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Novel Modulators of Human Sperm Motility

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Motility

Louise King

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**'Novel Modulators of Human Sperm Motility'**

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## Acronyms and Abbreviations

ACU: Assisted conception unit

AI: Artificial insemination

ALH: Amplitude of lateral head displacement

ANOVA: Analysis of variance

AR: Acrosome reaction

ART: Assisted reproductive technology

BCF: Beat-cross frequency (Hz)

BSA: Bovine serum albumin

Ca<sup>2+</sup>: Calcium ions

[Ca<sup>2+</sup>]<sub>i</sub>: Intracellular calcium ions

cADPR: Cyclic ADP ribose

CAM: Calcium/ calmodulin

CAMK II: Calcium/ calmodulin dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

CASA: Computer-aided sperm analysis

Ca<sub>v</sub>: Voltage gated calcium channels

cGMP: Cyclic guanosine monophosphate

CICR: Calcium induced calcium release

CNG: Cyclic nucleotide-gated

CM: Capacitating medium

CO<sub>2</sub>: Carbon dioxide

DDU: Drug discovery unit

DGC: Density gradient centrifugation

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

E<sub>m</sub>: Membrane potential

HCO<sub>3</sub><sup>-</sup>: Bicarbonate ions

HFEA: Human Fertilisation and embryology association

HOS: Hypo-osmotic swelling

HTS: High throughput screening

HVA: High voltage activated

ICSI: Intracytoplasmic sperm injection

IP<sub>3</sub>Rs: Inositol triphosphate receptors

IUI: Intrauterine insemination  
IVF: In-vitro fertilisation  
 $K^+$ : Potassium ions  
LIN: Linearity  
LVA: Low voltage activated  
 $Na^+$ : Sodium ions  
NAADP: Nicotinic acid–adenine dinucleotide phosphate  
 $NaHCO_3$ : Sodium bicarbonate  
NCM: Non- capacitating HEPES-buffered medium  
 $pH_i$ : Intracellular pH  
PKA: Protein kinase A  
PKC: Protein kinase C  
PTX: Pentoxifylline  
ROS: Reactive oxygen species  
r.p.m.: Revolutions per minute  
RT-PCR: Real time polymerase chain reaction  
RyRs: Ryanodine receptors  
sAC: Soluble adenylate cyclase  
SD: Standard deviation  
SEM: Standard error of the mean  
STR: Straightness (VSL/VAP)  
TMC: Total motile count  
tmACs: transmembrane adenylate cyclases  
UoD: University of Dundee  
VAP: Average path velocity  
VCL: Curvilinear velocity  
VSL: Straight-line (rectilinear) velocity  
WHO: World Health Organization  
WOB: Wobble (VAP/VCL)

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**Declaration**

I declare that the content of this project report is my own work and has not previously been submitted for any other assessment. The report is written in my own words and confirms to the University of Dundee's Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report.

Signature:

Date:

## **Summary**

Male factor is the underlying cause in 50% cases of infertility, and is most commonly characterised by asthenozoospermia (reduced sperm motility). Incredibly, there is currently no known treatment for this condition, and the only option is Artificial Reproduction Technology (ART) which is expensive and invasive. The development of a novel treatment would fulfil a therapeutic niche that needs to be met. Using calcium as a surrogate for motility, a Flexstation high-throughput screening assay (HTS) of 3312 drug discovery ion channel compounds was performed. The screen identified 14 hit compounds that increase intracellular calcium in human spermatozoa. This project investigated these compounds, along with, 6 hit compounds previously identified from a HTS of a Chemogenomics library (246 compounds) for their potential to enhance sperm motility. One compound, Trequinsin hydrochloride (Chemogenomics library), produced statistically significant increases in progressive motility ( $P=0.02$ ) instantaneously ( $t=0$  min), an effect that was sustained for 3 hrs. Trequinsin hydrochloride, a potent class 3 phosphodiesterase inhibitor, along with B1, an ion channel compound, produced statistically significant increases in penetrating ability of spermatozoa through viscous medium. These compounds were both examined on sub-fertile patient samples, with positive results, providing preliminary data on their clinical relevance.

# **CHAPTER 1**

## **General Introduction**

## **1.1 Male infertility**

Infertility is most commonly defined by the failure to conceive after a minimum of 12 months of regular unprotected intercourse (Rowe, 1993, Irvine, 1998, NICE, 2013). In the UK an estimated 3.5 million people suffer from infertility (HFEA 2011). Information for prevalence of infertility worldwide is based on data from either clinically based, (Thonneau et al., 1991, Hull et al., 1985) or population based, (Anderson et al., 2009, Walschaerts et al., 2013) studies and are in agreement that approximately one in six couples worldwide will have problems conceiving during their reproductive life (Irvine, 1998, Boivin et al., 2007). A common factor in each of these studies is that male factor infertility is the single most common diagnostic category (Hull et al., 1985, Thonneau et al., 1991, Schmidt et al., 1995), thus, male sub-fertility is a significant global problem and its prevalence is continuing to negatively impact on artificial reproductive technology (ART) (Barratt et al., 2011, Tomlinson et al., 2013). There is an urgent requirement to develop new tests for accurately diagnosing male infertility and new treatments that are specific to male factor conditions.

## **1.2 Diagnosis of male infertility**

### **1.2.1 Semen analysis**

It is difficult to diagnose male infertility and the universal approach that is most often adopted is using conventional criteria described by the World Health Organisation (WHO, 1987, WHO, 1992, WHO, 2010e). This criterion is solely based upon a descriptive semen analysis involving the assessment of morphology, motility and sperm concentration. However, it has generally been accepted that semen analysis is of little clinical value for diagnosing infertility (Tomlinson et al., 1999, Agarwal and Said, 2011) with the exception of circumstances where the man has very low numbers of motile sperm (Macleod and Gold, 1951a). Many groups have examined the robustness of semen analysis as a tool for diagnosing infertility with similar conclusions being

ascertained as the first study by Macleod and Gold in 1951 (Macleod and Gold, 1951a, Cooper et al., 2010). Since this conclusion that semen analysis is of limited value in the prediction of male fertility and fertilising ability, there has been a need to develop further assessments for evaluating male fertility. To fill this area methods have been developed that evaluate sperm functional competence.

### **1.2.2 Sperm Function Assays**

A number of assays have been developed that are capable of assessing sperm function which evaluates the capabilities of sperm to progress through the female reproductive tract and interact with the oocyte (Aitken, 2006, Lefievre et al., 2007, Barratt et al., 2011). However, currently these tests have yet to make a significant impact on management of infertility in a clinical setting (Lefievre et al., 2007, Barratt et al., 2011).

Sperm motility is one of the most important sperm functions generally analysed through semen analysis (Macleod and Gold, 1951a, Curi et al., 2003, Darszon et al., 2011).  $\text{Ca}^{2+}$  plays a fundamental role in motility, specifically in hyperactivated motility (Ho, 2001, Darszon et al., 2011, Barratt et al., 2011). Current knowledge on how  $\text{Ca}^{2+}$  is regulated by the cell has increased substantially and although there are still gaps in our understanding, clinical research suggests that  $\text{Ca}^{2+}$  regulation could be used as a means of identifying sperm dysfunction (Krausz et al., 1996, Costello et al., 2009, Barratt et al., 2011). Currently there are few options available for diagnosing male infertility both clinically and in the laboratory and for this reason semen analysis was used in this study, along with functional assays, including the Kremer mucus penetration test, to determine fertilising capabilities of spermatozoa.

The Kremer sperm penetration test examines the sperm-mucus interaction *in vitro*. It makes use of a glass-capillary tube filled with cervical mucus or a cervical mucus

substitute (methylcellulose) which is placed in contact with a sperm sample (Kremer, 1965, Ivic, 2002). The test predominantly highlights the proportion of progressively motile sperm in a sample identifying the number of sperm capable of mucus penetration (Katz et al., 1980). This is a simple and effective *in vitro* test known to provide important information about sperm function (Barratt et al., 1989, Abu-Heija et al., 1996, Ivic, 2002).

### **1.3 Asthenozoospermia**

Asthenozoospermia (low sperm motility), is defined clinically by a semen analysis where the total number of motile cells is less than 40% and/or the percentage of progressively motile cells is less than 32% (WHO, 2010b). A large retrospective study determined that 81% of infertile men are diagnosed with asthenozoospermia, 19% of these men had isolated asthenozoospermia and 63% had asthenozoospermia in combination with teratozoospermia (poor sperm morphology) and/or oligozoospermia (low sperm concentration) (Curi et al., 2003). A low sperm motility can be caused by structural (Chemes et al., 1998, Imai et al., 2001, Visser et al., 2011) or functional deficiencies (Makler et al., 1980), by a deleterious effect of seminal plasma (Mortimer et al., 1998) or by a combination of these factors. There is currently no drug a man can take, nor can be added to sperm *in vitro* to increase sperm motility (Barratt et al., 2011).

### **1.4 The Use of ART**

Currently the main treatment options available for motility dysfunction or sperm dysfunction, as a whole, is ART. ART comprises of a range of treatments according to the severity of sperm dysfunction, i.e. for mild male factor sub-fertility the treatment is IUI (Intra Uterine Insemination), for moderate IVF (In Vitro Fertilisation) and for severe ICSI (Intra Cytoplasmic Sperm Injection), all of which are expensive and invasive (Tournaye, 2012). The use of ART worldwide is increasing with an estimated 5

million babies being born worldwide using artificial reproductive technology (ART) (ESHRE, 2012). However, the cost of ART is a major deterrent to couples who need treatment in both developing and developed countries (Rauprich et al., 2010). Sperm movement characteristics and sperm morphology are significantly, positively correlated with fertilisation rates *in vitro* (Sukcharoen et al., 1995, Sukcharoen et al., 1996) suggesting that the ability to develop compounds that could increase sperm movement characteristics would have a positive effect on *in vitro* fertilisation rates.

### **1.5 Capacitation**

Capacitation (Austin, 1951, Chang, 1951) is the term given to the processes that sperm need to undertake *in vivo* in the female reproductive tract to acquire the ability to fertilise oocytes. Sperm capacitation was first discovered independently by Austin and Chang in rats and rabbits (Austin, 1951, Chang, 1951). The process of capacitation is poorly defined owing to there being no clear marker to indicate the occurrence of capacitation (Breitbart, 2002). However, capacitation has been characterized as a complex of changes (structural and functional) that occur in the spermatozoon after a period of time spend in the female reproductive tract (De Jonge, 2005). Some of these changes in mammals include: increases in intracellular  $\text{Ca}^{2+}$  and cAMP concentrations along with changes in the lipid composition of the plasma membranes resulting in a net cholesterol decrease (McLeskey et al., 1998, O'Flaherty et al., 2006, de Lamirande and O'Flaherty, 2008, Buzadzic et al., 2014). The removal of cholesterol and other sterols (de-capacitating factors) from the sperm plasma membrane, during capacitation, along with the non-covalently attached glycoproteins being released from the epididymis, produces a more fluid membrane environment that prime the spermatozoa for further fertilisation cues (Ikawa et al., 2010). During capacitation changes also occur in oxidative metabolism, tyrosine phosphorylation, sperm motility (altering to the hyperactivated state) and a drastic reorganisation, removal or modification of proteins

within the spermatozoa. Changes in the sperm plasma membrane are essential for the initiation of sperm motility, hyperactivation and induction of the acrosome reaction (Gadella and Visconti, 2006). The ability of the spermatozoa to hyperactivate and undergo the acrosome reaction are essential for fertilisation (Bavister, 2002, Liu et al., 2007). If spermatozoa are unable to capacitate they will be unable to fertilise an oocyte successfully without assistance (Fraser, 1998).

### **1.6 Initiation of sperm motility**

Mammalian spermatozoa are not motile in the male body they acquire motility after ejaculation (Lindemann and Kanous, 1989, Si and Okuno, 1995, Buzadzic et al., 2014). Motility is usually initiated by changes in environmental conditions, such as ionic concentrations or osmotic stimulation (Morisawa, 1994). During capacitation, early events which are regulated by a  $\text{HCO}_3^-$ /soluble adenylyl cyclase (sAC)/cAMP/protein kinase A (PKA) pathways initiate sperm motility (Lee and Storey, 1986, Boatman and Robbins, 1991b, Salicioni et al., 2007, Visconti, 2009). Sperm motility is initiated immediately after sperm are released from the epididymis after contact with high concentrations of  $\text{HCO}_3^-$  in the seminal plasma and female reproductive tract ( $\geq 20\text{mM}$ ) (Chen et al., 2000, Zhou et al., 2005, Xie et al., 2006).  $\text{HCO}_3^-$  plays an important role in mediating sperm motility and its actions are determined by activation of sAC, which initiate an increase in cytoplasmic cAMP levels (Chen et al., 2000, Xie et al., 2006, Battistone et al., 2013). This elevated cAMP level triggers activation of PKA which stimulates serine/threonine phosphorylation of target proteins, and ultimately, activates tyrosine phosphorylation of proteins located mainly in the flagellum in mammals (Carrera et al., 1996, Naz and Rajesh, 2004, Battistone et al., 2013). Knock out studies have been conducted on sAC and PKA null mice resulting in infertile males, however, addition of cell permeant cAMP reinstates flagella beat and sperm motility (Esposito et al., 2004, Xie et al., 2013). This indicates that the motility impairment noted is not due

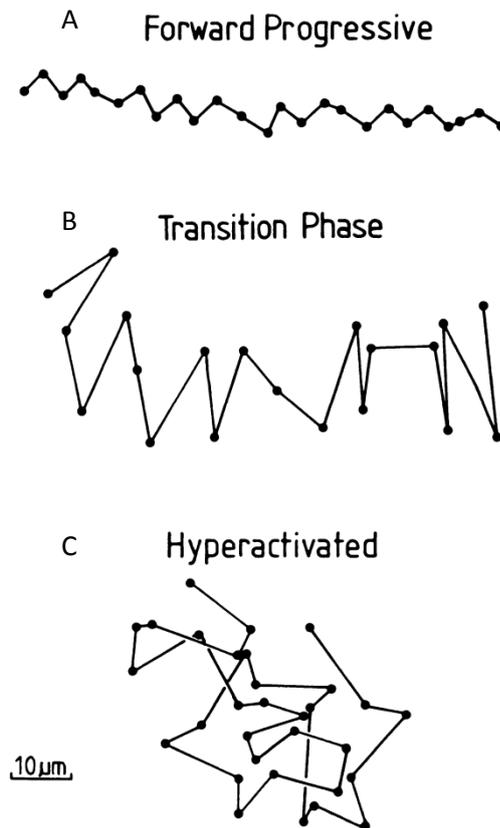
to a structural alteration or defect in the sAC null mice (Esposito et al., 2004, Xie et al., 2013).

As previously described, during capacitation, cAMP levels increase resulting in tyrosine phosphorylation, which, is one mechanism for maintaining sperm motility. In addition to this the membrane potential of the cell becomes hyperpolarised and intracellular pH ( $\text{pH}_i$ ) along with intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) increases in both mice (Visconti et al., 1995b), and humans (Brewis et al., 2000, Visconti et al., 2002). Mature sperm stored in the epididymis are quiescent due to the male reproductive tract having an acidic pH (~5.5 to 6.5). Sperm motility is initiated on ejaculation when sperm are mixed with seminal plasma (extracellular  $\text{pH} > 7$ ) which cause the  $\text{pH}_i$  to increase within the cell to approximately 6.8 (Hamamah et al., 1996), subsequently, while traversing the female reproductive tract, the  $\text{pH}_i$  is elevated further (Hamamah et al., 1996, Lishko et al., 2012b, Kirichok and Lishko, 2011). Intracellular alkalinisation is another important factor that regulates the initiation of sperm motility, capacitation and hyperactivation (Hamamah et al., 1996, Suarez, 2008, Lishko and Kirichok, 2010).

### **1.6.1 Hyperactivation**

Hyperactivation is the term given to the change in sperm swimming and is a concomitant event with capacitation in humans, it involves a change from symmetrical flagella beats to high amplitude, asymmetrical flagella bending (Suarez, 2008). It was first described in sperm from hamsters undergoing capacitation *in vitro* and similar patterns of movement were observed *in situ* through the walls of the oviductal ampulla of golden hamsters at the time of fertilisation suggesting a potential role in fertilisation (Yanagimachi, 1970). Hyperactivation has since been observed *in vitro* in spermatozoa from a variety of mammals including; rabbit (Johnson et al., 1981), mouse (Fraser,

1977), dog (Mahi and Yanagimachi, 1978), bull (Singh et al., 1983) and human (Mortimer et al., 1984). Hyperactivated motility is a property of the flagella although it is often measured according to changes in head movement. The changes observed between non-hyperactivated and hyperactivated spermatozoa are results of changes in the degree of axonemal bending along with changes in the propagation of flagella beats (Mortimer et al., 1997). Studies on human sperm capacitation have identified three main patterns of movement: (i) forward progressive motility (figure 1-1A), (ii) transition motility which is similar to progressive motility but has a more irregular track (figure 1-1B) and (iii) hyperactivated motility (figure 1-1C) (Mortimer and Mortimer, 1990). *In vitro*, hyperactivated spermatozoa have been described as swimming in ‘circles’ or having a ‘starspin’ motion (Ho and Suarez, 2001, Mortimer and Swan, 1995, Burkman, 1991). The hyperactivated movement pattern of human sperm is ‘biphasic’, similarly to rabbit sperm, meaning that the spermatozoa switch between a whiplash (hyperactivated) phase and a progressive phase (non-hyperactivated), depending on the environmental cues from the female reproductive tract and oocyte vestments, allowing the cell to alter its motility according to the requirements of the environment (Pacey et al., 1995, Mortimer and Swan, 1995, Mortimer and Mortimer, 1990, Olson et al., 2011).



**Figure 1-1 Schematic of motility patterns identified during capacitation.** (A) forward progressive motility, (B) transition phase – moving in a forward direction but with larger ‘whiplike’ action and (C) hyperactivated motility. Capacitated sperm are biphasic and switch between the three patterns of movement. (Mortimer and Mortimer, 1990)

Hyperactivation is crucial to sperm function aiding the sperm to travel along the female reproductive tract. The exact role that hyperactivation plays is still debated but, evidence suggests, it may facilitate migration through the highly viscous cervical mucus and enable penetration of the layers surrounding the oocyte (Suarez et al., 1991, Suarez, 2008, Ren et al., 2001). Experiments have also shown that hyperactivation may be required to detach the sperm from the oviductal epithelium in both animals and humans (Demott and Suarez, 1992, Pacey et al., 1995). Clinical studies in humans have provided data identifying that the percentage of hyperactivated sperm cells correlates with successful fertilisation *in vitro* (Sukcharoen et al., 1995). The central regulator of hyperactivated motility is an elevated  $[Ca^{2+}]_i$  level which is required for both initiation and maintaining the hyperactivated state (Alasmari et al., 2013a). There are at least two sources of  $Ca^{2+}$  that regulate the hyperactivated state in mammalian sperm these are;

entry through pH dependent CatSper channels located in the plasma membrane of the principle piece of the flagella (Ren et al., 2001, Olson et al., 2010, Ren and Xia, 2010) and mobilisation of stored  $\text{Ca}^{2+}$  from the neck/midpiece region of the sperm cell (Costello et al., 2009, Ho and Suarez, 2001).

## **1.7 The role of $\text{Ca}^{2+}$ signalling in sperm motility**

Sperm lack endoplasmic reticulum meaning functional regulation by transcription/translation will be very limited. Therefore, post-translational mechanisms primarily control all activities of the cell (Costello et al., 2009). One such mechanism involves changes in intracellular  $\text{Ca}^{2+}$  concentration.  $[\text{Ca}^{2+}]_i$  signalling is achieved through  $\text{Ca}^{2+}$  entering the cytoplasm (low  $\text{Ca}^{2+}$  concentration) from the extracellular space or intracellular organelles ( $\text{Ca}^{2+}$  concentration four orders of magnitude higher) (Costello et al., 2009). It is well established that  $\text{Ca}^{2+}$  permeable channels exist in the plasma membrane of sperm cells. To initiate signalling  $\text{Ca}^{2+}$  channels need to open to allow the flow of ions down their electrochemical gradient (Costello et al., 2009). Regulation of protein function through  $\text{Ca}^{2+}$  signalling plays a central role in controlling activities that are vital for sperm function, including motility, hyperactivation, chemotaxis and the acrosome reaction (Publicover et al., 2007, Darszon et al., 2011).

### **1.7.1 Calcium channels in sperm**

#### **1.7.1.1 Voltage gated $\text{Ca}^{2+}$ channels**

Voltage-gated  $\text{Ca}^{2+}$  channels ( $\text{Ca}_v$ ) produce increases in  $[\text{Ca}^{2+}]_i$  in response to membrane potential ( $E_m$ ) changes (Darszon et al., 2011). The channels have been divided into two separate groups: high voltage activated (HVA), defined as N-type, P/Q-type, and R-type  $\text{Ca}^{2+}$  currents which require strong depolarisation for activation (Tsien et al., 1991, Catterall and Few, 2008) and low voltage activated (LVA) which were initially classified as T-type because they open transiently, need weaker

depolarisations to open and inactivate faster at more negative potentials (Darszon et al., 2011).

G-proteins have been found to regulate  $\text{Ca}_v$  channels through many different pathways, this can be via direct activation with physical interactions between the channel and the G-protein or by indirect activation via second messengers and/or protein kinases (Dascal, 2001).  $\text{Ca}_v$  channels can be stimulated by several protein kinases including PKA, protein kinase C (PKC) and  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase II (CaMKII) (Welsby et al., 2003, Catterall, 2000, Catterall and Few, 2008, Dai et al., 2009).  $\text{Ca}^{2+}$ , itself, is known to inactivate  $\text{Ca}_v$  channels by associating with CaM. The mechanisms are best known for  $\text{Ca}_v1$  and  $\text{Ca}_v2$  channels, however it is possible that  $\text{Ca}_v3$  channel functions could also be influenced by CaM (Lopez-Gonzalez et al., 2001). RT-PCR experiments have revealed the presence of transcripts for  $\text{Ca}_v1$ , 2 and 3 in mouse spermatogenic cells and mature human sperm (Goodwin et al., 2000, Park et al., 2003, Serrano et al., 1999). Patch clamp studies have not been able to reveal  $\text{Ca}_v1$  or  $\text{Ca}_v2$  channels in spermatogenic cells however;  $\text{Ca}_v3$  currents have been well documented in spermatogenic cells in mice and humans using this technique (Darszon et al., 2006, Escoffier et al., 2007, Liévano et al., 1996). These studies suggest that these channels could be present in an electrophysiologically inactive state and may become activated during sperm maturation (Darszon et al., 2011).

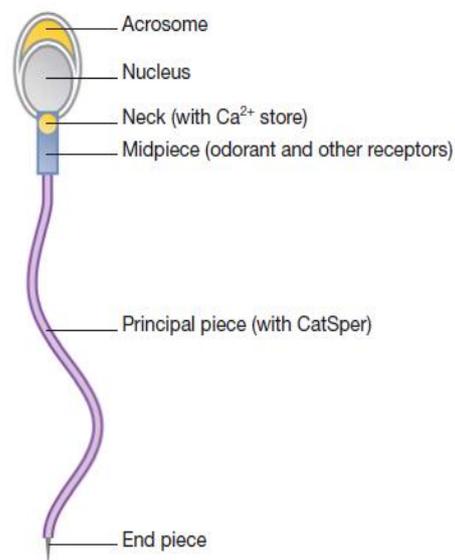
#### ***1.7.1.2 Voltage-gated proton channel $H_v1$***

In mammals the regulation of  $\text{pH}_i$  is fundamental for the initiation of sperm motility, capacitation, hyperactivation and the acrosome reaction (Yanagimachi, 1994). The proton selective, voltage-gated ion channel ( $H_v1$ ) was cloned in 2006 and it has been reported that voltage-gated proton channels are functionally expressed in mammalian sperm (Lishko et al., 2010).  $H_v1$  is abundantly expressed in human sperm cells and is located to the principal piece of the flagellum. This makes it perfectly located to activate

pH-dependent proteins of the axoneme and control motility (Ren et al., 2001, Kirichok and Lishko, 2011, Lishko and Kirichok, 2010). A role for H<sub>V</sub>1 in the regulation of [Ca<sup>2+</sup>]<sub>i</sub> homeostasis has been proposed involving active proton extrusion to maintain pH<sub>i</sub> balance after CatSper activation (Lishko et al., 2012b). Potentially, a depolarizing stimulus could also create an increase in pH<sub>i</sub> via the H<sub>V</sub>1 channel resulting in activation of CatSper and hyperactivated motility (figure 1-2) (Ren and Xia, 2010, Mannowetz et al., 2013).

### 1.7.1.3 *CatSper*

CatSper is a sperm specific cation channel that facilitates the entry of Ca<sup>2+</sup> into spermatozoa (Barratt and Publicover, 2012, Lishko et al., 2011). The CatSper channel is located in the principle piece of the flagellum and through this position it is able to have a key role in sperm motility (figure 1-2) (Barratt and Publicover, 2012, Ren et al., 2001).



**Figure 1-2 Diagram of labelled Human Spermatozoa indicating position of CatSper channel.** Yellow areas highlight stores of Ca<sup>2+</sup> (Barratt and Publicover, 2012)

The CatSper ion channel is comprised of four homologous pore-forming  $\alpha$  subunits (CatSper 1-4) and three auxiliary subunits, CatSper  $\beta$ ,  $\gamma$  and  $\delta$  (Brenker et al., 2012). The Channel was identified through the use of the Patch clamp technique. Research using this technique discovered that Ca<sup>2+</sup> entry into sperm triggered by progesterone is

through CatSper (Strunker et al., 2011, Lishko et al., 2011, Barratt and Publicover, 2012). An influx of  $\text{Ca}^{2+}$  is crucial for spermatozoa to be capable of fertilisation (Publicover et al., 2007) having been shown to play a role in hyperactivation, chemotaxis and the acrosome reaction (Publicover et al., 2007, Costello et al., 2009).

CatSper initiated increases in  $[\text{Ca}^{2+}]_i$  can be induced by some of the most important stimuli in sperm function including cyclic nucleotides, alkaline depolarisation, zona pellucida glycoproteins and bovine serum albumin, although many of the mechanisms of action are still not understood (Ren and Xia, 2010). The CatSper channel is pH sensitive and physiologically progesterone and prostaglandin have been identified as its most potent activators (Strunker et al., 2011, Brenker et al., 2012, Barratt, 2011). Progesterone has been shown to have an effect on CatSper *in vitro* at equivalent concentrations to the normal concentrations that the sperm would encounter *in vivo* (3-5 $\mu\text{M}$ ) (reviewed in (Baldi et al., 1998)). Both high and low concentrations of progesterone have been shown to rapidly increase  $[\text{Ca}^{2+}]_i$  through  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release (CICR) (Sagare-Patil et al., 2012). Activation by progesterone causes an influx in  $\text{Ca}^{2+}$  through the CatSper channel which triggers multiple  $\text{Ca}^{2+}$  dependent physiological responses that are essential for fertilisation (Lishko et al., 2011). It had previously been believed that progesterone acted to elevate cAMP levels, which in turn, elevated  $[\text{Ca}^{2+}]_i$  in human spermatozoa (Ren et al., 2001). However, Strunker *et al* were unable to confirm this suggesting that signalling in human spermatozoa is not through G-protein coupled receptor operated cAMP (Strunker et al., 2011). Strunker *et al* reasoned that the principle voltage gated  $\text{Ca}^{2+}$  channel in human sperm is CatSper.

Along with progesterone, voltage- sensitive  $\text{Ca}^{2+}$  selective currents have been shown to induce hyperactivation via CatSper. These currents are potentiated by a rise in intracellular pH suggesting that the alkalinisation that occurs during capacitation acts to

increase CatSper conductance shifting it to a more negative membrane potential (figure 1-3). This shift increases CatSper mediated  $\text{Ca}^{2+}$  entry, increasing  $[\text{Ca}^{2+}]_i$ , which in turn activates hyperactivation (Qi et al., 2007).

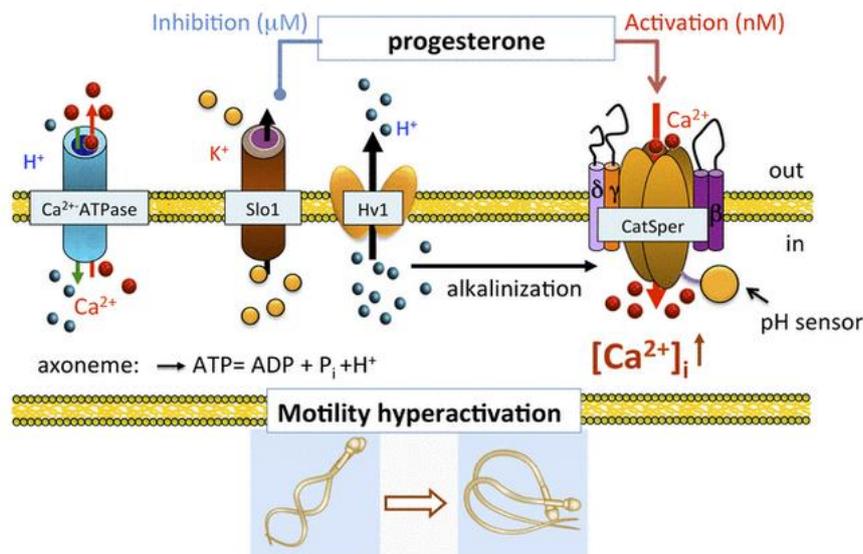
Knock out studies on mice identified that disruption to any of the four genes results in sperm abnormalities including decreased total motility, progressive motility and an inability to undergo hyperactivation rendering them infertile (Qi et al., 2007, Avidan et al., 2003). Currently in humans defects have been found on CatSper1, 2, 3 and 4 that have been linked to infertility (Visser et al., 2011). Failed fertilisations as a result of mutations were attributed to the sperm being unable to evoke a depolarisation, which is caused by  $\text{Ca}^{2+}$  entry, eliminating their ability to become hyperactivated (Hildebrand et al., 2010).

The CatSper associated progesterone receptor is sperm specific and structurally differs from the genomic form. This enables it to be a specific target for development of drugs to aid fertility or as a novel male non-hormonal contraceptive (Lishko et al., 2011, Carlson et al., 2009). Functional CatSper is only found in mature spermatozoa indicating that there should be no adverse effects anywhere else in the body if targeted specifically. HC-056456 (blocks alkaline KCl-evoked increases in  $[\text{Ca}^{2+}]_i$  and  $[\text{Na}^+]_i$ ) has been shown to be effective at blocking CatSper which resulted in treated sperm being unable to hyperactivate. Furthermore, addition of this compound to already hyperactivated sperm resulted in a complete reversal from the hyperactivated state back to a normal swimming pattern (Carlson et al., 2009). This indicates the importance of CatSper and  $\text{Ca}^{2+}$  in sperm motility.

#### **1.7.1.4 *KSper***

During capacitation sperm cells undergo intracellular alkalinisation, increases in  $[\text{Ca}^{2+}]_i$  and membrane hyperpolarization (Visconti et al., 2002). These processes are regulated

by sperm ion channels including H<sub>v</sub>1 (major H<sup>+</sup> channel) and CatSper (major Ca<sup>2+</sup> channel) in human sperm (Ren et al., 2001, Kirichok et al., 2006, Lishko and Kirichok, 2010, Lishko et al., 2011). However, until recently the identity of the main K<sup>+</sup> channel in human sperm remained unknown. K<sup>+</sup> channels are vital functional components of sperm physiology regulating membrane potential and cell motility (Mannowetz et al., 2013, Mansell et al., 2014). In mice an alkalinisation-sensitive, Ca<sup>2+</sup> insensitive sperm K<sup>+</sup> channel (Slo3) was found to be essential for male fertility and it had been assumed that the principle K<sup>+</sup> channel in human sperm would have a similar molecular identity (Schreiber et al., 1998, Santi et al., 2010). However, recent examination through the patch-clamp technique in human ejaculated spermatozoa found that human K<sup>+</sup> currents are insensitive to intracellular alkalinisation but dependent upon [Ca<sup>2+</sup>]<sub>i</sub> (Mannowetz et al., 2013). This suggested that the identity of human KSper (hKSper) may be distinct from that of murine and mice KSper which are represented by the Slo3 protein. The Slo1 channel contains structural additions allowing these channels to sense changes in voltage and [Ca<sup>2+</sup>]<sub>i</sub> (Schreiber et al., 1999). Staining with anti-Slo1 antibodies selectively stained the principle piece of the flagellum which is the same location as CatSper and H<sub>v</sub>1 (Mannowetz et al., 2013, Kirichok and Lishko, 2011). A proposed model for KSper channels indicates that KSper may act to coordinate the actions of H<sub>v</sub>1 and CatSper during capacitation resulting in elevated levels of [Ca<sup>2+</sup>]<sub>i</sub> and initiation of hyperactivated motility (figure 1-3) (Mannowetz et al., 2013).



**Figure 1-3 Role of human Ksper (Slo1) in sperm physiology and hyperactivation.**

As sperm travel through the uterus and fallopian tube they are exposed to progesterone (P) (pM – nM range), intracellular alkalinisation is evoked by Hv1 which partially activates CatSper. However, to fully activate CatSper the membrane must become depolarized. This is not achieved until sperm are in the proximity of the oocyte exposing them to micromolar concentrations of P which blocks hKsper resulting in membrane depolarization. This allows CatSper to become fully activated, triggering sperm hyperactivation and making fertilisation possible (Mannowetz et al., 2013).

### 1.7.2 Calcium stores in sperm

During the final stages of sperm differentiation, during nuclear volume reduction, excess material is packaged into the redundant nuclear envelope (RNE) of which an established function is unknown. This structure has been identified in sea urchin and mammalian spermatozoa and it has now been proposed that it functions as a  $\text{Ca}^{2+}$  store (Garnier-Lhomme et al., 2009, Darszon et al., 2011). For an organelle to be classified as having  $\text{Ca}^{2+}$  stores it must have two types of  $\text{Ca}^{2+}$  transporters; one enabling loading of the store and one enabling  $\text{Ca}^{2+}$  release from the store on demand (Costello et al., 2009).

Inositol trisphosphate receptors ( $\text{IP}_3\text{Rs}$ ) and ryanodine receptors ( $\text{RyRs}$ ) have been identified as the major intracellular  $\text{Ca}^{2+}$  channels located in neck/midpiece region of sperm (figure 1-2). The activity of these channels is dependent on the action of second messenger pathways such as; inositol triphosphate ( $\text{IP}_3$ ), cyclic ADP ribose (cADPR)

and nicotinic acid–adenine dinucleotide phosphate (NAADP) (Zhu et al., 2010a, Zhu et al., 2010b) and they are believed to play a role in sperm hyperactivation.

### **1.8 Flexstation screening assay**

The induction of high- throughput screening (HTS) in the 80's for drug discovery became a significant tool for identifying hits in pharmaceutical research (Terstappen et al., 2010). HTS is a well established process for lead discovery that is now being used for basic and applied research (Mayr and Bojanic, 2009). The process involves the use of automation, miniaturization assays and large scale data analysis to screen chemical libraries against a variety of extracellular and intracellular molecular targets in an attempt to identify novel chemotypes with a desired mode of action (Mayr and Bojanic, 2009). Recently the availability of large compound sources and identification of novel biological targets has dramatically changed the drug discovery process. HTS has evolved into a mature discipline that acts as a crucial source of chemical starting points for drug discovery (Mayr and Bojanic, 2009).

Complex systems such as spermatozoa that rely on post-translational modifications make discovery of new drugs for fertility fundamentally challenging. In an attempt to meet the unmet need for development of alternate treatment to ART, for male factor infertility, HTS offers a novel approach to drug discovery. HTS technology has evolved to allow for screening of focused libraries towards specific biological targets (Mayr and Bojanic, 2009, Miller, 2006). A screening library at the University of Dundee (UoD) Drug Discovery Unit (DDU) is currently being utilised in projects to identify new chemical starting points focused on ion channel targets for male factor infertility (Mok and Brenk, 2011). The passage of ions across a cell membrane is tightly regulated by ion channels. Ion channels are integral membrane proteins that are encoded by approximately 400 ion channel genes in the human genome (Mok and Brenk, 2011).

This family of membrane proteins is implicated in many important physiological functions in excitable and non-excitable cells and they underlie a wide range of diseases (Terstappen et al., 2010, Mok and Brenk, 2011, Lu and An, 2008). As a result, they are the third largest class of targets, after G-protein coupled receptors and protein kinases, in drug discovery (Lu and An, 2008, Mok and Brenk, 2011).

Martins da Silva *et al* (2012) developed and optimised a novel high throughput screening assay that assessed  $\text{Ca}^{2+}$  responses evoked by progesterone in human sperm (Martins da Silva et al., 2012). This HTS assay utilised a Flexstation microplate reader to measure increases in  $[\text{Ca}^{2+}]_i$  as a response to progesterone exposure (Martins da Silva et al., 2012). This has enabled screening of a chemogenomics library, a novel UoD DDU ion channel library and a library targeting GABA A receptors to identify compounds that increase  $[\text{Ca}^{2+}]_i$  in human sperm cells. The Flexstation assay uses  $\text{Ca}^{2+}$  as a surrogate for motility and is being used as the primary assay for identifying novel drugs that have the potential of modulating sperm motility.

### **1.9 Chemical stimulation of sperm motility**

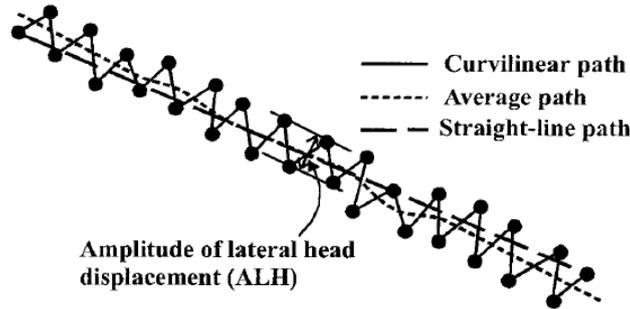
Previous attempts at improving ART fertilisation rates through chemical stimulation highlighted non-specific inhibitors of phosphodiesterases (PDEIs) e.g. pentoxifylline (PTX) as a potential treatment. PDEs hydrolyze the 3', 5'-phosphodiester bonds of cAMP or cGMP to regulate the effects of these important messengers (Lefievre et al., 2000). In 1990 Yovich *et al* (1990) released a study that identified significantly improved fertilisation rates after using PTX (Yovich et al., 1990). The use of PTX in sperm preparations, for couples undergoing fertility treatment, has also reduced the rates of failed fertilisation when dealing with male factor infertility (Yovich, 1993). PTX increased hyperactivated motility in human spermatozoa when under conditions that support capacitation (Kay et al., 1993). A further study reported higher pregnancy rates

after PTX treatment used in combination with IUI (Stone et al., 1999). However, results from PTX use were inconsistent with several groups identifying lower fertilisation rates *in vitro* along with the occurrence of a premature acrosome reaction (Tesarik et al., 1992, Ford et al., 1994, Yovich, 1993). PTX introduced the possibility of chemical stimulation for clinical application, although its use was discontinued, the potential for specific PDEi's in treating male factor infertility warrants further study.

### **1.10 Computer assisted sperm analysis (CASA) of sperm motility**

Dedicated computer assisted sperm analysis systems have been available commercially for 25 years (Mortimer and Mortimer, 2013a) and are capable of measuring sperm motility and sperm movement characteristics (kinematics) (Mortimer, 1997). Technical issues concerning their ability to accurately determine sperm concentration in semen (Mortimer et al., 1995, Fraser et al., 1997) has discredited their use since the beginning. However, in washed preparations CASA is capable of reliably and accurately determining sperm kinematics, percentage motility and concentration provided that recommended criteria are followed (Fraser and Group, 1998). CASA analysis has proved to be invaluable in research and in infertility diagnosis (Mortimer, 2000b). It has to be noted that averaged values of sperm kinematic measures from a population alone are meaningless and to identify sub-populations that are biologically accurate measures have to be determined on a cell-cell basis by combining several kinematic parameters (Fraser and Group, 1998, Mortimer and Mortimer, 2013a). The kinematic values determined by CASA for each individual sperm cell includes the velocity of movement, the width of the sperm head's trajectory and the frequency of the change in direction of the sperm head (David et al., 1981). The velocity parameters determined are the average path velocity (VAP), which is the average direction of movement measured for the distance travelled by the sperm cell in the observable area, curvilinear velocity (VCL) which refers to the total distance covered by the sperm head and straight-line velocity

(VSL) which is determined from the straight-line distance between the first and last points of the trajectory (figure 1-4).



**Figure 1-4. Schematic identifying motility trajectories as determined by CASA.**  
(Mortimer, 2000b)

The shape of the trajectory influences the velocity values and three further velocities; Linearity (LIN), Straightness (STR) and Wobble (WOB) can be calculated from the ratios of the velocity values (Mortimer, 2000b). These values are determined as follows:

$$\text{LIN} = (\text{VSL}/\text{VCL}) \times 100$$

$$\text{STR} = (\text{VSL}/\text{VAP}) \times 100$$

$$\text{WOB} = (\text{VAP}/\text{VCL}) \times 100$$

The CASA also determines the amplitude of lateral head displacement (ALH) from the VAP and is calculated as the total width of the lateral movement of the sperm head (figure 1-4). Beat cross frequency (BCF) is calculated by counting the number of times the curvilinear path crosses the average path per second. This value is useful when estimating the change in the flagella pattern but a limitation of this is the limited number of observations that can be made per second, if there are more beats per second than images captured per second then the BCF will be underestimated (Mortimer and Swan, 1999, Mortimer, 2000b). To be able to determine useful information from sperm kinematic analysis a range of normal kinematic values for movement need to be identified and matched to a proportion of sperm cells in a population that meet the

criteria (Mortimer, 2000b). Using this criteria and CASA it is then possible to determine the likelihood that a proportion of spermatozoa from a semen sample are capable of penetrating his partner's cervical mucus quickly and without having to obtain cervical mucus (Mortimer, 2000b). Motility parameters such as hyperactivated motility which, under normal circumstances, is only exhibited under capacitating conditions in human sperm, analysed at 60Hz, have to meet the criteria of  $VCL \geq 150 \mu\text{m/s}$  and  $LIN \leq 50\%$  and  $ALH \geq 7.0 \mu\text{m}$  (Mortimer et al., 1998). Non-capacitated human sperm cells or sperm cells in semen normally portray parameters of motility that are defined by CASA under slow ( $VAP \leq 5 \mu\text{m/s}$ ), medium ( $VAP$  between  $5\text{-}25 \mu\text{m/s}$ ), rapid ( $VAP \geq 25 \mu\text{m/s}$ ) and/ or progressively motile ( $VAP \geq 25 \mu\text{m/s}$ ,  $STR \geq 80\%$ ,  $ALH \geq 2.5 \mu\text{m}$  and  $< 7.0 \mu\text{m}$ ) (Mortimer and Mortimer, 2013a). The benefits of CASA analysis, both in research and in the clinical laboratory, is diverse and through correct practice is able to give greater understanding of the physiological implications of sperm motility. This makes it possible to quickly predict the ability of a man's sperm to penetrate his partner's cervical mucus and to identify sperm showing hyperactivated motility under capacitating conditions to help identify the best course of treatment (Mortimer, 2000b, Mortimer and Mortimer, 2013a).

### **1.11 Thesis Aims**

Previous research has identified the importance of  $\text{Ca}^{2+}$  signalling for sperm motility. Clinical studies have suggested a relationship between low intracellular  $\text{Ca}^{2+}$  and male factor infertility and numerous studies have highlighted the importance of sperm motility when correlated to fertilisation success. There are still many questions regarding  $\text{Ca}^{2+}$  signalling and regulation of motility in human sperm especially regarding the central role that  $\text{Ca}^{2+}$  signalling plays in sperm function.  $\text{Ca}^{2+}$  channels have been identified as potentially important targets for pharmacological interventions,

therefore, a series of experiments were designed and conducted with the following aims in mind:

1. To identify a sub-group from donor spermatozoa that can be used as a surrogate for patient samples.
2. To develop a standard operating procedure for screening compounds identified from the Flexstation high throughput screen to identify compounds that enhance motility.
3. To examine the effects of novel compounds mediating sperm motility on research sperm donors and sub-fertile patients.
4. To determine, by assessing data from two established clinics the incidence of asthenozoospermia in isolation and combination with other sperm parameters to identify patients who would benefit from treatment from therapeutic advancements and relate this to fertilisation success.

## **CHAPTER 2**

### **Methods and Materials**

## 2.1 Reagents and Stocks

Stock solutions of 1% dimethyl sulfoxide (DMSO) in distilled water were prepared on a monthly basis and stored at room temperature until use.

To make the stocks for PHA665752, inhibitor of MET kinase and Leelamine hydrochloride (CB<sub>1</sub> agonist), each were dissolved in DMSO to a concentration of 80mM and stored in the freezer (-20°C) (source and catalogue numbers see appendix). These 80mM stocks were further diluted in dH<sub>2</sub>O to make aliquots with a final concentration of 4mM. The final concentration of PHA665752 and Leelamine hydrochloride when in the sperm suspension was 40µM, which is the same concentration the compounds were screened at through the Flexstation assay screen, with final concentration of DMSO not exceeding 1%.

Stock solutions for GP1a (CB<sub>2</sub> agonist), JX401 (inhibitor of p38α), EO1428 (inhibitor of p38α and p38β) and Trequinsin hydrochloride (inhibitor of cGMP-inhibited phosphodiesterase, PDE3) were prepared by dissolving in DMSO (GP1a, EO1428 and Trequinsin hydrochloride 80mM, JX401 70mM), and stored in the freezer (-20°C) (source and catalogue numbers see appendix). Aliquots of these stocks were further diluted in DMSO to a final volume of 4mM, and stored in the freezer (-20°C) for no longer than 4 months. The final concentration of each; GP1a, JX401, EO1428 and Trequinsin hydrochloride, in sperm suspension was 40µM to ensure it matched the concentration of the compounds in the Flexstation assay screen, with a final concentration of DMSO not exceeding 1%.

Stock solutions for A1-H1 and A2-G2 were produced by the UoD DDU at a concentration of 10mM made up in 100% DMSO and further diluted in dH<sub>2</sub>O to a concentration of 1mM and stored at 4°C. The final concentration of each of these

compounds in sperm suspension was 10 $\mu$ M (same concentration as used in the Flexstation screen) with a final concentration of DMSO not exceeding 1%.

## **2.2 Media used for donor samples**

A non- capacitating HEPES-buffered medium (NCM) was used to measure samples under a non-capacitated state, consisting of: 1.8mM CaCl<sub>2</sub>, 5.4mM KCl, 0.8mM MgSO<sub>4</sub>.7H<sub>2</sub>O, 116.4mM NaCl, 1.0mM NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O, 5.55mM D-glucose, 2.73mM sodium pyruvate (C<sub>3</sub>H<sub>3</sub>NaO<sub>3</sub>), 41.75mM sodium lactate (NaC<sub>3</sub>H<sub>5</sub>O<sub>3</sub>), 25mM HEPES, 0.3% BSA dissolved in 10mls H<sub>2</sub>O (total volume: 50ml) (Matson and Tardif, 2012, Alasmari et al., 2013a). The pH was adjusted to 7.4 by the addition of 1M NaOH with an osmolarity of 290-320mOsm/kg. A synthetic tubal fluid (STF) was used as a capacitating medium (CM) (adapted from NCM). This was used to measure samples under capacitating conditions derived similarly to NCM but containing 26mM sodium bicarbonate (NaHCO<sub>3</sub>) instead of HEPES and 25mM NaC<sub>3</sub>H<sub>5</sub>O<sub>3</sub> instead of 41.75mM (in NCM). The STF was adjusted to pH 7.4-7.6 by the addition of 1M NaOH with an osmolarity of 290-320mOsm/kg (source and catalogue numbers see appendix).

## **2.3 Samples**

### **2.3.1 Ethical Approval**

Volunteer sperm donors (healthy men selected at random from the general public (mainly students) with no known fertility problems) were recruited in accordance with the Human Fertilisation and Embryology Authority (HFEA) Code of Practice (version 8) (covers donor recruitment) under local ethical approval (08/S1402/6). Written consent was obtained from patients in accordance with the HFEA Code of Practice (version 8) under the same ethical approval as stated above from the Tayside Committee of Medical Research Ethics B (patient and donor participation, see appendix for consent forms).

### 2.3.2 Semen Samples

Volunteer donor semen samples were produced by masturbation into a sterile plastic container following 2-3 days sexual abstinence. Samples were produced in the morning and brought to the lab within 1 hour (hr) of production. The samples were left to liquefy at 37°C for approximately 30 min before being analysed.

Semen samples collected from patients were produced in the Assisted Conception Unit (ACU) by masturbation into a sterile container, following 2-3 days abstinence, on the morning of treatment, and prepared by the ACU laboratory by density gradient centrifugation. 1.5ml gradients were layered using 40% and 80% PureSperm<sup>®</sup> with 2ml semen overlaid on top. After centrifuging at 300g for 20 min the 80% fraction was recovered and washed in 5ml SAGE<sup>®</sup> gamete buffer which was centrifuged at 500g for 10 min. If the sample was used in an ICSI procedure the required amount of 80% fraction was left in 100µl SAGE<sup>®</sup> gamete buffer. If used for IVF the 80% fraction was re-suspended in Quinn's Advantage<sup>®</sup> fertilisation medium supplemented with human serum albumin (HSA). Surplus sample not required for treatment were used in research. 'Morning' samples were prepared in the ACU laboratory by PureSperm<sup>®</sup> DGC (described above) (source and catalogue numbers see appendix). A proportion of the 80% fraction is removed to be used for treatment whilst the remainder of the 80% and the 40% fraction are combined back together and made available for research. The sample is then re-prepared in the research laboratory (to re-separate sperm into 40% and 80% fractions), by Percoll<sup>®</sup> density gradient (described below), and the 80% fraction collected. Samples used in chapter 3 represent patient samples donated to research following ACU embryologist preparation that were then re-prepared in the research laboratory. 'Afternoon' samples were prepared by embryologist preparation in the ACU (same as morning samples described above) however; these prepared samples were capacitated for a minimum of 3 hrs because they had been placed in the CO<sub>2</sub> incubator 2

hrs prior to being used for insemination (IVF) in the ACU lab. Following treatment, any remaining sample was made available for research but unlike the 'morning' samples it was not re-prepared before use (samples used in chapters 6&7).

Semen samples from patients attending the ACU are from couples attending for ART and are not necessarily all men with male factor infertility.

## **2.4 Sperm preparation**

Semen samples from donors were prepared using the density gradient centrifugation (DGC) method to isolate spermatozoa from seminal plasma. A maximum of 1.5ml of semen was gently added to the top layer of the density gradient (2ml 40% Percoll<sup>®</sup> with an underlay of 2ml 80% Percoll<sup>®</sup>) and centrifuged at 300g for 20 min. The supernatant was discarded followed by collection of the 40% layer and 80% pellet into separate wash tubes. Samples were washed in 5ml NCM (centrifuged at 500g for 10 min) (Mortimer and Mortimer, 2013b). After centrifugation, the supernatant was discarded and the pellet was re-suspended in NCM or CM (source and catalogue numbers see appendix).

## **2.5 CASA parameters adopted in all experiments**

Samples were diluted in NCM or CM to between  $\geq 2 \times 10^6$ /ml (lower limit, too few cells for CASA to distinguish between debris and sperm cells resulting in an increased error) (Garrett et al., 2003) and  $\leq 50 \times 10^6$ /ml (upper limit, measurement made above  $50 \times 10^6$ /ml are at a higher risk of collisions increasing error) (WHO, 2010c) allowing accurate reading on the CASA. Unless otherwise stated, motility was measured after centrifugation at time 0 min (time 0 min = addition of NCM/ CM to sample and motility measured instantly) then every 30 min for 3 hrs. Samples were kept in the appropriate incubator (5% CO<sub>2</sub> or normal) between readings. The slides used for readings were 4

chambered 20 $\mu$ M depth slides that incur no known drift (MicroCell counting chambers, Vitrolife, Inc. San Diego, CA, USA).

A minimum of 200 cells were counted per frame, with a minimum of 2 frames counted per chamber, to give a minimum total of 800 motile spermatozoa counted (unless otherwise stated) in every experiment. This number was chosen in order to achieve an acceptably low sampling error when evaluating motile cells (WHO, 2010d).

## **2.6 Gradient experiment (Chapter 3) to determine most suitable fraction to be used as a surrogate for patients**

Gradients were compiled from 1ml 80% Percoll<sup>®</sup> with an underlay of 1ml 90% Percoll<sup>®</sup>, followed by overlaying 2ml of 60%, 40% and 20% Percoll<sup>®</sup> (2ml of each added to reduce contamination between layers when collecting sample) with 1ml sperm added and then centrifuged at 300g for 20 min, as described previously. The supernatant was discarded followed by collection of each layer into separate wash tubes containing 5ml NCM. Each wash was centrifuged for 10 min at 500g. Samples were diluted in CM and readings taken at 0 min then every 60 min for 5 hrs. 400 cells counted per frame, 4 frames counted for each chamber giving a minimum total count of 1600 motile cells.

## **2.7 Assessment of calcium and motility induced by novel compounds**

### **2.7.1 Flexstation Assay Screen (experiments conducted by Sarah Matins da Silva in combination with the Dundee Drug Discovery Unit (DDU))**

Sperm samples were collected and prepared as previously described. Following preparation by DGC sperm cells were subjected to capacitating conditions for 2 hrs 30 min. Media and supernatant were removed and cells re-suspended in Flexstation assay buffer (1XHBSS, 20mM HEPES, 0.5mM probenecid, pH7.4) and concentration and volume ascertained. Approximately 110 million cells constituted in a 5ml volume were added to an equal volume of the Ca<sup>2+</sup> sensitive dye, Calcium 3, which had been made up

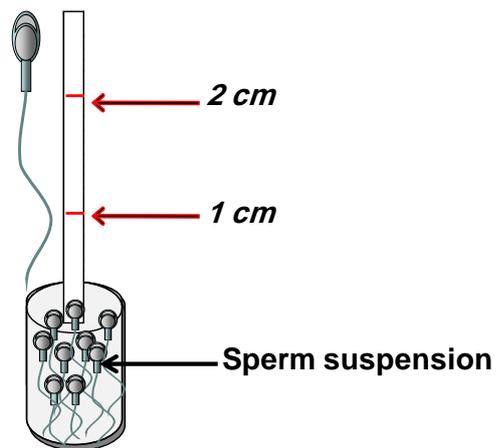
to twice the manufacturer's recommended concentration. Following an incubation at 37°C for 60 min, the sperm cells were recovered by centrifugation at 700g for 5 min at room temperature and re-suspended in Flexstation assay buffer at a concentration of  $5 \times 10^6$  cells / ml. The cells were plated out onto 384 well clear bottom, black wall plates at a concentration of  $2.5 \times 10^5$  cells / 50 $\mu$ l /well. The plates were then centrifuged at 700g for 5 min at room temperature to ensure the cells were at the base of each well. A plate was made up to contain test compounds at a 5x final concentration, with the use of 10 $\mu$ M progesterone as a control (plated by robotics at DDU). Both the assay and the plates containing compounds along with FLIPR tips were placed into the appropriate compartment of the Flexstation. The Flexstation recording was started and 12.5 $\mu$ l of test compound was added after 18 seconds (s). Ca<sup>2+</sup> dependent fluorescence of the Calcium 3 dye was measured for 100s as recommended by the manufacturer (excitation wavelength = 485nm, emission wavelength = 525nm, cut-off = 515nm). Hit compounds were identified by software analysis of the area under the curve function when compared to progesterone (positive control). These compounds were then examined for any effect they may have on motility (see below).

### **2.7.2 Preparation for motility assessment of novel compounds**

Following DGC preparation, each sample was diluted to a final concentration of 20million/ml. Samples were placed in appropriate incubators NCM-samples in normal (37°C, 15 min), CM-samples in 5% CO<sub>2</sub> (37°C, 2 hrs 30 min). 99 $\mu$ l of semen sample was then placed in labelled round bottom tubes with the addition of 1 $\mu$ l of appropriate drug. 1 $\mu$ l 1% DMSO was added to control tube as a vehicle control. 3 $\mu$ l of sperm suspension was then loaded onto preheated fixed chamber Microcell slides and CASA analysis conducted (described previously).

### **2.7.3 Sperm penetration into artificial viscous medium (Kremer test)**

Kremer penetration tests were carried out using methylcellulose (an artificial mucus substitute) with a viscosity of 4000 cp (MC4000). This was prepared in NCM containing 0.3% BSA at 10mg/ml. As previously described (Ivic 2002), 5 cm long, rectangular, flattened capillary tubes with a depth of 0.4mm (Camlab Limited Cambridge UK) were placed into the methylecellulose for 30 min at 37°C to allow the tubes to fill. Excess methylcellulose was gently wiped from the tubes and the upper ends of the capillary tubes sealed with plasticine. The open end of the tube was placed into the prepared sperm at a concentration of  $\sim 20 \times 10^6$ /ml for donor samples. Patient samples were capacitated in the ACU lab for a minimum of 3 hrs (as described above) they were then diluted in clinic media to a concentration of  $\sim 2 \times 10^6$ /ml. 1 $\mu$ l of compound was added to 99 $\mu$ l of sperm suspension, giving a final concentration in each case of 10 $\mu$ M A1 - E1, 40 $\mu$ M Trequinsin and 3.6 $\mu$ M progesterone. Controls contained 1% DMSO to account for any effects produced by DMSO itself. Sperm preparations and capillary tubes were then incubated in appropriate incubators (see above) for 1 hr. The capillary tubes were then removed and wiped to remove residual spermatozoa from the surface of the tubes. The open ends of the tubes were sealed with plasticine, and marks were made on the tubes at 1 cm and 2 cm. The tubes were then viewed on an Olympus CX41 microscope (20X objective final magnification x200) (Olympus Corporation, Tokyo, Japan), with the number of cells being counted at 1 and 2 cm distances from the base of the tubes (figure 2-1). Three fields of view were counted for three separate planes between the upper and lower wall of the capillary tube and an average obtained. Results were normalised to parallel untreated 1% DMSO controls to allow comparison between different experiments (source and catalogue numbers see appendix).



