study by Liu and Baker, it was found that addition of donor sperm to the oocyte resulted in many more sperm bound, indicating a primary problem with the partner's sperm and not with the oocyte itself (Liu and Baker, 2000). Failed fertilisation in IVF due to defective sperm-oocyte binding and penetration may be overcome in subsequent cycles by the employment of the ICSI technique, to bypass the functional requirement of the sperm to penetrate the oocyte. It is clear that fertilisation events are currently poorly understood. Further research must be carried out in order to characterise this, in order to understand the

causes leading to failed fertilisation, to predict its occurrence and ultimately identify appropriate treatment options for patients to avoid it.

1.1.3 Prevalence of male factor fertility

It is currently estimated that male factor infertility now contributes to up to half of all causes of infertility seen in the assisted conception clinic (Massart et al., 2012). Defects in sperm function are thought to be the most significant cause of male infertility (Hull et al., 1985), however there is currently no drug to stimulate *in vivo* or *in vitro* increase in sperm fecundity (Barratt et al., 2011). Multiple studies have reported a decrease in semen parameters with age or over time (Carlsen et al., 1992, Auger et al., 1995, Eskenazi et al., 2003, Rolland et al., 2012, Johnson et al., 2015). Currently, the sole assisted reproductive option for severe male factor infertility is ICSI, a highly invasive practice with risks of damage to oocytes, with potential increased risk of genetic/epigenetic conditions in children conceived (HFEA, 2009). Male factor infertility is a serious and widespread condition, currently lacking in adequate non-invasive treatment solutions in the ART repertoire.

1.2 Semen Analysis

To determine if male factor is the source of infertility, a basic semen analysis (SA) is performed according to WHO 2010 guidelines to determine the quality and quantity of sperm present in the ejaculate (Cooper et al., 2010, World Health World Health Organization, 2010). SA is currently the gold standard in assessing the function of the male reproductive system. Sperm concentration, total sperm count, volume of semen, percentage of motile sperm (progressive PR + non-progressive NP), percentage of

progressively motile sperm (PR), percentage of normal forms of sperm (including head, neck/midpiece/tail defects and cytoplasmic droplets), MAR (mixed antiglobulin reaction) test for antisperm antibodies (Scarselli et al., 1985) and pH tests on azoospermic samples are all analysed by the andrologist according to the WHO criteria for normal human semen (Cooper et al., 2010). Normal semen characteristics are provided in Appendix Table 1.

Samples exceeding the 5th centile values, and therefore found to be characteristically normal, tend to be allocated to IVF treatment. There are currently no medical interventions to improve fertility for male patients with a testicular or unknown cause of infertility (Khorram et al., 2001), therefore if a sample exhibits oligo- (low sperm concentration), astheno- (poor sperm motility) or teratozoospermia (poor sperm morphology), or a combination of these characteristics, ICSI treatment will be used to bypass the functional requirements of sperm and to improve the likelihood of successful fertilisation (Liu and Baker, 2002). ICSI is also used in cases where sperm are surgically retrieved from the testis, or if a previous IVF cycle has resulted in failed fertilisation of all oocytes incubated with sperm, indicating defective sperm-oocyte binding or an inability of the sperm to penetrate the oocyte (Khorram et al., 2001).

SA provides an indication whether male factor contributes to a couple's infertility and which of the assisted reproductive techniques is best suited to overcome this (Huang and Rosenwaks, 2012). However SA values of fertile males and men with male factor infertility display significant overlap between the groups: morphology has been demonstrated to have poor specificity (0.51), while sperm concentration also gives poor sensitivity between these groups (0.48) (Nallella et al., 2006). Furthermore, patients with impaired sperm function will not show obvious abnormalities during routine SA, therefore these couples could be described as having 'unexplained

infertility' (Sánchez et al., 2013). SA, although widely used, has limited diagnostic value and further research into identification of the potential causes underlying unexplained infertility must be performed, in order to more accurately interrogate gamete capability, and to tailor ART to treat infertility in these couples (Sánchez et al., 2013, Wang and Swerdloff, 2014).

1.3 Sperm function and quality tests

SA alone shows the quality of sperm motility and raw sperm concentration, and is taken as a surrogate assessment of fertility, however is unable to show functional abilities of the sperm (Pacey, 2012). The causes of sperm dysfunction are largely unknown, therefore several bioassays have been developed in an attempt to accurately analyse sperm function, and to diagnose male factor infertility, however these have not yet been widely adopted for clinical use (Sánchez et al., 2013, Wang and Swerdloff, 2014). These tests include the hypoosmotic swelling (HOS) test for vitality (Jeyendran et al., 1984), DNA damage analysis (Aitken et al., 2009, Robinson et al., 2012, Simon et al., 2013), hemizona binding assay (Oehninger et al., 2013) hyaluronic acid binding assay (Worrilow et al., 2013, Mokanszki et al., 2014), and analysis of reactive oxygen species (ROS) (Guthrie and Welch, 2012). Although many varied sperm function tests are offered as adjuncts to treatment in some clinics, there are very few clinical trials to back up the relevance of these tests to success in ART, are introduced to the repertoire without stringent testing being performed, and parameters have yet to be defined for the normal population (Harper et al., 2012). The tests selected for this thesis were thought to most accurately represent *in vivo* physiological

processes sperm encounter prior to fertilisation: penetration through viscous media, calcium response to progesterone, and initiation of hyperactivated motility.

1.3.1 Viscous media penetration test

In vivo, during sperm transit through the female reproductive tract, the motile cells encounter two areas containing highly viscous solutions: 1) the cervical mucus and 2) the cumulus-oocyte complex composed of hyaluronan. The Kremer penetration test was devised to assess the sperm's ability to penetrate these viscous fluids. Only competent, morphologically normal spermatozoa were found to be able to penetrate cervical mucus (Jeulin et al., 1985). The test (Kremer, 1965) originally used human cervical mucus obtained from healthy donors. However due to the ethical issues and impracticalities surrounding obtaining human cervical mucus, including cyclic variability in constitution and viscosity, and the lack of a suitable animal alternative, a synthetic cervical mucus substitute, methylcellulose, is now used (Ivic et al., 2002, Alasmari et al., 2013b). Its viscosity is 4000cP (centipoise) which closely resembles that of mid-cycle cervical mucus (Ishijima et al., 1986). The ability of the sperm to progress through cervical mucus is thought to be positively correlated with the fertility of these sperm (Berberoglugil et al., 1993), as well as their ability to penetrate cumulus cells held in a viscous jelly-like hyaluronan matrix *in vivo* in order to reach the oocyte.

1.3.2 Basal and stimulated hyperactivation

Significant levels of hyperactivated motility are available only after exposure to capacitating conditions (see section 1.5 and 1.6). This transition from activated to hyperactivated motility is postulated to enable sperm detachment from the oviductal epithelium, and to aid progression through visco-elastic obstacles encountered during sperm transit through the female reproductive tract, including cervical mucus and

cumulus cell matrix, as further described in section 1.6 (Suarez et al., 1991). Indeed, murine sperm that cannot hyperactivate, are unable to penetrate and therefore fertilise oocytes (Quill et al., 2003). Infertility patients have previously been found to exhibit reduced proportions of hyperactivated spermatozoa, which may contribute to their infertile status (Munire et al., 2004, Alasmari et al., 2013a).

Hyperactivation is dependent on increases in $[Ca^{2+}]_i$ (Suarez and Ho, 2003) either by activation of intracellular calcium stores or by activation of bivalent cation channels such as CatSper (Bedu-Addo et al., 2008, Suarez, 2008). Hyperactivated motility has previously been shown to be induced artificially by addition of calcium store mobilizing agents such as 4-aminopyridine (4-AP) (Gu et al., 2004, Alasmari et al., 2013a), which may be of significant clinical benefit for patients where the proportion of hyperactivated spermatozoa is significantly reduced.

1.3.3 Calcium response to progesterone

Many studies have correlated male factor infertility with sperm calcium response to progesterone: in oligozoospermic patients (Falsetti et al., 1993), patients with reduced/failed fertilisation (Krausz et al., 1995), and teratozoospermic patients (Oehninger et al., 1994). More recently, the calcium response to progesterone has been attributed to non-genomic CatSper channel activation by the steroid hormone, as described in section 1.7 (Strunker et al., 2011, Brenker et al., 2012). Absence of the functional CatSper channel complex in mice translated into inability to hyperactivate or penetrate the oocyte ZP (Quill et al., 2003), however contrary to the human model, CatSper does not appear to be activated by progesterone. In 2013, electrophysiological analysis of the sperm of a CatSper-deficient infertile patient revealed absence of the CatSper current under Cs^+ based conditions, however there were multiple other

abnormalities present (Smith et al., 2013). The CatSper channel therefore has significant importance in human male fertility, which certainly warrants further study (section 1.7 and section 1.9).

This thesis aims to utilise the techniques described above to investigate calcium pathway defects in patients attending for infertility treatment, to further interpret the role of calcium on male infertility.

1.4 Importance of ion channels in sperm function

Despite the presence of some mRNAs found within sperm cytoplasm (Jodar et al., 2013), mature ejaculated spermatozoa are thought to be both transcriptionally and translationally inactive. Sperm DNA is remodelled by exchanging histones for protamines via transition proteins during sperm development and differentiation, and is therefore rendered inaccessible to transcriptional cellular machinery (Johnson et al., 2011). Sperm also shed an abundance of cytoplasm through the maturation process, resulting in loss of intracellular organelles and subsequent characteristic streamlined sperm morphology. As a result, sperm rely on detection of cues from the surrounding environment of the female reproductive tract in order to alter their function, via the activation of ion channels present on the sperm phospholipid membrane. Capacitation, the acrosome reaction, initiation of hyperactivation, regulation of membrane potential and calcium response to progesterone all depend on appropriate ion channel function (Shukla et al., 2012). Defective ion channel function therefore inevitably results in male infertility, as seen in mice and men (Quill et al., 2003, Smith et al., 2013). The following sections will discuss the importance of suitable ion channel function on

regulation of sperm function, with particular emphasis on the regulation of intracellular calcium.

1.5 Capacitation

1.5.1 Introduction to capacitation

Immediately after ejaculation, spermatozoa are unable to fertilise an oocyte. The sperm cell must undergo several fundamental biochemical changes, both in natural conception and during IVF, before fertilisation is possible. Chang *et al* (1951) and (Austin, 1952) first discovered that *in vitro*-fertilisation would not occur unless the sperm had first been exposed to the female reproductive tract. The resultant biochemical and physiological changes in spermatozoa are collectively named 'capacitation', as the sperm has acquired the *capacity* for fertilisation (Austin, 1952). Edwards *et al* reported in 1969 that during IVF of human oocytes, human sperm required around 7 hours to capacitate before being able to penetrate the oocytes (Edwards et al., 1969).

1.5.2 Biochemistry of capacitation

The intracellular biochemical modifications associated with capacitation include an increased fluidity of the sperm plasma membrane, phosphorylation of protein serine/threonine and tyrosine residues, an increase in intracellular Ca^{2+}, cAMP and HCO_3^-, ability to undergo the acrosome reaction, a hyperactivated motility pattern and (somewhat controversially) ability to utilise chemotaxis to find the egg (Visconti et al., 1995a, Visconti et al., 1995b, Osheroff et al., 1999, Visconti et al., 1999, Fraser, 2010). Holding the sperm plasma membrane at a negative value (hyperpolarisation) is

also linked with capacitation, via postulated combined activation of the SLO1 and SLO3 K^+ channels (Lopez-Gonzalez et al., 2014, Mansell et al., 2014).

After ejaculation, sperm exhibit progressive and directional motility with symmetric, low-amplitude beats of the flagellum that changes to non-progressive hyperactivated motility (see section 1.6), and the ability to undergo the acrosome reaction (AR) following capacitation (Baldi et al., 1996, Baldi et al., 2000). When the spermatozoa undergoes AR, it reveals an inner plasma membrane, covered with enzymes to aid passage through the thousands of somatic cumulus cells (CC) surrounding the oocyte. The CC are held in a jelly-like matrix of hyaluronic acid; therefore one of the enzymes

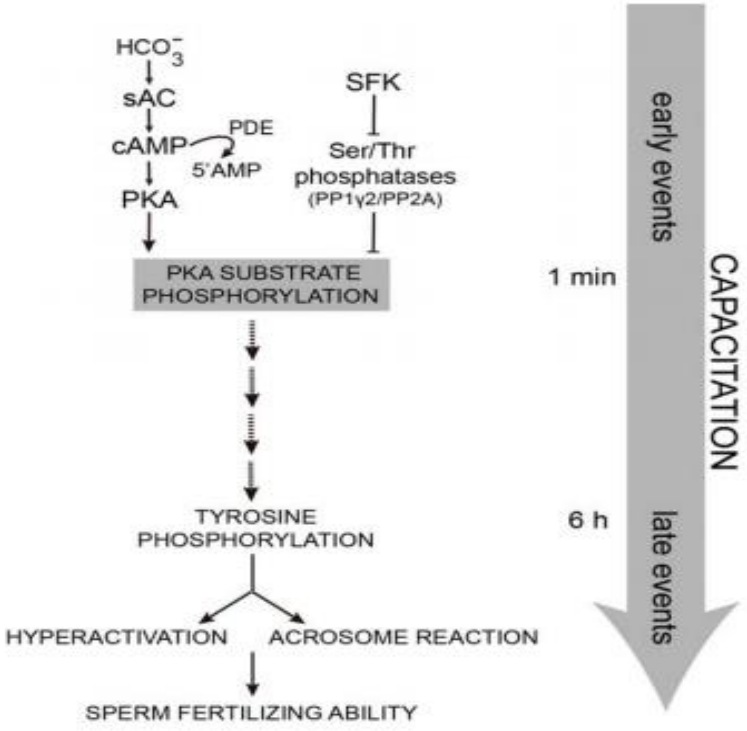


Figure 1.1 Early and late events in capacitation. Diagram showing the bicarbonate /PKA/ tyrosine phosphorylation pathway. Activation of the pathway leads to induction of hyperactivation and acrosome reaction: two fundamental parts of sperm fertilizing ability. Capacitation is separated into early and late events (Battistone et al., 2013).

exposed by the AR is hyaluronidase, Hyal5 and Ph-20 (Kim et al., 2008). Sperm must hyperactivate before they are able to penetrate through the CC, and reach the oocyte zona pellucida.

The influx of Ca^{2+} and HCO_3^- during capacitation, due to enhanced permeability of the more fluid plasma membrane, activates the soluble adenylyl cyclase (sAC) enzyme, which then produces cyclic AMP (cAMP) (Figure 1.1). cAMP is then able to activate PKA (protein kinase A) (Breitbart, 2002). Protein tyrosine phosphorylation is mediated by PKA activity, and Visconti et al (1995) showed that both protein tyrosine phosphorylation and capacitation are inhibited when PKA activity is inhibited. PKA is spatially organised within the sperm cell by A kinase-anchoring proteins (AKAPs), therefore is only present within specific subcellular compartments (Visconti et al., 1995b, Baldi et al., 2000). The extent of protein tyrosine phosphorylation increases with the sperm's duration of capacitation.

The proportion of acrosome reacted and hyperactivated sperm in a sample reflects the IVF success rate (Sukcharoen et al., 1995, Alasmari et al., 2013a), therefore the capacitating media must contain essential components such as Ca^{2+}, HCO_3^- and serum albumin, which aim to promote capacitation. This increase in HCO_3^-, from around 4mM in the epididymis to over 20mM in the capacitating media, mimics the HCO_3^--rich environment of the female reproductive tract (Gadella and Van Gestel, 2004). Moseley et al showed that using IVF media does indeed accelerate capacitation, with all the rates of tyrosine phosphorylation, hyperactivation and AR being faster than non-capacitating media (Moseley et al., 2005). The media used by the clinic for capacitating sperm before the IVF procedure, Quinn's Advantage® Fertilisation (HTF) Medium, has also been shown to enhance the zona pellucida-induced acrosome

reaction for human sperm when compared with human tubal fluid (HTF) medium (Liu et al., 2011).

The female reproductive tract regulates the process of capacitation by controlling production and location of factors implicated in capacitation, as well as storing sperm in the isthmus until needed in mammals, birds and reptiles (Holt and Fazeli, 2016). Furthermore the female reproductive tract has been reported to produce a chemoattractant gradient for the sperm to follow; in humans, this is thought to be progesterone released by the CC (Jaiswal et al., 1999, Teves et al., 2006).

1.5.3 Clinical relevance of capacitation

Failure to capacitate may be linked with incidence of male factor infertility (Munire et al., 2004, Buffone et al., 2009). Males whose sperm underwent the acrosome reaction prematurely were less likely to achieve a successful fertilisation with IVF, and more subfertile men had lost their acrosomes spontaneously compared to males with normal fertility (Pampiglione et al., 1993). Alasmari et al (2013a) also showed that the rate of hyperactivated activity in human sperm, an indicator of capacitation, correlated with fertilisation rate in IVF . Conversely, each stage of capacitation could be manipulated as a potential target for novel contraceptives. Contraceptives that could impair the normal process of sperm capacitation would impact sperm function so severely as to render the sperm infertile.

1.6 Hyperactivation

1.6.1 Role of hyperactivation

Hyperactivated motility has three main roles: to allow sperm to detach from the oviductal epithelium, migrate through the viscoelastic oviductal mucus *in vivo* and penetrate the layers of CC surrounding the oocyte *in vitro* and *in vivo* (Ho and Suarez, 2001, Suarez and Ho, 2003, Shukla et al., 2012). Around the time of ovulation, the sperm acquire hyperactive motility and changes in the sperm head plasma membrane, thus enabling their detachment from the epithelium in the storage region of the female reproductive tract (Simons et al., 2014). Hyperactivated sperm motility was first described in the golden hamster in 1970 (Yanagimachi, 1970).

Hyperactive motility has a significant role in zona pellucida penetration in a hamster model (Stauss et al., 1995). Furthermore sperm from mice lacking *CatSper2* are infertile due to their inability to hyperactivate and therefore penetrate the oocyte outer vestments (Quill et al., 2003). The importance of hyperactivated motility in human sperm has also been highlighted by its reduction in the sperm samples of infertile patients (Munire et al., 2004), and demonstration that a population of sperm displaying hyperactivated motility had a greater proportion of morphologically normal cells (Green and Fishel, 1999).

1.6.2 Identification of hyperactivated motility

Hyperactivated motility of the sperm is associated with a vigorous whiplash-like movement of the tail with high amplitude asymmetrical beating and non-progressive motility. The definition of hyperactivated sperm are those that have a curvilinear velocity (VCL)>150, linearity (LIN)<50 and amplitude of lateral head displacement

(ALH)>7 (Mortimer et al., 1998). HCO_3^- has been shown by Wennemuth et al to increase the flagellar beat frequency and therefore motility of murine sperm (Wennemuth et al., 2003). Progesterone gradients that mimic those found *in vivo* on the approach to the oocyte are also able to regulate the flagellar beat, via the rise in intracellular Ca^{2+} (Harper et al., 2004, Harper and Publicover, 2005), however do not initiate the AR.

1.6.3 Stimulation of hyperactivated motility with 4-AP

Gu et al (2004) showed that 4-AP strongly activated hyperactivated motility in sperm *in vitro*, principally by increasing intracellular Ca^{2+} concentration by mobilisation of $[Ca^{2+}]_i$ stores (Gu et al., 2004, Bedu-Addo et al., 2008). 4-AP was first found to do this in neural cells such as neurons and astrocytes, as well as skeletal muscle cells, before it was applied to sperm (Grimaldi et al., 2001). This result could be utilised in IVF procedures to increase the hyperactivated motility of sperm while it is in close proximity to the oocyte, and therefore increase the likelihood of sperm penetration and sperm-egg fusion. The amplitude of HA response to 4-AP in IVF patients was also correlated with fertilisation rate, and furthermore the proportion of IVF patients with HA not significantly stimulated by 4-AP was greater than in healthy volunteer donors (Alasmari et al., 2013a).

1.6.4 Hyperactivation and viscous media penetration

Strong hyperactivated movements of the flagellum functionally aid the sperm's journey through viscous media such as the oviductal or fallopian tube mucus, and disperse the CC that surround the oocyte for access to the ZP (Suarez et al., 1991). The Kremer penetration test (as mentioned in section 1.3), is a suitable assay for analysing sperm ability to penetrate mucus, as it involves sperm penetration of the

resembling viscous media methylcellulose. Sperm that are shown to penetrate methylcellulose in vitro are more likely to successfully bypass the cervical mucus, which is the first stage of sperm selection *in vivo*. Hyperactivated sperm have been found to be more able to penetrate mucus, as their whiplash movements of the tail in more fluid media become powerful progressive strokes in viscous media (Suarez et al., 1991), and furthermore both sperm concentration and progressive motility have been found to correlate with viscous media penetration (Mortimer et al., 1986).

1.6.5 Clinical relevance of hyperactivation

Hyperactivation of human spermatozoa is key for successful fertilisation of the oocyte, however is not routinely analysed in the clinical setting as a biomarker for capacitation and sperm function. Studies have shown that treatment of sperm with CatSper and calcium store-mobilising agents such as NH_4Cl, 4-AP and progesterone, along with phosphodiesterase inhibitor IBMX, are able to stimulate hyperactivation to varying degrees (Alasmari et al., 2013a). This study also revealed that failure to respond by increasing hyperactivation levels was more common in IVF patients than research donors, (10% versus 2%). Thus, further investigation should be made into the nature of these defects, particularly with reference to success at IVF.

1.7 CatSper

1.7.1 Introduction to the cation channel of sperm (CatSper)

CatSper channels are promiscuous, sperm-specific cation-permeable ion channels, located on the principal piece of the mature sperm flagellum, strongly potentiated by alkaline pH and activated by progesterone, ZP proteins, prostaglandins as well as the

environmental endocrine disruptor p,p'-DDE (Lishko et al., 2011, Strunker et al., 2011, Barratt and Publicover, 2012, Tavares et al., 2013). CatSper channels are permeable to Ca^{2+}, and the Ca^{2+} influx that occurs due to CatSper activation is necessary for hyperactivation of the sperm in mice (Quill et al., 2003). The CatSper channel contains four pore-forming domains coded by the genes *CatSper 1 CatSper 2, CatSper 3* and *CatSper 4* (Quill et al., 2001, Ren et al., 2001, Lobley et al., 2003). Three auxiliary subunits named CatSper β, CatSper γ and CatSper δ are also present (Liu et al., 2007, Wang et al., 2009, Chung et al., 2011).

In mice, all four knockouts of CatSper genes 1-4 produce the same phenotype: infertility due to an inability of the sperm to hyperactivate, and therefore inability to generate the necessary force for ZP penetration (Qi et al., 2007). The three auxiliary subunits are also necessary for channel function, as knocking out these genes also leads to a complete lack of sperm fecundity. The six transmembrane segments of CatSper have two distinct roles: segments 1-4 are involved in sensing voltage, resulting in the opening and closing of the pore, whereas segments 5 and 6 form the actual pore of the channel (Navarro et al., 2008). CatSper proteins 1-4 make up the pore-forming domain of the ion channel, and CatSper β, CatSper γ and CatSper δ are three auxiliary subunits associated with correct trafficking and assembly of the channel proteins (Liu et al., 2007, Wang et al., 2009, Chung et al., 2011). More recently, CatSper regulation by progesterone action has been found to be governed by the orphan enzyme ABHD2, which removes CatSper-inhibiting endocannabinoid 2-AG from the sperm membrane (Miller et al., 2016).

1.7.2 CatSper dysfunction in mammals

Although knock-out mice with CatSper 1-4 or δ null sperm appear to be morphologically normal, they are infertile. Their inability to sustain the CatSper current, $I_{catsper}$, is abolished therefore there is no increase in $[Ca^{2+}]i$ which regulates motility and is able to initiate hyperactivation, and therefore the ability to penetrate the ZP is abolished (Ren et al., 2001, Carlson et al., 2003, Quill et al., 2003, Qi et al., 2007), however sperm underwent the acrosome reaction as normal (Navarro et al., 2008). CatSper KO sperm display slower movements, and are less able to penetrate viscous medium, and indeed no eggs were fertilised when co-incubated with CatSper null sperm (Quill et al., 2003). However following removal of ZP from the oocyte by enzymatic action, the CatSper null sperm were still able to fertilise and activate the oocyte (Ren et al., 2001, Navarro et al., 2008).

In humans, expression of CatSper1 protein has been correlated with progressive and hyperactivated motility (Tamburrino et al., 2015), and possibly the progesterone-induced acrosome reaction (Tamburrino et al., 2014), with mutations in the gene causing human male infertility (Avenarius et al., 2009).

1.7.3 CatSper is a polymodal chemosensor

CatSper is known as a ‘polymodal chemosensor’ due to its ability to be regulated by many different molecules (Brenker et al., 2012). Among these proteins and subunits are multiple binding sites for molecules such as progesterone, prostaglandins, menthol and odorants such as bourgeonal (Lishko et al., 2011, Strunker et al., 2011, Brenker et al., 2012). These molecules are able to activate this cation channel, resulting in induction of hyperactivation, motility and chemotaxis. CatSper has been found to be

the ion channel responsible for controlling chemotaxis in sea urchin spermatozoa, via changing $[Ca^{2+}]_i$ (Seifert et al., 2015).

The steroid hormone progesterone is released by the somatic CC that surround the oocyte in the millimolar concentration, and is known to activate the CatSper channel and enable Ca^{2+} influx which aids the change in flagellar beat pattern from activated to hyperactivated motility (Lishko et al., 2011, Strunker et al., 2011). Progesterone may therefore also serve to trap sperm in the vicinity of the oocyte by inducing non-progressive hyperactivated movements, where the concentration is the highest, or chemotactically draw the sperm to the cumulus-oocyte complex up a progesterone concentration gradient, however the employment of chemotaxis by sperm is disputed (Jaiswal et al., 1999, Teves et al., 2006, Teves et al., 2009). Both of these proposed mechanisms enable the oocyte and the sperm to be in close proximity, thereby increasing the likelihood of a sperm penetrating the egg.

Cyclic nucleotides such as cAMP may indirectly activate CatSper channels by activating $Na^{+/}H^{+}$ exchangers, which modify the intracellular pH to make it more alkaline by the extrusion of hydrogen ions (Darszon et al., 2011). This consequently activates the pH-sensitive CatSper channels, and introduction of Ca^{2+} into the sperm via CatSper and then proposed Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores, such as the redundant nuclear envelope (RNE) and the acrosome (Olson et al., 2010). This then activates the sAC/cAMP/PKA pathway, which generates ATP. ATP is created by glycolysis, and is used as an energy substrate by dynein motor proteins on the axoneme within the flagellum, to allow extended periods of motility and hyperactivity. The axoneme is formed of microtubules which slide over each other, to produce flagellar bending and therefore sperm motility (Darszon et al., 2011).

Influx of Ca^{2+} at different concentrations regulates distinct sperm functions, for example both basal and hyperactivated motility. An increase in the level of extracellular Ca^{2+} is correlated with increased asymmetric flagellar bending, and therefore the hyperactivation dependent on CatSper channels in the sperm. 4-aminopyridine (4-AP) is also known to enhance hyperactivation, which may be due to the potentiation of the CatSper current. Costello (2010) showed that use of 4-AP caused the release of intracellular stores of Ca^{2+} at the neck/midpiece of the sperm, presumably at the RNE, which stimulated the deep flagellar bends associated with hyperactivated motility. 4-AP was also shown to activate CatSper in mice, leading to a rise in $[Ca^{2+}]i$ at the principal piece of the flagellum, and also a rise in [pH]i which is known to activate the pH-dependent channel (Chang and Suarez, 2011).

1.7.4 Effect of intracellular pH

Control of intracellular pH is particularly important in sperm motility. A high incidence of histidine residues within the CatSper ion channel may help to regulate intracellular pH (Ren et al., 2001, Navarro et al., 2008). An alkaline pH aids the activity of the CatSper channel to increase intraflagellar Ca^{2+} and induce hyperactivation (Kirichok et al., 2006). Alkaline pH is controlled in the sperm cell by Hv1 channels, which extrude protons from the cell to raise the $[pH]_i$. These channels are closely located to the CatSper channels, on the principal piece of the sperm flagellum, and the intracellular alkalinisation caused by Hv1 activates the pH-dependent CatSper channel (Lishko and Kirichok, 2010). Trimethylamine hydrochloride (TMA) is a compound that readily forms a tetramethylammonium ion and hydroxide ions in solution (Wu et al., 2012). Its basic property evokes an increased $[pH]_i$, which could therefore activate CatSper.

1.7.5 Potential contraceptive action

Drug discovery of ion channel blockers that specifically target the CatSper channel could be a potential target for a novel contraceptive, due to its critical role in male fertility (Carlson et al., 2009). As CatSper is specifically located in the testis and male germ cells, drug therapy may be targeted for localised effects, reducing the incidence of adverse side effects. Furthermore, channel antagonists could potentially be utilised by either men or women for a contraceptive purpose (Ren et al., 2001). Conversely, channel agonists or activators, such as progesterone, could be used to enhance the CatSper-dependent hyperactivation observed during capacitation, for beneficial results in either natural conception or in assisted reproductive technologies.

In summary, the CatSper channel is essential for successful fertilisation, due to the initiation of hyperactivated motility as seen in a murine model, and is certainly of interest in males with unexplained infertility or who have completely failed to fertilise oocytes after IVF. Differences in the levels of CatSper expression are relevant for patients where motility is compromised (Nikpoor et al., 2004). Furthermore, several studies have identified patients with specific CatSper defects: males with nonsyndromic male infertility (NSMI) tend to have a mutation in CatSper1, whereas males with an infertility and deafness phenotype (DIS- deafness-infertility syndrome) have been shown to have a defect in the CatSper2 gene (Hildebrand et al., 2010). Intracytoplasmic sperm injection (ICSI) can be used to create an embryo in males with defects in CatSper, as ICSI bypasses the requirement for ZP penetration. Theoretically, if in the course of infertility investigation a patient's sperm is identified to carry defects in CatSper, IVF may be surpassed in favour of ICSI, due to the likelihood of failed fertilisation in IVF. However investigation into the role of CatSper on human male infertility relies on the identification of natural CatSper knockout

males, which are thought to be rare. Furthermore the incidence of CatSper defects within the infertile population is not currently well understood, and therefore clearly requires further investigation.

1.8 The role of calcium in sperm function

$[Ca^{2+}]_i$ levels increase in sperm during capacitation (Baldi et al., 2000). There are two main mechanisms for the intracellular increase in Ca^{2+}: activation of CatSper, and activation of intracellular Ca^{2+} stores located in the acrosome and at the RNE (Bedu-Addo et al., 2007, Xia et al., 2007). The spatial organisation of these stores is thought to be critical to activate different functions due to Ca^{2+}: the acrosome reaction and initiation of hyperactivated motility.

