REGULATION OF MOTILITY AND Ca^{2+} SIGNALLING IN HUMAN SPERM

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A thesis submitted to

The University of Birmingham

for the degree of

DOCTOR OF PHILOSOPHY

School of Biosciences

College of Life and Environmental Sciences

The University of Birmingham

August 2016

Abstract

Human sperm motility is complex, involving several behaviours with different functions. The sperm 'selects' and switches between behaviours by using calcium signalling. For example, during the sperm's transit through the female reproductive tract, it undergoes molecular changes (capacitation, which is absolutely necessary for successful fertilisation). This is accompanied by the adoption of a whiplash-like behaviour called hyperactivation, which enables the sperm to penetrate the oocyte. Many infertile men have sufficient sperm, but their sperm have decreased in motility and/or have failed to adopt the appropriate behaviours, and so they fail to reach and/or fertilise the oocyte. In order to develop a treatment for these cases, it is important to understand the determination and regulation of sperm motility (Alasmari et al.,2013; Tamburrino et al.,2014).

In this project, I investigated the effects on human sperm behaviour of the preparation method (conventional density gradient method versus the direct swim up method) and manipulation of Ca^{2+} store mobilisation (5 mM 4-aminopyridine) and CatSper activation (3μM progesterone and 2μM prostaglandin E_1) and assessed the efficacy of the different behaviours for penetration through artificial viscous and viscoelastic environments composed of methylcellulose and polyacrylamide.

Levels of spontaneously-occurring hyperactivated motility were greater in density-gradient than swim-up prepared cells (6.25% vs 3.75% $p<0.05$) but swim-up cells performed better in the Kremer penetration test ($p<0.005$). 4-AP proved a potent inducer of hyperactivated motility ($p<0.0001$) but inhibited penetration into viscous medium (methylcellulose; $p=0.004$) in cells prepared by both methods. Similar results were observed with penetration into visco-elastic (polyacrylamide) medium. 2μM prostaglandin E_1 failed to stimulate hyperactivated motility in SU cells ($p>0.05$) but slightly increased hyperactivation in density gradient cells ($p<0.05$). Assessment of progesterone-induced $[Ca^{2+}]_i$ responses showed that

the proportion of responsive cells and the amplitudes of both transient and sustained responses were greater in swim-up cells than in density-gradient prepared cells ($p<0.05$). CatSper immunostaining showed greater levels of expression in swim-up cells ($p<0.05$).

Keywords: Male Infertility, Regulation of Motility, Computer assisted semen analysis (CASA), Kremer's penetration test, Capacitation, Hyperactivation, CatSper, Conventional Density Gradient (DG), Direct Swim-up (SU), 4-Aminopyridine (4-AP), Prostaglandin E_1 (PE_1), Progesterone (P4), Methylcellulose, Polyacrylamide.

Acknowledgments

I would like to thank my supervisor Dr Stephen Publicover for his valuable advice and support without which this thesis would not have been possible. Your valuable guidance helped me to gain skills (both research and managerial) that are the biggest assets in supporting my future career options.

I would also like to this opportunity to thank Prof C.L.R. Barratt from Reproductive and Developmental Biology, Medical school, Nine wells hospital, University of Dundee – DD1 9SY for providing his valuable advice with the penetration test.

Also, I would like to thank Dr Linda Lefievre from Reproductive Biology and Genetics group, Division of Medical Sciences – University of Birmingham for giving her valuable suggestions.

I would also like to thank School of Biosciences and School of Medical Sciences – University of Birmingham for providing the resources for continuing the project.

I would personally like to acknowledge research donors for their active participation in donating samples for the smooth progress of the research work.

I would also like to thank my colleagues Joa, Jenny, Cosmos, Elis and Sarah with whom I have shared the lab and the office space and had knowledgeable talks with them. I would also like to take this opportunity to thank support staff (floor maintenance and IT services) for providing up to date information on possible electric shutdowns and suggestions on data backup. Also, I would like to thank Ali and all the members on the 8th floor; I had a great time with them in sharing some valuable thoughts on career planning.

Last but not least I am incredibly grateful to my grandparents and my parents for supporting me throughout my studies and encouraging me in giving the confidence that I can fulfil my dreams and successfully move forward in life.

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LIST OF ABBREVIATIONS

2APB	2-aminoethoxydiphenylborate
4AP	4-aminopyridine
ADP	adenosine diphosphate
ALH	amplitude of lateral head displacement
AM	acetoxymethyl
AMP	adenosine monophosphate
AR	acrosome reaction
ART	assisted reproductive technology
ATP	adenosine triphosphate
BCF	beat frequency
BEB	blood epididymal barrier
BSA	bovine serum albumin
BTB	blood testis barrier
Ca^{2+}	calcium ions
$[Ca^{2+}]_i$	intracellular calcium concentration
$[Ca^{2+}]_{ex}$	extracellular calcium concentration
Cav	calcium voltage channel
cADPR	cyclic adenosine diphosphate receptor
cAMP	cyclic adenosine monophosphate
CCE	capacitative Ca^{2+} entry
CASA	computer aided semen analysis
CCE	capacitative Ca^{2+} entry
CICR	calcium induce calcium release
Cl^-	chloride ions

CO	cumulus oophorus
CO_2	carbon dioxide
cp	centipoise
CRAC	Ca^{2+} release activated Ca^{2+} channels
DAG	diacylglycerol
ddH_2O	double distilled water
DF	decapacitation factors
DG	density gradient
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBSS	earles balanced salt solution
Em_r	resting membrane potential
ER	endoplasmic reticulum
ES	equatorial segment
EVs	extracellular vesicles
FD	frequency distribution
FF	follicular fluid
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
GnRH	gonadotrophin-releasing hormone
HA	hyperactivation
HBS	HEPES buffered saline
HCO_3^-	bicarbonate ions
[HCO_3^-]	bicarbonate ion concentration
HCl	hydrochloric acid

HCM	human cervical mucus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFEA	human Fertilization and Embryology Authority
HVA	high voltage activated
Hz	hertz
IAM	inner acrosomal membrane
IBMX	3-isobutyl-1-methylxanthine
I_{CRAC}	calcium-release-activated calcium current
ICSI	intracytoplasmic sperm injection
IgG	immunoglobulin
IF	immunofluorescence
IP_3	inositol-1,4,5-triphosphate
IP_3R	inositol-1,4,5-triphosphate receptor
IUI	intrauterine insemination
IVF	*in vitro* fertilization
K^+	potassium ions
KCl	potassium chloride
kDa	kilo Daltons
KOH	potassium hydroxide
LH	luteinizing hormone
LIN	linearity
LVA	low voltage activated
min	minutes
ml	millilitres
mm	millimetres
mM	mill molar

mOsm	milli Osmole
mRNA	messenger ribonucleic acid
mV	millivolts
N	nucleus
Na+	sodium ions
NADH	nicotinamide adenine dinucleotide
NBC	Na^+/ HCO_3^- co-transporters
NaCl	sodium chloride
NCM	non capacitated medium
NCX	Na+-Ca^{2+} exchanger
NE	nuclear envelope
NGS	normal goat serum
nm	nanometre
nM	nanomolar
NO	nitric oxide
NPC	nuclear pore complexes
OAM	outer acrosomal membrane
ODF	outer dense fibers
Orai	CRAC PM Channel
P	prostasomes
pAB	primary antibody
PAP	prostate acid phosphatase
PAS	post acrosomal sheath
PBS	phosphate buffered saline
PDE	phosphodiesterase
PDEI	phosphodiesterase inhibitor

PE_1 prostaglandin E_1

pH_i intracellular pH

PHN posterior head/neck

PIP_2 phosphatidylinositol 4,5-bisphosphate

PKA protein kinase A

PKC protein kinase C

PKG protein kinase G

PM plasma membrane

PLC phospholipase C

PM plasma membrane

PMCA plasma membrane Ca^{2+} ATPase

PSA prostate specific antigen

PSCA prostate stem cell antigen

PT peri-nuclear theca

PTK protein tyrosine kinase

PVP polyvinylpyrrolidone

P4 progesterone

RNA ribonucleic acid

RNE redundant nuclear envelope

ROI region of interest

ROS reactive oxygen species

R normalised fluorescence intensity

R_{tot} fluorescence intensity mean

RT room temperature

RyR ryanodine receptor

sAB Secondary antibody

sAC soluble adenylate cyclase

S	sustain
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sEBSS	supplemented EBSS
sec	seconds
S.E.M	standard error of the mean
Ser/Thr	serine/threonine
SERCA	sarcoplasmic-endoplasmic Ca^{2+} ATPase
sGC	soluble guanylyl cyclase
SKF	SKF-96365
SOAF	sperm borne oocyte activating factors
SOC	store operated Ca^{2+} channel
SOCE	store operated Ca^{2+} entry
SPCA	secretory pathway Ca^{2+} ATPase
Src	serine kinase
STIM	stromal interaction molecule
STR	straightness
SU	swim up
T	transient
TBS	tris buffered saline
tmAC	transmembrane adenyl cyclase
TPBS	trisphosphate buffer saline
TRPC	transient receptor potential cation channels
TTBS	TBS with tween 20
TyPr	tyrosine protein phosphorylation
VAP	average path velocity
VCL	curvilinear velocity

Vm	membrane potential
VSL	progressive or straight line velocity
VOCC	voltage-operated Ca^{2+} channels
WHO	world health organization
w/v	weight per volume
ZP	zona pellucida
ΔF_{mean}	fluorescence intensity mean
ΔF_{max}	maximum fluorescence intensity
ΔF	fluorescence intensity
µm	micrometre
µl	microliter
µM	micromolar

Chapter 1: Introduction

1.1 The Human Reproductive System

Sexual reproduction is the process of producing offspring through fusion of germ cells (gametes) from male and female individuals. After gamete fusion (fertilisation), the new cell contains one set of chromosomes from each parent. Male and female have anatomically different reproductive organs (testes and ovaries respectively) that are adapted to produce different gametes (sperm from the male testes; oocytes from the female ovaries). In addition to producing gametes, male and female reproductive organs may produce hormones which regulate reproductive physiology and behaviour.

1.1.1 Anatomy of the Male Reproductive System

In the human, the two testes are placed externally and supported by the scrotum. In addition to the testes, the male reproductive system includes various ducts (epididymis, ductus deferens or vas deferens, ejaculatory duct, and urethra) that store male germ cells and assist in their maturation and transport. Associated glands (seminal vesicles, prostate gland and bulbourethral glands) nourish, protect and provide additional components of semen. The penis enables delivery of the semen, containing the male gametes, into the female tract.

1.1.1.1 Scrotum and Testes

The scrotum acts as a support structure for the testes. It consists of loose skin and an underlying subcutaneous layer that hangs from the root of the penis. The scrotal septum divides the scrotum into two sections, each containing a single testis. The function of the scrotum is to the maintain temperature (close to body temperature to suit sperm production) of the testes.

The testicles or testes are paired oval glands that measure about 5cm long and 2.5cm in diameter. Each testis weighs about 10-15g. During the seventh month of foetal development, the testes develop near the kidneys and begin to move into the scrotum through the abdominal wall. The testes are responsible for producing a male reproductive hormone called testosterone. This hormone is responsible for the development of masculine features and promotes sexual desire.

The testes contain a series of internal compartments called lobules, each of which contains one to three tightly coiled tubules called seminiferous tubules – the site of sperm production. At this location there occurs a complex process involving mitosis, meiosis of spermatogonia (precursor to the sperm cell) and further differentiation into mature sperm (spermiogenesis). Altogether, this differentiation is referred as spermatogenesis. For the anatomy of the testes and sperm cell differentiation in the seminiferous tubules, please see sections 1.3 and 1.4.

1.1.1.2 Penis

The main function of the penis is to act as a passageway for the ejaculation of seminal fluid and also to excrete urine. The penis contains different sections, such as the root, body and glans (head), and the urethra runs through the middle (Tortora, 2011).

The **body of the penis** is composed of corpus cavernosum – the tissue running along the sides of the penis (blood fills this tissue to cause an erection) – and corpus spongiosum – a

spongy tissue running along the front position of the penis and ending at the head of the penis. The urethra (spongy urethra) runs through corpus spongiosum to carry urine out of the body and, during erection, the corpus spongiosum is filled with blood.

The end section of the corpus spongiosum, which is slightly enlarged, is called the **head or glans of the penis**. In the **glans,** the urethra forms an opening called the external urethral orifice. In uncircumcised men, the glans is covered by the foreskin, or prepuce. The **root** consists of the blub – a posterior continuation of the base of the corpus spongiosum that aids in ejaculation – and crus (resembling a leg) – two separate and tapered portions of the corpus cavernosum – supports the weight of penis.

The male urethra carries urine from the bladder, passing through the prostate gland, deep muscles of the perineum and finally through the penis, a distance of about 20cm. The urethra also discharges seminal fluid (containing sperm).

1.1.1.3 Prostate Gland and its secretions

The prostate is about the size of a walnut and is located between the urinary bladder and the penis. The prostate gland secretes a thick white fluid (prostatic fluid) that mixes with the sperm produced in the testes) to make semen, or seminal fluid. Prostatic fluid accounts for 30% of seminal volume (the rest being 5% sperm cells, 60% fluid from seminal vesicles and secretions from the bulbourethral (or Cowper's) glands. Prostatic fluid contains high concentrations of citric acid, zinc and prostate specific enzymes, such as a prostate-specific antigen (PSA) and prostate acid phosphatase (PAP) (Veveris-Lowe et al., 2007, Graddis et al., 2011, Aalberts et al., 2014). Prostatic fluid is highly beneficial for fertility (Aalberts et al., 2014).

Prostasomes are extracellular vesicles (EVs) that originate from the prostate epithelium (Brody et al., 1983, Ronquist and Brody, 1985, Aalberts et al., 2014). Prostasomes contain Ca^{2+}, cholesterol and other small molecules (Kravets et al., 2000). They were first reported in prostatic secretions in the 1970s (Ronquist, 1977, Ronquist et al., 1978b, Ronquist et al., 1978a, Aalberts et al., 2014) and are about 150-200nm in diameter (Arienti et al., 1997). Prostasomes extracted from human semen contain prostate-specific proteins such as PAP, PSA, prostate stem cell antigen (PSCA) and prostate–specific transglutaminase. These proteins (PAP, PSA & PSCA) were referenced as biomarkers for prostate cancer study (Cho et al., 2010, Bjartell et al., 2011, Graddis et al., 2011, Zhao et al., 2012, Aalberts et al., 2014).

The enzyme aminopeptidase N (Arienti et al., 1997, Aalberts et al., 2014) is transferred to sperm cells by prostasomes. In human sperm the presence of aminopeptidase N was shown to regulate sperm cell motility (Subirán et al., 2008). Other than prostasomes, aminopeptidase N is also present in seminal fluid, sperm neck and sperm tail (Subirán et al., 2008, Aalberts et al., 2014). Prostasomes are involved in different physiological roles, such as protecting the sperm from the acid environment of the vagina, delaying the acrosome reaction (AR) and helping to dilute the sperm ejaculate (Kravets et al., 2000).

Considering prostasomes' biological significance, they might be useful in developing new contraceptive agents and improving invitro fertilisation (IVF) techniques, and further understanding of prostasomes might help us to treat male subfertility (Kravets et al., 2000).

Prostate-specific antigen (PSA) is a 33kDa glycoprotein that liquefies semen coagulum (Tosoian and Loeb, 2010, Aalberts et al., 2014). PSA is detectable in human seminal fluid, and elevated expression of PSA was observed in men with prostate cancer (Papsidero et al., 1980, Kuriyama et al., 1980, Tosoian and Loeb, 2010). PSA is used as an effective biomarker in prostate cancer screening (Papsidero et al., 1980, Catalona et al., 1991, Tosoian and Loeb, 2010, Shah and Zhou, 2016).