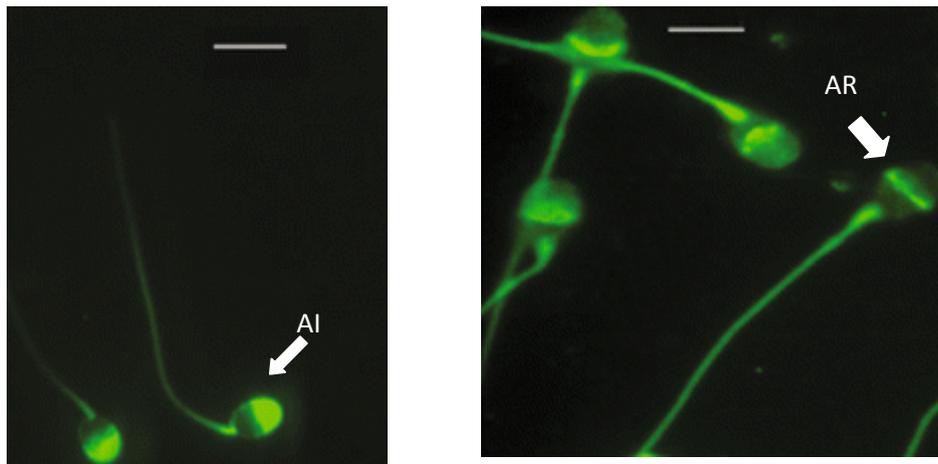
**Figure 2-1 Model depicting sperm penetration into artificial viscous medium (Kremer test).** Kremer capillary tubes filled with viscous medium were immersed in sperm suspensions in the presence or absence of compounds, and left at 37°C in a 5% CO<sub>2</sub> incubator for 1 hr to allow the migration of motile spermatozoa. Red lines on the tube show the locations at which sperm numbers were counted, i.e. 1 and 2cm from the base of the tube. (Alasmari, 2013)

## 2.8 Assessment of the Acrosome Reaction

Sperm obtained from donors was prepared by DGC as described previously. Sperm acrosomes were studied by staining with lectin from *Pisum sativum* (pea) (PSA) labelled FITC, as described by Liu et al (Liu and Baker, 1988). Prepared sperm were capacitated for 2 hr 30 min before being exposed to C1 or D1 at a final concentration of 10µM for 1 hr. A negative control was exposed only to CM (1 hr), a vehicle control was exposed to 1% DMSO (1 hr) and a positive control where sperm were induced to undergo the acrosome reaction using Ca<sup>2+</sup> ionophore (A23187) at a final concentration of 10µM for 15 min (Aitken et al., 1993, WHO, 2010a). Sperm were washed twice with 10ml 0.9% sodium chloride by centrifugation at 600g for 10 min. The sperm pellets were re-suspended in 20-30µl sodium chloride and smeared on microscope slides, allowed to air dry. The smear was fixed in 95% (v/v) ethanol for 30 min at 4°C.

After some initial experiments to optimise conditions, the final protocol involved using 12.5µg/ml PSA labelled with FITC in phosphate buffered saline (PBS) (PSA (FITC)) for 1 hr at 4°C in the dark. The slides were washed in PBS three times and mounted

using hydromount (source and catalogue numbers see appendix). At least 200 sperm were counted from each slide with 2 slides per treatment, 400 sperm counted total per compound, on an EVOS xl digital inverted microscope (Advanced Microscopy Group) using fluorescence setting with 400x magnification. Images were captured on the EVOS fully integrated colour camera. When more than half a sperm head was brightly fluorescing the acrosome was considered to be normal and intact (figure 2-2)



**Figure 2-2. Image depicting acrosome intact (AI) and acrosome reacted (AR) spermatozoa.** Acrosome intact spermatozoa were identified by more than half the sperm head brightly fluorescing whereas acrosome reacted spermatozoa were identified by having a fluorescing equatorial segment and no fluorescence elsewhere in the sperm head (Image courtesy of Jones et al., 2010).

## 2.9 Hypo-osmotic swelling (HOS) test

Agonists were examined to measure if they significantly compromised sperm viability by use of the hypo-osmotic swelling (HOS) test (Jeyendran et al., 1984). The HOS test measures sperm membrane integrity and for this test spermatozoa incubated with or without agonists were further incubated with hypo-osmotic medium (75 mmol D-fructose  $l^{-1}$  and 25 mmol sodium citrate dihydrate  $l^{-1}$ ) at 37°C in 5%  $CO_2$  for 30 min (source and catalogue numbers see appendix). Sperm with coiled tails were counted as viable and were counted from 2 separate aliquots with a minimum of 200 cells counted per replicate to reduce sampling error.

## 2.10 Statistical analysis

Normality of data (the supposition that the underlying random variable of interest is distributed normally, or approximately so) was assessed according to frequency distributions and the Kolmogorov-Smirnov test. Results are expressed as the mean  $\pm$  SD (standard deviation), mean  $\pm$  SE (standard error). Statistical comparisons were made using T-test and the analysis of variance (ANOVA) if the data were either originally normally distributed or normalised after transformation. However, some data sets were not able to be normalised following transformation thus; the statistical analysis of this data was performed using non-parametric tests including Mann Whitney U test and Kruskal – Wallis one way analysis of variance on ranks. Pearsons Chi squared analysis was conducted when analysing results from the Hypo-osmotic swelling (HOS) test. Linear regression analysis was employed when analysing semen data from two separate clinics. Significance was considered to be  $P \leq 0.05$ . All statistical analysis was performed using SPSS statistics 18.

## **CHAPTER 3**

**Identification of a sub-group of donor  
spermatozoa after density gradient  
centrifugation that is comparable to a  
patient sample**

### 3.1 Introduction

The availability of sub-fertile patient semen samples for research purposes is very limited and for this reason it would be invaluable to research to be able to isolate a sub-group of spermatozoa from donor samples that is similar to sub-fertile patients. Patient samples can be very variable having problems with low concentration, poor morphology, poor motility or combinations of these factors (Curi et al., 2003) making this task a difficult one. In an attempt to identify a 'surrogate' patient sample from normozoospermic donors density gradient centrifugation was used to separate donor samples into 6 fractions; 20%, 40%, 60%, 80% and 90%. Each fraction was then compared to the 80% fraction procured from sub-fertile patients for their motility characteristics. Motility is an important functional characteristic that is related to a spermatozoa's ability to successfully fertilise in IVF (Donnelly et al., 1998). Clear differences have been noted in sperm motility between sub-fertile and fertile men (Macleod and Gold, 1951b, Barratt et al., 2011) which corroborates the importance of identifying a surrogate population of donor sperm to be used in research if sub-fertile samples are not available. Poor sperm motility has been associated with defective mitochondrial DNA (Oaconnell, 2003) meaning that testing new drug therapies or preparation techniques on normozoospermic donors as a substitute for sub-fertile patients may give inaccurate results. To be able to accurately determine the effect of a new treatment on spermatozoa you need to be able to test on sperm that have similar qualities to that seen in patient samples.

DGC is currently the sperm preparation technique employed by assisted conception clinics for preparing spermatozoa before procedures. DGC was initially suggested to be used to enhance motility in cases of asthenozoospermia (Berger et al., 1985) and in the late 80's this technique was shown to enhance sperm penetration into human oocytes (Guerin et al., 1989). Previous studies have discovered that spermatozoa recovered from

different Percoll<sup>®</sup> fractions and incubated in capacitating media have the ability to maintain motility to different extents regarding which fraction the cells are recovered from (Saad and Guerin, 1992). Using this information this study attempted to isolate 6 different fractions (20%, 40%, 60%, 80% and 90%) from normozoospermic donor spermatozoa and compare the motility parameters from the spermatozoa recovered from each fraction to that of sub-fertile patient spermatozoa recovered from the 80% fraction. Previous studies have examined and analysed motility parameters from spermatozoa recovered from different fractions after DGC however, these studies were attempting to identify the fraction that contains the ‘best’ spermatozoa to be used clinically in ART (Saad and Guerin, 1992, Moohan and Lindsay, 1995, Forster et al., 1983, Yao et al., 1996). We have been unable to locate a study that compares fractions from donor spermatozoa with that of sub-fertile patients.

The main aim of this chapter is to isolate and identify a sub-group of spermatozoa from normozoospermic donors that has similar motility characteristics to spermatozoa recovered from patients undergoing fertility treatment.

## 3.2 Results

### 3.2.1 Comparison between sperm movement characteristics from normozoospermic donors after migration through 20%, 40%, 60%, 80% and 90% Percoll<sup>®</sup> gradients, with patient samples after an 80% Percoll gradient<sup>®</sup>

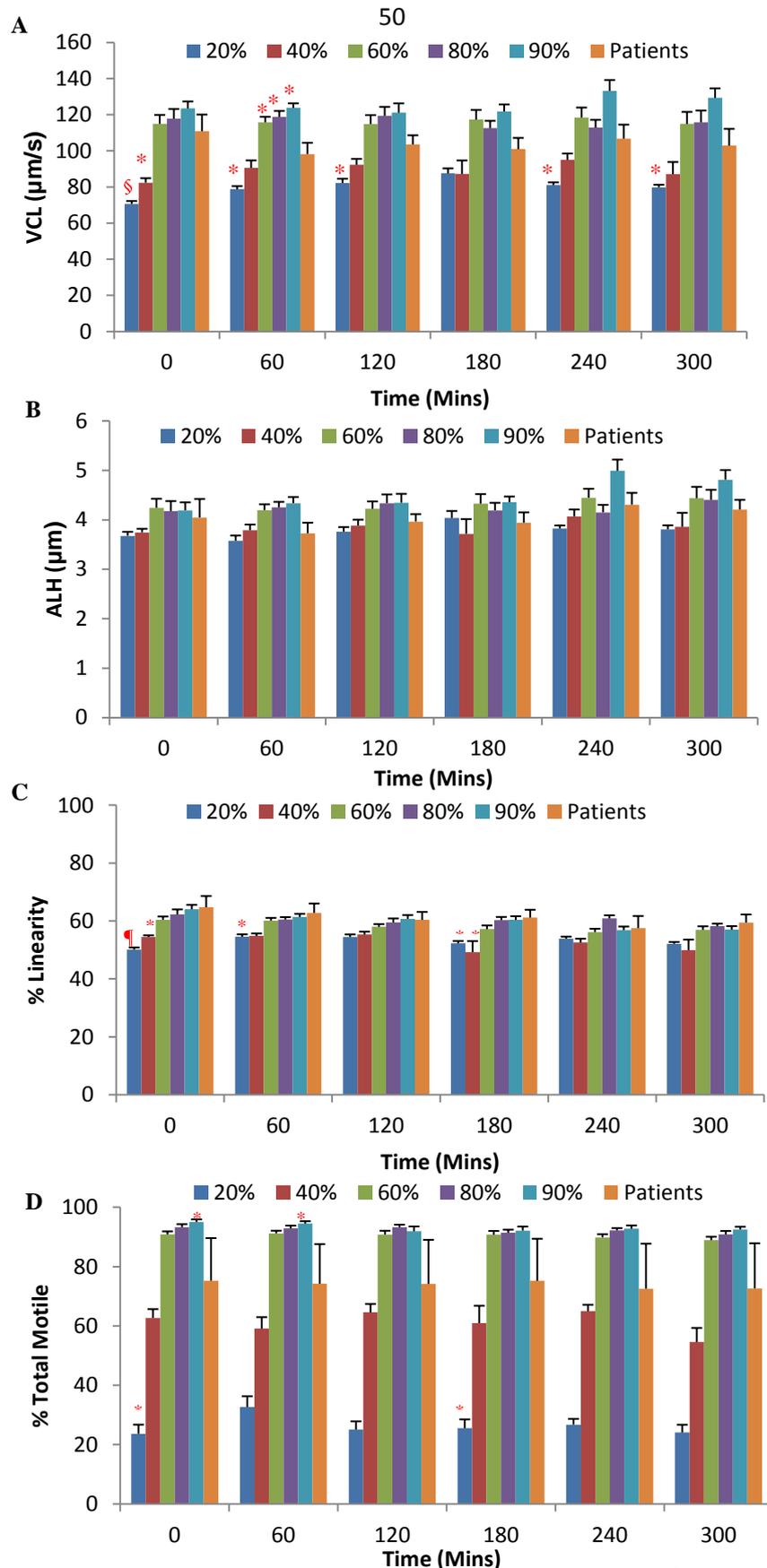
#### 3.2.1.1 *Movement of spermatozoa recovered from different fractions of Percoll<sup>®</sup> (donor) compared to 80% fraction (patient)*

In the motility assay the motility parameters measured were VCL, ALH, LIN, total motile, progressive motile and hyperactivation for each fraction acquired from donor samples. This information was then compared to the information obtained for the same parameters from the 80% fraction of sub-fertile patients. In this study we are looking for the donor fraction that most closely reflects the motility parameters of the sub-fertile sample meaning we are looking at fractions where there is not a significant difference. The 20% fraction showed significant differences to the sub-fertile sample concerning VCL across all time points (0 min  $P < 0.01$ , 60, 120, 240 and 300 min  $P < 0.05$ ) excepting after 180 min where no significant difference was noted (figure 3-1A and appendix). The 40% fraction was significantly decreased compared to the sub-fertile sample at 0 min ( $P < 0.05$ ) with no significant differences in VCL at any other time point (figure 3-1A and appendix). No significant difference was noted in the 60%, 80% and 90% fraction regarding VCL at 0, 120, 180, 240 or 300 min however, VCL was significantly increased in donor samples in all three fractions after 60 min (60%, 80%  $P < 0.05$ , 90%  $P < 0.01$ ) (figure 3-1A, and appendix).

Values for ALH were found to have no significant differences when compared to sub-fertile patient samples for any of the donor fractions analysed across the entirety of the 300 min time course (figure 3-1B and appendix).

Maximum linearity values were identified from spermatozoa recovered from the 60%, 80% and 90% fractions from donors and no significant difference was noted across the 300 min time course of each of these fractions when compared to the sub-fertile patient sample (figure 3-1C and appendix). The linearity values for the 20% and 40% fractions were similar (figure 3-1C) and significant decreases were found at time 0 min (20%  $P<0.005$ , 40%  $P<0.05$ ), 60 min (20%  $P<0.05$ ) and 180 min (20% and 40%  $P<0.05$ ) in donor samples compared to patients (figure 3-1C and appendix).

The percentage of total motile spermatozoa recovered were maximal in the 60%, 80% and 90% donor fractions but owing to the huge variation in the percentage of total motile spermatozoa recovered from the sub-fertile patients. Significant increases in motility from donor spermatozoa were noted at time 0 and 60 min in the 90% fraction ( $P<0.05$ ) compared to the 80% sub-fertile patient (figure 3-1D and appendix). The percentage of total motile in the 20% fraction were significantly lower than the sperm recovered from the sub-fertile patient sample at time 0 and 180 min ( $P<0.05$ ) but no significant differences were identified at any of the other time points (60, 120, 240 or 300 min) (figure 3-1D and appendix). The 40% fraction showed no significant differences at any time points when compared to the sub-fertile sample (figure 3-1D and appendix).

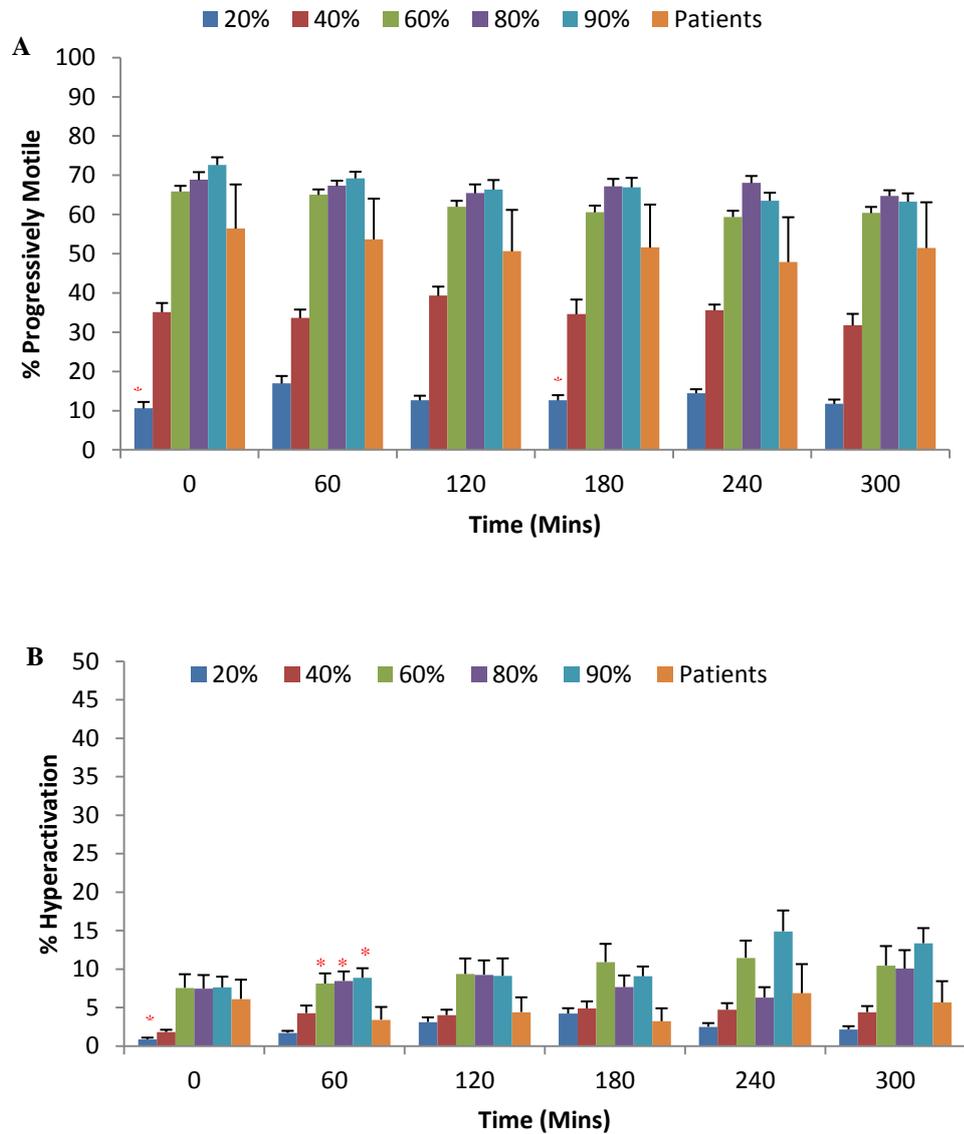


**Figure 3-1 Comparison between donor and sub-fertile samples in Percoll<sup>®</sup> fractions relative to incubation time under capacitating conditions.** Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction for VCL (**A**), ALH (**B**), LIN (**C**) and total motile (**D**). The result shown is the mean  $\pm$  SEM. N= 5 for both donors and patients, \* indicates significant difference ( $P < 0.05$ ), § indicates  $P < 0.01$ , ¶ indicates  $P < 0.005$  between each individual fraction and the patient sample (T-test and Kruskal-Wallace test).

The percentage progressively motile spermatozoa recovered from the 20% fraction were significantly lower than that of the sub-fertile patients at time 0 and 180 min ( $P < 0.05$ ) (figure 3-2A and appendix). Again there was a large variation in the percentage progressively motile cells recovered from the patient samples resulting in no significant difference being noted between the 40%, 60%, 80% or 90% fractions in comparison to the patient samples at any time point examined (figure 3-2A and appendix).

The percentage of hyperactivated spermatozoa was significantly lower in the 20% fraction at time 0 min in comparison to the sub-fertile sample ( $P < 0.05$ ) (figure 3-2B and appendix). A significant increase in the percentage of hyperactive spermatozoa was found in the 60%, 80% and 90% fractions after incubation for 60 min ( $P < 0.05$ ) compared to those recovered from the sub-fertile 80% fraction (figure 3-2B and appendix). There were no significant differences identified at any other time points (0, 120, 180, 240 or 300 min) for the 60%, 80% and 90% fractions. The 40% fraction showed no significant differences at any time point across the 300 min time course when compared to the sub-fertile samples (figure 3-2B and appendix).

Kruskall-Wallis statistical analysis showed that there was no significant difference in any of the parameters measured when looking independently at samples from 20%, 40%, 60%, 80%, 90% or patients across 300 min time course. This showed that incubation time didn't have an effect either positive or negative on the motility parameters measured (VCL, ALH, LIN, total motile, progressively motile and hyperactivation).



**Figure 3-2 Comparison between donor and sub-fertile samples in Percoll® fractions relative to incubation time under capacitating conditions.** Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction for progressively motile (A) and hyperactivation (B). The result shown is the mean  $\pm$  SEM. N= 5 for both donors and patients, \* indicates significant difference ( $P<0.05$ ) between each individual fraction and the patient sample (T-test and Kruskal-Wallace test).

### 3.3. Discussion

Density gradient centrifugation is a useful sperm preparation technique that allows for collection of motile sperm by separation into separate fractions even in cases of asthenozoospermia (Berger et al., 1985). This technique is currently used clinically to obtain the 80% fraction containing the 'best' portion of spermatozoa to be used for fertilisation in artificial reproductive technology (Forster et al., 1983, Yao et al., 1996, O'Connell, 2003). The data in this chapter corroborates that of other studies identifying that the proportion of spermatozoa collected after density gradient centrifugation from the higher percentage Percoll<sup>®</sup> fraction i.e. 90%, gives you the better overall motility parameters (Forster et al., 1983, Saad and Guerin, 1992, Yao et al., 1996). Although, the data in this study noted that there were no significant differences across any of the parameters measured between the 80% and 90% Percoll<sup>®</sup> gradient suggesting that an 80% gradient is of a high enough density to retrieve highly motile spermatozoa. In this study the patient samples did undergo a second Percoll<sup>®</sup> gradient before comparison with the donor samples. This could have had a detrimental effect on the motility of these samples however previous work in our laboratory has suggested that the effects of a second Percoll<sup>®</sup> gradient are negligible on motility. Previous studies available have compared motility parameters (Berger et al., 1985, Forster et al., 1983, Moohan and Lindsay, 1995, Saad and Guerin, 1992), morphology (Yao et al., 1996, Forster et al., 1983), vitality, interaction with zona-free hamster eggs (Forster et al., 1983) and DNA damage (O'Connell, 2003, Ghaleno et al., 2013) between differing fractions obtained after density gradient centrifugation from the same sample, however, we have been unable to identify any studies that compare fractions taken from normozoospermic donors with sub-fertile patient samples.

The results from this study show that, concerning VCL, the variability in the patient samples means it is not possible to discern which fraction is most similar with 40%, 60%, 80% and 90% all having values similar to that of the patient sample. With regard to ALH, it was again not possible to determine which fraction was comparable to sub-fertile samples with all fractions expressing similar values (including patients) reflecting findings of previous groups (Saad and Guerin, 1992). The data presented in this chapter identifies significant differences in more than one parameter and at more than one time point for both the 20% and 90% donor fractions when compared to the 80% sub-fertile fraction suggesting that they are not suitable comparable fractions to patient samples. The 40%, 60% and 80% fractions are all showing similarities to the 80% sub-fertile patient samples and from the data available in this study it is not possible to determine which of these three fractions is most similar to patients. However, in this study it was decided to use the 40% fraction as our poor motility group.

This study has looked specifically and solely at motility parameters and we are aware that to be able to accurately determine a sub-population of donor spermatozoa that is similar to that of sub-fertile patients, further in-depth analysis would need to be completed looking at morphology, DNA damage, vitality, penetration ability, acrosome reaction and also account for bacteria/debris (Aitken et al., 1995). This may be the reason that this study has not been attempted previously due to the many variables that need to be accounted for to make it a valid study. Another factor that may discourage people from completing this study could be the knowledge that variation between patient samples is large (Macleod and Gold, 1951b) and the ability to identify any useful data would depend on having a large sample size, which can be difficult to obtain.

## **CHAPTER 4**

# **Development of Standard Operating Procedure for Drug Screening**

## 4.1 Introduction

*In vitro* and *in vivo* sperm motility can be affected by a variety of factors some of which can be more easily controlled in an *in vitro* environment such as; temperature (37°C) (Esfandiari et al., 2002, WHO, 2010f), exposure to non-capacitating or capacitating conditions and types of apparatus used. As an attempt to increase the repeatability and reliability of any results obtained within this project, preliminary investigations were performed and environmental factors systematically examined thereby producing a protocol that limits/accounts for variation in results.

The focus of this chapter was to examine sperm motility under a variety of conditions addressing six key questions:

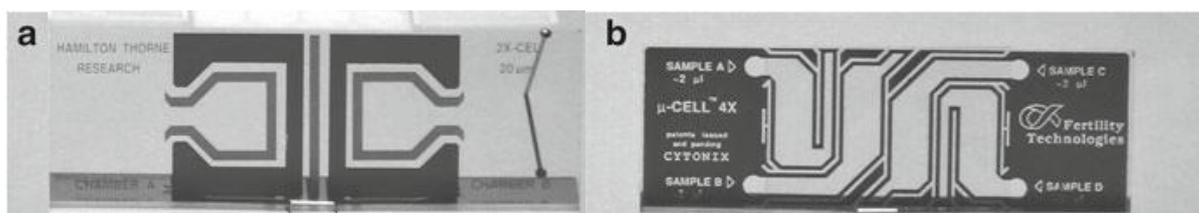
1. Given the variability within a prepared human sperm population, is it more accurate to assess motility parameters in 200 cells from 4 different aliquots compared to 400 cells from 2 different aliquots?
2. Does the use of slides with fixed coverslips give a more accurate representation of motility when compared to reusable slides and coverslips?
3. What is the effect of NCM and CM on sperm motility?
4. Do CM and NCM have a detrimental effect on sperm motility when incubated at 37°C for long periods of time?
5. Are motility responses consistent within and between ejaculates?
6. Does 1% DMSO have a detrimental effect on sperm motility under non-capacitating and/or capacitating conditions?

#### 4.1.1 Minimum number of cells counted to reduce sampling error

Spermatozoa are erratic and changeable and therefore huge variation in motility within a single sample can occur. Standard procedure describing analysis of motility parameters using CASA recommends that a minimum of 200 cells per frame from each replicate is measured to reduce sampling error (WHO, 2010d, Fraser and Group, 1998). However, it is not only important to identify a reliable method of evaluating motility, but to do so in the most economical way. To determine whether it was possible to read more than 200 cells from one replicate whilst maintaining similar variability within results, an investigation was devised using a sample split into two groups: 1) four separate aliquots of 200 cells measured per time-point and 2) two separate aliquots where 400 cells were measured per time-point (see chapter 2). Semen samples were prepared in the same way and cells diluted to the same concentration.

#### 4.1.2 Variation in motility assessed by different slide types

When assessing sperm parameters using CASA, there is large variation in sperm concentration and motility according to slide type used (Tomlinson et al., 2001). A comparison of CASA motility parameters was performed to assess differences between the two slide types available in our lab, which could account for variation within results, 2xCell Hamilton Thorne slides (20 $\mu$ m) with separate coverslips (figure 4a) and Microcell 4 fixed chamber slides (20 $\mu$ m) (figure 4b) were evaluated. The Hamilton Thorne slides are reusable and therefore washed and recycled, whereas the Microcell slides are disposable, single use only.



**Figure 4 Examples of slide types examined.** (a) 2xcell Hamilton Thorne slide with manual cover-slips (b) Microcell 4 fixed chambered slides. Image adapted from Spermatogenesis (Mortimer and Mortimer, 2013b).

4µl of prepared sperm was added to each chamber of the Hamilton Thorne slides and coverslips overlaid thereafter, whereas 3µl per chamber was added to the Microcell fixed chamber slides. A time-course experiment was run using a control sample in 1% DMSO. Samples were split and motility measured on each different slide type and results analysed. The hypothesis was that the Hamilton Thorne slides with the coverslips that are manually placed would have an increased chance of causing cells to stick to the slide resulting in a decreased total motility and progressive motility compared to the fixed chamber slides.

#### **4.1.3 Between-donor and within- donor biological variation**

If a series of samples are taken from the same person for semen analysis, the results of each of the independent deposits will not be exactly the same (Alvarez, 2003). The results may vary over time due to a variety of factors including; sexual abstinence period, transport of the sample to the laboratory and inherent biological variation (Alvarez, 2003). Spermatogenesis in mammals can range from 30 to 78 days and in humans the process takes over 70 days (Hess and Renato de Franca, 2008). To be able to account for any within-donor variability as a result of variation in spermatogenesis, repeat experiments were performed to compare the same sample over a 4 month period. Samples were subjected to capacitating conditions (2 hr 30 min, 37°C, 5% CO<sub>2</sub>) before motility assessments (CASA) were performed. Each sample was subjected to the same conditions to reduce variation from the external environment.

When the same tests are performed on different individuals the results once again would not be the same (Alvarez, 2003). In order to obtain robust and reliable results it is important to also be aware of between-donor variability. To be able to account for this a comparison in motility parameters between the same 5 donors examined for within-donor variability was conducted.

#### **4.1.4 Effect of DMSO on sperm motility**

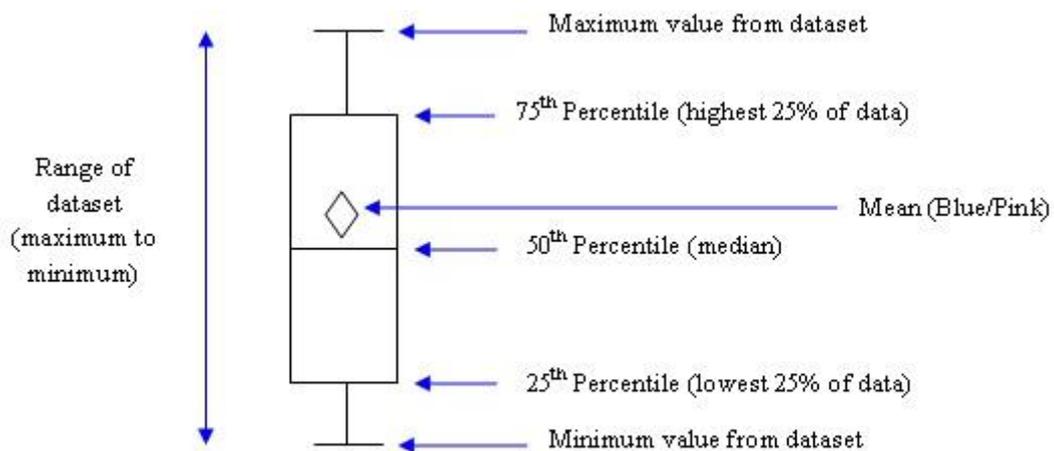
DMSO is a solvent used to solubilise and stabilise compounds generated by UoD DDU. In future experiments we planned to examine motility effects of DDU library compounds, and therefore investigated the effects of DMSO on human sperm. All DDU compounds were provided in solution in 100% DMSO, and these were further diluted to concentrations of 4mM or 1mM respectively (chapter 2). These prepared stocks were diluted 1:100 with the prepared sperm sample making final concentration of DMSO in solution no more than 1% (known non-toxic concentration (de Lamirande and Gagnon, 2002)). To ensure that 1% DMSO had no detrimental effect over time, serial motility assessments were made and compared with control (either NCM or CM alone) over a 3hr time course.

## 4.2 Results

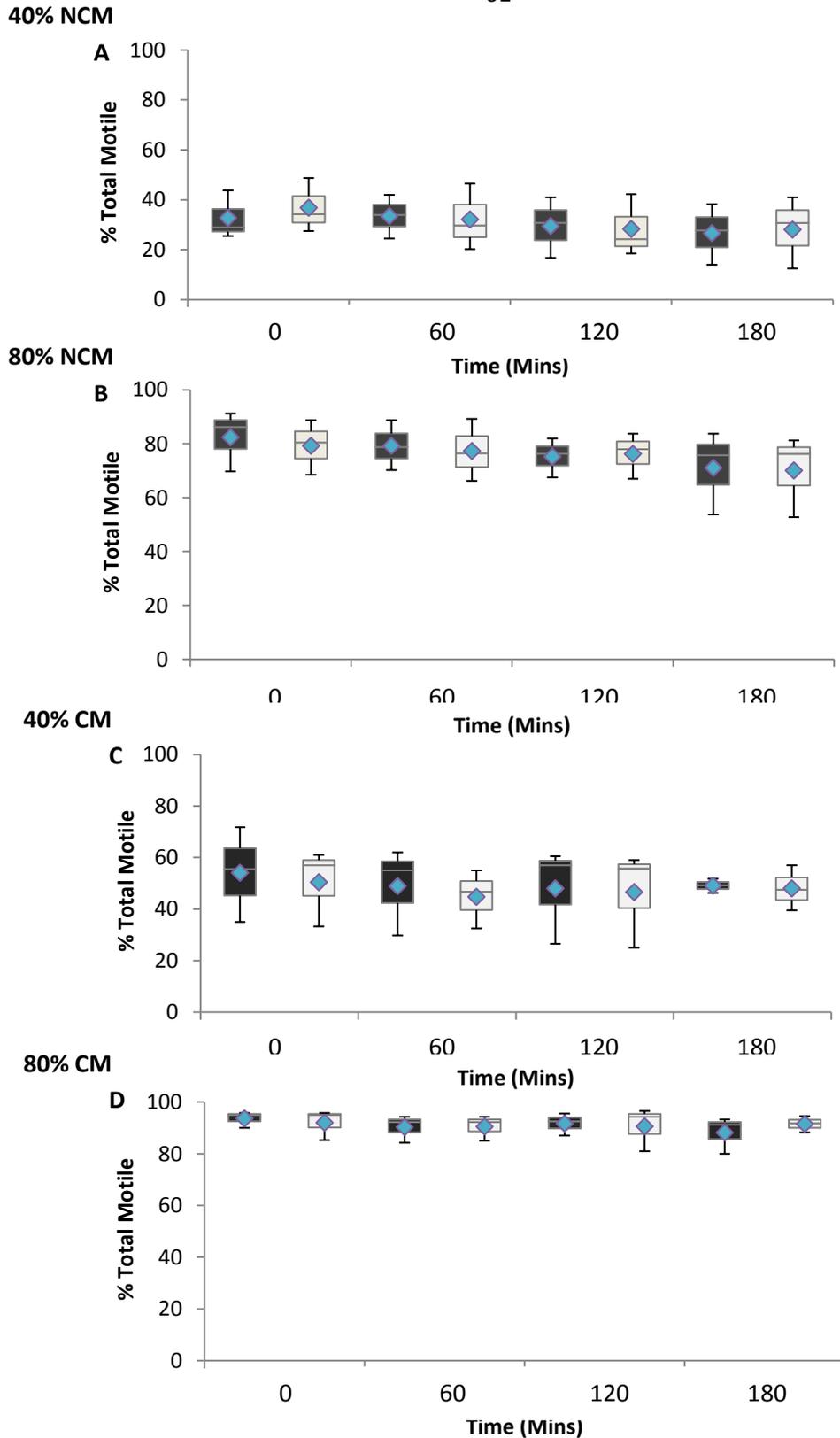
### 4.2.1 Development of methods for motility assessment by CASA

#### 4.2.1.1 Determination of the number of aliquots from a single sample that need to be taken to assess motility parameters

To determine the number of aliquots that need to be taken from a single sample in order to reliably assess motility parameters, a total of 800 motile cell counts were taken from either two aliquots from the same sample or four aliquots from the same sample. Motility parameters were measured from 40% and 80% fractions under non-capacitating and capacitating conditions to allow for any variation between fractions and types of media used. Figure 4-2 A&B shows the range for 800 motile cell counts in NCM and CM for 40% and 80% fractions. The range of results for reading cells from only two aliquots compared to four aliquots are very similar for both total motility and progressive motility (appendix). There is also no clear difference seen in ranges of total and progressive motility when reading 800 motile cells following incubation under capacitating conditions in either 40% or 80% fractions (figure 4-2 C&D and appendix). Results recorded for all variables also indicate that there is no statistical difference in range of all parameters measured at all time points (VCL, ALH and hyperactivation (appendix)).



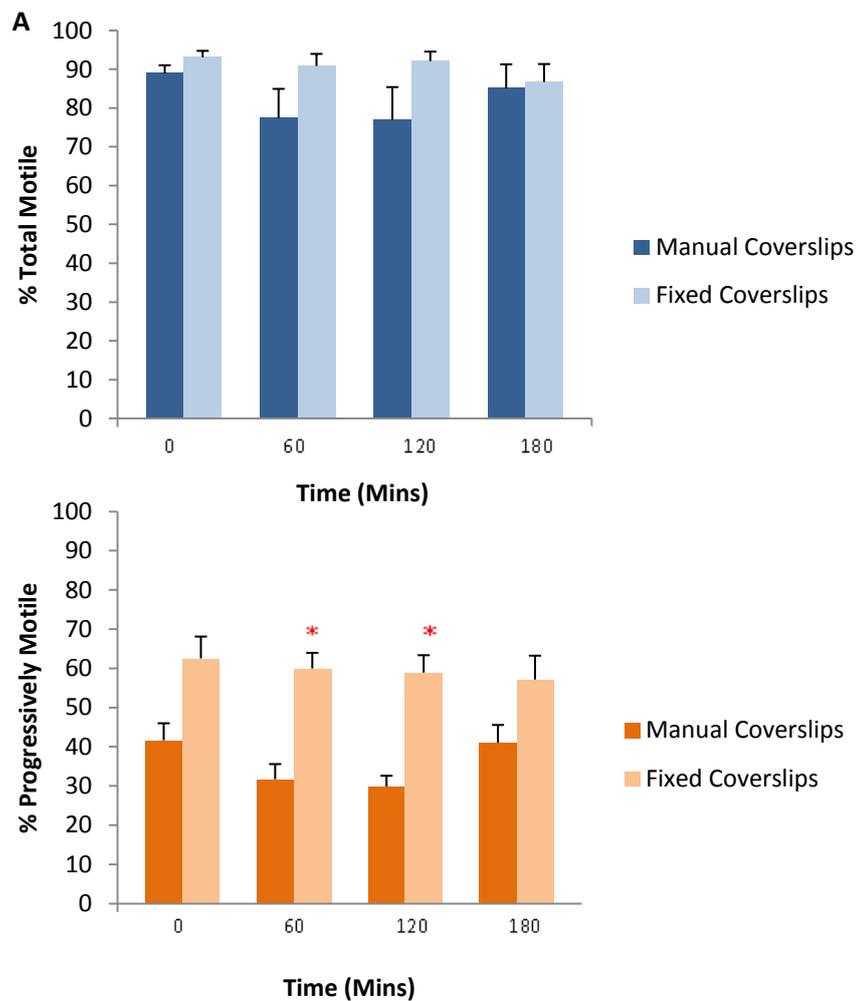
**Figure 4-1** Key for identifying features in Box Plots (figures 4-2 & appendix).



**Figure 4-2. Variation in range of results 4 aliquots vs 2 aliquots in non capacitating and capacitating buffer for % total motile (A) 40% NCM (B) 80% NCM (C) 40% CM (D) 80% CM.** The result shown is the mean (blue diamond)  $\pm$  the range for A-D, see **figure 4-1** for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating and capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)

#### 4.2.1.2 Determination of slide type required for CASA assessment of motility

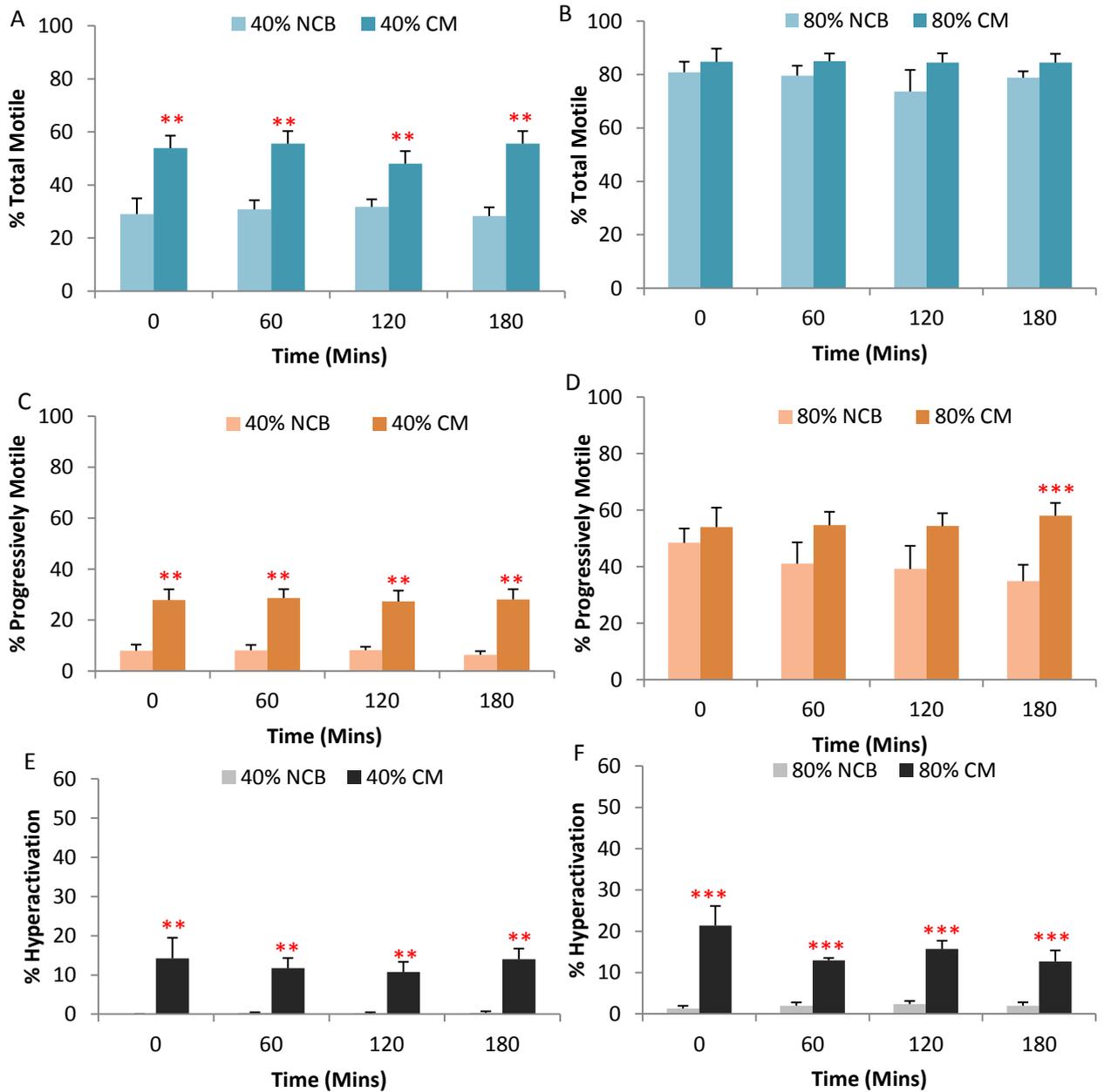
Hamilton Thorne 2X-Cell chambered slides (manual coverslips) were compared to MicroCell fixed counting chamber slides. Prepared sperm (80% fraction) were subjected to capacitating conditions and motility parameters, (VCL, ALH, hyperactivation (appendix), total motile and progressive motility (figure 4-3 A&B)), subsequently measured using CASA. 800 cells were counted for both slide types. Data from repeat experiments (n=3) was pooled, and results statistically analysed using Mann Whitney and Kruskal Wallace tests. Progressive motility was significantly increased in samples on the MicroCell fixed counting chamber slides compared to the Hamilton Thorne manual slides at both 60 min ( $P \leq 0.05$ ) and 120 min ( $P \leq 0.05$ ) (figure 4-3 B).



**Figure 4-3. Variation in motility between two slide types (A) % total motile (B) % progressively motile.** The result shown is the mean  $\pm$  SE measured at defined time points up to 180 min N= 3 for both parameters, \* indicates a significant increase between manual and fixed slides at independent time points. Significance was considered as  $P \leq 0.05$

#### **4.2.1.3. Assessment of motility parameters in capacitating and non-capacitating media**

To determine optimal incubation times under capacitating and non-capacitating conditions, prior to acquiring CASA readings, motility parameters were measured over a period of 180 min in both NCM and CM. As expected, in the 40% fraction there was a highly significant ( $P \leq 0.01$ ) increase across all motility parameters measured (VCL, ALH (appendix), total motile, progressively motile and hyperactivation (figures 4-4 A,C,E)) for samples incubated in CM compared to NCM across all time points. Similarly, the 80% fraction showed highly significant increases in VCL ( $P \leq 0.001$ ) and ALH ( $P \leq 0.05$ ) in CM compared to NCM across all time points (appendix). Hyperactivation also showed highly significant increases ( $P \leq 0.001$ ) at time 0, 60 and 120 min, with a  $P \leq 0.005$  at 180 min. There was no significant difference seen in total motility between the two media types with progressive motility only being significantly increased after 180 min ( $P \leq 0.005$ ) (Figures 4-4 B, D, F). Kruskal-Wallis statistical analysis showed that there was no significant difference in any parameters measured when looking independently at samples in NCM or CM (40% and 80% fractions) across 180 min time course.



**Figure 4-4. Expression of basal (control) motility values in non-capacitating (NCM) and capacitating media (CM) for 40% and 80% fractions.** % total motile: (A) 40% (B) 80%, progressively motile: (C) 40% (D) 80% and hyperactivation: (E) 40% (F) 80%. The result shown is the mean  $\pm$  SE for % total motile, % progressively motile and hyperactivation. In this study all motility parameters were measured over a period of 180 min under non-capacitating and capacitating conditions comparing the difference between the two media types.  $N=6$  for both parameters for the 40% fraction and  $N=5$  for NCM and  $n=9$  for CM for the 80% fraction, \*\* indicates a significant increase ( $P \leq 0.01$ ), \*\*\* indicates a significant increase ( $P \leq 0.005$  or  $P \leq 0.001$ ) between NCM and CM at independent time points (Mann Whitney test and Kruskal-Wallis test).

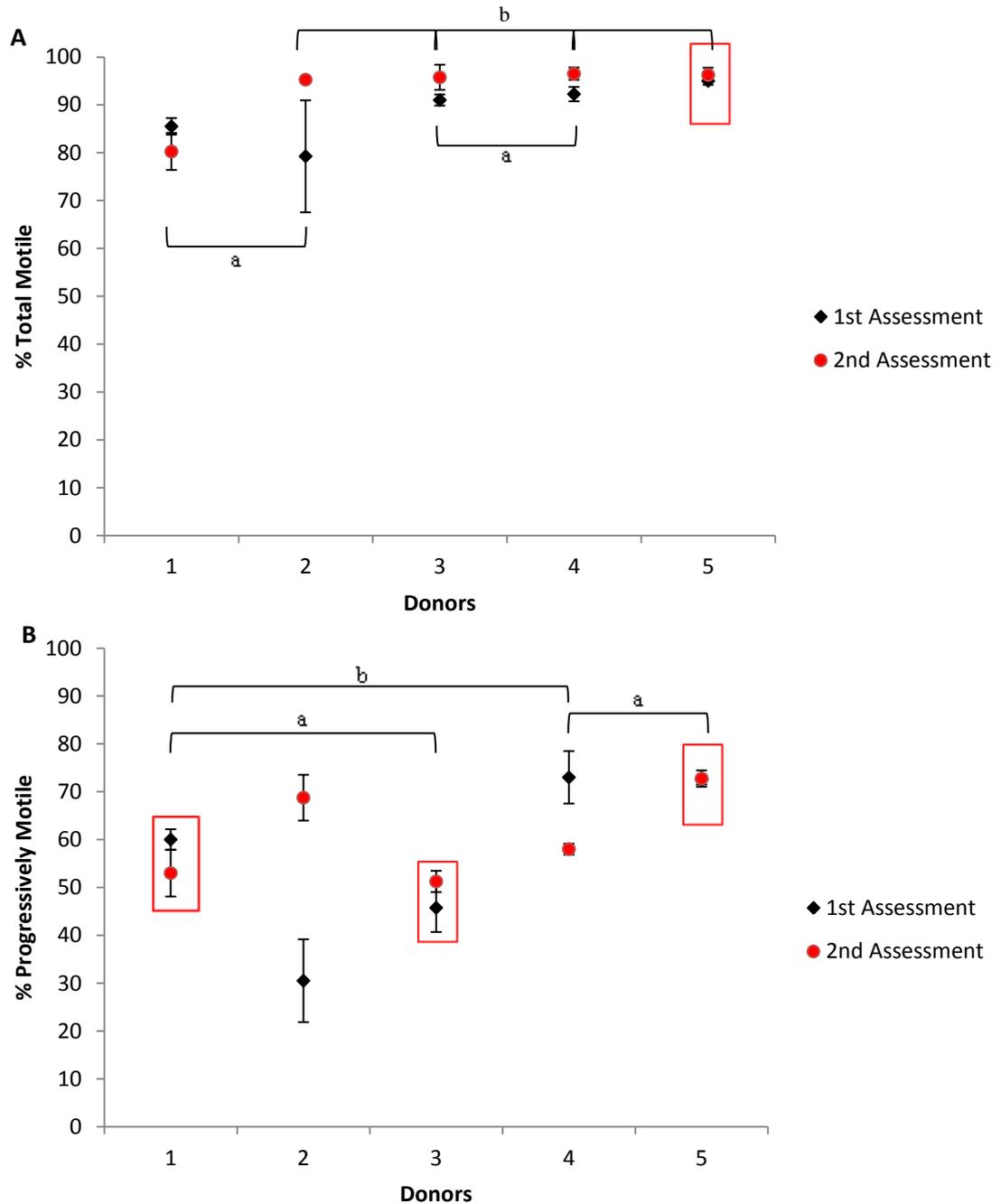
## **4.2.2 Development of methods for the assessment of motility for drug screening**

### **4.2.2.1. *Inter-ejaculate and Intra-ejaculate variation with respect to motility***

#### ***parameters measured on CASA***

In order to examine the variability in motility between different ejaculates from the same man, the percentage of motile cells and progressively motile cells were examined from 5 donors on two separate occasions. When evaluating within donor variation 2/5 donors showed similar motility in both ejaculates (no significant differences) (highlighted in figure 4-5A) however, 3/5 donors showed a significant increase ( $P \leq 0.05$ ) in the percentage of motile cells in the 2<sup>nd</sup> sample compared to the 1<sup>st</sup> sample assessed (figure 4-5A). With regard to progressive motility 2/5 donors showed significant differences ( $P \leq 0.05$ ) between the 2 samples produced with the remaining 3 donors indicating no significant changes (figure 4-5B).

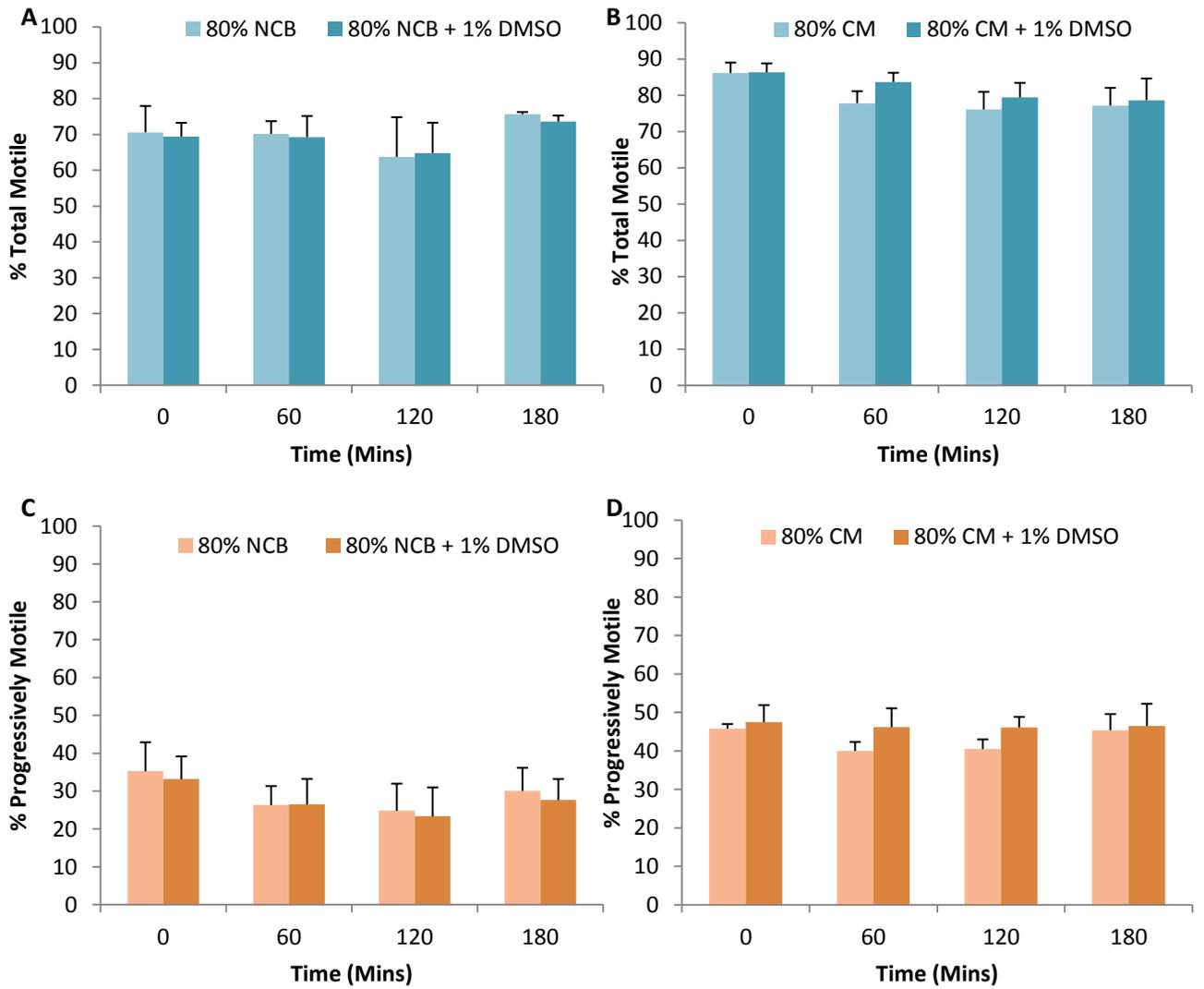
As expected, the variation between donor samples was found to be greater than the within sample variation. The percentage of motile cells was found to be significantly different ( $P \leq 0.05$ ) between every donor, with the exception of donor 1 (d1) in comparison to donor 2 (d2) and d2 in comparison to donor 5 (d5) (highlighted in figure 4-5A), when comparing the 1<sup>st</sup> sample from each donor. However, when evaluating the difference between the 2<sup>nd</sup> samples produced the only significant result was d1 which was significantly decreased ( $P \leq 0.05$ ) compared to the other 4 donors. In the case of progressively motile cells the 1<sup>st</sup> samples were all significantly different ( $P \leq 0.05$ ) to one another except in one instance, donor 4 (d4) compared to d5 (highlighted in figure 4-5B). The variation in the 2<sup>nd</sup> sample was not as great as the 1<sup>st</sup> sample with donors showing similar (non-significant) results (d1 compared to d3, d1 compared to d4 & d2 compared to d5). The remaining samples were significantly different to one another (figure 4-5B). The results from this study highlight the variability in assessments, both, within the same donor's ejaculate and between different donor samples.



**Figure 4-5. Inter-ejaculate variation in 5 donors following two assessments (A) % basal total motility (B) % basal progressive motility.** The result shown is the mean  $\pm$  SD for % total motile and % progressively motile. In this study, motility was assessed twice in 5 donors over a 4 month  $\pm$  1 week period. When evaluating **within** donor variation, where there is **NO** significant differences between the 2 assessments the result is highlighted with red boxes. When evaluating **between** donor samples 'a' denotes **NO** significant difference when comparing samples produced for the 1<sup>st</sup> assessment, 'b' denotes **NO** significant difference between donor samples when comparing samples produced for the 2<sup>nd</sup> assessment.

**4.2.2.2. Assessment of motility parameters in capacitating and non-capacitating media treated with 1% DMSO**

Compounds identified through HTS were prepared in solution with  $\leq 1\%$  DMSO. To determine if 1% DMSO is detrimental to motility parameters, a sample was treated under capacitating and non- capacitating conditions  $\pm 1\%$  DMSO for 180 min. This investigation enabled any adverse or influential reaction seen in sperm motility as a result of 1% DMSO treatment to be excluded. No significant difference was noted in any of the motility parameters measured (VCL, hyperactivation, total motile and progressively motile (figure 4-6 A-D and appendix)). The lack of significance, as analysed by Mann Whitney and Kruskal-Wallace tests, in both 40% (appendix) and 80% fractions (figure 4-6 and appendix) , in all media types examined (CM and NCM  $\pm 1\%$  DMSO), indicates that the addition of 1% DMSO to media does not impact on motility throughout 180 min incubation.



**Figure 4-6. Expression of basal (control) motility values in non-capacitating (NCM) and capacitating (CM) media treated with 1% DMSO for 80% fraction (A) % total motile NCM, (B) % progressively motile NCM, (C) % total motile CM and (D) % progressively motile CM. The result shown is the mean  $\pm$  SE for % total motile and % progressively motile. In this study all motility parameters were measured over a period of 180 min under non capacitating and capacitating conditions comparing the difference between treatment with 1% DMSO and non-treated media. N= 3 for NCM and NCM + 1% DMSO, N= 4 for CM and CM + 1% DMSO, no significant difference noted at any time point.**

### **4.3 Discussion**

The aim of these experiments were to examine the potential variables in the protocol proposed for screening sperm samples from donors and sub-fertile patients in order to improve experimental efficiency and to standardise laboratory protocol.

#### **4.3.1 Repeatability and Reliability of system**

##### ***4.3.1.1 Number of aliquots from a single sample that need to be taken to assess motility parameters***

Sample variation within an individual occurs as a natural part of any semen sample (Fraser, 2001, Alvarez, 2003). The reasons behind this variation include period of sexual abstinence, transport of the sample to the laboratory, analytical and systematic errors (CASA) and inherent biological variation (Fraser, 2001, Alvarez, 2003). Sperm preparation methods are designed to isolate high grade spermatozoa from low grade spermatozoa and debris (Mortimer, 2000a, Mortimer and Mortimer, 2013a) and thus reduce sample variation within a single sample, although these methods do not completely eliminate it. Individual samples contain inherent random fluctuations around a homeostatic set point which can be influenced by environmental and biological factors (Fraser, 2001), and not all ejaculates from the same individual or between different individuals will therefore be identical. This variation needs to be recognised and accounted for if comparisons within populations or between clinics are to be made (Fraser, 2001, Alvarez, 2003, Jørgensen et al., 2001, Castilla et al., 2006).