The balance between $[Ca^{2+}]_e$, present in the female reproductive tract or in media in vitro, and $[Ca^{2+}]_i$, is fundamental for normal sperm function (Darszon et al., 2011). Maintenance of a low $[Ca^{2+}]_i$ concentration is critical for sperm function, which relies on the entry of extracellular Ca^{2+} to modify sperm function (Bhoumik et al., 2014). Furthermore increases in calcium above physiological levels has been shown to inhibit sperm motility (Williams and Ford, 2001). Regulation of Ca^{2+} is thought to involve several Ca^{2+} channels, including cation channels of sperm (CatSper, see Section 1.6), voltage-operated Ca^{2+} channels (VOCCs), cyclic-nucleotide gated channels (CNG), and store-operated channels (Shukla et al., 2012). The two main stores of Ca^{2+} are able to mobilise and undergo Ca^{2+}-induced Ca^{2+} release (Bedu-Addo et al., 2008, Costello et al., 2009). The spatial differences between the two stores may indicate different functional roles and are thought to mobilise in response to different stimuli, therefore the acrosomal Ca^{2+} store may facilitate the acrosome reaction whereas the RNE store

is in close proximity to the midpiece and the flagellum and is likely to have a role in regulating flagellar movement and hyperactivation (Bedu-Addo et al., 2007). Regulation of Ca^{2+} involves Na^{+}-Ca^{2+} exchangers and Ca^{2+} pumps such as Ca^{2+}-ATPases which serve to buffer the Ca^{2+} within the cell, and are located on the plasma membrane (Jimenez-Gonzalez et al., 2006). Mitochondria, located in the midpiece of the sperm, also play a role in sequestering Ca^{2+}. These Ca^{2+} stores maintain the intracellular Ca^{2+} at a low level, so that when Ca^{2+} entry into the cell occurs this evokes a rapid rise in intracellular Ca^{2+}, which can then bring about many functional changes in sperm behaviour. An increase in Ca^{2+} concentration can either enhance or reduce sperm motility (Bhoumik et al., 2014), and its presence is necessary in media *in vitro* or in the human reproductive tract *in vivo*. Ca^{2+} binds to calmodulin which enables the dynein arms surrounding the microtubules within the sperm flagella to become activated, which results in flagellar movement, as described in mice (Schlingmann et al., 2007).

1.9 The role of calcium regulation in human male infertility

The increase in $[Ca^{2+}]_i$ in human sperm is integral for successful sperm function due to its ability to modify the sperm's motility, from an activated to a hyperactivated status, and stimulation of the acrosome reaction (See sections 1.5, 1.7). It is therefore widely believed that the Ca^{2+} response of human sperm is positively correlated with their fertility status. The steroid hormone progesterone is a putative activator of the cation channel of sperm (CatSper) (Lishko et al., 2011, Strünker et al., 2011). Defects in normal sperm Ca^{2+} response may result in loss of function of the sperm (Smith et al., 2013), and therefore will result in infertility due to inability of the sperm to

penetrate the egg. A proposed mechanism for the relationship between CatSper, calcium stores and sperm functions such as hyperactivated motility is presented in Figure 1.2.

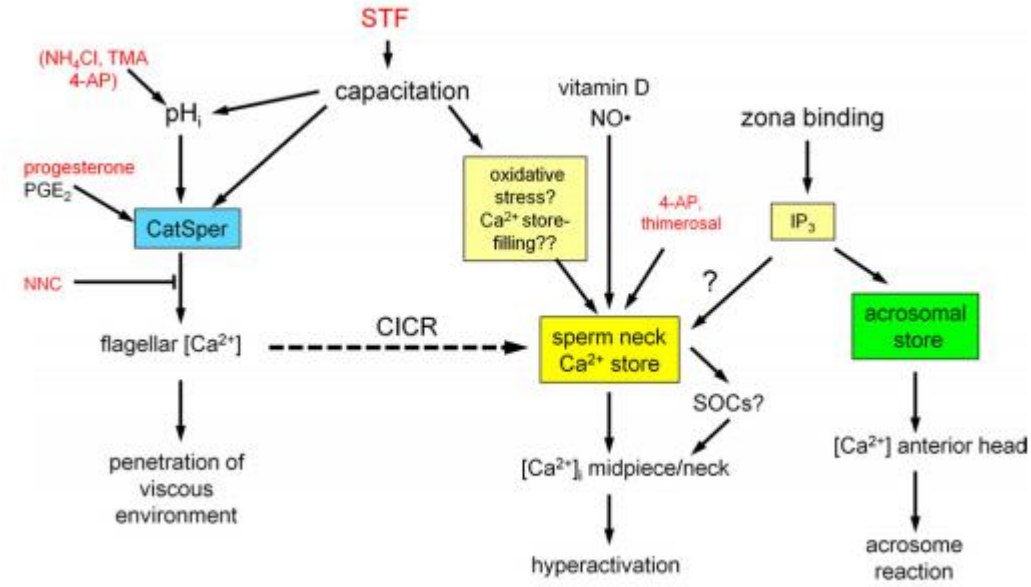


Figure 1.2 Calcium regulation in human spermatozoa. This diagram depicts the role of ⇧$[Ca^{2+}]_I$ from CatSper activation via progesterone and ⇧$[pH]_I$, and activation of sperm neck Ca^{2+} stores via 4-AP, on induction of hyperactivation and penetration of viscous environments. Both calcium stores at the acrosome and the neck/midpiece are activated respectively (Alasmari et al., 2013b).

Many studies have looked at the relationship between progesterone-stimulated Ca^{2+} influx, comparing healthy normozoospermic males and infertility patients. The question remains: is human sperm Ca^{2+} response to progesterone a useful biomarker

for the fertility status of sperm? And if so, should this procedure be included in the SA performed prior to treatment, to inform treatment choice?

Two studies in 1993 and 1994 looked at human sperm Ca^{2+} response to progesterone, comparing results between normal males and teratozoospermic patients. Shimizu et al performed experiments on sperm cells incubated with Fura-2 dye, which were then treated with 1microgram/mL progesterone (Shimizu et al., 1993). Sperm from infertile males with a large number of morphologically abnormal forms elicited a lower Ca^{2+} peak when stimulated with progesterone compared to fertile controls. Oehninger et al (1994) also found that men with severe teratozoospermia (less than 14% normal forms) showed abnormal basal Ca^{2+} levels and an abnormal Ca^{2+} response when stimulated with progesterone (Oehninger et al., 1994).

Falsetti et al (1993) also looked at the progesterone-stimulated Ca^{2+} increase and compared normozoospermic samples with oligozoospermic samples. Progesterone-stimulated Ca^{2+} increase significantly correlated with sperm motility, concentration, and good morphology. However the Ca^{2+} increase seen in oligozoospermic patients after treatment with progesterone was significantly lower than normozoospermic subjects. The addition of progesterone was also able to increase the number of acrosome reacted sperm in the normozoospermic samples, however had no significant effect on the oligozoospermic samples. Thus, the reduced ability of oligozoospermic samples to respond to stimuli such as progesterone may be the underlying factor causing their infertility. Significantly, there was also no discernible calcium response to progesterone in 4 of the 17 (24%) oligozoospermic patients (Falsetti et al., 1993).

Krausz et al (1995) compared Ca^{2+} response to progesterone in groups of low fertilisation (<50%) and high fertilisation (>50%). It was found that in patients with

fertilisation rates lower than 50%, the percentage of $[Ca^{2+}]_i$ and percentage of sperm that underwent acrosome reaction when treated with progesterone were significantly lower than in those patients who achieved >50% fertilisation rate. In 4 out of 5 cases of fertilisation failure, no increase in Ca^{2+} or acrosome was seen. The conclusions from this study were that Ca^{2+} increase and acrosome reaction in response to treatment with progesterone can have a good predictive value of fertilisation outcome (Krausz et al., 1995).

Espino et al showed that asthenozoospermic patients had lower Ca^{2+} responses to progesterone, and progesterone was not able to elicit an increase in progressive motility in these cells, compared with males with normospermia. Blockade of the progesterone receptor by c262 antibody or by RU-38486, a progesterone receptor antagonist, also reduced Ca^{2+} mobilization initiated by progesterone (Espino et al., 2009).

Meseguer et al found that there was a statistically significant difference in $[Ca^{2+}]_i$ between fertile and infertile males (Meseguer et al., 2004a). Furthermore, 5 years later, Huo et al also found that infertile patients have lower basal Ca^{2+} than fertile patients (Huo et al., 2009). Infertile patients have no significant difference between basal and peak Ca^{2+} levels as determined by confocal microscopy. This leads to the question of whether a defect of the sperm progesterone receptor was present in this subsection of infertile patients (Huo et al., 2009).

It has been previously shown that while IVF patients and donors show similar Ca^{2+} responses to progesterone, the Ca^{2+} response to progesterone for ICSI patients was significantly lower (Alasmari et al., 2013a). This difference in Ca^{2+} response between the two groups may have a significant basis for the infertility observed in the patient,

as progesterone and Ca^{2+} are known to be integral for sperm motility and therefore will have a knock-on effect on the sperm's fertility. The molecular reason behind their failed Ca^{2+} response is as of yet unknown, however it is accepted that progesterone is able to regulate CatSper, initiating Ca^{2+} influx into the cell, and the generation of hyperactivated movement. Absence of CatSper and therefore lack of entry of Ca^{2+} into the cell results in inability to produce hyperactivated movements of the flagellum in mice (Quill et al., 2003). Therefore it is important to recognise Ca^{2+} response to progesterone as an indicator of success or failure during use of ART, as it could indicate the function or dysfunction of CatSper within that sample.

Previous studies showing a reduced Ca^{2+} response to progesterone in infertile patients when compared with normozoospermic donors should be treated with caution. It is currently impossible to measure a cell's Ca^{2+} response to progesterone and then use the same cell to fertilise an oocyte, therefore we cannot truly know whether these cells that respond well are indeed the most fertile. Furthermore, the technique for measuring $[Ca^{2+}]_i$ measures an average fluorescence emitted by the bound/unbound Ca^{2+} when treated with progesterone. The data cannot distinguish the single cell's response to progesterone: whether some respond well and others not at all, or whether all cells respond but at a lower level in patient samples. Single cell Ca^{2+} imaging is required to inform whether all or a proportion of cells are responsive to progesterone, and whether this changes between normozoospermic and infertile patient samples.

1.10 The impact of cryopreservation on sperm function

The Italian scientist Lazzaro Spallanzani (1729-1799) first described the storage of human semen in snow in 1776, reporting that the sperm became motionless when

exposed to cold temperatures (Ombelet and Van Robays, 2015). Semen cryopreservation and thaw for artificial insemination was developed for commercial use in domestic animals, particularly in the dairy industry, as a method of enabling long-term/indefinite storage. Cryopreservation was noted for arresting metabolic cellular processes and allowing long-distance transport for semen samples from the most prolific breeders, or "stud" animals, to ensure continuation of their superior genetic material (Verberckmoes et al., 2004).

In humans, semen cryopreservation has been utilised in the ART repertoire since the 1960's (Sherman, 1973). The longest duration between semen being cryopreserved and achievement of pregnancy utilizing ICSI is 40 years (Szell et al., 2013), with further reports of pregnancies from IUI using sperm thawed after 28 years after cryopreservation (Feldschuh et al., 2005) and from ICSI 21 years after cryopreservation (Horne et al., 2004). Clinically, cryopreservation of semen is routinely utilised for fertility preservation under a variety of different circumstances. These circumstances include but are not limited to: pre-chemotherapy or radiotherapy, prior to surgery of the male genitalia (vasectomy, orchiectomy or gender reassignment), for quarantine of donor sperm and throughout IUI treatment cycles of multiple recipient patients, for storage pre- or posthumously, for males in dangerous professions such as the armed forces or working offshore, for treatment back-up if the sample on the day is expected to be poor, if the male partner is anticipated to not be present, or if the male partner experiences difficulty in producing a sample (Anger et al., 2003). The regular use of frozen-thawed sperm in IUI, IVF and ICSI evidently requires the best post-thaw recovery possible, in order to improve treatment success rates.

It has been well documented that cryopreservation of sperm results in a dramatic reduction in concentration and motility of the thawed samples, due to osmotic shock from the use of high osmolality cryoprotectants and membrane damage from intracellular ice crystal formation the freeze-thaw process (Boitrelle et al., 2012). Furthermore, sperm mitochondrial function, ability to acrosome react in response to progesterone and morphology were similarly shown to be reduced after cryopreservation (Rossato et al., 2000, O'Connell et al., 2002). However the impact of post-thaw sperm function on fertilisation is controversial; while some believe cryopreservation has no negative effect on fertilisation (Ashkenazi et al., 1991, Lansac et al., 1997, Marcus-Braun et al., 2004), others hold the opposite view (Smith et al., 1981, Subak et al., 1992, Di Santo et al., 2012). Cryopreservation has previously been shown to significantly negatively affect phospholipase Cζ (PLCζ) concentrations in human sperm (Kashir et al., 2011), however little is known about the effect of cryopreservation of semen on human sperm calcium regulation.

In 1994, it was identified that the intracellular calcium concentration in human sperm was increased following cryopreservation, and that around half the cryopreserved samples failed to produce an intracellular calcium response to progesterone compared to all of the fresh samples (McLaughlin and Ford, 1994). It was concluded that cryopreservation may result in the loss of the progesterone receptor due to cell membrane damage. It is now understood that the progesterone receptor is CatSper (Lishko et al., 2011, Strunker et al., 2011), and that CatSper integrity is required for fertilisation (Zhang et al., 2007, Avenarius et al., 2009, Smith et al., 2013). Furthermore, lack of CatSper 1 protein expression, and therefore functional channel activity, is correlated with asthenozoospermia in male infertility (Tamburrino et al., 2015) (Tamburrino et al., 2015). The effect of cryopreservation on human sperm

function, and in turn the effect on calcium regulation with particular reference to the CatSper channel, clearly requires further investigation.

1.11 Aims

Many studies have investigated the importance of Ca^{2+} signalling in response to progesterone and its impact on male infertility. However there are still gaps in our knowledge about CatSper, most importantly its role in male factor infertility. There are several key points that the experiments used in this study are going to address. Firstly, donor sperm with normal WHO parameters for semen (concentration, total concentration and motility) will be analysed for Ca^{2+} response to progesterone, hyperactivation with 4-AP, progesterone and TMA, and ability to penetrate mucus via the viscous media penetration test. Patient samples for IVF and ICSI will also be subjected to these tests.

This thesis will focus on the Ca^{2+} response to progesterone of sperm, more specifically:

- Investigation into incidence of calcium pathway defects in IVF patients and donors.
- To determine correlation between calcium pathway defects and fertilisation rate in IVF.
- Investigation into incidence of calcium pathway defects in patients who have undergone a cycle of IVF with low/failed fertilisation.
- To examine the effect of cryopreservation on CatSper channel function.

- Developing single cell Ca^{2+} imaging as a novel technique for investigating Ca^{2+} response to progesterone in IVF/ICSI patient samples.

1.12 Hypothesis

We hypothesise that the proportion of calcium pathway defects will be significantly elevated in patients undergoing assisted conception compared with donors, and this is a prominent factor affecting their likelihood of successful treatment outcome.

Chapter 2

Materials and Methods

2.1 Materials

Commercially available Quinn's Advantage Fertilization (HTF) Media (SAGE CooperSurgical, Origio, catalogue number ART-1021) was used as capacitating media for IVF patients in the ACU, and donors in the research laboratory. Serum protein substitute (SPS: SAGE CooperSurgical, Origio, catalogue number ART-3010-1, until the end of December 2013) or human serum albumin (HSA: SAGE CooperSurgical, Origio, catalogue number ART-3001-5, from January 1st 2014) was added to HTF media in a 1 in 10 dilution, to a final protein concentration of 5mg/ml or 3mg/ml respectively. Quinn's Sperm Washing Media (SAGE CooperSurgical Origio, catalogue number ART-1006) was used as non-capacitating media for ICSI samples. Components of these media are found in Appendix Table 2.1.

STF (synthetic tubal fluid) media based on Lishko et al, 2011, was used as a non-capacitating, phenol red free, protein-free media for re-suspending samples prior to calcium imaging in the FLUOstar Omega plate reader to reduce background fluorescence. Ionic concentrations were as follows (in mM): NaCl (98), KCl (4.7), $MgSO_4$ (0.2), $CaCl_2$ (2), HEPES (21), lactic acid (21), glucose (3) and Na pyruvate (0.3). pH was adjusted to 7.4 using 10M NaOH, with osmolality between 280-320mosmol. Components of this media are found in Appendix Table 2.1.

Stock reagents were dissolved in solvents such as dimethyl sulfoxide (DMSO, catalogue number D8418, Sigma Aldrich, UK), ethanol and deionized water. Stocks were created so that the concentration of solvent present in the final sperm suspension was equal to or less than 1%, which has been shown previously to not affect sperm acrosome reaction or capacitation (de Lamirande and Gagnon, 2002).

Stock progesterone (catalogue number P8783, Sigma Aldrich, UK) was dissolved in ethanol and stored in the freezer at -20°C at a concentration of 36mM. Stock progesterone was diluted in HTF before being added to sperm suspension for hyperactivation or calcium screening experiments, to make a final concentration of 3.6µM progesterone, similar to the concentration used previously (Meizel et al., 1997). The two-step dilution was performed to ensure ethanol concentration was just 0.01%. Concentrations of progesterone above 2µM were also found by Strunker et al to achieve maximal plateau phase calcium response to progesterone (Strunker et al., 2011). Diluted progesterone made fresh daily, and was warmed to room temperature before use.

Stock trimethylamine (TMA) was used as an intracellular alkylating agent (Cross and Razy-Faulkner, 1997), and was prepared by dissolving trimethylamine hydrochloride (catalogue number T72761, Sigma Aldrich, UK, formula weight 91.15g mol^{-1}) in DMSO at a 1M concentration, to be used in a 1:100 dilution for a 10mM final concentration. 10mM concentration was used as it was previously shown to be able to induce stable intracellular alkalinisation in bovine sperm (Galantino-Homer et al., 2004). Stock solutions were made monthly, kept in the fridge (5°C), and warmed to room temperature before use.

4-aminopyridine (4-AP, catalogue number 275875, Sigma Aldrich, UK) was prepared in deionized water to make 200mM stock concentration. It was added to the sperm in a 1:100 dilution to make a final concentration of 2mM; this concentration had been previously described as sufficient to instigate hyperactivated motility in human sperm (Gu et al., 2004). 4-AP stock was kept in the fridge (5°C) and warmed to room temperature before use.

Fura-2 acetoxymethyl ester stock (Fura-2/AM, catalogue number F-1221, Molecular Probes, Eugene Oregon, USA) was dissolved in DMSO and kept in the freezer at -20°C at a stock concentration of 500µM. 1µl of stock was added to 500µl of sperm suspension, for a final concentration of 1µM as previously described (Alasmari et al., 2013a). These steps were all carried out in the dark, to avoid photobleaching (Becker and Fay, 1987), and was warmed to room temperature before use.

Pluronic acid (Pluronic® F-127, catalogue number P2443, Sigma Aldrich, UK), a surfactant, was added to STF media (based on Lishko et al, 2011, a non-capacitating media free of BSA) at a 1%w/v solution, and was used to aid dye loading into the cells. This was made daily, and used at a final concentration of 0.05% as previously described (Strunker et al., 2011).

Stock manganese chloride (catalogue number 244589, Sigma Aldrich, UK) was dissolved in deionized water to a stock concentration of 100mM. 5µl was added to a 50µl well of sperm suspension in a 96 well plate, to make a final concentration of 9.1mM. This step was performed as previously described by Jörs et al in order to quench Fura-2/AM fluorescence, in order to obtain the background level of fluorescence (Jörs et al., 2006).

Fluo-4/AM was the single wavelength dye used for single cell imaging studies as described previously (Wood et al., 2003), with the aim of quantifying intracellular calcium changes. The dye was dissolved in DMSO and stored at -20°C at a stock concentration of 1mM. Fluo-4/AM was used at a final concentration of 1µM.

Stock Ca^{2+} ionophore A23187 (catalogue number C7522, Sigma Aldrich, UK) was dissolved in DMSO to a stock concentration of 5mM. Stock solution was dissolved 1:5 with HTF before use, then used at 100µM final concentration.

2.2 Ethical Approval and recruitment of patients/donors

Patients attending for IVF or ICSI treatment, or recalled for follow-up appointments with a consultant after a cycle of failed/low fertilisation, were recruited from the Assisted Conception Unit (ACU) Ward 35, Ninewells Hospital, to provide surplus samples to research, under the HFEA (Human Fertilisation and Embryology Authority) Code of Practice version 8 and ethical approval from Tayside Committee of Medical Research Ethics B (13/ES/0091) as previously described (Tardif et al., 2014). Patients were given information about the research projects carried out, and were given opportunities to ask questions and were advised that they could revoke their consent to research at any point. Patients were also asked if they were willing to be approached regarding the production of further samples for research. All patients willing to participate in research were allocated a research number to ensure anonymity: this was in the format of R0000 where the letter R was followed by 4 numbers. Numbers were assigned in ascending order chronologically.

Healthy young males were recruited by the research team under ethical approval 08/S1402/6 as donors (typically students) randomly selected from the population, with no known underlying fertility problems. During the initial consultation, potential donors were required to fill out questionnaires relating to fertile status (contribution to a pregnancy), use of drugs/medications, and other relevant sexual history. Potential donors then produced one sample for assessment, to ensure they met/surpassed WHO 2010 criteria for semen volume, sperm concentration and motility. After acceptance, donors were allocated a 3 digit code to provide anonymity, and all identifying information was only accessible by members of the laboratory team.

2.3 Donor Semen Samples – Production, Analysis and Preparation

Donors were requested to abstain for between 2 and 7 full days before production and to produce a sample by masturbation into a sterile container off-site (60ml Sterilin™ Thermo Scientific, polystyrene container with plastic lid). Donors were asked to bring their samples to Ninewells Hospital within one hour of production, and were requested to not expose the sample to any extremes of temperature, to note on the laboratory form if they had missed any fraction of the ejaculate and how many days abstinence had been observed (Bjorndahl et al., 2016). All donor sperm samples used in this study exceeded 2010 WHO criteria 5th centile for semen volume, sperm total/progressive motility and sperm concentration. The semen was allowed to liquefy for at least 15 minutes at 37°C, and a raw semen analysis using CASA (computer assisted sperm analysis) was undertaken within an hour of the time of ejaculation on a Hamilton Thorne CEROS microscope (Beverly, MA, USA) under phase contrast. Parameters were analysed as described in Appendix Table 2.2. Donors were compensated a fixed sum of £15 per donation.

Raw semen analysis was performed by CASA on donor samples according to WHO guidelines (WHO, 2010). Pre-warmed 2X-Cel chambers (Dual Sided Sperm Analysis Chamber, Hamilton Thorne Biosciences, Beverley, MA, USA) slides were loaded with 4µl of semen, and were covered with a pre-warmed coverslip to a set depth of 20µM. These were placed on a heated stage (37°C) to be analysed by a Hamilton Thorne CEROS machine attached to an Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan). Semen with a high sperm concentration (>100million/ml) was diluted with non-capacitating buffer in order to more accurately assess the concentration of sperm present. Concentrations of sperm per ml of semen, total

motility (%), progressive (% of cells which VAP>25µM/sec, STR>80%) and rapid motility (%) of sperm were analysed using CASA, with a negative phase contrast image at 100x final magnification. Other sperm motility parameters such as ALH, VCL, VSL, VAP, BCF, LIN and STR were also recorded (see Appendix Table 2.2).

To obtain the most mature and morphologically normal sperm from raw semen, sperm were separated from the seminal plasma by discontinuous density gradient centrifugation. 90% Percoll (catalogue number P1644, Sigma Aldrich, UK) was prepared using a 1:9 ratio of 10X STF (based on Lishko et al, 2011) to 100% Percoll. 40% and 80% Percoll were prepared by diluting the 90% Percoll with SW media (Mortimer and Mortimer, 2013). Up to 2ml of raw semen was gently layered on top of 1.5ml 40% Percoll, underlaid by 1.5ml 80% Percoll. The column was then centrifuged at 300g for 20 minutes in order to separate the sperm according to their density. The supernatant consisting of seminal plasma, bacteria, white blood cells and other debris was discarded. The pellet of highest quality (with the greatest density and compaction of DNA) was separated by the 80% fraction, as mature normal sperm have a density of >1.12g/ml, and the specific gravity of the 80% fraction is 1.10g/ml. The immature and morphologically abnormal sperm have densities less than 1.10g/ml, therefore are unable to penetrate the 80% layer, and were retained by the 40% layer (Mortimer and Mortimer, 2013). The seminal plasma and 40% layer were removed by sterile plastic Pasteur pipettes (Sigma Aldrich, UK), leaving just the 80% layer plus the pellet. This serves to reduce contamination of the pellet with seminal plasma containing bacteria or other cell debris, or sperm from the 40% layer. The pellet was removed from the tube and was washed in 4ml SW media by centrifugation at 500g for 10 minutes. The supernatant was removed and the pellet resuspended in HTF media and incubated at 37°C 5% CO_2 for a minimum of 2 hours.

2.4 IVF and ICSI patient Semen Samples – Production, Analysis and Preparation

In the ACU, IVF (meeting WHO (2010) normal semen analysis criteria and exceeding $1x10^6$ progressively motile sperm after preparation) and ICSI patients (those not meeting IVF criteria, or with other clinical indications) produced a fresh semen sample typically on-site on the morning of their procedure into a sterile Starstedt 100ml container. Semen was allowed to liquefy for a minimum of 15minutes in an incubator at 37°C. Raw semen analysis for patient samples was carried out in the clinic by an embryologist or laboratory technologist, using a haemocytometer for cell counts and a digital cell counter for motility analysis. Sperm were counted as being static (D, non-motile), slow (C, motile but not progressive), progressive (B) or rapid (A, movement > 25μm/sec). The sperm were then prepared by density gradient centrifugation using PureSperm® (catalogue number P5100, NidaCon International, AB Sweden) buffered with SW media. The 80% pellet was then washed using SW media and centrifuging 500g for 10 minutes, before being resuspended in bicarbonate-buffered Quinn's Advantage Fertilization (HTF) Media supplemented with 10% SPS/HSA and gassed with 6% CO_2 for 20 seconds for IVF samples. This HTF media was maintained in an incubator at 37°C, 6% CO_2 overnight previous to sperm preparation for treatment. Samples were resuspended in non-capacitating HEPES-buffered Quinn's Advantage Sperm Washing Media (SW) for ICSI procedures. Prepared samples were left at room temperature for up to 4 hours for ICSI, whereas in IVF, samples were maintained at room temperature for around three hours, depending on the time of insemination, before being placed into the CO_2 incubator to capacitate for one hour before insemination occurs. After insemination by IVF or ICSI, patient samples surplus to treatment requirements were retrieved for research purposes.

2.5 Screening for hyperactivation

A control reading of hyperactivation was obtained by acquisition of four fields of view of untreated sperm on the CASA, having been resuspended in HTF media and placed under capacitating conditions for 2 hours minimum. The drugs 4-amino pyridine (4-AP, final concentration 2mM), progesterone (final concentration 3.6µM) and trimethylamine hydrochloride (TMA, final concentration 10mM) were added to 100µl aliquots of sperm, and again four fields of view were analysed for hyperactivation at t0 after the drug was added. A minimum of 200 sperm were analysed per treatment, as per WHO guidelines for use of CASA (2010). The vehicle controls for these compounds (water, ethanol and DMSO for 4-AP, progesterone and TMA respectively) were also analysed to ensure that the vehicle was not having a detrimental effect on sperm motility.

2.5.1 Donor hyperactivation in response to progesterone, TMA and 4-AP and their respective vehicles ethanol, DMSO and water

Donor sperm were subjected to treatment with final concentrations of 3.6µM progesterone, 10mM TMA and 2mM 4-AP. Motility was analysed as soon as cessation of drifting on the slides had occurred (~30 seconds after compound addition), in order to visualise whether the calcium response to progesterone which immediately after addition and peaks after around 75 seconds, and lasts for a total of around 150-200 seconds, is correlated with any motility parameter. Furthermore as the HTF media used was bicarbonate-buffered, it was imperative to use the shortest time possible outside of the CO_2 incubator, in order to maintain the pH of the solution. CASA software was used to analyse the motility parameters, including percentage of sperm showing hyperactivated movement, with the requirements of curvilinear velocity

(VCL)>150, linearity (LIN)<50 and amplitude of lateral head displacement (ALH)>7 (Mortimer et al., 1998). Other sperm motility parameters compared between compound treatment groups and also between patients and donors, were VAP, VSL, VCL, ALH, BCF, STR, LIN, % motile, % rapid, % progressive and % hyperactivated (as described in Appendix Table 2.2). Vehicle controls (1% water control for 4-AP, 1% DMSO control for TMA and 0.01% ethanol control for progesterone) had no significant effect on any motility parameter studied (Figure 2.1).

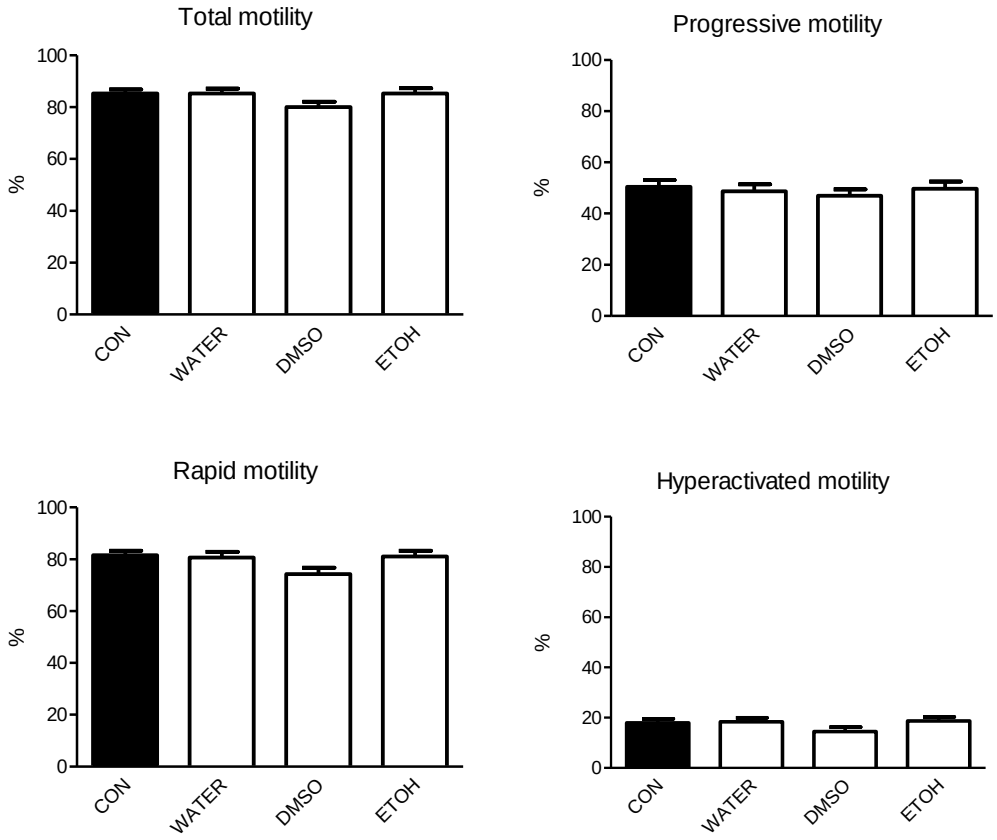


Figure 2.1 Effect of vehicle on motility. Motility in donor samples was not significantly affected in response to vehicle controls water, DMSO and EtOH. Bars represent average donor response ±SEM, n=30.

2.6 Calcium response to progesterone – FLUOstar Omega microplate method

2.6.1 Number of sperm per well

In previous studies, a minimum of 2 million sperm per well was found to be necessary for FLUOstar Omega calcium response to progesterone assessment (Alasmari et al., 2013b). To determine the number of sperm per well necessary for the present study, donor samples were adjusted to varying concentrations: 0.675 million, 0.125 million, 0.25 million, 0.5 million, 1 million, 2 million and 4 million. The response to progesterone was undetectable at 0.675 million cells. The response was difficult to detect with a low signal to noise ratio in 0.125 and 0.25 million cells. The signal was visible and quantifiable at 0.5 million, 1 million, 2 million and 4 million cells as displayed in Figure 2.2. However due to residual noise in the signal from 0.5, 1 and 2 million cells, 4 million cells was chosen to be the final working number of cells in the well. This cell count enabled a smoother curve and therefore a more accurate identification of the peak calcium response to progesterone.