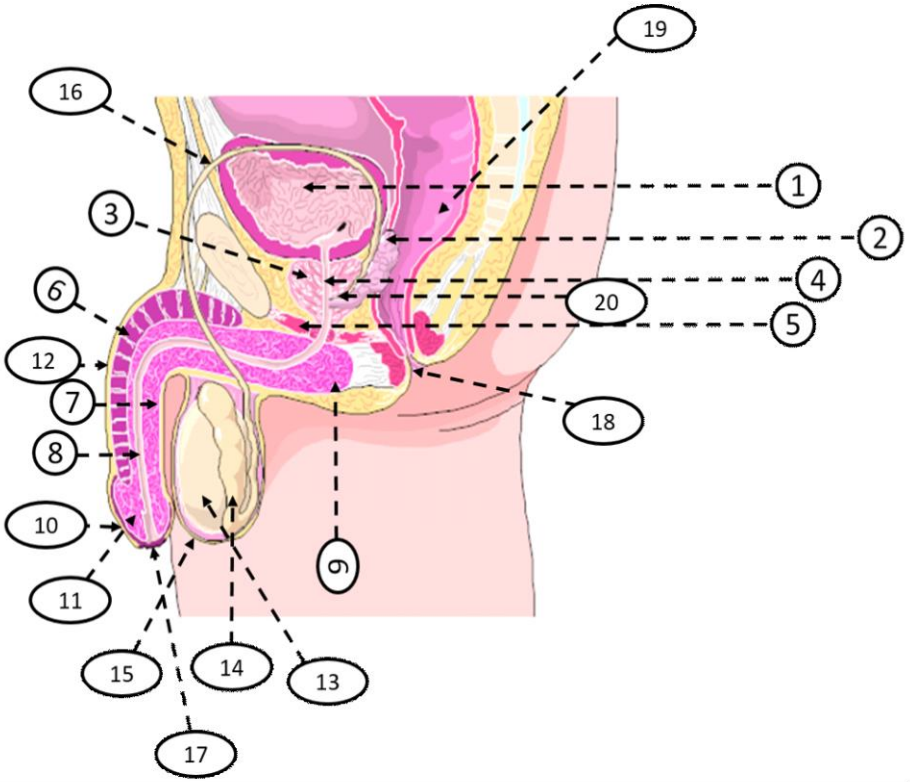


Figure 1. Anatomical image of the male reproductive system. Figure showing organs (testes), various channels or ducts (epididymis, vas deferens, urethra and ejaculatory duct), accessory sex glands (prostate and seminal vesicles) and supporting structures (scrotum and penis). Diagram adopted from smart draw source (www.smartdraw.com) and modified.

Key: -

1. Urinary bladder
2. Seminal vesicles
3. Prostate gland
4. Urethra (Prostatic urethra)
5. Deep muscle of perineum
6. Corpus cavernosum
7. Corpus spongiosum
8. Spongy urethra
9. Root
10. Foreskin (prepuce)
11. Glans (head)
12. Body
13. Testis
14. Epididymis
15. Scrotum
16. Vas deferens or ductus deferens
17. External urethral orifice
18. Anus
19. Rectum
20. Ejaculatory duct

1.1.2 Anatomy of the Female Reproductive System

The female reproductive system consists of two ovaries (female gonads), paired fallopian tubes or oviducts (ducts) made up of the isthmus, ampulla and infundibulum, and the uterus, cervix and vagina. Bartholin's gland (located near the opening of the vagina) lubricates the vagina by secreting mucus. The ovaries secrete progesterone and oestrogen (which are hormones necessary for ovulation and menstruation).

1.1.2.1 Vagina

The **vagina** (See fig. 2) is a tubular structure that accommodates the penis during coitus. It is a long (10cm) fibromuscular canal lined with a soft and elastic mucous membrane that extends from the lower section of the uterus (cervix) to the exterior of the body. The vagina also serves as a channel for the menstrual process (shedding of thickened uterus lining), and the gateway for childbirth. The vagina's environment is acidic ($pH \leq 5$), which protects against infections caused by pathogenic microbes (Suarez and Pacey, 2006). To protect sperm from the acidic environment, the semen is deposited at the cervix so that it will not spend a long period in the vagina (Sobrero and MacLeod, 1962, Suarez and Pacey, 2006).

1.1.2.2 Cervix

The **cervix** (See fig. 2) is located in the lower section of the uterus. It acts as a channel between the vagina and the uterus. The structure of cervix has two sections: the ectocervix and the endocervix (or endocervical canal). The opening in the middle of the ectocervix is called the external orifice (or os). Through this orifice, the cervix (lower section of the uterus) communicates with the vagina. The internal orifice (or os) is the passage between the cervix and the uterus. The cervix produces a secretory fluid called cervical mucus. Cervical mucus is composed of water, glycoproteins, lipids, enzymes and inorganic salts. Females secrete 20-60ml of cervical mucus every day (Tortora, 2011). The consistency of the cervical mucus

changes throughout the menstrual cycle. When approaching ovulation, the mucus is sticky in nature and white in appearance. At/near the time of ovulation cervical mucus is clearer, less viscous and more alkaline (pH 8.5), creating a friendlier environment that protects sperm from phagocytes and the environments of the vagina and uterus. It also provides energy for the sperm. After ovulation, the mucus again becomes sticky and more viscous and physically impedes sperm penetration. These variations in the mucus during the menstrual cycle are such that sperm migration occurs primarily during the time of ovulation (Morales et al., 1993, Suarez and Pacey, 2006). The physiological status of the cervix (Barratt and Cooke, 1991, De Jonge, 2005, Chakroun-Feki et al., 2009) and cervical mucus may also play a role in sperm maturation (capacitation), a series of functional changes (e.g. remodelling of plasma membrane) that gives sperm their fertilising potential before they fuse with the oocyte (Baldi et al., 2000, Chakroun-Feki et al., 2009) (For capacitation, please see section 1.7.1).

1.1.2.3 Uterus

The **uterus** (See fig. 2) is located between the urinary bladder and rectum. It is connected to the Fallopian (uterine) tubes and the vagina (via the cervix) and sperm deposited in the vagina must transit the uterus to reach the Fallopian tubes. The uterus is responsible for nourishing and protecting the foetus until birth. It also acts as a source of menstrual flow when there is no implantation. The inside of the body of the uterus is called the uterine cavity.

This **uterine cavity** (See fig. 2) is formed by the lateral walls of the uterus. The uterine walls consist of three layers of tissue: endometrium, myometrium, and perimetrium. The perimetrium is the outer layer of tissue around the uterus; posteriorly it forms a division between the uterus and urinary bladder. The middle layer is the myometrium and contains smooth muscle fibres to push out the foetus from the uterus. The inner layer of tissue is the

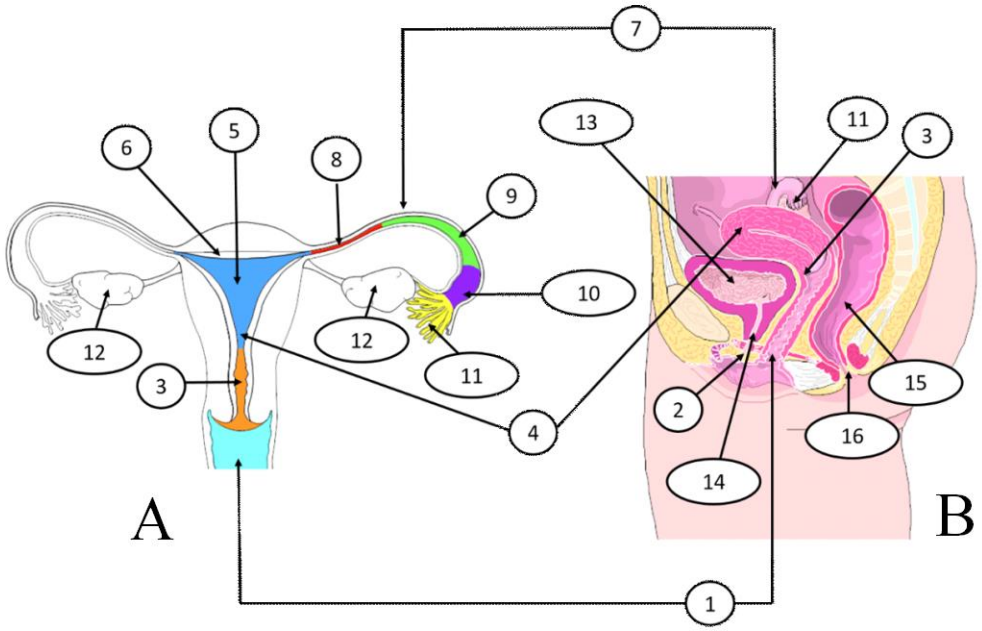
endometrium. It is the region where implantation takes place. If implantation does not occur, then the endometrium will shed and be discharged (menstruation).

1.1.2.4 Fallopian Tubes, Ampulla, Isthmus, Infundibulum and Fimbriae

There are two Fallopian (uterine) tubes or oviducts, which extend from the ovary to the uterus. At the junction with the uterus is the isthmus (see fig. 2) a short, narrow, thick walled portion of the Fallopian tube (Tortora, 2011). At the distal end of each Fallopian tube, there is a funnel-shaped portion called the infundibulum (see fig. 2). It is close to the ovary and ends in a group of a finger-like structure called fimbriae (see fig. 2), one of which is attached to the ovary (Tortora, 2011). Ovulated oocytes enter the Fallopian tube and fertilisation occur at the ampulla, the widest and longest section of the Fallopian tube (see fig. 2) (Tortora, 2011). The Fallopian tube thus provides a route for the sperm to reach the egg, the fertilisation site and the vessel that carries the fertilised egg to the uterus.

1.1.2.5 Ovaries

The ovaries are the female gonads. They are present on both sides of the uterus in the pelvic cavity and are attached to the uterus by ligaments which hold them in position. The ovaries produce female gametes (eggs) that are fertilised to produce an embryo. They secrete female sex hormones (progesterone and oestrogen), inhibin and relaxin that control the female reproductive cycle (Tortora, 2011).



Key: -

1. Vagina
2. External urethral orifice
3. Cervix
4. Uterus
5. Uterine cavity
6. Endometrium
7. Fallopian (uterine) tube
8. Isthmus
9. Ampulla
10. Infundibulum
11. Fimbriae
12. Ovaries
13. Urinary bladder
14. Urethra
15. Rectum
16. Anus

Figure 2. Anatomical image of the female reproductive system. A. Showing posterior view of different organs (vagina, uterus, cervix, different sections of the fallopian tubes and ovaries). **B** Showing sagittal section of female reproductive tract and other organs (urinary bladder, urethra, rectum and anus). Figures **A** and **B** were adopted from smart draw source (www.smartdraw.com) and modified.

1.2 Gametes

Gametes are sex cells, and are different from the body (somatic) cells (see Table 1). Male gametes are the **sperm,** and the female gametes are the **eggs or oocytes**. They are haploid, containing one set of genetic information. Fusion of male and female gametes (**fertilisation)** restores the diploid state and initiates the development of an embryo.

Different Characteristics	Somatic cell	Germline cell
Cell type	Somatic cell is a body cell Ex:- Skin, bone, blood and connective tissues	Germline cells are sex cells (gametes). Ex:- Sperm and Oocyte.
Chromosome number	Double the number of chromosomes compared to gametes (46)	Half the number of chromosomes compared to somatic cells (23)

Table 1. Showing differences between somatic cell and sex cells. Table showing difference in characteristics that exists between somatic and sex cells.

1.2.1 The Sperm Cell

The mammalian sperm is composed of a sperm head and flagellum or tail covered by a single continuous plasma membrane (also called as plasmalemma). In rodents, the sperm head is hook-shaped (falciform) and lacks a sperm centrosome and centriole. In contrast to this, the sperm of hoofed mammals (ungulates – e.g. cattle, goat, horse, etc.) has a spatula shaped head with a reduced form of centrosome with a single proximal centriole (Fawcett, 1975, Sutovsky and Manandhar, 2006). The normal human spermatozoon has an oval head shape with a distinct acrosomal region covering 40-70% of the anterior sperm head and a head length and width of 4-5µm x 2.5-3.5µm. The tail is approximately 45µm long (Eliasson, 1971, Mortimer and Menkveld, 2001) and is joined to the head by the mitochondrial midpiece, which is normally <1µm in width and approximately 1.5 times the head length. A cytoplasmic droplet (containing residual cytoplasm) may be attached at the midpiece, but if this is >30% the size of the normal head, it is considered as abnormal. (Eliasson, 1971, Mortimer and Menkveld, 2001).

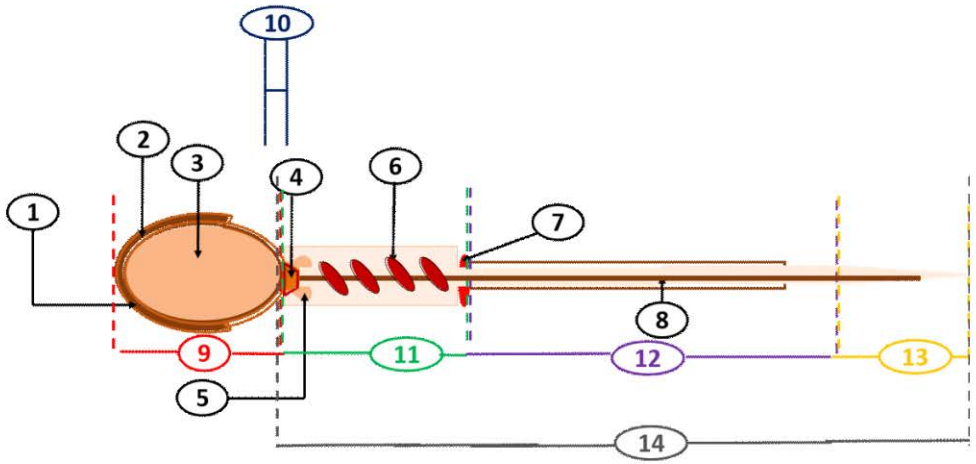


Figure 3. Showing the Human Sperm Cell with labelling of different regions: head and flagellum (connecting piece, mid piece, principle piece and end piece).

Key: -

1. Plasma membrane
2. Acrosome
3. Nucleus
4. Centriole
5. Cytoplasmic droplet
6. Mitochondria
7. Terminal disk
8. Axoneme
9. Head
10. Connecting piece
11. Mid piece
12. Principle piece
13. End piece
14. Flagellum (connecting, mid, principle and end pieces)

1.2.2 The Sperm's Architecture

The whole structure of the sperm is divided into two different sections: The **Head** and **Tail**, or **Flagellum.**

1.2.2.1 The sperm head

The sperm head, which contains the male nucleus, is divided into two regions the acrosome (bounded posteriorly by the equatorial segment (ES)) and the post acrosomal region. The acrosome is composed of inner and outer acrosomal membranes, the inner membrane overlying the anterior part of the nucleus. The narrow space between these membranes holds the dense acrosomal matrix. The post acrosomal region extends from the posterior end of the ES to the posterior ring. The ES carries receptor molecules that are involved in sperm-oolemma binding. The post acrosomal sheath (PAS) of the perinuclear theca (PT) contains a complex of sperm-borne, oocyte-activating factors (SOAF) (Sutovsky et al., 2003, Toshimori and Ito, 2003) that are dispersed across the ooplasm to trigger the signalling pathways that lead to oocyte activation and initiation of zygotic development (Sutovsky and Manandhar, 2002).

The sperm's nucleus contains highly condensed DNA. Histones of the sperm nucleus are replaced by protamines, in which the positively charged guanidinium group of arginine binds electrostatically to the negatively charged phosphate groups in DNA (Biegeleisen, 2006). Protamines are essential for sperm head condensation and DNA stabilisation. This compaction provides the shape suitable for sperm motility and migration through the egg's vestments (Brewer et al., 2002, Dadoune, 2003). The nucleus is surrounded by a nuclear envelope, which accumulates behind the nucleus (at the sperm neck) during nuclear condensation, forming the redundant nuclear envelope (RNE). Some nuclear pore complexes remain in the RNE and are observed at the base of the nucleus (Ho and Suarez, 2003). The

perinuclear theca (PT) forms a cytoskeletal shell that encapsulates and protects the sperm's nucleus.

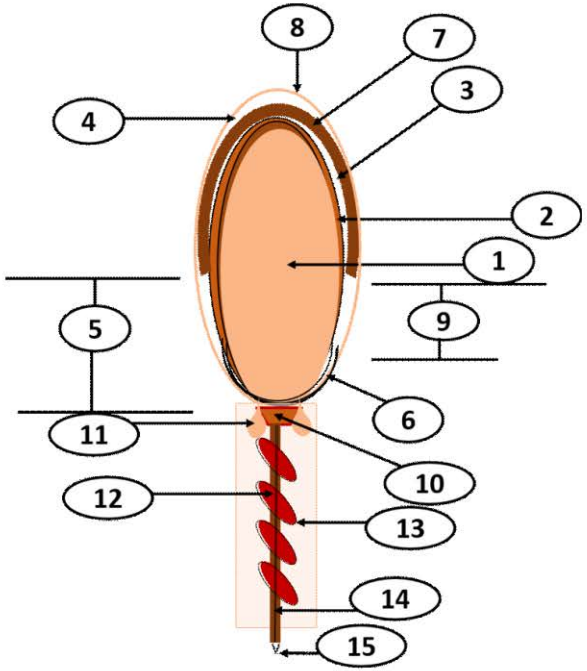
1.2.2.2 The flagellum

The sperm's tail or flagellum provides the motility that is required for the sperm to navigate the female reproductive tract. The axoneme, containing the motor proteins that provide movement runs centrally throughout the length of the flagellum and is surrounded by 9 outer dense fibres (see below; fig. 3).