Furthermore, the limitations of CASA technology must also be considered. Although CASA systems may be discredited as being poor determinates of semen concentration, they allow objective analysis of sperm motility kinematics as long as standard operating procedures are followed (Fraser, 2001, Mortimer and Mortimer, 2013a). The effect of sampling error on sperm concentration has previously been examined using motile

human sperm, analyzing a minimum of 200, 400, and 1,000 sperm (Tomlinson et al., 2010). Results were found to be similar regardless of the number of sperm assessed by this group (Tomlinson et al., 2010). To exclude variation as a result of the use of CASA this study followed expert recommendations on how to use CASA, limiting errors to a minimum (Fraser, 2001, Tomlinson et al., 2010, Mortimer and Mortimer, 2013a).

The standard procedure, as defined by ESHRE, states that at least 200 motile spermatozoa should be analysed per sample/aliquot when determining sperm motility characteristics using CASA (Fraser and Group, 1998, Tomlinson et al., 2010). Inaccuracies can occur as a result of inhomogeneity errors when using fixed chamber slides (Mahmoud et al., 1997) where the use of a smaller volume i.e. 6 $\mu$ l (2 aliquots) may not be as representative of a whole sample when compared to 12 $\mu$ l (4 aliquots) (Mahmoud et al., 1997, Tomlinson et al., 2001). This study has shown that if the sample is thoroughly mixed (flicking of the tube to re-suspend cells) before analysis, there is no significant difference in the variation in the sample whether you read 4 separate aliquots or 2 separate aliquots (figure 4-2). Under capacitating conditions the range in variation of the results in the 80% fraction is considerably narrower than that seen in the 40% fraction in both systems. This is as expected, because the 80% fraction contains a high proportion of motile and progressively motile spermatozoa (O'Connell, 2003, Mortimer and Mortimer, 2013c). Overall, the results of these experiments do not show any clear difference between the two approaches to CASA analysis when assessing cells under either capacitating or non-capacitating conditions (40% or 80% fractions), indicating that either approach could be used with minimal sampling error. As the more economical of the two systems it was decided to use the 2 replicate, 400 cells per replicate system in the final protocol.

#### **4.3.1.2 *Determination of slide type required for assessment of motility***

Variation in concentration between the two slide types was not measured in this study although it has previously been examined (Mahmoud et al., 1997, Tomlinson et al., 2001). The reusable Hamilton Thorne slides have been shown to overestimate sample concentration as cells may not spread evenly across the chamber (Mahmoud et al., 1997, Hoogewijs et al., 2012). This could impact on cell motility by 1) increasing cell clumping and 2) increasing the chance of adherence of cell to slides, despite the presence of BSA in the media (Mortimer and Mortimer, 2013a). Hamilton Thorne slides may be treated with a nitrocellulose and polyvinyl coating to prevent sperm adhesion and to decrease effects on motility (Chapeau and Gagnon, 1987) although, it is not advisable to treat fixed chamber slides in the same manner. Another factor that may contribute to sticking when using Hamilton Thorne slides includes frequent washing, slides are washed using hot tap water and vigorous rubbing, they are then rinsed with deionised water and allowed to air dry in an upright position. This process can result in smears and scratches along with other superficial damage to the slides and/or coverslips which can alter motility itself and increase mistakes when analysing on CASA. Both slide types use a small volume (3 $\mu$ l or 4 $\mu$ l for disposable and reusable slides respectively) which could explain some of the variability seen in the results (figure 4-3 A-B). However, samples were mixed before addition to slides to minimise variation. Variability (Mahmoud et al., 1997) seen on the reusable slides could be due to differences between chambers, presumably as a result of unequal pressure being asserted by the individual placement of coverslips. The two slide types used in this investigation are also loaded in different manners, the Hamilton Thorne 2xCell slides with separate glass coverslips are filled by 'drop loading' whereas the MicroCell 4 chambered fixed coverslips are filled by capillary loading (Mahmoud et al., 1997, Mortimer and Mortimer, 2013a). Fixed chamber slides that are filled by capillary flow

are subject to the Segre Silberberg effect (Douglas-Hamilton et al., 2005a, Douglas-Hamilton et al., 2005b, Leja, 2005, Mortimer and Mortimer, 2013c). Although the Segre Silberberg effect has been proven to affect concentration readings it has very little bearing on motility and thus would have minimal effect in this study (Mortimer and Mortimer, 2013c). It is known that slide types with manual coverslips incur drift and should to be left to equilibrate for 60secs before recording results (Mortimer and Mortimer, 2013c). The MicroCell 4 fixed chambered slides incur no known drift and readings can be taken instantly after addition of sample.

Disposable, single use, fixed chamber slides are becoming the preferred choice for many IVF laboratories (Tomlinson et al., 2001). They can be used to measure concentration and motility and have been shown to produce reproducible and consistently reliable counts when examined using latex beads and homogenised sperm samples (Mahmoud et al., 1997). There are clear advantages to using fixed chamber slides with consistent depths of 20 $\mu$ m for assessing sperm motility when compared to standard reusable glass slides with separate cover slips (Tomlinson et al., 2001) especially as sperm motion characteristics are heavily dependent on chamber material and depth (Le Lannou et al., 1992, Kraemer et al., 1998). In contradiction to this study, Tomlinson et al (2001) previously measured motility between reusable glass slides (Makler and Horwell) and fixed chambered slides (Cell Vu, MicroCell and Leja) with no significant differences noted in motility parameters. This conflict may be explained by methodological differences. As a result of this experiment, unless unavailable, the MicroCell 4 fixed chamber slides were used preferentially.

#### ***4.3.1.3 Assessment of motility parameters in capacitating and non-capacitating media***

Following ejaculation, capacitation (a complex series of molecular events that sperm undergo) occurs allowing sperm to be able to fertilise oocytes (Visconti et al., 2002).

Importantly this process can be replicated *in vitro* in specialised culture media, the composition of which is based on electrolyte concentration of oviductal fluid (Visconti et al., 2002). To enable capacitation to occur, capacitating media contains energy substrates, such as pyruvate, lactate and glucose, a cholesterol acceptor (serum albumin),  $\text{NaHCO}_3$ ,  $\text{Ca}^{2+}$ , low  $\text{K}^+$ , and physiological  $\text{Na}^+$  concentrations (Visconti et al., 2002). The key component that differs between the two media types used in this study (NCM and CM) is the addition of  $\text{HCO}_3^-$  to CM.  $\text{HCO}_3^-$  is essential for the development of capacitation and sperm motility (Boatman and Robbins, 1991a, Visconti et al., 1995a). An increased intracellular  $\text{HCO}_3^-$  and  $\text{Ca}^{2+}$  stimulate soluble adenylate cyclase activity in sperm which leads to an increase in tyrosine phosphorylation resulting in increased motility and hyperactivation (Visconti et al., 2002, Visconti et al., 1995a, Battistone et al., 2013). This explains the significant increase in all motility parameters measured (VAP, VSL, VCL, total motile, progressively motile and HA) in the 40% fraction across all time points and similarly (VCL, ALH, progressively motile (180 min) and hyperactivation) in the 80% fraction when samples are in CM compared to NCM (appendix).

Sperm velocity is highly temperature dependent and it is essential that experiments be carried out at  $37^\circ\text{C}$  to attempt to obtain as close to the physiological environment as possible (Appell and Evans, 1977, Mortimer and Mortimer, 2013c). The samples in NCM were incubated for 15 min after preparation (DGC) before readings were taken to allow the sample to return to  $37^\circ\text{C}$  before analysis. The cells in CM were incubated for 2 hrs 30 min at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  to allow capacitation before analysis. Motility parameters remained stable for both fractions in either media, over the entire period of analysis (180 min). This indicates that the spermatozoa were stable in both media types over a period of 3 hrs at  $37^\circ\text{C}$ . As a result of capacitation, there was a difference in the incubation times between samples in NCM and CM before motility analysis, however

both media results show no significant deterioration over time suggesting that no significant difference in the results would be seen for NCM samples if they had been incubated for 2 hrs 30 min in advance of analysis.

#### ***4.3.1.4 Determination of variability between ejaculates from the same donor and different donor samples***

Numerous studies have been published discussing the biological variation in semen parameters (Mallidis et al., 1991, Alvarez, 2003, Keel, 2006). The smallest between subject variability in this study was found for total motility which is in accordance with previous studies (Poland et al., 1985, Alvarez, 2003). The smallest within subject variability in this study was found for progressive motility which differs to previous studies (Poland et al., 1985, Alvarez, 2003) however, it has been previously reported that variations in semen parameters produce contradictory results between groups (Mallidis et al., 1991). The within subject variability in this study showed lower levels of variation than the between subjects variation which is in agreement with previous reports (Heuchel et al., 1983, Alvarez, 2003). The results from this study, although evaluating a limited number of donors, corroborates previous reports and reinforces the need to be aware of variation both within subjects and between donor samples when conducting experiments. Thus, in chapter 5, 6 and 7 where some donors were assessed on more than one occasion, each sample was treated independently.

#### ***4.3.1.5 Assessment of motility parameters in capacitating and non-capacitating media treated with 1% DMSO***

Dimethyl sulfoxide (DMSO) is a dipolar aprotic solvent (Rammler and Zaffaroni, 1967). Many compounds, therapeutic and toxic, that are not soluble in water are often soluble in DMSO. The physiologic and pharmacologic properties of DMSO are not completely understood (Brayton, 1986). DMSO is utilised in many biological

applications because it has many properties that are important for use, including rapid and enhanced penetration of other substances across biologic membranes; free radical scavenging; effects on coagulation and anticholinesterase activity. The systemic toxicity of DMSO is considered to be low making it suitable for addition to biological compounds (Brayton, 1986). The majority of HTS in drug discovery programmes use DMSO stock solutions because compounds are both highly soluble in DMSO and conferred long-term stability by its presence. However, to avoid adverse solubility enhancement of compounds and to reduce potential co-solvent effects, it is generally recommended that DMSO concentrations be kept to a minimum of  $\leq 1\%$  (Alsenz and Kansy, 2007).

It has been reported that media not exceeding 1% has no detrimental effect on sperm motility, capacitation or the acrosome reaction (de Lamirande and Gagnon, 2002). However, there is very little other information available describing the effect of DMSO alone on sperm, although in research it is readily used in combination with compounds added to sperm suspensions (de Lamirande and Gagnon, 2002, Lefievre et al., 2002, Marín-Briggiler et al., 2005, Agirregoitia et al., 2010b, Wang et al., 2013). The effect of DMSO on sperm motility was therefore examined, in order to account for any favourable or adverse reactions as a result of its presence if added whilst screening drug discovery compounds. No significant differences were seen in any of the motility parameters measured in any sample in either media with or without 1% DMSO over a 180min time course. This result corroborates previous studies, showing that presence of 1% DMSO has no effect on human sperm motility.

## **CHAPTER 5**

# **Motility Analysis of Sperm treated with Hit Compounds identified through a Flexstation High Throughput Screening Assay**

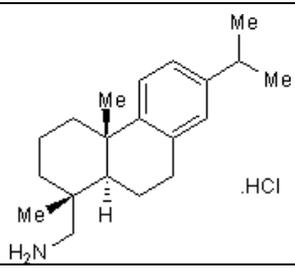
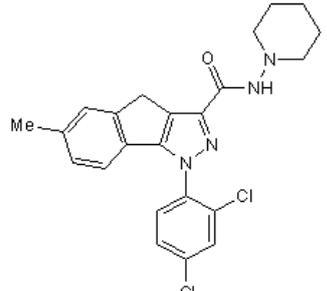
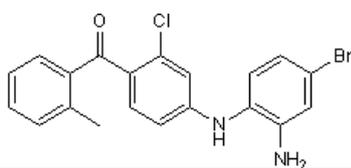
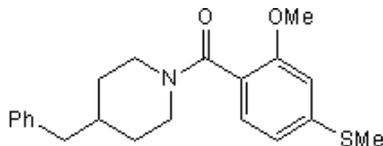
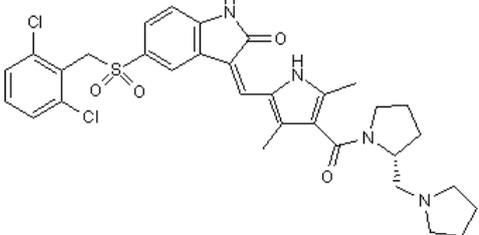
## 5.1 Introduction

Store operated  $\text{Ca}^{2+}$  channels at the neck/midpiece region of spermatozoa, through a CICR mechanism, have been identified as being regulators of sperm motility (Harper et al., 2004, Costello et al., 2009) along with the discovery of the sperm specific CatSper channel which is primarily associated with the development of hyperactivated motility triggered by depolarization- induced  $\text{Ca}^{2+}$  entry (Ren et al., 2001, Krasznai et al., 2006, Barratt, 2011). CatSper has enormous potential as a target for pharmaceutical manipulation due to its unique expression in sperm (Martins da Silva et al., 2012).

Martins de Silva *et al* developed a high throughput screening assay that identified compounds that increase  $[\text{Ca}^{2+}]_i$  in human sperm (Martins da Silva et al., 2012). Compounds from this high throughput assay screen were identified, using  $\text{Ca}^{2+}$  as a surrogate for motility, as potentially being able to enhance motility in human spermatozoa.

The Flexstation assay is a HTS assay developed using a Flexstation microplate reader to measure progesterone evoked increases in intracellular  $\text{Ca}^{2+}$  in motile human spermatozoa (Martins da Silva et al., 2012). Optimised conditions were then selected to successfully screen a Chemogenomics library from the UoD DDU, The Chemogenomic library examined Tocris compounds that were selected on the supposition they would be selective and potent ion channel compounds, based on evidence from literature. This library comprised of 246 compounds with progesterone being used as an internal control for the assay. This screen identified 27 active compounds that were capable of generating a peak  $\text{Ca}^{2+}$  response after exposure to prepared spermatozoa, including compounds which are known to modulate cellular  $\text{Ca}^{2+}$ . 6 of these compounds were subjected to in-depth spermatozoa motility assessments to try and identify novel compounds that have the ability to enhance sperm motility in an *in vitro* environment (Table 5.1). 5 of these compounds will be discussed in detail in this chapter with the 6<sup>th</sup>

compound Trequinsin hydrochloride being discussed in chapter 6. The benefits of the Chemogenomics screen for a complex system such as sperm is that it allowed for the identification of a range of pharmacologically active compounds that have known specific molecular targets giving you an indication of which proteins, receptors or enzymes may be in that system. This helps to define potential targets that may be activated/ inhibited when analysing the effects of unknown compounds.

Compound name	Primary action	Chemical structure
Leelamine Hydrochloride	CB1 agonist	
GP 1a	Highly selective CB2 agonist	
EO 1428	Selective inhibitor of p38 $\alpha$ and p38 $\beta$	
JX 401	Potent, reversible p38 $\alpha$ inhibitor	
PHA 665752	Potent and selective MET inhibitor	

**Table 5.1 Summary of active compounds identified in screen of the Chemogenomics library examined for their effects on sperm motility (Biosciences, 2013a-e)**

### 5.1.1 Cannabinoid agonists

Leelamine hydrochloride (Lylamine hydrochloride, (+)-Dehydroabietylamine) is a CB<sub>1</sub> receptor agonist (IC<sub>50</sub> 2.86µM) (Biosciences, 2013d) and GP1a is a CB<sub>2</sub> receptor agonist (Biosciences, 2013b) (Table 5.1). There are two different types of cannabinoid receptors that have been identified and cloned, both of which are members of the super-family of G-protein – coupled receptors (Howlett et al., 2002). CB<sub>1</sub> and CB<sub>2</sub> receptors are so called because they are the receptors that respond to cannabinoid drugs. The CB<sub>1</sub> cannabinoid receptor was first isolated in the brain and found mainly at the terminals of central and peripheral neurons but it has since been identified as being expressed in other tissues including spleen, peripheral leukocytes, uterus, testis, individual sperm cells, muscle cells and placenta (Howlett et al., 2002, Schuel et al., 2002, Rossato et al., 2005). It has been cloned from rat, mouse and human tissues and exhibits 99% homology across species (Howlett et al., 2002). The CB<sub>2</sub> receptor exhibits 48% homology with the CB<sub>1</sub> receptor and is primarily located to immune cells within and outside the central nervous system and has also been identified in human sperm cells (Ashton and Glass, 2007, Agirregoitia et al., 2010a).

Functional CB<sub>1</sub> and CB<sub>2</sub> receptors have been identified in mature human spermatozoa (Rossato et al., 2005, Agirregoitia et al., 2010a) along with mRNA and proteins for CB<sub>1</sub> and CB<sub>2</sub> receptors (Agirregoitia et al., 2010a, Amoako et al., 2013a). CB<sub>1</sub> has been shown to be localised to the plasma membrane of the acrosomal region, tail and midpiece of human sperm, whereas CB<sub>2</sub> has been found to be mainly localised in the post-acrosomal region, midpiece and tail of sperm cells (Agirregoitia et al., 2010a, Amoako et al., 2013b)

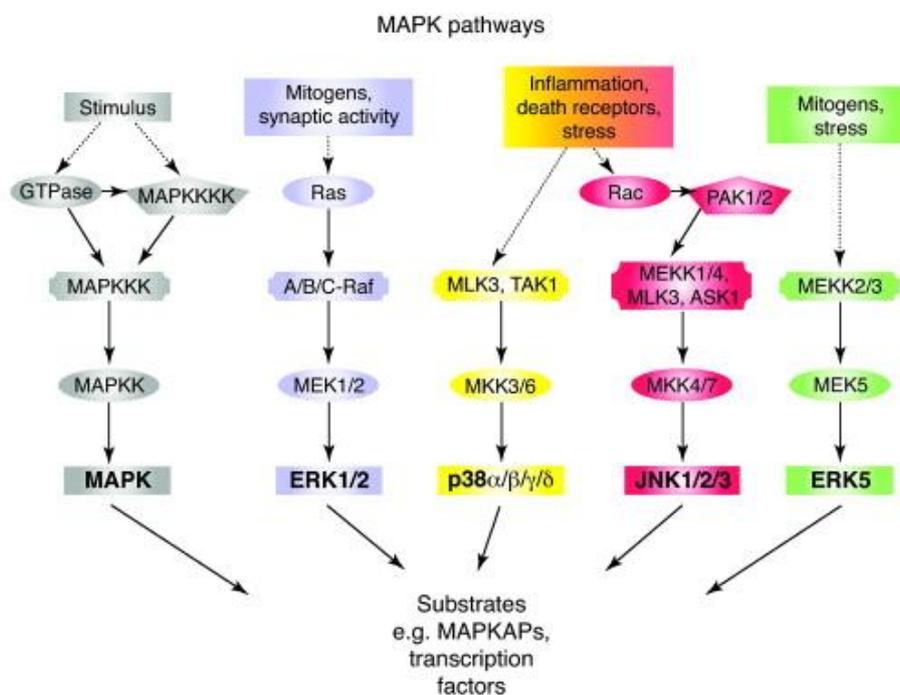
Compounds that act via the CB<sub>1</sub> and CB<sub>2</sub> receptors reproduce some of the biological actions of the cannabinoid drugs such as Δ<sup>9</sup>-tetrahydrocannabinol (THC) which is

derived from the natural *Cannabis sativa* (Howlett et al., 2002). Along with cannabinoids, a family of unsaturated fatty acid derivatives with actions similar to cannabinoids have been identified and are known as endocannabinoids. Cannabinoids and endocannabinoids exert their effects via activation of specific cannabinoid receptors (Rossato et al., 2005, Amoako et al., 2013b). Previous studies suggest that sperm function is regulated by endocannabinoids through a dual stage-dependent effect involving both CB<sub>1</sub> and CB<sub>2</sub> receptors (Agirregoitia et al., 2010a, Maccarrone, 2008).

CB<sub>1</sub> and CB<sub>2</sub> receptors have also been associated to transduction pathways coupled to pertussis toxin-sensitive G<sub>i</sub>/G<sub>o</sub> proteins. Through this they negatively affect adenylate cyclase activity and production of cAMP (Howlett et al., 2002), inhibit L, N, P and Q type voltage-gated Ca<sup>2+</sup> channels (Howlett et al., 2002, Howlett, 2005) and positively affect A-type and inwardly rectifying K<sup>+</sup> channels, phospholipase C and mitogen-activated protein kinase (Howlett, 2005, Pertwee, 1997). CB<sub>1</sub> receptors are also capable of coupling to G<sub>s</sub> proteins to activate soluble adenylate cyclase (Pertwee, 1997, Glass and Felder, 1997, Calandra et al., 1999). Additionally, there have been reports that CB<sub>1</sub> and CB<sub>2</sub> receptors enhance [Ca<sup>2+</sup>]<sub>i</sub> concentrations (Sugiura and Waku, 2000, Sugiura et al., 1997, Pertwee, 2005). This outlines the diversity of compounds acting on these receptors and the potential for different functional events. An understanding of this complex system is fundamental to male infertility and importantly has the possibility to provide potential targets for pharmacological intervention in male infertility (Amoako et al., 2013b).

### 5.1.2. Mitogen activated protein kinases

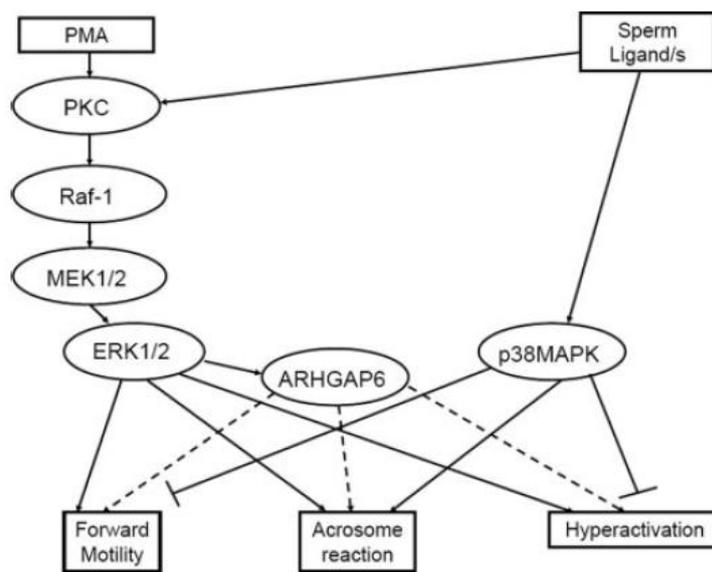
Mitogen activated protein kinases (MAPKs) are key regulatory enzymes that participate in cell growth, differentiation, survival, stress, cell cycle progression and apoptosis (Almog et al., 2008). MAPK cascades consist of levels of protein kinases that sequentially activate each other through phosphorylation. There are four major MAPK cascades known for mammals: ERK1-2, JNK 1-3, p38  $\alpha$ - $\delta$  and ERK5 (figure 5-1) (Gutkind, 1998, Pearson et al., 2001).



**Figure 5-1.** Key signalling components of the four major MAPK pathways in mammals (Berwick and Harvey, 2011)

EO1428 is a selective inhibitor of p38 $\alpha$  and P38 $\beta_2$  (IC<sub>50</sub> 0.039 $\mu$ M) (Biosciences, 2013a) and JX401 is a potent reversible inhibitor of p38 $\alpha$  (IC<sub>50</sub> 32nM) (Biosciences, 2013c) (Table 5-1). p38 MAPK is now known to be a stress related activated kinase which is initiated by a stress stimuli (Almog et al., 2008). The p38 MAPK cascade consists of sequential activation of MAP3Ks, MKK3/4/6, p38  $\alpha$ - $\delta$ , and several mitogen activating protein kinase activating protein kinases (MAPKAPKs) (figure 5-1) (Pearson et al., 2001, Almog et al., 2008).

During spermatogenesis MAPKs function to mediate cell division, differentiation, survival and death (Wong and Yan Cheng, 2005). The knowledge of the function of MAPK in motility, capacitation and the acrosome reaction in mature spermatozoan is limited and controversial (Weidinger et al., 2005). The role of p38 was unknown until Almog *et al* identified that p38 MAPK is not only expressed in mature spermatozoa but is primarily localised to the tail (Almog et al., 2008). Almog *et al* also discovered that ERK stimulated progressive and hyperactivated motility whereas p38 MAPK acted to inhibit motility (Almog et al., 2008) (figure 5-2). Both ERK 1/2 and p38 MAPK have also been identified, positively, in having a role in PKC-mediated acrosome reaction (Almog et al., 2008).



**Figure 5-2 Proposed model for p38 and ERK1/2 signalling in human sperm.** p38 MAPK and ERK1/2 have been identified in the tail of mature human sperm. ERK stimulates and p38 inhibits progressive and hyperactivated motility. Both p38MAPK and ERK1/2 are positively associated with the acrosome reaction. (Almog et al., 2008)

p38 MAPK is encoded by four genes that express nine isoforms with the main isoform found to be expressed in mature ejaculated human spermatozoa being p38 $\alpha$  (Pearson et al., 2001, Almog et al., 2008). As mentioned previously both JX401 and EO1428 are selective inhibitors for p38 $\alpha$  suggesting that these compounds should act to inhibit the p38 inhibition on sperm motility leading to increased progressive and hyperactivated

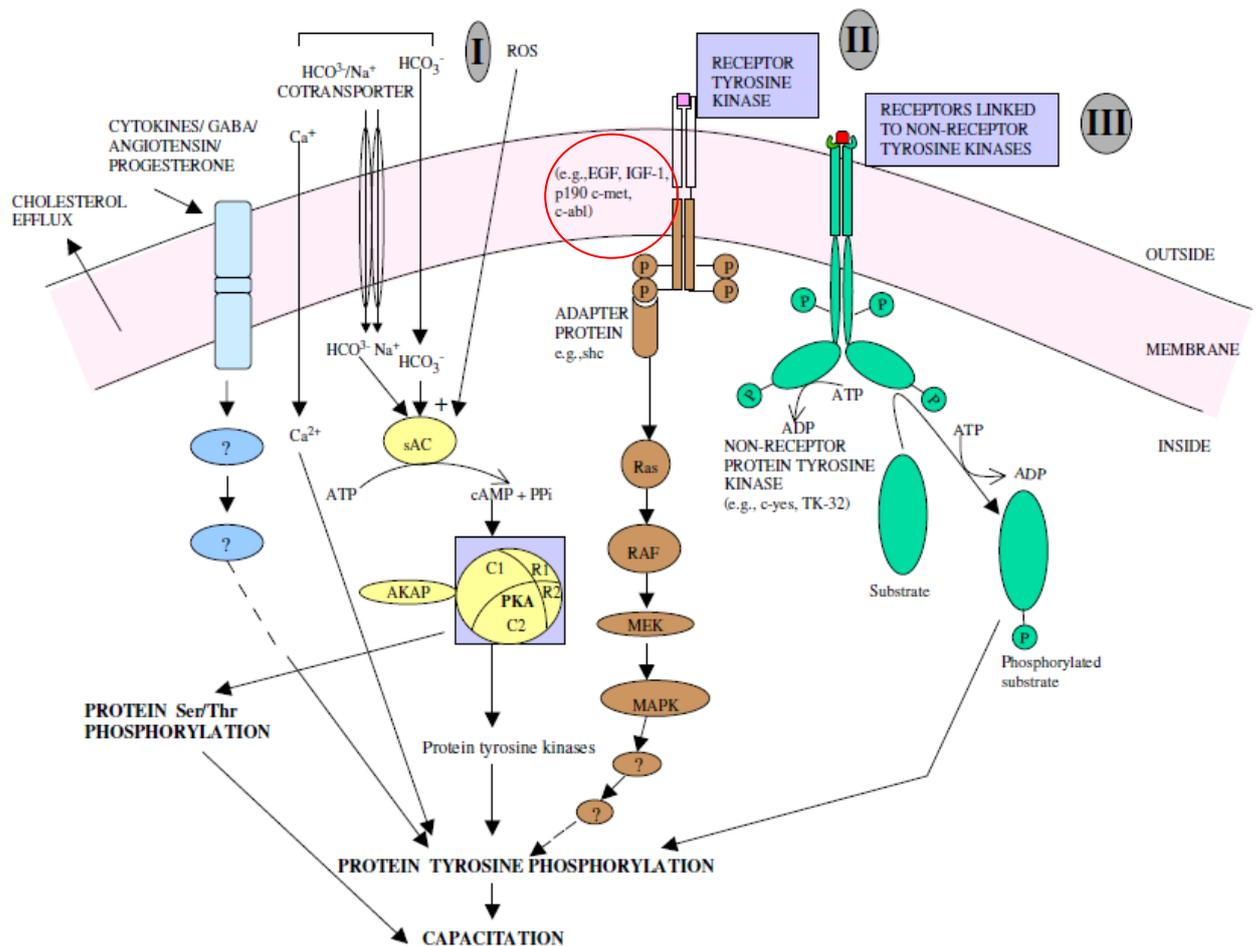
motility. p38 inhibitors have been used previously with positive effects on both progressive motility and hyperactivation (Almog et al., 2008).

### 5.1.3 c-MET kinase inhibitor

PHA665752 is a potent, selective 2<sup>nd</sup> generation ATP-competitive inhibitor of c-MET kinase, a receptor tyrosine kinase (Table 5-1) (IC<sub>50</sub> 9nM) (Biosciences, 2013e).

Tyrosine kinases are themselves receptors for some growth factors that regulate cell proliferation and differentiation (Ullrich and Schlessinger, 1990, Herness and Naz, 1999). The c-met proto-oncogene encodes a transmembrane glycoprotein p190<sup>MET</sup> tyrosine kinase receptor (Herness and Naz, 1999). The ligand for the c-met receptor has been identified and assigned as a scatter factor/ hepatocyte growth factor (SF/HGF) (Bottaro et al., 1991). SF/HGF has been identified as having potent motogenic, mitogenic and morphogenetic actions on epithelial cells and proteins have been identified in human amniotic fluid, human placental tissue, stromal components of human testis and in the ovary (Strain, 1993, Herness and Naz, 1999, Naldini et al., 1991, Lail-Trecker et al., 1998). Depuydt *et al* identified that the c-met receptor is localised in the human seminiferous epithelium and on mature spermatozoa (Depuydt et al., 1996). This same group have also shown that HGF is present in seminal plasma (Depuydt et al., 1997) and has identified relationships between the concentration of HGF in human seminal plasma and andrological diseases, including asthenozoospermia, suggesting a link between HGF and motility (Depuydt et al., 1998). SF/HGF has been found to be differentially expressed in segments of the male genital tract, highest at the cauda and distal corpus of the epididymis, in mice (Naz et al., 1994). Herness *et al* identified that the c-met receptor is present in human mature sperm cells predominantly in the acrosomal subcellular site (Herness and Naz, 1999). The c-met receptor is tyrosine phosphorylated/ autophosphorylated during capacitation through cross-talk

with other molecules (Naz, 1996) (figure 5-3). Protein tyrosine phosphorylation has a role in a variety of cellular functions including regulation of various receptors (Ullrich and Schlessinger, 1990). Tyrosine phosphorylation increases in human spermatozoa during capacitation and is an important regulatory pathway in events associated with capacitation. The signalling pathway of c-met kinases function on human sperm cells has not been elucidated but previous reports indicate that c-met and its ligand (SF/HGF) may have an important function in sperm motility owing to its motogenic properties (Naz et al., 1991, Naz, 1996, Herness and Naz, 1999, Naz and Rajesh, 2004).



**Figure 5-3. Heuristic model by Naz et al showing tyrosine phosphorylation signalling pathways in human sperm cells involved in capacitation.** There are three major pathways in the sperm cell; cAMP/PKA-dependent pathway (pathway I), receptor tyrosine kinase pathway (pathway II), and non-receptor protein tyrosine kinase pathway (pathway III). The pathway by which c-met kinases function is pathway II. These cascades are not mutually exclusive and it is possible they include cross-talk amongst several molecules. Many key molecules and receptors still need to be elucidated to fully understand the molecular mechanism and signal transduction cascade involved in capacitation. (Naz and Rajesh, 2004)

## 5.2 Experimental Design

Semen samples obtained from donors were prepared using DGC. Each sample was diluted in either NCM or CM as appropriate to a final concentration of 20 million/ml. Samples in NCM were placed in an incubator at 37°C for 15 min whereas samples in CM were placed in a 5% CO<sub>2</sub> incubator at 37°C for 2 hrs 30 min. 99µl of semen sample was placed in a round bottom tube followed by the addition of 1µl of the appropriate compound. 1% DMSO was added to the control tube to act as a vehicle control. After re-suspending cells, 3µl of sperm suspension was loaded onto a preheated fixed chamber slide and CASA analysis conducted at 30 min intervals over a period of 3 hrs. I will only be discussing total motility, progressive motility and hyperactivation in detail. The results for VCL and ALH are available in the appendix.

## 5.3 Results

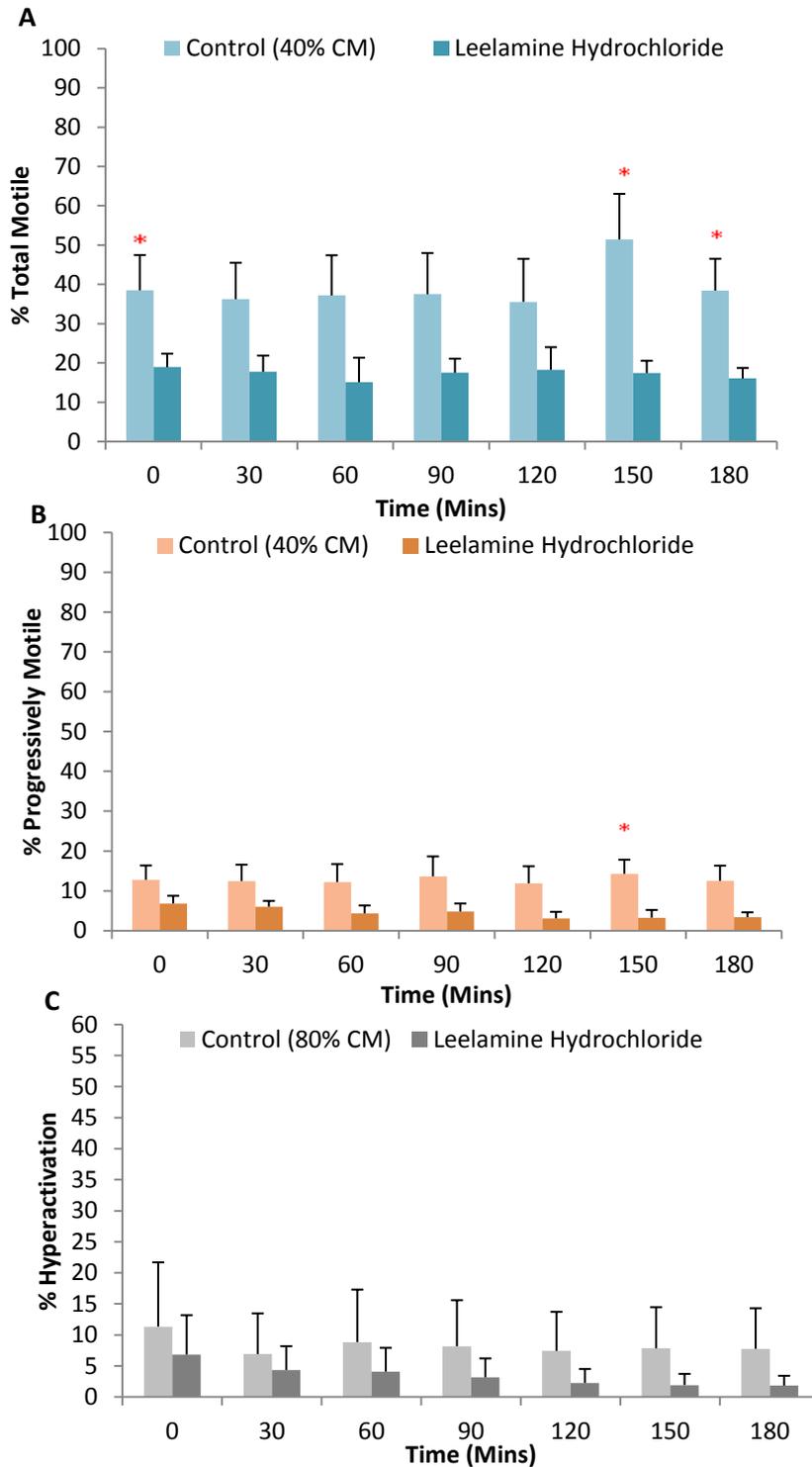
### 5.3.1 Motility assessment by CASA of five hit compounds

#### 5.3.1.1 *Leelamine Hydrochloride*

When spermatozoa were treated with Leelamine Hydrochloride (40µM), there was a decrease in total motility, progressive motility and hyperactivation when both the 40% and 80% fraction were assessed under non-capacitating conditions (appendix). Treated cells, after capacitation, from the 40% fraction showed significant changes instantly in the percentage of motile cells ( $P \leq 0.05$ ; figure 5-4A), whereas significant alterations in progressive motility were not observed until after 150 min ( $P \leq 0.05$ ; figure 5-4B).

Hyperactivation also decreased although this result was not significant (appendix).

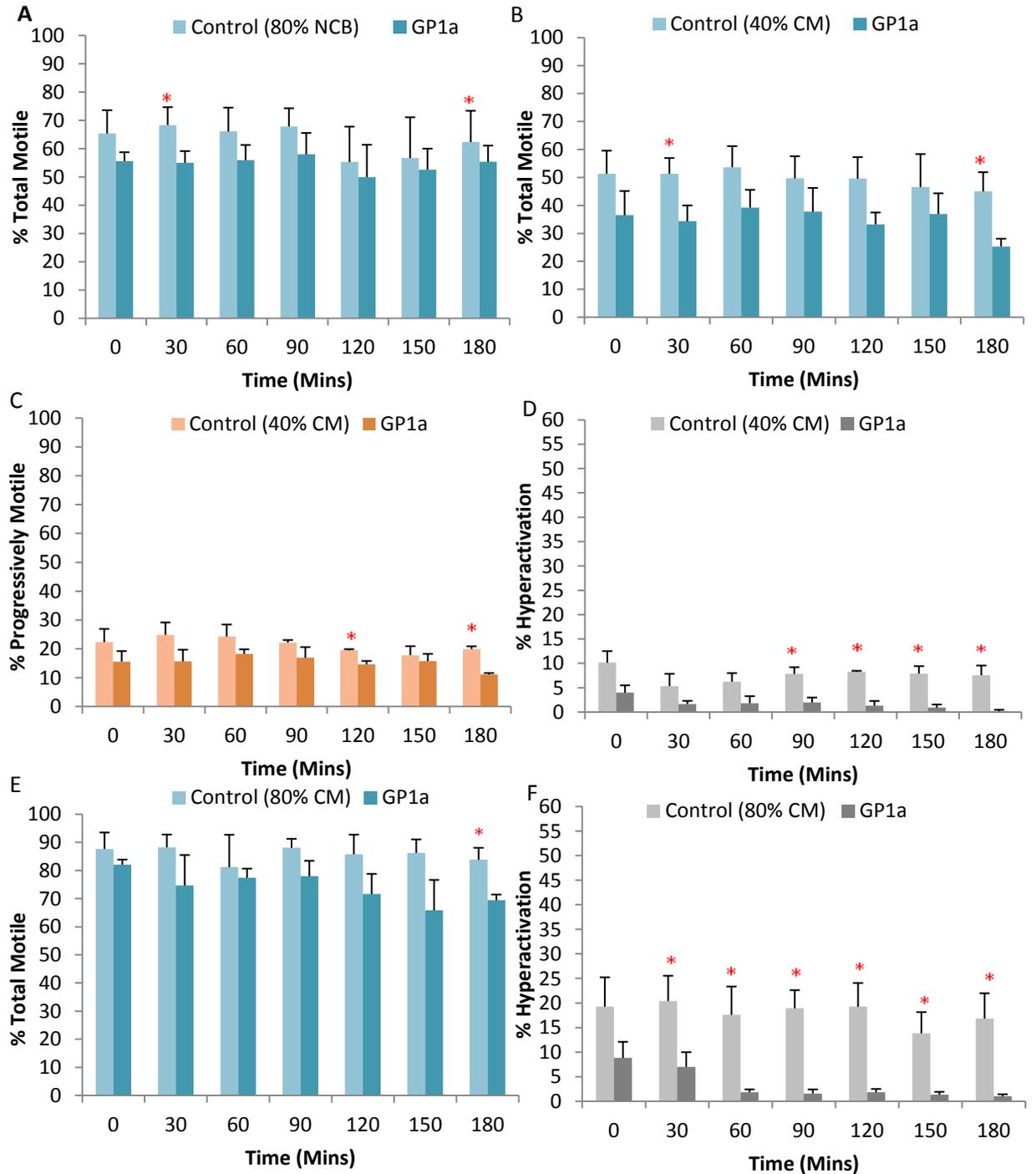
Results from the 80% fraction identified no significant differences between control and treated cells. However, a time- dependent decrease in hyperactivation was noted over the 3hr time course (figure 5-4C). The predominant effect exhibited by spermatozoa after prolonged exposure to this compound, in both the 40% and 80% fractions, under capacitating and non-capacitating conditions, was a decrease in all motility parameters assessed.



**Figure 5-4. Expression of basal (control) motility values compared to Leelamine Hydrochloride treated samples capacitating conditions.** (A) % total motile (40% fraction CM), (B) % progressively motile (40% fraction CM) and (C) % hyperactivation (80% fraction CM). The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min capacitating conditions comparing the difference between the control and treated samples.  $N=4$  for all parameters, \* indicates a significant increase ( $P \leq 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

### 5.3.1.2 *GP1a*

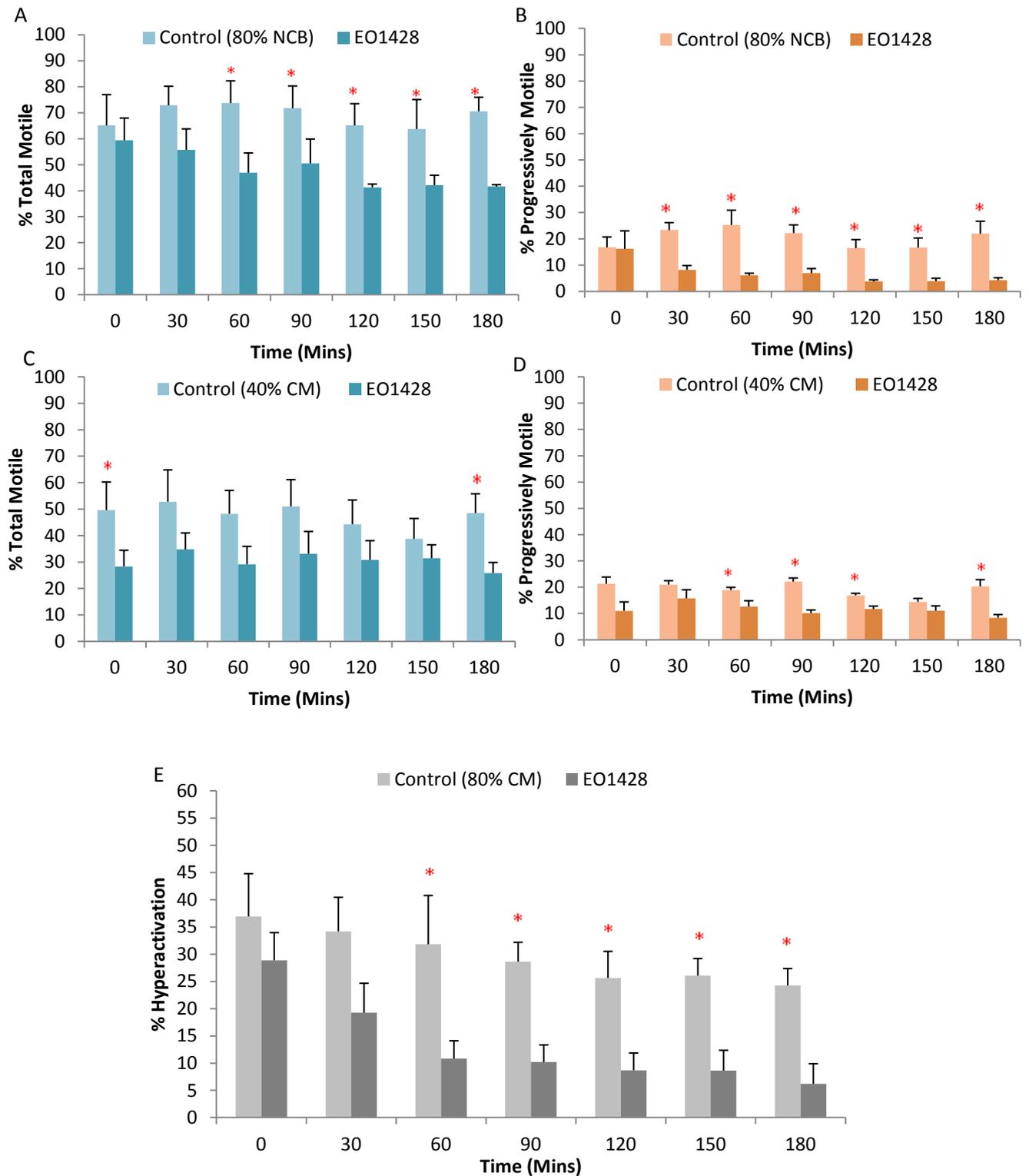
After evaluating the effects of a CB<sub>1</sub> agonist on sperm motility, GP1a, a CB<sub>2</sub> agonist, was examined (40μM). The results from cells treated with GP1a were similar to that of Leelamine hydrochloride with the trend exhibiting decreased motility. Once again, cells under non-capacitating conditions treated with the agonist displayed less motile cells than control in both the 40% (appendix) and 80% fractions ( $P \leq 0.05$  figure 5-5A). However, progressive motility and hyperactivation were not significantly changed from control (appendix). The most substantial changes were exhibited when the sperm cells had been exposed to GP1a after capacitation. In the 40% fraction total motile, progressively motile and hyperactivation, were all significantly decreased (figure 5-5B,C&D). Cells from the 80% fraction revealed time-dependent decreases in hyperactivation ( $P \leq 0.05$ ) after 30 min treatment (figure 5-5F). However, significant decreases in total motility were not observed until after 180 min treatment (figure 5-5E) with no changes in progressive motility observed (appendix).



**Figure 5-5. Expression of basal (control) motility values compared to treated samples in non capacitating (NCM) and capacitating (CM) media, (A) % total motile (80% fraction NCM), (B) % total motile (40% fraction CM), (C) % progressively motile (40% fraction CM), (D) % hyperactivation (40% fraction CM), (E) % total motile (80% fraction CM) and (F) % hyperactivation (80% fraction CM).** The result shown is the mean  $\pm$  SE for all parameters. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples.  $N=3$  for both parameters, \* indicates a significant increase ( $P \leq 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

### 5.3.1.3 EO1428

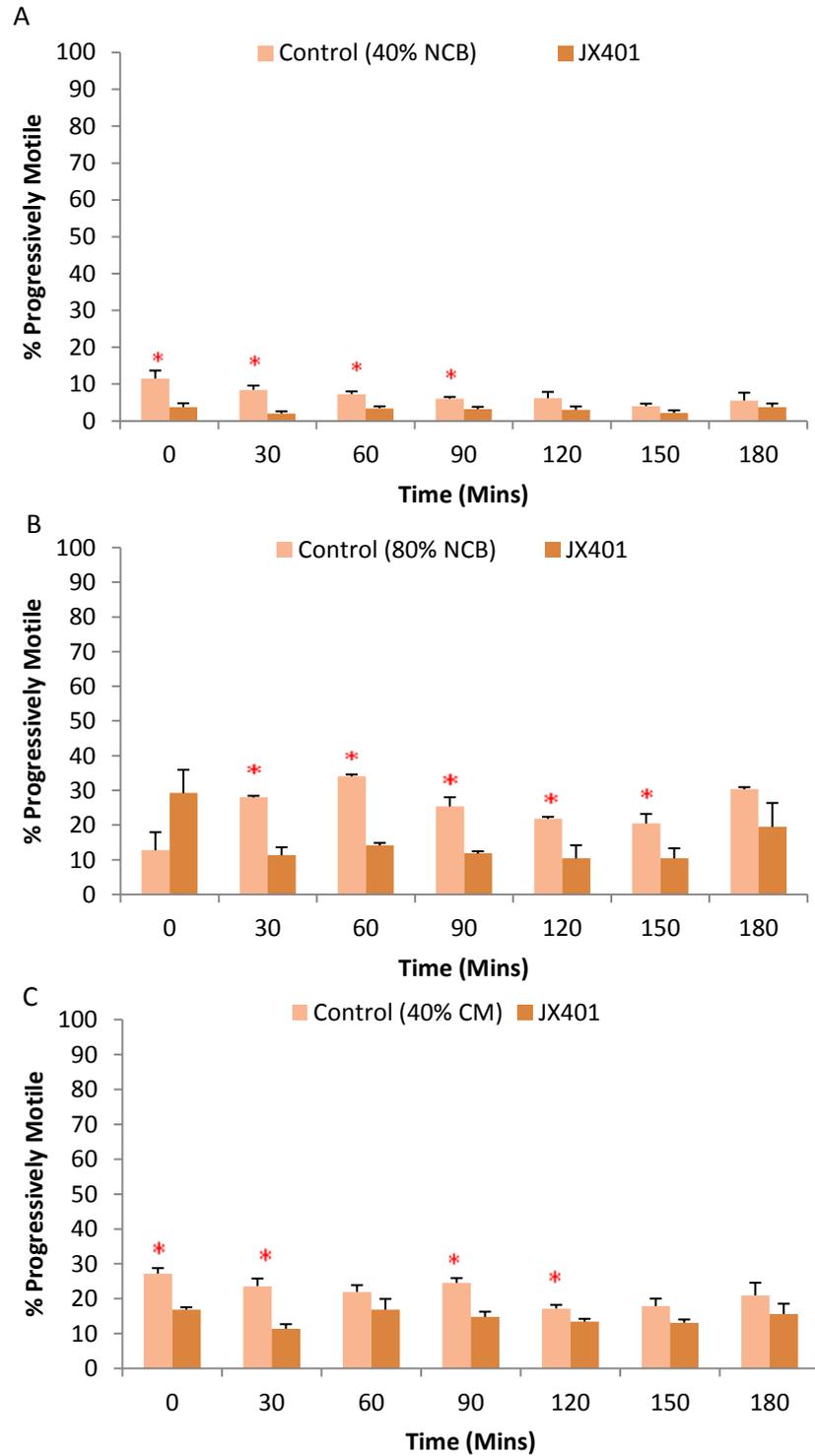
CASA analysis of sperm treated with EO1428 (40 $\mu$ M) under non-capacitated conditions showed significant decreases in total motility and progressive motility ( $P\leq 0.05$ , appendix) for cells from the 40% fraction. Treated samples from the 80% fraction displayed significant decreases in total motility ( $P\leq 0.05$ , figure 5-6A). The percentage of progressively motile cells was observed to decrease significantly after 30 min treatment and this decrease was sustained for the remainder of the time-course ( $P\leq 0.05$ , figure 5-6B). However, no change was observed in hyperactivation compared to control (appendix). Cells treated with this agonist, similarly to GP1a, exhibited a higher proportion of significant decreases in sperm motility in capacitated cells compared to non-capacitated. The 40% fraction under these conditions showed significant decreases in total motility, progressive motility and hyperactivation ( $P\leq 0.05$ , figure 5-6 C, D and appendix). Spermatozoa from the 80% fraction displayed significant reductions ( $P\leq 0.05$ ) in hyperactivation after 60 min an effect that was sustained over the remainder of the time-course (figure 5-6E). Kruskal Wallance statistical analysis indicated that hyperactivation was significantly lower in the treated spermatozoa ( $p=0.036$ ) over the 3hr time-course (figure 5-6E). Progressive motility appeared to increase compared to control after 60 and 90 min treatment however, this was not a significant increase and was not sustained (appendix). Total motility remained unchanged from control (appendix).



**Figure 5-6. Expression of basal (control) motility values compared to EO1428 treated samples in non-capacitating (NCM) and capacitated (CM) media.** (A) % total motile (80% fraction NCM), (B) % progressive motility (80% fraction NCM), (C) % total motile (40% fraction CM), (D) % progressive motility (40% fraction CM) and (E) % hyperactivation (80% fraction CM). The result shown is the mean  $\pm$  SE for all parameters. In this study all motility parameters were measured over a period of 180 min under non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 4 for both parameters, \* indicates a significant increase ( $P \leq 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

#### **5.3.1.4 JX401**

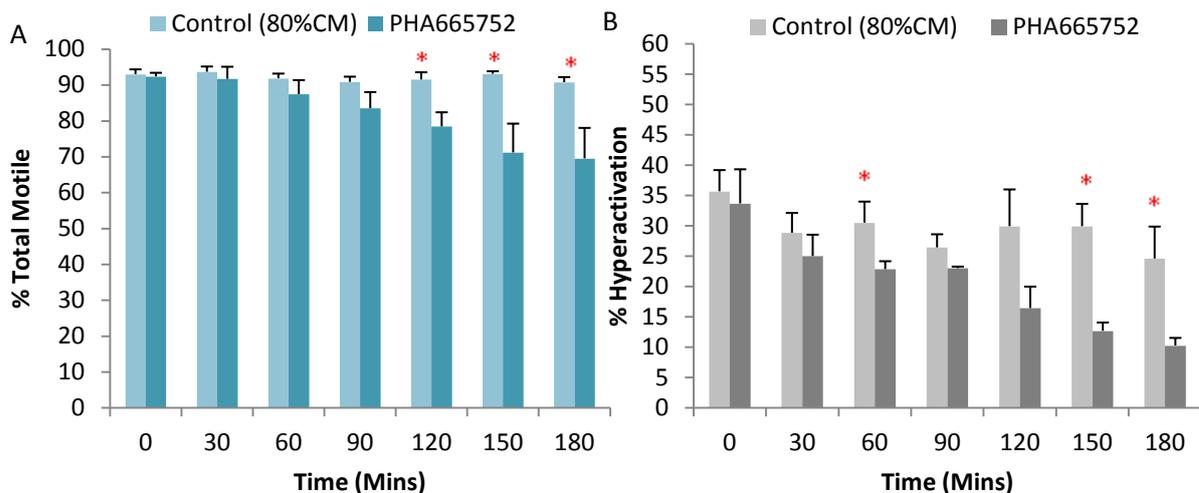
In motility parameters, under non-capacitating conditions, sperm progressive motility and hyperactivation were significantly decreased ( $P \leq 0.05$ ) for the 40% fraction (figure 5-7A & appendix respectively). No significant changes were observed in any other parameter over the time-course (appendix). For the 80% fraction both the percentage of total motile and progressively motile cells, treated with JX401 (40 $\mu$ M), showed significant decreases after 60 min (total motile) and 30 min (progressively motile) which is sustained for the 180min incubation (appendix and figure 5-7B). Hyperactivation was also found to be significantly lower after 30 min incubation (appendix). This compound differed to the others examined with the most significant results being produced under non-capacitating conditions instead of capacitating as shown previously. Cells from the 40% fraction, exposed to capacitating conditions, following treatment with this compound, exhibited reduced progressive motility instantly and this effect was sustained after 30, 90 and 120 min (figure 5-7C). However, no significant differences were found for any other kinematic parameter (appendix). Treated sperm collected from the 80% fraction revealed significant decreases in total motility and hyperactivation when compared to control (appendix) however, progressive motility remained unchanged from control (appendix).



**Figure 5-7. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) and capacitating media.** (A) % progressive motility (40% fraction, NCM), (B) % progressive motility (80% fraction, NCM) and (C) % progressively motile (40% fraction, CM). The result shown is the mean  $\pm$  SE for motility parameters measured. All motility parameters were measured over a period of 180 min under non-capacitating or capacitating conditions. N= 3 for all parameters, \* indicates a significant increase ( $P \leq 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

### 5.3.1.5 PHA665752

PHA665752 (40 $\mu$ M) was the only compound that produced no significant changes from control when motility parameters were examined for the 40% fraction under non-capacitating conditions (appendix). The 80% fraction under the same (non-capacitating) conditions exhibited significant decreases ( $P\leq 0.05$ ) in the percentage of motile cells after 90 min but was not sustained (appendix). No other parameters were significantly changed from control under these conditions (appendix). Cells that had been capacitated prior to treatment, when assessing motility, exhibited significant decreases for the percentage of total motile (180 min) and progressively motile (120 min) cells when compared to control in the 40% fraction (appendix). Similarly to GP1a and EO1428 the most significant decreases in kinematic parameters were identified from the 80% fraction after capacitation. PHA665752 was causative of decreases in hyperactivation, along with, a time-dependent decrease in the percentage of total motile cells ( $P\leq 0.05$ , figures 5-8A&B). Progressive motility exhibited no significant changes from control (appendix). Kruskal Wallance statistical analysis identified time-dependent significant decreases in spermatozoa treated with PHA665752 for hyperactivation (figures 5-8B).



**Figure 5-8. Expression of basal (control) motility values compared to treated samples in capacitating (CM) media in 80% fraction for (A) % total motile and (B) hyperactivation.** The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for both parameters, \* indicates a significant increase ( $P\leq 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallace test).

## 5.4 Discussion

The primary aim of this chapter was to examine the effects of compounds, identified from the Flexstation high-throughput screening assay, on motility parameters of human sperm from donors and sub fertile patients. However, the predominant effect seen as a result of addition of the 5 other agonists examined (Leelamine hydrochloride, GP1a, EO1428, JX402 and PHA665752) indicated significant decreases in all motility parameters.