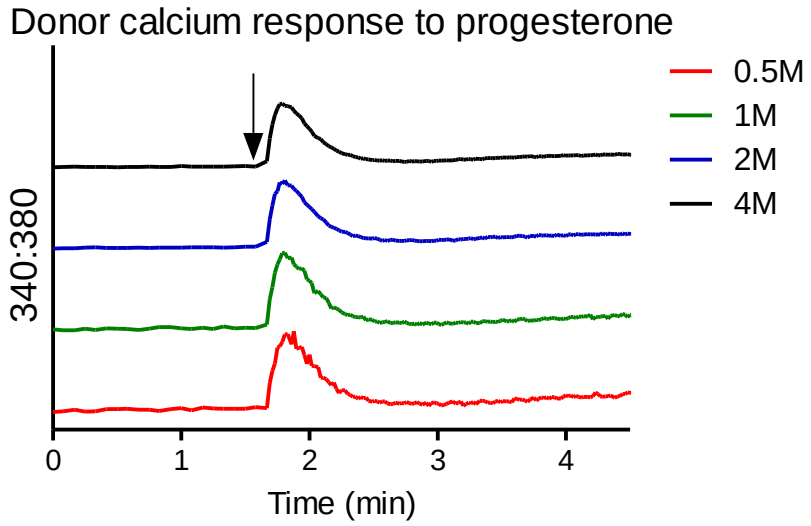


Figure 2.2 Calculation of numbers of sperm used for calcium response to progesterone. 0.5 million, 1 million, 2 million and 4 million sperm are able to produce a calcium response to progesterone however the signal to noise ratio was smallest when using 4 million cells (0.5 million n=7, 1 million n=8, 2 million n=6 and 4 million n=6). Arrow shows the addition of progesterone to a final concentration of 3.6µM. Graph baselines are staggered for clearer visualisation of the traces.

2.6.2 Capacitated versus non-capacitated sperm calcium response to progesterone.

All samples were capacitated for a minimum of two hours before use, unless otherwise stated, due to the previously reported potentiation of the calcium response to progesterone under capacitating conditions (Baldi et al., 1991, Bedu-Addo et al., 2005).

2.6.3 FLUOstar Omega method

Four million cells were prepared in 500µl HTF Media. 1µM Fura-2/AM and 0.05% Pluronic acid F127 were added for one hour at 37°C in the presence of 6% CO_2 to ensure continuing capacitation and bicarbonate buffered pH stability. The samples were centrifuged at 500g for 10 minutes to remove excess dye, before the pellet was removed and resuspended in 500µl STF media lacking BSA. A further centrifugation step at 500g for 5 minutes was performed, where again afterwards the supernatant was removed and replaced with 500µl STF media lacking BSA. After a further centrifugation step at 500g, the supernatant was then removed and the pellet resuspended in 45µl STF media, and was placed in one well of a 96-well black half-volume 4.5mm diameter 190µl volume Greiner plate. The FLUOstar Omega (BMG Labtech Offenburg, Germany) microplate reader machine was used to calculate the calcium response to progesterone. Progesterone was added at 3.6µM final concentration after 20 readings of baseline bound and unbound calcium (100 seconds). The calcium response to progesterone was observed by alternating fluorescence excitation at 380 and 340 nm, with emission at 520nm, and a normalised ratio of 340/380nm was used to obtain graphs. Manganese chloride was added at a final concentration of 9.1mM after the secondary progesterone response had plateaued in order to quench all Fura-2 fluorescent dye. This was then used to calculate the background level of fluorescence, which was subtracted from respective 340 or 380nm fluorescence intensities.

2.6.4 Vehicle control

In order to ascertain that progesterone itself was causing the rise in intracellular calcium, a vehicle control consisting of 0.01% ethanol in STF was used in the case of

three donors and the effect on calcium uptake was monitored. This was run in parallel with progesterone treated cells. As seen in Figure 2.3, the mean 380:340 peak ratio after vehicle addition was below the 5th centile cut-off for donor samples, and furthermore was significantly reduced at just 41% of the delta response obtained from progesterone addition. Presence of residual progesterone within the system could not be excluded. Thus it was concluded progesterone, and not vehicle, was significantly stimulating the increase in intracellular calcium.

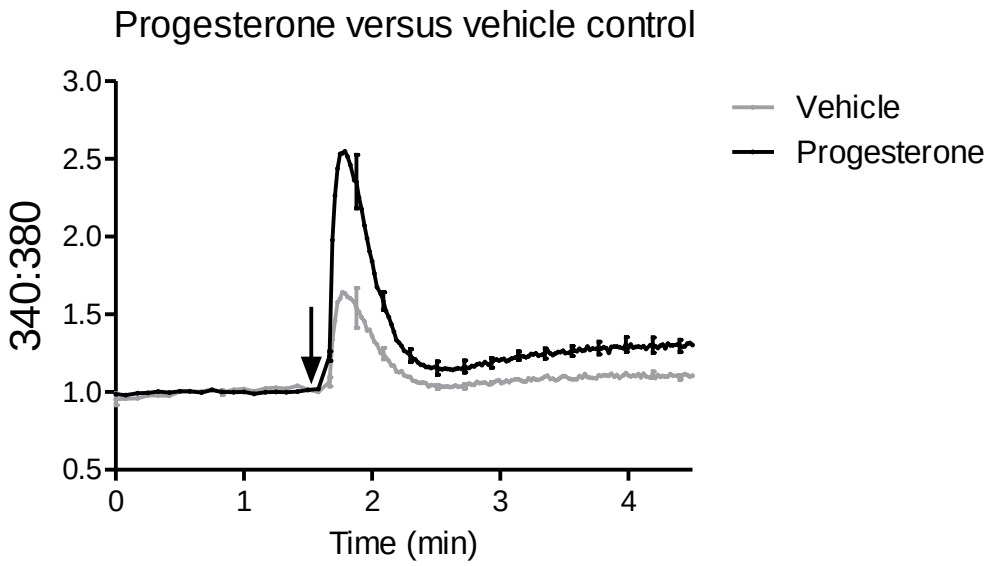


Figure 2.3 Addition of vehicle led to a significantly reduced calcium response. This was below the 5th centile cut-off value for donor samples, n=3 paired samples.

2.6.5 Donor inter-ejaculate variation

Individual donors were asked to provide multiple samples for analysis on separate occasions. Due to the variety in sperm concentration and sperm motility between ejaculates, it was important to decide whether sperm donors should be treated individually (multiple ejaculates pooled together), or if each individual ejaculate should be considered separately (result per ejaculate, regardless of donor origin).

Thus, multiple ejaculates from one specific donor were analysed for their post-preparation calcium response to progesterone. It was observed that there was a significant difference in the peak calcium response to progesterone when comparing results from different ejaculates from the same donor (an example of 4 ejaculates from 1 donor is visualised in Figure 2.4). Thus, calcium responses to progesterone from ejaculates from the same donor were classified as separate data, due to the high inter-ejaculate variability observed.

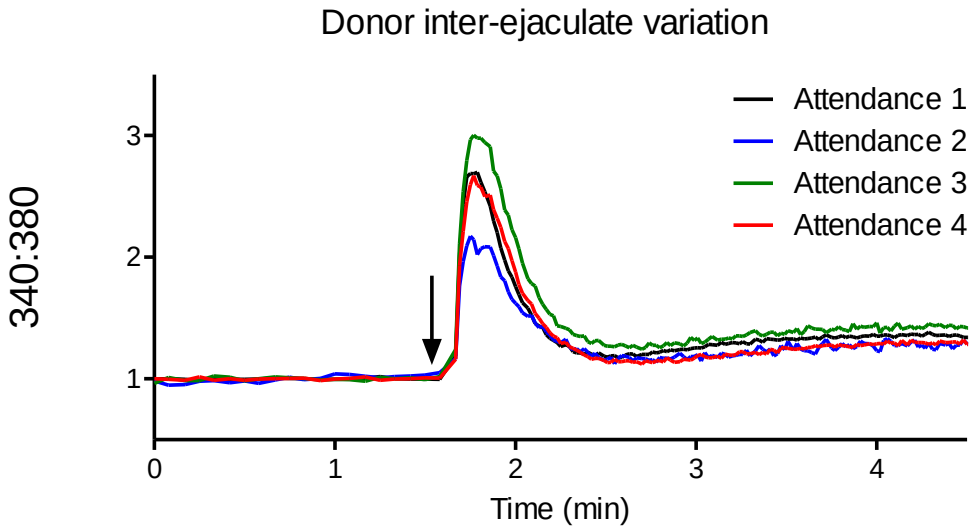


Figure 2.4 Variation in ejaculate response from a single donor. Donor inter-ejaculation variation in the peak calcium response to progesterone was sufficient enough to warrant treating each ejaculate as separate entities. The graph shows four different samples from the same donor. The arrow signifies addition of progesterone.

2.6.6 Defining cut-off values for poor responders

The frequency distributions for delta calcium response to progesterone were created for both IVF patients and donors (Figure 2.5). “Bin center” indicates the middle value

of the bin, or group of values: i.e. bin center of the values falling between 0.1-0.3 is 0.2. The cut-off value for poor responders was defined as being any delta value below the fifth centile for donors. This was calculated in Excel using the PERCENTILE function as being anything below a delta value of 0.64.

Three of the 44 donor samples examined had delta values below 0.64 (6.8%).

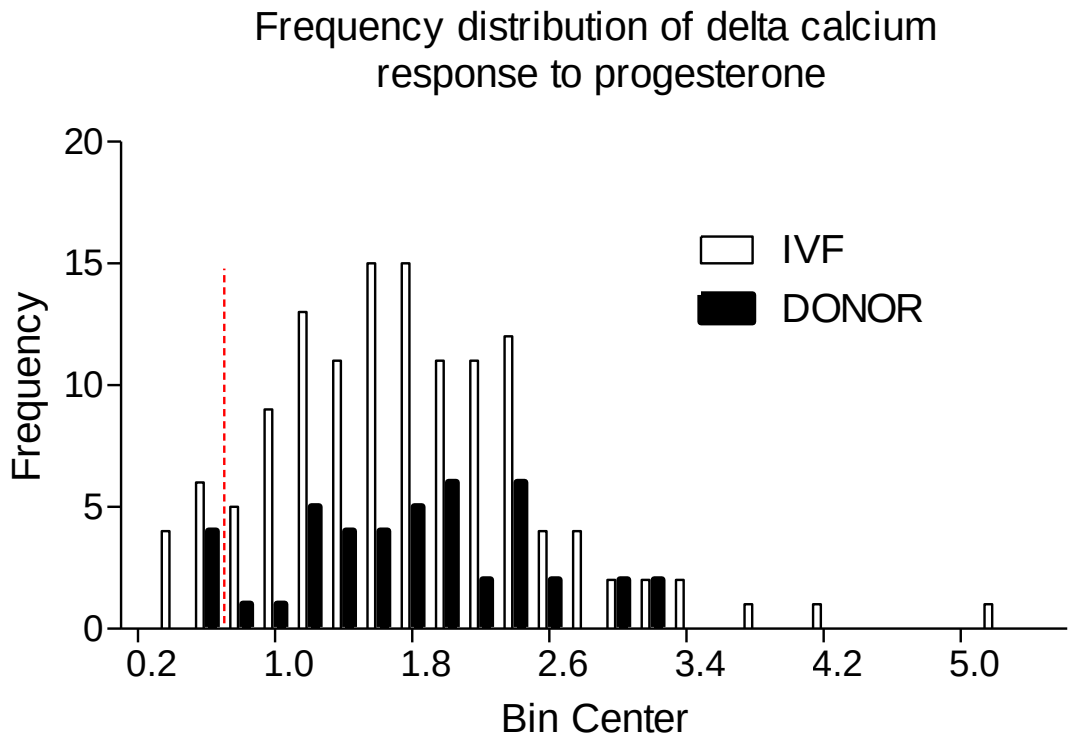


Figure 2.5 Frequency distribution of delta calcium response to progesterone. The histogram of delta calcium response to progesterone demonstrates that the frequency distribution of IVF patients and donors is similar, and normally distributed according to Kolmogorov-Smirnov normality test. The dotted red line represents the 5th centile. IVF n=129, donor n=44 ejaculates.

2.7 The Viscous Media Penetration Test

Methylcellulose, a synthetic cervical mucus solution (Ivic et al., 2002), was made by the addition of 10mg/ml of methylcellulose (catalogue number M0512, Sigma Aldrich, UK) to HTF media supplemented with 10% SPS or HSA as a protein source. Capillary tubes (Vitrotubes™, Rectangle Capillaries 0.4mm x 400mm, catalogue number 2540-050, VitroCom, New Jersey, USA) were inserted into the pre-warmed methylcellulose, incubated in 5% CO_2, which was then drawn into the tube by capillary action. The top end of the tube was sealed with Plasticine to prevent further capillary action once inserted into the sperm suspension. Tubes were labelled with the donor/patient code and treatment group, before being added to 100µl aliquots of sperm suspension in HTF media, and were maintained for an hour in conditions supporting capacitation, at 37°C and 5% CO_2. Sperm suspensions were either maintained untreated, treated with 0.01% ethanol as a vehicle control, or were treated with a final concentration of 3.6µM progesterone, to observe the effect of progesterone on penetrating ability of sperm.

After 1 hour, excess sperm were wiped off the tube and the open end was sealed with Plasticine. Marks were drawn on to the tube at 1cm from the end inserted into the sperm sample. 1cm was chosen as the distance measured as is the most informative and clinically relevant marker, as the hyaluronan matrix holding the cumulus cells does not exceed 1cm in distance from the oocyte. The tube was analysed for sperm penetration under 200x magnification, and CASA was used to analyse 4 fields of view at each distance measured, with three measures of depth adjusted manually for each field of view, in order to account for the depth of the tube. Average numbers of sperm at each centimetre mark for each treatment were recorded and compared. Motility

patterns for sperm swimming within the capillary tube were recorded by CASA, and compared between treatment groups (Chapter 3).

2.8 Cryopreservation and Thawing of Donor Samples

Semen was cryopreserved within one hour of ejaculation, after liquefaction was achieved. Prior to cryopreservation, both the cryoprotectant and liquefied semen were equilibrated to room temperature (21°C). An equal volume of cryoprotectant (Sperm Freezing Media, catalogue number 10670010, Origio, Medicult Media, Denmark) was added to the raw semen dropwise (~50µl) in a 50ml Falcon tube over a minimum of a 5 minute period, and was mixed continuously to avoid hypoosmotic shock (Stanic et al., 2000). 1ml of 1:1 semen:cryoprotectant was loaded per cryovial, and labelled with the donor number and date. The sample was placed in the fridge for 30 minutes before placing in vapour phase of the liquid nitrogen storage facility. The sample was maintained in vapour phase until use.

To thaw the sample, the cryovial was submerged in water at 30°C until thawed. A Pasteur pipette was used to place the whole thawed sample into a 50ml Falcon tube. The same volume of SW media as semen plus cryoprotectant was added dropwise, and mixed continuously to avoid osmotic shock, at room temperature for a minimum of 5min. Once mixed, up to 2ml of the sample was prepared over each 40%/80% Percoll gradient, and a wash step was performed as described in section 2.5.

This protocol was in accordance with the standard operating procedure used by Ninewells Assisted Conception Unit for semen cryopreservation and thaw.

2.9 Single cell calcium imaging.

Both IVF/ICSI patient and donor spermatozoa were subjected to single cell calcium imaging after treatment with progesterone. Donor samples were also investigated using 0.01% ethanol as a vehicle control. The spermatozoa were prepared by density gradient centrifugation and a wash step as described previously (Section 2.3). Following a minimum of 2 hours capacitation (excluding ICSI samples which remained non-capacitated), the spermatozoa were incubated with 1μM Fluo-4/AM (Shahar et al., 2011), for 30 minutes at 37°C, 5% CO_2. The sample was then centrifuged at 100g for 5 minutes to obtain a loose pellet. The supernatant with excess dye was removed and the pellet was resuspended in 100μl of fertilisation (HTF) media lacking HSA protein source, to allow for better cell adhesion to the coverslip.

100μl of 0.1% poly-D-lysine solution in HTF lacking HSA was pipetted onto the centre of a 24x50mm microscope cover glass slide and allowed to adhere at room temperature for 20 minutes. The surplus poly-D-lysine solution was removed. 45μl of sperm solution was added directly to the poly-D-lysine treated area, and incubated at room temperature for 10 minutes to promote settling and adhesion of the spermatozoa to the coverslip.

The coverslip was visualised on a Nikon Eclipse TE2000 microscope under 400X total magnification with oil immersion. Cell density was adjusted to around 200 sperm per field of view by dilution of the sperm pellet within the Eppendorf with HTF lacking HSA. The computer program MetaFluor (Molecular Devices, CA, USA) was used to analyse data. Regions of interest were drawn around each cell either manually or via the computer program, incorporating the spermatozoon head and midpiece. Each ROI was inspected for accuracy of spermatozoa head and midpiece selection and

incorporation, and were de-selected if cells moved into or out of the ROI during the acquisition. Images were acquired after a time lapse of 100 milliseconds, with a duration of exposure of 40 milliseconds. 100 intervals (44 seconds) of basal recordings were collected before the addition of final concentration 3.6μM progesterone. Following a further 150 intervals (66 seconds), a final concentration of 100μM calcium ionophore A23187 was added for maximum fluorescence intensity, and following 100 intervals (44 seconds), 45mM Ca^{2+} chelating agent EGTA (ethylene glycol tetraacetic acid) for minimum fluorescence intensity. After acquisition, an ROI was selected that did not contain any spermatozoa to detect background levels of fluorescence. Data was exported to EXCEL for background correction, normalisation and determination of response to progesterone.

Background correction was performed by taking the fluorescence intensity value at a given time point for an ROI placed manually over an area without any sperm present (Rx) and subtracting the value of the background fluorescence from the background detecting ROI for the given time point (BR). This was repeated for each cell observed. Normalisation of the data was performed by taking the average fluorescence intensity of the basal period of acquisition (Frest) and using the equation ((Rx – Frest)/Frest)*100).

2.10 Whole cell patch clamp electrophysiology

Electrophysiology was performed on some of the recalled patients by Dr Sean Brown and Dr Steven Mansell. The whole cell patch clamp protocol and solutions used were as previously published (Kirichok et al., 2006, Lishko et al., 2011, Mansell et al.,

2014). The ramp protocol (-92 to 68mV at 1Hz) with V_m held at -92mV between test pulses under physiologically relevant standard bath conditions was used to investigate Na^+, K^+ and Cl^- currents. The ramp protocol (-80 to 80mv) under Cs-based divalent free conditions with a holding potential of 0mV between pulses was used to investigate CatSper channel activity.

A minimum of three ramps per cell was performed on all patient samples.

2.11 IVF and ICSI fertilisation rate

Fertilisation data was obtained from IVF patients with 4 or more MII oocytes. The morning after oocyte retrieval and insemination, oocytes were stripped and identified to be 1) immature at the GV (germinal vesicle) or MI (metaphase I) stage, 2) mature but unfertilised at the MII (metaphase II) stage of maturation, 3) fertilised normally or abnormally. In this study, normal fertilisation was confirmed with the presence of two pronuclei (2PN) and two polar bodies (2PB). Abnormal fertilisation was not counted in our data. Fertilisation was expressed as a percentage of the number of oocytes at MII that were successfully fertilised (Number of oocytes fertilized/number of MII oocytes collected). For ICSI samples, the premise was the same however maturity status was acknowledged prior to injection, and only MII oocytes were injected, therefore fertilisation rates were determined according to the number of oocytes injected. Failed fertilisation was defined as no normal fertilisation with 4 or more metaphase II oocytes present. Low fertilisation was defined as <25% of 4 or more metaphase II oocytes normally fertilised.

Fertilisation data was requested retrospectively in batches of approximately 20 patients. The information was collected by a clinical support worker who un-blinded the research code, looked up the patient on the Assisted Conception Unit database and recorded the relevant information, and re-blinded the data before passing it to the research team, ensuring anonymity.

2.12 Statistics

The Kolmogorov-Smirnov test was used to analyse distribution of data, a *P*-value of $P>0.05$ was accepted as data being normally distributed. To compare normally distributed data, an independent samples two-tailed Student's t-test or one-way ANOVA (analysis of variance) was used. Paired Student's t-tests and Wilcoxon signed-rank tests were used as parametric and non-parametric tests respectively, where samples were paired. To compare non-parametric data, a Mann-Whitney or Kruskal-Wallis test was used. Correlation analysis was performed to compare the relationship between normally distributed variables, with a Pearson product-moment correlation coefficient to analyse the goodness of fit. Spearman's rank correlation coefficient was used to analyse the correlations between non-parametric data.

Data was processed and statistically analysed in GraphPad Prism and Microsoft Excel.

Chapter 3

Investigation into the incidence of calcium pathway defects in IVF patients and donors

3.1.1 Introduction

Electrophysiological study of the CatSper channel by whole cell patch clamp technique is the gold standard for assessment of ion channel function (Kirichok et al., 2006), however this technique is low-throughput with few cells able to be studied per sample, and relies on Cs^+ based divalent free solutions rather than studying Ca^{2+}. Therefore other methods must be used to indirectly assess CatSper function, and in the present study this is performed by using calcium response to progesterone as a surrogate. CatSper function is particularly relevant in patients undergoing IVF, where absence or dysfunction of the channel could result in inability to hyperactivate and therefore fertilise oocytes as is the case in mice (Quill et al., 2003). Although Alasmari et al (2013a) described that calcium regulation in human sperm differs between patients attending the assisted conception clinic in Ninewells Hospital, Dundee, and healthy donors randomly selected from the population, this study focused mainly on utilising agents such as 4-AP to modify sperm hyperactivated motility.

Many studies have previously correlated the human sperm calcium response to progesterone with male infertility and therefore likelihood of success in IVF (as mentioned in section 1.9). However these studies have failed to identify the incidence of calcium pathway defects in response to progesterone in these patients. The molecular or physiological causes behind why these patients have a decreased calcium response to progesterone is also largely unknown, particularly as the majority of these studies were performed before the discovery that CatSper is potentiated by progesterone (Lishko et al., 2011, Strunker et al., 2011).

3.2 Aim

This study aimed to use several different screening methods in order to elucidate the prevalence of defective calcium regulation and CatSper function in IVF patients and donors. These methods were: calcium response to progesterone, viscous media penetration test and induced hyperactivation with 4-AP.

3.3 Hypothesis

We hypothesized that there would be a greater incidence of calcium pathway defects in IVF patients when compared with donors, and furthermore poor response in any of these screening tests will be negatively correlated with sperm function, manifested in poorer fertilisation rates in IVF.

3.4 Experimental design

Donor and patient samples were prepared and capacitated as previously described. Samples were analysed for cell concentration and motility, before being split into portions for separate experiments, depending on cell number. The priority was to perform sperm calcium response to progesterone (IVF n=129 men, donor n=44 from 30 men), followed by hyperactivation in response to stimulation by 4-AP (IVF n=68 men, donor n=35 from 21 men) then progesterone (IVF n=48 men, donor n=34 from 21 men), then TMA (IVF n=48 men, donor n=34 samples from 21 men), and lastly the role of progesterone on progression through viscous media (IVF n=17 men, donor

n=18 from 15 men). For the IVF patients, all screening results were correlated with fertilisation rate achieved in IVF.

3.5 Results

3.5.1 Donor initial semen parameters and IVF patient/donor prepared sperm parameters

The average semen parameters for motility and concentration recorded for donors exceeded WHO 2010 criteria (mean concentration 119.9M/ml, mean total motile cells 66.5%, mean progressively motile cells 38.2%, mean rapidly motile cells 56.2% (47 ejaculates from 20 donors) Appendix Table 3.1).

For samples prepared by density gradient centrifugation, the concentration of sperm in the donor samples was significantly higher than in the IVF samples (38.1M/ml versus 17.1M/ml, *P*= <0.0001, donor n=48 ejaculates from 20 donors, IVF n=68 men). Total motility (85.3% versus 79.5%, *P*=0.014), rapid motility (81.6% versus 74.9%, *P*=0.008) and hyperactive motility (18% versus 13.5%, *P*=0.021) parameters were all observed to be significantly higher in donor samples compared to IVF samples, however there was no difference observed in progressive motility (50.5% versus 50.3%, *P*=0.95), as displayed in Appendix Table 3.2.

3.5.2 IVF versus donor sperm calcium response to 3.6µM progesterone

Analysis of the data for the basal calcium ratio, transient peak calcium and delta response (peak - basal) indicated that there was no significant difference between

values for IVF patients and donors in terms of the calcium response to progesterone, as displayed in Table 3.3, Figures 3.1 and 3.2. However there was a significant increment in the amplitude of the plateau phase for samples taken from IVF patients (Mann Whitney test, *P*=0.031), and the time taken to reach peak calcium response to progesterone was also increased by 2.02 seconds (Mann Whitney test, *P*=0.0004) for samples taken from IVF patients. Furthermore, the total duration of calcium response to progesterone was significantly increased in the IVF patient sample population (Students t test, unpaired samples *P*=0.029).

Parameter	**Donor ±SD**	**IVF ±SD**	***P* value**
Peak (340:380)	2.81 (0.7)	2.69 (0.8)	0.37
Basal (340:380)	1.48 (0.2)	1.51 (0.3)	0.64
Plateau (340:380)	1.39 (0.2)	1.45 (0.2)	**0.031**
Time to peak (s)	8.1 (2.9)	10.12 (3.8)	**0.0004**
Duration of peak (s)	61.81 (9.2)	65.97 (11.3)	**0.029**
Delta (340:380)	1.81 (0.7)	1.78 (0.8)	0.78

Table 3.3 Calcium response to progesterone kinetics for IVF patients and donors. Plateau calcium response, time to peak and duration of peak were all significantly increased in IVF samples, whereas peak calcium response, basal calcium, delta response were not significantly different between groups (n=129 for IVF samples, and n=44 samples from 30 donors).

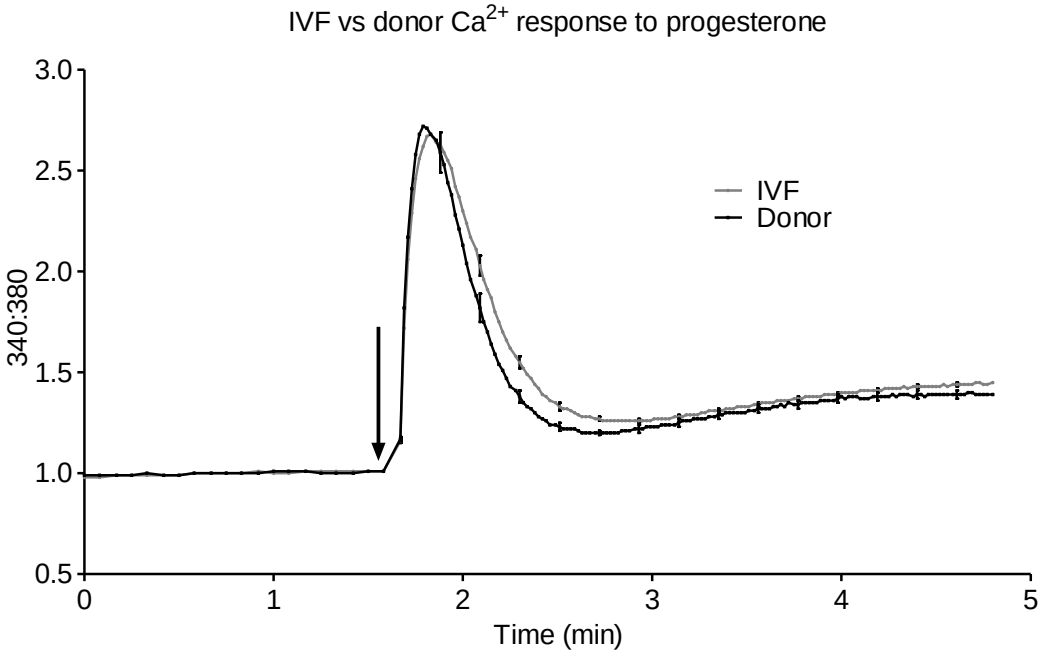


Figure 3.1 IVF versus donor calcium response to progesterone. Mean calcium response to progesterone traces for IVF and donor samples are virtually indistinguishable from one another. Error bars represent SEM, arrow indicates time of 3.6µM final concentration progesterone addition. IVF sample n=129, donor n=44 samples from 30 men.

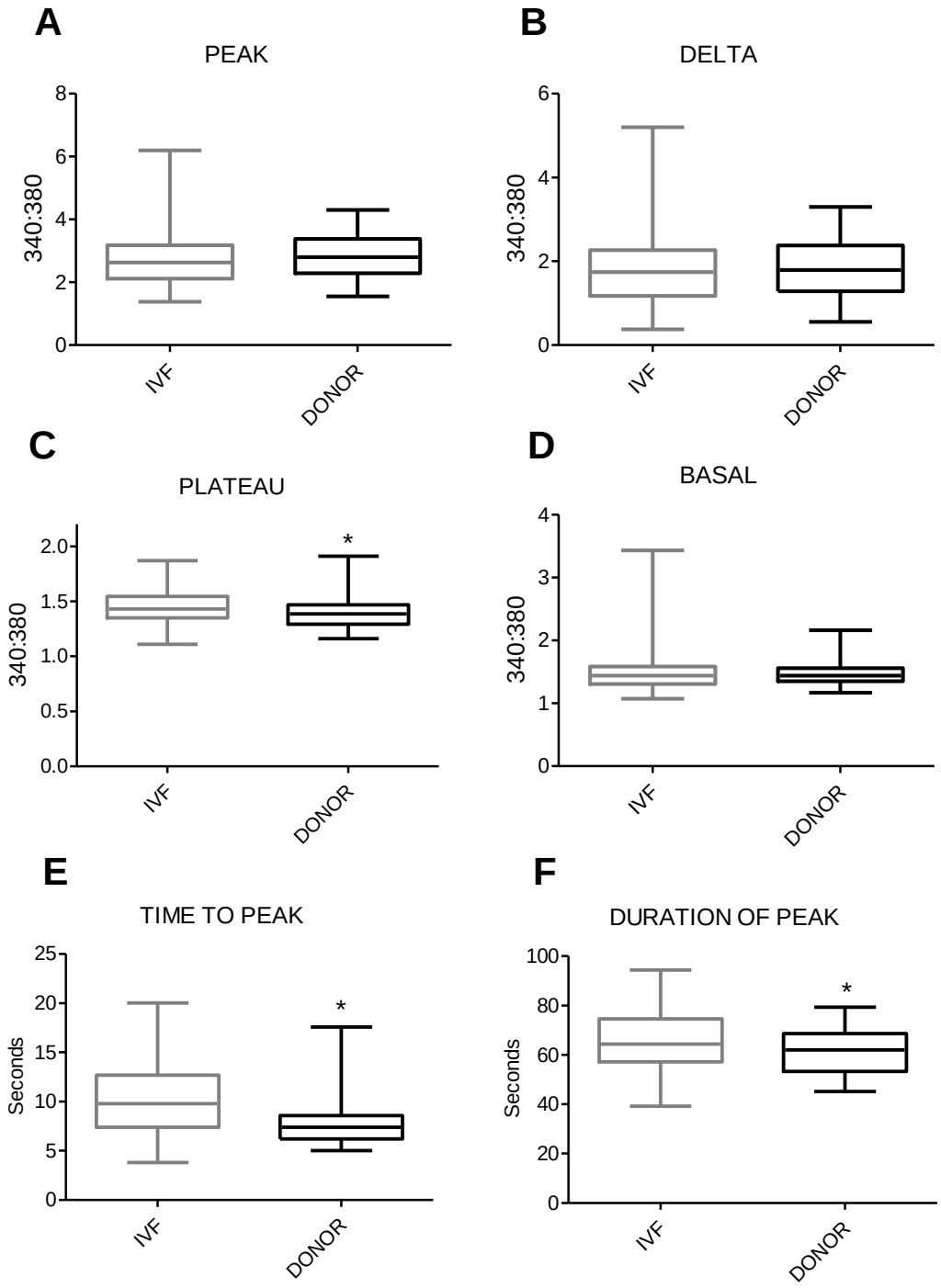


Figure 3.2 Graphical representation of data shown in Table 3.3. Plateau phase calcium response, time to and duration of peak were significantly increased for IVF patients when compared with donor controls. Peak, basal and delta calcium response to progesterone were not significantly different between groups. IVF sample n=129 and donor sample n=44.