Four sections are recognised within the structure of the he flagellum: the connecting piece, the mid piece, the principal piece and the end piece (Mortimer, 1997). The **connecting piece** (see fig. 3) is a short linking segment between the flagellum and head. The connecting piece contains the proximal centriole, which acts as the origin of the pair of central microtubules of the axoneme. The **mid piece** (see fig. 3) contains a mitochondrial sheath in the form of a helix wrapped around the axoneme (see below) which generates the energy that is required for sperm motility.

The **principal piece** is separated from the mid piece by an annulus or Jensen's ring, a ring of dense material found distal to the mitochondrial organelle. The principal piece is covered by a fibrous sheath composed of two longitudinal columns which surround the centrally positioned axoneme (see below). A fibrous sheath may act as a scaffold to sequester and immobilise important signalling molecules such as protein kinases that are required for the process of sperm capacitation and hyperactivation before fertilization (Eddy et al., 2003). The last piece of the flagellum, the **end piece,** contains only the motor proteins of the axoneme surrounded by a plasma membrane (Turner, 2006).

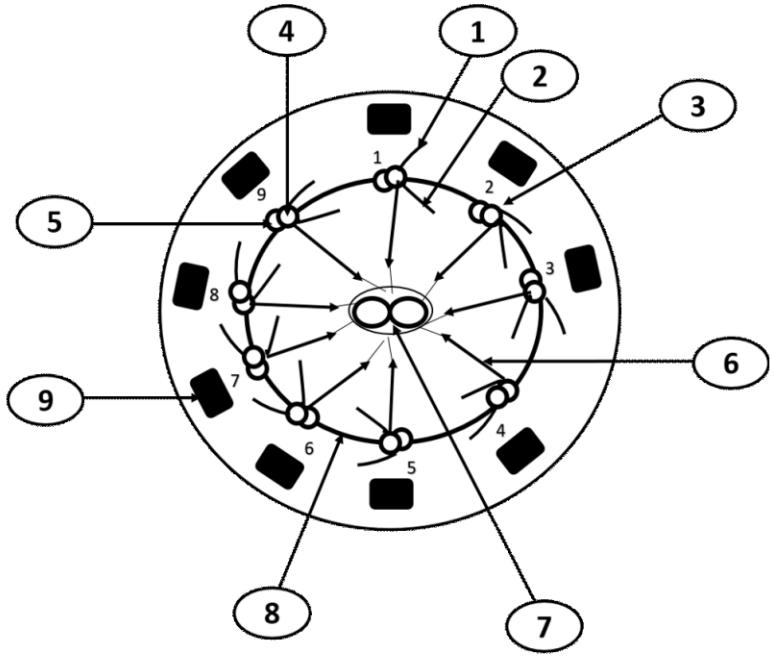
Sperm motility is provided by the axoneme, composed of two central microtubules which are connected by linkages (Pedersen, 1970) surrounded by nine microtubule doublets (in a 9+2 arrangement). Two central microtubules are surrounded by a central sheath composed of the spiral of two fibres. As explained by (Fawcett, 1965) and (Mortimer, 1997), each microtubule doublet consists of A and B subunits. Subunit A is attached to the "C" shaped B subunit. A multisubunit ATPase complex was formed when the dynein arms were attached to the A-subunit. This multisubunit ATPase complex converts chemical energy into the kinematic energy. This transfer of energy causes a sliding between the adjacent microtubule doublet further potentiating an axonemal bending thereby initiating a tail movement. Nexin link (an elastic element that regulate the shear forces when doublet sliding happens) between A and B subunit helps in connecting the adjacent microtubule. Outer dense fibres (ODFs) extend to almost 60% of the length of the principal piece of the flagellum, with two short fibers (**3** and **8**; 6μm), three medium length fibres (**2**, **4** and **7**; 17-21μm) and four long fibres (**5**, **6** and **9**; 31-32μm and fibre **1**; 35μm).



Key: -

1. Nucleus
2. Nuclear envelope
3. Sub-acrosomal space
4. Peri-acrosomal space
5. Post-acrosomal region
6. Post-acrosomal sheath
7. Acrosome
8. Plasma membrane
9. Equatorial segment
10. Centriole
11. Cytoplasmic droplet
12. Mitochondria
13. Outer dense fibres
14. Central pair of tubules
15. Axoneme

Figure 4. Showing Human sperm Head and Flagellum. Figure showing different sections of the head (nucleus, sub- and peri-acrosomal space, acrosome and equatorial segment) and flagellum (centriole, cytoplasmic droplet, mitochondria and axoneme).



Key: -

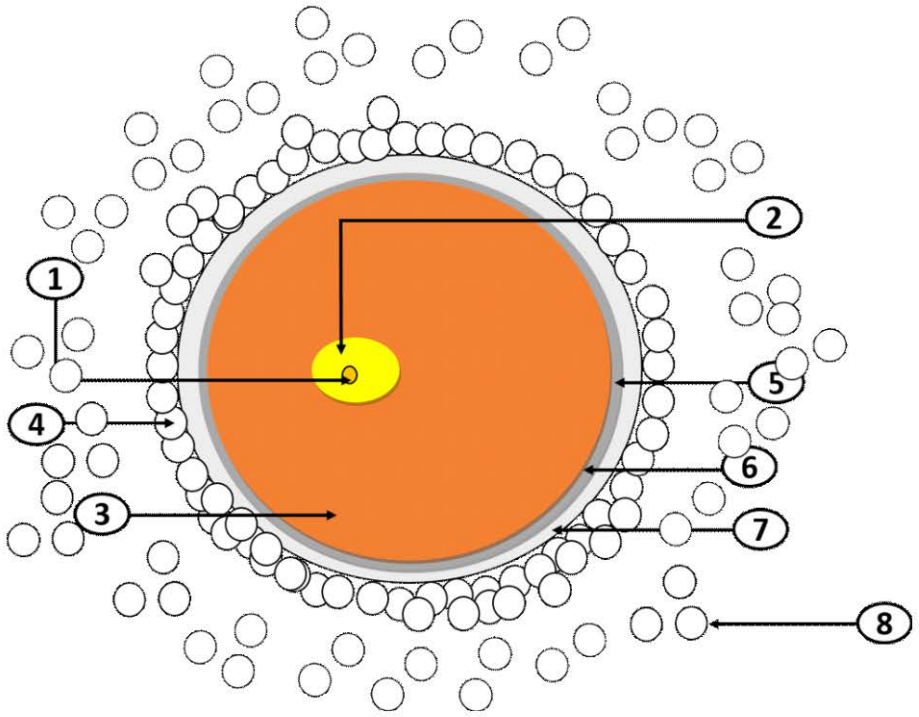
1. Outer dynein arm
2. Inner dynein arm
3. Microtubule doublets (9)
4. A subunit
5. B subunit
6. Radial spoke
7. Central pair of tubules (2)
8. Nexin bridge
9. Outer dense fibres

Figure 5. Showing Axoneme Structure in Human Sperm. Figure showing the structure of axoneme containing a 9 (microtubule doublets) + 2 (central pair of tubules) arrangement that provides flagellum movement for sperm.

1.2.3 The Egg Cell or Oocyte

Oocytes are the largest cells in the human body. They are non–motile and navigate the reproductive tract on currents created by ciliary activity and smooth muscle contractions. Upon ovulation, a mature oocyte is surrounded by several layers including the zona pellucida (ZP), corona radiata and cumulus layer which together comprise the oocyte-cumulus complex. The ZP in humans consists of four proteins (ZP1, ZP2, ZP3 and ZP4) (Petit et al., 2014, Louros et al., 2016). In contrast to human, only three ZP proteins are present in mouse zona (ZP_1, ZP_2 and ZP_3) (Wassarman, 1995a, Conner et al., 2005, Goudet et al., 2008, Petit et al., 2014). Sperm must be able to recognise and bind to the glycoproteins of the ZP and in order to penetrate and successfully fuse with the oocyte. This interaction is species-specific, and sperm does not bind to the ZP of another species (e.g. when the sperm is from a human and the oocyte is from a mouse).

The ZP is surrounded by the cumulus oophorus (CO), a hyaluronan-rich extracellular matrix. The innermost layer of the cumulus oophorus is the corona radiata, which is directly adjacent to the ZP. The cumulus cells may attract sperm through chemotaxis (Eisenbach, 1999a) that alters sperm movement (Eisenbach, 1999b). Only a small fraction (10%) of mammalian sperm show chemotaxis, which is believed to occur only in capacitated (matured) sperm cells (refer to capacitation section) (Eisenbach, 1999a). Different constituents of follicular fluid (FF), such as heparin, progesterone (P4) and atrial natriuretic peptide have been proposed to act as chemoattractants (Eisenbach, 1999b).



Key: -

1. Nucleolus
2. Nucleus
3. Cytoplasm
4. Corona radiata
5. Perivitelline space
6. Oocyte plasma membrane
7. Zona pellucida
8. Cumulus oophorus

Figure 6. Showing the Cumulus–oocyte Complex, showing different sections (nucleolus, nucleus, cytoplasm and plasma membrane) and different layers (zona pellucida, corona radiata and cumulus oophorus). The nucleus is protected by the zona pellucida, which is surrounded by the corona radiata and several layers of cumulus cells that constitute the cumulus–oocyte complex.

1.3 The testis and spermatogenesis

The testis is the site of sperm production. It is divided into a series of internal compartments called lobules which are separated by septa. Each of the lobules contains one to three tightly coiled seminiferous tubules. These coiled tubules have a central lumen and an external basement membrane and contain two types of cells: sperm-forming cells (spermatogenic cells); and Sertoli cells or supporting cells (Jégou, 1992).

Sertoli cells, which are embedded among the spermatogenic cells in the tubules, extend from the basement membrane to the lumen of the tubule and support germ cell growth, division and maturation. Sertoli cells are joined to one another by tight junctions (Jégou, 1992, Griswold, 1995, Griswold, 1998), forming the impermeable blood-testis barrier (BTB). Substances must pass through the Sertoli cells before they reach the germ cells and the tubule lumen. The BTB ensures the separation of sperm-forming cells from the blood, thereby protecting developing sperm cells from an immune response against the sperm cells' surface antigens. Sertoli cell-secreted proteins include proteases that are necessary for tissue remodelling during spermiation (Griswold, 1998).

In addition to their role in sperm production, Sertoli cells control the release of mature sperm into the seminiferous tubules and produce fluid for sperm transport, including bioactive peptides and nutrients. Sertoli cells also secrete the hormone inhibin, part of the hormonal feedback loop that controls spermatogenesis, and regulate the effects of testosterone and follicle stimulating hormone (FSH) (Griswold, 1995). In the interstitial spaces between adjacent seminiferous tubules, there are clusters of cells called interstitial endocrinocytes (Leydig cells). Leydig cells are stimulated by luteinizing hormone (LH) and, in response,

they secrete testosterone, which is responsible for the development of masculine characteristics.

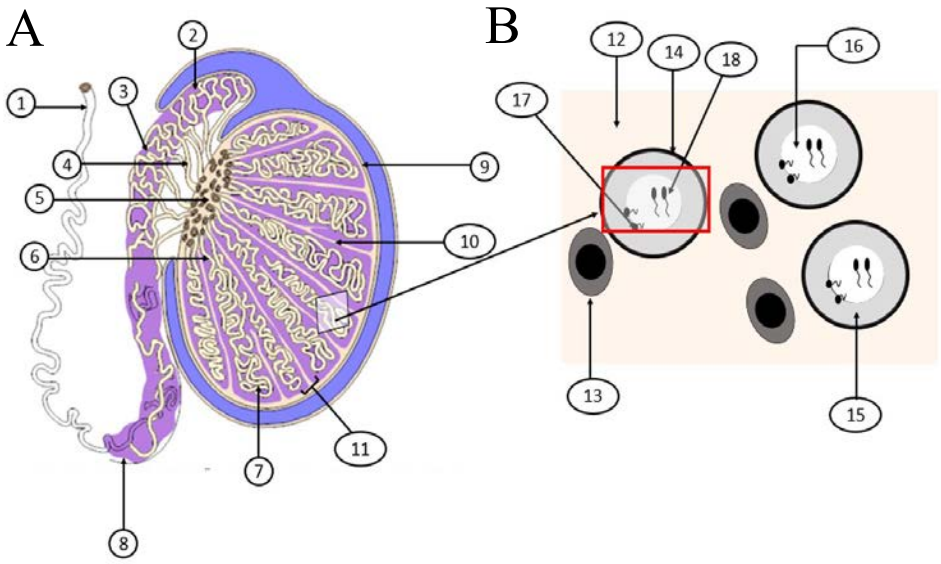


Figure 7. Anatomy of a Testis & Seminiferous Tubules. A Showing anatomy of testis with different sections (1 to 11 in key). **B** Showing the cross section of a seminiferous tubule with the lumen containing sperm cells that evolved from spermatids containing a haploid (n=23) number of chromosomes. Figure **B** also shows the interstitial space containing Leydig cells and a basement membrane. Diagram **A** adopted from smart draw source (www.smartdraw.com) and modified.

Key: -

1. Vas deferens
2. Head of the epididymis
3. Body of the epididymis
4. Efferent ducts
5. Rete testis
6. Mediastinum testis
7. Seminiferous tubules
8. Tail of the epididymis
9. Tunica albuginea
10. Septum
11. Lobules
12. Interstitial space
13. Leydig cells
14. Basement membrane
15. Seminiferous tubules
16. Lumen of the seminiferous tubules
17. Spermatid (n)
18. Sperm cell (n)

Mammalian gametogenesis involves meiotic division meiosis to form mature, haploid sex cells. The cell division (oogenesis and spermatogenesis) in these gametes occurs at different times during development and achieves different end points. In females, oogenesis commences in the foetus before birth (Griswold, 2016) in order to produce a finite number of oocytes that can be used periodically during the defined reproductive lifetime. At birth 1 million or more ovarian follicles are present in the ovaries but this reduces to between 10,000 to 100,000 follicles at age 30, 1,000 to 10,000 at age 40 and finally, during the arrest of the menstrual cycle (menopause) the number of ovarian follicles declines to less than 1,000 (Virant-Klun, 2015). In males, this process of reduction division (2n to n) doesn't commence until postnatal life at the onset of puberty. The main goal of gametogenesis in males is to produce millions of sperm cells, and any misregulation in reduction division could lead to infertility and germ cell cancers (Feng et al., 2014). In humans, the daily sperm production per testis is about 45 million (JOHNSON et al., 1980, Griswold, 2016), which means ~1,000 sperm are produced per second (Griswold, 2016).

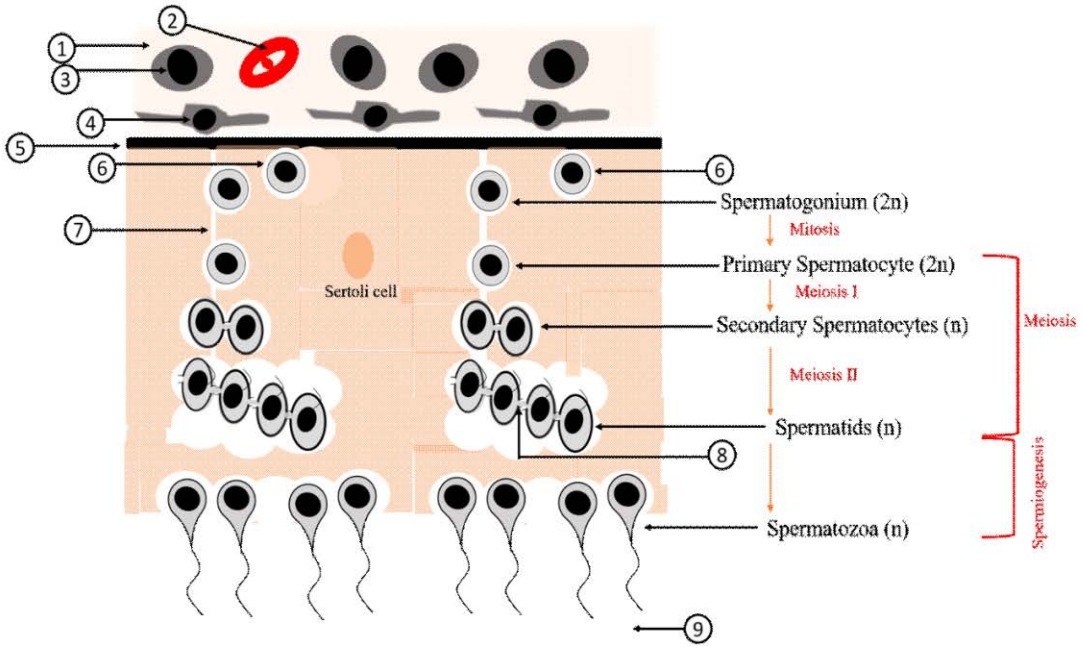
1.3.1 Spermatogenesis and Spermiogenesis

The process that involves mitosis, meiosis and further differentiation of spermatids to mature sperm (spermiogenesis) is referred to as spermatogenesis (de Kretser et al., 1998). The three key stages in spermatogenesis (spermatogonia, spermatocytes and spermatids) occur in order, with undifferentiated diploid cells adjacent to the basement membrane and mature gametes adjacent to the lumen of the tubule (fig. 8). The spermatogonial population of the mammalian testis arises from primordial germ cells which migrate into the developing testis during foetal life; later they transform into gonocytes (spermatogonia) which are surrounded by immature Sertoli cells.