### **Cannabinoid receptor agonists increase $[Ca^{2+}]_i$ but fail to enhance sperm motility**

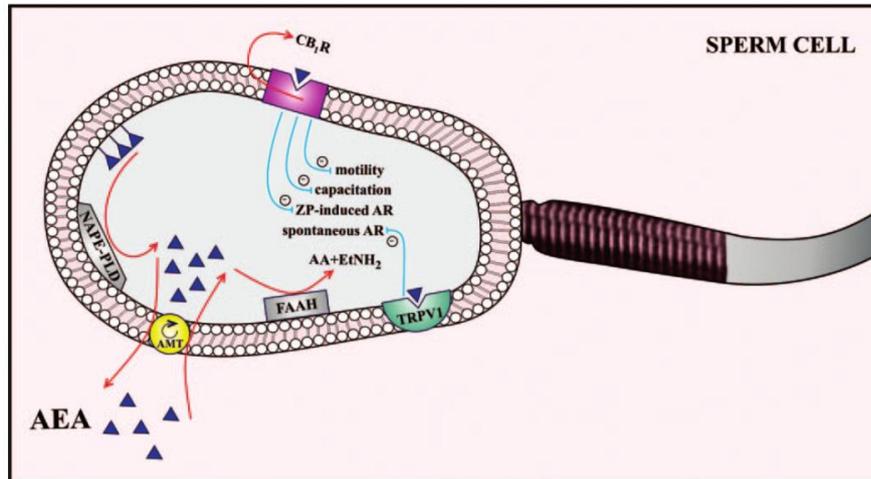
The results of this chapter showed that motility parameters of human sperm, specifically the percentage of total motile cells, were decreased when exposed to both a CB<sub>1</sub> agonist; Leelamine hydrochloride, and a CB<sub>2</sub> agonist; GP1a. This result reflects previous studies examining the effects of cannabinoids on sperm function (Rossato et al., 2005). Endocannabinoids (a group of endogenous, lipid mediators) have been found to play a role in motility and negatively affect fertilising potential of human sperm (Francavilla et al., 2009). The two best characterised members of the endocannabinoid family are *N*-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG, a monoacylglycerol) both of these endocannabinoids are capable of working at the type 1 (CB<sub>1</sub> receptor) and type 2 (CB<sub>2</sub> receptor) cannabinoid receptors (Howlett et al., 2002, Francavilla et al., 2009). Increases in intracellular  $Ca^{2+}$ , as a result of cannabinoid agonist addition (Howlett et al., 2002), induces production of AEA which in turn through a dual stage-dependent effect activates both CB<sub>1</sub> and CB<sub>2</sub> receptors (Marzo et al., 2005, Maccarrone, 2008, Agirregoitia et al., 2010a). The most well know endocannabinoid is *N*-arachidonylethanolamine (anandamide, AEA) (Devane et al., 1992). The biosynthesis of AEA is complex but most of the pathways involved in the synthesis of AEA are by a specific phospholipase (NAPE-PLD) with AEA being synthesised from the precursor *N*-acylphosphatidylethanolamine (NAPE) (Okamoto et

al., 2007). The effects of AEA are terminated when AEA is taken up through an AEA membrane transporter after which it is degraded to ethanolamine (EtNH<sub>2</sub>) and arachidonic acid (AA) by the fatty acid amide hydrolase (FAAH) (Cravatt and Lichtman, 2002, Amoako et al., 2013b).

Significant levels of AEA have been found to be present in human seminal plasma, as sperm leave seminal plasma and approach the egg in the female reproductive tract; they are exposed to progressively decreasing AEA concentrations (Rossato et al., 2005, Amoako et al., 2010). It has been theorised that the high AEA concentrations observed in seminal plasma could act to maintain sperm in a quiescent metabolic state that can be activated within the female reproductive tract (Schuel et al., 2002). Once a threshold level of AEA is reached membrane hyperpolarization is triggered increasing the influx of Ca<sup>2+</sup>. This theory is supported by differential expression of NAPE-PLD and FAAH that finely control levels of AEA in mouse oviduct (Wang et al., 2006). In this respect, decreasing AEA concentration in the female reproductive tract secretions could reduce its inhibitory effect on sperm, thus rendering them suitable for capacitation and gaining their fertilisation potential (Rossato et al., 2005). The existence of this AEA gradient suggests a modulatory role of the endocannabinoid system on human sperm function and could modulate sperm cell motility (Agirregoitia et al., 2010a, Amoako et al., 2013b).

Leelamine was found from the Flexstation screen to increase [Ca<sup>2+</sup>]<sub>i</sub> in human sperm cells which potentially increases production of AEA leading to increased levels of AEA and a decrease in sperm function, including sperm motility, by promoting inhibition through activation of the CB<sub>1</sub> receptor (mechanisms described previously, figure 5-9). Similarly, the incubation of sperm cells with a selective CB<sub>2</sub> receptor agonist GP1a, also exhibited significant decreases in motility parameters which corroborates a previous

study by Agirregoitia *et al* who identified a shift in the proportion of cells from A grade to B grade on addition of CB<sub>2</sub> agonist (Agirregoitia *et al.*, 2010a). These results suggest that cannabinoids do have a role in controlling sperm motility however it may be more beneficial for their potential therapeutic applications to further study their possible use as contraceptives rather than motility enhancers.



**Figure 5-9. The endocannabinoid system and sperm function.** Alterations in  $[Ca^{2+}]_i$  prompt AEA production which binds to the extracellular site of CB<sub>1</sub> (CB<sub>1</sub>R) leading to inhibition of sperm motility, acrosome reaction and capacitation. AEA is broken down via the synthesizing phospholipase NAPE-PLD and the AEA hydrolyzing FAAH which cleaves AEA into ethanolamine (EtNH<sub>2</sub>) and AA. Image from Wang *et al.* (Wang *et al.*, 2006)

### Mitogen activated protein kinase inhibitors fail to enhance sperm motility

Treatment with both JX401 and EO1428 was found to significantly decrease total motility, progressive motility and hyperactivation of treated cells under all conditions examined. Numerous groups have published information regarding MAPK cascades involvement in spermatogenesis and spermatozoa functions, however this data is controversial and information regarding the nature of endogenous and physiologically relevant sperm- ligands, that are capable of activating MAPK's including p38, remain unknown (Weidinger *et al.*, 2005, Wong and Yan Cheng, 2005, Almog and Naor, 2008). However, previous groups making use of p38 inhibitors in assessing sperm motility noticed increases in sperm motility and hyperactivation (Almog *et al.*, 2008) which contradicts the results of this study. As described previously p38 $\alpha$  has been

identified to be located at the sperm tail and has been proposed to negatively affect progressive motility and hyperactivation (figure 5-3) (Almog et al., 2008). This suggested that the use of specific p38 $\alpha$  inhibitors would have the opposite effect allowing for improved motility and hyperactivation. As this was not seen to be the case in this study, but has been seen previously (Almog et al., 2008), it suggests the possibility that these compounds may be toxic to spermatozoa (Li et al., 2009). p38 $\alpha$  is broadly expressed and is the most abundant p38 family member found to be present in most cell types (Porras et al., 2004). Due to the limited understanding of p38 signalling pathways in sperm it could be possible that inhibition of p38 $\alpha$  could be detrimental not only to sperm motility but to other vital functions associated with cell survival. Almog et al proposed that p38 works in association with ERK1/2 to regulate sperm motility with ERK1/2 promoting and p38 inhibiting motility (Almog et al., 2008) however, Weidinger et al published an opposite report that concluded that ERK1/2 inhibited sperm motility (Weidinger et al., 2005). In this instance, if p38 and ERK1/2 are regulating motility, and ERK1/2 is actually inhibiting motility, then it is possible that p38 might be promoting motility and addition of p38 inhibitors would surmise that you would see a decrease in sperm motility under these circumstances. An earlier study, on demembrated fowl sperm, had suggested that the MAPK pathway had a function in sperm motility after noticing inhibited flagella motility on addition of MAPK inhibitors (Ashizawa et al., 1997). p38 is known to play a vital role in germ cell development, specifically in spermatogenesis having a role in disturbing the blood testes barrier to allow preleptolene spermatocytes to migrate to the adluminal compartment (Almog and Naor, 2010). Previous studies on mice have also indicated the importance of p38 through targeted inactivation of the p38 $\alpha$  gene which resulted in embryonic death due to a placental defect (Adams et al., 2000). If p38 $\alpha$  inhibitors were proposed for use clinically in increasing sperm motility the potential negative effects on the prospective

embryo (Adams et al., 2000) would need to be considered suggesting that p38 $\alpha$  inhibitors may not be of use in pharmacological intervention without causing systemic toxicity and embryonic death (Adams et al., 2000, Li et al., 2009).

### **C-met inhibitors decrease motility when spermatozoa are in a capacitated state**

The effect of c-met on human ejaculated spermatozoa is still not clear with published results being controversial (Wiltshire et al., 2000, Catizone et al., 2006). C-met receptor induces its biological activities through binding its ligand; HGF/SF which has potent mitogenic, motogenic and morphogenetic activities (Herness and Naz, 1999, Kitamura et al., 2000, Wiltshire et al., 2000). Transgenic studies have shown that in mice targeted HGF gene disruption is lethal *in utero* (Uehara et al., 1995) identifying the importance of action through this ligand and its associated c-met receptor. This study suggests that inhibition of the c-met receptor would prevent HGF binding resulting in a similar effect to that seen in the knock out study. Naz *et al* have previously examined the relationship between HGF sperm motility in murine models and their results suggested HGF may be involved in the attainment of motility in mammalian sperm (Naz et al., 1994). HGF has also been reported to have a positive effect on epididymal sperm motility in rats (Catizone et al., 2006). This same group identified c-met receptors to be localized both on the head and the flagellum of isolated epididymal spermatozoa and suggested in rats that HGF/c-met are related to epididymal acquisition and maintenance of sperm motility (Catizone et al., 2006). This data contrasts with studies on human sperm where it has been reported that HGF does not significantly maintain or alter human sperm motility (Kitamura et al., 2000, Wiltshire et al., 2000). This conflicting data suggests that the effects of HGF/c-met function differently in rodents than in humans (Catizone et al., 2006). The data available from studies on human ejaculated spermatozoa report that HGF has no active role in controlling sperm motility however both studies suggest the possibility of HGF playing a role in epididymal maturation and maintenance of motility

(Kitamura et al., 2000, Wiltshire et al., 2000). The results of this study corroborate that of Wiltshire *et al* and Kitamura *et al* suggesting that HGF/c-met do not appear to control ejaculated human sperm motility. Use of PHA665752 (c-met inhibitor) when in combination with spermatozoa under non-capacitating conditions exhibited no changes to motility parameters compared to control. This inhibition of c-met would have no effect on sperm motility if HGF/c-met are not associated with controlling motility. However, these results are not conclusive and under capacitating conditions the percentage of motile, progressively motile and hyperactivated cells displayed time-dependent decreases which could support the role for c-met in maintaining motility with the use of inhibitors preventing this action. Further evidence supporting that HGF may have a supporting role in maintaining sperm motility is that the c-met receptor has been found to be tyrosine phosphorylated/ autophosphorylated during capacitation, an effect that is enhanced by HGF/SF (Herness and Naz, 1999). Tyrosine phosphorylation of sperm membrane proteins is vital in development of fertilising capacity, the c-met receptor may have involvement in this process through its ability to be phosphorylated/ autophosphorylated at tyrosine residues (Herness and Naz, 1999). Inhibition of c-met would inhibit tyrosine kinase activity decreasing hyperactivation (Bajpai and Doncel, 2003) a result which is corroborated by this study. Tyrosine phosphorylation is a hallmark of capacitation in mammalian sperm (Visconti et al., 1995b, Signorelli et al., 2012) and leads to increases  $[Ca^{2+}]_i$  and activation of CatSper channels (Wennemuth et al., 2003, Signorelli et al., 2012) suggesting that c-met could play a role in increasing sperm motility. Inhibition of c-met would therefore decrease motility in capacitated sperm as seen in this study. The results of this study and previous publications suggest that C-met inhibitors would not be beneficial clinical treatments for sperm motility (Depuydt et al., 1998, Herness and Naz, 1999, Kitamura et al., 2000, Wiltshire et al., 2000).

## **CHAPTER 6**

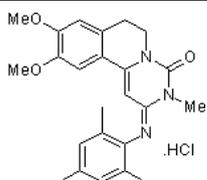
### **Motility Analysis of the PDE inhibitor**

### **Trequinsin Hydrochloride**

## 6.1 Introduction

### 6.1.1 Phosphodiesterase inhibitor

Trequinsin hydrochloride is a potent phosphodiesterase inhibitor of cGMP-inhibited phosphodiesterase (PDE3) (Biosciences, 2013f) (IC<sub>50</sub> 250pM) (Table 6-1).

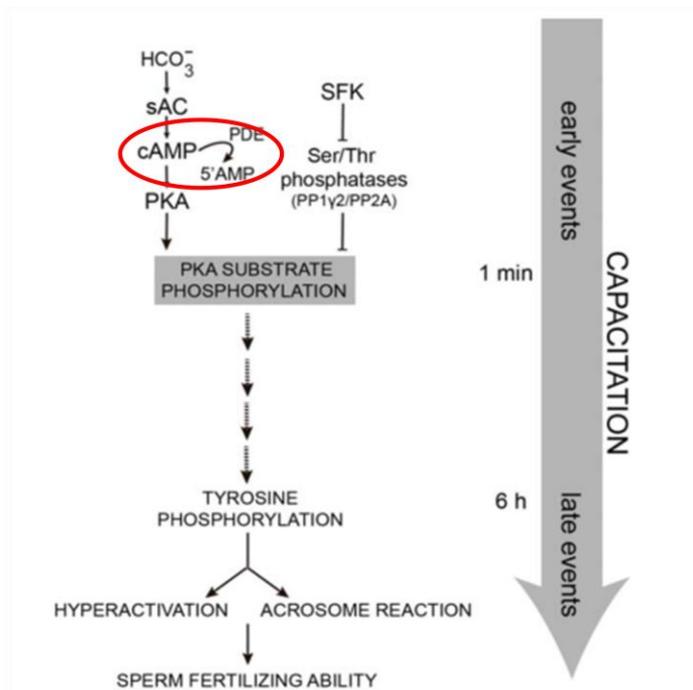
Compound name	Primary action	Chemical structure
Trequinsin Hydrochloride	Selective inhibitor of PDE3	

**Table 6.1 Summary on Trequinsin Hydrochloride identified from screen of the Chemogenomics library** (Biosciences, 2013f)

Cyclic adenosine monophosphate (cAMP) plays an important role in the signalling pathways that regulate sperm motility (Tash, 1990), capacitation and the acrosome reaction (Visconti et al., 1995c). Levels of cAMP are regulated by two key enzymatic reactions: (i) soluble adenylyl cyclase which produces cAMP from adenosine triphosphate (ATP) and (ii) cyclic nucleotide phosphodiesterases (PDEs). PDEs degrade cAMP to 5'AMP and are important in controlling cyclic nucleotide levels in spermatozoa (Lefievre et al., 2002). There are at least 11 identified families of phosphodiesterases, many with several different isoforms, which are classified according to kinetics, mechanism of regulation, affinity and substrate specificity (Beavo, 1995, Soderling and Beavo, 2000). PDEs are highly specific for hydrolysis of cAMP (PDE 4, 7 and 8), cyclic guanine monophosphate (cGMP) (PDE 5, 6 and 9) or they hydrolyze both cAMP and cGMP (PDE 1, 2, 3, 10 and 11) (Lefievre et al., 2002). Lefievre *et al* (2000) identified that the PDEs present in spermatozoa have a higher affinity for cAMP after observing that the rate of hydrolysis of cAMP in sperm by PDEs is 3 fold higher than that of cGMP (Lefievre et al., 2000). Currently PDE 1 (Vasta et al., 2005), PDE 2, PDE 3, PDE 4 and PDE 5, PDE 8 (Richter et al., 1999) and PDE 11 (Yuasa et al., 2001) have been identified in mammalian and human testis and spermatozoa along with mRNA transcripts being identified for six types of PDEs (Fisch

et al., 1998, Lefievre et al., 2000, Lefievre et al., 2002, Richter et al., 1999, Yan et al., 2001, Fisher et al., 1998, Fawcett et al., 2000, Coskran et al., 2006).

As mentioned, PDE3 has mixed specificities with cGMP acting to inhibit the hydrolysis of cAMP by PDE3 (Lefievre et al., 2002). The activity of PDE3 is regulated by feedback mechanisms involving cAMP dependent PKA phosphorylation (Visconti et al., 1997) (figure 6-1).



**Figure 6-1. Signaling pathways involved in sperm capacitation.** Two pathways involved in human sperm capacitation, one corresponds to the activation of PKA by cAMP, and the other involves downregulation of Ser/Thr phosphatases by the Src family kinase (SFK). Both pathways lead to PKA substrate phosphorylation followed by tyrosine phosphorylation resulting in capacitation. PDEs play a role in the pathway by degrading cAMP to 5'AMP (red circle). (Battistone et al., 2013)

cAMP is a key second messenger in protein tyrosine phosphorylation, motility and capacitation (Tash, 1990). Previous studies investigating the effects of PDE3 specific inhibitors have identified that they act by increasing the level of cAMP in sperm (Lefievre et al., 2002). This, combined with the discovery that Trequinsin hydrochloride increases levels of intracellular  $\text{Ca}^{2+}$ , led to the investigation of the effect of Trequinsin hydrochloride on sperm motility.

## 6.2 Experimental Procedure

Experimental procedure for sperm preparation and CASA analysis is identical to that as described for compounds in chapter 5.

Kremer mucus penetration tests were conducted using methylcellulose prepared in NCM. Capillary tubes were placed in the methylcellulose for 30 min at 37°C allowing the tubes to fill. One end was then sealed and the open end was placed in prepared sperm (donor and patient samples, see chapter 2 for details). Sperm preparations and capillary tubes were incubated in appropriate incubators (CO<sub>2</sub> or normal) for 1 hr. The capillary tubes were then removed and the open ends sealed with plasticine. The tubes were marked at 1 cm and 2 cm points before being viewed on a microscope using a 20X objective. Results were normalised to parallel untreated 1% DMSO controls.

Assessment of the acrosome reaction was conducted by staining with PSA labelled FITC. Sperm were prepared through DGC, capacitated for 2 hrs 30 min before treatment with the appropriate drug for 1hr. CM only was used as a negative control (1 hr), 1% DMSO was used as a vehicle control (1 hr) and Ca<sup>2+</sup> ionophore was used as a positive control (15 min). Sperm were washed twice and re-suspended in sodium chloride before being smeared on a microscope slide. After air drying the smear was fixed in 95% (v/v) ethanol before the addition of PSA labelled FITC. After 1 hr the slides were washed three times with PBS and mounted. Analysis was conducted using the fluorescence setting on an EVOS xl digital inverted microscope.

The hypo-osmotic swelling test was used to examine sperm vitality after drug treatment. Sperm suspensions were further incubated with hypo-osmotic medium and sodium citrate dihydrate at 37°C in 5% CO<sub>2</sub> for 30 min. Sperm with coiled tails were counted as viable.

For further details refer to chapter 2.

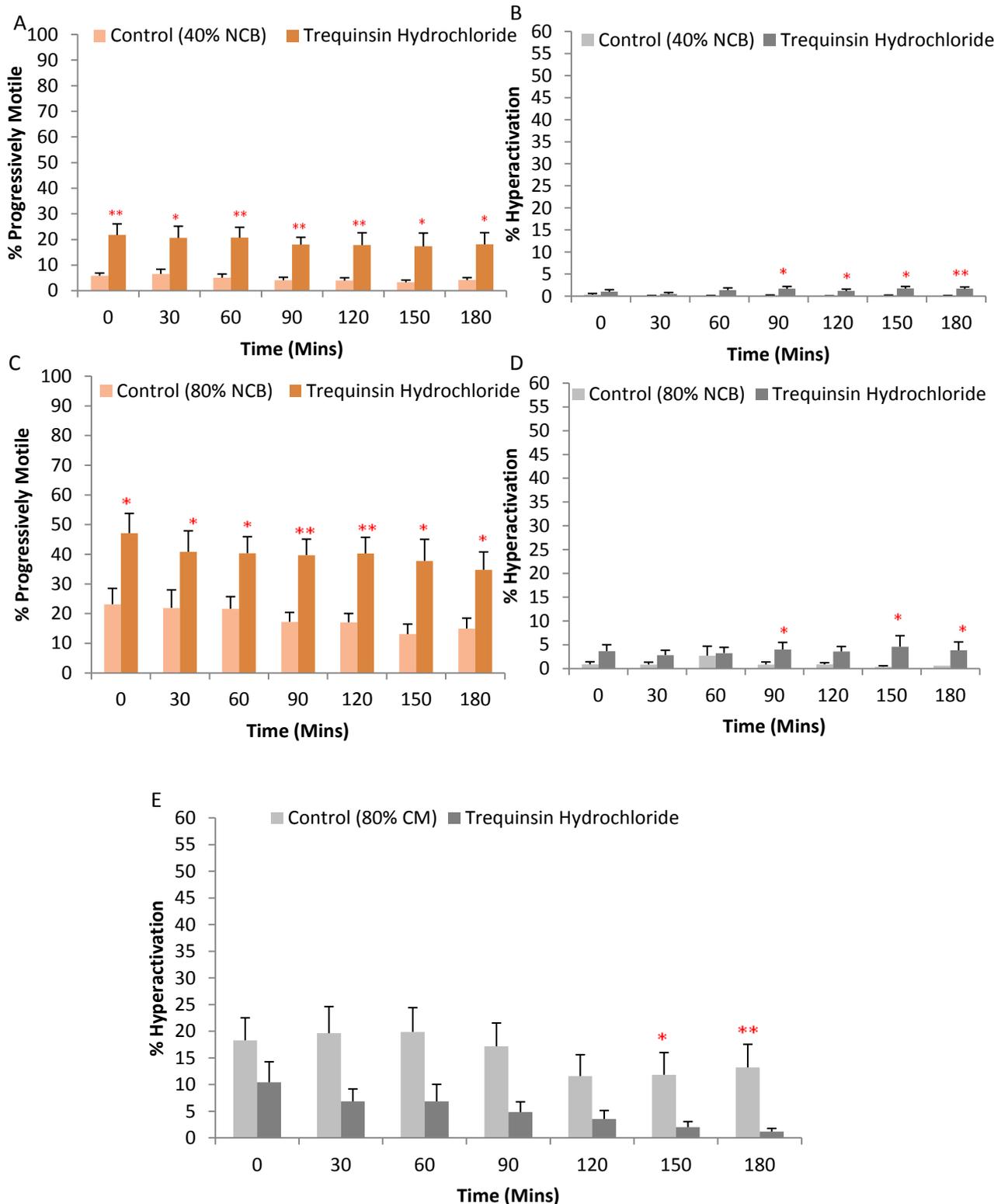
## 6.3 Results

### 6.3.1 CASA motility analysis of Trequinsin Hydrochloride

It was clear that sperm samples under non-capacitating conditions that were then exposed to Trequinsin exhibited the most dramatic responses from both the 40% and 80% fraction. Progressive motility was significantly increased instantaneously after treatment with the compound ( $P=0.002$ ) and this increase was sustained for the entire time-course ( $P\leq 0.05$ ) (figure 6-2A). The greatest increment in hyperactivation, when stimulated by Trequinsin, was found in the 40% fraction under non-capacitating conditions after 90 min ( $P\leq 0.05$ ) which continued to be significantly increased for the remainder of the time-course ( $P<0.005$ ) (figure 6-2B). However, total motility remained unchanged from control (appendix).

Spermatozoa from the 80% fraction (high quality sperm) exhibited a pronounced increase in progressive motility when treated with Trequinsin, after incubation at 37°C, in non-capacitating conditions. This statistical increase in progressive motility was sustained for the entirety of the 3 hr time-course (0-60 min, 150 & 180 min  $P\leq 0.05$ , 90 & 120 min  $P<0.005$ ) (figure 6-2C). Similarly to the 40% fraction the percentage of hyperactivated cells in the 80% fraction was significantly higher than control after 90 min incubation with the compound ( $P\leq 0.05$ , figure 6-2D). Furthermore, hyperactivated motility was increased after 150 min and 180 min treatment ( $P<0.005$  &  $P\leq 0.05$  respectively). This increase in progressive motility and hyperactivation supports the knowledge that PDE inhibitors increase cAMP leading to capacitation (Lefievre et al., 2000, Lefievre et al., 2002). However, similarly to the 40% fraction there was no significant change in total motility for this parameter (appendix). When sperm cells were exposed to Trequinsin after capacitation the resulting effect on motility was the opposite of non- capacitating conditions. The 40% fraction exhibited significant decreases in motility after 180 min incubation with a steady decline evident over the

entire time-course for the percentage of total motile, progressively motile and hyperactivated cells (appendix). Similarly, to the 40% fraction, the 80% fraction demonstrated a noticeable decline in motility which was most evident and significant in the percentage of hyperactivated cells after 150 and 180 min treatment ( $P=0.041$  &  $P=0.004$  respectively) (appendix and figure 6-2E). No change was observed for both the 40% and 80% fractions after capacitation for the percentage of total motile and progressively motile cells (appendix).

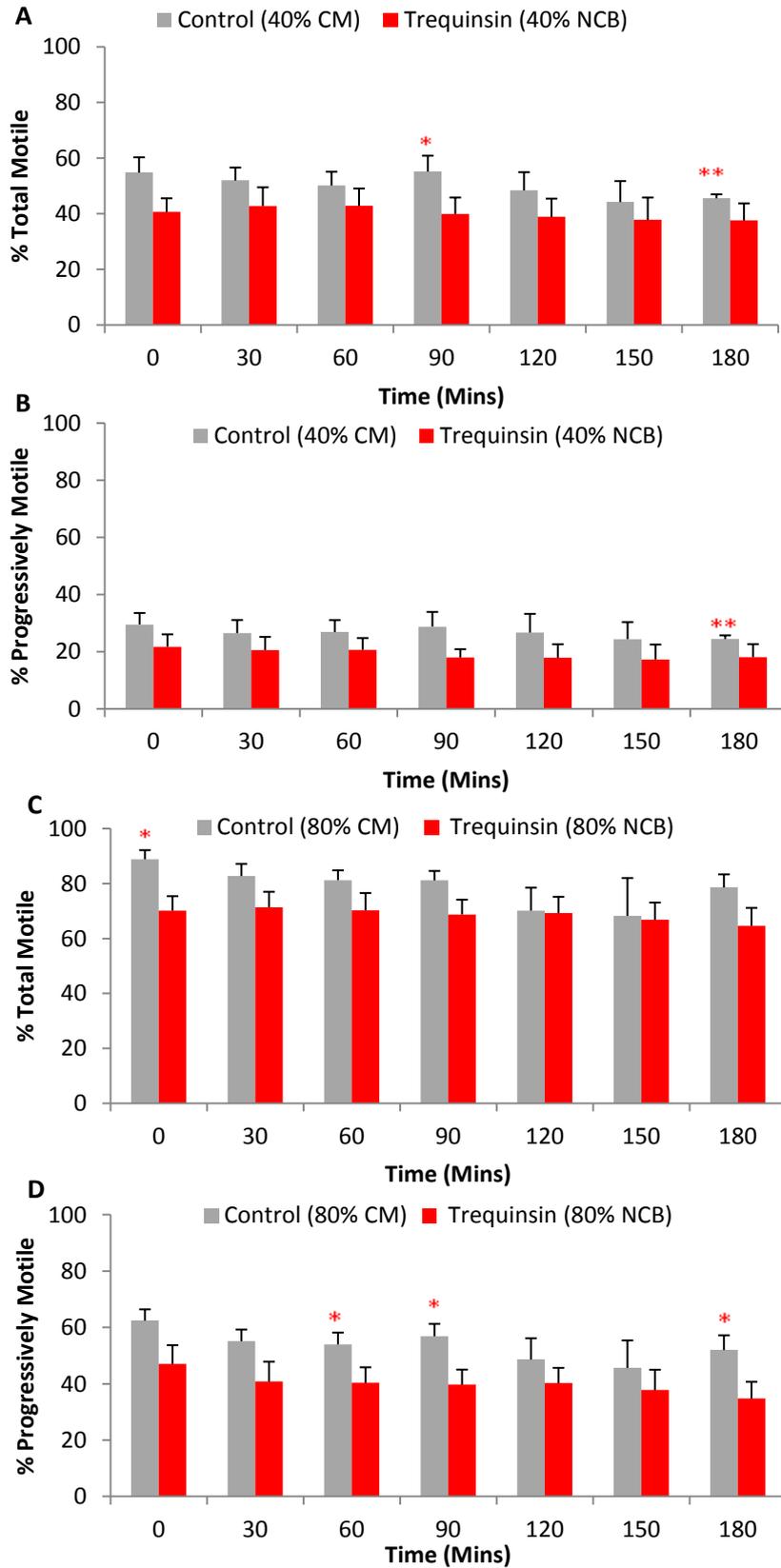


**Figure 6-2. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) and capacitating (CM) media for (A) % progressively motile (40% fraction NCM), (B) hyperactivation (40% fraction NCM), (C) % progressively motile (80% fraction NCM), (D) hyperactivation (80% fraction NCM) and (E) hyperactivation (80% fraction CM).** The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 6 for both parameters, \* indicates a significant increase ( $P \leq 0.05$ ), \*\* indicates  $P \leq 0.005$  between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

The dramatic opposing effects seen in response to treatment with Trequinsin under non-capacitating and capacitating conditions directed analysis to compare the Trequinsin stimulated increase, in both the 40% and 80% fraction, under non-capacitating conditions, to that of the controls after capacitation (figure 6-3). When comparing the motility parameters from sperm cells, in the 40% fraction, no significant difference was noted between capacitated control and the treated non-capacitated sperm cells suggesting that the treated non-capacitated sperm are now acting similarly to a capacitated sample (figure 6-3A&B). Treatment with Trequinsin increased progressive motility in the treated, non-capacitated cells to within the range of control capacitated cells on instant addition of the agonist. These parameters remained similar to the capacitated control throughout the time-course until it reached 180min incubation when it was found that all parameters assessed exhibited a significant decrease compared to capacitated control ( $P \leq 0.05$ , figure 6-3A&B and appendix). The percentages of total motile cells were also increased in the treated, non-capacitated sample to the same as the capacitated control instantaneously although, at the 90min and 180min time-points the percentage of total motile cells was significantly decreased in the treated samples ( $P \leq 0.05$  and  $P \leq 0.005$  figure 6.-3A). This could indicate that the action of the compound may not last past the 3hr time course and would need testing for a longer period of time to examine any lasting motility alterations.

The same comparison was run for the 80% capacitated control and the 80% non-capacitated treated sample. The similarities in the 80% fractions were not as great as those noted for the 40% fraction. After 0 min the only parameter that was similar to the capacitated control was progressive motility. The percentage of total motile cells were similar to the capacitated control after 30 min incubation and remained this way until the 3 hr time point when it was found to have significantly decreased ( $P=0.41$ , figure 6-

3C). The percentage of progressively motile cells after 30 min treatment was observed to be similar to the capacitated control however; hyperactivation was significantly higher in the capacitated control at this point ( $P \leq 0.05$ , appendix). Hyperactivated motility displayed variable results that were only similar to the capacitated control after 90, 150 and 180 min (appendix). The percentage of progressively motile cells, after treatment with Trequinsin, increased instantaneously, an effect that was sustained with the exception of 90 and 180 min incubations (figure 6-3D).



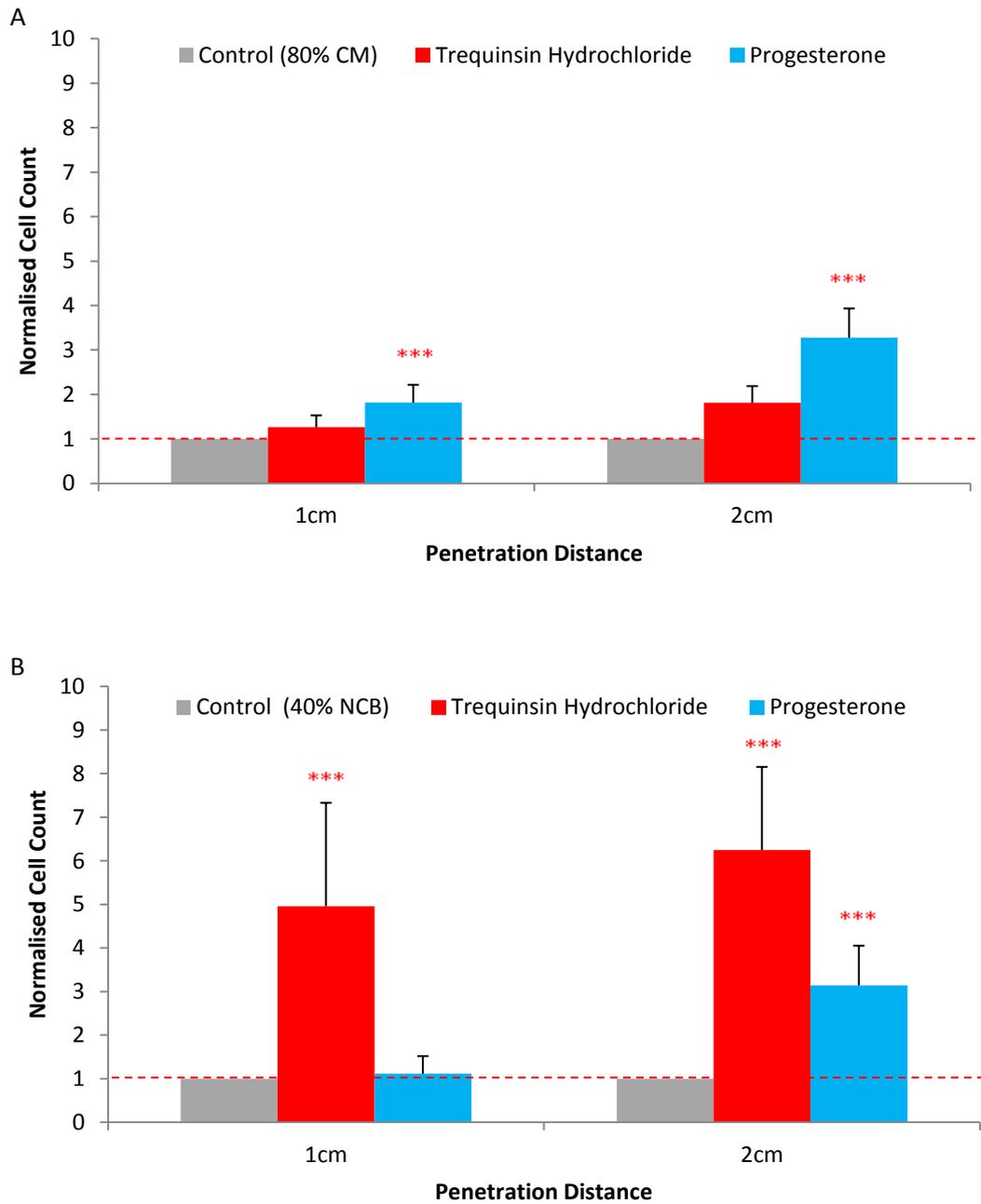
**Figure 6-3. Comparing basal (control) motility values under capacitating conditions to Trequinsin treated non-capacitated samples.** (A) % total motile (40% fraction), (B) % progressively motile (40% fraction), (C) % total motile (80% fraction) and (D) % progressively motile (80% fraction). The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min.  $N=6$  for all parameters, \* indicates a significant increase ( $P \leq 0.05$ ), \*\* indicates  $P \leq 0.005$  between control and treated samples at independent time points (T-test, Mann Whitney test and Kruskal-Wallis test).

### **6.3.2 Examination of sperm motility through an artificial viscous medium: methylcellulose (Kremer mucus penetration test)**

To examine the ability of sperm treated with Trequinsin Hydrochloride to penetrate cervical mucus, a viscous medium methylcellulose, was used as a cervical mucus substitute in the Kremer mucus penetration assay. Previous studies have reported that sperm penetration into mucus correlates with their functional capacity and fertilisation rates both *in vivo* and *in vitro* (Aitken et al., 1992, Barratt et al., 1989, Eggert-Kruse et al., 1989). Sperm cells were incubated with Trequinsin for 1hr before cell penetration was analysed.

The initial Kremer penetration assay was conducted on capacitated sperm from the 80% fraction using progesterone as a positive control. Progesterone has previously been shown to increase sperm penetration under these conditions (Alasmari, 2013) and was used as a positive control in the Flexstation assay screen. Capacitated cells from the 80% fraction, incubated with Trequinsin, failed to increase penetration into the viscous medium. However, under the same conditions 3.6 $\mu$ M progesterone stimulated significant increases in cell penetration at both 1 cm and 2 cm ( $P \leq 0.005$ , figure 6-4A).

CASA analysis identified significant increases in motility in the 40% fraction under non-capacitating conditions although, this was not reflected in 80% capacitated samples. To examine whether this effect was the same in this functional motility assay analysis was conducted on cells from the 40% fraction under non-capacitating conditions. Under these conditions Trequinsin treated cells significantly increased penetration at both distances ( $P \leq 0.005$ , figure 6-4B). This result corroborates the CASA analysis indicating that Trequinsin is capable of increasing motility in non-capacitated cells. Treatment with progesterone under these conditions did not significantly change the proportion of cells penetrating at 1 cm although, at 2 cm the proportion of cells was significantly increased ( $P \leq 0.005$ , figure 6-4B).

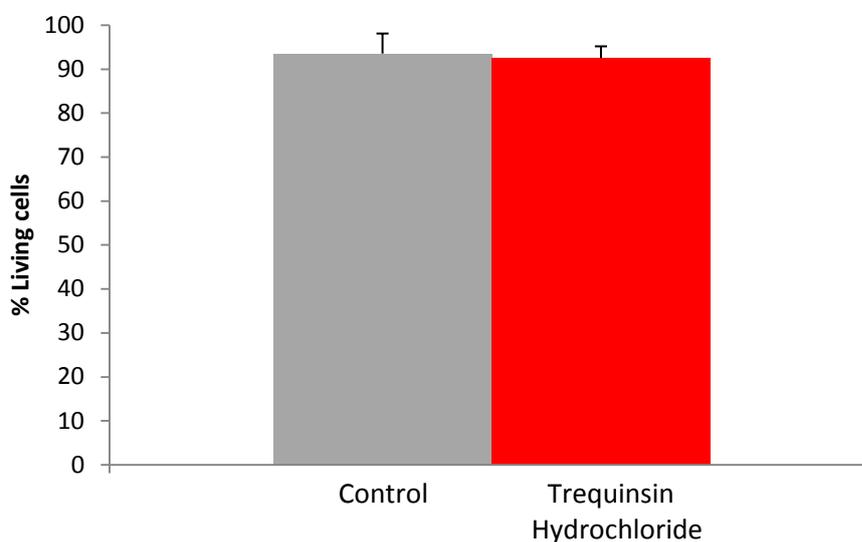


**Figure 6-4. Trequinsin induced penetration of sperm cells into viscous medium.** Cells from (A) 80% fraction under capacitating conditions and (B) 40% fraction under non-capacitating condition were evaluated for the number of sperm penetrating to 1 cm and 2 cm. The cells were counted and normalised to values in untreated controls (red dashed line).  $N=5$  for all experiments and all values are the mean  $\pm$  SE. \*\*\*- indicates a significant difference from the control, considered to be  $P \leq 0.005$  &  $P \leq 0.001$  as assessed by non-parametric Mann Whitney U test.

### 6.3.3 Vitality analysis (HOS test) of treated sperm cells

This study utilised the hypo-osmotic swelling test in an attempt to analyse the effect seen in capacitated sperm after 3 hrs incubation with Trequinsin. Both the CASA analysis and Kremer mucus penetration test identified a decrease in sperm motility in the 80% fraction under capacitating conditions. To ensure that addition of this compound to human sperm is not toxic over long periods of time a vitality assessment was conducted.

Donor spermatozoa were capacitated for 2 hrs 30 min before being incubated with Trequinsin for a period of 3 hrs. Following incubation the vitality of the sperm cells was assessed. The result identified a mean of 92.5% of living cells with the results ranging from 88% - 99% from the 3 donor samples assessed (figure 6-5). This result was not significantly different from control suggesting that this compound is not killing the cells.



**Figure 6-5. Vitality analysis after incubation with Trequinsin.** Trequinsin Hydrochloride (red bar) was assessed for its effects on sperm vitality after incubation with sperm cells for a period of 3 hrs. All values are the mean  $\pm$  SE,  $n=3$  for each experiment. No statistical difference was noted from control (grey bars). Analysis was conducted using Pearson's Chi squared test and significance was assessed as  $P \leq 0.05$ .

### **6.3.4 Response of patient samples to incubation with Trequinsin Hydrochloride**

To evaluate what effect Trequinsin may have in a clinical setting the same analysis were performed on treated patient samples.

#### **6.3.4.1 CASA Motility analysis of patient samples pre-treated with Trequinsin**

Patient samples provided by the ACU, after capacitation, were treated with Trequinsin. All samples examined were from patients attending the ACU for IVF and motility analysis was conducted over a period of 2 hrs using CASA (see chapter 2 for detailed methods). Results for each patient were analysed independently (Table 6.2). Treated patient samples exhibited significant increases in VCL (8/9) and ALH (9/9) resulting in significant increases in hyperactivated motility (Table 6-2). Following an increase in ALH, LIN was significantly decreased in 8/9 patients along with progressive motility. However, one patient did show significant increases (P8) in progressive motility but this result was highly variable for this patient across the time-course (Table 6-2). The results for all samples, regarding total motility, were variable however the predominant response showed no change in the percentage of motile cells (Table 6-2). Significant increases were observed from this CASA analysis for hyperactivation but only 2/9 patients exhibited significant increases in all the four time points, with 1 patient exhibiting significant increases after 60 min. 3/9 patients displayed no change in hyperactivation with the remaining 3 patients exhibiting variable results (Table 6-2).

Overall the result of treatment on patients with Trequinsin hydrochloride was better than that of the donor response under the same conditions (80% fraction, capacitated). Donors exhibited significant increases in VCL and hyperactivation which is opposite to results obtained for the patient samples under the same parameters (Table 6-2). There also were no significant differences noted in ALH for treated donors where there were clear increases in ALH in patient samples.

Trequinsin Hydrochloride												
	VCL				LIN				ALH			
Patient No.	0	30	60	120	0	30	60	120	0	30	60	120
P1	✓	✓	✓	✓	✗	✗	✗	✗	✓	✓	✓	✓
P2	-	-	✓	✓	-	-	-	-	✓	✓	✓	✓
P3	-	✓	✓	✓	-	✗	✗	✗	-	✓	✓	✓
P4	-	-	-	-	✗	✗	✗	✗	✓	✓	✓	✓
P5	✓	✓	✓	✓	✗	✗	✗	✗	✓	✓	✓	✓
P6	✓	✓	-	✓	-	✗	-	✗	-	✓	-	✓
P7	-	-	✓	-	✗	-	✗	✗	-	-	✓	✓
P8	✓	-	-	-	✗	✗	✗	✓	✓	✓	✓	-
P9	✓	-	-	-	-	-	✗	✗	-	-	✓	✓
	Total Motile				Progressively Motile				Hyperactivation			
Patient No.	0	30	60	120	0	30	60	120	0	30	60	120
P1	-	-	✗	✗	✗	✗	✗	✗	✓	✓	✓	✓
P2	✗	-	✗	-	✗	-	✗	-	-	-	✓	✓
P3	✗	-	-	✗	-	-	-	✗	-	-	✓	-
P4	-	✗	✗	✗	✗	✗	✗	✗	-	-	-	-
P5	✗	-	-	-	✗	-	✗	-	✓	✓	✓	✓
P6	-	-	-	-	-	✗	-	-	-	-	✗	-
P7	-	✗	-	-	✗	-	-	-	-	-	✓	-
P8	-	✓	-	✓	-	✓	✗	✓	-	-	-	-
P9	-	✗	✗	✗	-	-	✗	✗	-	-	-	-

Key

✓=Increase

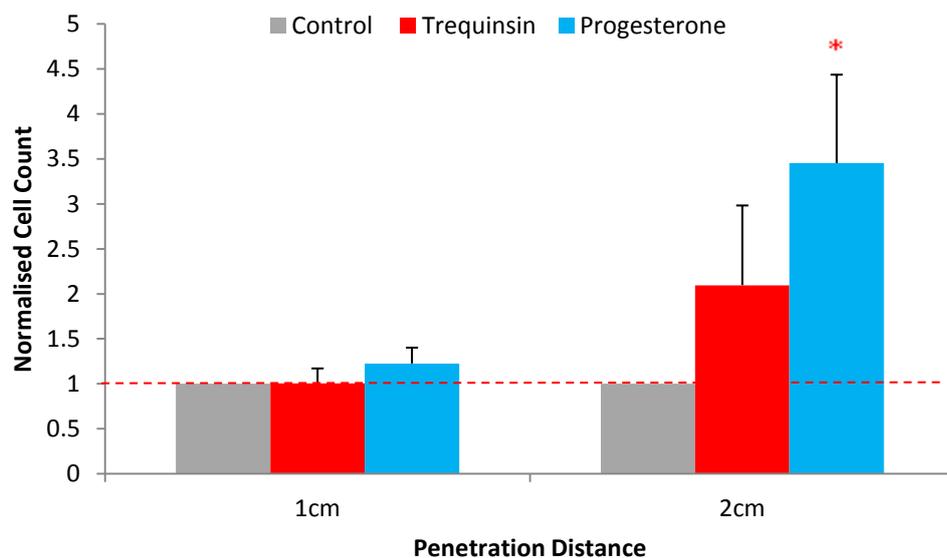
✗ = Decrease

- = No change

**Table 6.2 Identification of motility kinematics analysed by CASA, from patient samples, after incubation with Trequinsin Hydrochloride.** Motility parameters after stimulation with Trequinsin Hydrochloride were evaluated from 9 patients undergoing IVF at Ninewells ACU. The table identifies the 6 motility parameters evaluated for each patient over a period of 2 hrs. Statistical significance (see key) was determined from comparison with control  $\pm$  SD. If the agonist response was greater than or less than control it was considered as a significant result.

### 6.3.4.2 Kremer mucus penetration assay to assess functional motility of patient sperm treated with Trequinsin Hydrochloride

Donor samples exhibited significant increases in sperm penetrating ability when under non-capacitating conditions. When evaluating capacitated cells an increase compared to control was observed however it was not significant. The patient samples available for analysis were all IVF so had been capacitated for treatment. Although, we have previously shown that Trequinsin does not seem to influence capacitated cells as much as non-capacitated it was necessary to use these patient samples. The result from this assay confirmed the results observed from donor samples with no change compared to control at the 1 cm distance and a slight increase at the 2 cm distance (figure 6-6). However, this increase similarly to donors did not reach a significant level. Progesterone once again was used as a positive control. In this assay progesterone did not significantly increase penetrating ability at 1 cm in contrast to the donors but it did significantly increase the proportion of cells penetrating at 2 cm the same as donors (figure 6-6).



**Figure 6-6. Agonist induced sperm penetration into viscous medium.** Sperm penetration into viscous medium upon application of 40 $\mu$ M Trequinsin Hydrochloride or 3.6 $\mu$ M progesterone as measured by counting cells at 1 cm and 2 cm. The number of sperm penetrating to 1 cm and 2 cm was assessed and normalised to values in untreated controls (red dashed line). All values are the mean  $\pm$  SE (n=9 experiments performed with 9 different patient samples). \*- indicates a significant difference ( $P \leq 0.05$ ), \*\*\*- indicates a significant difference ( $P \leq 0.005$ ) from the control, as assessed by non-parametric analysis Mann Whitney U test.

### 6.3 Discussion

#### **Trequinsin hydrochloride enhances motility, assessed by CASA, and increases penetration into a cervical mucus substitute**

A variety of cellular functions are regulated by cyclic guanine monophosphate (cGMP) or cyclic adenosine monophosphate (cAMP) levels (Richter et al., 1999). Cyclic nucleotide functions are dependent on a dynamic intracellular equilibrium between synthesis and degradation (Rossi et al., 1985). The use of nonselective PDE inhibitors have been shown to affect capacitation (Visconti et al., 1995c, deLamirande et al., 1997), sperm motility (Tesariki et al., 1992, Jaiswal and Majumder, 1996) and acrosome reaction (Tesarik et al., 1992).

Trequinsin hydrochloride is a selective potent inhibitor of cGMP-inhibited phosphodiesterase (PDE3). In this study it significantly increased progressive motility and hyperactivation in non-capacitated samples. Lefievre *et al* (2002) identified PDE3A isoforms in ejaculated human spermatozoa however, immunocytochemistry experiments indicated it to be localised to the postacrosomal segment of the sperm head (Lefievre et al., 2002). This same group tested the involvement of PDE3 in sperm function using Milrinone (a selective PDE3 inhibitor) which increased cAMP levels in sperm but not to the extent to induce capacitation or have any effect on motility or tyrosine phosphorylation (Lefievre et al., 2002). However, the data presented in this study contradicts these results with dramatic increases in motility and hyperactivation as a result of treatment with Trequinsin. The positioning of PDE3 at the postacrosomal region of the sperm head does not suggest a role in sperm motility and from the work of Lefievre *et al* (2002) it is reported that the use of PDE3i alone does not increase cAMP levels enough to induce capacitation or alterations in sperm motility (Lefievre et al., 2002). The results from the Flexstation identified Trequinsin as being a compound that increased  $[Ca^{2+}]_i$  levels of sperm to an equivalent concentration of that seen after treatment with progesterone suggesting that capacitation may have been triggered in

these sperm cells (Baldi et al., 1991). Elevation of  $[Ca^{2+}]_i$  is essential for changes in flagella function as a manifestation of capacitation (Lishko et al., 2012b). This knowledge led to the possibility that Trequinsin was functioning not only through PDE3 but through another signalling pathway. This theory was tested through the use of the patch clamp technique (performed by Steven Mansell) and identified Trequinsin as having activation kinetics almost identical to progesterone (data not shown) suggesting that Trequinsin may act via the CatSper cation channel. Activation of CatSper, by Trequinsin, would lead to an elevation in  $[Ca^{2+}]_i$  and promote hyperactivated motility (Mannowetz et al., 2013), both of which are reported in this study. However, cells that had previously been capacitated before exposure to Trequinsin exhibited a time dependent decrease in motility especially in the percentage of hyperactivated cells. Human sperm flagella are long and thin ( $40\mu\text{M}$  and  $<2\mu\text{M}$  respectively) and because diffusion is inversely proportional to the area through which a substance diffuses it takes molecules many seconds to traverse through the flagellum from the mid-piece to the end-piece (Lishko et al., 2012b). Thus, ATP produced in the mitochondria of the midpiece, which is needed for sperm motility, especially hyperactivation, takes a long time to reach the end of the flagellum (Lishko et al., 2012b). To be able to compensate for this delay, flagella movement especially at the tip of the sperm tail is generated through glycolysis (Miki et al., 2004, Mukai and Okuno, 2004, Williams and Ford, 2001). Glucose is required to sustain an optimal ATP concentration in human sperm motility and is essential in supporting hyperactivated motility (Williams and Ford, 2001). Human sperm obtain a high proportion of their energy from glycolysis of glucose to lactate and a marked decline in vigorous motility was identified when sperm cells were incubated over long periods of time (Williams and Ford, 2001, Chulavatnatol and Haesungcharern, 1977). This decrease in motility was attributed to the stress of prolonged incubation which resulted in a decreased concentration of ATP and ADP.

The use of Trequinsin under capacitating conditions would have required an instantaneous release of ATP to increase and sustain hyperactivation of cells that had previously been capacitated. Williams and Ford (2001) accounted for leakage of adenine nucleotides from dead/dying cells concluding that the loss of ATP/ADP wasn't owing to cell death (Williams and Ford, 2001). Results from this study also showed that Trequinsin did not alter sperm vitality suggesting that the decrease in motility observed under capacitating conditions was not because the compound is detrimental to sperm survival. This same effect had previously been observed with use of caffeine to artificially stimulate motility resulting in an increased demand for energy (Chulavatnatol and Haesungcharern, 1977). If the glycolysis of endogenous substrate is not fast enough the result is a depletion in ATP level detrimental to motility (Chulavatnatol and Haesungcharern, 1977), an effect that was noted in both donor and patient samples which had been capacitated before treatment. However, supplementation of glucose after incubation in all studies mentioned re-stored motility to normal levels suggesting this detriment is not permanent (Miki et al., 2004, Mukai and Okuno, 2004, Williams and Ford, 2001, Chulavatnatol and Haesungcharern, 1977).

Similarly to progesterone (Alasmari et al., 2013b), Trequinsin initiated an increase in penetrating ability of sperm through a cervical mucus substitute, when under non-capacitating conditions. This result supports the determination of Trequinsin hydrochloride functioning via the CatSper channel. This compound has the potential of being beneficial therapeutically in increasing sperm motility however, further research needs to be conducted to confirm the motility effects observed under capacitating conditions are not irreversible and to see whether further supplementation with glucose prevents this effect.

## **CHAPTER 7**

# **Investigating the effects of novel ion channel compounds on sperm motility**

## 7.1 Introduction

Discovery of new drugs targeting sperm motility to facilitate conception is particularly challenging. Two commonly used methods in drug discovery are computational chemistry and molecular modelling, however HTS has broadened these approaches to allow for identification of hits through screening extensive chemical or focused libraries (Miller, 2006).

Sperm are highly complex systems that rely on post-translational modifications as their primary signalling mechanism. Ionic gradients and ion channels are fundamental resources allowing changes in electrical potential and the concentration of second messengers (Visconti et al., 2011). They regulate intracellular signalling pathways and control physiological changes within the cell (Darszon et al., 2011).  $\text{Ca}^{2+}$  plays a pivotal role in fertilisation and plays crucial roles in maturation, motility and the acrosome reaction in invertebrate and vertebrate spermatozoa (Yanagimachi, 1994). Over the past 10 years the fundamental role of ion channels and particularly  $\text{Ca}^{2+}$  to sperm physiology has been consolidated (Kirichok et al., 2006, Darszon et al., 2008). Intracellular  $\text{Ca}^{2+}$  in sperm appears to be regulated by at least two processes; mobilisation of stored  $\text{Ca}^{2+}$  in the neck/midpiece region and/or through  $\text{Ca}^{2+}$  permeable ion channels and transporters (Ho and Suarez, 2001, Ren et al., 2001, Costello et al., 2009).

Ion channels are integral proteins on the membrane of the cell that regulate ion transmission across the cell (Mok and Brenk, 2011). Ion channel targets remain unexploited in drug development with only 10% of drugs currently on the market known to bind to ion channels (Terstappen et al., 2010). Two sperm specific ion channels, CatSper and KSper (Slo3/Slo1) have been identified as the primary spermatozoal ion channels (Ren et al., 2001, Mannowetz et al., 2013, Santi et al., 2010, Schreiber et al., 1998). CatSper is a pH-regulated  $\text{Ca}^{2+}$  channel that is present on the

flagella of human sperm. Recently,  $\text{Ca}^{2+}$  induced entry by progesterone has been identified as being via CatSper channels (Lishko et al., 2011, Strunker et al., 2011). CatSper are crucial to sperm motility and are fundamental to male fertility (Ren et al., 2001).

The knowledge of the importance of ion channels and in particular  $\text{Ca}^{2+}$  in sperm function led to the development of an ion channel assay based on a HTS approach. The HTS assay used was similar to that described for screening the chemogenomics library (chapter 5) however, this assay utilised an in-house ion channel focussed library assembled by the UoD DDU. This library of putative ion channel ligands was assembled from the ChEMBL database and from this library 3312 compounds were screened (Mok and Brenk, 2011). The HTS assay using a Flexstation microplate reader measured the agonist responses of the screened compounds compared to progesterone as a putative control. 70 of the compounds from the primary screen were examined further for >90% purity as demonstrated by chromatography- mass spectrometry (LCMS), reducing the number of compounds to 48 that evoked a concentration dependent increase in  $[\text{Ca}^{2+}]_i$ . The molecular and chemical structures of these compounds were further examined and 14 ion channel compounds were selected to undergo in-depth spermatozoa motility assessments.

The 14 compounds will be identified as A1- H1 and A2- F2. Of the 14 compounds subjected to motility analysis the 5 most promising compounds (A1-E1) will be discussed in detail in this chapter with motility data for the remaining 9 (F1-H1 & A2-F2) being accessible in the appendix.

## **7.2 Experimental Procedure**

Motility analysis was conducted on spermatozoa from the 40% fraction, under non-capacitating conditions, after DGC, from donor spermatozoa. This fraction was used as a surrogate for a patient sample (chapter 3). The 80% fraction, from a donor sample, under capacitating conditions, was evaluated to see if the compounds were capable of increasing motility in the high-density portion of strongly motile sperm (Oaconnell, 2003). Non-capacitated samples were left in the incubator at 37°C for 15 min before treatment with the compounds whereas capacitated samples were left in the 5% CO<sub>2</sub> incubator for 2.5 hrs before treatment with the compounds (for further details see chapter 2). Analysis was conducted at 30 minute intervals for a period of 3 hrs. We will only be discussing total motility, progressive motility in detail in this chapter. The results for VCL, ALH and hyperactivation are available in the appendix.

Kremer mucus penetration tests, acrosome reaction analysis and vitality tests were conducted in an identical manner to that described in chapter 6.

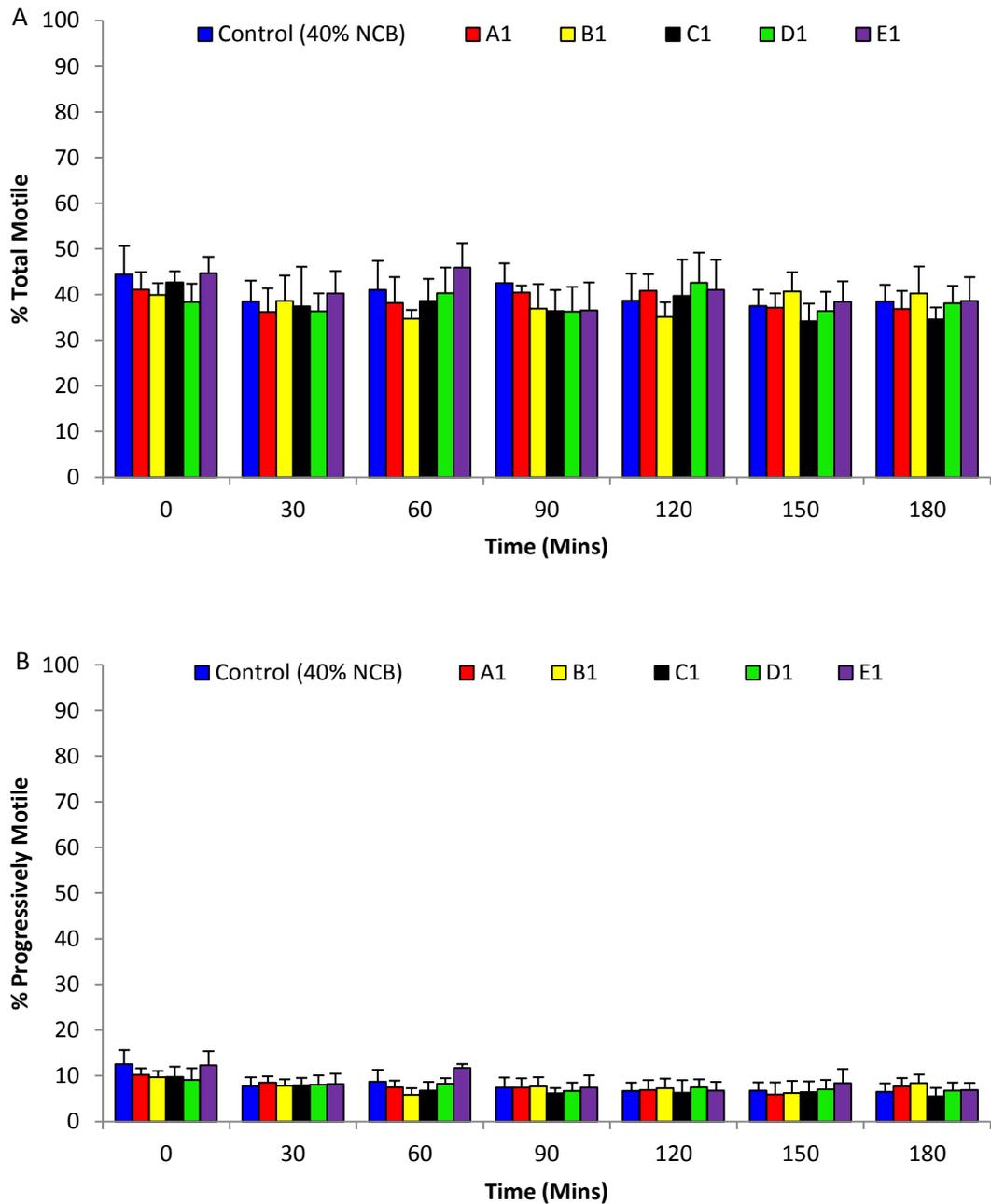
## **7.2 Results**

### **7.2.1 Motility assessment by CASA of five hit compounds**

#### ***7.2.1.1 Effect of 5 compounds on motility of 40% fraction in non-capacitating buffer***

A1 exhibited no significant differences to control in any of the motility parameters evaluated by CASA when the spermatozoa from the 40% fraction were evaluated under non-capacitating conditions (appendix). Total motility and progressive motility were unaffected by the compound at any of the time points analysed (figure 7-1 A&B).