3.5.3 Hyperactivation in response to progesterone, TMA and 4-AP

3.5.3.1 Donor versus IVF patient hyperactivation to 3.6μM progesterone, 10mM TMA and 2mM 4-AP

Both donors and IVF patient samples were subjected to hyperactivation analysis under control conditions (IVF sample n=68, donor sample n=35), or after treatment with 3.6μM progesterone (IVF sample n=48, donor sample n=34), 2mM 4-AP (IVF sample n=68, donor sample n=35) and 10mM TMA (IVF sample n=48, donor sample n=34), as shown in Figure 3.3. Under control conditions, and after 4-AP treatment, the mean donor hyperactivation response was significantly higher than the mean IVF patient hyperactivation response (P=0.021 and P=0.048 respectively). Neither TMA nor progesterone had any significant effect on enhancing hyperactive motility in either donor or IVF patient sperm samples (Figure 3.3).

Total motility and rapid motility were not significantly altered after addition of any of the treatments, for either IVF patients or donors (Figure 3.3). As 4-AP was the only treatment to stimulate an effect on hyperactivated and progressive motility parameters, it will be discussed in further detail in section 3.4.2.

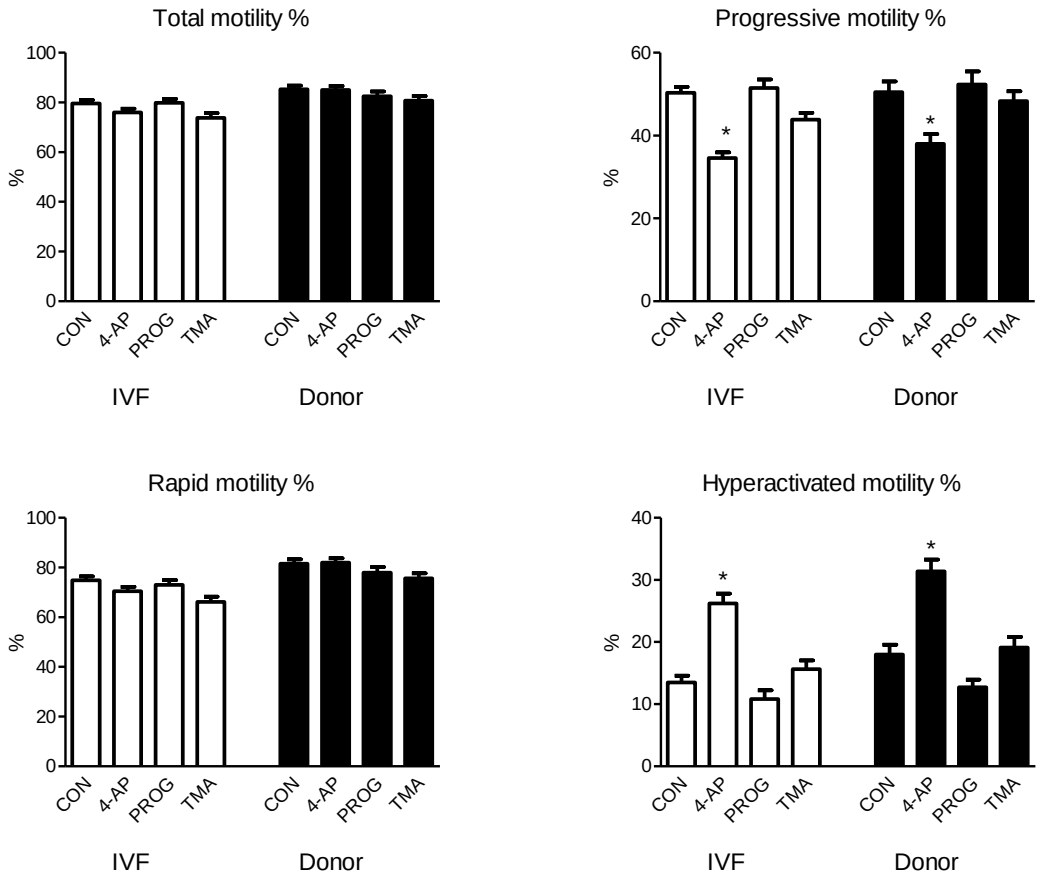


Figure 3.3 Motility parameters after treatment with 2mM 4-AP, 3.6µM progesterone or 10mM TMA. CASA motility analysis revealed that only treatment with 4-AP was able to significantly reduce progressive motility and increase hyperactivated motility. Treatment with progesterone and TMA revealed no significant change in motility in any parameter studied. Error bars represent SEM.

3.5.3.2 Hyperactivation and motility patterns in response to 2mM 4-AP treatment

For both donors and IVF patients, 4-AP induced a rapid and significant average increase in hyperactivated motility (Figure 3.3). 4-AP also significantly increased the sperm VAP, VCL and ALH while decreasing BCF, STR, LIN and progressive motile %. Cells from 45 of the 68 (66%) IVF patients tested were able to significantly respond on addition of 4-AP, by displaying a significantly increased proportion of hyperactive cells. Cells from 2 of the 68 patients (3%) displayed a significant reduction in hyperactivation after 4-AP treatment, whereas cells from the remaining 21 patients (31%) showed no significant change in hyperactivation after treatment. For donors, sperm from 28 of the 35 samples (80%) were able to significantly respond to 4-AP in terms of increased hyperactivation. Cells from the remaining 7 samples (20%) were unable to significantly respond to 4-AP treatment. Significantly, there were no donor samples that exhibited a reduced hyperactivated motility in response to 4-AP, unlike their IVF patient counterparts. A histogram depicting the percent change in hyperactivation after 4-AP treatment for IVF patients is presented below (Figure 3.4) similarly to methods described previously (Alasmari et al., 2013b).

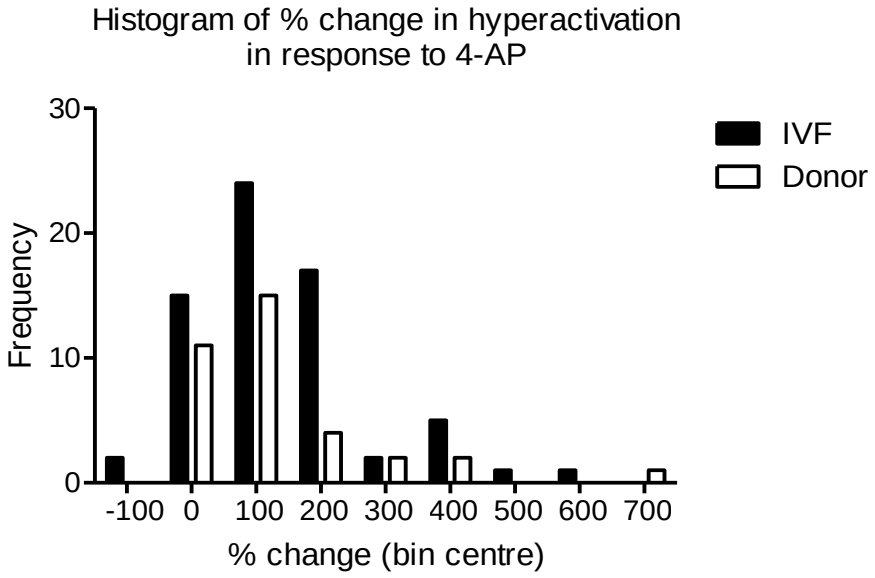


Figure 3.4 Histogram of proportional change (%) in hyperactivation in response to 2mM 4-AP treatment. Distributions of % change in hyperactivation between IVF patients and donors in response to 4-AP were not significantly different, although no donors exhibited a negative response to 4-AP.

3.5.4 Viscous media penetration test: IVF patients and donors

Cells from 17 IVF patients and 18 donors were subjected to the viscous media penetration test, under control, vehicle and progesterone-treated conditions. Cells from 15 of the 17 IVF patients (88%) were able to significantly respond to progesterone in terms of increased cell number at 1cm in the viscous media penetration test, with only 2 patients (12%) had no significant response to progesterone. For the donors, cells from 15 of the 18 donors (83%) were significantly able to respond to progesterone at 1cm, and 3 (17%) were not able to respond to progesterone at all. Mean changes in cell density for IVF patients and donors under control, vehicle and 3.6μM progesterone treatment are displayed in Figure 3.5.

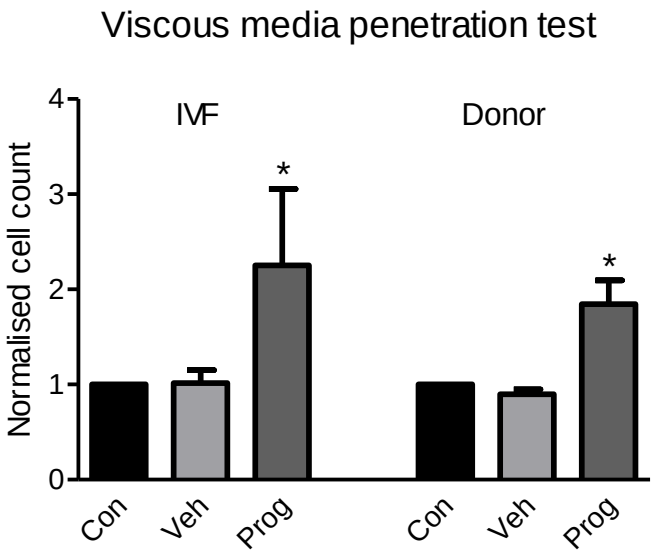
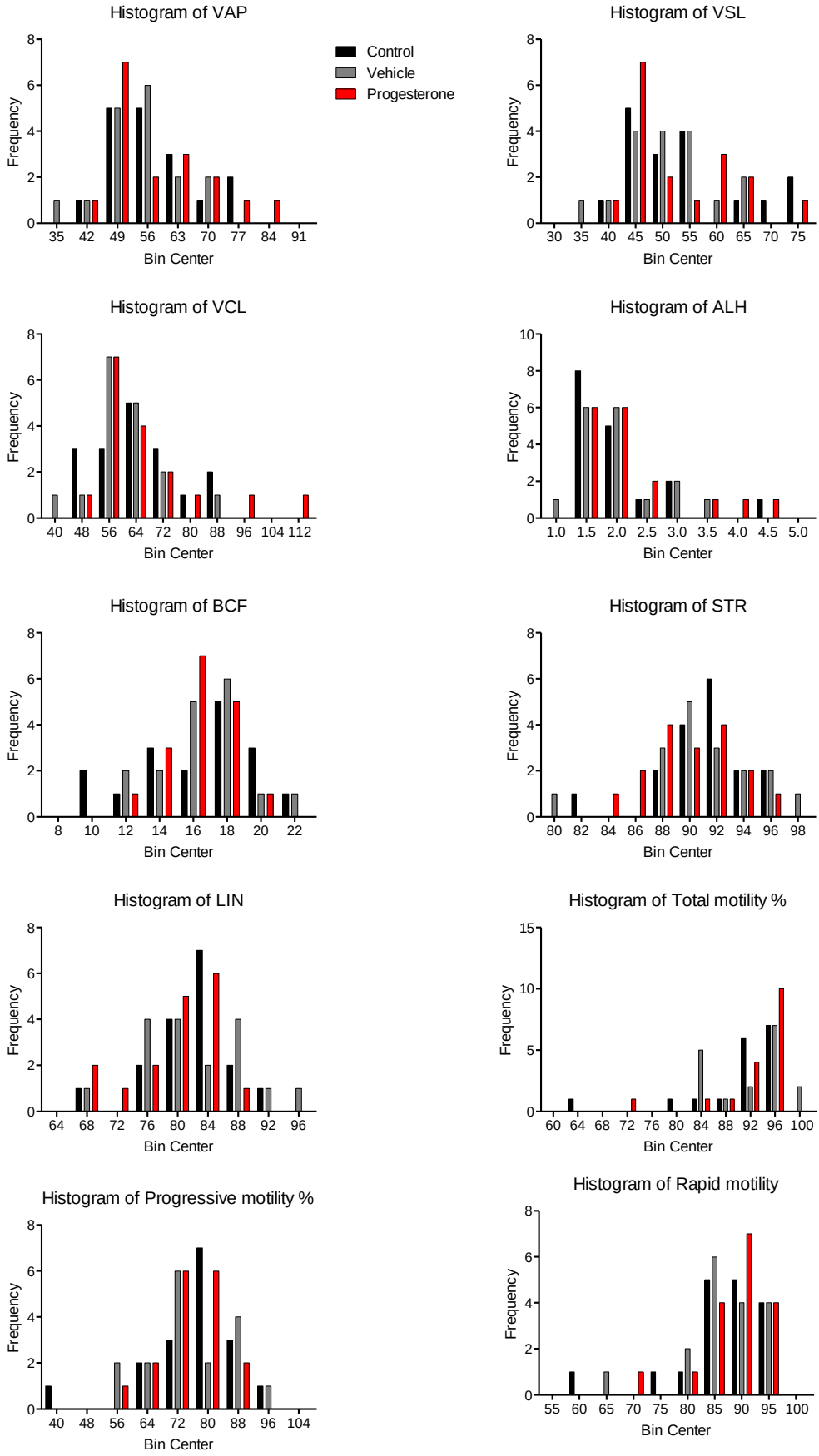


Figure 3.5 Viscous media penetration test in IVF patients and donors. Progesterone treatment yielded an increase in cell count. Bars show average normalised cell count at 1cm within the viscous media. Donor n=18, IVF n=17.

3.5.5 Motility characteristics of sperm within synthetic cervical mucus

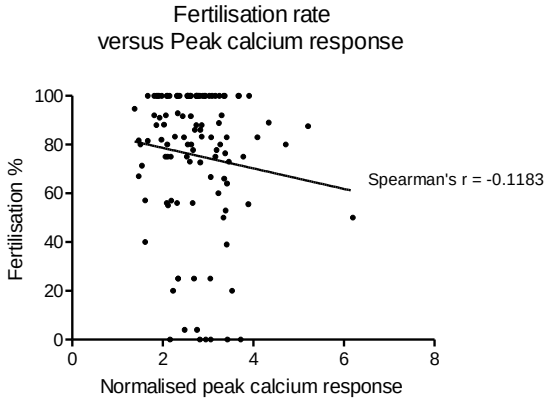
Detailed motility characteristics for those sperm able to penetrate viscous media after control, vehicle control or progesterone treatment were compared between treatment groups for both IVF patients (n=17, Figure 3.6) and donors (n=18). This was performed using CASA by directly observing sperm swimming within the viscous media, following incubation of the samples for one hour. There was no significant difference in the distribution of any of the motility parameters examined within the viscous media, for either donors or IVF patients, under control, vehicle or progesterone-treated conditions, therefore the data is solely presented for IVF patients as described in Figure 3.6.

Figure 3.6 (overleaf). Motility of sperm within viscous media. Motility kinetics analysis by CASA revealed that there was no significant differences in any motility parameters for the sperm observed within the viscous media penetration test, when treated with vehicle or progesterone and compared with control. There were no significant differences between motility of IVF or donor sperm sample motility (graph shows motility kinetics for IVF patients), n=17 for IVF patients.


Histogram of VAP
Control
Vehicle
Progesterone
Frequency
Bin Center
Histogram of VSL
Histogram of VCL
Histogram of ALH
Histogram of BCF
Histogram of STR
Histogram of LIN
Histogram of Total motility %
Histogram of Progressive motility %
Histogram of Rapid motility

3.5.6 Screening results: correlation with fertilisation rate in IVF

All motility parameters under each treatment group during hyperactivation screening, cell count, degree of cellular penetration into methylcellulose, kinetics of sperm swimming within the methylcellulose, and calcium response to progesterone parameters were correlated with fertilisation success at IVF. None of these parameters were significantly positively or negatively correlated with fertilisation success, according to the Spearmans's rank correlation coefficient (P>0.05). Examples of two of these correlations are given in Figure 3.7 below.



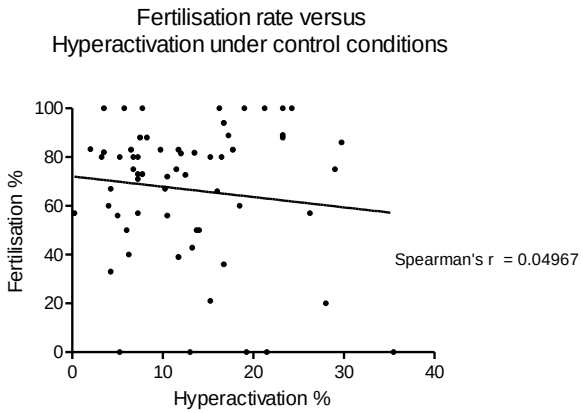


Figure 3.7 Correlating fertilisation rate with observed parameters. When comparing fertilisation rate of MII oocytes with peak calcium response and hyperactivation under control conditions, there was no significant correlation between the parameters, according to Spearman's rank.

3.6 Discussion

The primary aims of this chapter were to investigate the roles of 4-AP, progesterone and TMA on hyperactivation, the calcium response to progesterone and the viscous media penetration test on donor and IVF patient samples. Control data was obtained from donors to establish "normal" responses to hyperactivation screening, calcium response to progesterone and the viscous media penetration test. Patients were screened continuously throughout the project, in order to identify patients currently undergoing a treatment cycle that had a defective response to our analyses. Patient fertilisation success was also correlated with outcomes from the screening process.

Up to 50% of the patients attending the Assisted Conception Unit are classified as having male factor infertility as defined by WHO criteria (Cooper et al., 2010). It was expected that IVF patient samples would have poorer motility parameters in the prepared capacitated sample than donor samples, due to their subfertile status. This was observed in the present study, where prepared sperm from donors had significantly greater sperm concentrations, sperm total motility, rapid motility and hyperactivated motility when compared with their IVF patient counterparts. This finding has corroborated the data presented in previously published papers (Alasmari et al., 2013a), that IVF patient motility is significantly lower than that of donors.

After treatment with 2mM 4-AP, sperm hyperactivation was significantly increased compared to control levels of hyperactivated motility for both donors and IVF patients, as previously described (Gu et al., 2004). However the mean proportion of hyperactivated cells was reduced following 4-AP treatment in IVF patients when compared with progesterone, again verifying results identified previously (Alasmari

et al., 2013a). A lower proportion of sperm samples from IVF patients (66%) were able to significantly respond to 4-AP, compared with sperm samples from donors (80%). As the samples were prepared in a similar manner and allowed to capacitate for the same length of time before being subjected to analysis, the difference in hyperactivation between donors and IVF patients could be indicative of the subfertile status of the patients (Munire et al., 2004).

Neither progesterone (contrary to data published by Alasmari et al., 2013a) nor TMA were able to significantly stimulate an increase in hyperactivation in either donors or IVF patients. No motility parameters in either of the treatment groups were significantly different from control or the vehicle control. It is interesting to observe that although treatment with progesterone increases sperm ability to enter and progress through cervical mucus, there is no apparent change in motility detectable by CASA. Furthermore, although the time of analysis after progesterone treatment was chosen to coincide with the rapid transient increase of intracellular calcium as observed by fluorimetric methods, there was no corresponding increase in hyperactivation.

The transient peak sperm calcium response, delta response, or basal calcium ratio were not significantly different between the donor and IVF patient population. Furthermore, raw calcium levels were not significantly different for both the 340 and 380 bound and unbound fluorescence intensities in IVF patient samples when compared with donor samples. The parameters that were found to be significantly different between the groups were an increased time to peak calcium response to progesterone, an increased duration of calcium response and an increased plateau phase calcium response in IVF patient samples. This was contrary to findings published in previous studies, where peak calcium response to progesterone was significantly reduced within IVF patient sperm samples (Falsetti et al., 1993, Alasmari et al., 2013a). In the present study, the

majority of possible confounding factors were controlled for: for example concentration of spermatozoa studied, dye concentration, incubation time and progesterone concentration. The one factor that was difficult to control for was IVF patient participation: only IVF patients with >4 million spermatozoa in the prepared sample received by the research laboratory from the ACU could be used in this experiment. This meant that a proportion of IVF patients with sperm number of <4 million were not studied, thus excluding IVF patients with oligozoospermia. No ICSI samples were able to be investigated by this method, due to the restriction of cell numbers, therefore development of a more sensitive method of detection of calcium response to progesterone would clearly be beneficial in this case.

Throughout the duration of the study, there was one incidence of an IVF patient sample which exhibited a complete absence of calcium response to progesterone when investigated with this technique. Such an outcome had not previously been identified, and this case is discussed in more detail in Chapter 4.

The present study showed that similar proportions of IVF patients and donors, 15/18 donors (83%) and 15/17 IVF patients (88%) showed greater numbers of sperm at 1cm after progesterone treatment, compared with control and vehicle control. However there was no significant change in motility parameters after treatment with progesterone to suggest the reason why this was possible, and furthermore there was no significant variation in motility parameters between IVF patient and donor sperm samples measured within the viscous media.

Results from the motility analysis, calcium response to progesterone and viscous media penetration test were correlated with the fertilisation rate of all MII oocytes collected. There was no significant correlation, either positive or negative, between

any of the parameters studied and the fertilisation rate. This is in contrast to previous studies where parameters such as progesterone induced calcium responses and hyperactivation in response to 4-AP were found to be significantly correlated to IVF fertilisation rates (Alasmari et al., 2013a). This finding could be explained by the fact that only samples of >4 million cells could be examined for calcium response to progesterone, thus skewing the population of IVF patient samples studied. The one incidence of completely failed calcium response to progesterone did also experience total failed fertilisation, which may indicate that any calcium response above cut-off value does not affect fertilisation ability.

Limitations of the current study include the difficulty in obtaining IVF patient samples of sufficient quality in order to carry out the screening tests. Many of the patient samples received were of insufficient sperm numbers for techniques that required a certain number of cells (such as 4 million cells required for the calcium response to progesterone assay). Furthermore, poor sperm concentration hampered the ability to perform multiple screening techniques on the same sample, and as such the calcium response to progesterone took precedence over hyperactivation assay and viscous media penetration test.

Future experiments may include further development and sensitization of the screening techniques described in this chapter, to aid in the examination of spermatozoa from patients and donors with lower sperm counts or poorer recovery after density gradient centrifugation.

3.7 Conclusion

Although there was no significant correlations between any of the parameters investigated in this study with fertilisation rates at IVF, cells from IVF patients had a significantly lower level of basal hyperactivation, and defects were present in an individual patient when investigating the calcium response to progesterone. Samples from IVF patients and donors performed similarly in the viscous media penetration test.

The study identified one male with significant abnormalities, which will be discussed in more detail in Chapter 4.

Chapter 4

Analysis of calcium response to progesterone in patients recalled due to previous failed/low fertilisation treatment outcomes

4.1 Introduction

The live birth rate per cycle of IVF or ICSI started remains relatively low at 32.8% for women under 35 (HFEA, 2014). Adverse treatment outcomes affecting all stages of assisted reproduction contribute to the low birth rate (Huang and Rosenwaks, 2012). This chapter will focus on one of the arguably most significant stages of ART and IVF in particular: fertilisation of the oocyte. The incidence of total fertilisation failure (TFF) remains around 5-10% in all IVF cycles and 1-3% of all ICSI cycles (Barlow et al., 1990, Ruiz et al., 1997, Bhattacharya et al., 2001, Repping et al., 2002), with recurrence rate of a further TFF outcome in a subsequent treatment cycle estimated to be 30-65% (Roest et al., 1998).

Failed fertilisation in IVF or ICSI may be attributed to a number of causes (Barlow et al., 1990, Liu and Baker, 2000, Mahutte and Arici, 2003), yet in the clinical ART laboratory setting, it is generally not known whether failed fertilisation can be directly linked to either the male or female partner. As male causes cannot be detected by basic semen analysis, research tools such as the hemizona binding assay have previously been used to diagnose male factor infertility. However, they are currently rarely used despite their apparent value, partly due to the employment of ICSI technique for cases of male factor infertility (Oehninger et al., 2014). The present study used the screening research techniques described in Chapter 3 as a novel approach in investigating sperm function in semen samples from males who had an IVF, or ICSI treatment cycle where sperm numbers were suitable for analysis, where the outcome was failed or low fertilisation. These patients were recalled for a consultation, and were requested to provide a further semen sample for research. This research could provide valuable information regarding the direction of the next treatment cycle for the patient.

4.2 Aim

The aims of this chapter were to investigate semen samples from patients that had suffered a previous low or TFF in IVF or ICSI, and to use different research techniques to identify defects in sperm function leading to male factor infertility.

4.3 Hypothesis

Incidence of sperm dysfunction and specifically calcium pathway defects will be significantly higher in patients who have experienced at least one cycle of TFF, than IVF patients and donors.

4.4 Methods

Sixteen IVF patients and two ICSI patients were asked to attend a follow-up appointment with Dr Sarah Martins da Silva following failed or low fertilisation in a previous treatment cycle, and provided at least one semen sample for research. Semen samples were produced and prepared as previously described. Calcium response to progesterone, basal and stimulated hyperactivation and the viscous media penetration test were all performed as previously described. Electrophysiological techniques performed by Dr Sean Brown to investigate both Ca^{2+} and K^{+} channel conductance, was applied to capacitated spermatozoa from eight of the patients as previously described.

4.5 Results

Clinical features of the recalled patients are presented in Table 4.1. Of the 18 patients, the majority (12/18, 67%) were described as having unexplained infertility, with 4 suffering from PCOS (polycystic ovarian syndrome, 22%). One patient had male factor infertility due to a vasectomy reversal, and one had a recorded cause of infertility due to being an offshore worker (infertility due to the male partner's job obstructing coitus in fertile period). Average duration of infertility was 2.9 years ±1.3 SD. Mean male age was 36 years ± 5.6 SD, while mean female age was 34 years ± 5.1 SD.

Patient code	Recorded cause	IVF/ICSI	Duration	Male age	Female age
Patient A	Unexplained	IVF	2	43	39
Patient B	Unexplained	IVF	5	24	25
Patient C	Male factor	ICSI	3	40	36
Patient D	Offshore worker	IVF	-	-	-
Patient E	Unexplained	IVF	2.5	42	38
Patient F	Unexplained	IVF	2	34	30
Patient G	Unexplained	IVF	2.25	37	36
Patient H	Unexplained	IVF	2	36	36
Patient I	Unexplained	IVF	-	33	28
Patient J	Unexplained	IVF	5	36	39
Patient K	PCOS	IVF	-	36	36
Patient L	PCOS	ICSI	5	44	31
Patient M	Unexplained	IVF	1.5	31	33
Patient N	PCOS	IVF	3	27	25
Patient O	Unexplained	IVF	1.5	34	34
Patient P	Unexplained	IVF	2	33	37
Patient Q	Unexplained	IVF	3	40	44
Patient R	PCOS	IVF	4	42	36

Table 4.1 Clinical features of recalled patients. Recorded cause of infertility, treatment, duration of infertility, male and female age. "-" denotes that details are not known for this parameter.

4.5.1 Calcium response to progesterone

Each of the 18 sperm samples were analysed for their calcium response to progesterone by FLUOstar Omega microplate method. All samples except one, which will be discussed in more detail in section 4.5.6, were able to significantly respond to progesterone (17/18, 94%). When comparing recall patients with IVF patients and research donors, there was no significant differences in the peak, delta or plateau calcium response to progesterone (Figure 4.1, one-way ANOVA, P=0.0525, P=0.0546 and P=0.0635 respectively), or in the duration of the response (Figure 4.1, one-way ANOVA, P=0.0850). However, the time taken to achieve peak response was significantly different among the three groups (Figure 4.1, one-way ANOVA, P=0.0028).

Tukey's multiple comparison test revealed a significant increase in time to peak for recall patients when compared with donors, and the same phenomenon between IVF patients and donors (as previously described in section 3.3), but there was no significant difference between recall and IVF patients.

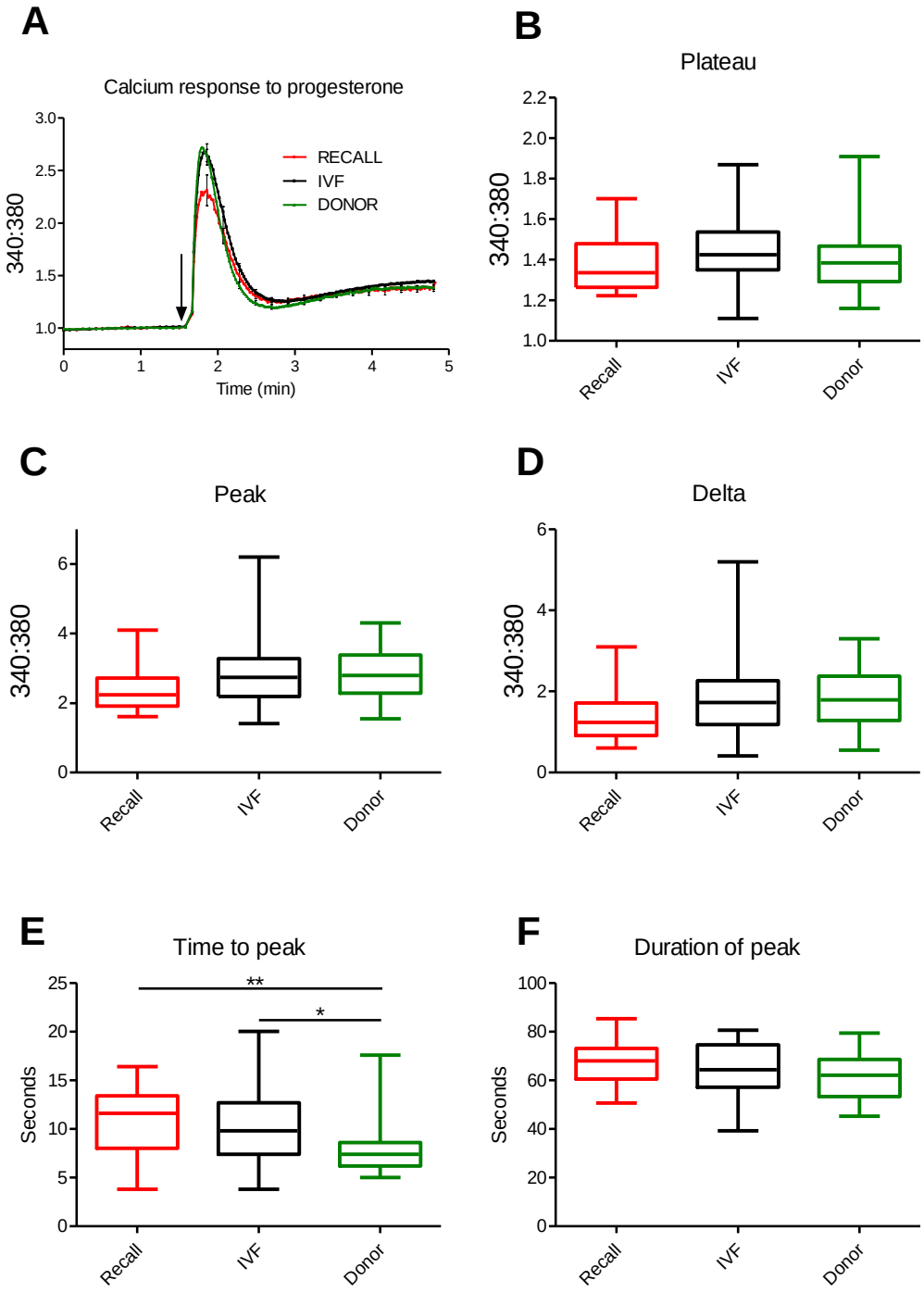


Figure 4.1 Calcium response to progesterone in donors, IVF and recalled patients. There was no significant difference in B) plateau phase, C) peak or D) delta calcium response to progesterone or F) duration of peak between Recall patients, IVF patients or donor samples. However E) time to peak was significantly increased for IVF patients and Recall patients when compared with donor samples. Error bars on A) indicate SEM. Arrow indicates time of progesterone addition.