The spermatogonial cells undergo two different processes (i) Some spermatogonial cells remain at the basement membrane and this cell that stay at the basement membrane won't participate in the cell division. (ii) The other set of spermatogonial cells move away from the basement membrane and participate in the cell division (mitosis) containing a diploid number (2n) of chromosomes (2n=46). The newly divided cells are called as the primary spermatocytes. Primary spermatocytes participate in further cell division (meiosis) containing a haploid (n) number of chromosomes (n=23). These newly divided spermatocytes are called as secondary spermatocytes.

Spermatids were formed as a result of further differentiation of secondary spermatocytes. These cells (spermatids) are haploid (n) cells, and four haploid cells were formed at the end of cells division. The end stage of spermatogenesis is called spermiogenesis where it involves the changeover of each spermatid into elongated spermatids (where it acquires both acrosome and flagellum) and finally developed into a sperm cell (spermatozoa).

Sertoli cells remove the excess cytoplasm, and the sperm cells are now released from their connections to the Sertoli cells; a process called **spermiation**. After the completion of spermiation, the sperm enter the lumen of the seminiferous tubules.



Key: -

1) Interstitial space
2) Blood capillary
3) Leydig cell
4) Myoid cell (smooth muscle-like cell)
5) Basement membrane
6) Spermatogonium
7) Blood–testis barrier (BTB)
8) Cytoplasmic bridge
9) Lumen of the seminiferous tubule

Figure 8. Showing Sperm Cell Differentiation (Spermatogenesis). Figure showing different events in spermatogenesis. Some spermatogonia remain at the basement membrane (BM), acting as precursor stem cells for future cell division and subsequent sperm production. The rest of the spermatogonia detach from the BM and squeeze through the tight junctions of the BTB and undergo mitotic differentiation into **primary spermatocytes** containing a diploid (2n) number of chromosomes. Primary spermatocytes undergo meiosis I to produce **secondary spermatocytes.** At this stage, the diploid (2n) number of chromosomes are reduced to a haploid (n) of chromosomes. Secondary spermatocytes undergo meiosis II to form four haploid cells (n) called **spermatids.** Now, the spermatids develop into sperm; this process is called **spermiogenesis**. Finally, the sperm lose their connections to the supporting cells (Sertoli cells); this process is called **spermiation**. After this, the sperm enter the lumen of the seminiferous tubules.

1.4 Post-testicular sperm processing

After entering the lumen of the seminiferous tubules (see fig. 7B), fluid secretions of the Sertoli cells push the sperm towards the short ducts of the testes. These short ducts lead to the **rete testis**, which is responsible for carrying the sperm from the seminiferous tubules into a series of coiled efferent ducts, or vasa efferentia. The vasa efferentia act as a bridge that connects the rete testis and the long, highly convoluted tube called the **epididymis** (Turner, 2008).

The epididymis has several functions, such as transporting, concentrating and storing the sperm (Turner, 2008). Apart from this, it also acts as the site of sperm cell maturation (Brooks, 1983, Turner, 2008). Anatomically, the epididymis is divided into three different segments: **the initial segment** or **caput** (the head of the epididymis, which also contains the end sections of the vasa efferentia), **the middle segment** or **corpus (**the body of the epididymis**)** and **the terminal segment** or **cauda (**the tail of epididymis**)** (See fig. 9).

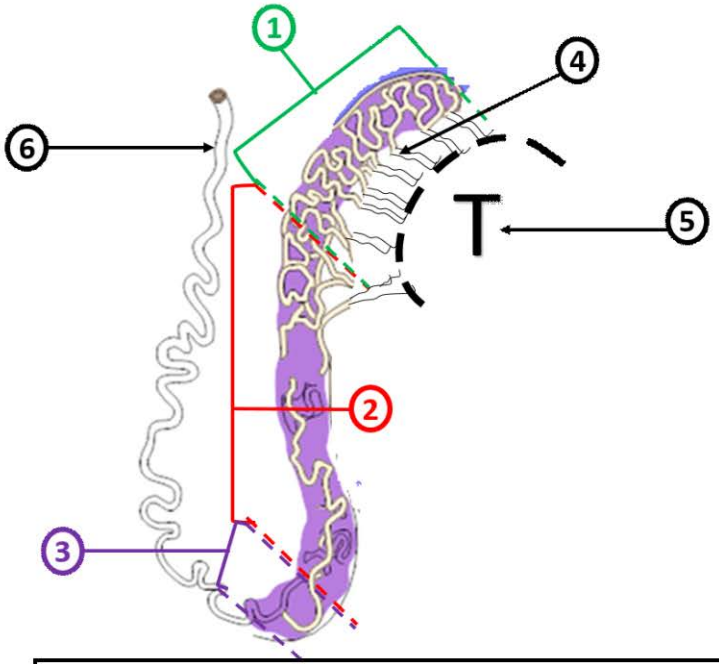
The caput and the vasa efferentia are responsible for fluid absorption, absorbing 90% of the fluid that carries the sperm. This is important because accumulation of fluid can eventually increase pressure inside the caput. If the fluid is not absorbed in time, that could affect the seminiferous tubules, which may eventually stop spermatogenesis (Turner, 2008, Johnson, 2012).

At this stage, the sperm is less concentrated and fluid absorption continues in the epididymis, which increases the concentration, and from here onwards, the sperm move to later segments (the corpus and cauda) through epididymal tubule contractions. At this stage, the sperm are incapable of movement and don't have reproductive competence (Johnson, 2012). They start to mature as they reach the mid corpus of the epididymis (Bedford, 1994).

At the corpus of the epididymis, the apical borders of the epididymal epithelial cells show cell-cell tight junctions containing a number of cell adhesion molecules. These cell adhesion molecules impose the blood-epididymal barrier (BEB), which is a specialised immune-privileged microenvironment that separates the sperm from other body cells (Hinton, 1985, Turner, 2008). Sperm at the mid corpus epididymis can undergo maturation (capacitation) process in the female reproductive tract that helps sperm to acquire the potential ability to fertilise oocytes and forward pattern of swimming. In mid corpus epididymis they do not swim actively in vivo, the activeness in motility was observed after they release from male reproductive tract. (Bedford, 1994, Johnson, 2012).

The last segment or cauda of the epididymis stores the mature sperm which have active motility. Along with active sperm population unexpectedly there also exists a population of high proportion of immotile cells (Yeung et al., 1993, Bedford, 1994).

From the epididymis, the sperm moves into the vas deferens (or ductus deferens) which is about 45cm long. The vas deferens starts at the posterior border (border of the tail (cauda)) of the epididymis (see fig. 9), moves through the spermatic cord and enters the pelvic cavity, where it loops over the ureter and passes over the posterior end of the urinary bladder (see fig. 1). It transports sperm from the epididymis towards the urethra by peristaltic muscular contractions. Other than sperm transport, it also stores sperm just like the epididymis (cauda region), and any stored sperm that has not been ejaculated is reabsorbed.



Key: -

1. Head (caput)
2. Body (corpus)
3. Tail (cauda)
4. Vasa efferentia
5. Testis
6. Vas deferens

Figure 9: Showing the Human Epididymis. Figure showing different sections of the human epididymis. The human epididymis contains three segments: **1. Head (caput),** involved in fluid absorption and carrying spermatozoa; **2. Body (corpus)** and **3. Tail (cauda),** involved in storing sperm at a high density in the lumen. Apart from sperm storage, the epididymis is the site of sperm maturation, where sperm acquire changes in concentration, an increase of membrane fluidity, nuclear condensation, the ability to swim progressively and the potential to fertilise oocytes. Diagram adopted from smart draw source (www.smartdraw.com) and modified.

1.4.1 Epididymal Maturation

In the epididymis, sperm undergo biochemical, morphological and functional (motility) changes. This whole process is called **maturation**. This process is crucial for human sperm to acquire reproductive competence (Johnson, 2012). The table below shows different maturational changes of sperm in the human epididymis.

Type of change	Details	Reference
Morphology	Immature sperm showing acorn-shaped heads due to epididymal dysfunction. % of sperm with normal heads increases with maturation.	(Ludwig and Frick, 1987) (Yeung, 2006)
Sperm modelling	Completion of sperm modelling: nuclear condensation, acrosomal shape gets completed. Squeezing in cytoplasmic droplet.	(Johnson, 2012)
Concentration	Head of epididymis (caput & vasa efferentia) containing 50x10^6/ml in fluid. At the distal duct (cauda) concentration increases to 50x10^8/ml, with densely packed sperm observed in ejaculate.	(Johnson, 2012)
Motility	Sperm obtained from caput/efferent ducts of epididymis are weakly motile. An increase in motility is observed as they pass through the corpus & cauda of the epididymis. Progressive velocity (VSL) and linearity (LIN) is higher at corpus and cauda.	(Yeung et al., 1993) (Cooper, 2002) (Yeung, 2006)
Sperm–Zona binding	Difference in binding capacity. Caput-derived sperm are unable to bind to zona. Cauda derived sperm can bind to zona.	(Cooper, 2002) (Yeung, 2006)
Sperm vitellus binding and fusion	Mature human epididymal sperm (possibly at the cauda) show increase	(Cooper, 2002)

Table 2: Showing Different Maturational Changes to Human Sperm in the Epididymis.
Sperm cells were detached from the sperm supporting cells (Sertoli cells) and the fluid secretions from the Sertoli cells push the sperm towards the rete testis, and from here the sperm are carried through the epididymis. In the epididymis, human sperm undergo a process called maturation, where they acquire reproductive competency and swim progressively. These maturational changes are accompanied by changes in morphology, sperm modelling, concentration, motility, an increase in binding capacity between sperm and zona, an increase in %AR (acrosome-reacted sperm), an ability to penetrate eggs and an increase in fertilisation (F) and pregnancy rates (PR).

1.4.1.1 Role of Epididymosomes in Epididymal Maturation

As the sperm leave the testis and arrive in the epididymis, they are in a state of limited functional capabilities and are not reproductively competent. To achieve reproductive competency, they need to undergo the maturation process. Epididymosomes are thought to play an important role in further sperm maturation in the epididymis. These are microvesicles (~0.1 to 1µm in diameter) (Cornwall, 2009, Aalberts et al., 2014) and are thought to be shed from the epididymal plasma membrane. They transfer some specific epididymal membrane proteins to the sperm's cell surface.

Protein	Role in epididymal maturation	Reference
P34H	Enables sperm cells to bind to the zona pellucida.	(L gar et al.,)
Disintegrin and Metalloproteinase 7 (ADAM 7 or GP83)	Proposed to play role in sperm–oocyte interaction	(Sun et al., 2000; Oh et al., 2009)
Enzymes involved in polyol pathway Aldose reductase (reduces glucose to sorbitol) Sorbitol dehydrogenase (oxidises sorbitol to fructose, an energy source for sperm)	Role in sperm cell motility	(Frenette et al., 2006)

Table 3: Table showing involvement of epididymosomes in epididymal maturation. Epididymosomes are microvesicles that are shed from epididymal plasma membrane and play important role in epididymal maturation. They (epididymosomes) transfer epididymal membrane proteins (P34H , GP83 & ADAM 7) and enzymes like aldose reductase and Sorbitol dehydrogenase (synthesized by epididymal epithelium) to sperm cell surface which helps sperm to undergo maturation in male reproductive tract that enhance reproductive competency.

1.4.2 Ejaculation

After the sperm leave the vas deferens, they enter into the ejaculatory duct, which is about 2cm long, and is the union of the seminal vesicle duct and the ampulla of the vas deferens. This ejaculatory duct is located just above to the base of the prostate gland, and terminates at the prostatic urethra. The prostatic secretions from the prostate gland also move to the prostatic urethra.

The seminal ducts secrete an alkaline, viscous fluid containing prostaglandins, fructose and clotting proteins. The alkaline nature of this fluid helps it to neutralize the vagina's acidic environment and so protects the sperm. It also contributes to neutralizing the acidic environment in the male's urethra. Prostaglandins contribute to sperm motility, and clotting proteins help to coagulate semen after ejaculation.

This secretion (seminal vesicles) constitutes the majority of **semen**. Secretions from the prostate gland also decrease the bacterial content in the semen, and it contains PSA that usually breaks down clotting proteins in the seminal vesicles. The bulbourethral glands secrete fluid into the urethra that neutralizes acids from the urine and also lubricates (fluid rich in muco proteins) the urethral lining at the end of the penis and avoids sperm damage upon ejaculation.

Semen is made up of fluid secretions from the seminal vesicles (60%), secretions from the prostate gland (30%), bulbourethral secretions and sperm cells (5%) (Owen and Katz, 2005). The pH of semen is alkaline (7.2-7.7), secretions from the prostate gland give semen a milky appearance, and secretions from seminal vesicles and bulbourethral makes the sperm sticky in nature.

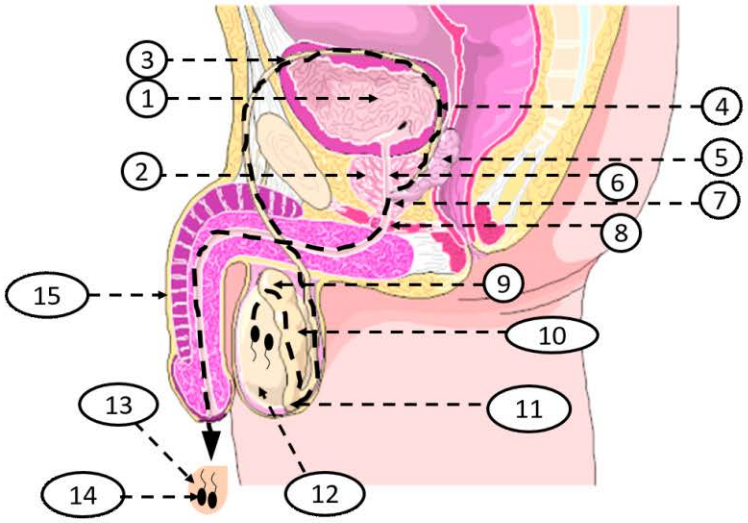
1.4.2.1 Prostasomes in Semen/Ejaculate

Prostasomes (P) are extracellular vesicles (EVs) that originate from the prostate epithelium (see Section 1.1.1.2.1). The table below shows the role of prostasomes in providing functional competence (Kravets et al., 2000, Aalberts et al., 2014) to sperm through their presence in seminal fluid. These functional attributes are of great importance in providing sperm with the capability to successfully transit the female reproductive tract and successfully fertilise the oocyte.

Prostasomes role	Functional importance	Reference
Sperm transit in female tract	Sperm fusion with **prostasomes (P)** protects sperm from the vagina's acidic environment.	(Arienti et al., 1997; Arienti et al., 1999; Kravets et al., 2000)
Low pH	**(P)** ensure that the low pH has a limited effect on sperm motility.	(Arienti et al., 1999; Kravets et al., 2000)
Enzyme transfer	Enzymes (aminopeptidase N, and dipeptidyl peptidase) may modify the composition and biological properties of sperm membranes. They probably playing role in sperm maturation.	(Arienti et al., 1997a; Arienti et al., 1997b; Kravets et al., 2000)
AR (Acrosome reaction) delay	**(P)** are rich in cholesterol which inhibits the sperm's response to progesterone. They increase plasma membrane fluidity, thereby delaying AR.	(Arienti et al., 1998; Carlini et al., 1997; Kravets et al., 2000)
Immune modulation	Seminal fluid acts as an immunosuppressive agent and protects sperm against immune responses encountered in the female tract.	(Kelly, 1995; Kravets et al., 2000)
Prostate specific antigen (PSA)	Breaks down the clotting proteins and liquefies the semen.	(Lilja and Laurell, 1984; Kravets et al., 2000)
Clinical Applications – Oligoasthenozoospermic (OAT) (lower motile conc) condition	**(P)** are shown to reactivate motility in OAT condition. Motility is further enhanced by adding glucose in the presence of magnesium ions.	(Fabiani et al., 1995; Kravets et al., 2000)
Prostate cancer diagnosis	PSA is used as a biomarker for prostate cancer screening.	(Papsidero et al., 1980; Catalona et al., 1991; Tosoian and Loeb, 2010; Shah and Zhou, 2016)

Table 4: Table Showing Different Roles of Prostasomes in Contributing Functional Competence to Sperm. Prostasomes are extracellular vesicles that originate from the prostate epithelium. The table shows the different roles of prostasomes in providing functional competency to sperm. Apart from their role in achieving sperm functional competence they also have clinical applications (reactivating motility in OAT condition and as a biomarker (PSA) to screen for prostate cancer).