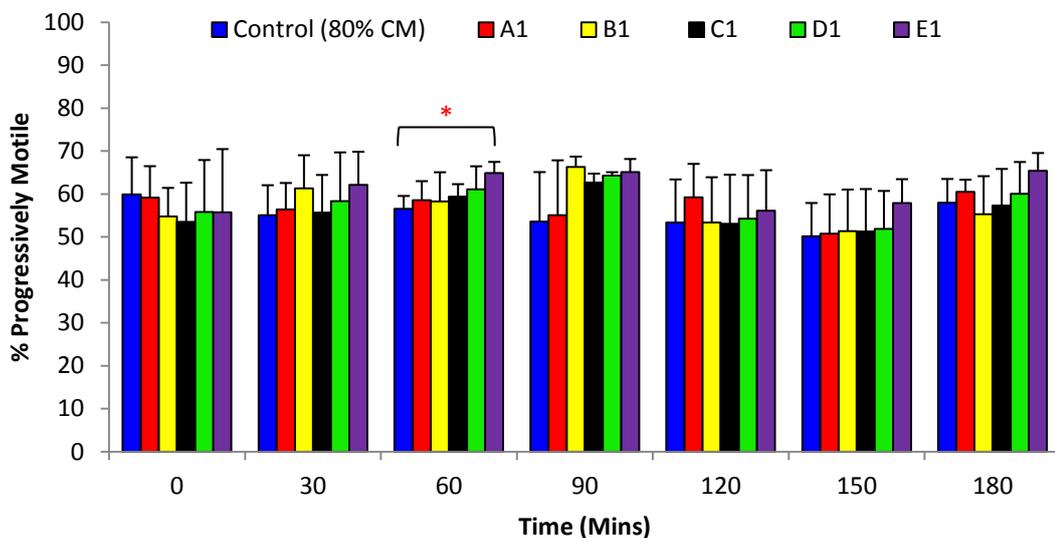
Spermatozoa incubated with B1 had a similar response to A1 with total motility and progressive motility exhibiting very little change in flagella motility between the treated sample and control (figure 7-1 A&B). The effects of C1, D1 and E1 on motility were very similar to that of A1 and B1 with no significant changes seen in treated cells (figure 7-1 A&B).



**Figure 7-1. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) % total motile and (B) % progressive motility.** The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, \* indicates a significant increase ( $P \leq 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

### 7.2.1.2 Motility resulting from exposure to 5 novel compounds in the 80% fraction after capacitation

Exposure of spermatozoa from the 80% fraction, which had been capacitated before treatment, exhibited no significant changes from control for either total motile or progressive motility after treatment with A1, B1, C1 or D1 (appendix, figure 7-2). However, sperm incubated for 60 min with E1 were found to be significantly more progressively motile than control cells ( $P \leq 0.05$ ) (figure 7-2) although, this was the only time point to exhibit significant increases. Examination of motility by use of CASA did not highlight any compounds that had a sustained, positive, significant effect on motility however; none of the compounds appear to be having a negative effect as was seen from compounds identified from the Chemogenomics screen (chapter 5).

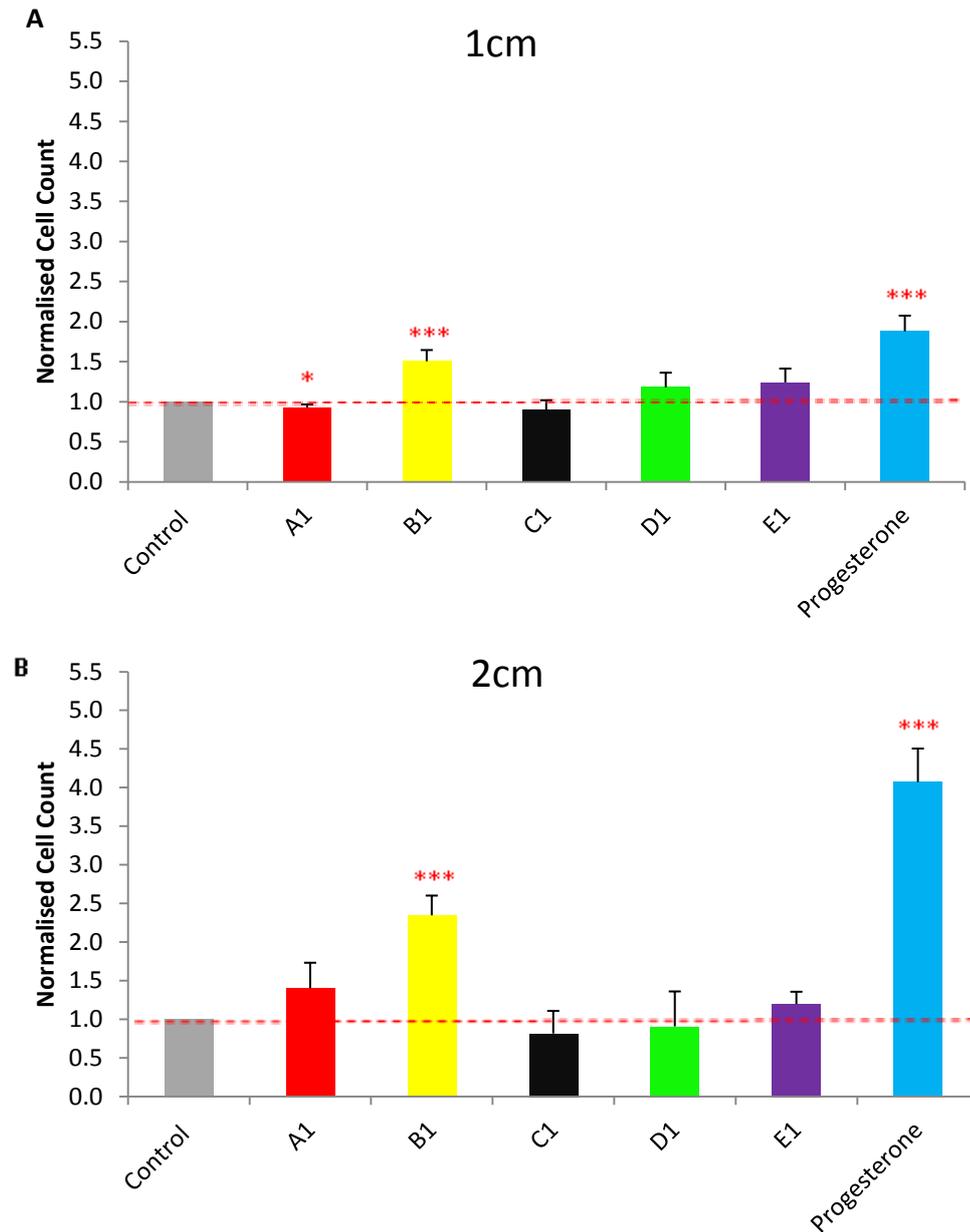


**Figure 7-2. Expression of basal (control) motility values compared to treated samples in capacitating media (CM), 80% fraction for % progressive motility.** The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, \* indicates a significant increase ( $P \leq 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

### **7.2.2 Evaluation of sperm motility into artificial viscous medium (Kremer test)**

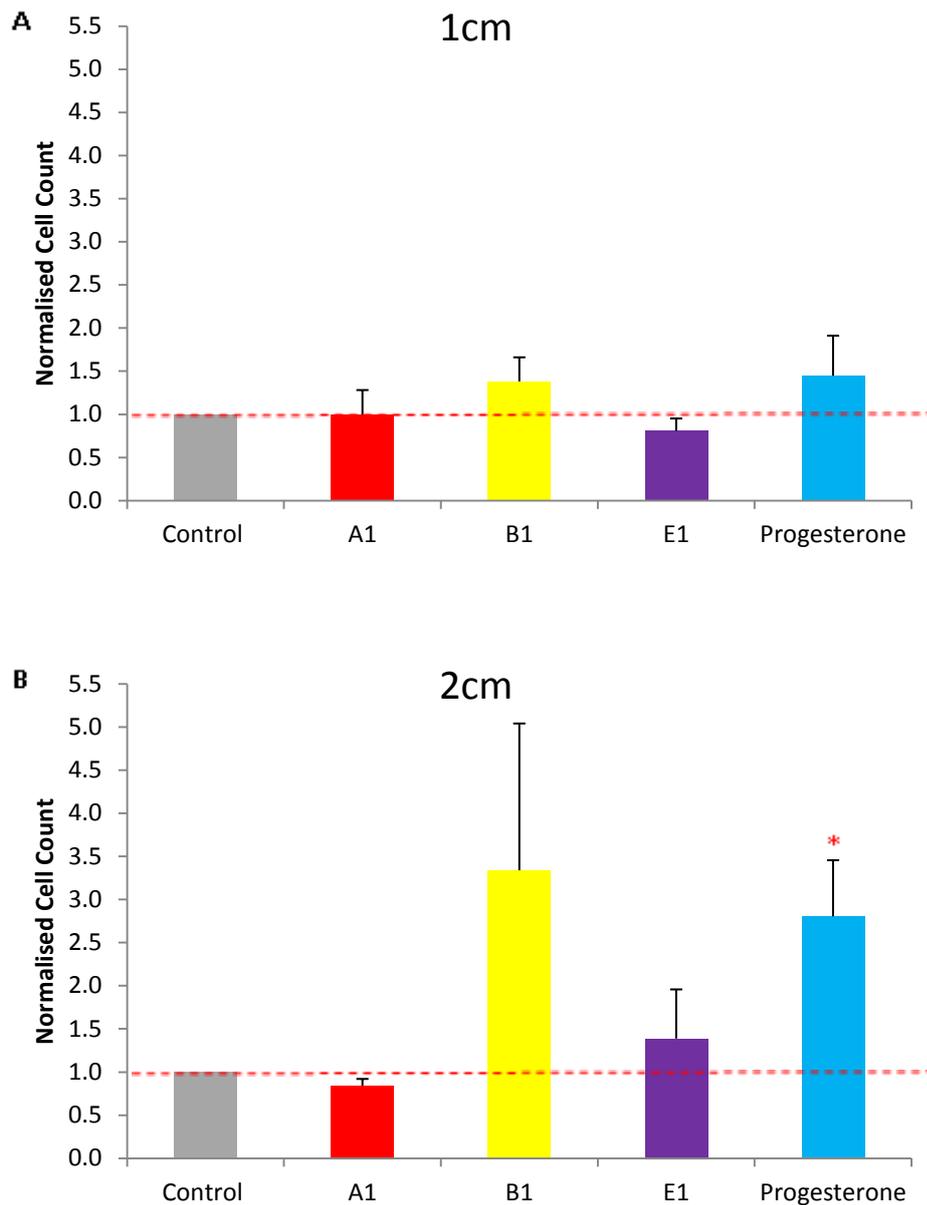
To further evaluate sperm motility a functional assay that measures penetrating ability of cells into a viscous substrate (methylcellulose – alternative to cervical mucus) was conducted (Kremer, 1965, Ivic, 2002). To determine whether the motility response following the addition of agonists leads to impaired or improved penetration into viscous medium, donor samples were incubated with agonists for 1hr before cell penetration was analysed.

The initial analysis was conducted on the 80% fraction following capacitation. Once again progesterone was used as a positive control alongside the treated compounds (see chapter 6.3.2). Exposure of cells prepared in CM to C1, D1 and E1 failed to increase cell penetration into methylcellulose. Stimulation with B1 and 3.6 $\mu$ M progesterone significantly enhanced penetration into viscous medium at distances of both 1 cm and 2 cm (B1  $P \leq 0.005$ , progesterone  $P \leq 0.001$ ; figure 7-3 A&B). A1 significantly decreased penetration at 1 cm ( $P \leq 0.05$ ) however; it appeared to increase penetration at 2 cm although this did not reach statistical significance (figure 7-3 A&B).



**Figure 7-3. Agonist induced penetration of cells into viscous medium.** Cells from the 80% fraction under capacitating conditions were evaluated for the number of sperm penetrating to 1 cm (**A**) and 2 cm (**B**). The cells were counted and normalised to values in untreated controls (red dashed line). Agonists include: A1, B1 (n=5 experiments with 5 different donors), C1, D1 (n=3 experiments with 3 different donors), E1 (n=5 experiments with 5 different donors) and progesterone (n=11 experiments with 6 different donors). All values are the mean  $\pm$  SE. \*- indicates a significant difference from the control, considered to be  $P \leq 0.05$ , \*\*\*- indicates significance of  $P \leq 0.005$  &  $P \leq 0.001$  as assessed by non-parametric Mann Whitney U test.

Sperm motility, when measured by CASA, was not found to increase as a result of agonist exposure (figure 7-1& 7-2) however, the penetration assay found B1 and progesterone significantly enhanced penetrating ability of sperm cells when under capacitating conditions (figure 7-3 A&B). To examine whether this effect was the same in our surrogate patient sample (40% fraction under non-capacitating conditions) the penetration assay was conducted on A1, B1, E1 and progesterone. It was decided to examine A1 under these conditions due to the increase in penetration seen at the 2 cm position under capacitating conditions (figure 7-3B) and E1 was selected following a significant increase in progressive motility being exhibited through CASA analysis (figure 7-2). Sperm cells exposed to C1 and D1 were not found to enhance motility or penetrating ability through CASA analysis or the Kremer assay and for this reason were not evaluated under these conditions. Sperm cells exposed to A1 and E1 showed no significant changes in penetrating ability compared to control at either distance. B1 and progesterone were both found to enhance penetrating ability at 1 cm however; this was not a significant result (figure 7-4A). At the 2 cm position B1 increased sperm cell penetration however; the results between donors were highly variable and did not produce a significant result. Progesterone produced a significant increase in the number of sperm cells able to penetrate the viscous medium at the 2 cm position under these conditions ( $P \leq 0.05$ ; figure 7-4B).

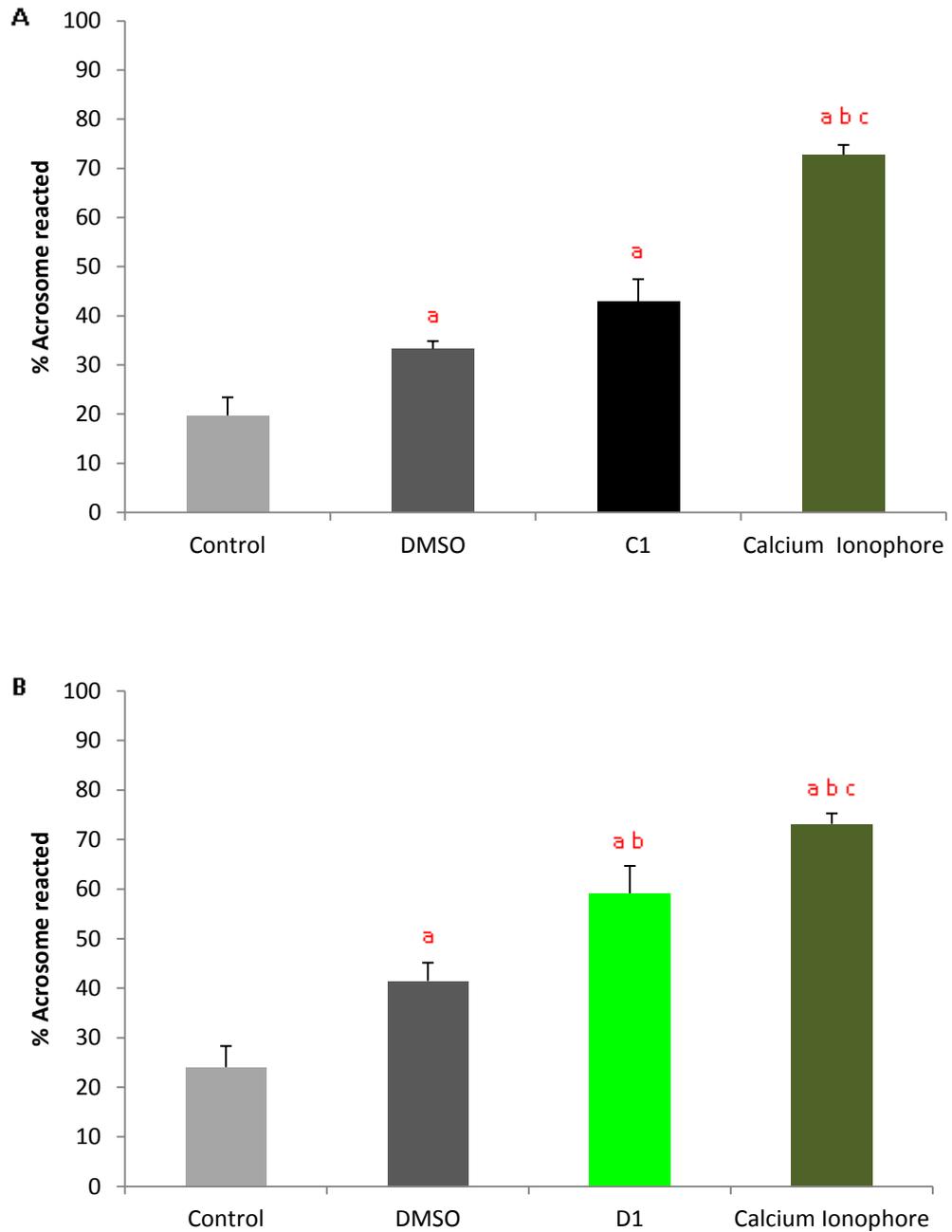


**Figure 7-4. Agonist induced penetration of cells into viscous medium.** Cells from 40% fraction under non-capacitating conditions were evaluated for the number of sperm penetrating to 1cm (**A**) and 2cm (**B**). The cells were counted and normalised to values in untreated controls (red dashed line). Agonists include: A1 (n=4 experiments with 4 different donors), B1 (n=5 experiments with 5 different donors), E1 (n=4 experiments with 4 different donors) and progesterone (n=18 experiments with 5 different donors). All values are the mean  $\pm$  SE. \* indicates a significant difference from the control, considered to be  $P \leq 0.05$  as assessed by non-parametric Mann Whitney U test.

### 7.2.3 Evaluating acrosome reaction in response to agonist stimulation

The Flexstation assay highlighted C1 and D1 as increasing intracellular  $\text{Ca}^{2+}$  however, this increase in  $\text{Ca}^{2+}$  does not appear to affect motility measured, by CASA or the Kremer mucus penetration test. This raises the question as to what this increase in  $[\text{Ca}^{2+}]_i$  can be attributed to. The knowledge of a  $\text{Ca}^{2+}$  store in the acrosomal region (Walensky and Snyder, 1995) led to the examination of the acrosome of cells after exposure to the aforementioned compounds.

The mean percentage of sperm with reacted acrosomes was significantly increased compared to control when cells were exposed to 1% DMSO ( $P \leq 0.05$ ; figure 7-5 A&B). The response to 1% DMSO was analysed due to these DDU compounds being in combination with DMSO. C1 induced the acrosome reaction in 42% of sperm cells which was a significantly higher percentage of cells compared to control, however, this result was not significantly different to the DMSO induced reaction indicating that this significant increase was most likely not a response solely of the agonist in question (figure 7-5A). D1 provoked the acrosome reaction in 59% of sperm cells which was a significantly higher proportion of cells than both the control and DMSO treated ( $P \leq 0.05$ ; figure 7-5B). This result suggests that D1 could potentially be inducing a premature acrosome reaction which would be detrimental to the fertilising potential of spermatozoa exposed to this compound. To ensure that the experiment was running successfully the effect of  $\text{Ca}^{2+}$  ionophore (A23187), a known inducer of the acrosome reaction (Aitken et al., 1993), was conducted in parallel to agonists. In both sets of experiments  $\text{Ca}^{2+}$  ionophore significantly enhanced the percentage of acrosome reacted cells when compared to control ( $P \leq 0.05$ ; figure 7-5A,  $P \leq 0.001$ ; figure 7-5B), DMSO ( $P \leq 0.05$ ; figure 7-5A,  $P \leq 0.001$ ; figure 7-5B), C1 ( $P \leq 0.05$ ) and D1 ( $P \leq 0.05$ ) independently (figure 7-5 A&B).

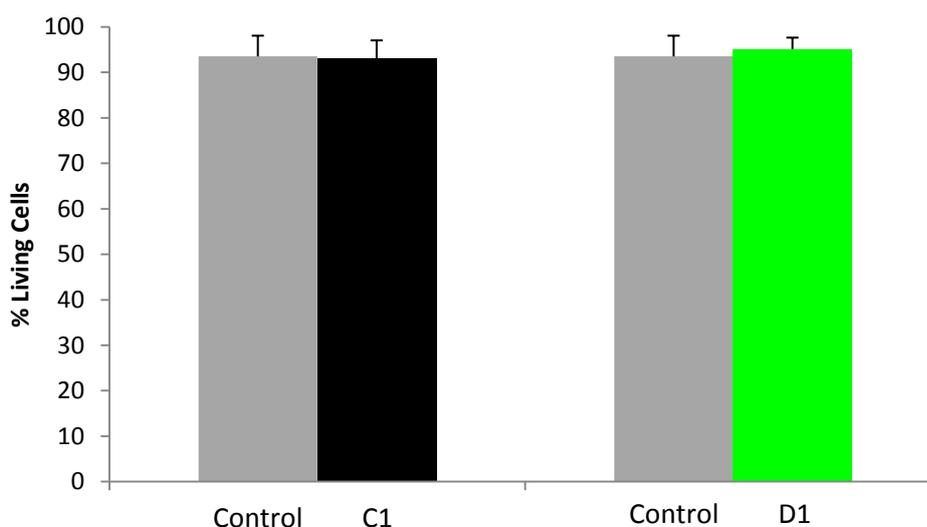


**Figure 7-5. Agonist induced acrosome reaction.** Staining with PSA-FITC allowed for evaluation of the percentage of sperm cells that become acrosome reacted in response to C1 (**A**) and D1 (**B**). All values are the mean  $\pm$  SE,  $n=5$  for all experiments. Significance was considered as  $P \leq 0.05$  indicated by **a** (significantly different from control), **b** (significantly different from DMSO) and **c** (significantly different to agonist C1 or D1). Statistical significance evaluated by T-test or non-parametric Mann Whitney U test.

#### 7.2.4 Vitality assessment of sperm cells exposed to agonists

To further identify where this increase in  $[Ca^{2+}]_i$  measured by the Flexstation assay may have come from or could be attributed to the Hypo-osmotic swelling test (HOS) was conducted to test vitality after exposure to the agonists. It is known that as cells die they tend to show an increase in  $[Ca^{2+}]_i$  as a result. To be able to assess whether this increase seen in the Flexstation was a result of the cells dying the HOS test was conducted on cells stimulated by both C1 and D1. Capacitated sperm cells were exposed to either C1 or D1 for a period of 3 hrs, equivalent to the amount of exposure assessed in the CASA motility assay, and the number of living cells assessed.

The HOS test identified that neither C1 nor D1 appeared to have a detrimental effect on sperm vitality. The result for C1 indicated a mean of 93.5% of living cells with the results ranging from 85% - 99% from the 3 donor samples assessed (figure 7-6). Sperm cells exposed to D1 showed similar results to C1 with a mean of 95% living cells and a range of 90% - 98% from the 3 samples assessed (figure 7-6). Neither C1 nor D1 were significantly different from control (figure 7-6) suggesting that neither of the compounds were toxic to sperm cells.



**Figure 7-6. Vitality analysis after agonist exposure.** C1 (black bar) and D1 (green bar) were assessed for their effects on sperm vitality after incubation with sperm cells for a period of 3 hrs. All values are the mean  $\pm$  SE,  $n=3$  for each experiment. Neither C1 nor D1 were statistically different from control (grey bars). Analysis was conducted using Pearson's Chi squared test and significance was assessed as  $P \leq 0.05$ .

### **7.2.5 Effect of ion channel agonists on patient samples**

Analysis of ion channel compounds on motility of donors conducted through CASA analysis did not highlight any compounds that consistently increased any of the motility parameters evaluated (section 7.2.1). However, the Kremer penetration assay identified B1 along with progesterone as significantly stimulating penetration through the cervical mucus substitute methylcellulose (section 7.2.2). To determine whether this was a true agonist response CASA motility analysis and the Kremer penetration assay were conducted on patient samples after exposure to B1.

#### ***7.2.5.1 CASA motility analysis of patient samples***

Patient samples provided by the ACU were incubated with B1 and motility kinematics measured by CASA over a period of 2 hrs. All samples examined were from patients undergoing IVF at the ACU and the results from each sample will be assessed independently (table 7.1).

Incubation with B1, when compared with control, resulted in a huge variation in responses, in all parameters, between the 9 patients analysed. Significant increases were found at points in every parameter evaluated but none of these significant increases were consistent between samples (table 7.1). The only parameter that contained a sustained statistical increase over the 2 hr time course was found for the percentage of total motile cells, however, this was limited to only one sample (P8, table 7.1). P1 and P6 showed significant decreases in VCL compared to control that were sustained for the 2 hrs evaluated. The predominant result after stimulation with B1 in all parameters was no significant change from control which corroborates the data from the donor analysis with this compound.

B1												
	VCL				LIN				ALH			
Patient No.	0	30	60	120	0	30	60	120	0	30	60	120
P1	x	x	x	x	-	✓	-	-	-	x	x	-
P2	-	✓	x	-	-	-	x	✓	-	-	-	x
P3	-	-	-	-	-	✓	-	✓	-	x	x	x
P4	✓	-	-	-	-	✓	-	-	✓	-	-	-
P5	-	-	-	-	-	-	-	-	-	-	-	-
P6	-	x	x	x	-	-	-	-	-	-	-	-
P7	-	-	-	-	-	-	-	-	-	-	-	-
P8	-	-	-	✓	-	-	-	✓	-	✓	-	-
P9	-	-	-	-	-	-	-	-	-	-	-	-
	Total Motile				Progressive Motility				Hyperactivation			
Patient No.	0	30	60	120	0	30	60	120	0	30	60	120
P1	-	x	-	-	-	-	x	-	x	-	-	x
P2	-	-	-	✓	-	-	x	✓	-	-	✓	-
P3	-	x	✓	-	-	✓	x	-	-	-	-	-
P4	-	-	-	-	-	-	-	-	-	-	-	-
P5	-	-	-	-	-	-	-	-	-	-	-	-
P6	-	-	-	x	-	-	✓	x	-	-	-	-
P7	-	-	-	-	-	-	-	-	-	-	-	-
P8	-	✓	✓	✓	-	-	-	✓	-	-	-	✓
P9	-	-	-	x	-	-	-	-	-	-	-	-

Key

✓=Increase

x = Decrease

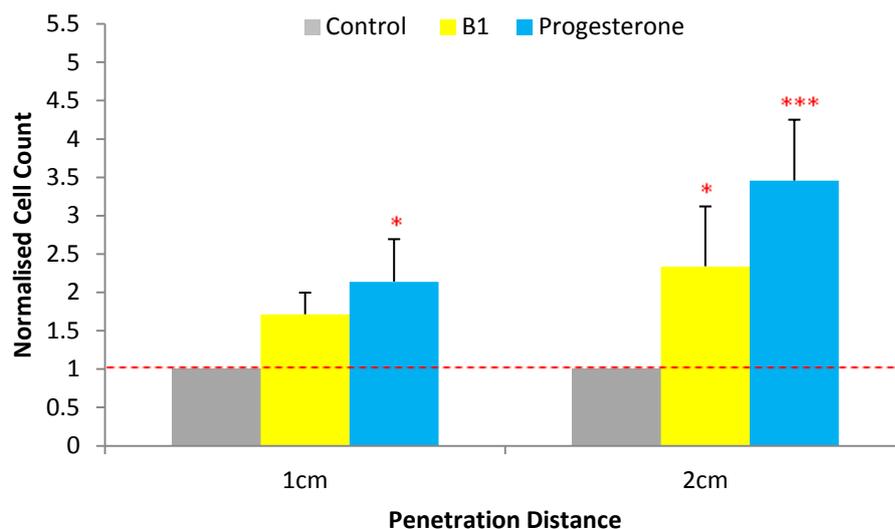
- = No change

**Table 7.1 Identification of motility kinematics, measured by CASA, from patient samples after exposure to B1.** Motility parameters after stimulation with the agonist B1 were evaluated from 9 patients undergoing IVF at Ninewells ACU. The table identifies the 6 motility parameters evaluated for each patient over a period of 2 hrs. Statistical significance (see key) was determined from comparison with control  $\pm$  SD. If the agonist response was greater than or less than control it was considered as a significant result.

### 7.2.5.2 Assessing functional motility of patient samples stimulated by B1

Section 7.2 identified that treatment with B1 and progesterone significantly enhances penetration of donor cells into methylcellulose (B1  $P \leq 0.005$ , Progesterone  $P \leq 0.001$ ,  $n=5$  figure 7-3 A&B). To ensure this result was not only valid in WHO normal donor samples the same assay was conducted on patient samples. The patients were all undergoing IVF at the ACU at Ninewells hospital. The results from this assay were consistent with the donor assay with cells exhibiting significantly increased penetrating ability after incubation with B1 and progesterone ( $P \leq 0.05$  at 1 cm,  $P \leq 0.005$  at 2 cm; figure 7-7). However, B1 was only found to show a significant increase in the number of cells penetrating to the 2 cm position ( $P \leq 0.05$ ; figure 7-7) instead of at both positions as seen with donor samples (figure 7-3).

When the behaviour of sperm swimming within viscous medium was investigated using CASA, neither B1 nor progesterone produced significant alterations in the speed of progression through the viscous medium (appendix). This observation is consistent with the effects of B1 seen in standard medium (figure 7-1 & 7-2).



**Figure 7-7. Agonist induced sperm penetration into viscous medium.** Sperm penetration into viscous medium upon application of  $10\mu\text{M}$  B1 or  $3.6\mu\text{M}$  progesterone measured by counting cells at 1 cm and 2 cm. The number of sperm penetrating to 1 cm and 2 cm was assessed and normalised to values in untreated controls (red dashed line). All values are the mean  $\pm$  SE ( $n=9$  experiments performed with 9 different patient samples). \*- indicates a significant difference ( $P \leq 0.05$ ), \*\*\*- indicates a significant difference ( $P \leq 0.005$ ) from the control, as assessed by non-parametric analysis Mann Whitney U test.

### 7.3 Discussion

The aims of this study were: 1) to examine whether the ion channel compounds increased sperm motility when evaluated by CASA analysis, 2) analyse whether agonist treated sperm were capable of penetrating viscous medium more effectively than untreated controls, 3) to establish the effects of agonists on sperm vitality and the ability to undergo the acrosome reaction and 4) after identifying promising compounds, to examine the robustness of the results by applying agonists to patient samples.

#### **B1 activation promotes sperm penetration into viscous medium but does not influence motility kinematics specifically**

The results from this chapter did not identify any ion channel compounds that consistently increased motility parameters, of donor or patient sperm, over a 3hr time-course when analysed by CASA. This prompted examination of these agonists using an alternative motility assay, the Kremer mucus penetration test. Interestingly, the results from this assay identified that B1 and progesterone significantly enhanced penetrating abilities of sperm through a cervical mucus surrogate (methylcellulose) although, it did not significantly change any motility kinematic parameters.

A previous study has described that direct activation of CatSper channels by progesterone has insignificant effects on hyperactivation when assessed by CASA analysis, however, progesterone was found to strongly enhance sperm penetrating abilities into methylcellulose (Alasmari et al., 2013b). This effect is similar to this study with the ion channel compound B1 showing insignificant effects on motility and hyperactivation when evaluating cells under both non-capacitating and capacitating conditions. However, this same compound significantly enhances spermatozoa's penetrating ability into viscous medium under both conditions. The Flexstation assay identified B1 as increasing  $[Ca^{2+}]_i$  in sperm cells similarly to progesterone. These

findings could indicate that B1 may be acting via the sperm specific,  $\text{Ca}^{2+}$  permeable, CatSper cation channel in a similar manner to progesterone, although, further motility analysis needs to be conducted making use of CatSper ion channel blockers to confirm this theory. Stimulation of human sperm with progesterone has been shown to activate CatSper situated in the flagellum (Strunker et al., 2011, Lishko et al., 2011). The associated increase in  $[\text{Ca}^{2+}]_i$  is amplified and propagated forward by CICR at the sperm neck (Costello et al., 2009, Olson et al., 2010, Lefievre et al., 2012). Previous studies on animals and human have reported active  $\text{Ca}^{2+}$  propagation from the tail to the midpiece and head in response to varying stimuli (Xia et al., 2007, Suarez et al., 1993). This  $\text{Ca}^{2+}$  propagation has an active role in the regulation of sperm behaviour. The theory behind B1 possibly acting via CatSper instead of acting via activation of  $\text{Ca}^{2+}$  stores results from previous work identifying that agonists that actively induce  $\text{Ca}^{2+}$  increase only from  $\text{Ca}^{2+}$  stores leads to a visible increase in hyperactivation as measured by CASA, whereas, the use of CatSper blockers on the same compounds resulted in no visible alteration in hyperactivation when measured by CASA (Alasmari, 2013). This information leads us to surmise that if B1 solely activated release of  $\text{Ca}^{2+}$  from internal stores you would have seen an increase in hyperactivation using CASA, which is not the case in this study. This suggests to me that it is more likely that B1 may act on CatSper with motility findings reported in this chapter being similar to that of this previous study with regard to compounds such as progesterone. Progesterone was found to regulate CatSper without showing an increase in hyperactivation that was measurable by CASA, similarly to B1. However, treated cells did exhibit significant increases when it came to the ability of spermatozoa to penetrate through methylcellulose (Alasmari, 2013, Alasmari et al., 2013b) which is a similar trait to that observed when spermatozoa are treated with B1. To be able to confirm this theory the next step for B1 would be to attempt to make use of known CatSper blockers on treated cells and measure  $[\text{Ca}^{2+}]_i$

alterations and motility through both CASA and the Kremer mucus penetration test see if you still see the increase in penetrating ability.

It has been reported that for sperm to be effective at penetrating through mucus they require the amplitude of lateral head movements to be  $\geq 2.5\mu\text{M}$  but  $< 7.0\mu\text{M}$  (Bjorndahl et al., 2010). Similarly Tesarik et al described that for human sperm to be capable of penetrating the cumulus matrix they required small amplitudes of lateral head displacement with a predominantly linear movement but an increased beat cross frequency (Tesařík et al., 1990). Alasmari et al describe the main functional effect of activating CatSper is an enhanced penetration into viscous medium (Alasmari et al., 2013b) which is an effect exhibited by B1 treated sperm. This enhanced penetration into viscous medium without enhanced motility kinematics, when measured by CASA, may result from the limitations of the CASA system which only measures movement of the sperm head and would be unable to distinguish subtle alterations in flagella beat that would enhance entry into viscous medium (Alasmari et al., 2013b). A group had previously investigated the effects of a caged progesterone analog (a known CatSper activator) on flagella bending of human sperm and they identified that after the progesterone activated CatSper it induced changes in curvature at the distal flagellum (Servin-Vences et al., 2012), these changes would not have been recognised by CASA owing to its inability to measure anything but alterations in movement at the sperm head. This would explain why no visible changes were noticed in motility when measured by CASA alone.

This compound has the potential to be beneficial in a clinical setting acting to increase penetrating ability of spermatozoa with no detrimental effects on motility. This could be beneficial in treatments such as IUI in aiding the sperm to swim through and penetrate the zona pellucida and oocyte vestments to bind to the oocyte.

## **CHAPTER 8**

**Investigating the incidence of sub-fertile patients, from andrology clinics, who could benefit clinically from sperm stimulants**

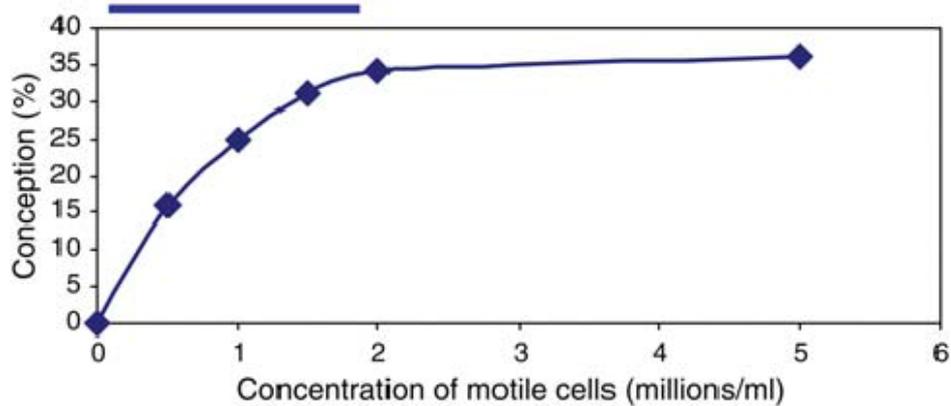
## 8.1 Introduction

Standard semen analysis is a subjective technique and has been found to be associated with large inter-laboratory variation making it difficult to predict accurate values (Larsen et al., 2000, Hargreave and Elton, 1983). Previously there had been controversy over the use of WHO reference values for diagnosis of infertility owing to the population they were created from being a 'normal population' of healthy men, rather than from men with proven fertility, raising concerns about the possibility of men conceiving who have semen analysis values below the WHO cut-off values (Lewis, 2007, Jedrzejczak et al., 2008, Ombelet et al., 1997). The current WHO 2010 reference values have now corrected for this and the reference values generated are from healthy donors with known fertility (time to pregnancy  $\leq 12$  months) (Cooper et al., 2010). The lower reference limits (LRL) outlined by WHO 2010, especially where motility is concerned (total motility; LRL 40% and progressive motility; LRL 32%), are similar to those proposed by John MacLeod and Ruth Gold in 1951 (Macleod and Gold, 1951b) suggesting that these new values are more robust than previous editions (Publicover and Barratt, 2011).

Sperm motility is one of the fundamental requirements for sperm to be capable of successful fertilisation *in vivo* and *in vitro* (Donnelly et al., 1998). MacLeod and Gold (Macleod and Gold, 1951b) identified that there are clear differences in sperm motility between sub-fertile and fertile men which has since been corroborated by numerous groups (Barratt et al., 2011). For this reason it is one of the key characteristics that is measured through semen analysis to provide diagnostic and prognostic information for natural conception and ART (Publicover and Barratt, 2011). Along with motility, sperm morphology and concentration are also important factors affecting fertility that are measured during a routine semen analysis (Guzick et al., 2001). Proposed treatment plans for male factor infertility are dependent on the results from two independent

semen analyses (two samples produced on separate occasions with a minimum of 2 days and maximum of 7 days abstinence) with IUI being the initial treatment option used in an attempt to alleviate mild male factor infertility (Tournaye, 2012). To be eligible for IUI treatment the female partner has to have a normal fertility status and the unprocessed semen sample must contain a total motile count (TMC) of at least  $1 \times 10^6$ - $3 \times 10^6$  which corresponds to mild oligoasthenozoospermia and a minimum of  $0.8 \times 10^6$  progressively motile cells must be recovered after sperm preparation (Tournaye and Cohlen, 2012, Tournaye, 2012, van Weert et al., 2004). Conception rates through IUI are very poor which reduces its use as the first-line treatment (van Weert et al., 2004, Campana et al., 1996) however, compared with IVF there is good evidence to suggest that IUI is a cost-effective treatment that should be initiated before more invasive and expensive treatments are considered (Goverde et al., 2000, Karande et al., 1999).

The range and distribution of semen profiles of men attending different infertility clinics is considerable. It is difficult, and maybe impossible, to identify a standard patient profile from semen analysis data owing to patients representing different clinical populations i.e. patients are a mixture of primary and tertiary referrals, NHS or private. Although this is the case, using semen analysis data from the two centres as a guide, it is possible to predict the percentage of patients who may benefit from *in vitro* drug stimulation to increase the concentration of motile cells. Publicover and Barratt (2011) constructed a graph using data adapted from Hargreave and Elton (1983) to predict conception according to the concentration of motile cells (figure 8-1) (Publicover and Barratt, 2011, Hargreave and Elton, 1983). This graph identifies reduced conception rates below 2 million motile cells/ml before a plateau occurs where the numbers of motile cells do not have an effect on conception. The area that is important for targeting for novel drug stimulation, according to this graph, would be patients who have less than 2 million motile cells/ml.



**Figure 8-1** Figure courtesy of Publicover and Barratt denoting likelihood of conception with regard to the concentration of motile cells. Data adapted from Hargreave and Elton (1983) depicting a plateau in conception rates with higher concentrations of motile cells. Reduced conception rates are noted below 2 million motile cells/ml (blue bar) this is the target area where improving motility by use of motility enhancing drugs would have most effect on improving fertility (Publicover and Barratt, 2011).

Using the data from Publicover and Barratt (2011) and the knowledge that an unprocessed semen sample must contain a TMC between  $1 \times 10^6$ -  $3 \times 10^6$  to qualify for IUI (Tournaye and Cohlen, 2012), as a first-line treatment, this study aims to identify the benefits of a specific treatment i.e. novel drug therapies that can be used to increase sperm motility. The clinical use of sperm stimulants may be enough to increase the TMC of some patients, who previously were not eligible for IUI owing to a low TMC, to allow them to use less invasive treatments as a first treatment option.

## 8.2 Experimental design

The analysis evaluated the semen characteristics of 3239 patients from clinic A and 552 patients from clinic B. Patients where all the data was missing were excluded from the study (31 patients' clinic A, 0 patients' clinic B). Patients were divided into categories according to the WHO 2010 reference parameters for semen analysis with patients being classed as oligozoospermic (concentration of <15 million/ml), asthenozoospermic (total motility <40% and/or progressive motility <32%), teratozoospermic (normal forms <4%) or a combination of two or three of these factors, cryptozoospermic (<1 million/ml), normozoospermic (concentration >15 million/ml, total motility >40%, progressive motility >32% and >4% normal forms) and finally azoospermic.

For statistical analysis all parameters were analysed, utilizing frequency distributions and P-P plots (probability plots for assessing how closely two data sets agree), for normality. Semen analysis data from both of the clinics was not found to be normally distributed and after transformation the data remained non-normal. For the purposes of correlation/regression data is not required to be normally distributed, however, assumptions are made about "error" which is the difference between the observed and predicted values (also called residuals). Scatter plots and correlation analysis was conducted on three pairs of variables; 1) % motile with % normal forms, 2) % motile with concentration and 3) % normal forms with concentration. Linear regression analysis was then conducted on these same variables and the "error" was analysed for normality by use of histograms.

Cumulative frequency distributions depicting the concentration of motile cells were generated for the two datasets. Azoospermic patients were excluded from the dataset. To focus the dataset only patients with a concentration of 6 million motile cells per ml (calculated from WHO 2010 lower reference limits: concentration <15 million/ml, total motility <40%) were included in the analysis (clinic A n= 687, clinic B n=175).

### 8.3 Results

According to the WHO 2010 reference parameters for semen analysis, for clinic A, 56% of the patients were identified to be normozoospermic with 6% exhibiting solely motility dysfunction (asthenozoospermia) and a further 10% showing poor motility combined with either poor sperm morphology, low concentration or a combination of all three factors (teratozoospermia, oligozoospermia or oligoasthenoteratozoospermia) (figure 8-2). This differed to the data collected from clinic B with only 24% of patients being considered normal and 4% exhibiting isolated asthenozoospermia with a further 25% exhibiting poor motility in combination with other factors (figure 8-3). With the exception of the patients characterised as being normal the highest percentage of patients in both clinics were identified as being teratozoospermic 10% and 22%, clinic A and B respectively (figure 8-2 and 8-3). When the three single parameters (oligozoospermia, teratozoospermia and asthenozoospermia) were compared with one another the correlation between the parameters was found to be highly significant ( $P \leq 0.000$ ) for both clinic A and B although, after visually comparing the parameters in a scatter plot you would not expect this to be a highly significant result (figure 8-4 A-C and 8-5 A-C). Correlation can be statistically significant simply because of the large number of cases, not because there is a meaningful bivariate relationship which appears to be the case for these datasets. Bearing this in mind, when looking at the correlations, the linear regression model identifies the percentage of variance explained by the model, which for clinic A is 11%, suggesting that both morphology and sperm concentration influence progressive motility and vice versa. Similarly for the dataset for clinic B linear regression analysis identified that both independent variables (sperm concentration and morphology) contribute to the model of motility 16% of the time. These percentages suggest there is a correlation between the three factors although it is minimal.

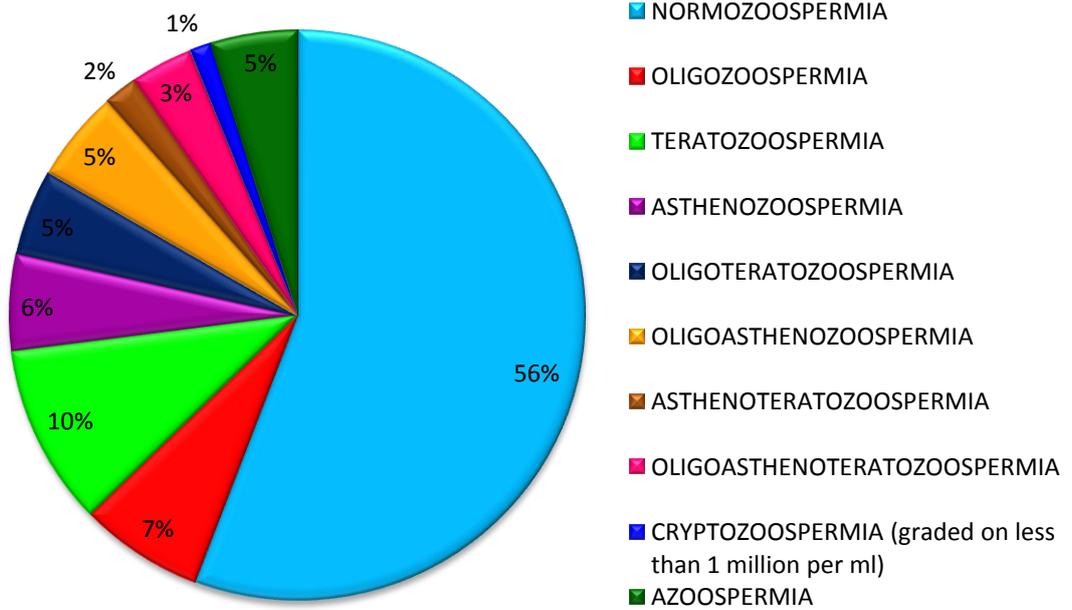


Figure 8-2 Classification of patients according to WHO reference parameters (WHO, 2010e) for semen analysis for patients attending clinic A (2002-2003) .

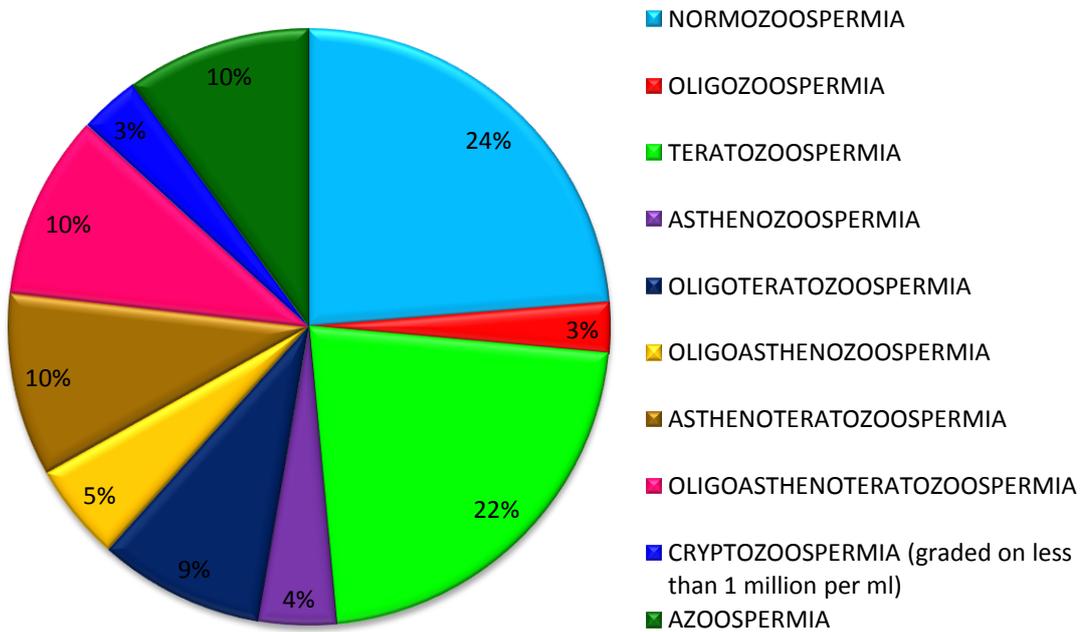
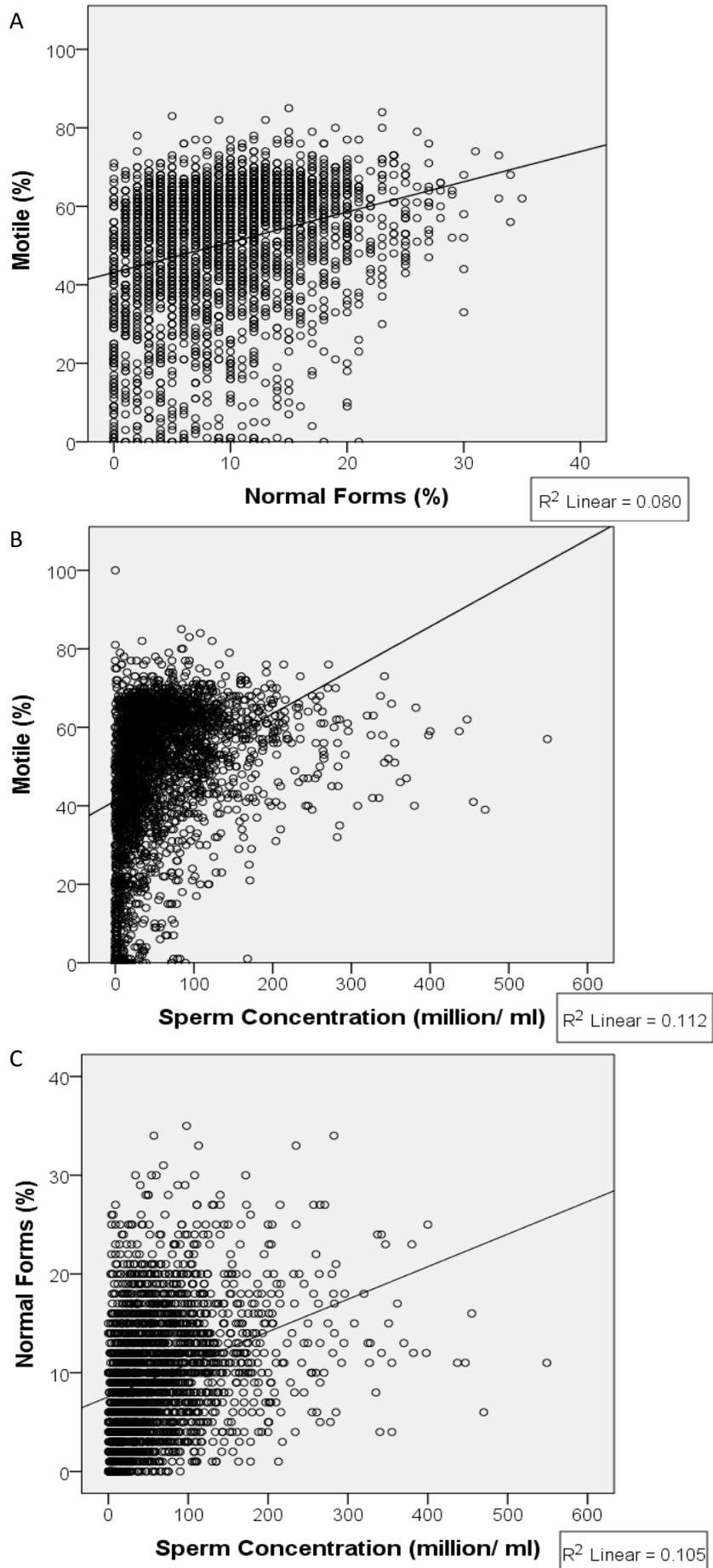
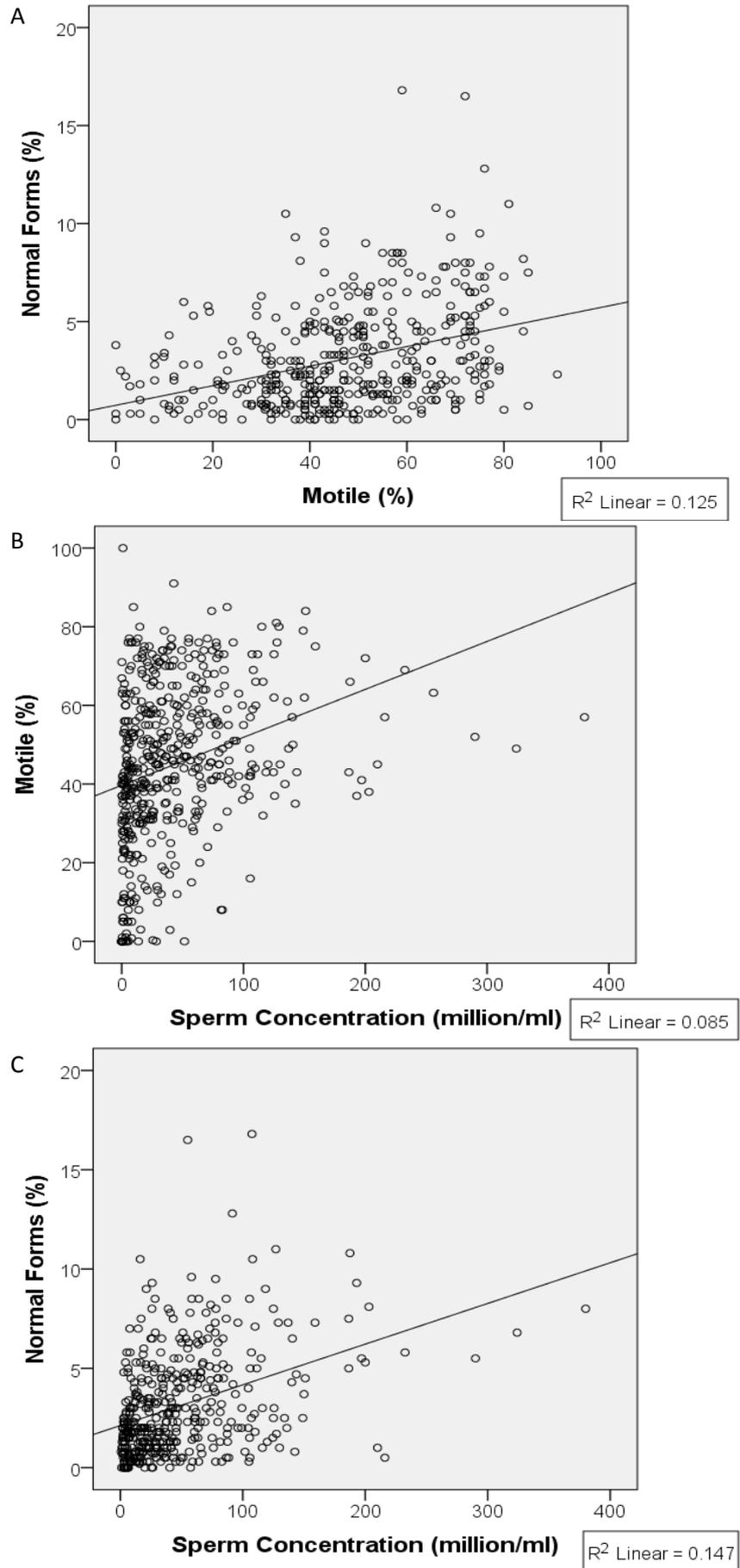


Figure 8-3. Classification of patients according to WHO reference parameters (WHO, 2010e) for semen analysis for patients attending clinic B (2011)



**Figure 8-4 Scatter plots identifying correlations for Clinic A.** 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C). N= 3239



**Figure 8-5 Scatter plots identifying correlations for Clinic B.** 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C). N= 552

In an attempt to corroborate this, data from both clinics was further separated into two groups with a lower limit and upper limit: 1) semen with a concentration of less than, and including, 15 million/ml (WHO 2010 criteria) and 2) semen with a concentration of less than, and including, 100 million/ml. The same analysis as before was then conducted on the same pairs of variables within these two groups. When these same parameters (oligozoospermia, teratozoospermia and asthenozoospermia) were compared within their segregated groups the lower limit (less than 15 million/ml) once again identified a highly significant correlation ( $P \leq 0.000$ ) for both clinic A and clinic B although, after visually comparing the parameters the scatter plots do not show a meaningful linear relationship (appendix). Linear regression analysis identifies that the total variance explained by the model is 12.2% with regards to clinic A and only 6% with regard to clinic B once again confirming a small correlation between the three parameters.