4.5.2 Viscous media penetration test

Five of the 18 recall patients had sufficient sample in order to study their response in the viscous media penetration test (Figure 4.2). Numbers of sperm that were able to reach 1cm in the synthetic cervical mucus-filled capillary tubes were compared between control, vehicle and 3.6μM progesterone-treated groups (Figure 4.2). Of the 5 patients, 3 (60%) were able to significantly respond to progesterone, whereas 2 (40%) were unable to respond significantly to progesterone.

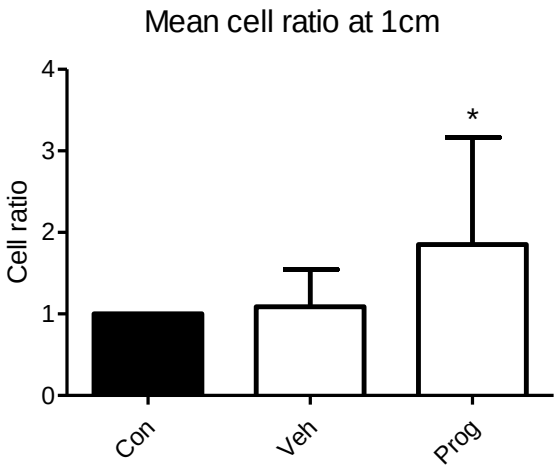


Figure 4.2 Viscous media penetration of recall patient samples. The mean cell ratio at 1cm was significantly increased on addition of progesterone (n=5). Error bars indicate SEM.

4.5.3 Hyperactivation in response to 4-AP, TMA and progesterone

Seven of the 18 patients had sufficient sample to investigate their hyperactivation response to stimuli: 4-AP, progesterone and TMA. Overall, there was no significant difference between any motility parameter studied between treatment groups (representative graphs displaying hyperactivation, VAP, ALH and LIN motility

parameters are shown in Figure 4.3 below). Only two of these seven patients (29%) were able to significantly positively respond to 4-AP (One-way ANOVA with Tukey's multiple comparison test), a significantly lower proportion than for donor or IVF patient sperm samples.

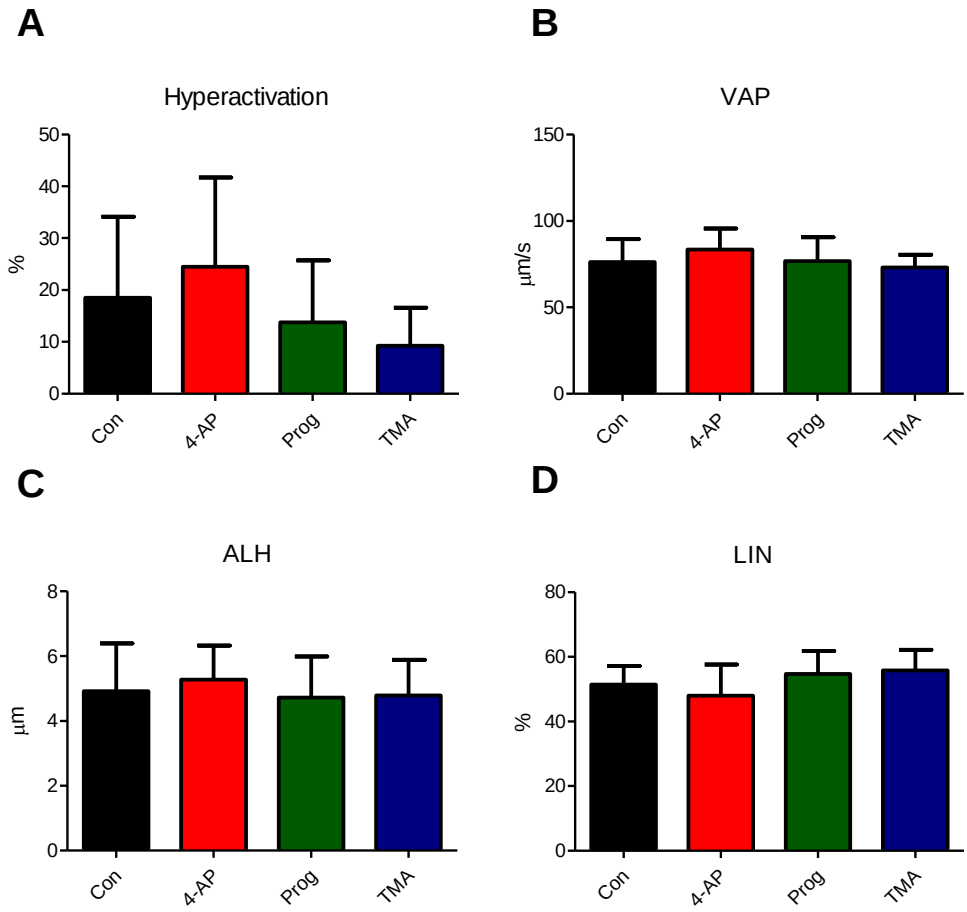


Figure 4.3 Motility parameters for recall patients. There was no significant difference in hyperactivation, VAP, ALH or LIN motility parameters between control, 4-AP, progesterone and TMA treatment groups for Recall patients (n=7).

4.5.4 Whole-cell patch clamp electrophysiology

Whole-cell patch clamp was performed on seven of the 18 patients. Two of these patient samples who produced abnormal responses to this assay will be discussed in further detail in sections 4.5.5 and 4.5.7. The remaining five samples were able to elicit a significant outward K^+ current upon application of ramp stimulus under quasi-physiological conditions (Figure 4.4).

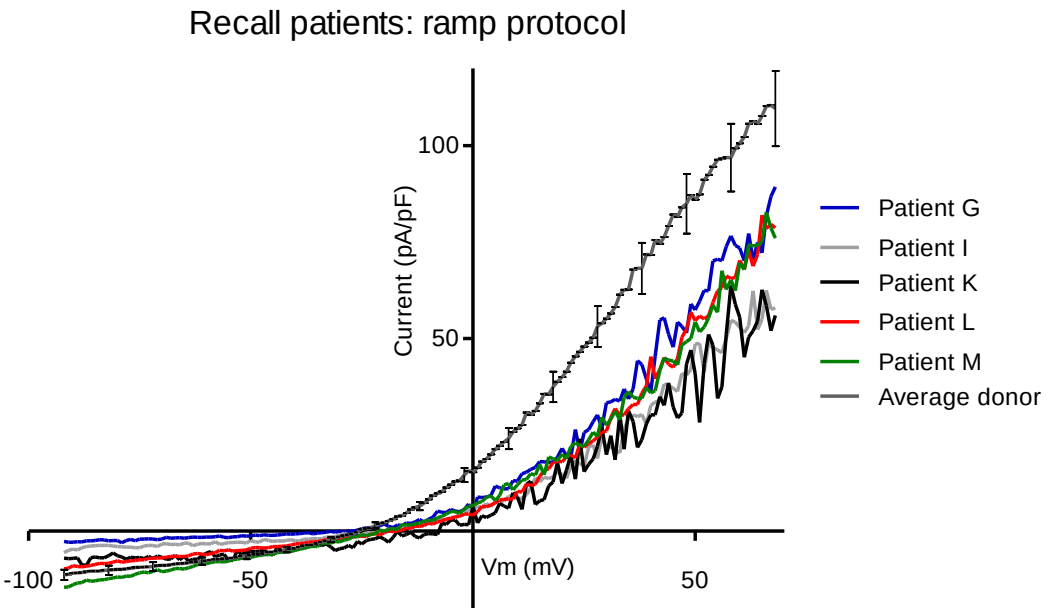


Figure 4.4 Electrophysiological recording of sperm from recall patients. Sperm cells of Patients G, I, K, L and M were able to elicit significant outward currents in response to ramp stimulus, similar to donor control cells. Patient cell n = 1, apart from Patient M (n = 3 cells). Donor cell number was 47. Error bars on the donor trace represent SEM.

4.5.5 Patient A

Patient A attended the "Failed fertilisation clinic" after a total failed fertilisation outcome in an IVF treatment cycle. Patient A and his partner were allocated to IVF treatment as semen parameters were normal, according to WHO 2010 semen analysis, and their infertility (duration of >2 years) was unexplained. Patient A had good initial semen parameters on the first day of experimentation: the concentration of sperm in seminal plasma was 121M/ml, semen volume was 4ml, 76% motile and 49% progressively motile. The prepared sample of spermatozoa had a concentration of 74.8M/ml, total motility of 97% and progressive motility of 76%.

The viscous media penetration test, calcium response to progesterone and analysis of hyperactivation both basally and in response to 4-AP and progesterone were studied. The calcium response to progesterone was studied on two separate ejaculates more than 8 weeks apart. Although the second semen sample exhibited a concentration of 11.8M/ml, lower than the 95th centile of WHO 2010 criteria (15M/ml), total motility and progressive motility were normal (43% and 38% respectively). The result observed was consistent between these two separate ejaculates.

The sperm cells of Patient A were unable to respond to progesterone in the population calcium assay or in the viscous media penetration test (Figure 4.5, B and C). Patient A was the only recall patient of 17 studied to present with this phenomenon. The repeat sample 8 weeks later, run parallel to a donor control sample, confirmed the previous findings (Figure 4.5, D). The cells of Patient A were subjected to calcium ionophore A23187 treatment following pre-incubation with Fura-2/AM, which elicited a sustained rise in intracellular calcium, indicating dye loading had taken place (data not shown).

The sperm cells of Patient A showed normal basal levels of hyperactivation on motility analysis, with a significant positive response upon addition of 2mM 4-AP (Figure 4.5 A).

Results from electrophysiological analysis of the sperm under Cs^+ based divalent free conditions and depolarizing ramp protocol revealed that CatSper current ($I_{CatSper}$) was absent when compared with normozoospermic donors (maximum outward current 4.0 ± 0.4 pA/pF versus 204.6 ± 17.7 pA/pF respectively, $P<0.01$), as shown in Figure 4.6.

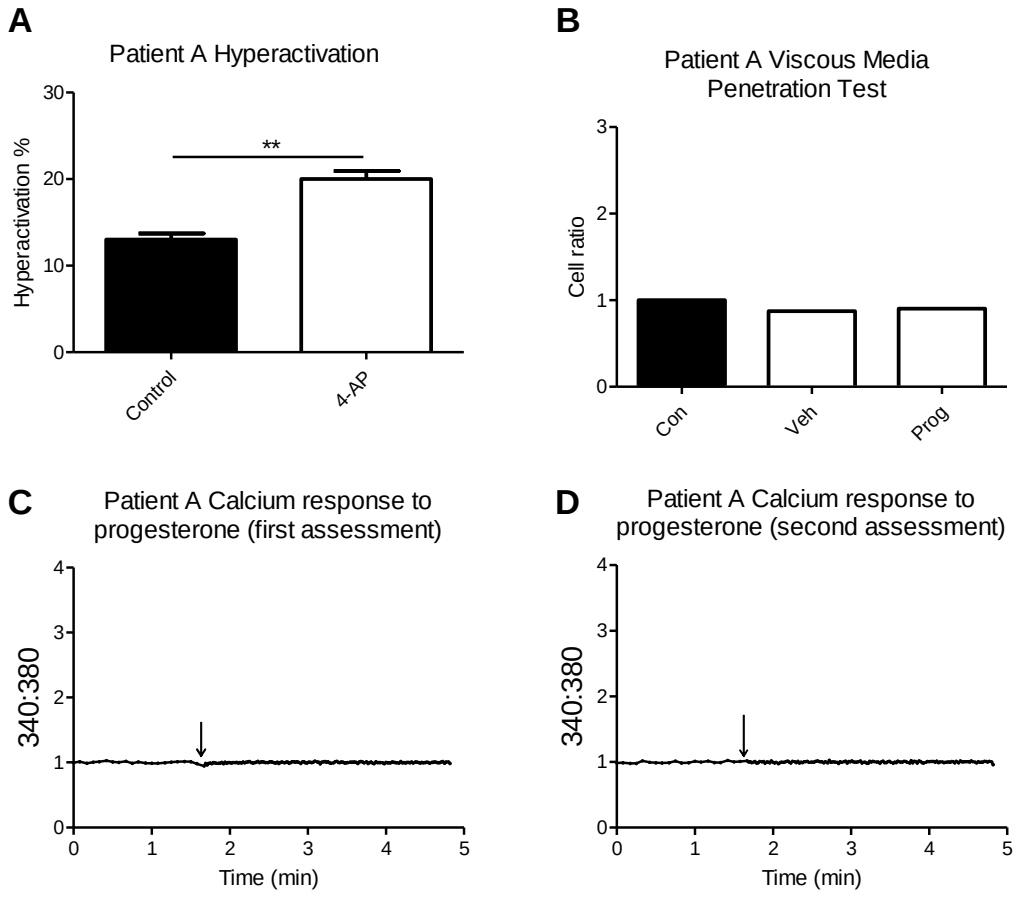


Figure 4.5 Patient A. Sperm cells from Patient A were able to A) significantly respond to 4-AP by increasing hyperactivation, however were unable to respond to progesterone in the B) viscous media penetration test or C) initial or D) second fluorimetric assay. Arrow indicates progesterone addition.

Patient A was recalled to the Sperm Studies Clinic due to experiencing total failed fertilisation of all 9 oocytes inseminated via IVF. Following these findings and the normal recommendations following a failed fertilisation in IVF, Patient A and his partner returned for a further cycle where ICSI was employed. This resulted in 5 out of 6 eggs injected displaying normal fertilisation (2PN, 2PB). However, embryo development failed to progress past day 3, therefore embryo transfer was not performed.

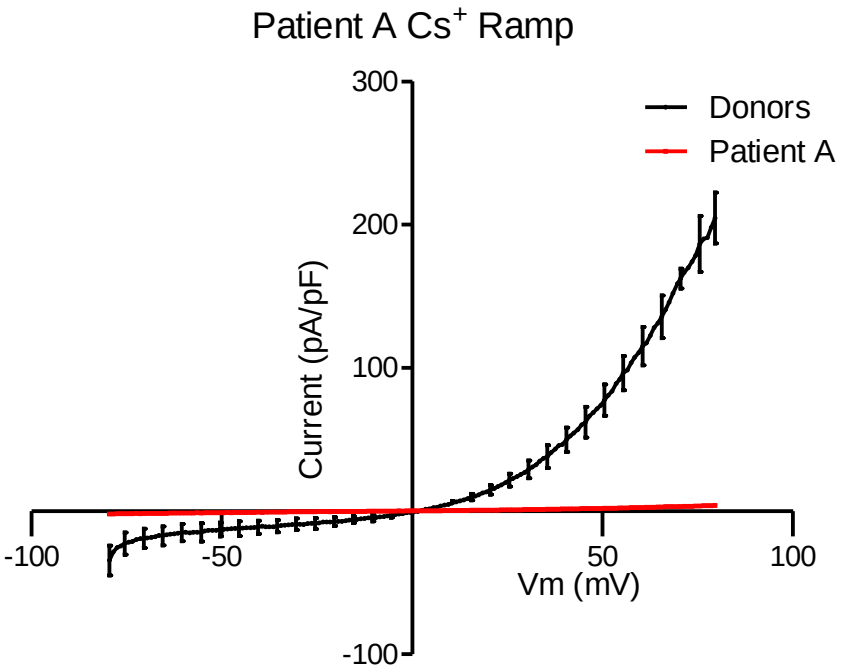


Figure 4.6 Electrophysiological analysis of sperm from Patient A. Sperm cells from Patient A showed no discernible CatSper current, in comparison with donor sperm cells under Cs^+-based divalent-free conditions (n=7). Error bars represent SEM.

4.5.6 Patient B

Patient B produced his sample on the day of IVF treatment; and surplus sample was donated to research following oocyte insemination. The concentration of the prepared sample was 17.6M/ml, volume was 0.7ml, 82% total motility, 50% progressive motility, 29% hyperactivated motility. The viscous media penetration test, calcium response to progesterone and hyperactivation to 4-AP were performed. It was observed that the sperm cells of Patient B was unable to respond to progesterone both in the fluorimetric technique (Figure 4.4E, no peak observed) and in the viscous media penetration test (Figure 4.4C, one way ANOVA with Tukey's multiple comparisons); however 4-AP was still able to induce an increase in hyperactivation over basal levels (Figure 4.4A, Student's paired t-test, P=0.0106). This was in accordance with the results previously observed for Patient A. Furthermore, again it was noted that in this IVF all 9 oocytes had failed to fertilize (no PN structure, <2PB).

However when Patient B was recalled to provide a further sample for research 7 months later, it appeared that CatSper function had been restored as the sample was able to significantly respond to progesterone when assayed in both the viscous media penetration test and calcium response to progesterone (Figure 4.4 D, F respectively). Conversely in this sample, sperm cells were unable to significantly respond to 2mM 4-AP (Figure 4.4B). This sample was used in ICSI treatment, where 2 of the 5 MII oocytes inseminated resulted in normal fertilisation (40%), however embryo transfer did not result in a pregnancy.

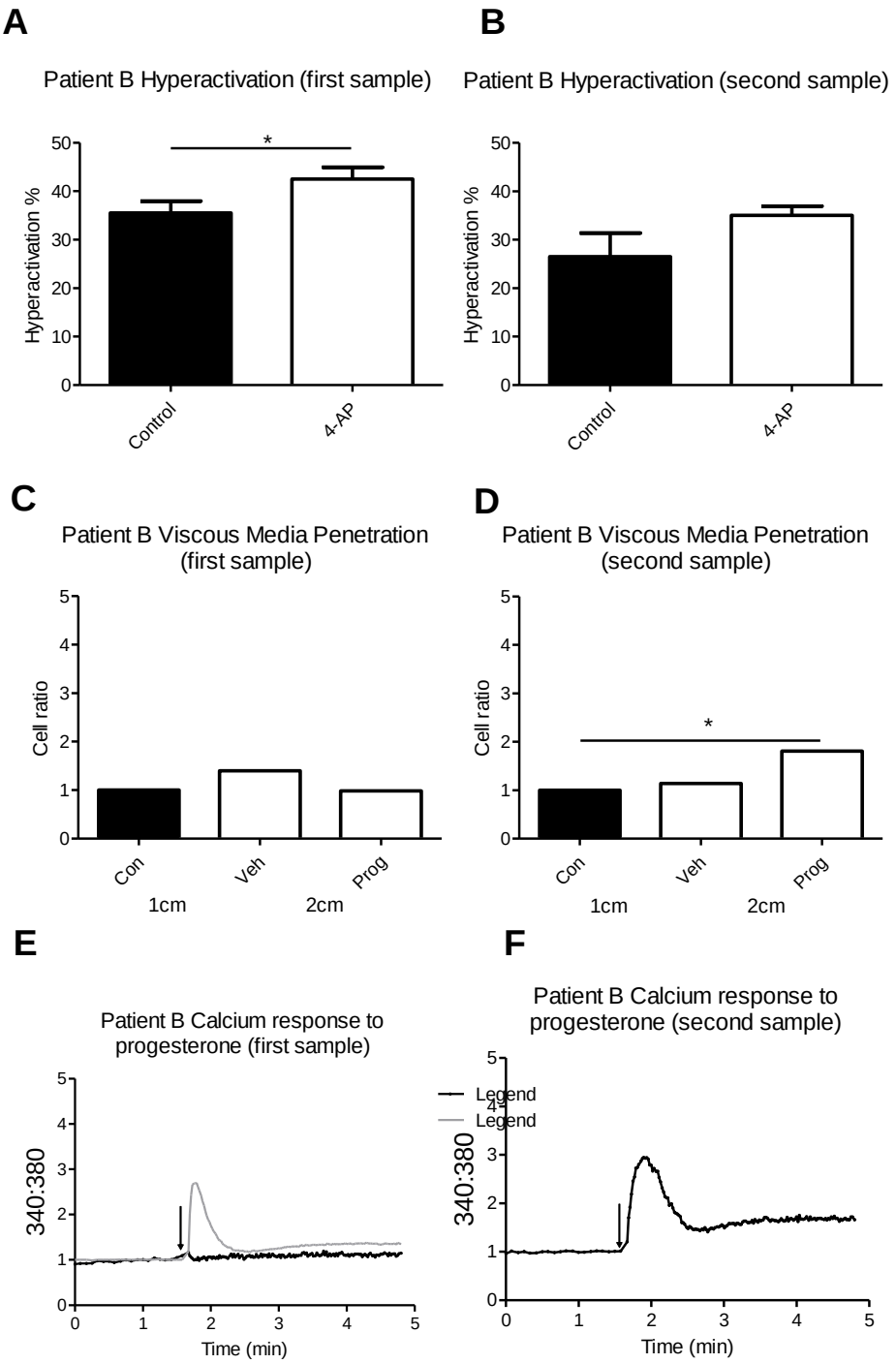


Figure 4.7 Patient B. Analysis was performed on two occasions. Cells from the initial sample were tested for hyperactivation (A), viscous media penetration (C) and calcium response to progesterone compared to the control donor sample run concurrently represented by the grey trace (E). Cells from the second sample were again tested for hyperactivation (B), viscous media penetration (D) and calcium response to progesterone (F). Arrow indicates progesterone addition.

4.5.7 Patient C

Patient C attended the ACU with his partner for a treatment cycle, and consented for his surplus sample to be donated to research. Patient C and his partner had a duration of infertility of 3 years, with the cause given as male infertility, although his vasectomy reversal was successful, as indicated by the presence of motile sperm in his ejaculate. The volume of prepared sperm received was 0.3ml, with a concentration of 22.3M/ml, total motility 75% and progressive motility 53%. Although this patient exhibited a normal count and motility of sperm, his vasovasostomy had led to the production of anti-sperm antibodies, thus resulting in agglutination and aggregation of the sperm. Therefore this patient was allocated to ICSI treatment.

The calcium response to progesterone was originally carried out on Patient C's non-capacitated sample, leading to a reduced peak response (Figure 4.7A). Hyperactivation in this patient was normal, with a significant response to 2mM 4-AP (Figure 4.7C). Furthermore, following fertilisation check, it was found that a total failed fertilisation of all inseminated oocytes had occurred. This was unexpected as his semen parameters were largely normal; additionally this patient had two children from a previous relationship.

The patient produced a further sample for research. Prepared sperm parameters were normal: concentration was 50.3M/ml, motility 93% and progressive motility 62%. The sample was capacitated, and the calcium response to progesterone was found to be normal (Figure 4.7B). However upon applying the whole-cell patch clamp technique to the sperm, absence of the potassium current was discovered (Figure 4.7D).

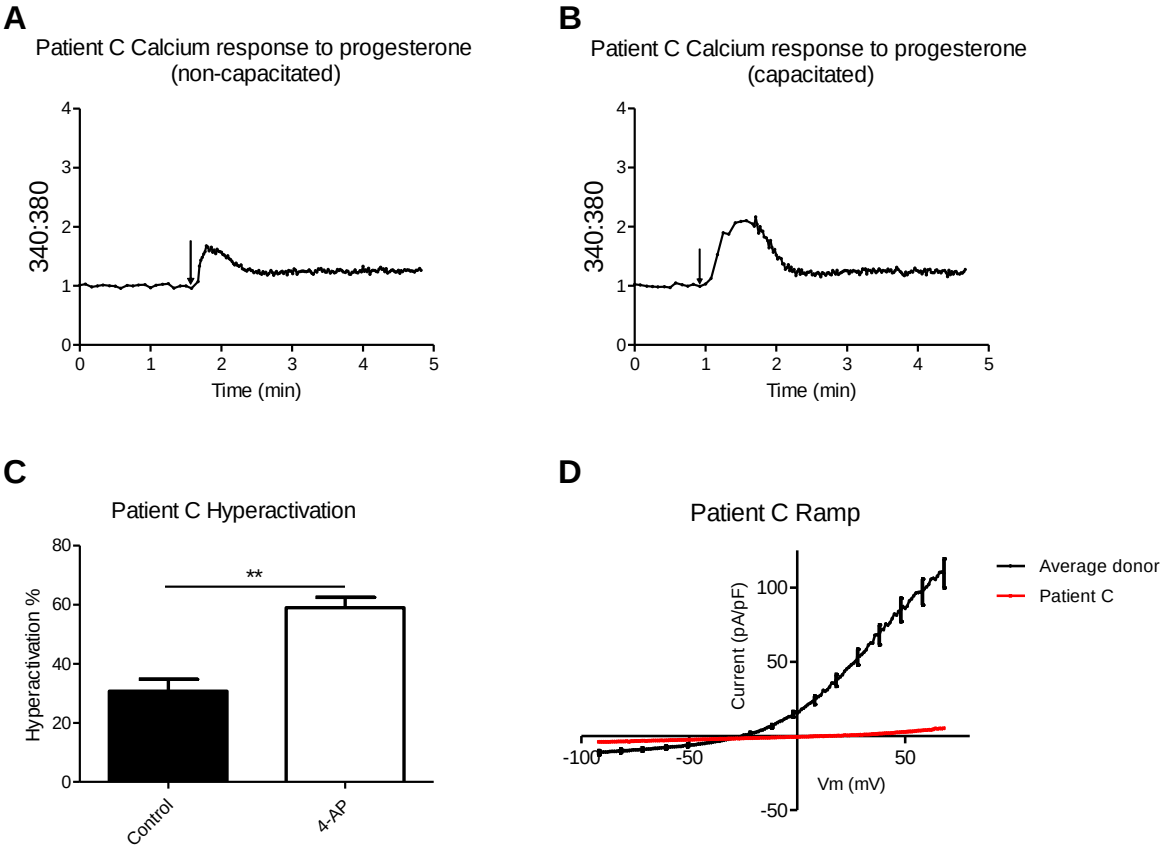


Figure 4.8 Patient C. The sperm cells of Patient C was significantly able to respond to progesterone when both A) non-capacitated, although this response was low, and B) capacitated. Cells of Patient C were furthermore able to C) significantly respond to 2mM 4-AP. However, electrophysiological analysis revealed the absence of a significant potassium current (D).

4.6 Discussion

Failed fertilisation is a significant negative outcome of IVF and ICSI cycles, often unpredictable by conventional basic semen analysis and occurs without obvious cause or explanation (Mahutte and Arici, 2003). This is stressful, difficult and frustrating for both the patient and the embryologist performing the ART procedure. Currently, semen analysis for the assessment of male fertility is limited by the inadequacy of counting sperm and assessing their motility to try and predict sperm function (Wang and Swerdloff, 2014), yet this represents the current clinical standard. Previous research techniques used in the clinical setting to assess male infertility, such as the hemizona assay, have fallen out of favor in recent years and are no longer routinely carried out (Oehninger et al., 2014). Therefore this study took a novel approach and, for the first time, aimed to use published research techniques (Alasmari et al., 2013a, Alasmari et al., 2013b) to analyse sperm from male patients who had already experienced failed/low fertilisation in IVF or ICSI for the presence of calcium pathway defects.

Patient A and Patient B both failed to elicit a peak in intracellular calcium following treatment with progesterone. Furthermore both patients also failed to significantly enhance penetration into the viscous media penetration test when stimulated with progesterone. Interestingly for Patient A, CatSper dysfunction persisted over the 8 weeks between analyses, however the same was not the case for Patient B where CatSper function was restored on second analysis 7 months later and following antioxidant treatment. The initial failure to respond to progesterone in these experiments suggests the aberrant expression or complete absence of the human sperm cation channel, CatSper, which has been shown to be activated by progesterone.

Several men with an infertility phenotype have also previously been found to have deletions or aberrant expression of the CatSper gene (Avidan et al., 2003, Nikpoor et al., 2004, Qi et al., 2007, Avenarius et al., 2009, Hildebrand et al., 2010, Smith et al., 2013). Considering that sperm from males who were previously found to have CatSper defects were also severely astheno-teratozoospermic (Smith et al., 2013), it was unclear whether poor motility/morphology or homozygous deletion of *CatSper2* was the principal cause of infertility in that case. In the present study, both Patients A and B had normal semen parameters according to WHO (2010). Furthermore, sperm from both men were able to significantly respond to the putative calcium store releasing agent 4-AP as described previously (Alasmari et al., 2013a), indicating that their inability to fertilize oocytes was not due to a calcium store defect.

The hypothesis that CatSper dysfunction was to blame for the failed fertilisation in Patient A's IVF treatment cycle was further confirmed by the successful fertilisation of 5/6 oocytes after ICSI treatment: where there is no functional requirement for the human sperm to penetrate the oocyte and therefore no requirement for functional CatSper. One interesting finding in this study was that both patients unable to respond to progesterone were still able to robustly hyperactivate, contrary to previous findings in mice (Ren et al., 2001, Quill et al., 2003). However, mice do not exhibit the same CatSper progesterone responsiveness as humans, therefore species differences will reveal separate phenotypes of the sperm deficient in the functional CatSper channel.

K^+ channels function to maintain the membrane potential (V_m) in human spermatozoa at negative values (hyperpolarized). Slo3 has been found to be the primary K^+ channel in mice, together with the auxiliary subunit LRRC52 (Navarro et al., 2007, Zeng et al., 2015). Spermatozoa from animals lacking either of these channels show significantly depolarized membrane potential, which was shown to have a significant

negative effect on motility and ability to hyperactivate, and furthermore resulted in a reduction in fertility (Santi et al., 2010). However previously, little was known of the role of K^+ channels on human sperm function. The activity of K^+ channels was investigated in the current study by whole-cell patch clamp technique. In a novel finding, spermatozoa examined from one ICSI patient were found to have a severely defective K^+ current, suggesting absence of the KSper potassium channel. This finding elaborates on the knowledge of functional importance of KSper on human sperm function, however could also be indicative of more severe sperm dysfunction as the failed fertilisation in this case was encountered in an ICSI cycle, where the functional requirements of sperm were abolished.

There were several patients who did not show an abnormal response in any of the experiments performed. As male factor infertility is thought to account for up to 50% of problems in conceiving in infertile couples (Health, 2009), it is plausible that the failed fertilisation observed was of a female-derived or oocyte origin. Otherwise, the failed fertilisation could have been the result of a sperm factor as yet unidentified by the limitations of our experimental techniques.

The main limitation of this study was the select number of research techniques able to be employed, coupled with the limited concentration of sperm obtained from the recalled patient samples. This often gave an incomplete view of where the cause of failed fertilisation lay, which required the patient to, if willing, submit a further sample for research. Furthermore, if a patient had failed fertilisation after IVF, ICSI would be recommended in the next cycle, which limited the predictive value of our research. The study would have also benefited from receiving several samples from each patient, to exclude the possibility of inter-ejaculate variability.

4.7 Conclusion

In conclusion, the present study describes the novel finding of two normozoospermic males exhibiting defective CatSper function, thus leading to failed fertilisation in their IVF treatment cycles. Furthermore, this study presents the first description of a male ICSI patient with severe K^+ channel dysfunction.

There are many varied reasons behind total failed fertilisation that are not normally recognized in a typical treatment cycle. The belief is that around half of these outcomes originate from the male (sperm), and half from the female partner (oocyte). By examining the sperm of males who have previously endured total failed fertilisation in a treatment cycle, we have identified several causes of male origin including CatSper defects and potassium channel deficiency. There were however, many others with no obvious defects identifiable with the research techniques employed. Further studies must work on identifying these causes, to develop novel screening techniques to detect sperm dysfunction or male infertility prior to treatment.