The penis acts as a passageway for the ejaculation of seminal fluid and excretion of urine. Upon sexual stimulation, semen is released from the urethra to the exterior. The fast reflex action upon ejaculation is controlled by a lumbar portion of the spinal cord. The smooth muscle **urethral sphincter** located beneath the bulbourethral glands closes, preventing semen from entering the urinary bladder and urine from being expelled upon ejaculation. After ejaculation, the sperm in the seminal plasma enters the female reproductive tract. The sperm's journey into the female reproductive tract is explained in Section 1.5



Key:-

1. Urinary bladder
2. Prostate gland
3. Vas or ductus deferens
4. Sperm ejaculatory path (dotted line)
5. Seminal vesicle
6. Urethra
7. Ejaculatory duct
8. Bulbourethral gland
9. Caput (head) of the epididymis
10. Corpus (body) of the epididymis
11. Cauda (tail) of the epididymis
12. Testis
13. Semen/Ejaculate
14. Sperm cells
15. Penis

Figure 10: Figure Showing the Ejaculatory Path of Human Sperm in the Male Reproductive Tract. Human sperm moves from the testes to the epididymis, where they undergo maturation. After maturation, the sperm leaves the epididymis and enters the vas deferens where they are transported to the urethra (prostatic). Different accessory sex glands (comprising of seminal vesicles, prostate and bulbourethral gland) move secretions to the prostatic urethra where they mixes with sperm to form semen. After this, the semen enters the penis which acts as a passageway for the ejaculation of semen and the excretion of urine. Diagram **A** adopted from smart draw source (www.smartdraw.com) and modified.

1.5 The Sperm Cells' Journey into the Female Reproductive Tract

In humans, the seminal fluid (containing sperm) is deposited in the female anterior **vagina** in contrast to other species like pigs, where the seminal fluid is deposited directly in the uterine cavity, bypassing the vagina altogether (Hunter, 1981, Stephen, 1986). As the vagina is exposed to the exterior environment, it is prone to infections. Therefore, to combat infections, the vaginal environment is acidic in nature, pH ≤ 5 (Suarez and Pacey, 2006), which acts as an antimicrobial for many disease-causing pathogens. This acidic environment is maintained by the production of lactic acid by anaerobic lactobacilli present in vaginal epithelial cells feeding on glycogen (Boskey et al., 2001, Suarez and Pacey, 2006).

Before entering the cervix, sperm must navigate through the vagina's acidic environment. Seminal fluid has a pH of 7.2-7.8 (Johnson, 2012) and, when it enters the vagina, it neutralises the acidic pH (it increases pH from ≤ 5 to 7.2 (Suarez and Pacey, 2006) within 8 sec of seminal fluid entry), helping the sperm to survive.

Within minutes of entering the vagina, the sperm enters the **mucus-filled cervical canal** (Suarez and Pacey, 2006), which lies deep within the vagina (Suarez, 2010). The nature of the mucus in the cervix will change depending on where the female is in her menstrual cycle. After ovulation, the cervical mucus becomes sticky and hostile, which physically impedes sperm penetration. This sticky mucus depends on the secretion of different hormones (estrogen and progesterone).

A high amount of estrogen production during mid menstrual cycle ensures the mucus is less viscous and greater in quantity (an increase of water content leads to hydrated mucus). Unlike estrogen, progesterone ensures the mucus is stickier and decreased in water content (Davajan and Nakamura, 1973) (Suarez and Pacey, 2006).

Here, the cervical mucus selectively filters out sperm cells which are abnormal in morphology and have weak motility. Sperm cells with an abnormal shape swim at a slower pace in mucus, even if they have a normal flagellum, which is explained by them encountering greater resistance from the mucus. Sperm in cervical mucus moves primarily through the spaces/gaps between mucus micelles and sperm progression depends upon the size of these spaces (Chretien, 1989). The size of these gaps are smaller than the size of sperm head, and therefore the sperm has to push its way through the mucus during its transit through the female reproductive tract (Chretien et al., 1975, Katz and Berger, 1979, Poon and McCoshen, 1985).

Apart from the cervix's sticky nature, it also mounts an immune response as the sperm passes through the vagina and enters the cervix, in the form of leukocytes, particularly neutrophils and macrophages (Pandya and Cohen, 1985, Suarez and Pacey, 2006). Neutrophils migrate through the mucus layers of the human cervix (Parkhurst and Saltzman, 1994).

Only sperm with normal morphology, and which is highly motile can resist the leukocytic defences (phagocytosis), and all other sperm and microbial pathogens are unable to migrate through the cervical mucus. From the cervix, the sperm moves to the **uterus**. Smooth muscle contractions in the **uterine wall** help transport the sperm through the uterus to the uterotubal junctions, which form the gateway to the oviducts.

In humans, the uterotubal junction is an average of 1.2cm in length (Suarez, 2010). Once the sperm passes through the uterotubal junction, they are held in a sperm storage reservoir at the **isthmus** where they encounter high mucus containing narrow lumen (Eisenbach, 1999b), which slows down their forward progression and they (the sperm) bind strongly to the oviductal epithelium. In order to successfully transit through the female reproductive tract and fertilise the oocyte, the sperm undergo a maturation process known as **capacitation** (see Section 1.6).

When ovulation is completed, a change in sperm motility behaviour is observed: hyperactivated motility (for hyperactivation please see sections 1.6 and 1.8) associated with asymmetrical flagellar beat (Suarez et al., 1991). Only sperm which has shown functional competence (that have undergone capacitation) can successfully detach from the oviductal epithelium and move away from the isthmus–ampulla junction and successfully penetrate through the cumulus oophorus, bind to the ZP (Yoshida et al., 2008) and undergo acrosome reaction (AR) and finally fertilise the oocyte.

A diagrammatic representation of the sperm cells journey into the female reproductive tract along with the fertilisation was shown at the end of section 1.6.

1.6 Fertilisation

Fertilisation is the process by which a spermatozoon successfully fuses with an oocyte. For a sperm cell to attain fertilisation capacity, it must undergo capacitation, a maturation process that occurs in the female reproductive tract. **Capacitation** and associated events (**hyperactivation** (HA), **thermotaxis**, **chemotaxis** and the **acrosome reaction** (AR)) are required for the sperm successfully to participate in fertilisation.

1.6.1 Capacitation

Austin and Chang first demonstrated the requirement for sperm maturation at the beginning of the 1950s. They showed that sperm must remain in the female reproductive tract for a period of time of time before acquiring fertilising capability. This phenomenon is known as **capacitation** (Austin, 1951, Chang, 1951b, Austin, 1952). Later Yanagimachi performed an assessment to study the fertilising capacity of human sperm (Yanagimachi et al., 1976).

The physiological changes that are observed during capacitation in the female reproductive tract (or under in vitro conditions in a medium containing Ca^{2+}, bicarbonate (HCO_3^-) and bovine serum albumin (BSA)) involve a number of sequential and parallel process. Some of these processes take place as soon as the sperm is ejaculated (called the **fast/early phase** of capacitation) but other processes occur over a longer period of time and are classified as the **slow/late phase** of capacitation (Visconti et al., 2002, Bedu-Addo et al., 2005).

These capacitation events include cholesterol efflux, an increase in plasma membrane fluidity, increase in $[Ca^{2+}]_i$, an increase in intracellular pH (pHi), enhanced activity of soluble adenylate cyclase (sAC) and elevated levels of cyclic adenosine monophosphate (cAMP), increased activity of protein kinase A (PKA), increased phosphorylation of serine/threonine and tyrosine residues and plasma membrane hyperpolarisation (Tash and Means, 1983, Leclerc et al., 1996b, Cross, 1998, Visconti and Kopf, 1998, Osheroff et al., 1999b, LefiÈVre

et al., 2002, Visconti et al., 2002, O'Flaherty et al., 2004, Moseley et al., 2005, Bedu-Addo et al., 2005, Visconti, 2009, Battistone et al., 2013).

1.6.1.1 Fast/early phase of capacitation

As the sperm leaves the epididymis and comes into contact with seminal fluid, a vigorous movement of the flagellum (motility activation) starts immediately because of the presence of HCO_3^- and Ca^{2+} in the seminal fluid. HCO_3^- and Ca^{2+} are essential for the increase in sperm motility (Rojas et al., 1992, Osheroff et al., 1999a, De Vries et al., 2003, Jaiswal and Conti, 2003, Luconi et al., 2005, Visconti, 2009).

During fast/early phase capacitation HCO_3^- is transported across the plasma membrane (PM) using Na^+/HCO_3^- (bicarbonate) co-transporters (NBC). NBC is referred to as "electrogenic" (1 Na^+: at least 2 HCO_3^-) (Jentsch et al., 1984, Romero, 2001). HCO_3^- entry into the cell is associated with an increase in intracellular pH and stimulates an increase in cyclic adenosine monophosphate (cAMP) (reaches maximum within ~60sec) through the activation of atypical soluble adenylyl cyclase (sAC) (Visconti, 2009). cAMP, in turn, activates PKA that phosphorylates amino acid (serine/threonine) residues on target proteins in the flagellum. PKA-dependent phosphorylation begins within ~90sec of HCO_3^- activation, leading to an increase in beat frequency and motility activation and contributing to numerous signalling processes in the sperm (Xie et al., 2006, Visconti, 2009).

1.6.1.2 Slow/late phase of capacitation

As in fast/early phase capacitation, Ca^{2+}, HCO_3^- and activation of sAC with a consequent increase in [cAMP] and PKA activation, are essential contributors (Visconti, 2009, Bailey, 2010). Slow/late phase capacitation involves cholesterol efflux from the PM, which can be

achieved in vitro by the addition of BSA or β-cyclodextrins to the capacitation media (Salicioni et al., 2007, Visconti, 2009). Cholesterol removal from the PM activates PKA phosphorylation of amino acid (serine/threonine) residues, which further causes an increase in protein tyrosine phosphorylation (TyPr). It is well documented that TyPr is downstream from the PKA pathway, and the increase in TyPr also depends on the presence of BSA, HCO_3^- and Ca^{2+} (Salicioni et al., 2007, Visconti, 2009). The absence of any one of these constituent's failure of the slow/late phase of capacitation. Diagrammatic representation of both fast/early and slow/late phase capacitation events is shown in Figure 11 **A** & **B**.

In sperm of mice, cows and horses, population measurements of resting membrane potential (Em_r) of non-capacitated sperm lie between -35 and -45mV, but after capacitation the Em_r was observed to be -65mV (Espinosa and Darszon, 1995, Zeng et al., 1995, Muñoz-Garay et al., 2001, Demarco et al., 2003, Hernández-González et al., 2006, Santi et al., 2010, De La Vega-Beltran et al., 2012, López-González et al., 2014). In mouse sperm changes in K^+, $Na^{+,}$ and Cl^- permeability are believed to contribute to this PM hyperpolarisation, thereby increasing cholesterol efflux and membrane fluidity for lipid raft reassembly (Cross, 1998, Xie et al., 2006, Cross, 2004).

Measurements of Em of non-capacitated human sperm populations are around -40mV (Linares-Hernández et al., 1998, López-González et al., 2014) which increased to about -58mV in capacitated cells (Patrat et al., 2002, López-González et al., 2014). Both Slo1 and Slo3 K^+ channels may contribute to capacitation-associated hyperpolarisation of human sperm whereas only Slo3 channels are implicated in the hyperpolarisation in mouse sperm (López-González et al., 2014).

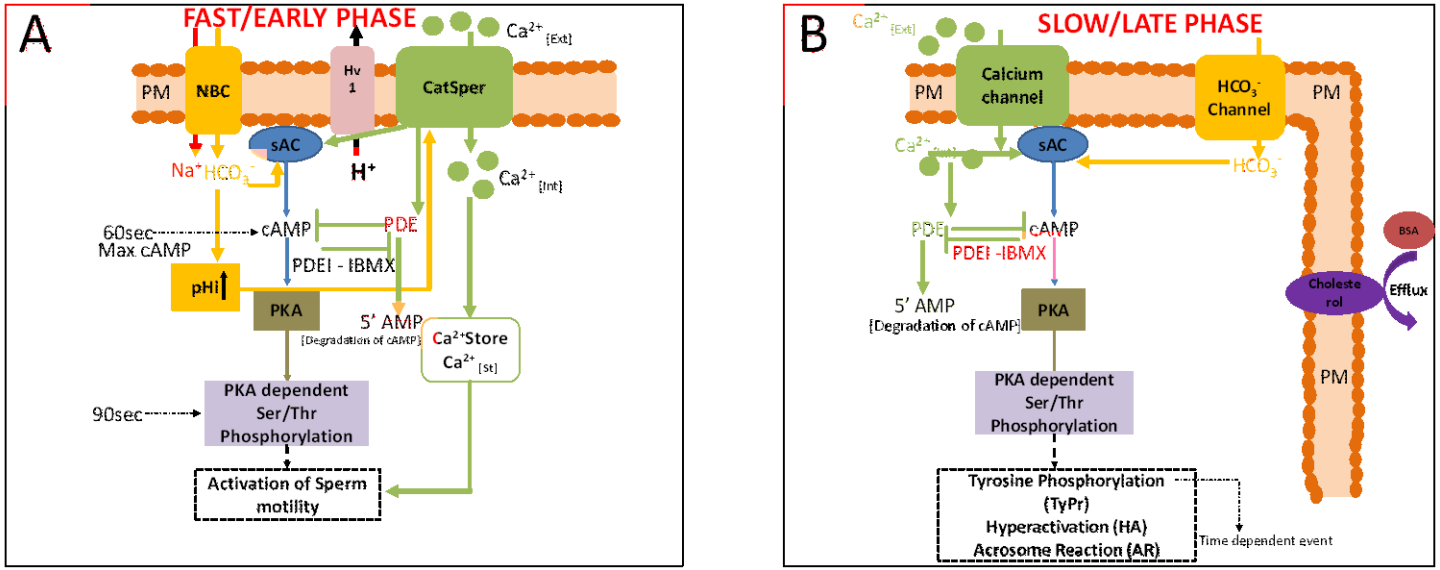


Figure 11: showing different fast/early and slow/late phase events associated with sperm capacitation.

Figure **A** shows the biochemical pathway relating to **fast/early phase capacitation**. In fast/early phase capacitation, sodium bicarbonate cotransporters in the plasma membrane (PM) transport Na^+ and HCO^{3-} into the cell, causing an increase in intracellular pH and activating soluble adenylyl cyclase (sAC). sAC can also be activated by an increase in $Ca^{2+}[i]$ through the sperm calcium channel (CatSper). Activation of sAC further leads to production of cyclic adenosine monophosphate (cAMP) (happens in 60sec). Excess levels of calcium activates phosphodiesterase (PDE) that hydrolyses cytoplasmic cAMP to 5' AMP (degradation of cAMP). Use of phosphodiesterase inhibitors (PDEI) (e.g. 3-isobutyl-1-methylxanthine (IBMX)) inhibits PDE activity and increases cAMP that activates protein kinase A (PKA), PKA responsible for phosphorylation of amino acid (serine/threonine) residues on target proteins.

Figure **B** shows the biochemical pathway relating to **slow/late phase capacitation**. In slow/late phase capacitation, cholesterol efflux from the plasma membrane (PM) by bovine serum albumin (BSA) ensures PM remodelling that changes the influx of calcium and HCO^{3-}. Both fast/early and slow/late phase capacitation events are centrally regulated by PKA. Different events such as Protein tyrosine phosphorylation (Increases with time), Hyperactivation and Acrosome reaction are associated with slow/late phase capacitation.

1.6.2 Hyperactivation

Hyperactivation (HA) is a whiplash-like form of sperm motility (behaviour) (de Lamirande and Gagnon, 1993) that is necessary for fertilisation and is considered an important marker for capacitation. (Ho and Suarez, 2001) observed that bovine sperm showing hyperactivated motility have an asymmetrical beating with deeper bends and, in in vitro conditions, they appear to be swimming vigorously in circles. It has been characterised in human sperm and is characterised and assessed by it kinematic characteristics (VCL, ALH and LIN) recorded by computer-assisted semen analysis (CASA, see below) (Mortimer and Mortimer, 1990, Burkman, 1984, Mortimer et al., 1997, Aitken et al., 1985). HA was first described by Yanagimachi when he stated that movement of hamster spermatozoa was extremely active after they had completed capacitation (YANAGIMACHI, 1970). Human sperm samples with good fertilisation rates in vitro show significant changes in HA during capacitation; whereas those samples demonstrate poor hyperactivation are associated with poor fertilisation rates, therefore hyperactivation is critical for fertilisation (Coddington et al., 1991, Pilikian et al., 1991, Quill et al., 2003, Suarez, 2008, Singh and Rajender, 2015, Alasmari et al., 2013a).