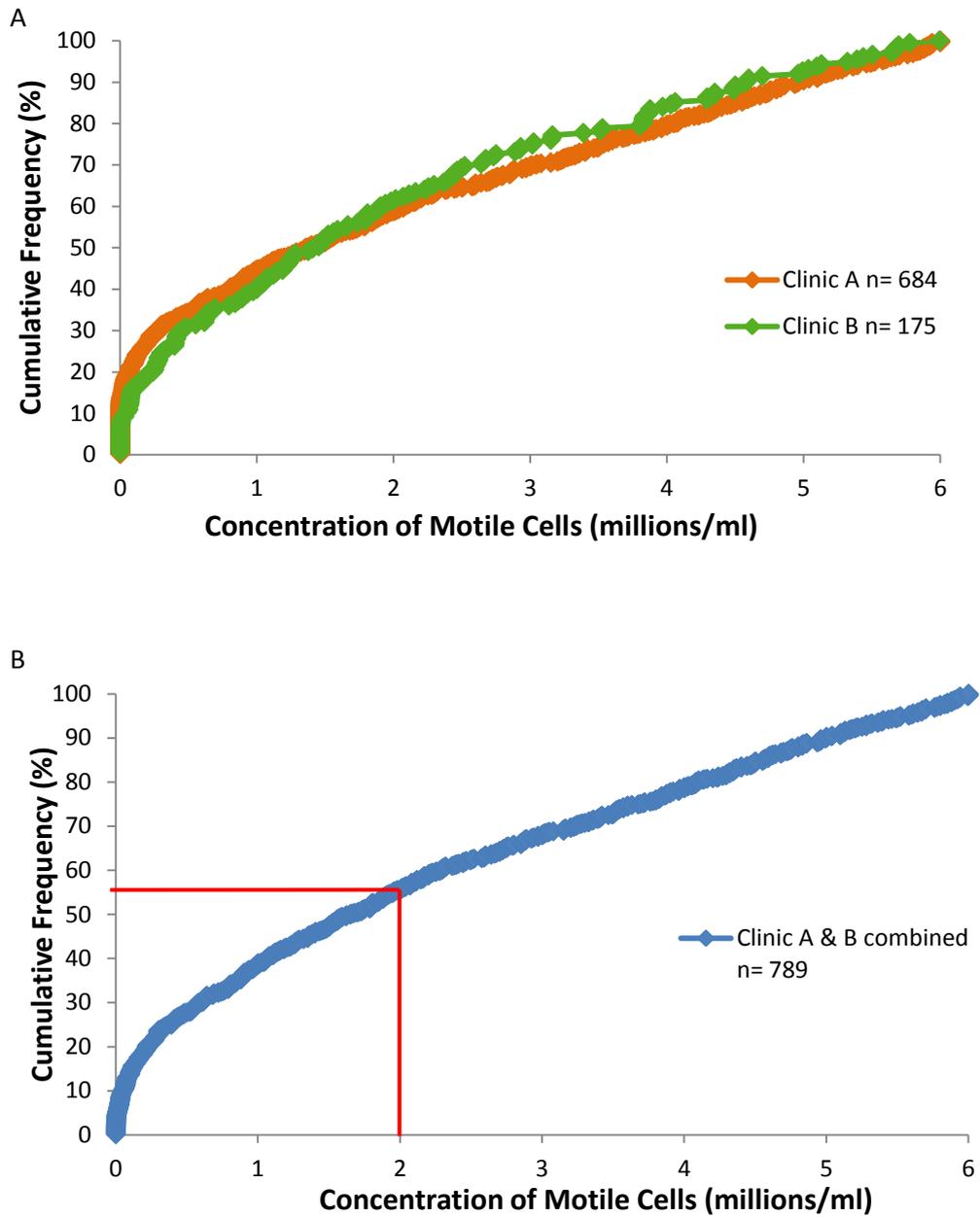
For the high concentration group, once again the correlations for both clinics are highly significant (clinic A;  $p \leq 0.000$  - all parameters, clinic B;  $p \leq 0.01$  - sperm concentration and motility,  $p \leq 0.000$  motility and morphology and concentration and morphology). Linear regression analysis identified 6.5% variance is due to the model for clinic A and 11.4% for clinic B. This data highlights the variability between patients with the total percentage of variance explained by the model being greatest in the low concentration group for clinic A (12.2% vs 6% clinic B) whereas, the greatest variance explained by the model is greater in the high concentration group for clinic B (11.4% vs 6.5% clinic A). This data suggests that although a correlation does appear to exist between the three parameters it is only minimal identifying that each factor can be assessed independently and is not reliant on the other two parameters. This data suggests therapeutic advancements in motility are not restrained by the concentration and quantity of morphologically normal sperm.

As mentioned previously Publicover and Barratt (2011) identified that to increase the likelihood of a successful conception the ideal concentration of motile cells is  $\geq 2$  million/ml (Publicover and Barratt, 2011). Below this value small changes in the concentration of motile cells can have a noticeable effect on the chance of conception making it an ideal target area for motility enhancers. Using the data collected from the two centres it was possible to identify patients who, according to the graph from Publicover and Barratt (2011) (figure 8-1), would be suitable for and may benefit from *in vitro* drug treatment to increase motility. Data from clinic A highlighted 13% of patients (n=3015, azoospermic patients excluded) having a concentration of motile cells  $\leq 2$  million per ml with clinic B exhibiting 22% of patients (n=494, azoospermic patients excluded), under the same category, with the potential of benefiting from motility stimulants.

Data collected previously in this study (chapter 6) identified a potential therapeutic drug treatment (Trequinsin Hydrochloride) that can increase motility *in vitro*. This compound was capable of increasing total motility and specifically progressive motility by approximately 20%. Given this knowledge it is possible to predict that in patients with poor/low motility you would also see a 20% increase in motility of cells treated with this compound. Using guidelines from Ninewells Assisted Conception Unit it is possible to calculate the percentage of patients from both clinics who, after treatment with motility enhancing drugs, could be eligible for less invasive treatment options than currently used. Patients with concentrations of motile cells  $\leq 1$  million/ml would normally be treated with ICSI. The data for clinic A identified 1% (n=3015) whereas clinic B identified 3% (n=494) of patients after treatment with motility enhancing drugs, that could be eligible for IVF before the more invasive treatment of ICSI. To be eligible for IUI patients need to have a concentration of motile cells  $\geq 5$  million/ml, using this value as a guideline; it was possible to calculate that 3% of patients from clinic A

(n=3015) and 19% of patients from clinic B (n=494), after treatment, would be eligible for IUI instead of IVF.

Looking at sub-fertile patients (below WHO normal semen parameters) it was possible to generate a cumulative frequency curve identifying the percentage of patients from both clinic A and clinic B independently that have the potential of benefiting from treatment with motility stimulants (figure 8-6A). Looking at the curves from both clinics it is clear to see that they both follow a similar pattern indicating that the sub-fertile patients have similar profiles regardless of the spatio-temporal differences in data collection (figure 8-6A). Because the patient profiles follow a similar trend the data from the two clinics was combined to produce a single curve (figure 8-6B). In this analysis we are looking specifically at the potential of improving motility of patients who have a concentration of  $\leq 2$  million/ml motile cells and using the single curve it is possible to identify that approximately 60% of the sub-fertile patients from the two clinics combined fit this profile (figure 8-6B indicated by red line).



**Figure 8-6** Cumulative frequency graph depicting the percentage of sub-fertile patients from clinic A and B independently (azoospermic patients included) (A) and combined (azoospermic patients excluded) (B). These graphs highlight the percentage of patients with below WHO normal values for concentration of motile cells. Using the graphs it is possible to determine the percentage of patients who could benefit from motility enhancing stimulants (red line).

## 8.4 Discussion

Motility dysfunction is major problem with regard to male infertility (Hull et al., 1985) with previous studies identifying isolated asthenozoospermia as the most frequent semen abnormality (Milardi et al., 2012). Similarly to Curi *et al* (2003) these dataset identified a higher percentage of patients with motility dysfunction in combination with factors such as poor morphology and/or low concentration than patients presenting with solely poor motility highlighting that novel treatments to improve motility could have a beneficial effect not only for couples with isolated motility problems but also with combined factors (Curi et al. 2003). This is further supported by this dataset and the findings of Macleod and Gold (1951) that sperm concentration contributes to sperm motility and this study further identifies sperm morphology as a contributing factor (Macleod and Gold, 1951b). The main aim of this study was to identify the sub-set of patients who would benefit clinically from treatments that specifically enhance motility. Combining the data from the two clinics 15% (n=3509) of patients could benefit from motility stimulants, according to the graph by Publicover and Barratt (2011) (Publicover and Barratt, 2011). By looking specifically at the sub-fertile patients (Below WHO normal concentrations of motile cells) it is possible to identify that almost 55% of these patients would be eligible for treatment with motility stimulants (excluding the azoospermic patients). This highlights the potential clinical benefits of successful treatment with motility enhancing stimulants.

The ability to increase success rates after IUI and make IUI more accessible to more patients through the use of motility stimulants would decrease the financial (Van Voorhis et al., 2001) and emotional burden on patients and healthcare providers. The proposed motility stimulants could significantly increase the number of progressively motile cells produced meaning that men who previously had below the threshold number of motile cells for IUI (between  $1 \times 10^6$ - $3 \times 10^6$  unprocessed sample) could now

be eligible for treatment (Tournaye and Cohlen, 2012, Tournaye, 2012, Van Voorhis et al., 2001). However, this data is based on semen analysis meaning it is not possible to guarantee that these samples would produce a minimum of  $0.8 \times 10^6$  total motile cells post preparation as required for IUI (Tournaye, 2012). Combining data from both Clinic A (1.8%) and Clinic B (1.2%) it has been possible to predict that 1.3% of patients, after treatment with motility enhancing drugs, could be eligible for IVF before the more invasive treatment of ICSI. Similarly it is possible to predict that a combined total of 5% (n=3509) of patients could now be considered for IUI as a first line treatment instead of IVF (Clinic A 2.6% n=3015, Clinic B 19.2% n=494). From this study it is only possible to predict the percentage of patients who previously were not eligible for IUI but after treatment with motility stimulants could become eligible. It is not possible to predict the potential beneficial effects motility stimulants could have on conception rates after treatment through IUI. However, there is good evidence that IUI is a cost effective treatment option that is less invasive than IVF and ICSI (Tournaye, 2012, Van Voorhis et al., 2001, Comhaire et al., 1995). With the discovery of novel compounds that enhance motility the success rates for fertilisation through IUI should increase making it a more viable option for more couples as an initial therapy for couples with male factor infertility.

There are a number of assumptions made from this data to be able to come to the conclusions drawn in this study. It is important to remember that even through improvement of motility you may not improve conception rates. There are a number of factors that contribute to a successful fertilisation that are not accounted for in this study including DNA abnormalities, inability to capacitate at the correct time, premature acrosome reaction and problems with phospholipase C Zeta activation to name but a few (Amdani et al., 2013, Donnelly, 2000).

## **CHAPTER 9**

### **Conclusion and Future work**

The primary aims of this thesis were to: (1) identify a sub-group of spermatozoa from donor samples that can be used, in research, as a representative sample for patient samples, (2) develop, and (3) implement a protocol for screening compounds, identified from a Flexstation screen, that have the possibility of enhancing motility through increasing  $[Ca^{2+}]_i$  in sperm and finally, (4) establish the incidence of asthenozoospermia in patients who have previously undergone fertility treatment to identify patients who would benefit from therapeutic advancements in this area.

### **Key findings and future work**

By establishing the optimum conditions *in vitro* (chapter 4) it was possible to develop a robust system for screening of motility enhancing compounds. Six compounds identified from the Chemogenomics library and fourteen compounds from the UoD DDU ion channel library were then tested using the standard operating procedure developed in chapter 4. The majority of these compounds were found to have no beneficial effects on sperm motility posing questions as to why these compounds increase  $[Ca^{2+}]_i$  in sperm. To be able to further examine the effects of these compounds studies need to be conducted examining vitality and presence of the acrosome. With respect to chapter 5, the data highlights the importance of one compound identified from the Chemogenomics library; Trequinsin Hydrochloride. This data identified that Trequinsin increases  $[Ca^{2+}]_i$  resulting in a significant increase in motility when measured by CASA and Kremer mucus penetration assays under non-capacitating conditions. This study, through the use of patch clamping, identifies CatSper activation by this PDEi suggesting that CatSper is an unidentified target of PDEi's in human sperm cells.

Trequinsin hydrochloride, after patch clamping, identified changes in human sperm potassium currents (hKSper). hKSper have been shown to originate in the sperm flagellum and have been found to support CatSper activity (Mannowetz et al., 2013). The human sperm principle potassium channel has been identified as Slo1 and is dependent on intracellular  $\text{Ca}^{2+}$  concentrations (Mannowetz et al., 2013). Lishko *et al* and Strunker *et al* independently discovered that CatSper is activated by progesterone by shifting Catsper activation to more hyperpolarised membrane potentials, increasing intracellular  $\text{Ca}^{2+}$  resulting in hyperactivated motility (Lishko et al., 2011, Strunker et al., 2011, Lishko et al., 2012a). Activation of CatSper is vital for fertilisation and since potassium channels are well known to regulate membrane potentials Lishko *et al* proposed their role in capacitation and specifically supporting CatSper induced hyperactivation (Navarro et al., 2007, Mannowetz et al., 2013). Slo1 has been found to be inhibited in a dose dependent manner by micromolar concentrations of progesterone which in an *in vivo* environment suggests that once the spermatozoa get in close proximity to the oocyte the hKSper channel will become blocked by increasing concentrations of progesterone, leading to membrane depolarization. This shift in membrane potential activates CatSper channels allowing for an influx of  $\text{Ca}^{2+}$  which in turn elevates  $[\text{Ca}^{2+}]_i$ . This triggers  $\text{Ca}^{2+}$  dependent processes such as hyperactivated motility enabling spermatozoa to become capable of fertilisation (Mannowetz et al., 2013). This study proposes Trequinsin Hydrochloride functions in a similar manner to progesterone acting to inhibit Slo1 triggering membrane depolarisation thereby supporting full activation of CatSper. These events trigger hyperactivation allowing for penetration of the oocyte vestements and successful fertilisation.

The improvement in sperm motility attributed to treatment with B1 (chapter 7) were less pronounced than those seen in response to Trequinsin (chapter 6) with significant increases only visible when analysed by the Kremer mucus penetration assay. This

suggests that although it is possible B1 is activating CatSper it may not be an effective activator of sperm motility. B1 may only function via the CatSper channel which would allow for changes in flagella pattern to strongly enhance sperm penetration through viscous media but may not be capable of triggering  $\text{Ca}^{2+}$  release from internal stores preventing inducement of hyperactivation (Alasmari et al., 2013b). Thus it could be speculated that addition of this compound to spermatozoa from a therapeutic view point would not be wholly beneficial. If this compound were to be used in IUI procedures it appears it would be successful in aiding sperm penetration through cervical mucus but would not affect the sperms ability to, on reaching the oocyte, successfully penetrate the oocyte vestements to complete fertilisation.

One key issue that has been highlighted in this study is that use of 1% DMSO induces a premature acrosome reaction in cells treated with this compound for a long period of time (chapter 7). This is a similar problem to that found with use of pentoxifylline treatment previously (Tardif et al., 2014). One solution to this problem could be to examine the effects on motility of sperm preparations with a limited incubation with the motility enhancing compound which is then washed off. The motility of these sperm can then be examined after the wash to see if the motility enhancement is sustained reducing the risk of a premature acrosome reaction.

Whilst the work in this study has highlighted two potential compounds that successfully increase sperm motility; 1) Trequinsin hydrochloride (commercially available) and 2) B1 (previously unknown compound) these compounds have only been examined a limited number of times on both donor and patient samples. To categorically confirm their potential for increasing motility further studies are required. Furthermore, this work would need to be expanded on in order to confirm the proposed models in this study. Through the use of activators, inhibitors and/ or patch clamp studies experiments

need to be conducted to confirm that Trequinsin and B1 activate CatSper specifically and to investigate their effects on the human sperm potassium channel Slo1. These compounds also warrant closer examination to evaluate any unwanted effects such as; premature acrosome reaction or adverse effects caused by long incubations with the compounds. These compounds were identified as increasing  $[Ca^{2+}]_i$  through a Flexstation Assay Screen to ensure this response is genuinely repeatable it would be beneficial to conduct fluorimetric population measurements on treated samples in parallel with motility analysis. To further clarify whether these compounds are activating CatSper alone or have any currently unidentified effects on  $Ca^{2+}$  stores the use of single cell imaging of samples would enable identification of  $Ca^{2+}$  store mobilisation as a result of treatment with these compounds. It would also be beneficial to conduct single cell  $Ca^{2+}$  imaging on sperm treated by each of the compounds to allow for exact identification of the increase in  $[Ca^{2+}]_i$  seen as a result of drug stimulation.

This study has helped to develop a standard operating procedure for efficiently and reliably analysing the motility effects of sperm treated with compounds identified from the Flexstation screen. The results identified that in the future it would be beneficial to only analyse the effects of potential compounds on capacitated sperm. However, it is beneficial to analyse the effects on both the 40% fraction and 80% fraction from donor sperm. To ensure that the positive or negative effects on sperm are a true result of treatment with the compounds, in the future, the effect of leukocytes especially in the 40% fraction needs to be accounted for.

Male factor infertility is the cause of sub-fertility in 50% of cases with sperm dysfunction being the single most common factor responsible for male infertility (Hull et al., 1985, Thonneau et al., 1991). ART is currently the main way of treating male factor infertility but it is expensive and invasive (Comhaire et al., 1995, Van Voorhis et

al., 2001). The ability to treat more patients with IUI as a first line treatment for male factor infertility, through the use of motility stimulants, would make fertility treatment more accessible to couples worldwide who are not eligible for state funded treatments. This study identifies for the first time that through the use of motility enhancers it may be possible to treat a further 5% (chapter 8) of patients attending two independent fertility clinics with IUI before the more invasive procedure of IVF. It is also conceivable that these stimulants could not only increase the access to IUI (through making more patients eligible for treatment) but could potentially improve pregnancy rates within this treatment. This would make IUI a cost-effective, sustainable treatment option. This study has highlighted the percentage of sub-fertile patients from two independent fertility clinics. Through the use of a cumulus frequency curve it is possible to identify the percentage of patients, according to their concentration of motile cells, which could benefit from potential novel treatments. The similarity in the trend exhibited by the sub-fertile patients from the two independent clinics (chapter 8) suggests that this graph could be used in the future as a guideline for identifying target groups of patients for future studies.

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# **CHAPTER 10**

## **Appendix**

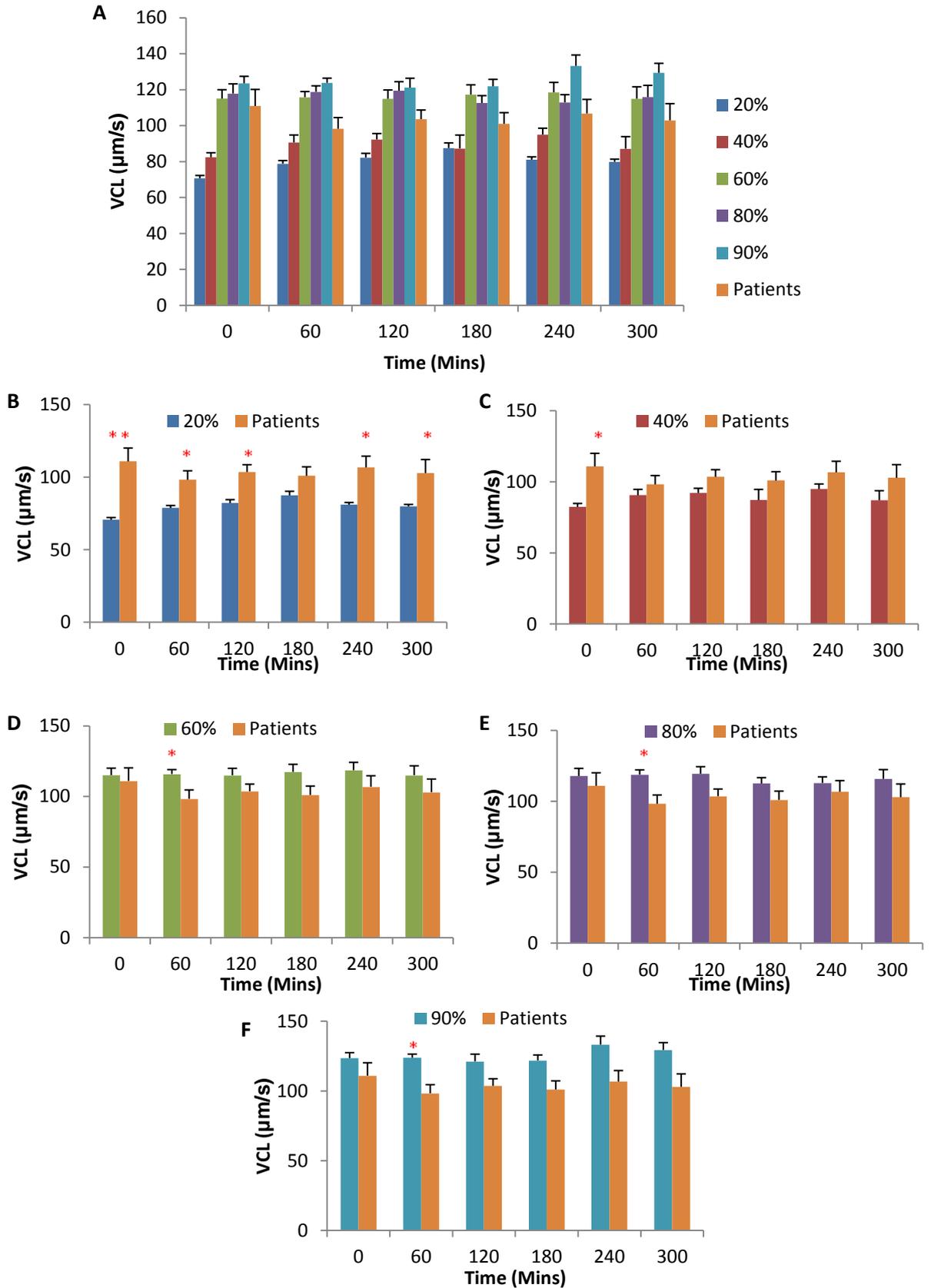
### 10.1 Components, product list and catalogue numbers for Media and Compounds used.

Compound	NCM (mM)	CM (mM)	Source	Catalogue N <sup>o</sup>
CaCl <sub>2</sub>	1.8	1.8	Sigma-Aldrich	C3306 - 100
KCl	5.4	5.4	Sigma-Aldrich	P5405 – 250g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.8	0.8	Sigma-Aldrich	M2773 - 500g
Sodium chloride	116.4	116.4	Sigma-Aldrich	S5886 – 1Kg
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	1.0	1.0	Sigma-Aldrich	71505 - 250g
D- glucose	5.55	5.55	Sigma-Aldrich	G6152 – 500g
Sodium lactate	41.75	25	Sigma-Aldrich	L7900 – 100ml
Sodium pyruvate	2.73	2.73	Sigma-Aldrich	P2256 – 25g
HEPES	25	-	Sigma-Aldrich	H3375 – 1Kg
Sodium bicarbonate	-	26	Sigma-Aldrich	S5761 -500g
BSA	0.3%	0.3%	Sigma-Aldrich	A3059 – 100g

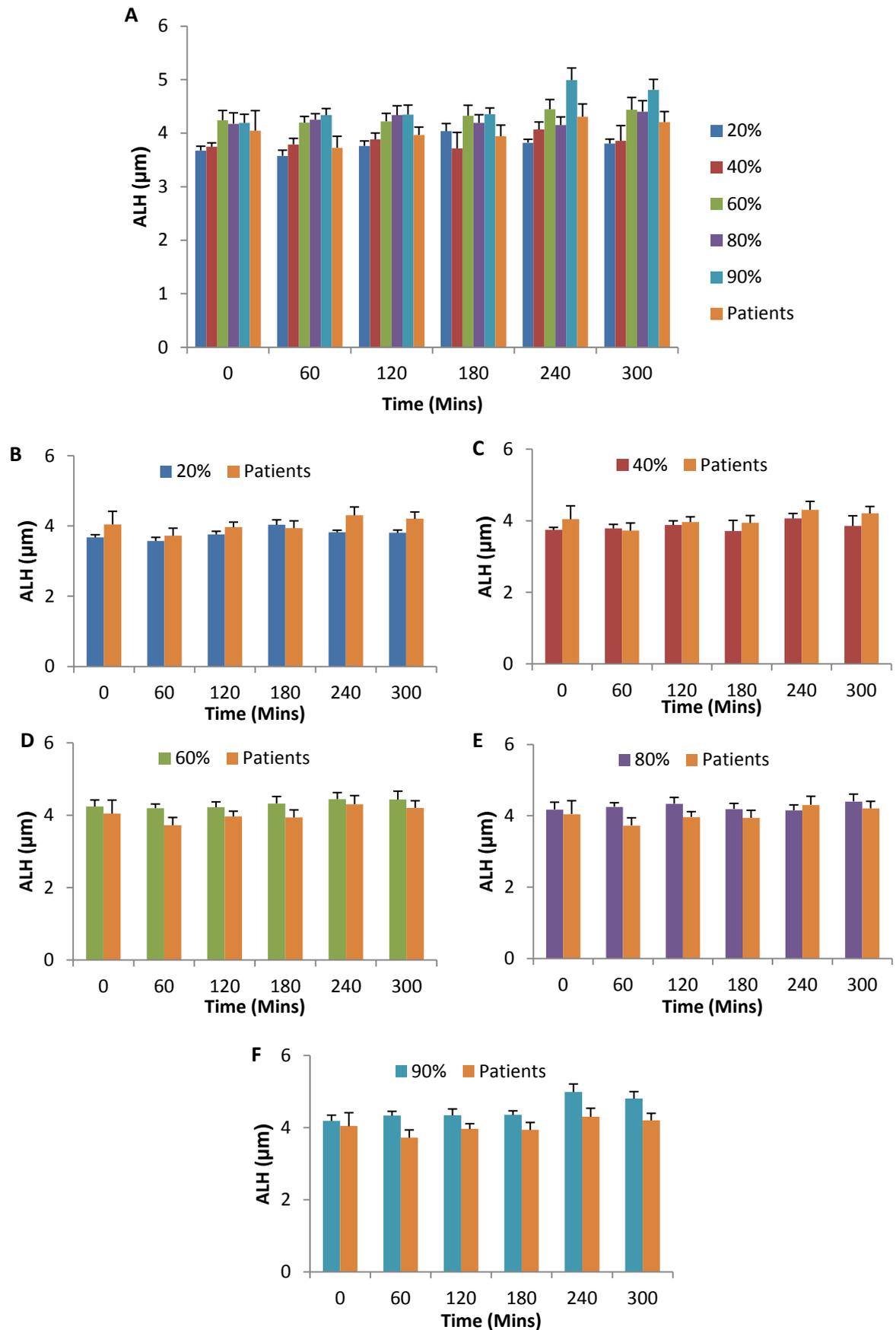
Compound	Source	Catalogue N <sup>o</sup>
DMSO	Sigma-Aldrich	673439
PHA665752	Tocris	2693
Leelamine Hydrochloride	Tocris	2139
GP1a	Tocris	2764
JX401	Tocris	2657
EO1428	Tocris	2908
Trequinsin Hydrochloride	Tocris	2337
PureSperm 40% and 80%	Hunter Scientific	PS40-100 PS80-100
SAGE Gamete buffer	Origio	ART-1005
Quinns Advantage Fertilisation Medium	Origio	ART-1020
Human Serum Albumin (HSA)	Origio	ART-3001
Percoll	Sigma-Aldrich	P1644
Calcium 3	Molecular Devices	
Methylcellulose	Sigma-Aldrich	M0512
PSA FITC	Sigma-Aldrich	L0770
Calcium Ionophore	Sigma-Aldrich	C7522
PBS	Sigma-Aldrich	P4417
Hydromount	National Diagnostics	HS-106
D-fructose	Sigma-Aldrich	F0127
Sodium citrate dihydrate	Sigma-Aldrich	W302600

**Table 10.1** Identification of compounds and the product source for media which were utilised in all experiments conducted within this project.

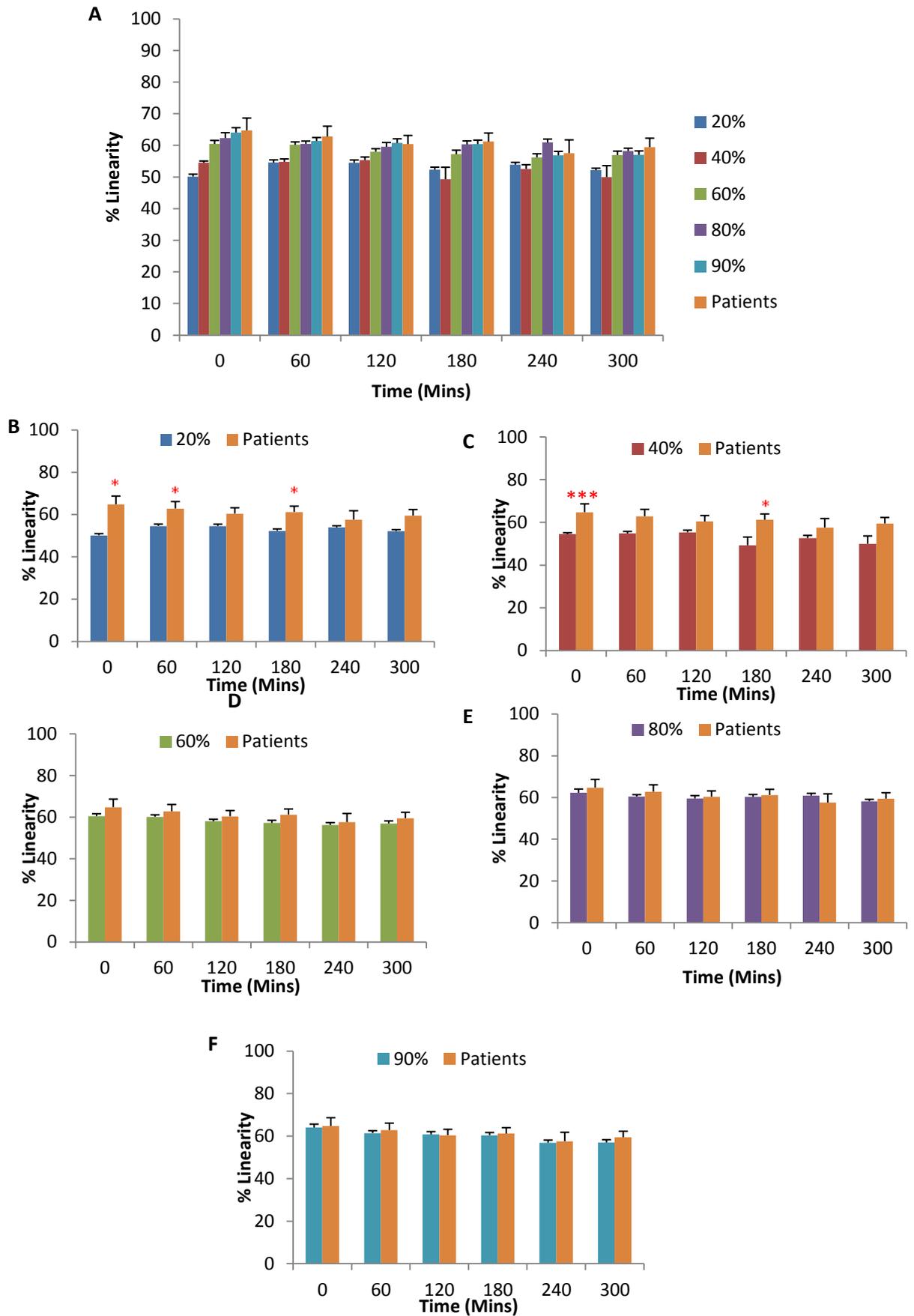
## 10.2 Comparing donor fractions to 80% fraction recovered from patients.



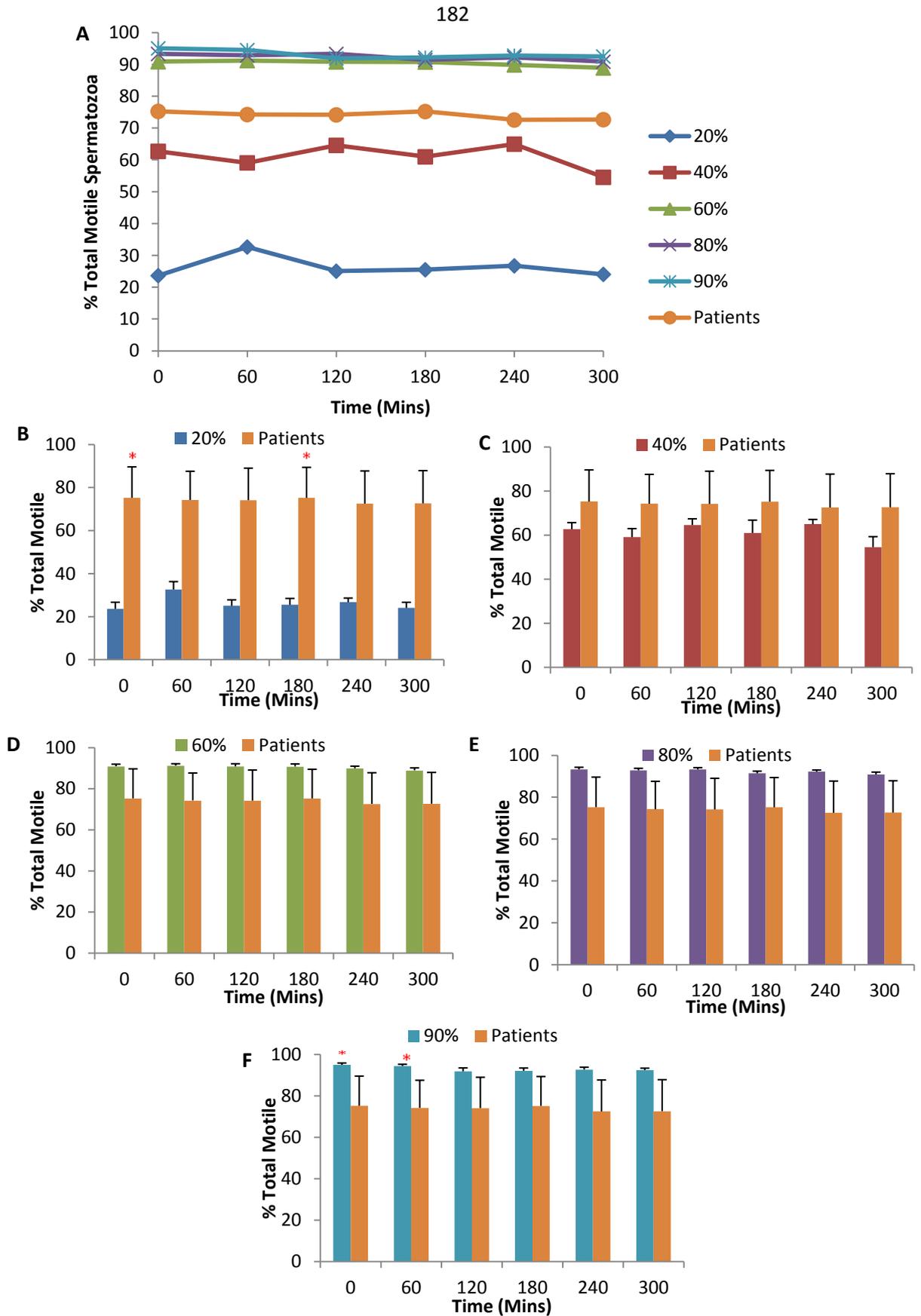
**Figure 10-2. Comparison between donor and sub-fertile samples in Curvilinear Velocity (VCL) in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction (B) 20% donor, 80% sub-fertile (C) 40% donor, 80% sub-fertile (D) 60% donor, 80% sub-fertile (E) 80% donor, 80% sub-fertile (F) 90% donor, 80% sub-fertile. The result shown is the mean  $\pm$  SEM. N= 5 for both donors and patients, \*indicates significant difference ( $P < 0.05$ ) \*\*indicates  $P < 0.01$  (T-test and Kruskal-Wallis test).**



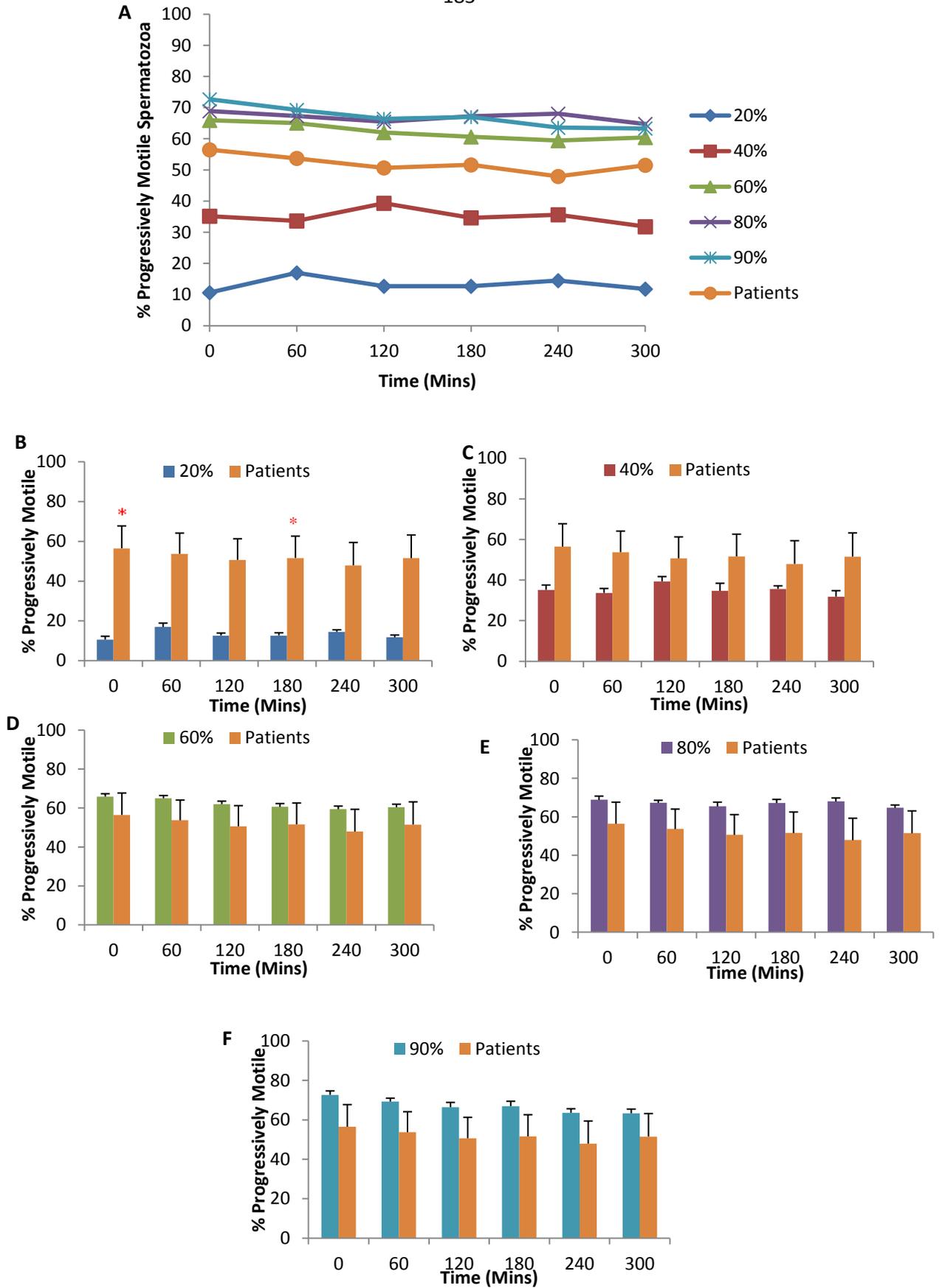
**Figure 10-2. Comparison between donor and sub-fertile samples in amplitude of lateral head displacement (ALH) in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction (B) 20% donor, 80% sub-fertile (C) 40% donor, 80% sub-fertile (D) 60% donor, 80% sub-fertile (E) 80% donor, 80% sub-fertile (F) 90% donor, 80% sub-fertile. The result shown is the mean  $\pm$  SEM. N= 5 for both donors and patients, \*indicates significant difference ( $P < 0.05$ ) (Mann Whitney test and Kruskal-Wallis test).**



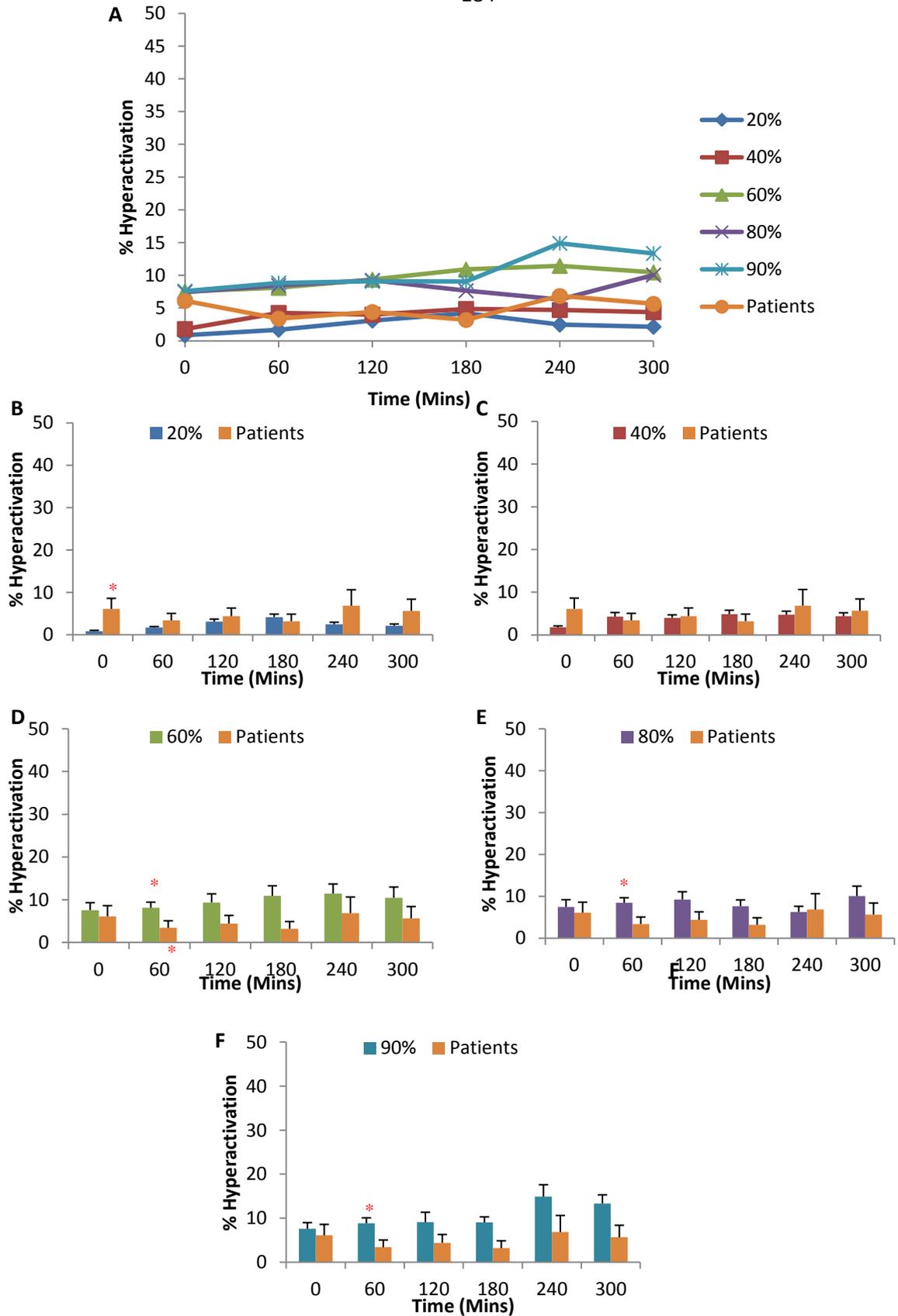
**Figure 10-3. Comparison between donor and sub-fertile samples in linearity (LIN) in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction (B) 20% donor, 80% sub-fertile (C) 40% donor, 80% sub-fertile (D) 60% donor, 80% sub-fertile (E) 80% donor, 80% sub-fertile (F) 90% donor, 80% sub-fertile. The result shown is the mean ± SEM. N= 5 for both donors and patients, \*indicates significant difference ( $P<0.05$ ) \*\*\*indicates  $P<0.001$  (T-test and Kruskal-Wallis test).**



**Figure 10-4. Comparison between donor and sub-fertile samples in % total motile in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction (B) 20% donor, 80% sub-fertile (C) 40% donor, 80% sub-fertile (D) 60% donor, 80% sub-fertile (E) 80% donor, 80% sub-fertile (F) 90% donor, 80% sub-fertile. The result shown is the mean  $\pm$  SEM. N= 5 for both donors and patients, \*indicates significant difference ( $P < 0.05$ ) (Mann Whitney test and Kruskal-Wallis test).**

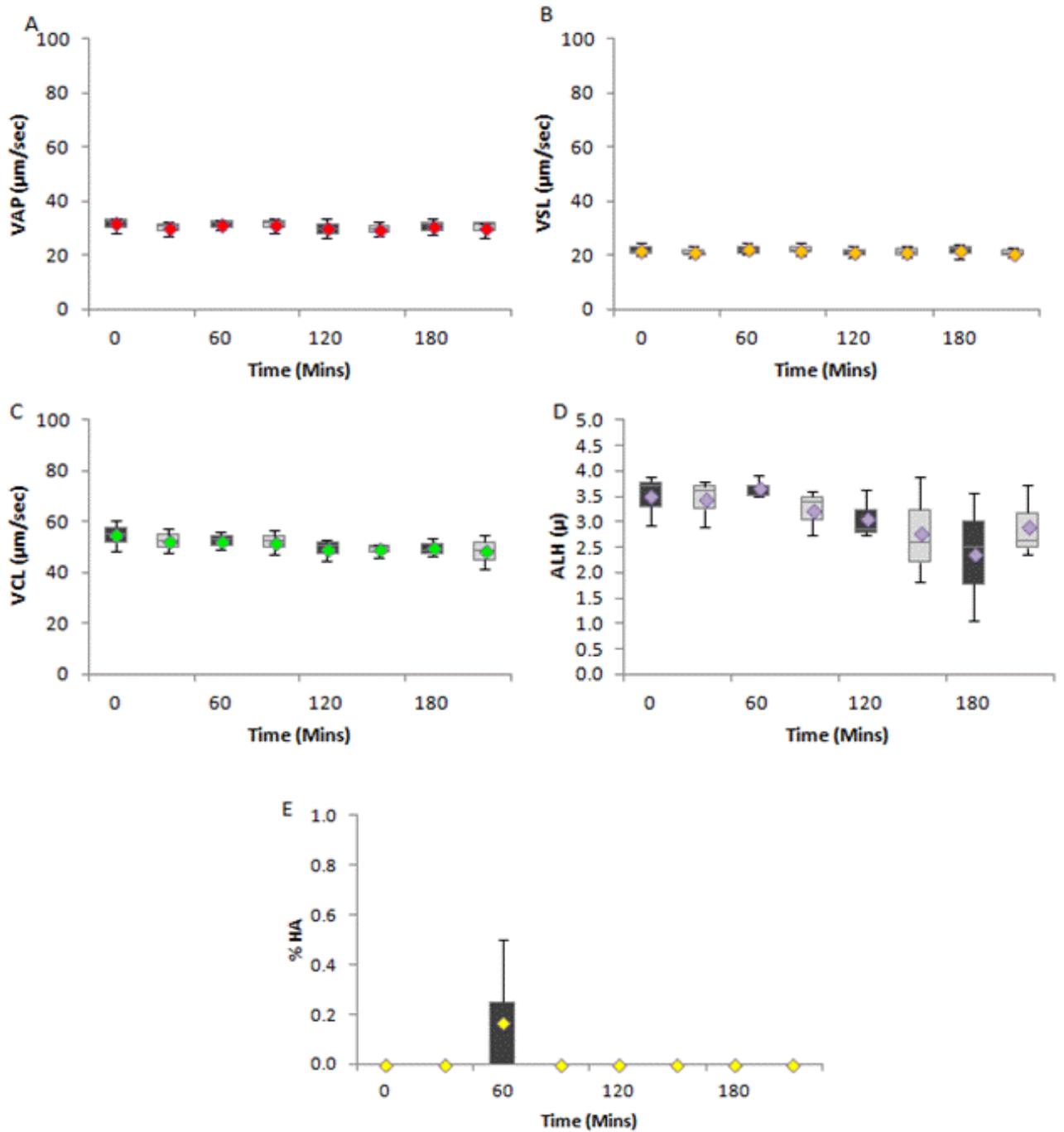


**Figure 10-5. Comparison between donor and sub-fertile samples in % progressive motility in Percoll<sup>®</sup> fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction (B) 20% donor, 80% sub-fertile (C) 40% donor, 80% sub-fertile (D) 60% donor, 80% sub-fertile (E) 80% donor, 80% sub-fertile (F) 90% donor, 80% sub-fertile. The result shown is the mean  $\pm$  SEM. N= 5 for both donors and patients, \*indicates significant difference ( $P < 0.05$ ) (Mann Whitney test and Kruskal-Wallis test).**

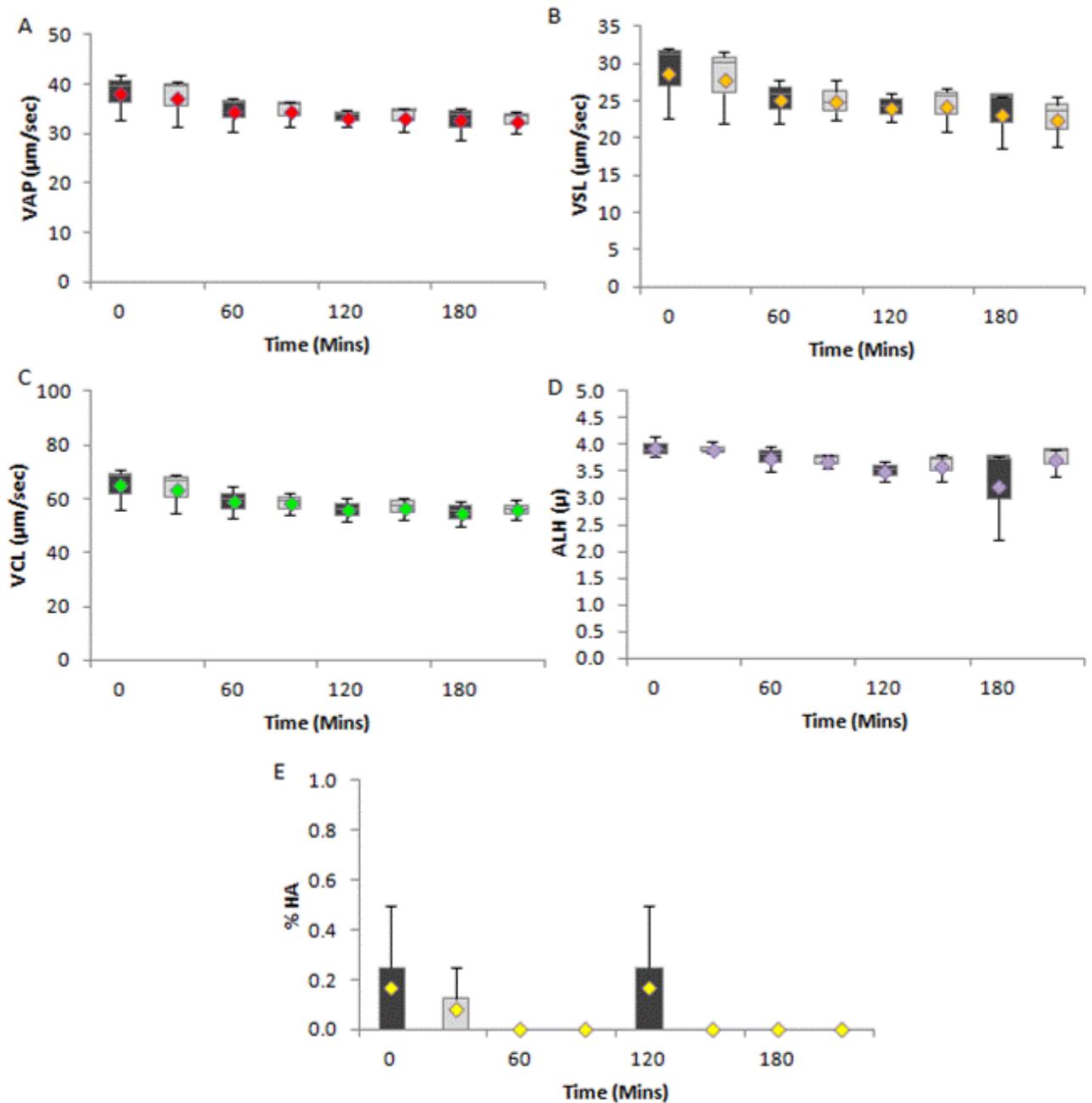


**Figure 10-6. Comparison between donor and sub-fertile samples in % hyperactivation in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction (B) 20% donor, 80% sub-fertile (C) 40% donor, 80% sub-fertile (D) 60% donor, 80% sub-fertile (E) 80% donor, 80% sub-fertile (F) 90% donor, 80% sub-fertile. The result shown is the mean ± SEM. N= 5 for both donors and patients, \*indicates significant difference ( $P < 0.05$ ) (Mann Whitney test and Kruskal-Wallis test).**

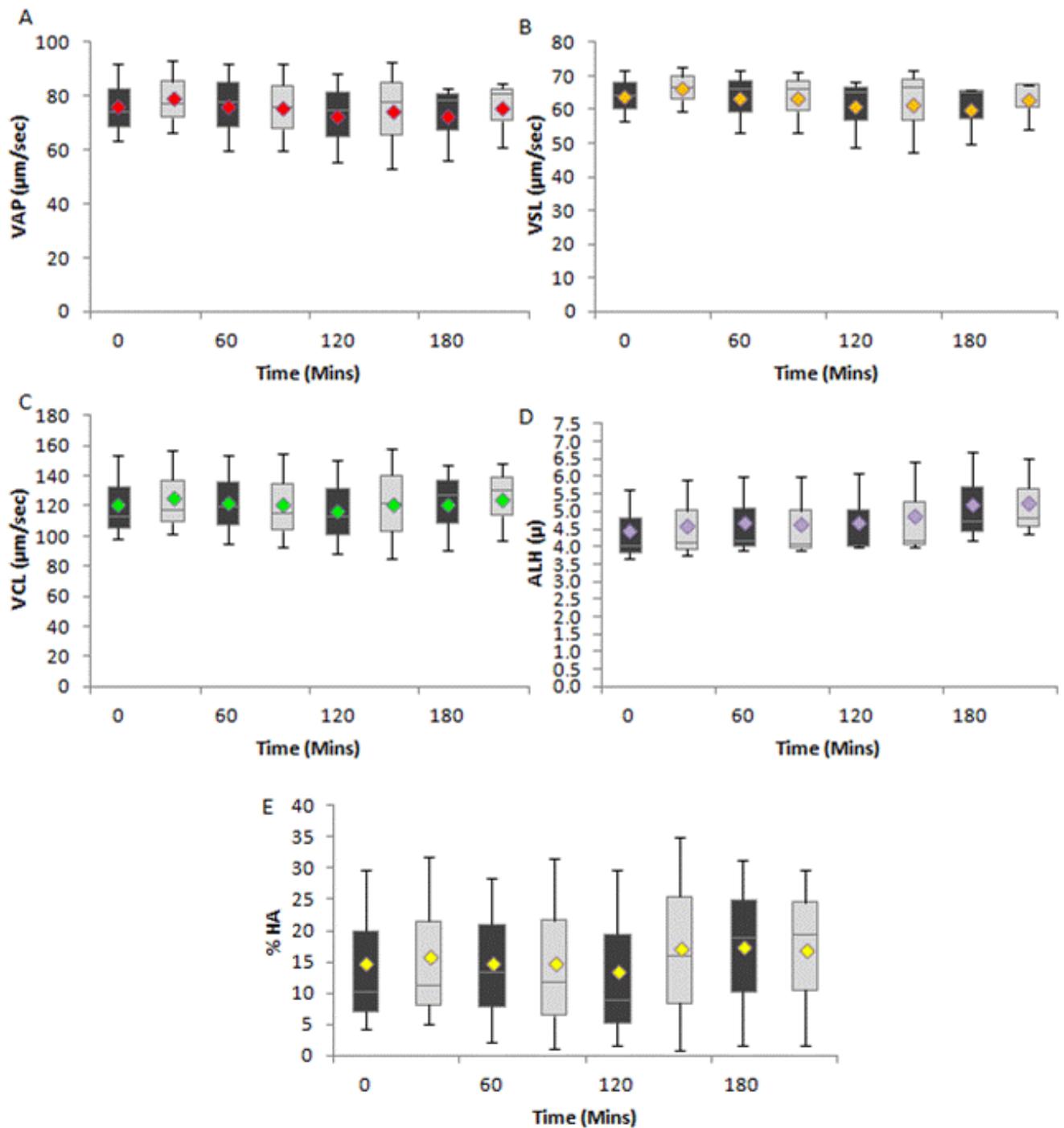
### 10.3 Determination of the number of aliquots from a single sample that need to be taken to assess motility parameters