Chapter 5

Development of imaging groups of individual cells in response to progesterone in donors, IVF and ICSI patients

5.1 Introduction

Previous studies examining the intracellular calcium response to progesterone in IVF and ICSI patients investigated a largely heterogeneous population of spermatozoa (Alasmari et al., 2013a, Alasmari et al., 2013b). While population-based fluorescent imaging techniques are valuable for observing ionic kinetics occurring over time, they provide an average cellular fluorescence intensity which is ultimately unable to resolve whether each individual cell is responsive, or to what extent. Furthermore, population assays tend to require large numbers of sperm in order to produce repeatable and reliable results (as described in Chapter 3), which can lead to samples of lower sperm concentrations (<4M/ml for IVF, and the vast majority of ICSI samples) being unsuitable for analysis and thus remain unstudied. Therefore there is a clear need to utilise a more sensitive technique with improved resolution, in order to further distinguish calcium responses to progesterone between spermatozoa of patients and donors, particularly those patients with poor sperm concentrations.

Calcium imaging of groups of individual cells in response to progesterone in human spermatozoa has previously been extensively described for donor samples (Kirkman-Brown et al., 2000, Harper et al., 2003, Kirkman-Brown et al., 2003, Kirkman-Brown et al., 2004, Bedu-Addo et al., 2007, Nash et al., 2010, Alasmari et al., 2013b, Mata-Martinez et al., 2013). The technique has been developed over the past 20 years to monitor changes in free intracellular calcium ion concentrations (Tesarik et al., 1996), and has observed phenomena such as calcium oscillations in individual sperm cells in response to stimuli such as progesterone: this would not have been possible in whole-population assays. However, the single-cell response to progesterone in spermatozoa from patients attending for assisted conception has not been previously described.

5.2 Aims

This study aims to describe the development of methods for use of single-cell calcium imaging. Furthermore, the study aims to investigate variation in calcium response to progesterone in individual cells from donor sperm samples, IVF sperm samples and ICSI sperm samples.

5.3 Hypothesis

The hypothesis states that the calcium response to progesterone within individual spermatozoa will reveal significant variation in proportion of responsive cells, and amplitude of response, between donors, IVF and ICSI patients.

5.4 Development of the materials and method

5.4.1 Materials

The single wavelength dye Fluo-4/AM (Molecular Probes, Thermo Fischer Scientific, MA, USA) 494nm excitation, 506nm emission, was used as the fluorescent calcium indicator. Uneven dye loading between cells was corrected by normalising the trace to 1 using the equation given in Chapter 2. Fluo-4 with AM ester was chosen over the salt form, as the hydrophobic dye was passively membrane-permeable, and was able to concentrate within the intracellular cytoplasm (Paredes et al., 2008). Fluo-4/AM can be used at a final concentration of 1-10μM (Invitrogen, 2010), and the concentration of fluorescent calcium indicator used was 1μM (Shahar et al., 2011).

Poly-D-lysine: varying concentrations of poly-D-lysine were investigated (1% w/v, 0.1% w/v, 0.01 w/v and 0.001% w/v). The aim was to obtain sperm heads loosely adhered to the glass coverslip, with tails minimally motile. It was found that the concentration that had the best compromise between maximum sperm "sticking" and minimal free-swimming sperm was 0.1% poly-D-lysine, so was used for all future experiments.

Progesterone was administered after 100 intervals (44 seconds) of basal calcium fluorescence recording. Progesterone was solubilised in 100% ethanol at 36mM concentration before it was diluted in either Quinns HTF media lacking protein, or STF as used in FLUOstar Omega, to a final concentration of 36µM. When added in a 1:10 ratio, this gave a final concentration of 3.6µM progesterone. As STF gave a large "mixing artefact" due to the difference in media being added (STF did not contain Phenol red), Quinns HTF was used for the duration of the study. The final concentration of progesterone was maintained at 3.6µM, equivalent to the concentration used in the FLUOstar Omega.

Ca^{2+} ionophore A23187: Addition of ionophore A23187 was incorporated in the experimental design in order to calibrate the fluorescence to calculate calcium concentration (Bootman et al., 2013). Addition was performed 150 intervals (66 seconds) after progesterone addition. However, response to ionophore varied drastically within a sample, and between samples from different individuals. Therefore it was difficult to establish a protocol for accepting or rejecting individual cellular responses to ionophore. Use of ionophore for max-min calculations, was not performed however is still included on the graphs for information purposes.

5.4.2 Method

Open system: use of an open imaging system was used, to avoid tubing contamination by agonist in perfusion systems, and to replicate the conditions of the FLUOstar fluorescence microplate reader described in Chapter 3. Thus, sperm were centrifuged after dye incubation, before being resuspended in 45µl protein-free HTF media. 45µl of the sperm solution was added to the glass coverslip for imaging. Addition of 5µl of 36µM progesterone was performed to a final concentration of 3.6µM progesterone, mimicking the volumes of solution used in the FLUOstar system.

Semen preparation: previous studies have utilised swim-up harvesting procedure (Kirkman-Brown et al., 2000, Kirkman-Brown et al., 2004), whereas other used density gradient (Tesarik et al., 1996). In the present study, due to all clinical samples being prepared for IVF and ICSI by density gradient centrifugation, this was also used in the research lab to prepare donor samples to ensure continuity and comparability.

Temperature: due to the use of an open coverslip system sensitive to evaporation and previous studies using 25°C as the set temperature, 25°C was used as the set temperature in the present study (Plant et al., 1995, Kirkman-Brown et al., 2000, Kirkman-Brown et al., 2004).

Regions of interest (ROI) were produced automatically by the computer software MetaFluor. These shapes covered the entire sperm head. It was important for the ROIs to always be checked manually for fidelity as some ROIs were placed erroneously by the software, and were removed and re-drawn manually if they did not meet the expected standard.

Experimental timings: timing of drug/stimuli addition was a compromise between avoiding media evaporation and resultant increase in osmolality, and recording all kinetic events after progesterone addition (transient and plateau phase calcium response) and ionophore/EGTA addition within a suitable timeframe. Total recording time was 3 mins 40 seconds, with 500 recordings being taken 440ms apart. Basal recordings of fluorescence intensity were performed for 100 intervals (44 seconds). Progesterone addition was performed, and recording proceeded for 150 intervals (66 seconds), which was deemed an acceptable length of time according to Chapter 3 where peak transient calcium response to progesterone occurred within one minute of addition. Ionophore addition was performed, and recording proceeded for 100 intervals (44 seconds). EGTA was added and recording proceeded for a further 150 intervals (66 seconds).

Dye loss: occasionally, sperm were seen to show a decrease in fluorescence intensity over time. This was previously observed, and reported as dead or dying cells being unable to maintain dye loading and thus dye leakage occurred (Tesarik et al., 1996, Kirkman-Brown et al., 2000). Any cells visualised to be doing this, either during data acquisition or after data processing were excluded from analysis.

Negative control: 0.01% final concentration of ethanol in HTF was used as negative control in samples from three donors. Ethanol was unable to stimulate any significant calcium responses after addition. Examples of the single cell traces obtained from one of these donors is shown below (Figure 5.1).

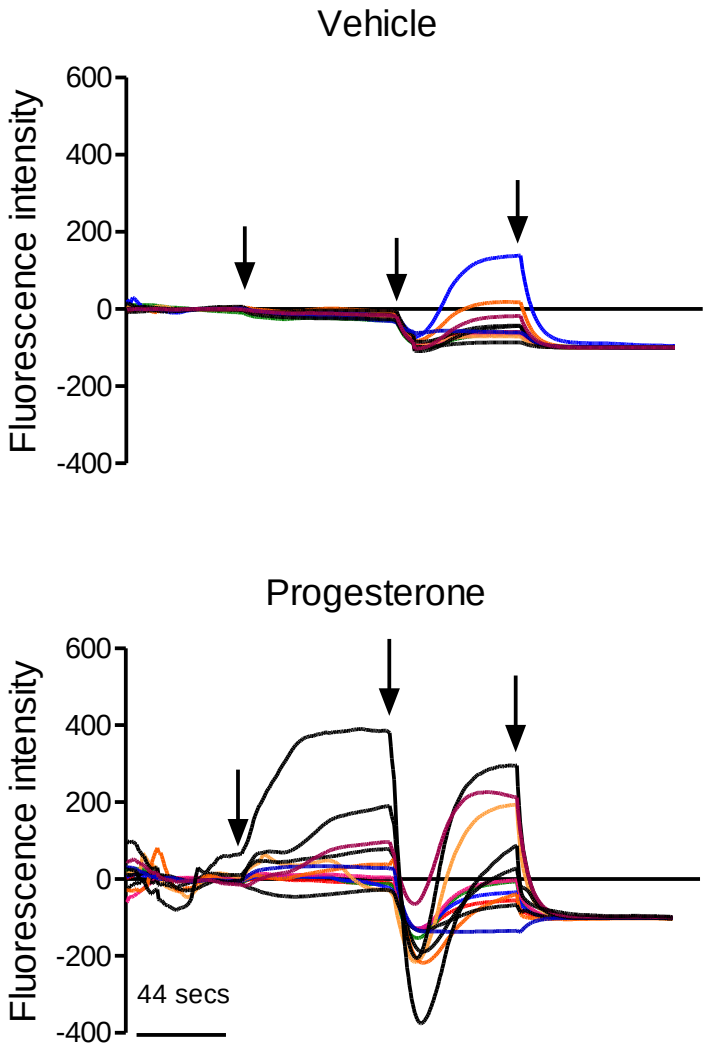


Figure 5.1 Single cell calcium response to vehicle. Treatment with vehicle (indicated by first arrow on top panel) did not cause a significant change in intracellular calcium. The arrows indicated vehicle/progesterone, ionophore A23187, and EGTA addition respectively.

5.5 Results

5.5.1 Capacitation of the samples

While IVF samples from the Assisted Conception Unit were received in capacitating medium for over 4 hours, ICSI samples were maintained in non-capacitating gamete buffer. Due to the experimental constraint of low sperm numbers in ICSI samples, care was taken to obtain as many sperm as possible for evaluation. Re-suspending the ICSI samples in capacitating medium risked losing sperm in the wash steps. Therefore capacitated and non-capacitated donor samples were compared for analysis of whether capacitation status affected the proportion of cells responding to progesterone and their average area under the curve. Capacitation status did not affect average area under the curve, and the proportion of sperm responding to progesterone was significantly higher when not capacitated in donor samples, therefore ICSI samples were maintained in non-capacitated medium with no detrimental experimental effect. As described in Figure 5.2, there was no significant difference in the average area under the curve between capacitated and non-capacitated donor samples (Mann Whitney test, P=0.14), however the proportion of cells able to positively respond to progesterone was significantly higher in non-capacitated samples (Student's unpaired t-test, P=0.021).

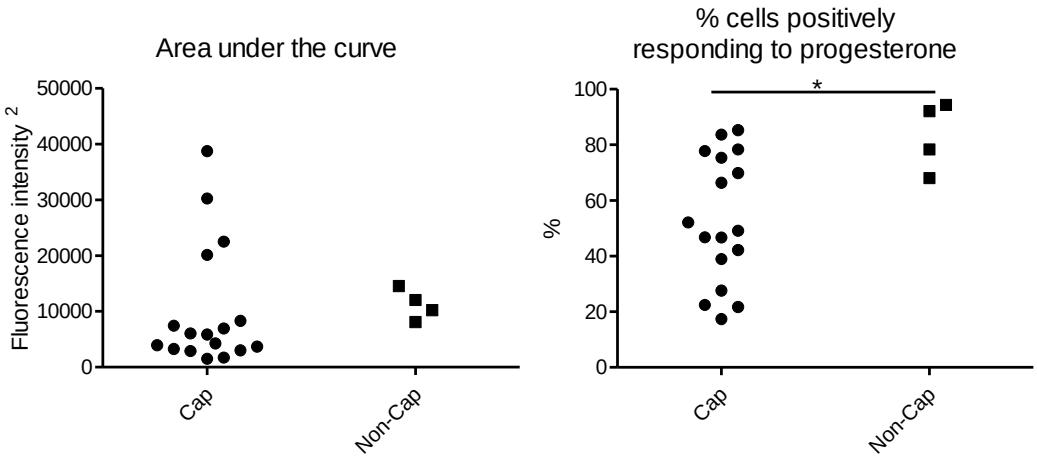


Figure 5.2. Capacitated versus non-capacitated single cell calcium response to progesterone. Comparison of mean area under the curve after 3.6μM progesterone addition and percentage of donor sperm cells responding to progesterone in capacitated (cap) and non-capacitated (non-cap) samples. There was no significant difference in area under the curve between the two treatment groups (*P*=0.14), however a significantly higher proportion of cells were able to respond to progesterone when maintained in non-capacitating conditions (*P*=0.021).

5.5.2 Example traces from one donor, one IVF patient and one ICSI patient

21 IVF patient sperm samples and 10 ICSI patient sperm samples were evaluated for their calcium response to progesterone. This was compared with 17 samples from 9 donors. Representative example traces from one IVF patient, one ICSI patient and one donor sample are shown below (Figure 5.3).

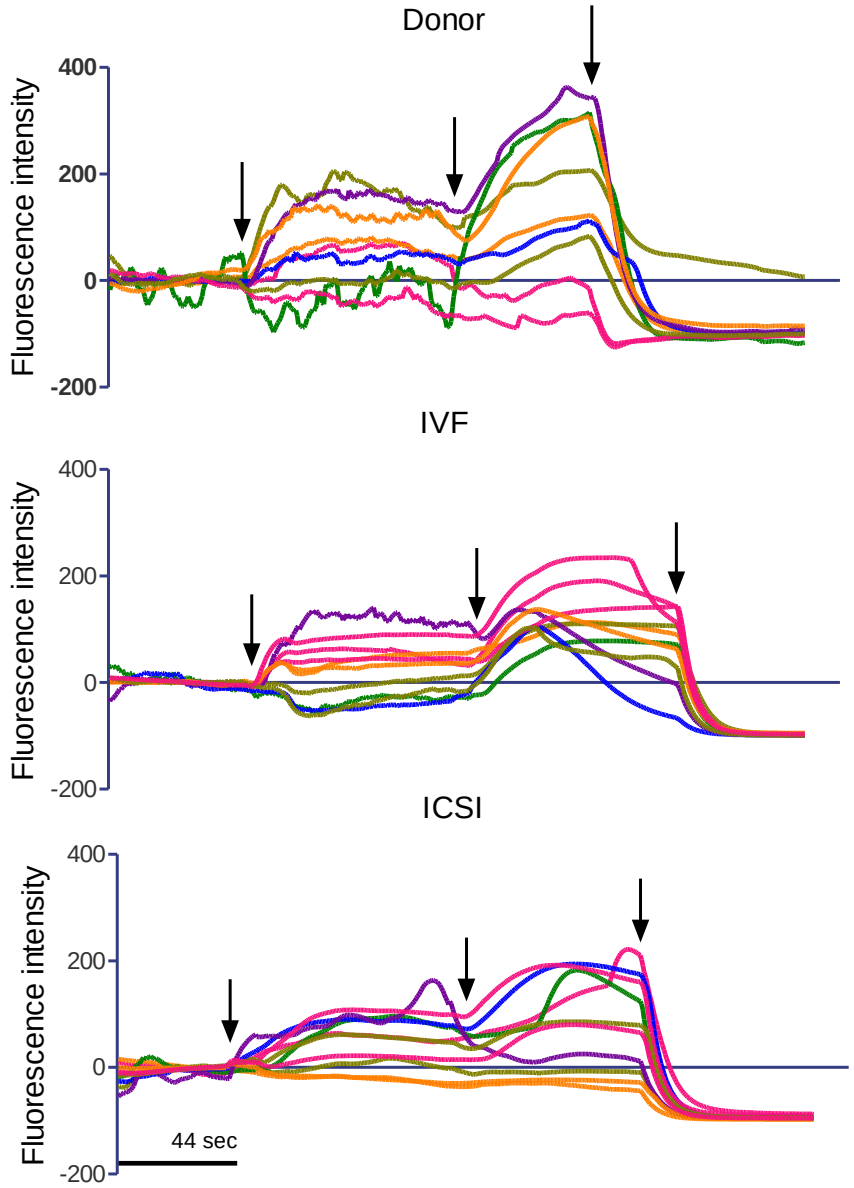


Figure 5.3 Example traces of individual cells from a donor, an IVF patient and an ICSI patient. Addition of progesterone, ionophore A23187 and EGTA are indicated by each of the arrows respectively. The total duration of each recording was 3 minutes 40 seconds.

5.5.3 Proportion of sperm responding to progesterone

Comparisons were made between the proportion of sperm responding to progesterone for IVF and ICSI patients and normozoospermic donors. As described in Figure 3.2, single cell calcium imaging revealed there was no significant difference in the proportion of responsive sperm: an average of 53% for donors, 52% for IVF patients and 46% of ICSI patient sperm responding after addition of progesterone (Figure 5.4, one-way ANOVA, *P*=0.698).

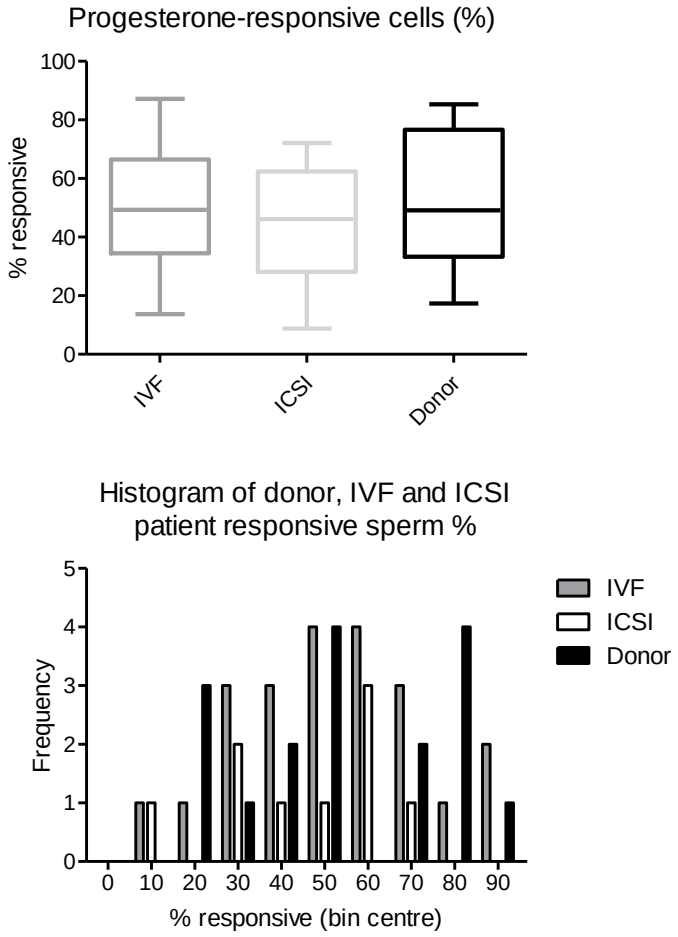


Figure 5.4 Proportion of progesterone-responsive cells. There was no significant difference in the proportion or distribution of sperm cells positively responding to progesterone between donors, IVF and ICSI patients (*P*=0.698).

The 5th centile for the proportion of sperm from donor samples positively responding to progesterone was 20.9%. One IVF patient (1 of 21, 4.8%), one ICSI patient (1 of 10, 10%) and one donor (1 of 17, 5.9%) had samples with proportions lower than 20.9% responding to progesterone.

5.5.4 Area under the curve analysis

To quantitatively analyse the extent to which individual cells were able to produce a calcium response to progesterone, area under the curve analysis was performed. Cells where the area under the curve yielded a negative value were excluded from further analysis. Analysis showed that there was no significant difference in the average area under the curve between IVF patients, ICSI patients and donor samples (Figure 5.5, Kruskal Wallis test, P=0.7099).

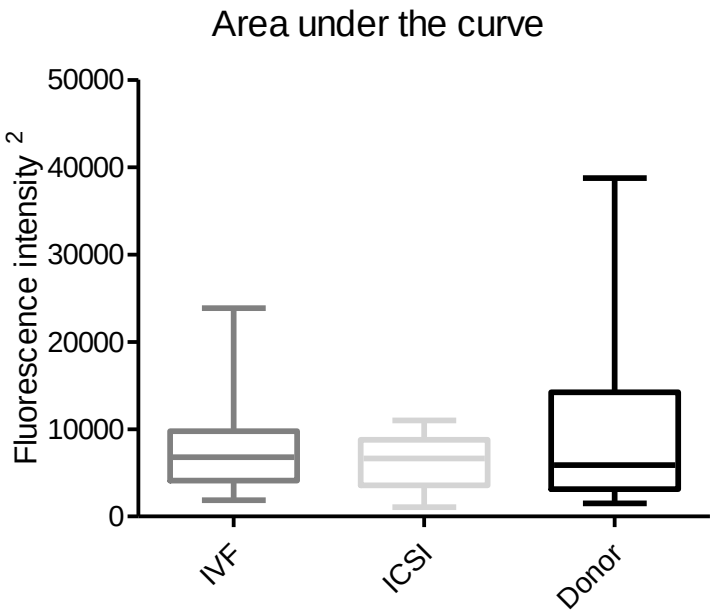


Figure 5.5 Area under the curve analysis. There was no significant difference in area under the curve between IVF patients, ICSI patients and donors (*P*=0.7099).

The 5th centile for fluorescence intensity was 1698.6 relative fluorescence units. One ICSI sample (1 of 10, 10%) and one donor sample (1 of 17, 5.9%) exhibited average fluorescent intensity of less than 1698.6 relative fluorescence units. No samples from IVF patients were below the 5th centile.

5.5.4.1 Area under the curve frequency distributions in individual patients

The frequency distributions from the area under the curve for single cells responding positively to progesterone in individual IVF and ICSI patients are plotted in Figure 5.6. The frequency distributions display that while the majority of single cells have a fluorescence intensity^2 of between 0-6000RFU^2 in both IVF and ICSI patients, a small proportion of cells display a significantly greater response (cells present in IVF samples 1 and 2, ICSI samples 1 and 3, up to 28,000RFU^2).

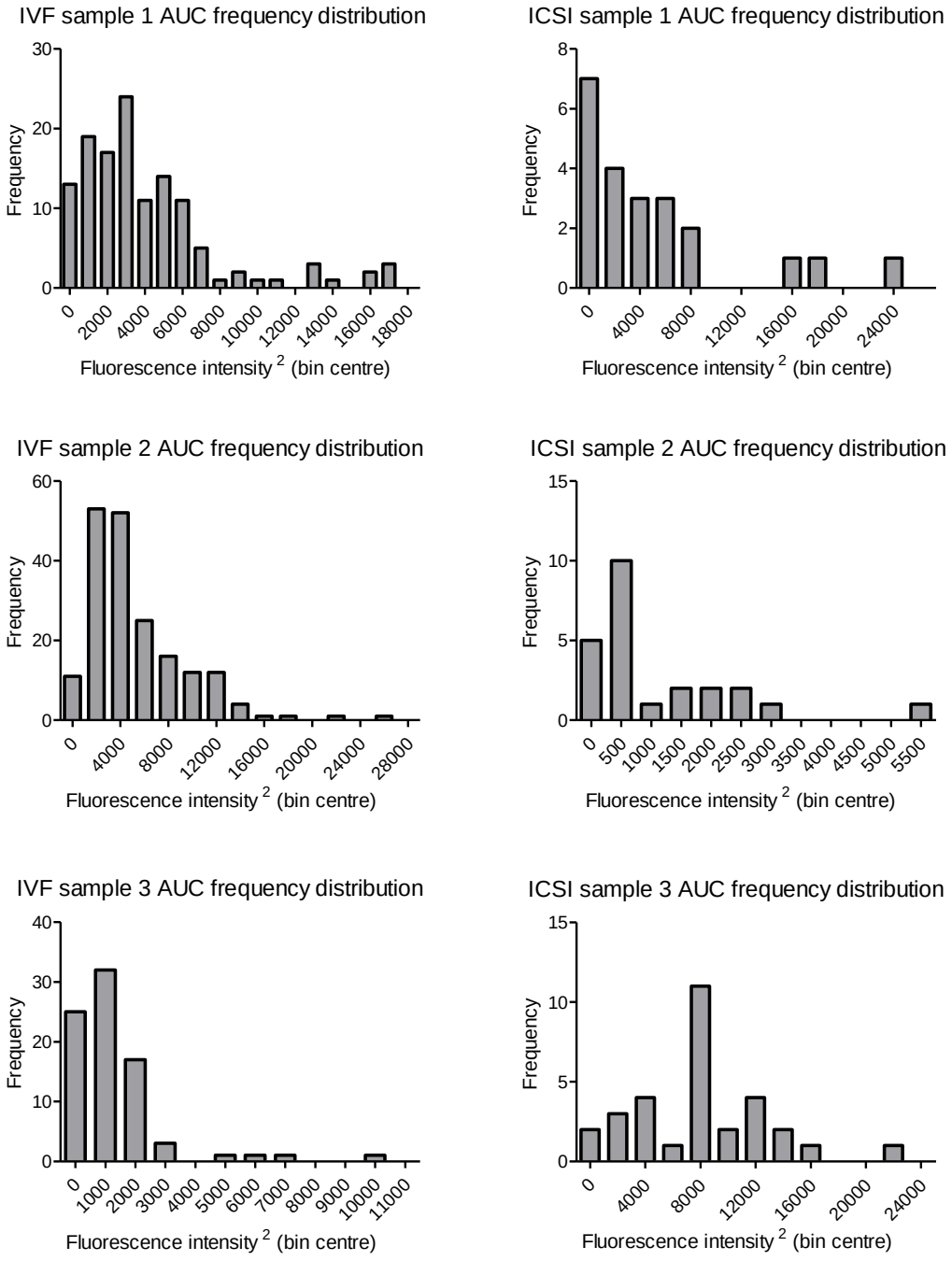


Figure 5.6. Frequency distributions for the area under the curve of individual sperm cells. The distribution of fluorescence intensity^2 values for different patients is variable.

5.5.5 Absence of the second phase

Due to the relatively short period of time monitoring calcium response to progesterone (66 seconds) 3 of the patient samples were selected for extended analysis of the calcium response to progesterone. In this experiment, calcium ionophore A23187 and EGTA were not added.

Over the 176 seconds of recording with progesterone present, there was no significant secondary phase response in any of the samples studied (Figure 5.7), contrary to findings reported in previous studies (Kirkman-Brown et al., 2000). Furthermore no significant evidence of calcium oscillations was observed contrary to previous reports (Harper et al., 2004, Kirkman-Brown et al., 2004). However the duration of recording in the present study was shorter than previously reported (3.5 minutes as opposed to >10 minutes).

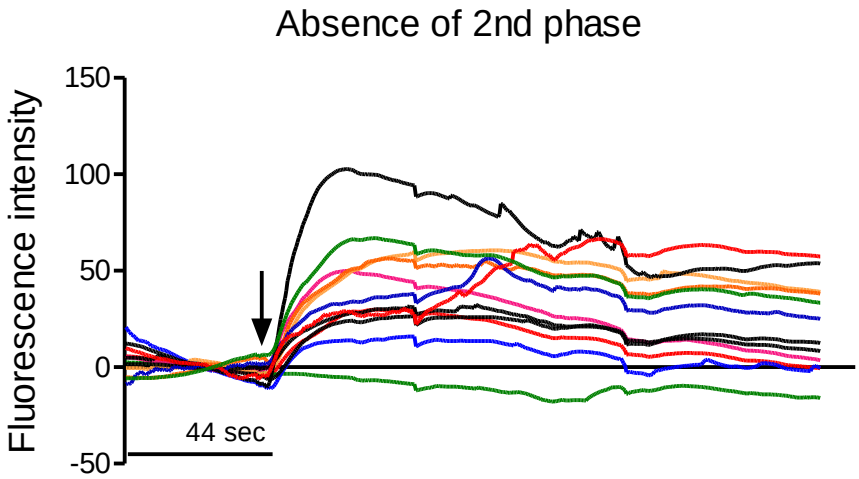


Figure 5.7 Second phase of calcium response to progesterone. There was no obvious second phase to the calcium response to progesterone over the duration of recording. Arrow indicates time of progesterone addition.

5.5.6 Correlation with IVF and ICSI fertilisation rate

There was no significant correlation between the average fluorescent intensity of responsive sperm nor the proportion of sperm that responded to progesterone and the fertilisation rate of solely MII oocytes or all collected oocytes after IVF treatment. This is in agreement with the results presented in the previous study (Chapter 3), where there was no significant correlation between any of the parameters observed for the population calcium response to progesterone assay (Figure 5.8).

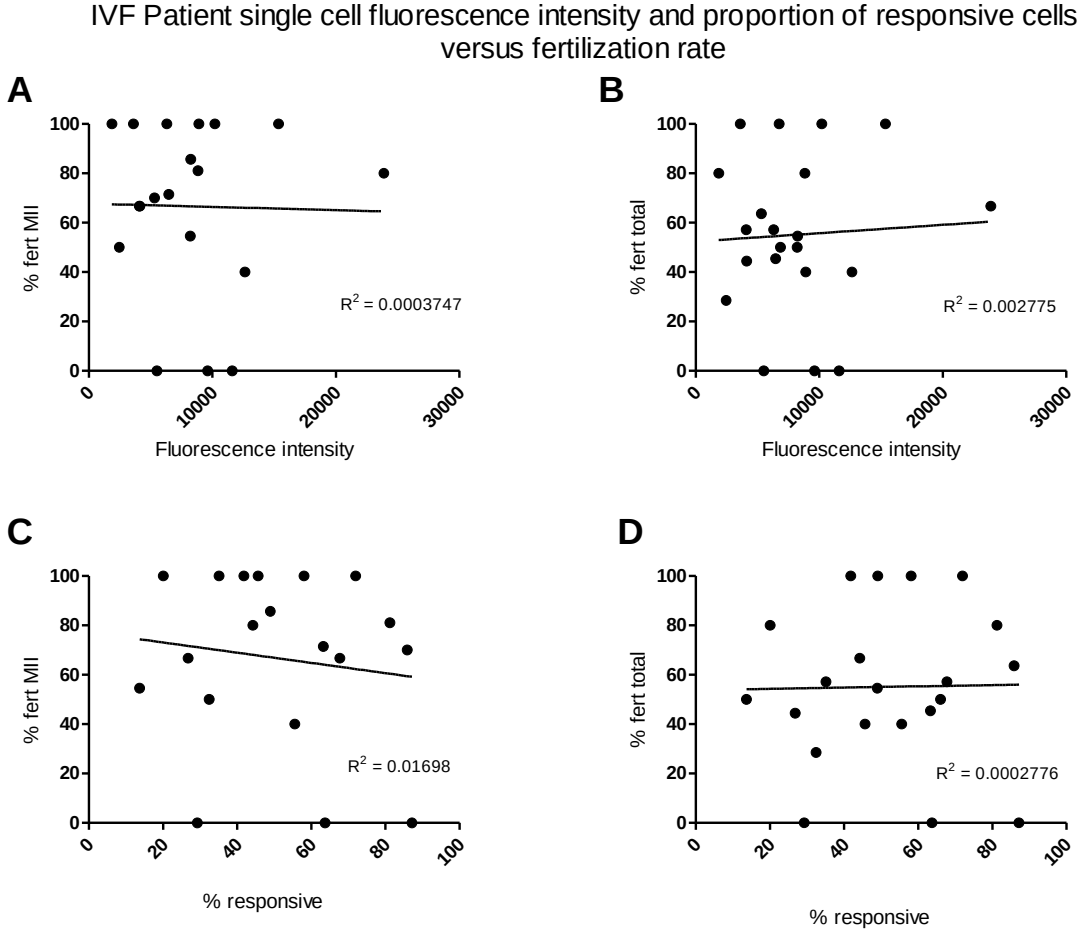


Figure 5.8 Correlation between IVF fertilisation rate and single cell calcium imaging results. No significant correlation was observed between IVF patient single cell fluorescence intensity nor proportion of responsive cells to IVF fertilisation rate.

Interestingly, although there was no significant correlation between ICSI patient average fluorescent intensity and fertilisation rates or proportion of responsive sperm versus fertilisation of MII oocytes only, there was a significant positive correlation between the proportion of responsive sperm and total fertilisation rate (Figure 5.9, Pearson product-moment correlation coefficient r=0.7802, *P*=0.0131).