Motility characteristics of hyperactivated sperm differ in a number of ways from those of sperm showing activated motility and CASA is used to assess these characteristics in a population of sperm. When analysing using CASA, human sperm showing curvilinear velocity (VCL) $\geq$ 50µ m/s, linearity (LIN) <50%, increased amplitude of lateral head displacement (ALH) $\geq$7µm (Mortimer, 2000b) are considered as hyperactivated.

1.6.2.1 Types of hyperactivated motility

(i) **Hyperactivated Non-Progressive Motility**

In this type of human sperm motility, the flagellum beats highly asymmetrically, particularly in the proximal section, resulting in continuous tumbling or turning so that the cell fails to progress. CASA measurements from the cells show a high VCL and ALH but very low LIN and VSL. Such hyperactivated motility is considered as an essential process for sperm to detach from the oviductal epithelium, successfully penetrate the ZP and fuse with an oocyte. Regulation of hyperactivated motility is shown in figure18.

(ii) **Hyperactivated Progressive or Transitional Motility**

In this type of motility, the flagellum bends strongly (particularly in the proximal region), but beating is less asymmetric such that the sperm head follows a highly tortuous but progressive path (fig 12).

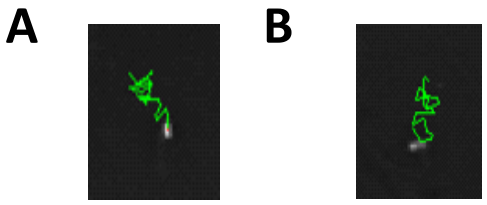


Figure 12: Showing different types of human sperm motility tracks. Image showing motility tracks of **A** Hyperactivated-progressive and **B** Hyperactivated non-progressive (green) human sperm. The tracks were analysed by the CASA. Sperm cell with (VCL) = 150µm/s, linearity (LIN) <50%, increased amplitude of lateral head displacement (ALH) =7µm (Mortimer, 2000) are considered as hyperactivated. When motility characteristics (VCL, ALH and LIN) were assessed along with progressive or straight line velocity (VSL), human sperm cell showing hyperactivated non-progressive motility (**B**) with a high VCL and ALH but very low LIN and VSL. In the case of hyperactivated progressive motility (**A**) show low VCL, ALH but high LIN and VSL (Compare to **B**).

1.6.3 Thermotaxis and Chemotaxis

In mammals, there may be guidance mechanisms that help sperm to reach the oocyte in the fallopian tube. (Eisenbach and Giojalas, 2006). Thermotaxis refers to sperm movement directed by a temperature gradient (Bahat and Eisenbach, 2006). Under in vivo conditions, sperm movement was observed from a cooler sperm reservoir (**Isthmus of the fallopian tube, 31°C**) to the warmer sperm fertilisation site (**Ampulla of the fallopian tube, 37°C**) (fig 13). Thermotaxis is believed to be a long-range guidance mechanism, which means it is probably generated at the time of ovulation and enables sperm to travel a distance of 3-5cm in humans (from the isthmus to the oocyte at the fertilisation site) (Harper, 1982, Tur-Kaspa, 1992). Human sperm are reported to sense temperature differences as small as 0.5°C, or maybe even less (Bahat et al., 2003, Bahat and Eisenbach, 2006).

The thermotaxis response in human sperm is thought to be operated by phospholipase C (PLC) and inositol triphosphate receptor (IP_3R) calcium channel (Bahat and Eisenbach, 2010). PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol (IP_3) and diacylglycerol (DAG). The released IP_3 binds to theIP_3R located in the internal calcium store to release stored calcium, which finally increases intracellular calcium. As the calcium in the store gets depleted this activates the store-operated channel (SOC) that pumps in extracellular calcium to refill the store (a mechanism known as store-operated calcium entry) (Dutta, 2000, Bahat and Eisenbach, 2010).

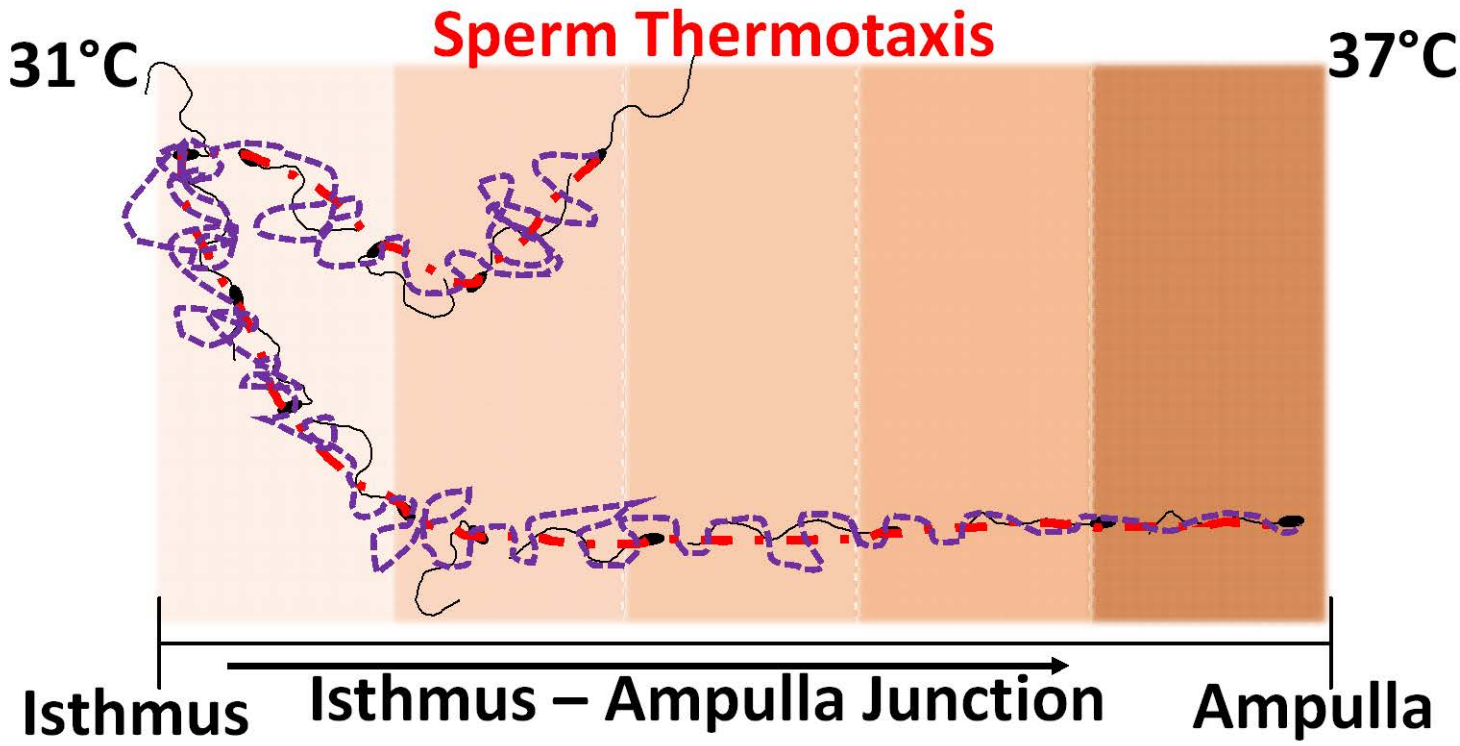


Figure 13: Human sperm guidance mechanism, showing human sperm response to a temperature gradient by thermotaxis. During a temperature shift from 31°C to 37°C a change in human sperm behaviour was observed. At 31°C, the sperm showed more hyperactivated motility, and as the temperature increased, the sperm's ability to show hyperactivated motility was decreased (Boryshpolets et al., 2015). The violet dotted line indicates the trajectory of the head and the red dotted line indicates average path. Under in vivo conditions, this temperature shift was observed between isthmus, isthmus-ampulla junction and ampulla acting as guidance mechanism in helping sperm to reach the oocyte.

Unlike thermotaxis, in **chemotaxis** only capacitated sperm cells respond to a concentration gradient of a **chemoattractant** (Eisenbach, 1999b, Eisenbach, 1999a, Eisenbach and Giojalas, 2006). As explained in above the section (section 1.6.2) hyperactivated sperm motility shows an asymmetrical flagellar beat pattern which causes a change of direction, and as chemotaxis is a response to a chemical gradient induced by a chemoattractant, the sperm may use hyperactivation to achieve chemotaxis. Chemotactically dominated hyperactivated motility might show a reduced flagellar beat response sufficient to cause turning and direct sperm towards the source of the attractant.

In humans, follicular fluid (FF) (Ralt et al., 1991, Ralt et al., 1994, Cohen-Dayag et al., 1995, Wang et al., 2001) and cumulus oophorous (CO) (Sun et al., 2005, Tamba et al., 2008) attract sperm. The active agent in FF is progesterone (P4) (Wang et al., 2001, Chang and Suarez, 2010), and CO cells secrete P4 (Oren-Benaroya et al., 2008, Chang and Suarez, 2010) to produce a gradient that which may act as a chemoattractant in guiding sperm towards the oocyte. In mammals, P4 induces sperm chemotaxis at very low (picomolar) concentrations (Teves et al., 2006, Teves et al., 2009) and is the only chemoattractant that is secreted by the CO (Guidobaldi et al., 2008, Teves et al., 2009).

On the basis of pharmacological manipulation a very complex mechanism has been proposed for the chemotactic response to progesterone, involving activation of tmAC (transmembrane adenyl cyclase)-cAMP-PKA pathway followed by TyrP at equatorial segment and flagellum, calcium mobilisation through IP_3R and SOC and finally activation of soluble guanylyl cyclase (sGC)-cAMP-protein kinase G (PKG) (Teves et al., 2009).

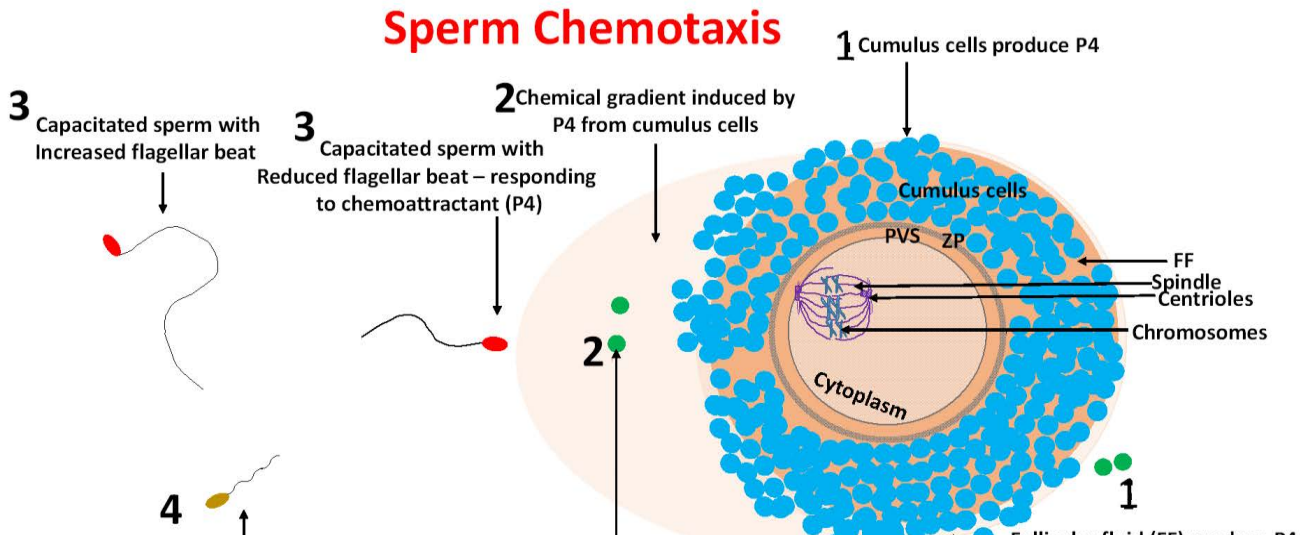


Figure 14: Figure showing human sperm chemotaxis. 1. Showing progesterone (P4) secreted by both cumulus cells and follicular fluid (FF). 2. P4 acts as a chemoattractant and induce a chemical gradient that guides sperm towards oocyte. 3.Change in capacitated human sperm motility behaviour from intense hyperactivated motility to motility with a reduced flagellar beat in response to the chemical gradient induced by chemoattractant (P4). 4. Showing non-capacitated human sperm not responding to the chemical gradient induced by chemoattractant (P4). FF – Follicular fluid, P4 – Progesterone, PVS – Perivitelline space and ZP – Zona pellucida.

1.6.4 Acrosome Reaction

The acrosome reaction (AR) is a capacitation-dependent exocytotic process (Abou-Haila and Tulsiani, 2000, Patrat et al., 2000, Breitbart, 2002) that is required for fertilisation. Only acrosome-reacted sperm can pass through zona pellucida (ZP), bind to the oocyte's PM and finally fuse with the oocyte. Various enzymes are involved in the sperm's penetration through the oocyte envelope. They are mainly hydrolytic (hyaluronidase) and proteolytic (acrosin) enzymes (Harper et al., 2008). The PM of the anterior region of the sperm's head fuses with the outer acrosomal membrane (OAM) which leads to membrane vesiculation and the exposure of the inner acrosomal membrane (IAM). The AR may have induced by the ZP, which acts as a ligand for the various sperm PM receptors (Cross et al., 1988, Patrat et al., 2000, Gupta et al., 2009) necessary for fertilisation. Binding of the sperm to the ZP induces an increase in intracellular calcium $[Ca^{2+}]_I$ that initiates sperm AR at the ZP's surface (Darszon et al., 2011). Under in vitro conditions, this exocytosis process is induced by the sperm's exposure to progesterone (P4) (Turner et al., 1994, DasGupta et al., 1994, Patrat et al., 2000). Calcium channels specific to sperm cell signalling (CatSper) are involved in P4-induced AR (Tamburrino et al., 2014).

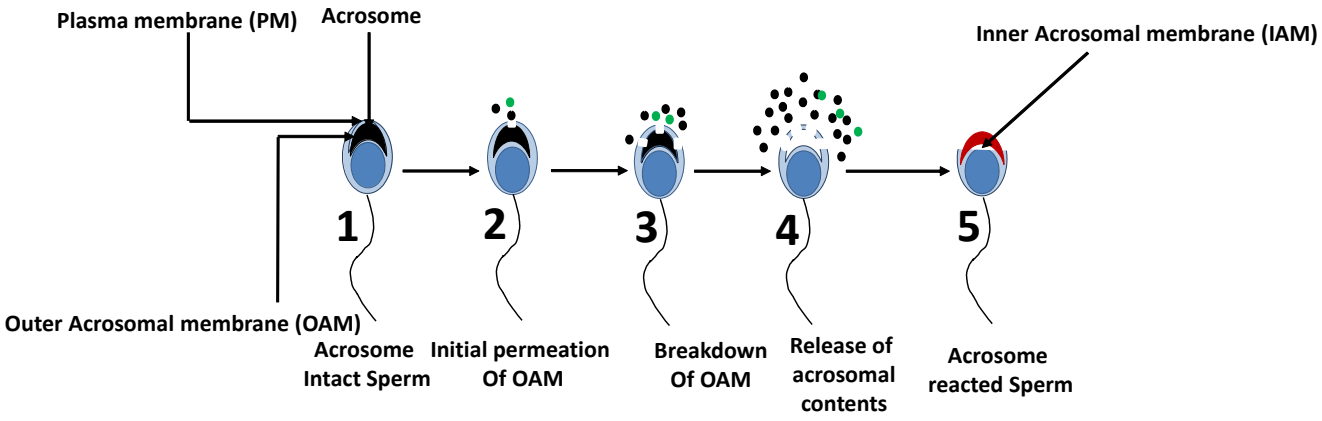


Figure 15: showing acrosomal exocytosis involved in acrosome reaction in human sperm. **1.** Showing an intact outer acrosomal membrane (OAM), looking like a cap-like structure, and the sperm's plasma membrane. This is under normal conditions. **2.** Showing initial permeation of OAM when sperm cells were exposed to AR-inducing stimulus. **3.** Showing breakdown of OAM **4.** Release and exposure of acrosomal contents (acrosin, hyaluronidase and **calcium**) into the immediate vicinity **5.** Resulting in an acrosome-reacted sperm capable of binding to the zona pellucida (Harper et al., 2008).