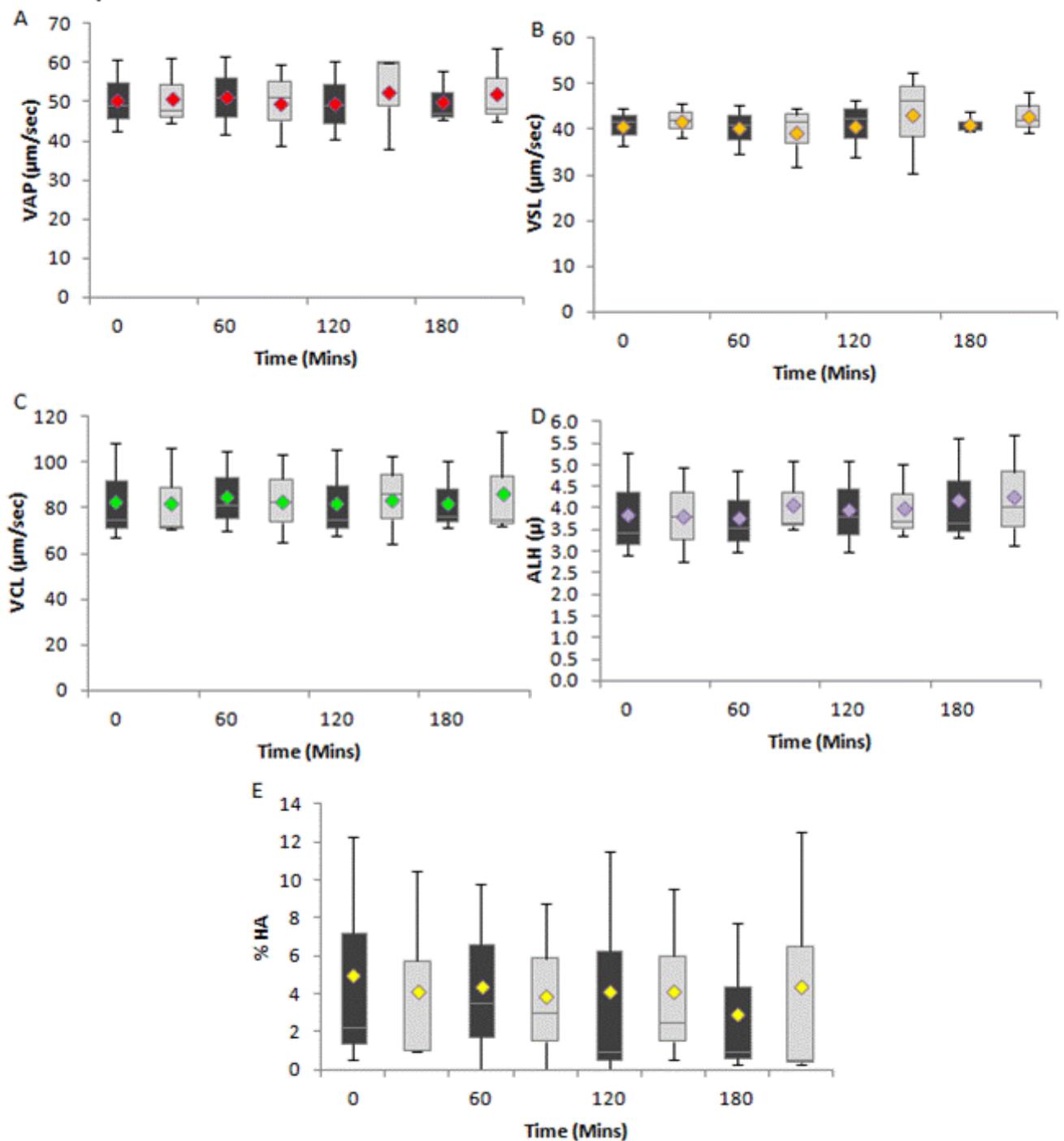
**Figure 10-7** Variation in range of results, 4 aliquots vs 2 aliquots, for the 40% fraction of spermatozoa in non-capacitating buffer (A) VAP, (B) VSL, (C) VCL, (D) ALH and (E) % hyperactivation. The result shown is the mean (coloured diamonds)  $\pm$  the range for A-E, see figure 4-1 for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)



**Figure 10-8** Variation in range of results, 4 aliquots vs 2 aliquots, for the 80% fraction of spermatozoa in non-capacitating buffer (A) VAP, (B) VSL, (C) VCL, (D) ALH and (E) % hyperactivation. The result shown is the mean (coloured diamonds)  $\pm$  the range for A-E, see figure 4-1 for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)

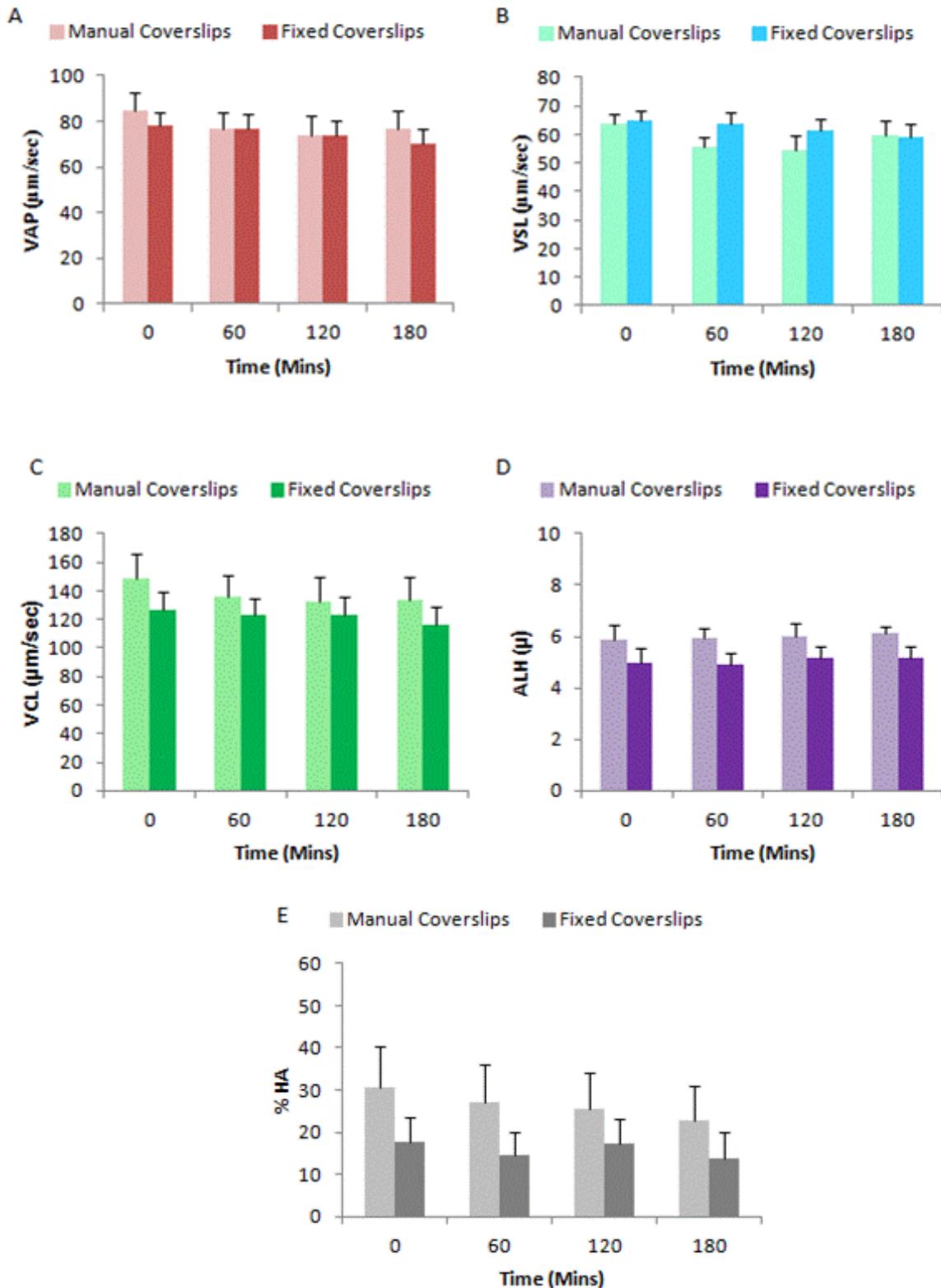


**Figure 10-9** Variation in range of results, 4 aliquots vs 2 aliquots, for the 40% fraction of spermatozoa in capacitating conditions (A) VAP, (B) VSL, (C) VCL, (D) ALH and (E) % hyperactivation. The result shown is the mean (coloured diamonds)  $\pm$  the range for A-E, see figure 4-1 for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)



**Figure 10-10** Variation in range of results, 4 aliquots vs 2 aliquots, for the 80% fraction of spermatozoa in capacitating conditions (A) VAP, (B) VSL, (C) VCL, (D) ALH and (E) % hyperactivation. The result shown is the mean (coloured diamonds)  $\pm$  the range for A-E, see figure 4-1 for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)

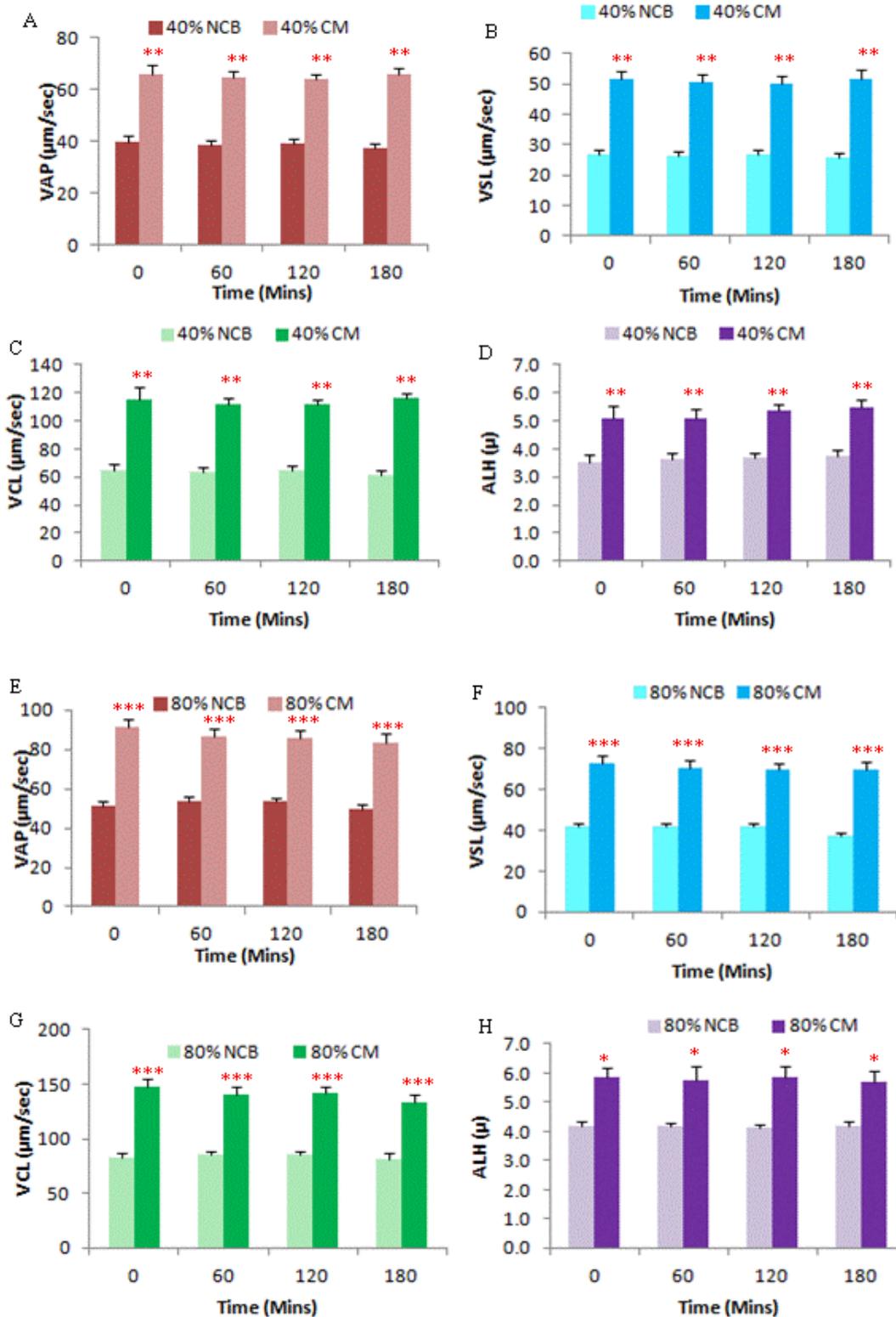
### 10.4 Determination of slide type required for CASA assessment of motility



**Figure 10-11 Variation in motility between two slide types: Hamilton Thorne glass slides with manual glass coverslips and fixed chamber slides (A) VAP, (B) VSL, (C) VCL, (D) %**

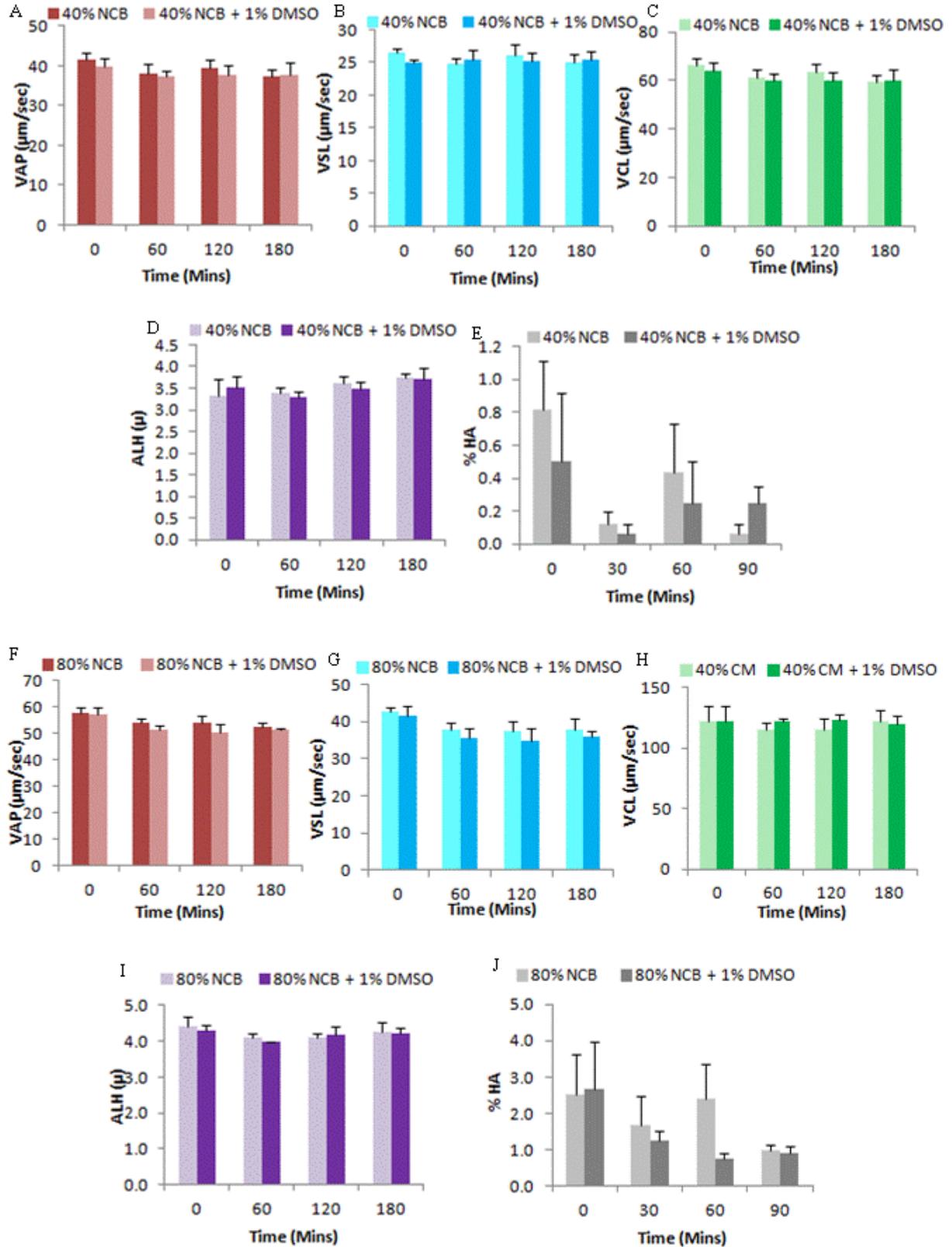
hyperactivation. The result shown is the mean  $\pm$  SE measured at defined time points up to 180 min N=3 for both parameters, \* indicates a significant increase between manual and fixed slides at independent time points for 80% fraction of sperm under capacitating conditions. Significance was considered as  $P < 0.05$

### 10.5 Assessment of motility parameters in capacitating and non-capacitating media

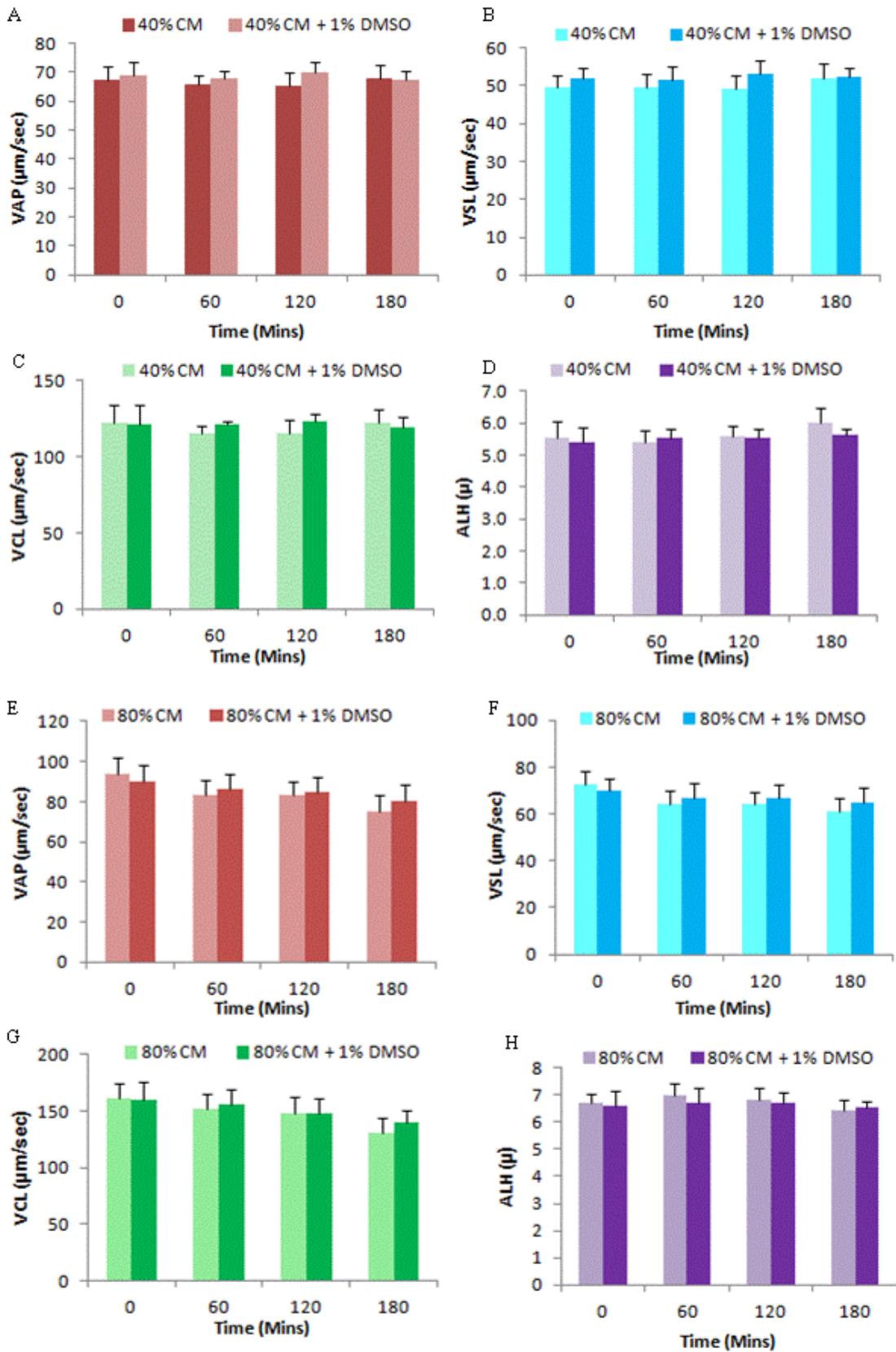


**Figure 10-12** Expression of basal (control) motility values in non-capacitating (NCM) and capacitating media (CM) for 40%; (A) VAP (B) VSL (C) VCL and (D) ALH and 80% fractions; (E) VAP, (F) VSL, (G) VCL and (H) ALH. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating and capacitating conditions comparing the difference between the two media types. N= 5 for NCM and N= 9 for CM, \*\*\* indicates a significant increase ( $P < 0.005$  or  $P < 0.001$ ) between NCM and CM at independent time points (Mann Whitney test and Kruskal-Wallis test).

### 10.6 Effect of 1% DMSO on treated sperm samples

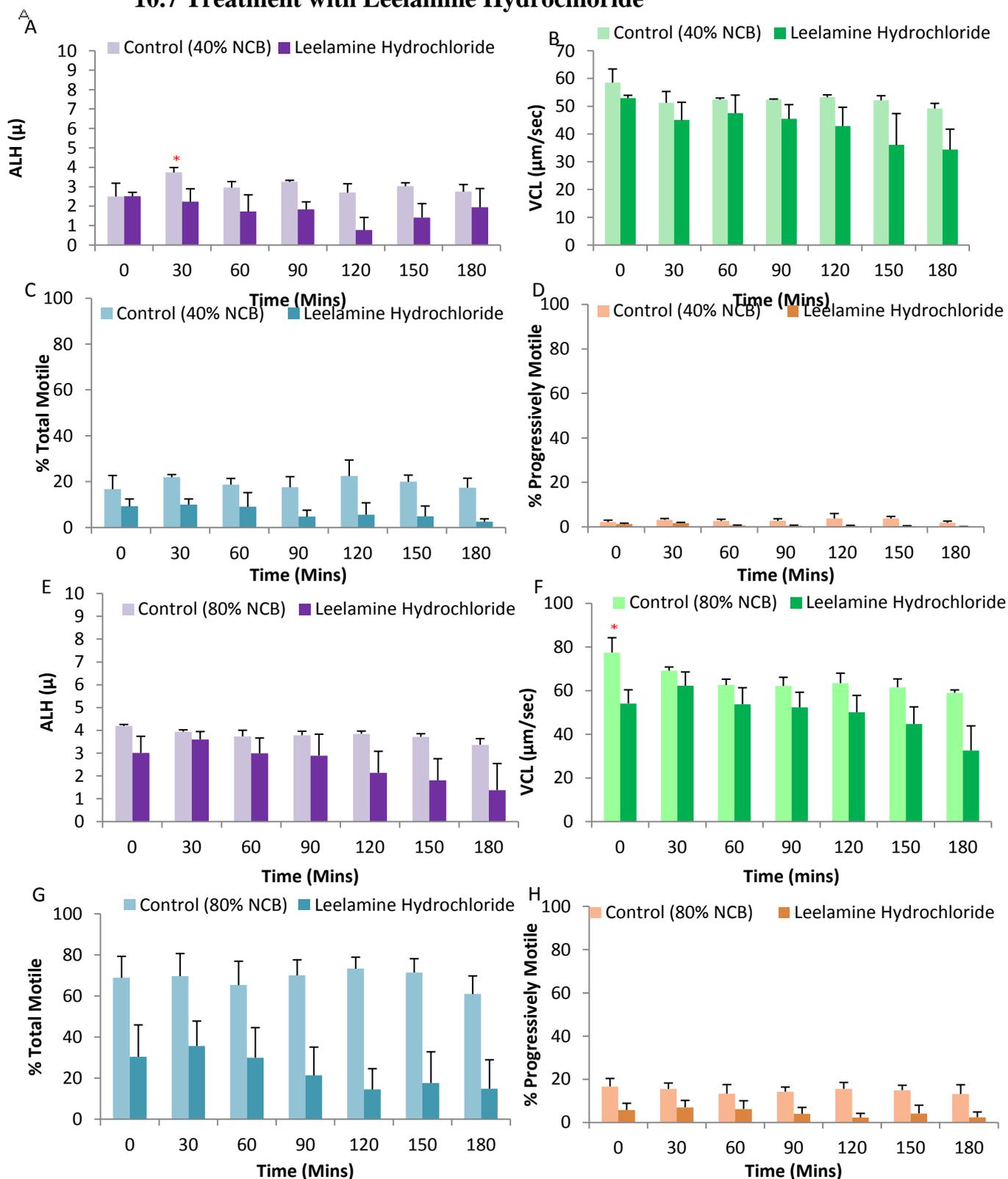


**Figure 10-13** Expression of basal (control) motility values in non-capacitating media (NCM) treated with 1% DMSO in 40% fraction (A) VAP, (B) VSL, (C) VCL, (D) ALH, (E) % HA and 80% fraction (F) VAP, (G) VSL, (H) VCL, (I) ALH, (J) % HA. The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under non- capacitating conditions comparing the difference between treatment with 1% DMSO and non-treated media. N= 4 for NCM and NCM + 1% DMSO, no significant difference noted at any time point.

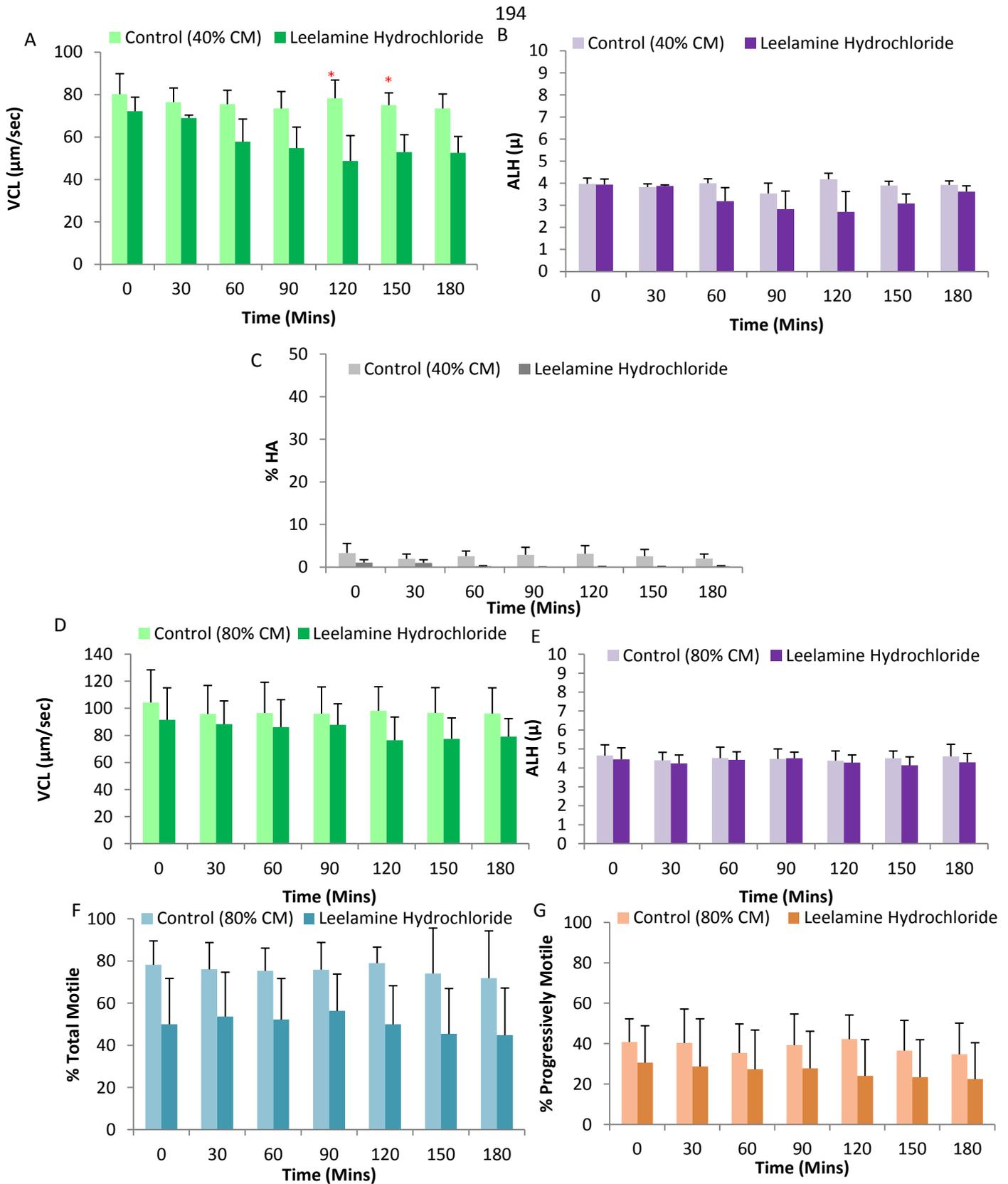


**Figure 10-14 Expression of basal (control) motility values capacitating media (CM) treated with 1% DMSO in 40% fraction (A) VAP, (B) VSL, (C) VCL, (D) ALH and 80% fraction (E) VAP, (F) VSL, (G) VCL, (H) ALH. The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between treatment with 1% DMSO and non-treated media. N= 4 for CM and CM + 1% DMSO, no significant difference noted at any time point.**

### 10.7 Treatment with Leelamine Hydrochloride

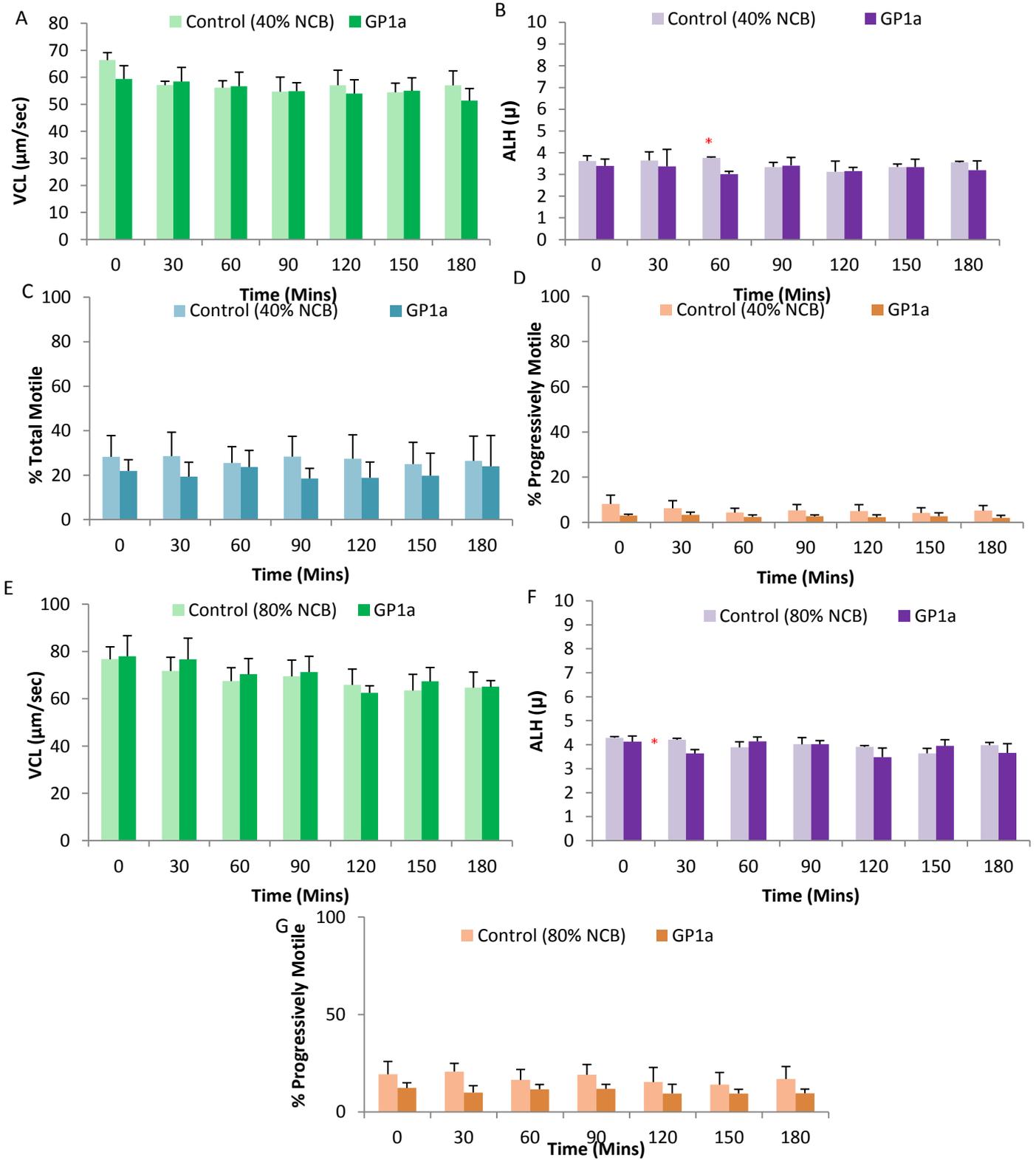


**Figure 10-15 Expression of basal (control) motility values compared to treated samples (Leelamine Hydrochloride) in non-capacitating (NCM) media, 40% fraction (A) ALH, (B) VCL, (C) % total motile, (D) % progressively motile and 80% fraction (E) ALH, (F) VCL, (G) % total motile, (H) % progressively motile. The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 4 for both parameters, \* indicates a significant increase ( $P < 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).**

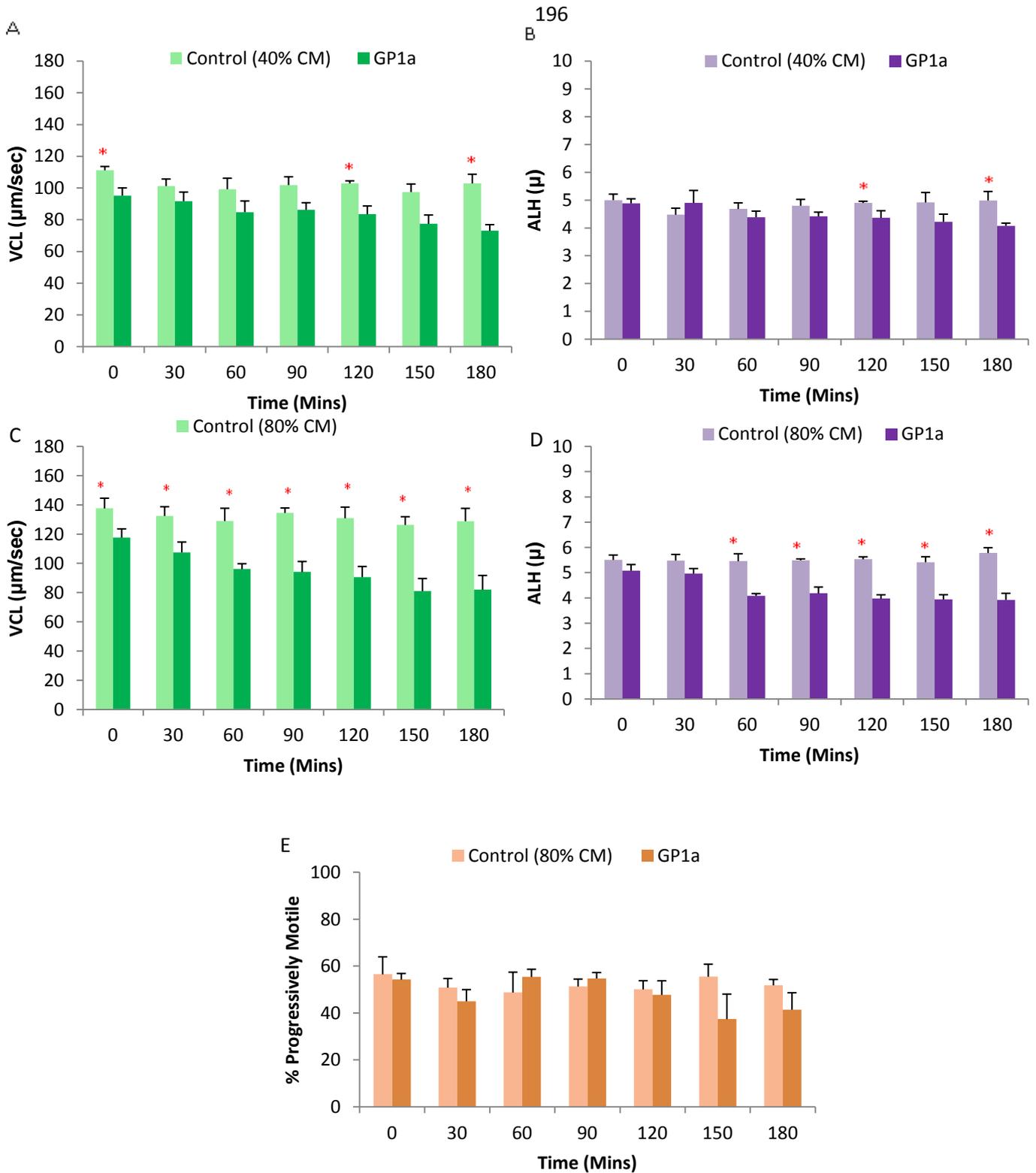


**Figure 10-16** Expression of basal (control) motility values compared to treated samples (Leelamine Hydrochloride) in capacitating (CM) media, 40% fraction (A) VCL, (B) ALH, (C) % HA and 80% fraction (D) VCL, (E) ALH, (F) % total motile, (G) % progressively motile. The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 4 for both parameters, \* indicates a significant increase ( $P < 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

### 10.8 Treatment with GP1a

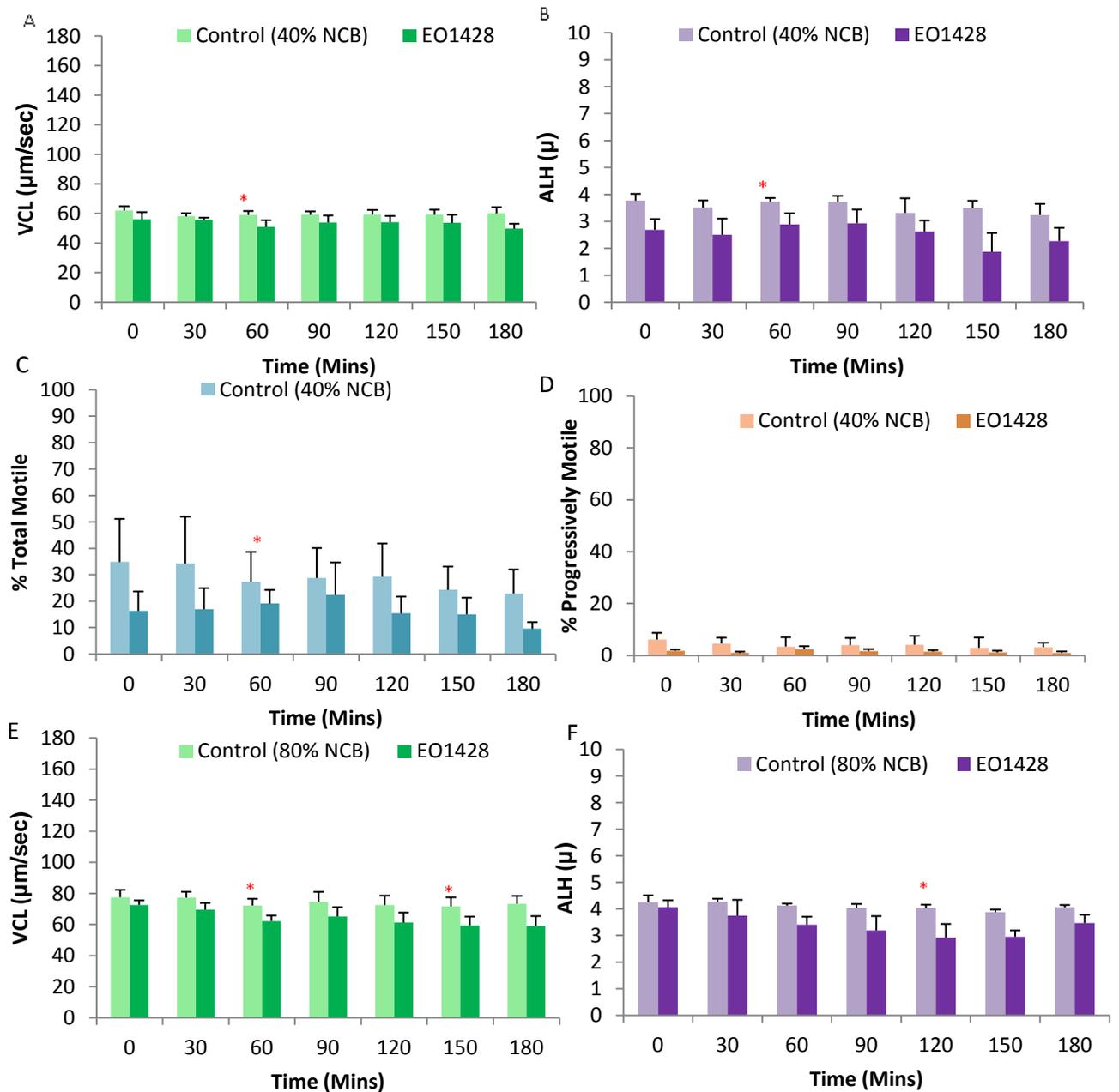


**Figure 10-17 Expression of basal (control) motility values compared to treated samples (GP1a) in non- capacitating (NCM) media (40% and 80% fraction). For 40% fraction (A) VCL, (B) ALH, (C) % total motile, (D) % progressively motile and for 80% fraction (E) VCL, (F) ALH and (G) % progressive motility. The result shown is the mean  $\pm$  SE for all parameters. In this study all motility parameters were measured over a period of 180 min under non- capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 3 for all parameters, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallace test).**

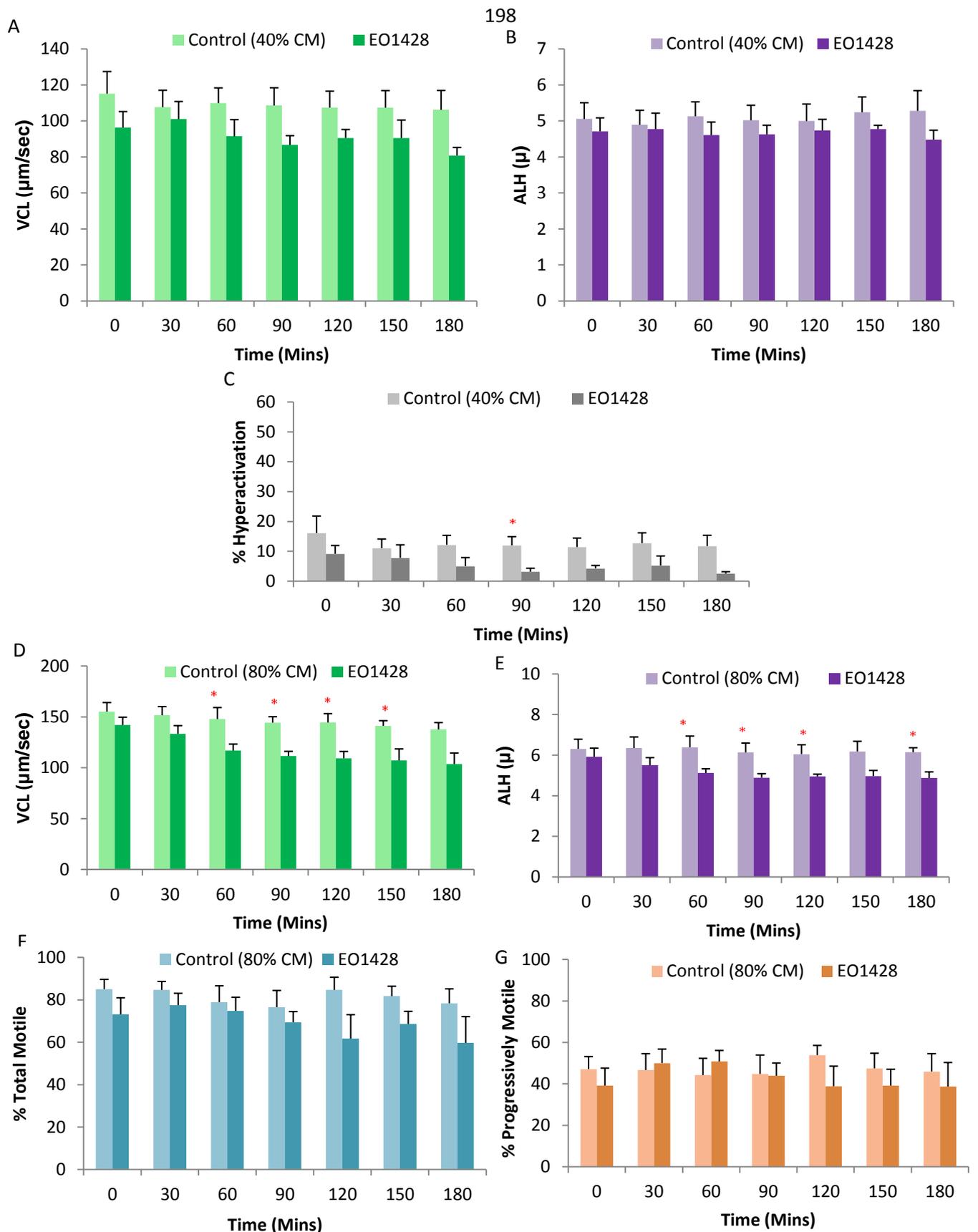


**Figure 10-18** Expression of basal (control) motility values compared to treated samples (GP1a) in capacitating (CM) media (40% and 80% fraction). For 40% fraction (A) VCL, (B) ALH and for 80% fraction (C) VCL, (D) ALH and (E) % progressive motility. The result shown is the mean  $\pm$  SE for all parameters. In this study all motility parameters were measured over a period of 180 min under non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 3 for all parameters, \* indicates a significant increase ( $P < 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

## 10.9 Treatment with EO1428

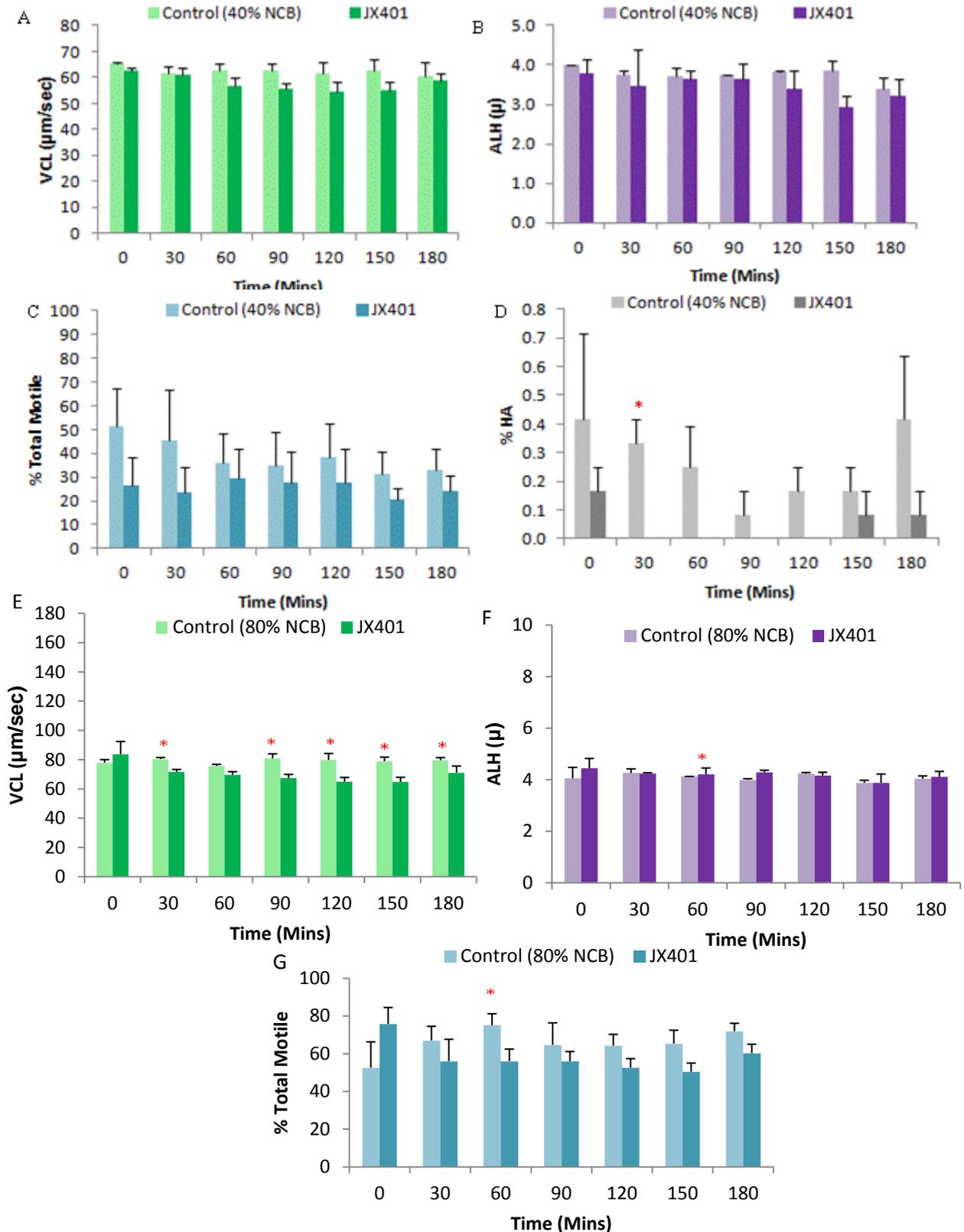


**Figure 10-19. Expression of basal (control) motility values compared to treated samples (EO1428) in non-capacitating (NCM) media.** Data for 40% fraction under non-capacitating conditions for (A) VCL, (B) ALH, (C) % total motile and (D) % progressively motile. Data for 80% fraction; (E) VCL and (F) ALH. The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples.  $N=4$  for all parameters, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

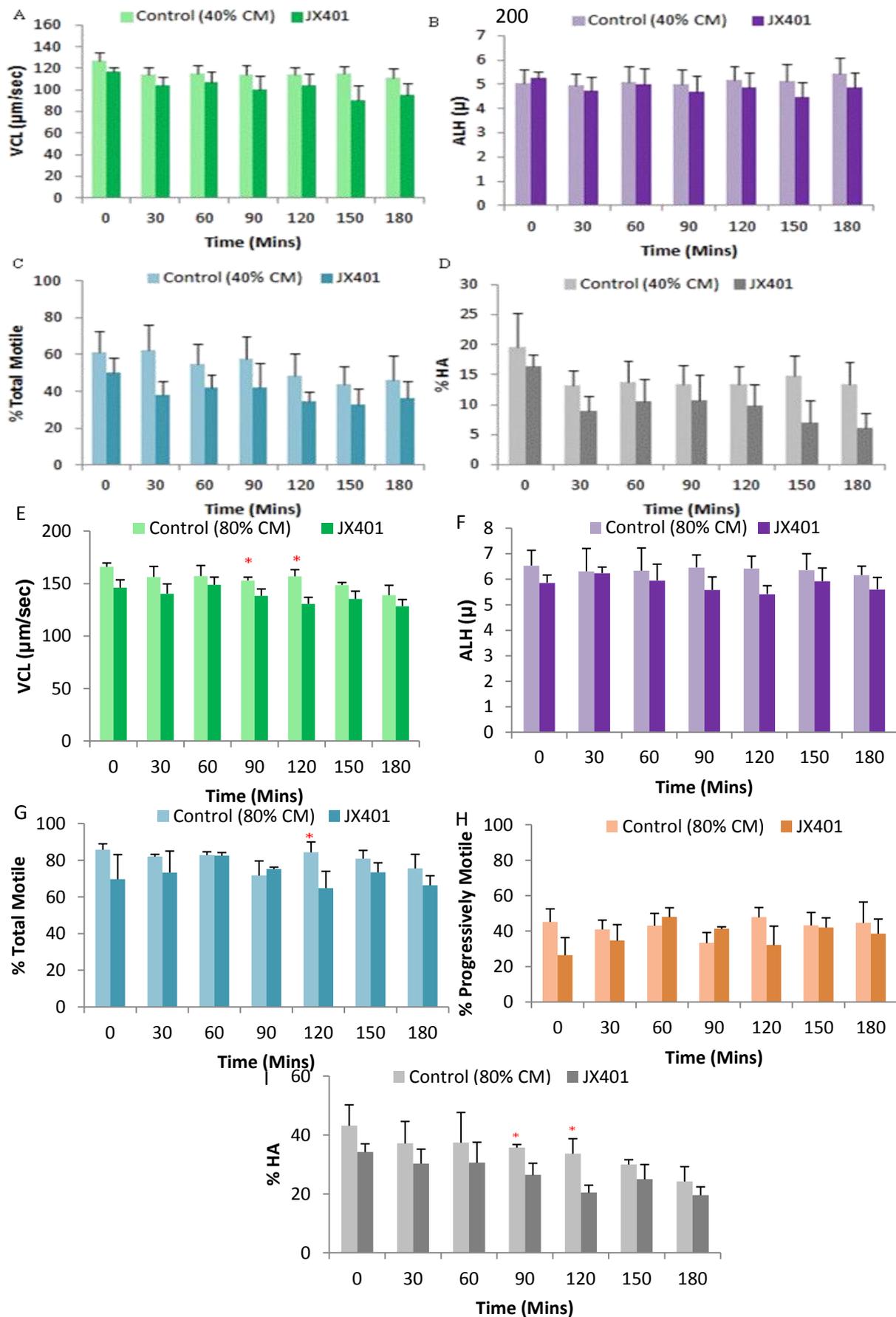


**Figure 10-20. Expression of basal (control) motility values compared to treated samples (EO1428) in capacitating (CM) media.** Data for 40% fraction for (A) VCL, (B) ALH, (C) % hyperactivation. Data for 80% fraction; (D) VCL, (E) ALH, (F) % total motile and (G) % progressively motile. The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples.  $N=4$  for all parameters, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

## 10.10 Treatment with JX401

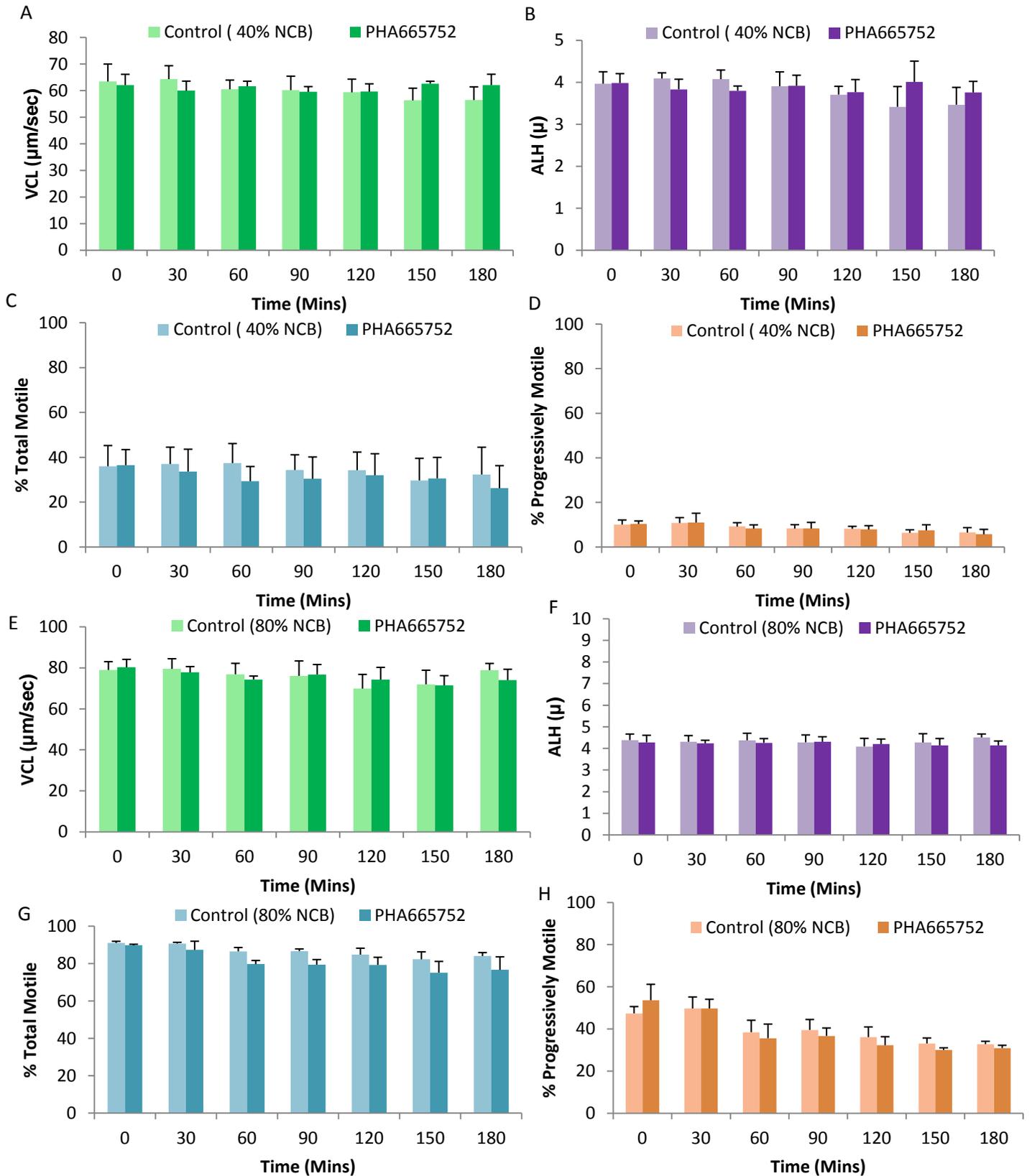


**Figure 10-21. Expression of basal (control) motility values compared to treated samples (JX401) in non-capacitating (NCM) media in 40% fractions for (A) VCL, (B) ALH, (C) % total motile, (D) hyperactivation and 80% fractions (E) VCL, (F) ALH and (G) % total motile.** The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples.  $N=3$  for both parameters, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

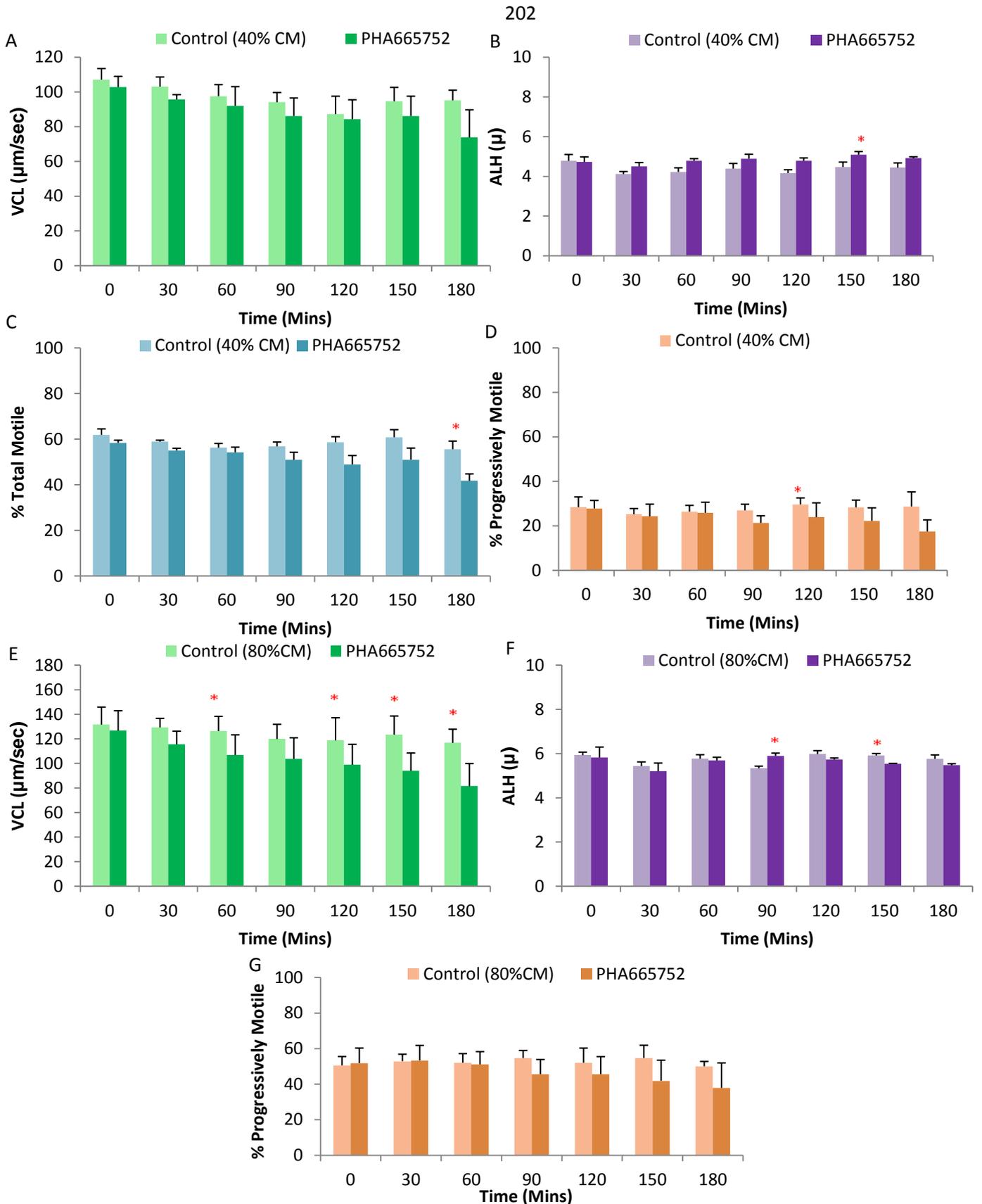


**Figure 10-22. Expression of basal (control) motility values compared to treated samples (JX401) in capacitating (CM) media in 40% fractions for (A) VCL, (B) ALH, (C) % total motile, (D) hyperactivation and 80% fractions (E) VCL, (F) ALH, (G) % total motile, (H) % progressively motile and (I) hyperactivation.** In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for both parameters, \* indicates a significant increase ( $P < 0.05$ ) between control and treated samples at independent time points.