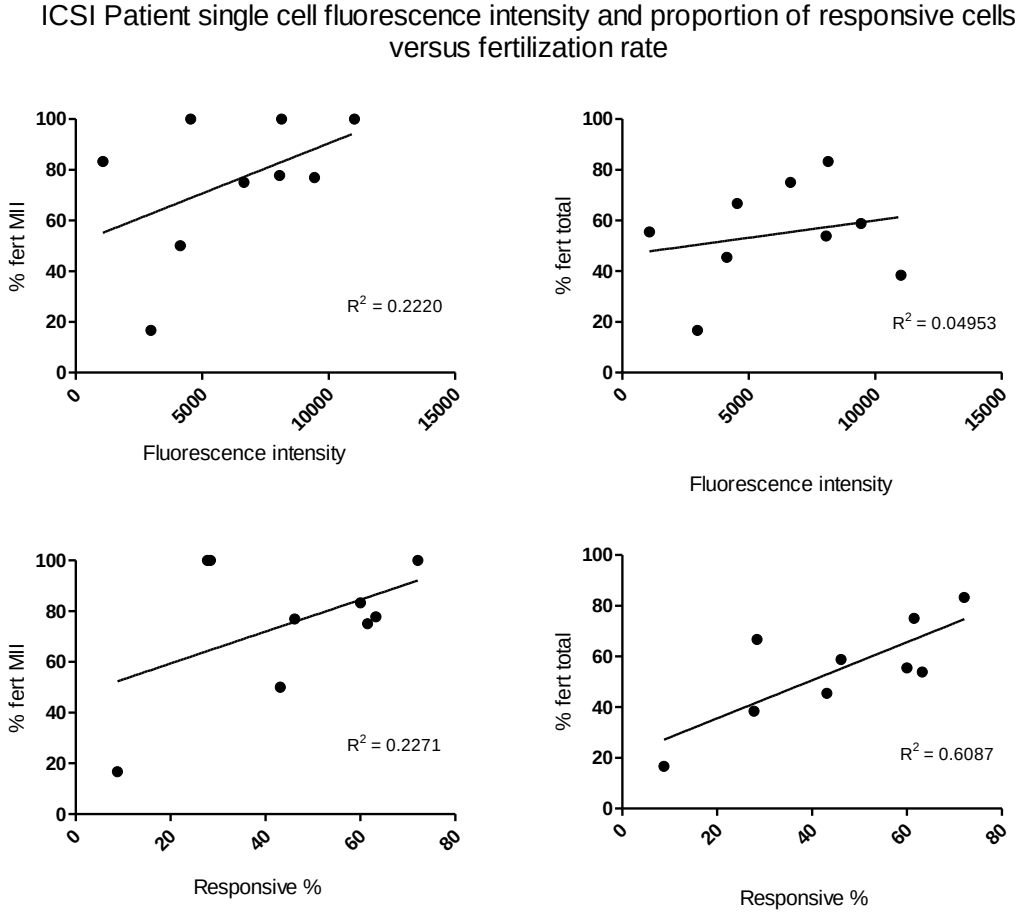


Figure 5.9 Correlation between ICSI fertilisation rate and single cell calcium imaging results. There was no significant correlation between average fluorescent intensity and fertilisation rate of ICSI samples, however there was significant positive correlation between the proportion of responsive cells and the total fertilisation rate (Pearson's r = 0.7802, *P*= 0.00131, R^2 = 0.6087).

5.6 Discussion

The aims of the present study were to describe the development of methods for the single cell imaging system, and to use this system to investigate, for the first time, changes in single sperm calcium responses to progesterone from IVF and ICSI patients. In the present study, methods were described for the set-up of this single cell calcium imaging experiment, including experimental timings, conditions, reagents used, protocol devised and rationale behind these decisions.

Previous population-based studies on sperm calcium response to progesterone did not show a significant difference between donors and IVF patients, however ICSI samples produced a significantly lower response to progesterone (Alasmari et al., 2013a). This was not the case in the present study, as ICSI samples produced comparable proportions of cells forming a positive calcium response to progesterone, with comparable average area under the curve. ICSI samples routinely contain lower concentrations of sperm, sperm with poor motility, poor morphology or a combination of two or more of these indications. However from the data discussed in the present study, this does not seem to have a detrimental effect on the ability of these cells to be able to respond to progesterone. A caveat to this conclusion is that only the ICSI samples received post-ICSI procedure with sufficiently concentrated and motile spermatozoa were suitable for study: ruling out those samples with severe oligozoospermia where <10 sperm were seen in a 4µl aliquot, or severe asthenozoospermia where motility was significantly impaired, or a combination of the two. Thus the ICSI samples studied were of a specific sub-group, where male infertility pathogenesis was less pronounced. It would be of interest to develop a more

sensitive method to study the most severely oligo/asthenozoospermic samples for their ability to respond to progesterone.

The present study indicates that around 50% of both IVF/ICSI patient and donor sperm cells have the capacity to produce a significant calcium response to progesterone, although in previous studies the proportion of responsive cells was reported to be around 70% (Kirkman-Brown et al., 2000). This may be due to the difference in imaging techniques between the present study and that of Kirkman-Brown et al: open coverslip method and perfusion chamber method respectively. Furthermore, the method used to separate sperm from seminal plasma was via density gradient centrifugation, not swim-up as used by Kirkman-Brown et al; therefore it is possible that the populations of cells studied by the present and previous studies were different.

The present study found no correlation between IVF fertilisation rates and proportion of cells responsive to progesterone, or extent of response. This is not altogether surprising; as discussed in Chapter 3 (screening of patients), there was no correlation between any of the parameters studied and IVF fertilisation rate. However as discussed previously, a total lack of response to progesterone was indicative of total fertilisation failure. None of the samples studied in the present study exhibited a total lack of response, therefore the hypothesis that this would lead to fertilisation failure could not be tested.

Interestingly, it was found that although there was no significant correlation between ICSI patient average fluorescent intensity and fertilisation rates or proportion of responsive sperm versus fertilisation of MII oocytes only, there was a significant correlation between the proportion of responsive sperm and total fertilisation rate. This suggests that although ICSI bypasses the requirement of normal sperm function to

fertilise the oocyte, there may be some fundamental biological components within the sperm cell unable to be identified by the ICSI practitioner, yet linked with the ability of the sperm to produce a calcium response to progesterone, that are necessary for successful fertilisation. Unfortunately this effect was not observed when looking at the fertilisation rate of only MII oocytes, although this may be owing to the small sample size of ICSI patients with 4 or more oocytes to analyse for fertilisation rate (n = 9). Further investigation of ICSI patient sperm samples would strengthen the observations reported in this study.

5.7 Conclusion

The present study described a suitable method for screening sperm samples, particularly those with lower sperm numbers such as those seen in ICSI patient samples. The data presented here show that there is no significant difference between IVF, ICSI or donor samples in terms of proportion of sperm that positively respond to progesterone, or to what extent they respond (area under the curve). Furthermore, the data suggests that there is no correlation between proportion of responsive cells, nor extent of response in IVF patients, however total ICSI fertilisation rates are significantly correlated with the proportion of sperm significantly responsive to progesterone.

Chapter 6

Donor semen cryopreservation and its effect on sperm calcium response to progesterone

6.1 Introduction

Cryopreservation and thaw of human semen are fundamental and widely used techniques within the ART repertoire: to preserve reproductive capacity by enabling indefinite sperm storage in LN_2 for a wide range of medical conditions and thaw for use in IUI, IVF and ICSI cycles (Anger et al., 2003). Cryopreservation of semen is necessary for quarantine of reproductive donor semen samples, and storage of semen pre-chemotherapy. However, the quality of sperm recovered post-thaw is unpredictable (Lee et al., 2012), and varies significantly between samples (Nallella et al., 2004). Relatively few studies have recently investigated how sperm function deteriorates after cryopreservation and thaw.

In 1994, it was discovered that cryopreservation significantly affected calcium response to progesterone post-thaw in donor semen samples (McLaughlin and Ford, 1994). Furthermore, cryopreservation has been shown to significantly negatively impact human donor sperm ability to penetrate bovine cervical mucus (Esfandiari et al., 2000), and also affects sperm morphology and motility (O'Connell et al., 2002). However these studies were carried out prior to knowledge of the role of the progesterone-sensitive CatSper channel on human sperm function (Smith et al., 2013). Considering that the human sperm calcium response to progesterone is of significant clinical importance (described in Chapters 3 and 4), the effect of cryopreservation on human sperm calcium regulation, and sperm function in general, clearly requires further investigation.

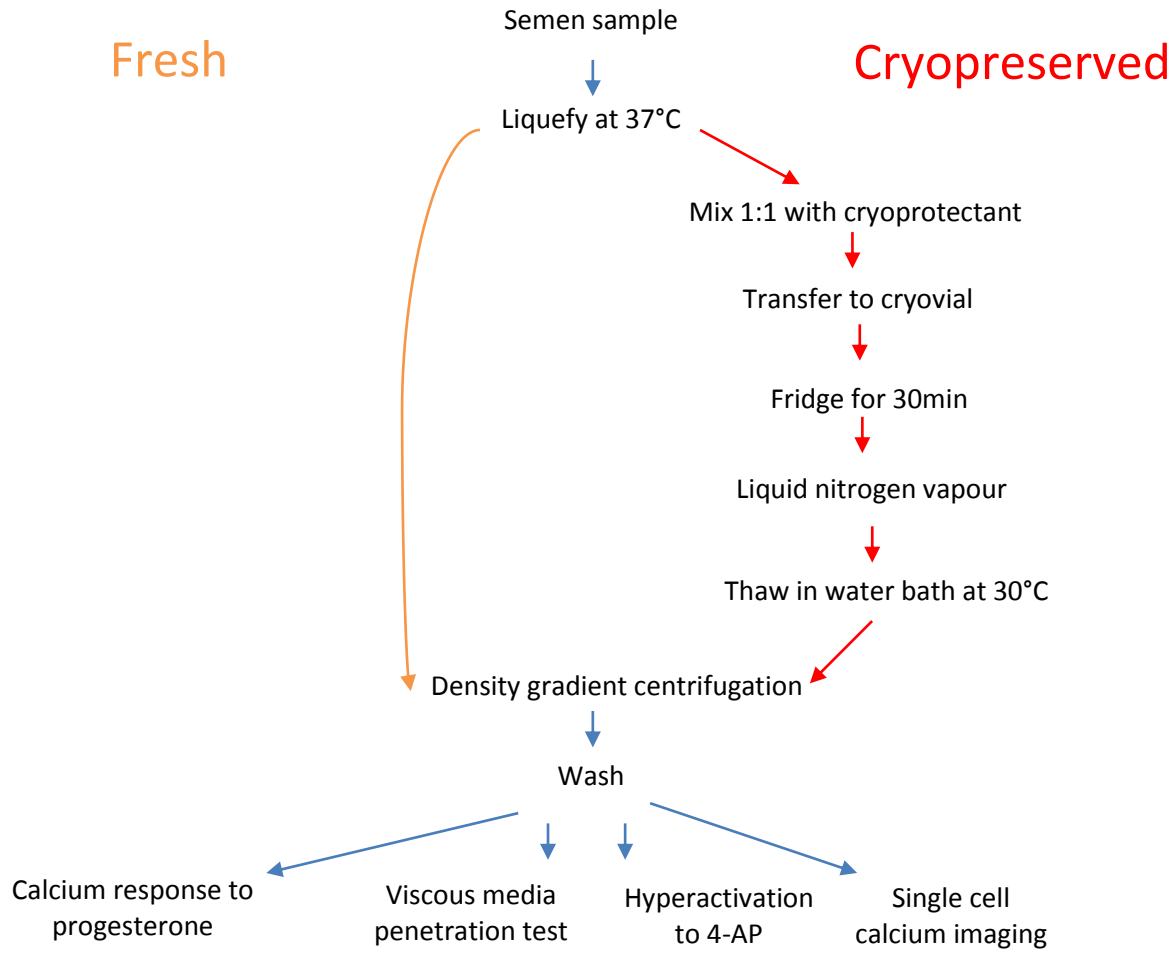
6.2 Aims

The study aimed to investigate fresh and cryopreserved samples by applying previously described screening methods (Chapter 3), in order to determine if cryopreservation has a detrimental effect on calcium signalling after thaw.

6.3 Hypothesis

We hypothesised that cryopreservation and thaw of semen would have a significant detrimental effect on the regulation of calcium signalling in human spermatozoa.

6.4 Experimental design



Briefly, thirty four normozoospermic (exceeding WHO 2010 criteria for normal semen concentration and total motility) donor semen samples were divided into two aliquots: half was prepared fresh and half was cryopreserved and thawed before preparation, in a similar manner to previous studies (Rossato et al., 2000, Meseguer et al., 2004b). The fresh and frozen thawed semen samples were then subjected to one or more analysis techniques dependent on post-preparation sperm concentration: calcium response to 3.6μM progesterone, viscous media penetration test and hyperactivation in response to 2mM 4-AP. Calcium response to progesterone was performed using two methods: whole population analysis by FLUOstar Omega, and calcium imaging of individual cells.

6.5 Results

6.5.1 Sperm concentration and motility post-cryopreservation

Thirty four semen samples were obtained from 21 donors. Due to significant inter-donor and intra-ejaculate variation (see Section 2.7.4), all donor samples were considered as separate entities unless otherwise stated. After semen preparation, all fresh samples and thawed samples met the >1 million motile sperm criteria to be theoretically suitable for IVF in ACU Dundee. From examining the collective data obtained after basal and 2mM 4-AP induced motility analysis (Appendix Figure 6.1 A-E displays data for sperm concentration, total motility, progressive, rapid, and hyperactivated motility, n=9 paired samples from 8 donors), fresh and frozen thawed samples showed no significant differences in concentration or any motility parameter

studied by computer aided semen analysis as displayed in Appendix Table 6.1 and Appendix Figure 6.1.

6.5.2 Calcium response to progesterone – FLUOstar Omega population-based method

Calcium response to progesterone was performed as described previously. Four million cells were used for either fresh or cryopreserved samples. The number of cells investigated per sample was not controlled by total motility, as previous experiments showed that there was no significant correlation between motility and peak calcium response to progesterone. Furthermore, motility analysis revealed that there was no significant difference in motility between prepared fresh and prepared cryopreserved samples (Appendix Figure 6.1).

Of the 34 paired fresh and frozen thawed samples, all were able to produce a calcium response to progesterone. None of the donors completely failed to respond to progesterone after cryopreservation, although the peak calcium response for one donor in both the fresh and cryopreserved fraction of the sample was below the 5th centile for donors (as defined in Section 2.7.5). Overall as seen in Figure 6.1, there was a significant decrease in the peak (Figure 6.1 C, Student's paired t-test, P=0.0046), delta (Figure 6.5.2 D, Student's paired t-test, P=0.0046) and plateau phase calcium response (Figure 6.5.2 E, Wilcoxon signed rank test, P=0.0126) to progesterone after cryopreservation. There was a significant increase in the basal calcium ratio (Figure 6.1 B, Wilcoxon signed rank test, P=0.0009), and the time to peak calcium response (Figure 6.1 F, Wilcoxon signed rank test, P=0.0279). The difference in the duration of response between fresh and frozen-thawed samples failed to reach statistical significance (Figure 6.1 G, Student's paired t-test, P=0.0521).

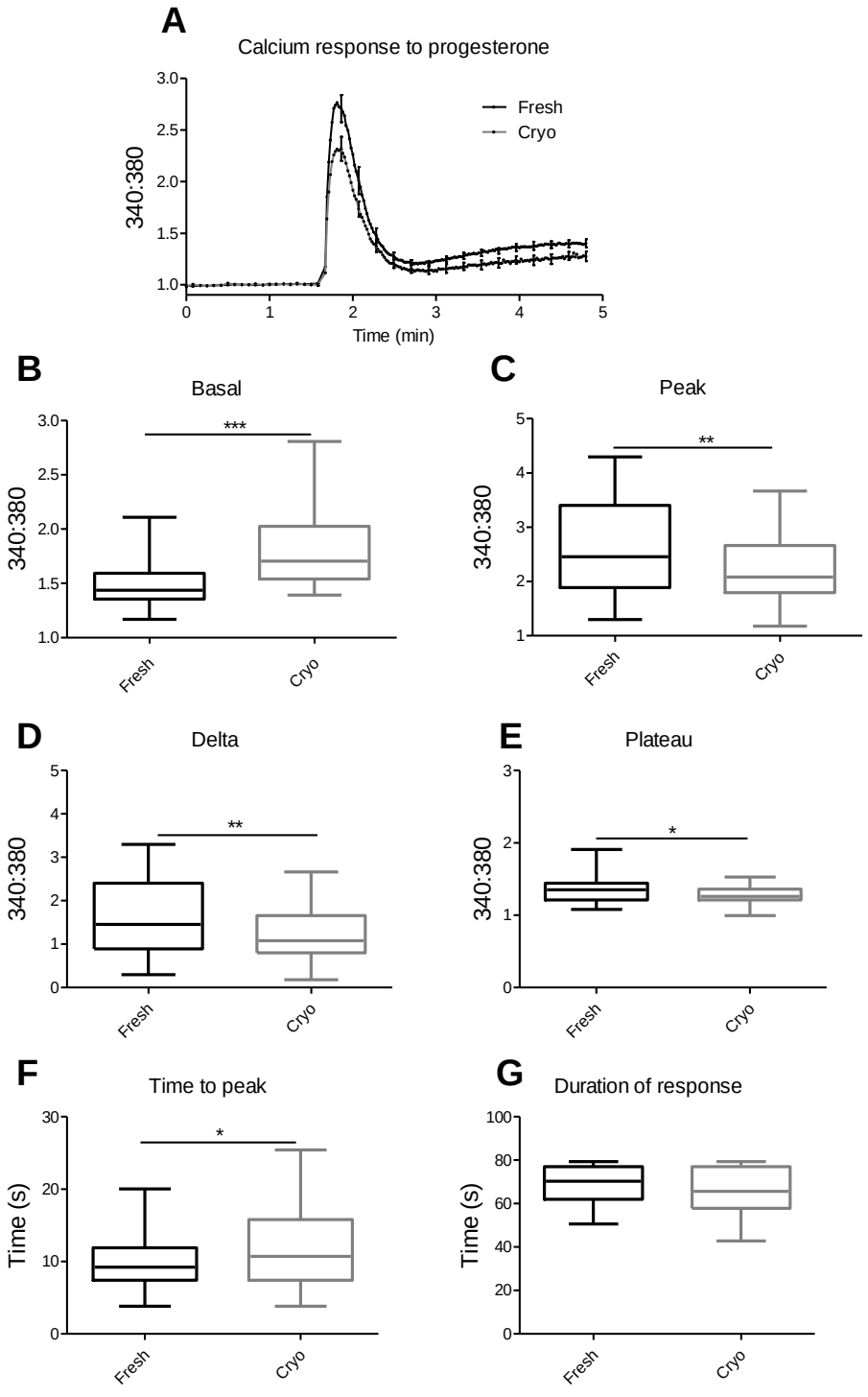


Figure 6.1 Fresh versus cryopreserved calcium response to progesterone. Peak calcium response to progesterone, basal calcium level, delta response, plateau phase, time taken to reach peak and duration of peak were examined. N=34 paired donor samples.

6.5.3 Variation in calcium response to progesterone between individual donors

Although the mean calcium response to progesterone was significantly reduced in sperm exposed to cryopreservation and thaw, this effect varied considerably between donors. For 26 of the 34 donor samples examined (76%), there was no significant difference in the calcium response for the fresh versus frozen-thawed sample (Figure 6.2, represented by the trace of Donor 1, >75 but <125% of the fresh response). However, for 6 of the 34 donors samples (18%), peak calcium response recorded after cryopreservation was significantly reduced (Figure 6.2, representative trace presented of Donor 2, <75% of the fresh response,). Interestingly, two of the samples with reduced response came from the same donor. For two donors (6%), their frozen thawed response was significantly greater than fresh (>125% of the fresh response, data not shown).

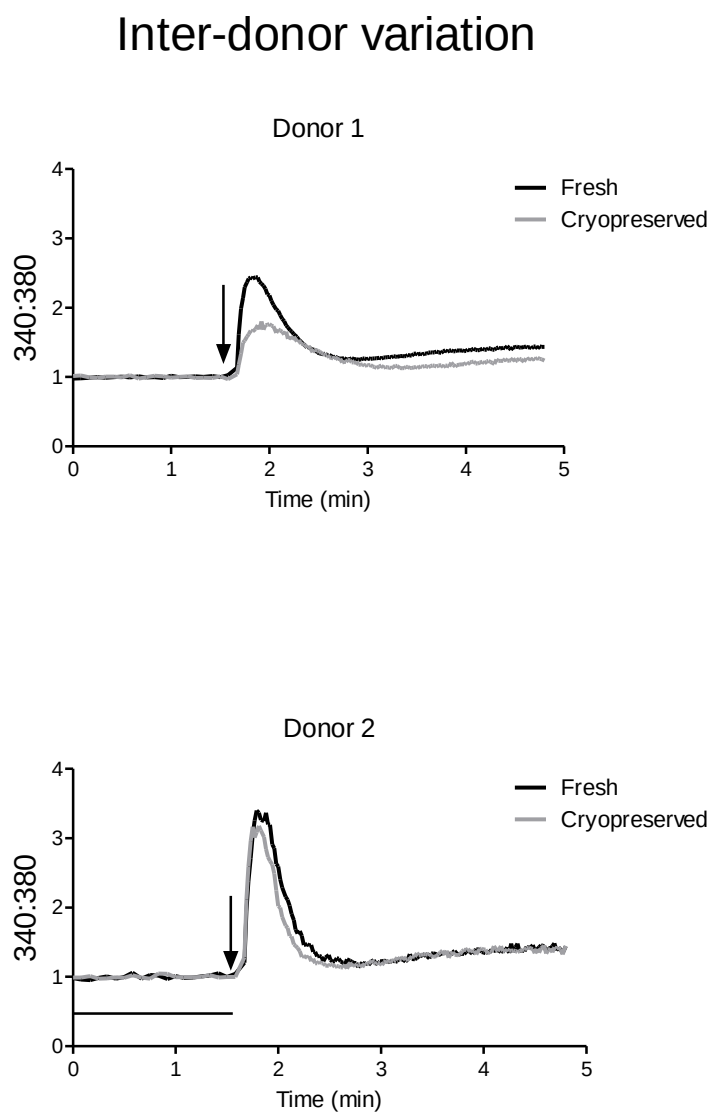


Figure 6.2 Variation in response to cryopreservation between individual donors. Representative individual donor samples demonstrate the variability of proportionate change of peak calcium response to progesterone after cryopreservation. Some samples show no significant change, whereas others exhibit a drastic reduction (<75% of fresh) in peak response. The line represents 100s, the arrow represents time of progesterone addition.

6.5.4 Calcium response to progesterone – single cell calcium imaging

As described in Chapter 5, the single cell calcium imaging technique was performed on paired prepared fresh and prepared frozen-thawed donor samples, in order to ascertain whether the change in calcium response to progesterone was due to a change in the proportion of responsive cells or in the percentage increment in response to progesterone between samples. As described in Figure 6.3, there was no significant difference in the average area under the curve of progesterone-responsive cells in both treatment groups (Figure 6.3 A, 7682 ±8324SD versus 7462 ±5800SD, Wilcoxon matched pairs test, P=0.8926, n=12). When examining the proportion of cells positively responding to progesterone, there was also no significant difference between them (Figure 6.3 B, 50.63 ±25.01SD versus 56.17 ±25.25SD, Student's paired t-test, P=0.5924, n=12).

When analysing individual samples, four samples (33%) exhibited significantly reduced mean fluorescent intensity when stimulated with progesterone after cryopreservation, four samples (33%) showed the opposite where mean fluorescent intensity was significantly increased when stimulated with progesterone after cryopreservation, and four samples (33%) showed no significant difference.

When analysing the mean basal fluorescence (background corrected), there was no significant difference observed between fresh and cryopreserved samples (Figure 6.3 C, Wilcoxon matched pairs test, P=0.5693).

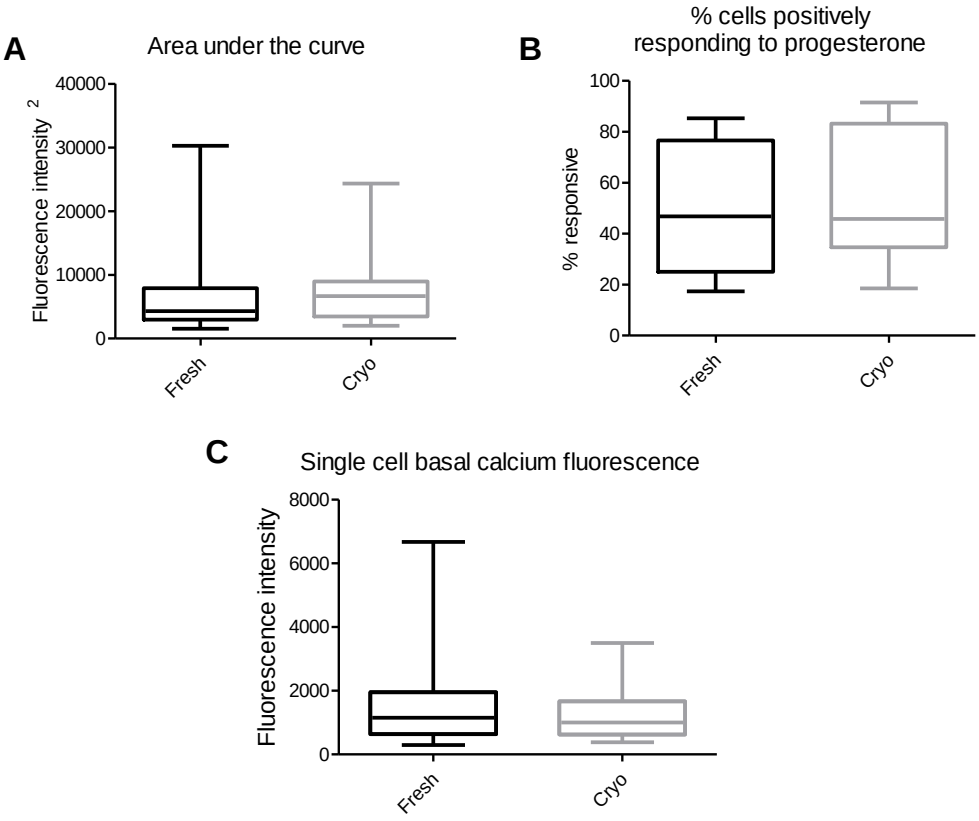


Figure 6.3. Single cell calcium response to progesterone after cryopreservation. Single cell calcium imaging revealed no significant difference between fresh and cryopreserved samples in average area under the curve or proportion of cells responding to progesterone.

6.5.5 Viscous media penetration test

Fresh and frozen thawed samples of suitable cell number (were subjected to the viscous media penetration test (Ivic et al., 2002, Alasmari et al., 2013b), where 1-2 million cells were incubated under control, vehicle (0.01% ethanol) and 3.6µM progesterone conditions. Sperm from both fresh and frozen thawed samples were able to penetrate into the methylcellulose, however there was a significant reduction in cell numbers at 1cm under control conditions after cryopreservation and thaw, as under fresh conditions median cell number was 38.5 ±20.39SD, whereas cryopreserved

median cell number was 22.14 ±19.1SD (Figure 6.4, Wilcoxon signed rank test, $p<0.0001$, n=9 paired fresh and cryopreserved samples from 8 donors). The data for all conditions at 1cm for fresh and cryopreserved samples is found in Table 6.1.

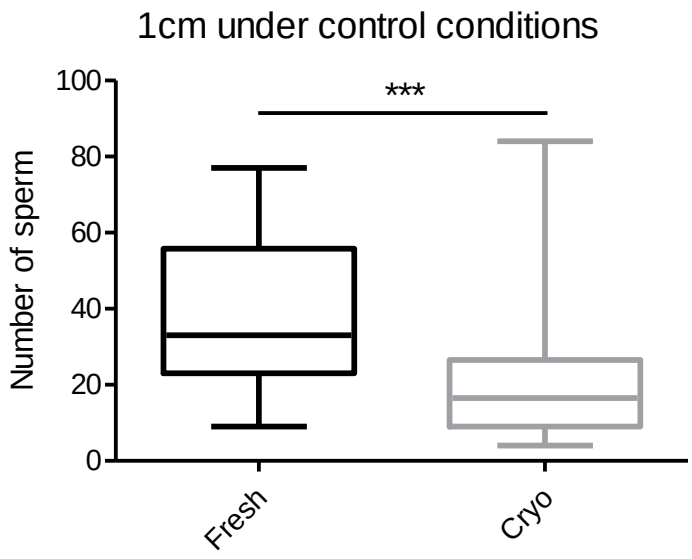


Figure 6.4 Viscous media penetration in fresh and cryopreserved samples. Fresh samples had a significantly greater cell density at 1cm under control conditions when compared to cryopreserved samples (Wilcoxon signed rank test, $p<0.0001$, n=9 paired fresh and cryopreserved samples from 8 donors).

	Fresh	**Cryopreserved**
Control 1cm	38.5 (20.4)	22.1 (19.1)
Vehicle 1cm	40.1 (19.2)	23.8 (16.1)
Progesterone 1cm	86.1 (58.7)	36.4 (22.8)

Table 6.1 Numbers of sperm reaching 1cm under control, vehicle and progesterone treated conditions. The figures represent mean number of sperm with SD in brackets. N=9.

Furthermore, fresh samples were able to significantly respond to progesterone in terms of increased cell number ratio found at 1cm in the methylcellulose following 1hr of incubation, however frozen thawed samples were unable to significantly respond to the addition of progesterone at 1cm (Figure 6.5).

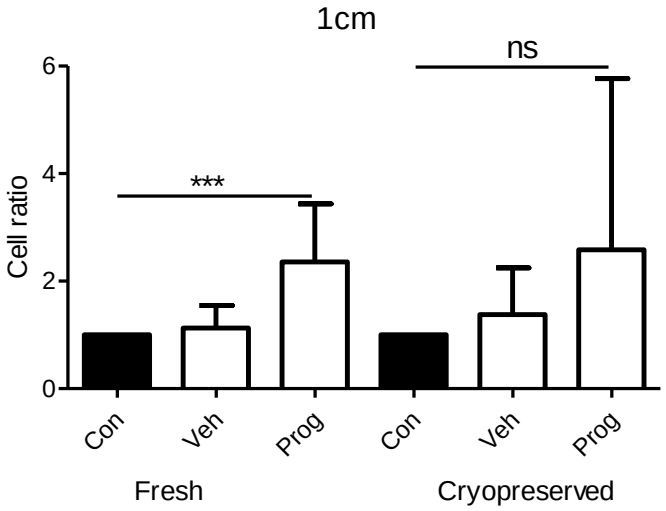


Figure 6.5 Viscous media penetration in response to progesterone pre-treatment. Fresh sperm samples were on average able to respond significantly to progesterone at 1cm in the viscous media penetration test, however cryopreserved samples were unable to respond to progesterone on average (n=9 pairs from 8 donors).

6.5.6 Hyperactivation

Fresh and frozen thawed spermatozoa were analysed for hyperactive motility using CASA. 2mM 4-AP was also used to stimulate hyperactivated motility. When looking at all samples together, both fresh and frozen thawed samples exhibited normal levels of hyperactivation after exposure to capacitating (control) conditions, and both were

also able to significantly respond to 4-AP (Figure 6.6, A) control 17.64% ±11.69SD and 4-AP 33.83% ±11.41SD, Wilcoxon signed rank test *P*=0.0039, and B) control 13.25% ±5.64 and 4-AP 22.72% ±11.04, Student's paired t-test *P*=0.0170 respectively, n=9 paired samples from 8 donors).