1.6.4.1 Zona pellucida and the AR

After penetrating the cumulus oophorous the sperm comes into contact with the ZP, that surrounds the oocyte in all vertebrates (Conner et al., 2005). Sperm-ZP binding is a highly species-specific process (Petit et al., 2014). This means that human sperm can't recognise mouse oocyte because they are unable to recognise the ZP's glycoproteins. Other than being species-specific, the ZP also protects the oocyte and prevents the occurrence of polyspermy (Conner et al., 2005). In the mouse, the ZP contains three glycoproteins: ZP_1, ZP_2 and ZP_3 (Wassarman, 1995b, Lefièvre et al., 2004, Wassarman, 2008, Litscher et al., 2009). ZP_3 acts as a sperm receptor and induces the AR. ZP_2 acts as secondary sperm receptor in sperm-oocyte binding (Rankin and Dean, 1996, Rankin et al., 1996). In contrast to the mouse, in humans there are four ZP glycoproteins: ZP_1, ZP_2, ZP_3 and ZP_4 (Lefièvre et al., 2004, Gupta et al., 2009, Gupta and Bhandari, 2011, Gupta, 2015). ZP_1, ZP_3 and ZP_4 induce the AR, and all four ZP glycoproteins are involved in sperm-oocyte binding. ZP_2 plays an important role in avoiding polyspermy (Gupta, 2015). In both mouse and human sperm induction of the AR is associated with biphasic (combination of transient and sustained) calcium influx, but it was best characterised in the mouse (Bailey and Storey, 1994, Shirakawa and Miyazaki, 1999, Patrat et al., 2000). In acrosome-intact mouse sperm, the zona receptor present on the outer acrosomal membrane binds to ZP_3 (oocyte) which results in an opening of T-Type voltage-operated calcium channel (VOCC) and a transient influx of calcium (last ~50sec) (Arnoult et al., 1996, O'Toole et al., 2000) followed by a slower sustained calcium $[Ca^{2+}]_i$ elevation. Prolonged ZP_3 interaction maintains this sustained calcium response. Sperm binding with ZP_3 leads to activation of phospholipase $C\gamma_1$ ($PLC\gamma_1$), resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and resulting in the production of inositol triphosphate (IP_3). This, in turn, binds to IP3R (IP_3R channel gets activated) and mobilises calcium from the acrosome (Roldan and Shi, 2007).

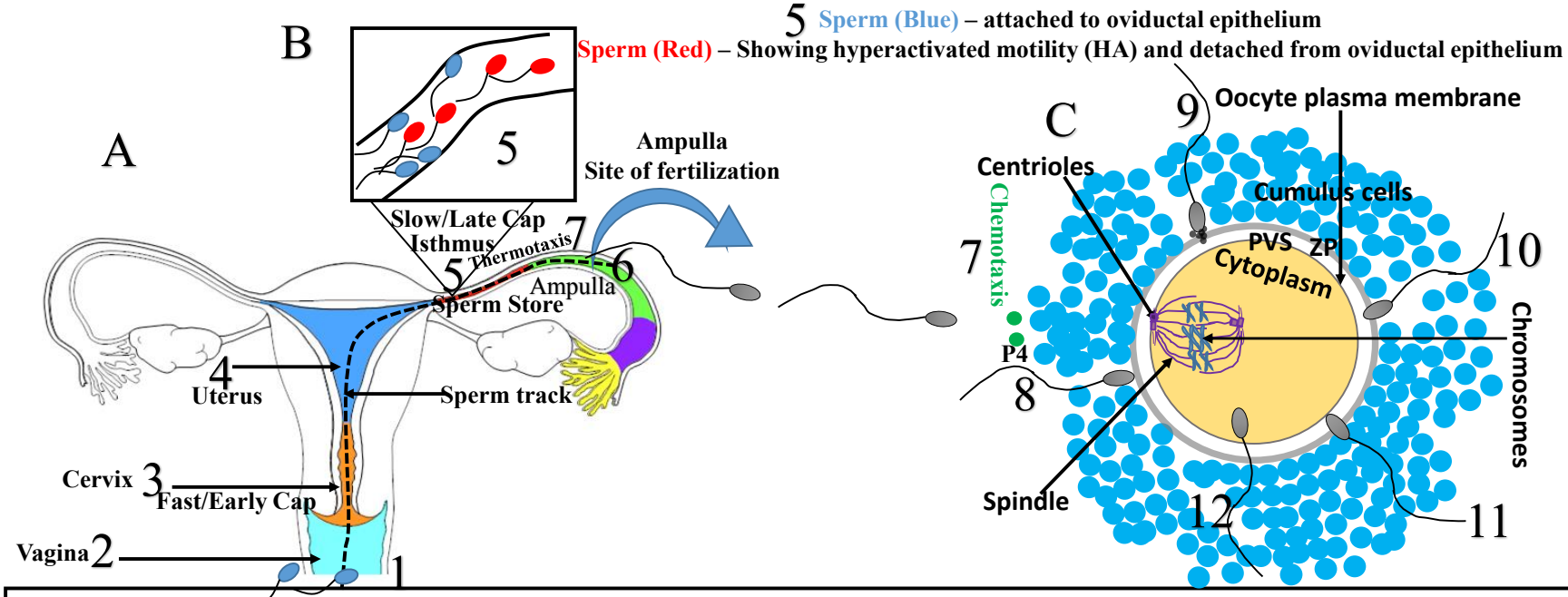


Figure 16: Showing human sperm transit in to female reproductive tract and Fertilization. Figure A Showing sperm journey in to female reproductive tract. 1. After coitus sperm journey begins in to female reproductive tract. **2**.The first point of entry is **vagina**. The environment (pH) in vaginal is acidic and as sperm in seminal fluid enters vagina it (seminal fluid) neutralises acidic environment protecting sperm from harsh vaginal environment. **3**. After vagina sperm moves in to **cervix mucus** where they begin fast/early phase capacitation. At this stage activation of sperm motility was observed. **4.** After cervix sperm move to **uterus** it induces a host reaction where leukocytes out number sperm (100:1) engulf abnormal sperm. **B 5**. **Isthmus** - Sperm are attached to oviductal epithelium and this stage sperm becomes quiescent (sperm in blue) and they are stored in readiness for ovulation. As the ovulation approaches sperm undergo a maturation process (slow/late phase) called capacitation that makes sperm to acquire reproductive competency and also shown hyperactivated motility that helps sperm in detaching from oviductal epithelium (sperm in red). **C 6** After isthmus sperm moves to **ampulla** – point of fertilisation. **7** For fertilisation to happen sperm undergo guidance mechanisms (**Thermotaxis (Temp gradient),** **Chemotaxis (Cumulus cells – P4)**) that pulls sperm towards oocyte and 8. HA enables sperm penetration of the surrounding layer of cumulus cells and sperm attaches to zona pellucida (ZP). **9** After attaching to ZP sperm undergo acrosome reaction (AR) and**10**along with HA enable sperm penetrate ZP.**11**Sperm binding to oocyte plasma membrane**12**and finally fusion of two gamete complete fertilization process.

1.7 Intracellular calcium signalling toolkit

In somatic cells, long-term regulation of cellular activity happens through control of genetic expression in response to signals from other cells or signals from the extracellular space. This control of gene expression occurs through transcription (DNA to RNA), translation (RNA to protein) and regulation of protein products. Signals from extracellular components, like hormones and cell growth factors, are involved in modification of final product (proteins) functions. One such mechanism is through changes in the intracellular calcium [Ca^{2+}_i] concentration (Costello et al., 2009).

Unlike somatic cells, the mature haploid sperm cell has a reduced cytoplasmic volume and loss of endoplasmic reticulum (ER). In addition replacement of histones with protamines and tight condensation nuclear DNA make the prevent the occurrence transcription and translation. To achieve successful regulation of cellular activity, sperm use modification of protein functions, termed **"post-translation modification"**. Post-translation modifications, therefore, are involved in controlling all cellular activities in a sperm cell.

In sperm cells, calcium signalling is particularly important in the regulation of different sperm functions, such as HA, chemotaxis and the AR (Publicover et al., 2007) and may be involved in penetration of mucus. SU cells performs better in viscous medium penetration compared to DG cells (see chapter-3) because they more effectively express CatSper and CatSper-mediated Ca^{2+} signals (Chapter 6). Defects of Ca^{2+} calcium in human sperm are associated with male infertility (Krausz et al., 1995, Baldi et al., 2000, Espino et al., 2009, Costello et al., 2009, Darszon et al., 2011, Williams et al., 2015). This chapter discusses the presence of the PM and intracellular calcium channels (See Figure 17) in human sperm.

1.7.1 Calcium channels in the PM

In the human sperm cell, four calcium channels are involved in PM calcium influx. These channels enable the flow of calcium into the cell to increase cytoplasmic $[Ca^{2+}]_{i.}$ These four PM-positioned calcium channels are storage operated calcium channels, transient receptor protein channels, voltage operated calcium channels and sperm-specific calcium channels.

1.7.1.1 Storage-operated calcium channels

An increase in intracellular calcium levels is achieved by Ca^{2+} influx through the PM and the release of calcium from intracellular stores. Both of these calcium pathways are necessary to achieve and control a variety of cellular functions in a cell. The release of calcium from intracellular stores is accomplished by IP_3-induced calcium release from stores (somatic cells ER; Acrosome/RNE – sperm cell) coordinated with Ca^{2+} influx through the PM, is achieved by process called storage-operated calcium entry (SOCE) or capacitive calcium entry (CCE) (Putney, 2009, Darszon et al., 2011). The calcium channels that are responsible for SOCE are called storage-operated calcium channels (SOCs). These SOCs are located in the PM of human sperm (Costello et al., 2009), and this SOCE pathway in a somatic cell is important (Davis et al., 2016) to regulate cellular functions.

SOCE-associated calcium influx across the PM causes a small storage-operated current, called the calcium-release-activated calcium current (I_{CRAC}) (Soboloff et al., 2006, Frischauf et al., 2008). Two membrane proteins play an important role in calcium entry into the cell through storage-operated calcium-release-activated calcium (CRAC) signalling (Derler et al., 2016). These membrane proteins are the stromal interaction molecule (STIM) and Orai (CRAC PM channel).

The STIM acts as ER located Ca^{2+} sensor (Putney, 2007, Fahrner et al., 2013) and the Orai membrane protein is involved in the calcium selective ion channel in the PM (Prakriya et al.,

2006, Hogan et al., 2010, Fahrner et al., 2013). Upon IP_3 binding to IP_3R induced calcium mobilisation from ER to cytoplasm that creates calcium depletion from the store. As the calcium gets depleted from the ER, the STIM1(an isoform of STIM which is the ER calcium sensor) detects the calcium content and the STIM1 gets redistributed close to the PM (Cahalan, 2009) where it couples and activates Orai1 (an isoform of Orai) (Cahalan, 2009, Fahrner et al., 2013). This results in the activation of the CRAC channel, which causes influx extracellular calcium into the cell (Soboloff et al., 2006). Other than STIM1 and Orai, the transient receptor potential channel (TRPC) is involved in CCE, where it forms protein clusters with Orai and helps in refilling the store (Elaib et al., 2016).

All three membrane proteins, STIM1, Orai and TRPC, are found in human sperm (Castellano et al., 2003a, Darszon et al., 2012, Lefievre et al., 2012, Correia et al., 2015). STIM1 is found in the neck/midpiece and the acrosome region of the head. Unlike in somatic cells, in human sperm cells, redistribution of STIM towards the PM won't happen; instead STIM is localised near the PM as calcium storage organelles (e.g. acrosome, neck/midpiece (Lefievre et al., 2012)) and SOCE could activate immediately (Correia et al., 2015)**.** Treatment of human sperm with 2-aminoethoxydiphenylborate (2-APB) initiates CCE, which promotes the interaction of STIM1 with storage-operated calcium channels (Lefievre et al., 2012). This activation by 2-APB significantly increases the P4-induced transient calcium response at sperm neck but does not affect the calcium responses in the sperm flagellum that are dependent on CatSper (Correia et al., 2015).

1.7.1.2 Canonical transient receptor potential channels

Canonical transient receptor potential channels (TRPC) are classified into TRPC, TRPV, TRPM, TRPP, TRPML and TRPN all together forms TRP superfamily (Huang, 2004). TRPC channels were first reported in Drosophila, where TRP gets activated in response to

continuous light exposure of Drosophila photoreceptor cells (Huang, 2004). Activation of TRPC results in the influx Ca^{2+}, raising $[Ca^{2+}]_{i.}$

TRPC1, TRPC3, TRPC6 and TRPC7 are found in human sperm (Castellano et al., 2003b). They are localised in the head and the flagellum, suggesting they may play an important role in HA (Trevino et al., 2001, Castellano et al., 2003b) and other events of fertilisation (e.g. capacitation and AR). Human sperm treated with SKF96365 (TRP channel inhibitor) show decreased motility in a dose-dependent manner, suggesting that the TRP channel is involved in sperm motility (Castellano et al., 2003b).

1.7.1.3 Voltage-operated calcium channels

VOCCs (Ca_v) are a family of voltage-operated calcium channel that contribute to the increase in $[Ca^{2+}]_i$ during PM depolarisation (Darszon et al., 2011). VOCCs are classified into two different functional groups: (i) High voltage activated (HVA); and (ii) Low voltage activated (LVA) (Darszon et al., 2011). With respect to their biophysical and pharmacological characteristics, both HVA and LVA VOCCs are divided into different types of currents: L, N, P, Q and R (Birnbaumer et al., 1994, Dunlap et al., 1995, Publicover and Barratt, 1999, Darszon et al., 2011). Strong depolarisation is necessary to activate HVA-VOCC channels, in contrast to LVA-VOCCs, which are activated by weaker depolarisation but inactivate rapidly and at less depolarised potentials compare to HVA VOCCs (Darszon et al., 2011).

The VOCC or Ca_v is formed by an α subunit and is encoded by a family of 10 genes ($Ca_v1.1$-1.4; $Ca_v2.1$-2.3; $Ca_v3.1$-3.3) grouped into three sub-families (Ca_v1; Ca_v2 and Ca_v3). Ca_v1 with its four genes $Ca_v1.1$-1.4 conducts L-type current, Ca_v2 with its three genes conducts P- and Q- ($Ca_v2.1$), N- ($Ca_v2.2$) and R- ($Ca_v2.3$) type currents. Ca_v3 with its three genes ($Ca_v3.1$; $Ca_v3.2$ and $Ca_v3.3$) conducts T-type currents (Darszon et al., 2011). Ca_v channels can be

regulated by activation of a G-protein-coupled mechanism involving PLC, DAG and activates PKC.

mRNA and or protein for all three Cav subfamilies are reportedly present in mammalian sperm. $Ca_v1.2$, Cav2.2, $Ca_v2.3$ (Goodwin et al., 2000), $Ca_v1.3$ and Cav2.3 (Trevino et al., 2004) have been detected in human sperm. In mouse sperm $Ca_v3.3$ is present in the midpiece and $Ca_v3.2$ is localised at the principle section and posterior sperm head (Serrano et al., 1999).

1.7.1.4 CatSper channel

CatSper is a cationic channel of sperm cells (Ren et al., 2001, Quill et al., 2001, Kirichok and Lishko, 2011, Brenker et al., 2012, Singh and Rajender, 2015). CatSper channels are expressed exclusively in the plasma membrane (PM) of the sperm flagellar principle (Quill et al., 2001, Ren et al., 2001, Qi et al., 2007, Singh and Rajender, 2015, Tamburrino et al., 2015). This channel is pH sensitive (Kirichok et al., 2006) and voltage-gated (Quill et al., 2001, Shukla et al., 2012, Singh and Rajender, 2015). Upon activation by increasing intracellular pH or by progesterone or prostaglandins (Lishko et al., 2011, Publicover and Barratt, 2011, Strunker et al., 2011, Brenker et al., 2012), CatSper mediates Ca^{2+} entry to raise $[Ca^{2+}]_{i,}$(Ren et al., 2001, Publicover et al., 2007, Navarro et al., 2008, Ren and Xia, 2010).

CatSper channels are made up of four homologous alpha subunits and three additional auxiliary subunits. They are CatSper α (α_{1-4}), CatSper β, CatSper γ and CatSper δ (Lishko et al., 2010, Singh and Rajender, 2015). CatSper α 1 (Ren et al., 2001, Singh and Rajender, 2015), CatSper α 3 and α 4 (Jin et al., 2005, Qi et al., 2007, Singh and Rajender, 2015) are expressed in late stage spermatids. CatSper2 protein is expressed in early stage sperm cell division (pachytene spermatocytes) (Quill et al., 2001, Schultz et al., 2003, Singh and

Rajender, 2015). The three auxiliary subunits - CatSper β (Liu et al., 2007), CatSper γ (Wang et al., 2009) and CatSper δ (Chung et al., 2011) - are expressed in spermatocytes and spermatids (Singh and Rajender, 2015).

CatSper is required for hyperactivated motility, successful detachment from the oviductal epithelium and fertilisation (Qi et al., 2007, Ho et al., 2009, Singh and Rajender, 2015, Ernesto et al., 2015). Absence of any of the four CatSper alpha subunit proteins results in failure of channel expression, which will in turn affect sperm hyperactivated motility (Qi et al., 2007) and the fertilising potential in human sperm (Williams et al., 2015).