### 10.11 Treatment with PHA665752

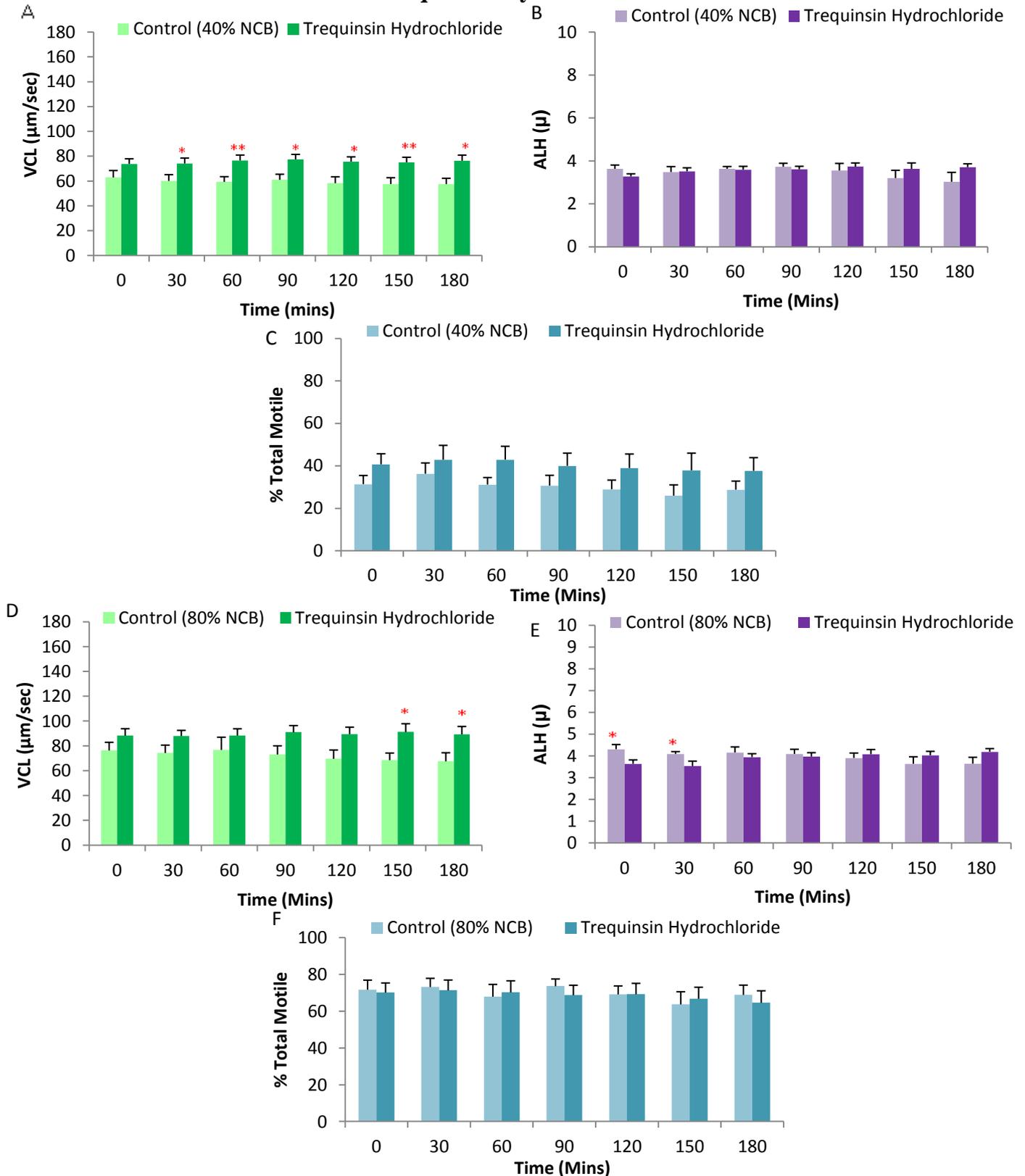


**Figure 10-23. Expression of basal (control) motility values compared to treated samples (PHA665752) in non-capacitating (NCM) media in 40% fraction for (A) VCL, (B) ALH, (C) % total motile, (D) % progressive motility and 80% fraction for (E) VCL, (F) ALH, (G) % total motile and (H) % progressively motile. The result shown is the mean  $\pm$  SE for motility parameters measured. All motility parameters were measured over a period of 180 min under non-capacitating conditions. N= 3 for all parameters, \* indicates a significant increase ( $P < 0.05$ ) between control and treated samples at independent time points.**

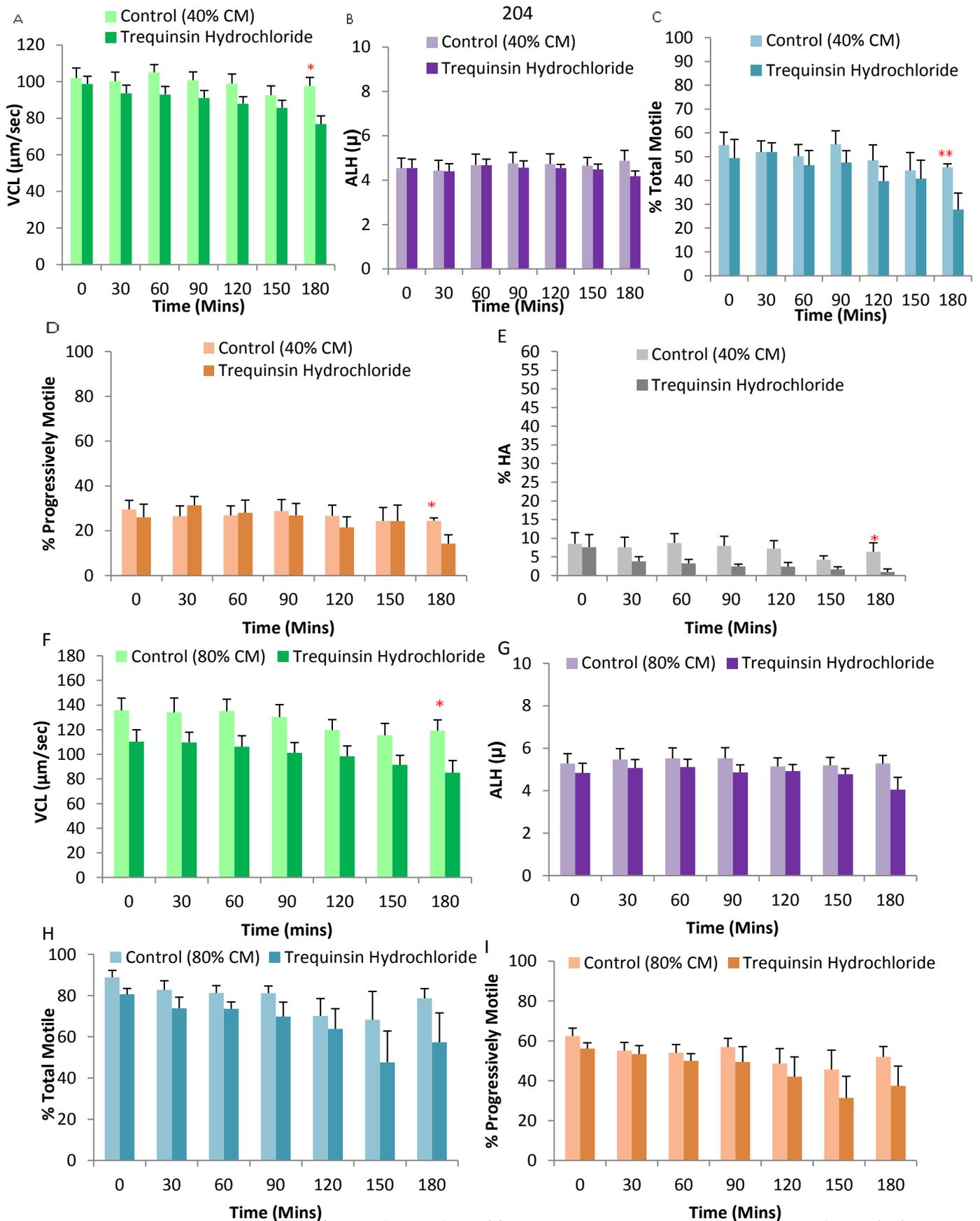


**Figure 10-24. Expression of basal (control) motility values compared to treated samples (PHA665752) in capacitating (CM) media in 40% fraction for (A) VCL, (B) ALH, (C) % total motile and (D) % progressively motile and 80% fraction (E) VCL, (F) ALH and (G) % progressively motile.** The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples.  $N=3$  for all parameters, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

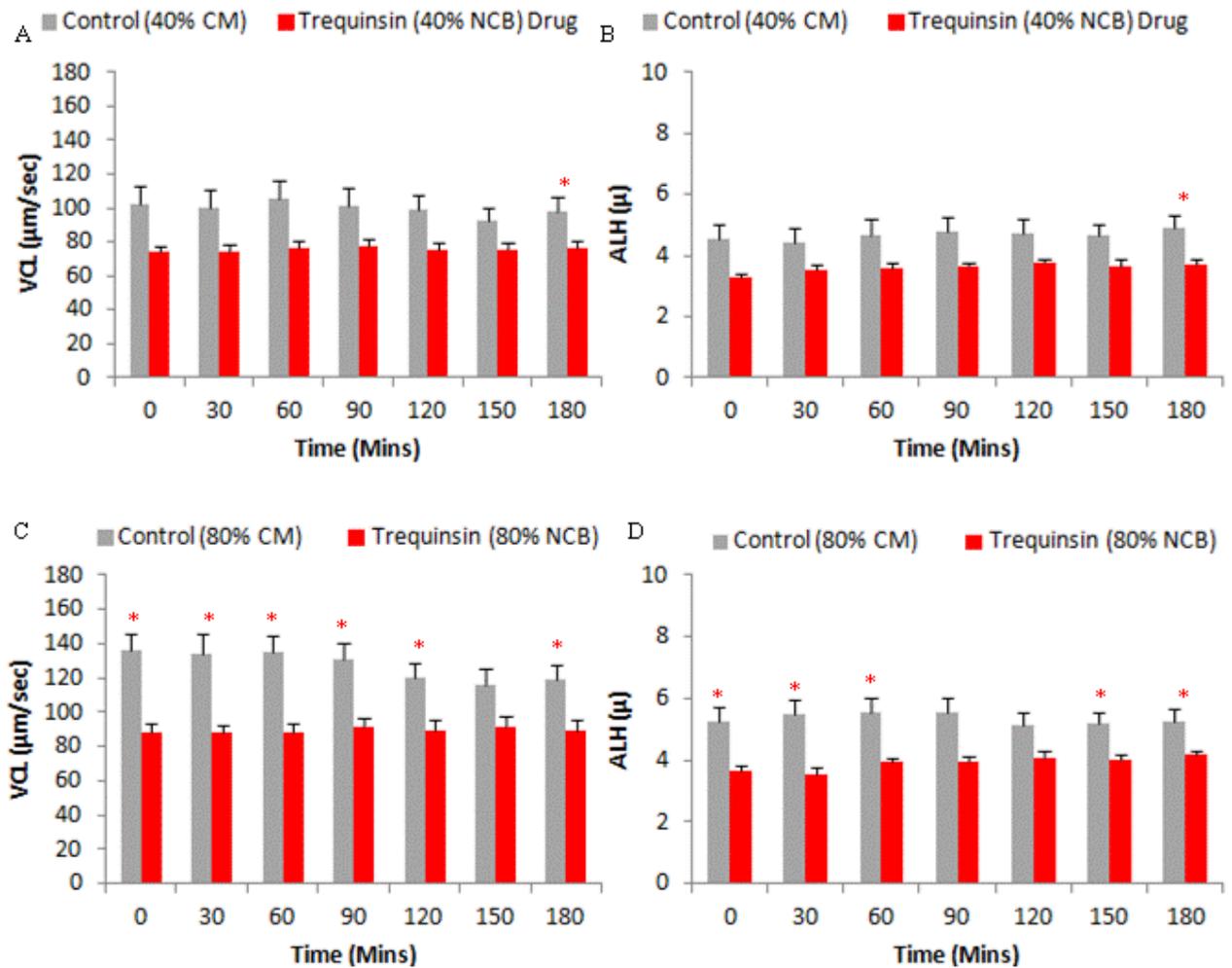
### 10.12 Treatment with Trequinsin Hydrochloride



**Figure 10-25 Expression of basal (control) motility values compared to treated samples (Trequinsin Hydrochloride) in non-capacitating (NCM) media for 40% fraction (A) ALH, (B) VCL and (C) % total motile and 80% fraction (D) VCL, (E) ALH and (F) % total motile. The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 6 for all parameters, \* indicates a significant increase ( $P < 0.05$ ), \*\* indicates  $P < 0.005$  between control and treated samples at independent time points (T-test, Mann Whitney test and Kruskal-Wallis test).**

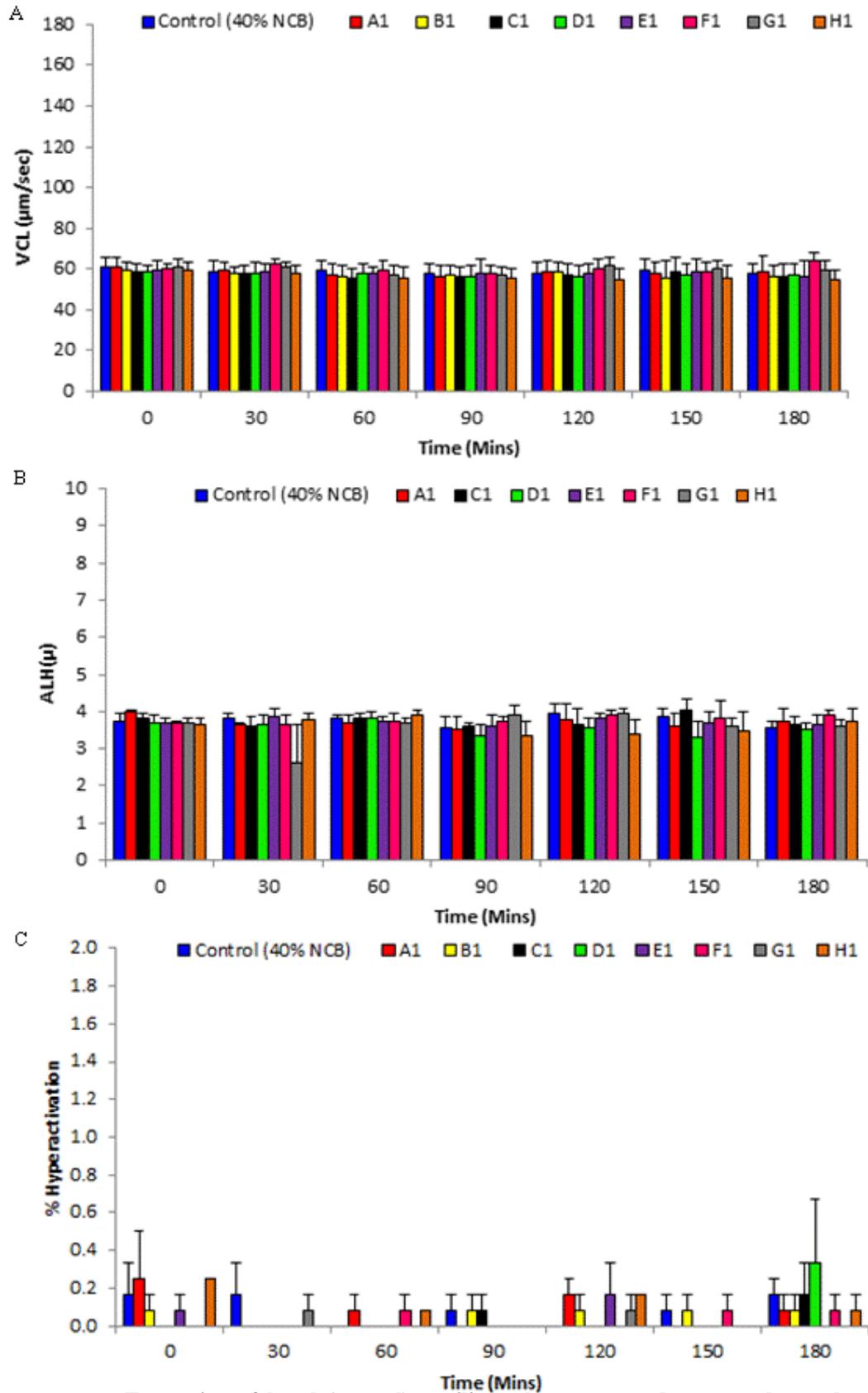


**Figure 10-26** Expression of basal (control) motility values compared to treated samples (Trequinsin Hydrochloride) in non-capacitating (NCM) media for 40% fraction (A) VCL, (B) ALH, (C) % total motile, (D) % progressively motile and (E) hyperactivation and 80% fraction (F) VCL, (G) ALH, (H) % total motile and (I) % progressively motile. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 6 for all parameters, \* indicates a significant increase ( $P<0.05$ ), \*\* indicates  $P<0.005$  between control and treated samples at independent time points (T-test, Mann Whitney test and Kruskal-Wallis test).

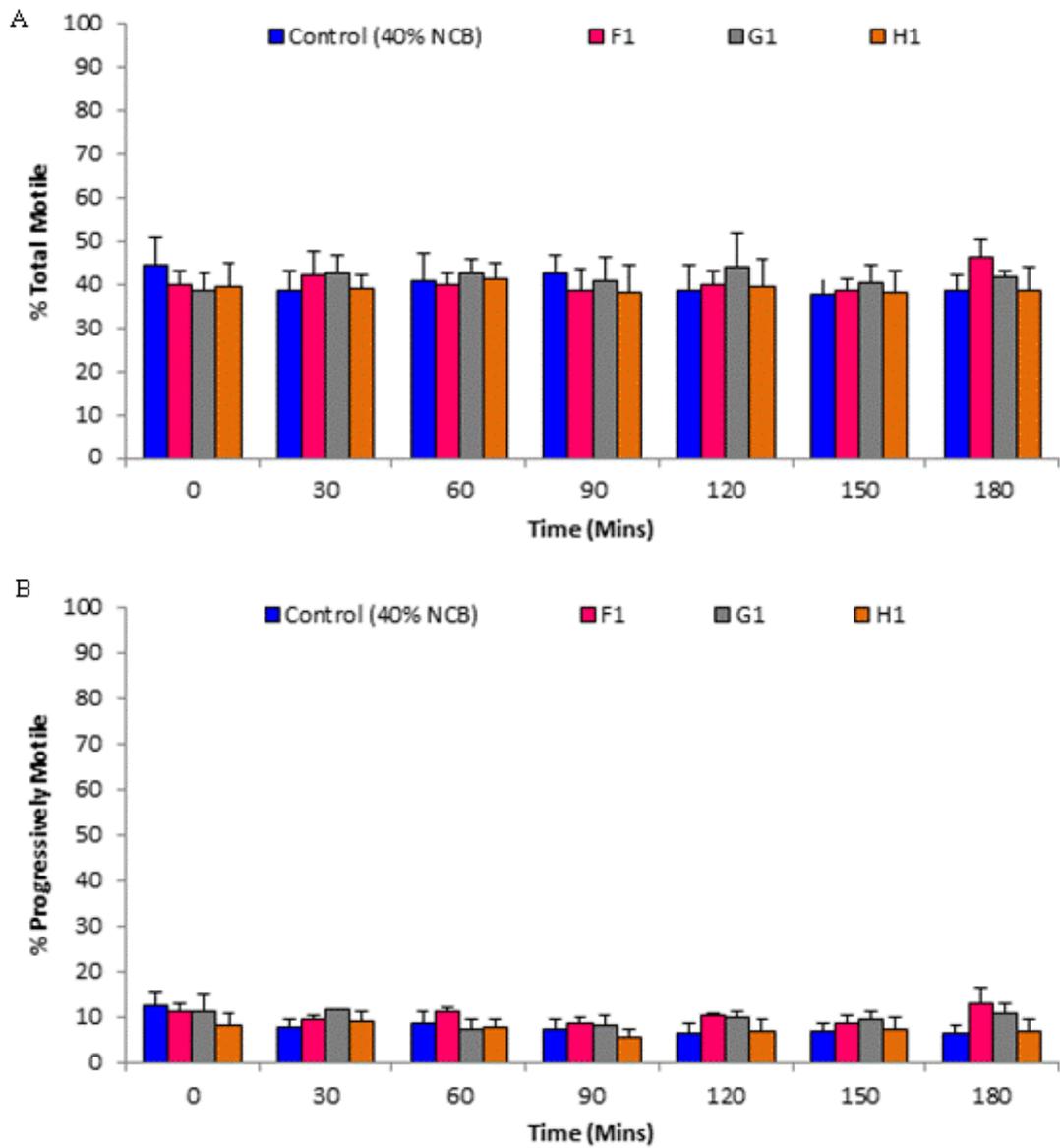


**Figure 10-27. Comparing basal (control) motility values under capacitating conditions to Trequinsin treated non-capacitated samples for 40% fraction (A) VCL, (B) ALH and 80% fraction (C) VCL and (D) ALH.** The result shown is the mean  $\pm$  SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min. N= 6 for both parameters, \* indicates a significant increase ( $P < 0.05$ ), \*\* indicates  $P < 0.005$  between control and treated samples at independent time points (T-test, Mann Whitney test and Kruskal-Wallis test).

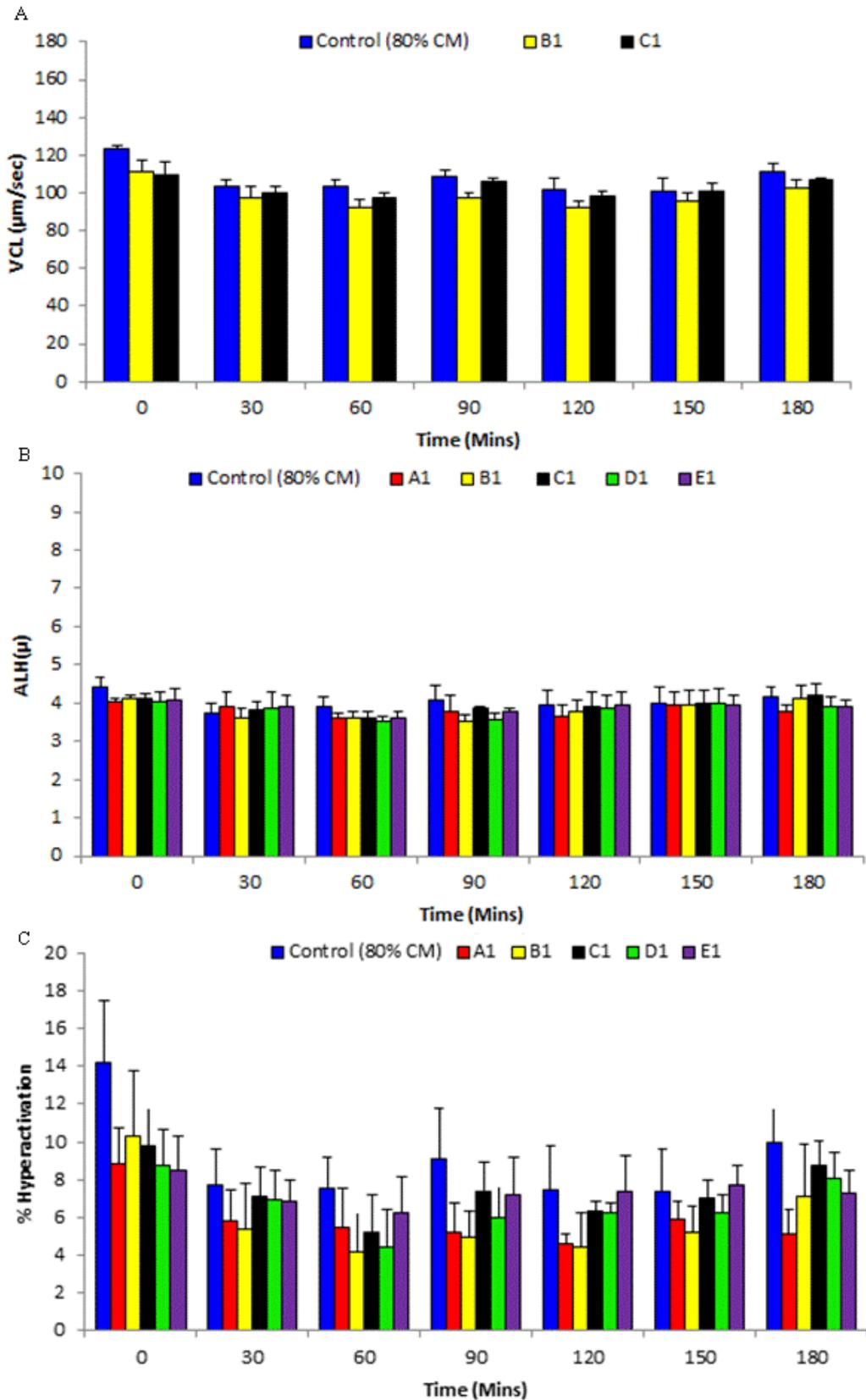
### 10.13 Treatment with Novel Ion Channel Compounds (A1-H1)



**Figure 10-28. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) VCL, (B) ALH (C) % hyperactivation. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).**



**Figure 10-29. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) % total motile and (B) % progressively motile.** The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, \* indicates a significant increase ( $P < 0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).



**Figure 10-30 Expression of basal (control) motility values compared to treated samples in capacitating media (CM), 80% fraction (A) VCL, (B) ALH and (C) % hyperactivation. The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).**

## 10.14 Progesterone Metabolites (Neurosteroids)

### 10.14.1 Introduction

Progesterone induces hyperactivation, stimulates the acrosome reaction and increases binding and penetrating capabilities of spermatozoa in hamster oocytes (Uhler et al., 1992, Calogero et al., 1996, Bronson et al., 1999, Sueldo et al., 1993). The physiological relevance of this has been proven through sperm incubation with follicular fluid which controls sperm function in relation to the steroid content. When steroids were removed from the follicular fluid the related sperm functional effects (hyperactivation and the acrosome reaction) disappeared (Calogero et al., 2000). Progesterone has been found to significantly increase the number of hyperactivated spermatozoa and is capable of generating an almost three-fold increase in the percentage of hyperactivated sperm in a sample even at low concentrations (Uhler et al., 1992, Calogero et al., 2000, Calogero et al., 1996). A large number of studies have shown that progesterone is capable of modulating sperm function by interacting with a membrane binding site similar to the neuronal  $\gamma$ -aminobutyric acid (GABA<sub>A</sub>) receptor (Calogero et al., 2000). Activation of the neuronal GABA<sub>A</sub> receptor results in an influx of chloride ions through open chloride channels leading to hyperpolarization in the neuron. Progesterone and its metabolites have been implicated in the interaction of GABA with its GABA<sub>A</sub> receptor in the central nervous system (Calogero et al., 2000, Lan et al., 1991, Majewska et al., 1986). The presence of GABA in human semen and specifically the identification of GABA<sub>A</sub> binding sites on human sperm plasma membranes had not been elucidated until 1995 however in 1993 Wistrom and Meizel observed the involvement of a putative GABAergic receptor in the progesterone induced acrosome reaction (Aurell and Meizel, 1993, Aanesen et al., 1995). However, the GABA<sub>A</sub> receptor identified on sperm is different to the neuronal GABA<sub>A</sub> receptor and results in an efflux of chloride ions rather than an influx as seen in neuronal tissue (Aurell and Meizel, 1993). The presence of GABA<sub>A</sub> receptors and their biological competences has

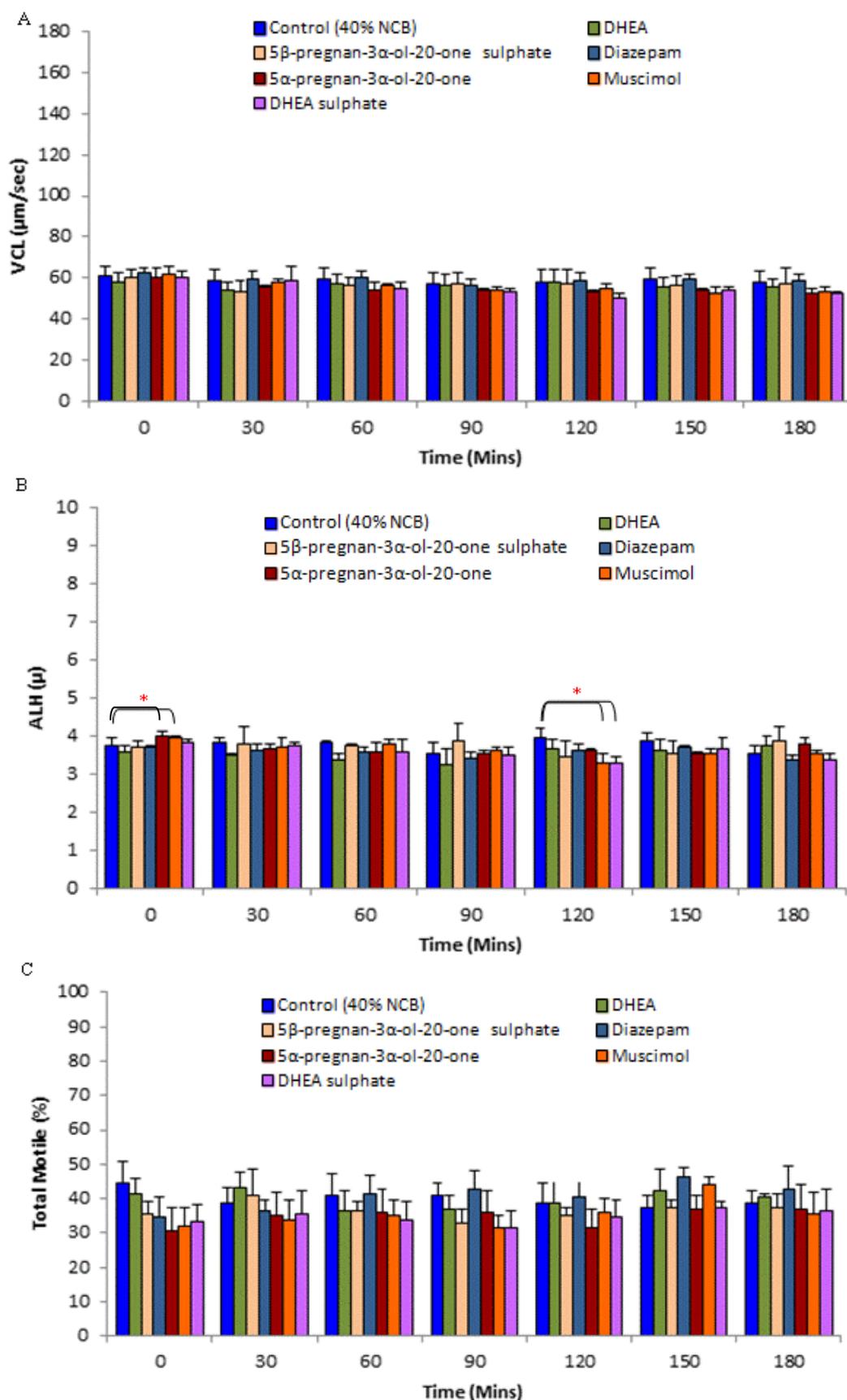
been substantiated by a numerous studies and it has been shown that GABA independently is capable of initiating the acrosome reaction in human sperm (Aurell and Meizel, 1993, Aanesen et al., 1995, Ritta et al., 1998). Calogero *et al* identified that GABA is also able to modulate sperm kinematic parameters in normal men and this modulation involved the GABA<sub>A</sub> receptor specifically (Calogero et al., 1996). However, the effect of GABA on sperm motility is still controversial with Aanesen *et al* not being able to identify any effect of GABA on sperm motility (Aanesen et al., 1995).

The Dundee drug discovery unit had previously produced a library similar to the Chemogenomics library (chapter 5 and 6) which consisted of neurosteroids which are metabolites of progesterone. These compounds are also allosteric modulators of GABA<sub>A</sub> receptors which included the GABA<sub>A</sub> agonist muscimol and the modulator diazepam along with DHEA, 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one and 5 $\beta$ -pregnan-3 $\alpha$ -ol-20-one and DHEA sulphate. From the knowledge of the effect of GABA and progesterone on sperm function via the GABA<sub>A</sub> receptor it was decided to include some compounds from this library in the motility assessment. These compounds were evaluated identically to the compounds from the Chemogenomics library and were selected for their ability to increase intracellular Ca<sup>2+</sup>.

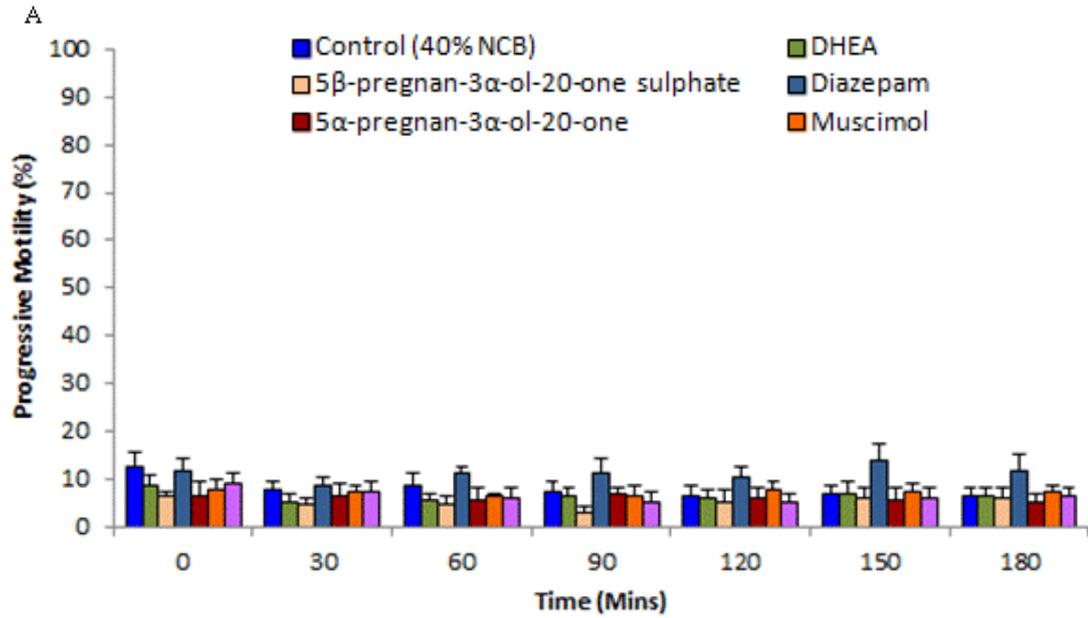
### 10.14.2 Results

Prepared sperm cells were collected from the 40% fraction after density gradient centrifugation and placed in appropriate incubators under non-capacitating conditions. Following this, cells were treated with the appropriate compound and motility analysis conducted on addition of compound and then at 30 min intervals over a period of 3 hrs (see chapter 2, methods).

No significant changes were identified from treatment at any time point, in any of the kinematic parameters assessed (VCL, ALH, total motile and progressively motile) for DHEA,  $5\beta$ -pregnan-3 $\alpha$ -ol-20-one and diazepam (figures 10-31 A-C and 10-32 A). However, muscimol and  $5\alpha$ -pregnan-3 $\alpha$ -ol-20-one were found to significantly increase ALH instantaneously (0 min,  $P < 0.05$ , figure 10-31 B) although this effect was not sustained past this point.  $5\alpha$ -pregnan-3 $\alpha$ -ol-20-one and DHEA sulphate were then found to significantly decrease ALH after 120 min incubation ( $P < 0.05$ , figure 10-31 B). No other significant changes were noted for these compounds in any other parameter at any other time point.



**Figure 10-31. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) VCL, (B) ALH and (C) % total motile.** The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples.  $N=3$  for all compounds, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).



**Figure 10-32. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) % progressive motility.** The result shown is the mean  $\pm$  SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples.  $N=3$  for all compounds, \* indicates a significant increase ( $P<0.05$ ) between control and treated samples at independent time points (Mann Whitney test and Kruskal-Wallis test).

### 10.14.3 Discussion

As mentioned previously the effect of compounds affecting sperm motility through GABA<sub>A</sub> receptors is controversial. The results from this study overall corroborate the results from Aanesen *et al* (1995) identifying very few significant changes in motility (Aanesen *et al.*, 1995). However, muscimol was found to stimulate ALH in this study which is similar to the results observed by Calogero *et al* (1996) (Calogero *et al.*, 1996). The study by Calogero *et al* (1996) found treatment with muscimol also increased VCL, the percentage of active sperm and hyperactivation which was not seen in this study (Calogero *et al.*, 1996). Ritta *et al* (1998) also identified significant increases in hyperactivation as a result of muscimol treatment suggesting under the correct conditions this compound has the potential to promote hyperactivated motility (Ritta *et al.*, 1998). The reason for this discrepancy is most likely owing to the difference in experimental protocol with samples in this study being from the 40% fraction under non-capacitating conditions.

Numerous studies have linked compounds that act via GABA<sub>A</sub> receptors to the acrosome reaction and a proposed model for this suggests the chloride flux in spermatozoa, plays a role in the regulation of the secondary Ca<sup>2+</sup> transient, essential for the acrosome reaction (Tesarik, 1996, Aurell and Meizel, 1993, Shi and Roldan, 1995). The chloride induced depolarization which results from binding to the GABA<sub>A</sub> receptor is thought to open an additional set of channels that are voltage regulated, leading to a secondary Ca<sup>2+</sup> transient and the initiation of the acrosome reaction (Tesarik, 1996, Meizel *et al.*, 1997). Meizel *et al* (1997) were the first group to detect a ligand mediated Ca<sup>2+</sup> wave in sperm and they proposed that the efflux of chloride ions as a result of GABA<sub>A</sub> receptor binding of progesterone influences Ca<sup>2+</sup> influx during the acrosome reaction (Meizel *et al.*, 1997). This Ca<sup>2+</sup> influx could explain the increased [Ca<sup>2+</sup>]<sub>i</sub> measured by the Flexstation when spermatozoa were treated with these compounds. It

would be beneficial to complete acrosome studies on these compounds to confirm this theory. Further analysis was not conducted on these compounds owing to the minimal changes in motility expressed as a result of incubation with these compounds.

## 10.15 Clinic A and B datasets

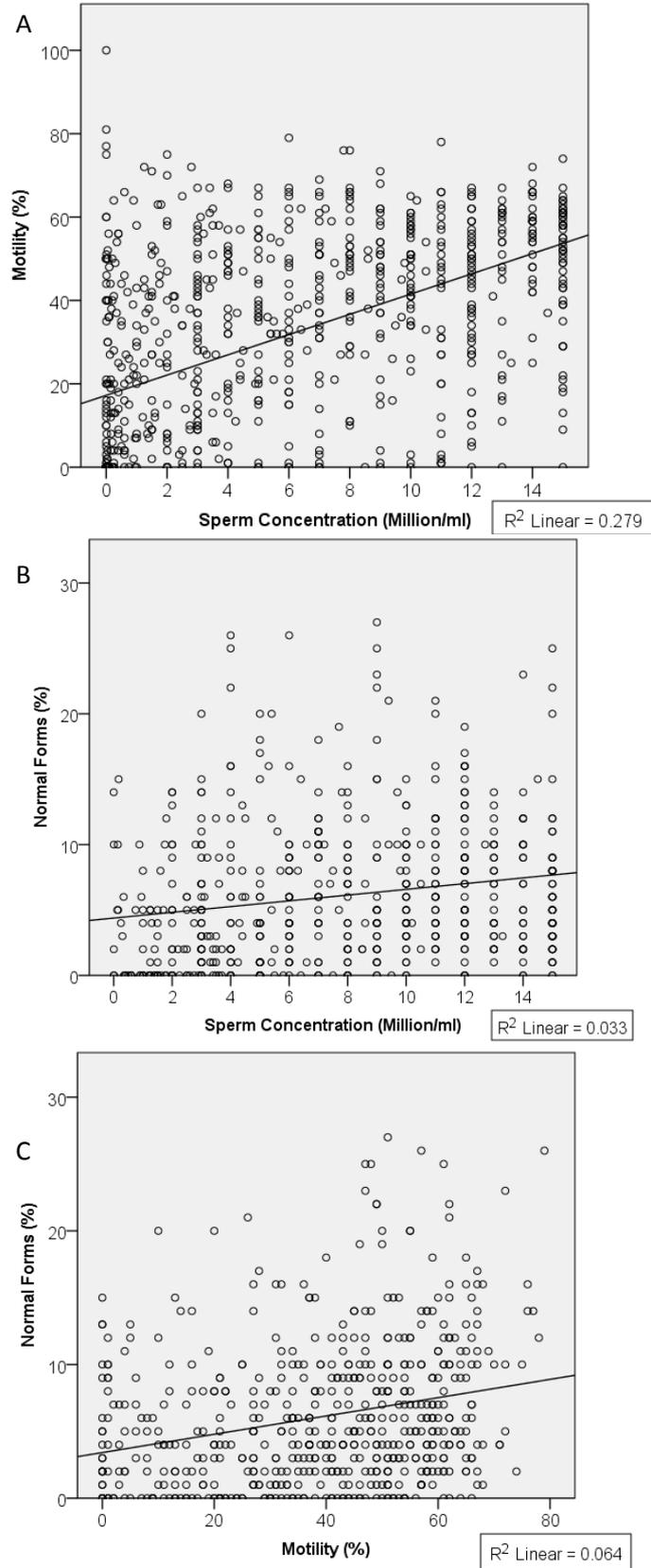
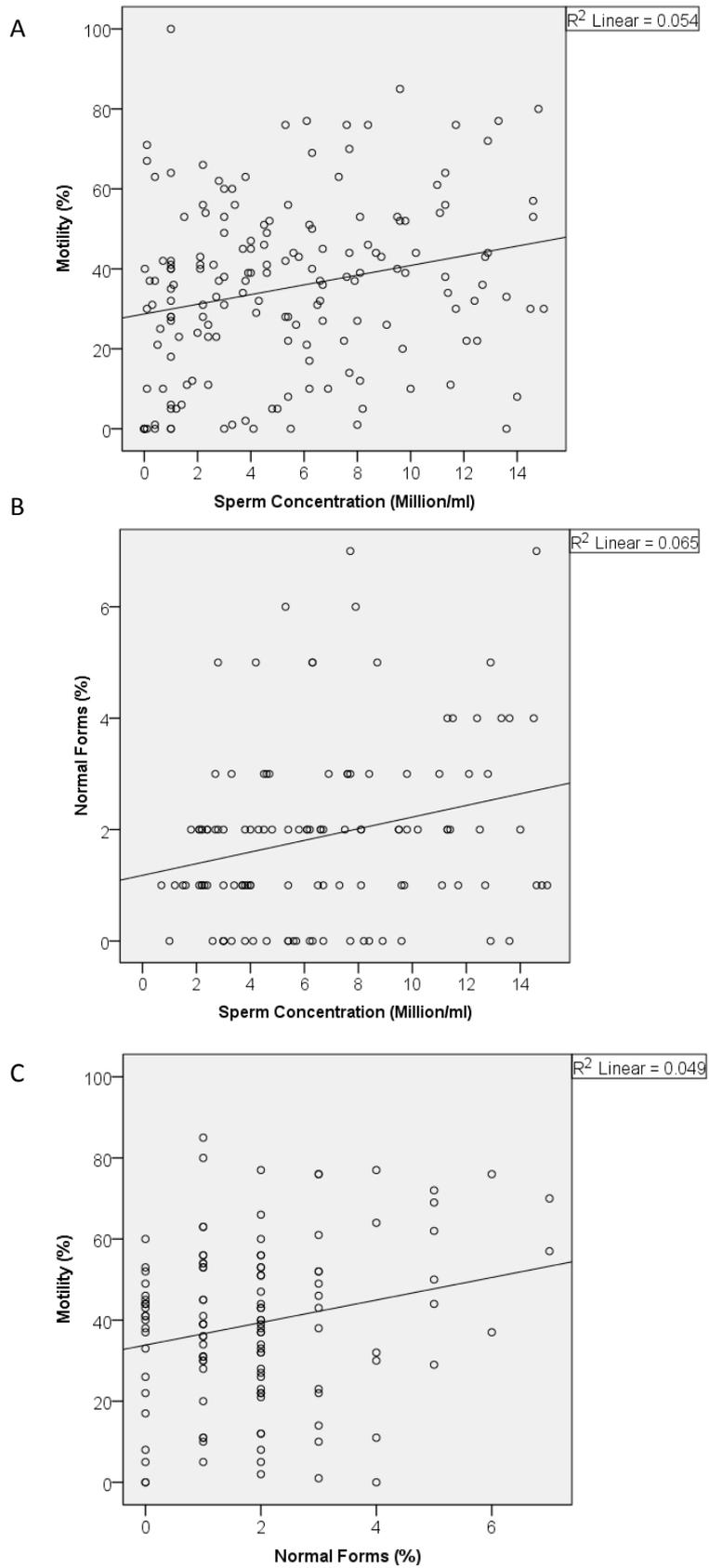
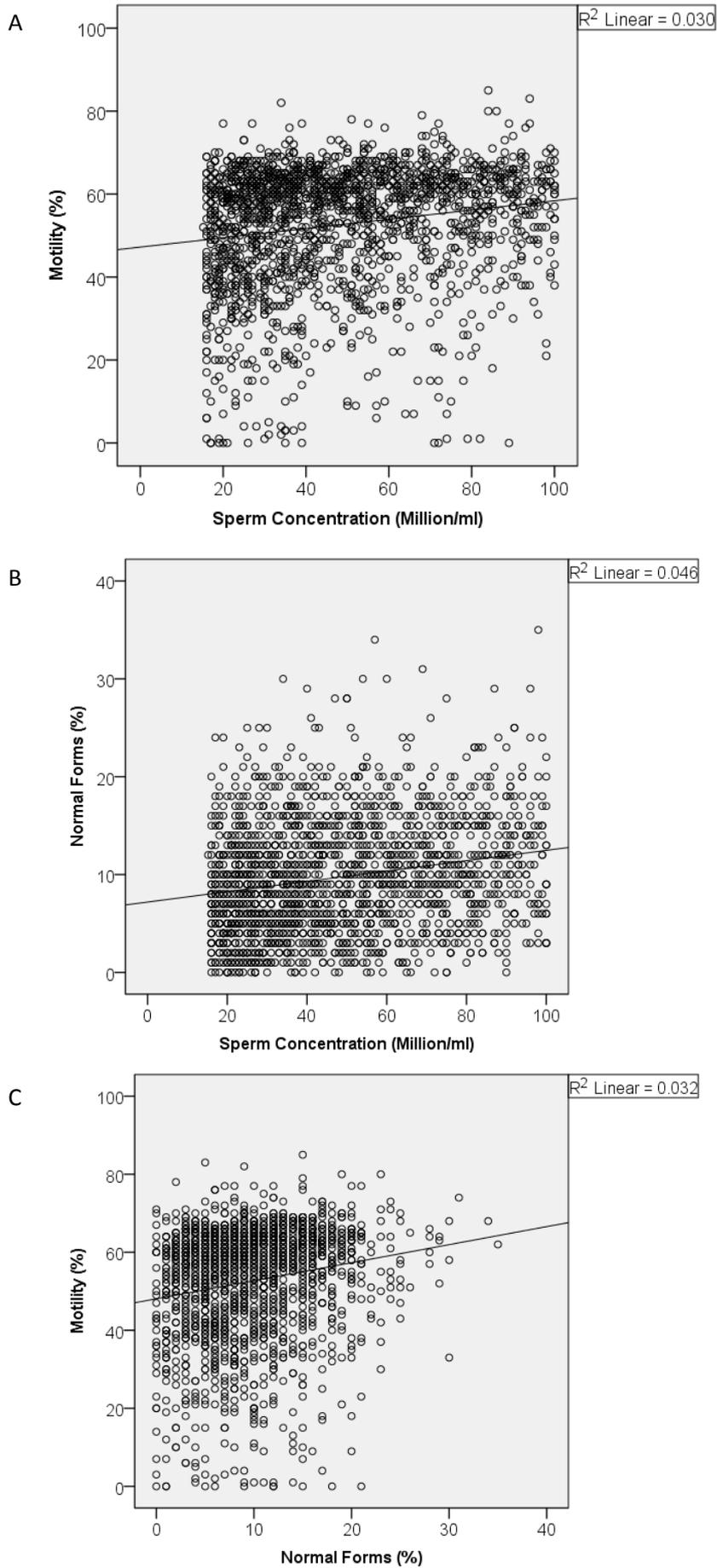


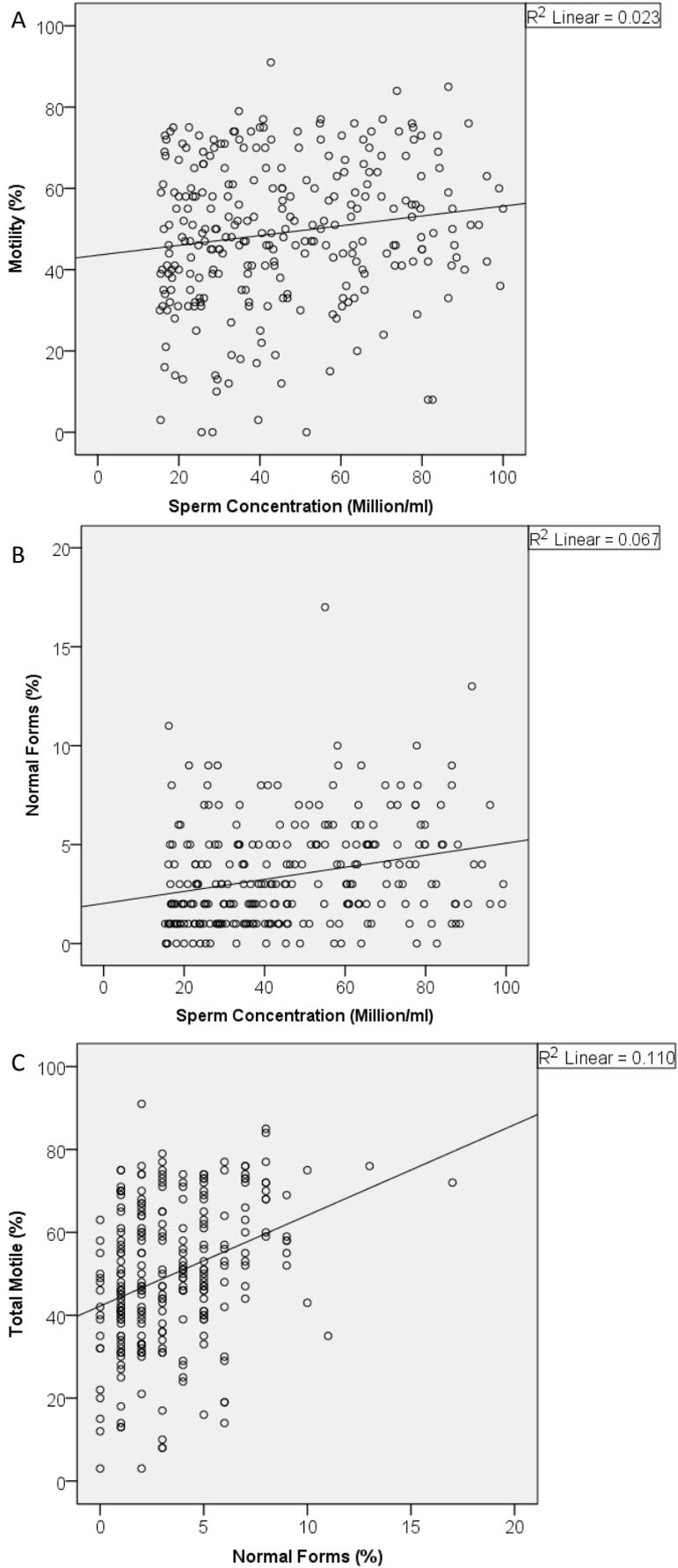
Figure 10-33 Scatter plots identifying correlations for Clinic A for the lower limit group ( $\leq 15$  million/ml). 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C).



**Figure 10-34** Scatter plots identifying correlations for Clinic B for the lower limit group ( $\leq 15$  million/ml). 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C).



**Figure 10-35 Scatter plots identifying correlations for Clinic A for the upper limit group ( $\leq 100$  million/ml).** 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C).



**Figure 10-36 Scatter plots identifying correlations for Clinic B for the upper limit group ( $\leq 100$  million/ml). 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C).**

### 10.16 Consent form for patient/donor participation in research

ASSISTED CONCEPTION  
UNIT

NHS TAYSIDE

WARD 35

NINEWELLS HOSPITAL

DUNDEE DD1 9SY



Direct line (01382) 632111

Fax (01382) 633853

#### CONSENT FORM FOR PATIENTS/DONORS

[producing extra semen samples]

**Title of research:** Understanding the regulation of human sperm function and the development of novel treatments for male infertility.

First of all we would like to thank you very much for taking part in our research project.

The aim of this study is to understand how a sperm cell is activated in response to secretions from the female tract - progesterone and nitric oxide and to understand if this activation is abnormal in some men. In addition we would like to test enzyme inhibitors to see if we can enhance sperm motility and hope that in the future we may be able to develop drugs which may be able to improve IVF success.

You may decline to take part, or withdraw at any time without this affecting, in any way, your treatment and care now or in the future.

I have fully understood what will be involved in the project. This study involves me producing a semen (sperm) sample by masturbation in the Assisted Conception Unit or by arrangement at home, for the research purposes of the project. In the future there may be requests for further semen samples.

Signed.....

Name (block capitals).....

Date.....

Witnessed.....(name).....Signature

If you have any further queries or questions you can contact either: Mr Steven Mansell (01382 660111 ext. 33605) or Nurse Evelyn Barratt, [e.barratt@dundee.ac.uk](mailto:e.barratt@dundee.ac.uk)

ASSISTED CONCEPTION UNIT  
WARD 35



NHS TAYSIDE  
NINEWELLS HOSPITAL  
DUNDEE DD1 9SY

### Patient/donor consent form

#### Research to improve our understanding of how human sperm function

I have read the research study information sheet and have had the opportunity to ask questions YES / NO

I understand that I can withdraw consent or change my mind at any time YES / NO

Whether I participate or not, I understand that this will have no impact on my treatment or care either now or in the future YES / NO

I consent to allowing researchers to use left-over semen following diagnosis or treatment YES / NO

I am prepared to be contacted for a further sample, if required YES / NO

If yes: contact details

SIGNED.....

NAME (BLOCK CAPITALS).....

DATE.....

WITNESSED.....(NAME).....(SIGNATURE)

If you have any further questions or queries you can contact either nurse Evelyn Barratt (07528 558624 or email [e.barratt@dundee.ac.uk](mailto:e.barratt@dundee.ac.uk)) or Dr Sarah Martins da Silva (01382 660111 bleep 4535 or email [s.martinsdasilva@dundee.ac.uk](mailto:s.martinsdasilva@dundee.ac.uk))

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