However when taking into account individual donor sperm responses to 4-AP, 8/9 (89%) donors were able to significantly respond in fresh samples, whereas just 4/9 (44%) donors were able to respond to 4-AP after cryopreservation. This indicates that half of the donors whose sperm originally significantly responded to 4-AP, lost this ability after cryopreservation and thaw.

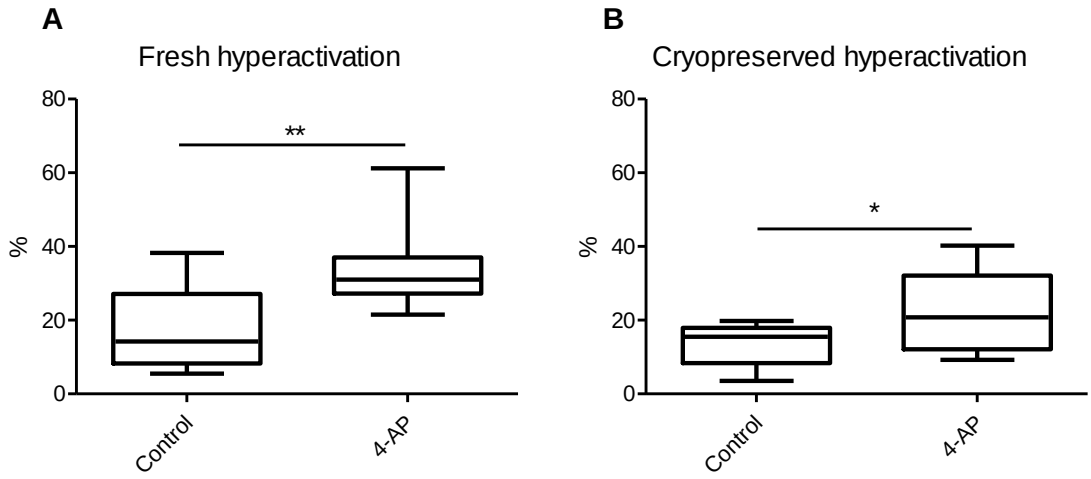


Figure 6.6 Hyperactivation in response to 2mM 4-AP in fresh and cryopreserved samples. Sperm from both fresh and cryopreserved samples were able to significantly respond in terms of increment in hyperactivated motility in response to 4-AP (n=9 paired samples from 8 donors).

6.5.7 Cumulative poor responders

There were 5 semen samples where if the sample responded poorly post-thaw to any of the tests subjected to, it would also perform poorly under one or more of the other tests. Taking the example of one of the donors whose fresh sample was able to significantly respond in the tests to both progesterone and 4-AP: fresh basal hyperactivation was significantly higher than cryopreserved basal hyperactivation, the cryopreserved sample showed no significant increment in hyperactivation in response to 4-AP, the cryopreserved sample calcium response to progesterone was just 68% of the fresh sample, and the cryopreserved sample was unable to significantly increment numbers of sperm in the viscous media under progesterone treatment.

6.6 Discussion

Multiple studies have shown that cryopreservation of semen leads to a decrease in sperm motility, concentration, morphology, mitochondrial activity and increases nuclear vacuolization when compared to their freshly prepared counterparts (Lin et al., 1998, Rossato et al., 2000, O'Connell et al., 2002, Boitrelle et al., 2012). However fresh semen analysis can seldom predict the quality of sperm recovered post-cryopreservation and thaw (Meseguer et al., 2004b). Often in cases where time is sensitive, such as in the case of a pre-chemotherapy male, semen is cryopreserved without regard for sample quality, as ICSI may be utilised if the thawed sample quality is poor. However, although prospective sperm donors for reproductive purposes submit a semen sample for a “test thaw” to analyse sperm quality in terms of concentration and motility post-cryopreservation before acceptance as a donor, the thawed sperm function is not analysed, even though cryopreservation has been

demonstrated to have a significant detrimental effect on other aspects of sperm function such as the concentration of the sperm-derived oocyte activating factor PLCζ (Kashir et al., 2011).

The data in the present study is based upon responses of normozoospermic (normal concentration and motility by WHO 2010 standards) research donor semen samples to cryopreservation and thaw. The data suggest that in contrast to previous studies (O'Connell, et al., 2002, Boitrelle, et al., 2012) cryopreservation has little significant negative effect on post-preparation sperm motility or concentration, however it exhibits a significant negative effect on normal sperm function: in particular the calcium signalling pathway and response to the stimulant progesterone. Cryopreservation also significantly impedes the ability of the sperm to penetrate a viscous media. These findings have serious implications for fertilisation both *in vitro* and *in vivo*, as evidenced by multiple previous studies (Falsetti et al., 1993, Shimizu et al., 1993, Krausz et al., 1995), sperm must maintain functional calcium signalling in response to progesterone in order for fertilisation to occur.

As described previously (McLaughlin and Ford, 1994), in the present study cryopreserved sperm samples exhibited a significantly higher basal calcium ratio than their fresh counterparts, indicating a greater proportion of bound to unbound Fura dye. This in turn suggests that there was a greater concentration of free intracellular calcium within cryopreserved sperm cells. However this was not the case in single cell calcium imaging, where there was no significant difference between mean basal fluorescence (background corrected), between fresh and cryopreserved samples.

Similarly to previous research (Rossato et al., 2000), the mean peak calcium response to progesterone was significantly reduced after cryopreservation and thaw. Further,

18% of donor samples in the present study exhibited a significant reduction in calcium response to progesterone after cryopreservation, however, 76% of donor samples showed little or no change following the cryopreservation procedure. As the technique used to freeze, thaw and prepare the sperm was identical for all samples, it stands to reason that an inherent sperm factor causes this change.

Considering that two of the samples producing a reduced response to progesterone after cryopreservation were obtained from the same donor, it suggests that some donor sperm samples may be particularly susceptible to cryo-damage, due to genetic or epigenetic differences between subjects. It may therefore be prudent to assess individual donor susceptibility to cryo-damage in the laboratory workup before accepting them as donors for reproduction purposes, to avoid lower success rates with donors susceptible to the damage.

Single cell calcium imaging showed that there was no significant difference in the proportions of sperm that were able to respond to progesterone between the fresh and frozen-thawed samples. However four individual samples within the twelve studied had a significant reduction in cellular fluorescence intensity post progesterone addition following cryopreservation, in agreement with results reported for samples in the FLUOstar. Intriguingly, a further third of these samples showed a significant increase in cellular fluorescence intensity post progesterone addition. It is of unknown cause, as only 6% of the samples showed a significantly increased calcium response to progesterone in the population assay.

Esfandiari et al described in 2000 the significant negative effect of cryopreservation and thaw on the ability of sperm penetrating bovine cervical mucus. The further finding of the current study that under fresh sample conditions sperm are significantly

able to penetrate viscous media to a depth of 1cm in the viscous media penetration test however significantly fewer numbers of cryopreserved and thawed sperm are able to penetrate artificial viscous media at 1cm under control conditions correlates with these findings. This is of significant clinical importance: taking into consideration the widespread use of cryopreserved semen samples in IUI and IVF where although the cervical mucus challenge is artificially bypassed, sperm still have a fundamental role in penetrating the visco-elastic hyaluronan matrix surrounding the oocyte.

Analysis of average basal hyperactivation of capacitated fresh and frozen-thawed sperm revealed no significant difference between the groups. However four individual samples showed a significant reduction in basal hyperactivation post-thaw. Eight of the nine fresh donor samples studied were able to significantly respond to 2mM 4-AP by significantly increasing the proportion of hyperactivated sperm. However in the cryopreserved group, only four of the nine samples were able to elicit the same significant response. As 4-AP is thought to exert its function via the mobilisation of calcium from intracellular stores in human sperm (Alasmari et al., 2013b), this result suggests that these membrane-bound calcium stores are dysfunctional or disrupted following cryopreservation in around half of all semen samples.

A retrospective study found that fertilisation rates using fresh ejaculated sperm in ICSI were higher than using cryopreserved spermatozoa, even though the functional ability of the sperm is not required (Borges et al., 2007). Therefore, with respect to the current study, limitations include the lack of gold-standard experimental technique to prove correlation between poor post-thaw sperm function and fertilisation ability: determining sperm functional ability by either *in vitro* fertilisation to determine fertilisation rate or IUI to prove fertilisation by pregnancy achievement. These experiments, although informative, would be wholly unethical due to the findings of

the present study and that of Borges *et al.*, of the negative impact on sperm function by cryopreservation.

Further limitations include the reduction in viable cell number post-cryopreservation and thaw which in turn limited the number of different experiments able to be performed on each individual sample. It should be noted that the cryopreservation procedure itself results in an increased duration of sperm exposed to semen of around 40 minutes, and also exposes the sperm to cryoprotectant for the same duration of time, excluding the time in liquid nitrogen storage. Failure to retrieve sufficient numbers of sperm post-thaw occurred in occasional individual cases resulted in reduction of potential n-numbers of paired samples to 34 detailed in this study. Furthermore, due to the timeline of performing the experiments, all hyperactivation assays, population-based calcium response to progesterone assays, and viscous media penetration tests were performed prior to single cell calcium imaging, preventing comparison of an individual sample performance in population versus single cell calcium response to progesterone.

The semen samples analysed in the present study met the WHO 2010 criteria for normal values of sperm concentration and motility. It is crucial to consider that many of the semen samples cryopreserved within the Assisted Conception Unit are from patients who may exhibit oligo-, astheno-, teratozoospermia or a combination of the three. It has been previously reported that infertile patient semen samples with <50M/ml sperm concentration and <40% motility had significantly poorer post-thaw recovery (Mossad et al., 1994), therefore further investigation must be performed in order to establish post-thaw calcium response to progesterone outcome in these samples with poorer fresh semen parameters. These samples may be susceptible to

further cryo-damage and loss of function, which would significantly alter treatment outcome, and may lead to more invasive techniques such as ICSI.

6.7 Conclusion

In summary, cryopreservation does not affect sperm motility parameters however does have a novel significant negative impact on the human sperm calcium response to progesterone, and ability of these sperm to penetrate artificial viscous media following progesterone treatment. However this effect is highly variable between individual donors, and the impact on fertilisation potential following cryopreservation is as of yet unknown.

Chapter 7

General Discussion

7.1 Review of the thesis aims and findings

7.1.2 Thesis aims

The aims of the present study were 1) to investigate incidence of calcium pathway defects in IVF patients and 2) patients recalled due to failed/low fertilisation, 3) to investigate the impact of cryopreservation on calcium pathway function, and 4) to initiate and develop the use of single cell calcium imaging as a more sensitive method for detecting calcium pathway dysfunction in IVF/ICSI patients and donors.

7.1.3 Key findings

The present study describes that, in contrast to previous studies, there was no significant difference in the parameters of the calcium response to progesterone between IVF patients and donors (Chapter 3). However the present study does confirm findings from a previous study (Alasmari et al., 2013a) that a reduced proportion of IVF patients (66%) were able to significantly respond to 4-AP treatment in terms of increase in hyperactivated motility compared with donors (80%), although these proportions were different than previously reported. The present study describes the novel attempt to use sperm penetration of viscous media under treatment with the known CatSper agonist, progesterone as previously described (Alasmari et al., 2013b), as a method of screening IVF patients for calcium pathway defects, and defects in sperm function.

The present study describes, for the first time, the identification of two normozoospermic IVF patients with a complete absence of the ability of their sperm to produce a calcium response to progesterone. Previously, spermatozoa from one

male with homozygous *CatSper2* deletion had been characterised with absence of CatSper current under electrophysiological measurement, however the spermatozoa had a myriad of other defects including asthenoteratozoospermia (Smith et al., 2013). Furthermore, the function of CatSper in this study was solely investigated using divalent-free Cs^+ currents, not the direct monitoring of Ca^{2+} as performed in the current study. The normozoospermic patients described in the current study had unexplained total failed fertilisation of all inseminated oocytes during IVF. Both of these patients also failed to respond to progesterone treatment, in terms of enhancing cell numbers penetrating viscous media. Furthermore, both of these patients exhibited normal basal hyperactivation once exposed to capacitating conditions, unlike descriptions of sperm from mouse CatSper knockouts (Quill et al., 2003), thus the link between CatSper function and hyperactivation/fertilisation is complex in the human. Increase in hyperactivation via the calcium store-mobilising agent 4-AP was noted for both patients, indicating sufficient calcium store function. For one of the patients, the finding of CatSper channel dysfunction was confirmed by electrophysiological analysis, under Cs-based divalent free conditions. This finding is the first example of defective CatSper function in patients with normal semen parameters, and reinforces the message that CatSper deficiencies are not detectable by routine semen analysis techniques. Furthermore, the present study is the first to describe reversible CatSper function in an IVF patient, which then led to failed fertilisation when function was abolished. Reversible specific modification of the CatSper channel to alter function could therefore present a promising target for development of a novel contraceptive action, for use by either the male or female partner, as data from the current study suggests that sperm with non-functional CatSper are unable to fertilise oocytes. Novel CatSper antagonists which specifically inactivate the channel function would

potentially be beneficial, although specificity is key in this case, to reduce the risk of off-target effects on other calcium channels present systemically.

The present study also demonstrated, in a novel manner, the detrimental effect of semen cryopreservation on calcium regulation and CatSper function post-thaw. Although none of the samples studied had CatSper function completely abolished after cryopreservation and thaw, the peak and plateau phase calcium response to progesterone were significantly reduced. Looking at fresh and cryopreserved samples from individual males revealed that some donors were particularly susceptible to cryodamage, while others showed little noticeable difference. This could indicate genetic/epigenetic variances between individuals, resulting in the observed cryodamage. This is a significant finding, particularly within the clinical context where semen cryopreservation and thaw is routinely used for procedures such as IUI and IVF. Furthermore, while basal hyperactivation was not significantly different between fresh and cryopreserved samples, cryopreservation reduced the proportion of samples able to significantly respond to 4-AP (89% for fresh samples versus 44% for cryopreserved samples with 4-AP treatment). This is a potentially significant finding, as increment of hyperactivation has been previously correlated with IVF fertilisation rate (Alasmari et al., 2013a), although this correlation was not observed in the present study (Chapter 3).

The present study also demonstrated that single cell calcium imaging is an effective method for analysis of calcium response to progesterone in IVF and ICSI patient samples. Although fewer sperm are analysed per experiment (~200 versus 4 million for population-based methods), the technique benefits from increased sensitivity and specificity for single cell studies. This is particularly of use for ICSI samples where low sperm numbers render population methods impractical, but where significant

variation in the value of area under the curve between cells is evident (Chapter 5). The increased resolution obtained by analysing single cells could further localise and quantify (by proportions of responsive cells, or extent of response to progesterone as shown in Chapter 5) defective calcium regulation within a range of IVF and ICSI patients, as well as investigating patients with experience of a total failed fertilisation cycle.

7.2 Clinical relevance

This study investigated spermatozoa from male IVF and ICSI patients undergoing ART in Dundee. There were two main pools of patients investigated. The first pool were those undergoing routine IVF/ICSI who donated the surplus sample to research, not to directly benefit themselves but to potentially benefit others via the findings of the screening research carried out. The second pool were those patients who had experienced a failed/low fertilisation in a previous ART cycle, who donated a further semen sample solely for the investigation into causes of male factor infertility affecting this couple.

The screening research carried out in Chapter 3 and Chapter 4 identified two men with defective calcium response to progesterone, and subsequent failed fertilisation. It is key to note that conversely, no patients were identified with defective calcium response to progesterone, with subsequent normal fertilisation. This study indicated that for the remainder of the IVF patients studied, there was no correlation found between any of the parameters studied with fertilisation rates in IVF. Furthermore, less than 1% of the samples studied had a severely defective calcium response to progesterone. The incidence of defects in calcium response to progesterone is low, and

the overwhelming majority will be "normal" with regard to calcium pathway function. However, screening a portion of cells from the IVF sample by FLUOstar after semen preparation and capacitation however prior to insemination could direct the embryologists to ICSI insemination rather than IVF, if a defect was noted. This could result in the avoidance of failed fertilisation outcome for these patients, which would clearly have significant financial, clinical and emotional benefits for both the patients and clinical staff. This would be entirely possible in the ACU in Ninewells, where there is access to a FLUOstar machine in the research laboratory which is located within the same hospital. If all IVF patients cannot be screened, then priority should be for those who have unexplained infertility, as there was a disproportionate number of patients suffering from unexplained infertility in Chapter 4, two of whom had defective calcium response to progesterone.

The research in Chapter 4 presents a very different story: where defects in calcium pathways or impaired K^+ conductance were noted for a larger proportion of the patient samples investigated. For the defects that may be rectified by ICSI in the following cycle, such as absent calcium response to progesterone, the information gained by this screening technique would provide valuable information to the patient about why the failed/low fertilisation occurred. Furthermore it would provide vital information to the clinician regarding the direction that further treatment should take: for example an IVF attempt using an oocyte donor, where CatSper dysfunction was observed in the male partner, would be futile.

Many research techniques are offered as adjuncts during infertility treatment, whether they are based upon sound scientific evidence with clear clinical benefit, or not (Harper et al., 2012). The patients are often charged for carrying out these extra techniques, unless they are part of an ongoing clinical trial. Thinking about the

screening methods used in the current thesis, it is unlikely that all NHS assisted reproduction clinics will have access to the equipment required (for example a FLUOstar, or single cell calcium imaging microscope setup). However, all clinics should have access to cryopreservation facilities for semen. If a clinician wanted to examine a semen sample for calcium pathway abnormalities, for example after a failed fertilisation in IVF where semen parameters were normal, they could ship the sample in LN_2 for analysis by our laboratory. The cost would be relatively cheap (the most expensive step being shipping the sample), it would involve using the process of semen cryopreservation and shipping which is used routinely in the clinical laboratory, and the answer could be given within a day of receipt of the sample. This rapid turnaround of results could be particularly useful for those patients already in the early phases of a treatment cycle, prior to oocyte retrieval and fertilisation stages. Chapter 6 demonstrated that although there is a significant reduction in peak calcium response to progesterone after cryopreservation when examined by FLUOstar Omega plate reader, none of the 34 samples examined completely lost the ability to respond to progesterone. Furthermore, using single cell calcium imaging, there was no significant difference in proportion of responsive cells to progesterone or extent of the response. Thus, either of these non-invasive, rapid techniques could be used to accurately analyse an external patient's calcium response to progesterone.

7.3 Further study

Chapter 3 examined the effect of CatSper agonists (progesterone, and TMA via intracellular alkalization) and calcium store mobiliser (4-AP) on IVF patients and donors. There are many other compounds found to have effects on calcium pathways

in sperm: for example the activation of CatSper via prostaglandins, odorants (Brenker et al., 2012) and endocrine disruptors such as p,p'-DDE (Tavares et al., 2013). Therefore investigation must focus on determining the action of these other compounds on calcium regulation, particularly with reference to their involvement in male factor infertility. Also of significant interest may be the role played by follicular fluid on stimulating modifications in intracellular calcium, as a method more representative of the *in vivo* environment the sperm are exposed to while traversing the human female reproductive tract. Furthermore, investigation into drug discovery may reveal compounds that by acting on sperm calcium regulation are able to provide suitable therapeutic aid for patients suffering from poor sperm motility, as drugs targeting other fundamental sperm functions such as phosphodiesterase activity have already shown potential benefit in Andrology, IVF and ICSI patients (Tardif et al., 2014). Thus, activators of calcium pathways in human sperm could be harnessed for therapeutic benefit, rather than being used for diagnostic analysis as described in the present study.

For the patient samples received in Chapter 4, further study of further semen samples from this select group of patients who had experienced low/failed fertilisation would benefit from more sensitive techniques such as single cell calcium imaging in response to progesterone, in order to ascertain whether the pattern of response or proportion of responding cells were significantly different from IVF patients and donors. This would also enable the examination of spermatozoa from semen samples of low sperm concentration, which in the current study was not possible using the FLUOstar Omega microplate reader.

Other future work should include further developments of the techniques described in Chapter 5. For example, use of a perfusion chamber with a closed bath would

circumvent the difficulties with manual addition of stimuli, avoid excessive evaporation, and would therefore extend the duration of recording to 10 – 30 minutes as described in other studies (Kirkman-Brown et al., 2000, Kirkman-Brown et al., 2004). This would enable the study of the secondary phase of the intracellular calcium response to progesterone, and furthermore may enable the observation of calcium oscillations after progesterone treatment, which may be of clinical interest in the IVF and ICSI patient samples (Harper et al., 2004, Kirkman-Brown et al., 2004). As previously mentioned, evolution of this method to allow analysis of severely oligo/asthenozoospermic samples, or samples from patients recalled due to a failed/low fertilisation treatment outcome, would be beneficial.

For the development of understanding of the impact of cryopreservation on the ultimate human sperm function, fertilisation of the oocyte, the gold standard would be to assess an aliquot of fresh and cryopreserved semen for calcium response to progesterone, and inseminate half of the oocytes with fresh prepared spermatozoa and half with thawed spermatozoa. This is not ethical, due to the fact that cryopreservation introduces abnormalities into the sperm that could negatively impact fertilisation (Esfandiari et al., 2000, O'Connell et al., 2002, Boitrelle et al., 2012). One way around this conundrum is to use a situation where cryopreservation and thaw is unavoidable, and furthermore no male factor infertility is present: such as using semen from a donor that would be used for reproductive purposes (for example use in IUI treatment cycles). This would not have a significant negative effect on the clinical treatment of the patients, as the standard donor tends to produce enough semen for many more vials of cryopreserved semen than are required for the limit of 10 families. Furthermore, a test thaw is always carried out prior to the donor being accepted, to assess the quality of his sample post-thaw, therefore examination could be performed on this sample.

Although a direct comparison with pregnancy rates with insemination using fresh semen is not possible, the technique could identify donors with increased susceptibility to cryodamage and thus increased time to pregnancy in inseminated patients. The reduction in calcium response to progesterone following cryopreservation as compared to the fresh aliquot could be compared with number of insemination cycles required to achieve a pregnancy. It must be noted that female infertility could be a confounding factor in this case, therefore care must be taken when interpreting the results.

7.4 Conclusions

The conclusions from this study are that while dysfunction of CatSper is rare in the general population and in the IVF patient population, the incidence rises when patients with total failed fertilisation are examined. Patients with unexplained total failed fertilisation may benefit from the use of single cell calcium imaging as a more sensitive method to screen for calcium defects within individual cells from a population. As cryopreservation does not significantly affect either the extent of calcium response to progesterone within the population of cells, or proportion of responsive cells, semen samples may be cryopreserved and transported for analysis by our laboratory for further investigation into calcium pathway defects.

Publications

Williams HL, Mansell S, Alasmari W, Brown SG, Wilson SM, Sutton KA, Miller MR, Lishko PV, Barratt CL, Publicover SJ and Martins da Silva S. Specific loss of CatSper function is sufficient to compromise fertilizing capacity of human spermatozoa. Hum Reprod 2015;30:2737–2746.

Brown SG, Publicover SJ, Mansell SA, Lishko PV, Williams HL, Ramalingam M, Wilson SM, Barratt CLR, Sutton KA and Martins Da Silva S. Depolarization of sperm membrane potential is a common feature of men with subfertility and is associated with low fertilisation rate at IVF. Hum Reprod 2016, first published online 6th April 2016.

I collaborated with Dr Sarah Martins da Silva to write a book chapter for joint publication between Royal College of Obstetricians and Gynaecologists and British Fertility Society: "Chapter 3: Semen analysis and sperm function tests" (in press).

Presentations

Fertility 2015 (7-9 January, ICC Birmingham) oral presentation entitled "Identification of putative CatSper defects in two IVF patients".

CMDN (College of Medicine, Dentistry and Nursing) poster presentation (February 2015) entitled "Putative CatSper defects in two IVF patients".

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Appendix

Characteristic	5th centile lower reference limits
Volume	1.5ml
Concentration	15M/ml
Count (per ejaculate)	40M/ejaculate
Total motility	40%
Progressive motility	32%
Normal forms	4%

Appendix Table 1. Semen characteristics. Replicate table of "normal" semen characteristics as defined by WHO (2010) 5th centile lower reference limits.

Quinn's Advantage Fertilization HTF Universal Medium	Quinn's Sperm Washing Medium	HTF (Lishko et al 2011)
Sodium chloride	Sodium chloride	Sodium chloride (97.8mM)
Potassium chloride	Potassium chloride	Potassium chloride (4.69mM)
Sodium bicarbonate	Sodium bicarbonate	
Glucose, D- (+)	Glucose	Glucose (2.78mM)
Sodium Pyruvate	Sodium pyruvate	Sodium pyruvate (0.33mM)
Phenol Red	Phenol red	
Gentamicin	Gentamicin	
Alanyl-glutamine	Alanyl-glutamine	
Taurine	Taurine	
EDTA	EDTA	
Potassium phosphate, monobasic, anhydrous	Potassium phosphate, anhydrous	
Magnesium sulphate heptahydrate	Magnesium sulphate	Magnesium sulphate (0.2mM)
Asparagine	Calcium chloride	Calcium chloride (2.04mM)
Aspartic acid	HEPES	HEPES (21mM)
Glycine	Sodium lactate (DL)	Lactic acid (21.4mM)
Proline	Human serum albumin	0.3% BSA
Serine		NaOH to pH-balance
Calcium lactate (L+)		
Citric acid		
Serum protein substitute (5mg/ml)		

Appendix Table 2.1. Components in media used in the study. Components of Quinn's Advantage Fertilisation (HTF) Universal Medium, Quinn's Sperm Washing Medium, and HTF (based on Lishko et al 2011) are listed for comparison.

	Definition	Measurement units
VAP	Average path velocity	µM/sec
VSL	Straight line velocity	µM/sec
VCL	Curvilinear velocity	µM/sec
ALH	Amplitude of the lateral head displacement	µM
BCF	Beat cross frequency	Hz
STR	Straightness	%
LIN	Linearity	%
Total Motile	Total % of motile cells	%
Progressive	% of progressive cells	%
Rapid	% of rapid cells	%
Hyperactivation	% of hyperactivated cells	%

Appendix Table 2.2 CASA motility parameters. Sperm motility parameters measured by computer assisted sperm analysis (CASA), and their measurement units.

	Concentration (M/ml)	Total Motile %	Progressive Motile %	Rapid Motile %
Donor semen	119.9 (11.4)	66.5 (2.5)	38.2 (2.1)	56.2 (2.5)

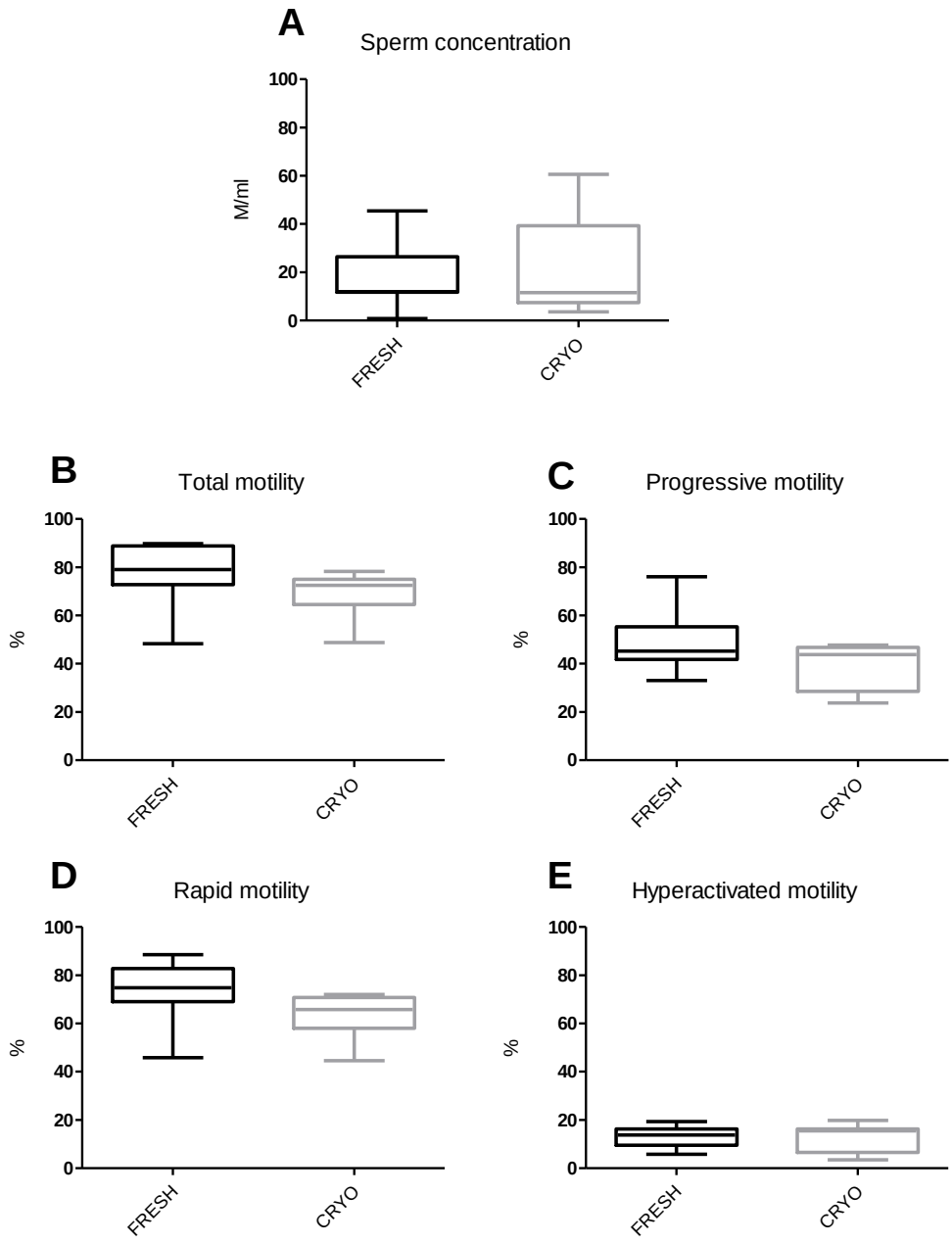
Appendix Table 3.1 Mean sperm concentration and initial motility parameters for donor semen ±SEM. Donor sample n=47 ejaculates from 20 donors.

	Concentration (M/ml)	Total Motile %	Progressive Motile %	Rapid Motile %	Hyperactive %
Donor	38.1 (5)	85.3 (1.5)	50.5 (2.6)	81.6 (1.7)	18 (1.6)
IVF	17.1 (1.3)	79.5 (1.5)	50.3 (1.5)	74.9 (1.5)	13.5 (1.1)
p value	**<0.0001**	**0.014**	0.95	**0.008**	**0.021**

Appendix Table 3.2 A comparison of sperm concentration and motility parameters for prepared (resuspended in capacitating HTF media) samples ±SEM. Donor n=48 ejaculates from 20 donors. IVF patient n=68.

Parameter	Fresh	Cryopreserved
VAP	83.6 (6.8)	75.2 (8.9)
VSL	69.1 (5.7)	60 (9.3)
VCL	126.4 (17)	126.8 (14.6)
ALH	4.7 (0.7)	5 (0.4)
BCF	20.5 (3.6)	22.6 (4)
STR	80.9 (6)	77.6 (4.7)
LIN	57.4 (9.3)	50 (6.1)
Concentration	18.5 (14.4)	22.4 (20.8)
Motility	76.9 (14)	68.9 (9.9)
Progressive	49.4 (13.7)	38.6 (9.4)
Rapid	73.5 (13.8)	63.3 (9.5)
Hyperactive	12.79 (4.50)	12.54 (5.92)

Appendix Table 6.1. Motility parameters for fresh and cryopreserved sperm samples. There was no significant difference in any motility parameter observed between fresh and cryopreserved prepared sperm samples. Table shows mean values, the number in brackets denotes 1SD.



Appendix Figure 6.1 Motility parameters for fresh and cryopreserved samples. There was no significant difference in any motility parameter observed between fresh and cryopreserved prepared sperm samples, n=9 paired samples from 8 donors.