CatSper channel is activated by different agonists such as progesterone (P4) (Lishko et al., 2011, Publicover and Barratt, 2011, Brenker et al., 2012) and Prostaglandins (Lishko et al., 2011, Brenker et al., 2012). These hormones (progesterone and prostaglandins) are produced by cumulus cells that surrounds the oocyte and follicular fluid (FF). P4 stimulates calcium increase in a non-genomic fashion (Publicover et al., 2007, Strunker et al., 2011) that ensures rapid calcium influx. An increase in intracellular pH also activates the CatSper channel that ensures rapid influx of extracellular calcium (Strunker et al., 2011).

1.7.2 Calcium pumps

The main purpose of calcium pumps is to maintain basal (resting) levels of $[Ca^{2+}]_i$ and to return $[Ca^{2+}]_i$ to basal levels after the completion of necessary cell functions in somatic and sperm cells. This is achieved either by pumping calcium into the stores or into the extracellular environment. This calcium clearance mechanism is achieved by ATP-driven Ca^{2+} pumps (Ca^{2+} ATPases) and/or Na^{+} - $Ca2^{+}$ exchanger (NCX) that eject calcium from the cytoplasm into the extracellular environment or move calcium from cytoplasm into the intracellular compartments (Michelangeli et al., 2005, Jimenez-Gonzalez et al., 2006).

Plasma membrane Ca^{2+} ATPase (PMCA) is the largest Ca^{2+} ATPase pump with a molecular weight ranging between 130 and 140Kda (Carafoli and Brini, 2000, Jimenez-Gonzalez et al., 2006) It is located exclusively in the principle piece in mammalian sperm (Wennemuth et al., 2003, Okunade et al., 2004, Schuh et al., 2004, Jimenez-Gonzalez et al., 2006). PMCA4 (isoform of PMCA) knockout in mice are incapable of inducing HA motility (Okunade et al., 2004). More than 90% of the PMCA proteins contain a PMCA4 isoform (Okunade et al., 2004, Jimenez-Gonzalez et al., 2006).

Sarco/Endoplasmic reticulum Ca^{2+} ATPase (SERCA) is localised in the ER and ensures the refilling of the calcium store. SERCA1 (isoform of SERCA) is expressed in skeletal muscle, SERCA2 is expressed in tissues, and SERCA3 has limited expression (Correia et al., 2015). SERCA2 is localised at the acrosome and midpiece of the human sperm (Lawson et al., 2007, Correia et al., 2015). Apart from the SERCA pump, another calcium pump called secretory pathway Ca^{2+} ATPase (**SPCA**) is present in the Golgi apparatus in somatic cells (Wootton et al., 2004, Correia et al., 2015). In human sperm, SPCA1 has been detected in the redundant nuclear envelope (RNE) of human sperm (Harper et al., 2005, Correia et al., 2015).

Na^{+}-Ca^{2+} exchanger (**NCX**) has a coupling ratio of 1:3, exchanging one Ca^{2+} for three Na^{+} across the PM. In forward mode this occurs as extrusion of one Ca^{2+} for entry of three Na^{+}, and is thus driven by the membrane Na^{+} gradient (Jimenez-Gonzalez et al., 2006)). NCX1.3 and NCX1.7 are found in rat testis (Quednau et al., 1997) and observed to be localised in the PM of the flagellum (Bradley and Forrester, 1980). NCX is present in the PM of human sperm and any loss or inhibition of NCX activity could lead to a disturbance in the distribution of Na^{+}-$Ca2^{+}$ ions and inhibit human sperm motility (Krasznai et al., 2006, Peralta-Arias et al., 2015).

1.7.3 Mobilisation of stored calcium through intracellular store channels

Ca^{2+} stores are involved in the calcium signalling process in somatic cells. In somatic cells, Ca^{2+} is stored primarily in the endoplasmic reticulum and is mobilised upon activation of intracellular Ca^{2+} channels by secondary messengers. Apart from the ER, other organelles, such as the sarcoplasmic reticulum (SR), mitochondria, nuclear envelope and Golgi apparatus, have all been identified as intracellular calcium stores in somatic cells (Michelangeli et al., 2005). There are at least two intracellular channels that release stored Ca^{2+}: inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs), both of which have been identified in mammalian sperm (Walensky and Snyder, 1995, Naaby-Hansen et al., 2001, Costello et al., 2009, Correia et al., 2015).

1.7.3.1 Inositol 1,4,5-trisphosphate receptors

IP_3R-associated calcium signalling exists in somatic and sperm cells, and involves the generation of the diffusible secondary messenger inositol 1,4,5-trisphosphate (IP_3) (Vermassen et al., 2004, Jimenez-Gonzalez et al., 2006) (Parys and De Smedt, 2012).. Binding of IP_3 to IP_3R activates the release of calcium from stores into the cytoplasm. IP_3R activity is also regulated by calcium itself: when cytoplasmic calcium concentrations are low (~300nM), then IP_3 binding affinity to IP_3R increases and results in the mobilisation of calcium from stores to the cytoplasm. The presence of higher concentrations of calcium in the cytoplasm inhibits IP_3R activity. Three isoforms of the IP_3R family have been identified: IP_3R_1, IP_3R_2 and IP_3R_3.

Three isoforms of IP3R occur, with 75% sequence similarity to one another (Taylor et al., 1999, Jimenez-Gonzalez et al., 2006). It is the IP_3R_1 isoform that is expressed in the anterior region of the acrosome in mammalian sperm (Kuroda et al., 1999). IP_3R, G-protein and phospholipase-α (PLCα) are present in mammalian sperm, and these proteins are involved in

agonist-induced IP_3 production (Walensky and Snyder, 1995, Kuroda et al., 1999, Jimenez-Gonzalez et al., 2006). Dissociation of the G-protein subunit Gα upon receptor activation leads to activation of phospholipase $C\beta_1$ ($PLC\beta_1$), which results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and the production of IP_3 and diacyl glycerol (DAG). The IP_3 binds to and activates IP_3R to release calcium from stores (anterior region of acrosome) into the cytoplasm.

1.7.3.2 Ryanodine receptors

Ryanodine receptors (RyRs) are so-called because of their activation by the alkaloid ryanodine (Van Petegem, 2012). The molecular mass of these receptors is about ~2.2MDa (Lai et al., 1988, Lanner et al., 2010). Similarly, to IP_3Rs, RyRs are an intracellular calcium channel located on the ER/SR membrane that is involved in releasing stored calcium into the cytoplasm. The family of RyRs contains three different isoforms: RyR_1, RyR_2 and RyR_3. All of these are homotetrameric proteins that are activated by Ca^{2+} and regulated by proteins (e.g. calsequestrin (CSQ)-SR luminal calcium binding protein (Zhang et al., 2015) and Calreticulin in ER (Ellgaard and Helenius, 2003)). RyR_1 is expressed primarily in skeletal muscle, RyR_2 is in cardiac muscle and RyR_3, which has the widest distribution, is expressed in brain tissue. (Brini, 2004, Jimenez-Gonzalez et al., 2006).

In somatic cells (e.g. cardiac muscle), calcium entering the cytoplasm through plasma membrane channels activates ryanodine receptors, causing Ca^{2+}-induced Ca^{2+} release (CICR) (Zhang et al., 2015). In addition, RyRs are activated by cyclic-adenosine diphospho-ribose (cADPR) which thus acts as a secondary messenger (Ogunbayo et al., 2011).

If RyR activation leads to depletion of stored Ca^{2+} (and a significant reduction in the calcium gradient across RyRs) this may lead to closure of the channel. As a result, the cytoplasmic calcium drops down within 15ms, eventually preventing the CICR mechanism. Low store

(SR) calcium will stop the reopening of the RyR channel, resulting in a termination of store calcium release (Guo et al., 2012, Laver et al., 2013, Zhang et al., 2015).

Ryanodine receptors isoforms RyR_1 and RyR_3 are expressed in mouse spermatocytes and spermatids, but no expression of RyR_2 was observed in mouse sperm (Giannini et al., 1995). Later investigations into RyR localisation detected only RyR_3 in mature sperm (Treviño et al., 1998). RyR_3 expression could be detected in both acrosome-intact and acrosome-reacted mouse sperm (Treviño et al., 1998) from studies performed using RT-PCR and RyR_3-specific RyR antibodies.

Investigations into RyR localisation in human sperm, performed by Harper et al. (2004), using a fluorescent ryanodine analogue (BODIPY-FL-X-ryanodine) detected RyRs primarily at the posterior head/neck (PHN) region though some atining of the acrosome was also reported. These authors also reported that calcium oscillations in human sperm were independent of IP_3Rs, but the oscillations could be modified by application of ryanodine: with a low dose of ryanodine application, an increased frequency of intracellular calcium oscillations was observed and at higher doses of ryanodine application, a decrease in frequency of intracellular calcium oscillations was observed.

High doses of RyR application can also affect spermatogonial proliferation and increase cell meiosis (Chiarella et al., 2004). Observations highlighted here suggests that RyR plays an important role in sperm development and potentially in secondary messenger-mediated calcium mobilisation from store (PHN) to the cytoplasm when sperm are exposed to P4.

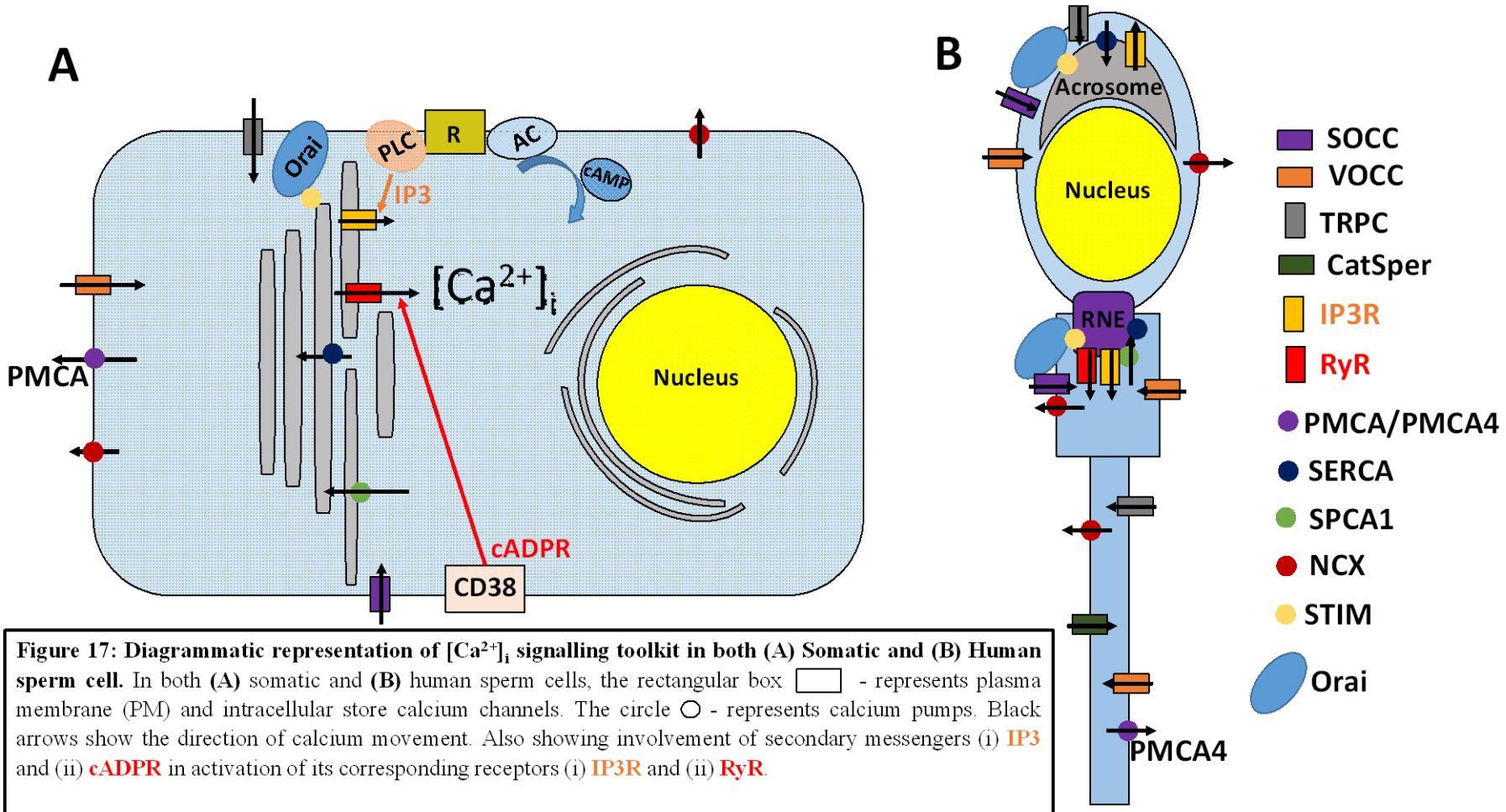


Figure 17: Diagrammatic representation of $[Ca^{2+}]_i$ signalling toolkit in both (A) Somatic and (B) Human sperm cell. In both **(A)** somatic and **(B)** human sperm cells, the rectangular box ▭ - represents plasma membrane (PM) and intracellular store calcium channels. The circle ○ - represents calcium pumps. Black arrows show the direction of calcium movement. Also showing involvement of secondary messengers (i) **IP3** and (ii) **cADPR** in activation of its corresponding receptors (i) **IP3R** and (ii) **RyR**.

1.8 Role of calcium in regulation of motility

Events such as HA, chemotaxis and the AR are all associated with $[Ca^{2+}]_i$ signalling in human sperm, which is necessary to facilitate successful fertilisation. Signalling of $[Ca^{2+}]_i$ is achieved by the influx of calcium from the extracellular milieu to the intracellular cytoplasm through the CatSper channels. Intracellular calcium signalling can also be achieved by calcium flow from the intracellular store to the cytoplasm. The mobilisation of calcium from either stores or CatSper results in initiating different characteristic behaviours (Alasmari et al., 2013b) (see Figure 18) that play a key role in sperm transportation and fertilisation. During the sperm's journey in the female tract, it encounters viscous and viscoelastic environments. To penetrate successfully through these environments, the sperm selects and switches between different behaviours for which calcium signalling is crucial. After migrating through these environments, calcium is also essential to initiate particular behaviour that helps it to detach from the oviductal epithelium, penetrate through the cumulus matrix and ZP and to fertilise the oocyte. To artificially (in vitro) induce different behaviours, sperm were treated with different drugs like 4-Aminopyridine (4-AP), Progesterone (P4) and Prostaglandin (PE1). Further explanation about 4-AP was enclosed below.

Please see Figure 19 for an illustration of the mobilisation of calcium from stores and CatSper channel in human sperm.

4-Aminopyridine (4-AP)

4-Aminopyridine (4AP), also known as Fampridine (Goodman et al., 2009), is used primarily as a broad spectrum potassium (K^+) channel blocker (Aronson, 1992, Ishida and Honda, 1993, Grimaldi et al., 2001, Bhaskar et al., 2008, Goodman et al., 2009). It bears a single amino group and is a weak base (Gobet et al., 1995), which can exist either in an ionized or non-ionized form (Choquet and Korn, 1992). Therapeutically 4AP is used to treat neurological disorders such as multiple sclerosis (MS), a disease that effects the central

nervous system (Judge and Bever Jr, 2006). Clinical studies conducted on MS patients has shown an improvement in the neurological condition when treated with the 4-AP (Goodman et al., 2009) by blocking the Potassium (K^+) channel present in the neurons. Application of this drug clearly improved the walking ability in the MS patients. Although this drug has a therapeutic importance, it can cause some side effects such as epilepsy (partial seizures), anxiety and respiratory distress (Peña and Tapia, 1999, Pena and Tapia, 2000, Sun et al.,

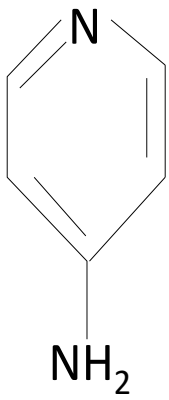


Figure 18. Showing the structure of the 4-Aminopyridine/Fampridine (4-AP). Showing the structure of the 4-Aminopyridine/Fampridine (4-AP) with amino group ($-NH_2$).

$[Ca^{2+}]_i$ and activity, since CatSper channels (sperm-specific Ca^{2+} channels) are voltage sensitive. However, KSper activity and sperm Vm are insensitive to 4AP (Mansell et al, 2014).

In addition to its role as a K^+ channel blocker, 4-AP is able to raise $[Ca^{2+}]_i$ in a range of cell types, apparently by mobilising stored Ca^{2+} (Gobet et al., 1995, Grimaldi et al., 2001, Bhaskar et al., 2008, Alasmari et al., 2013b). Grimaldi et al (2001) reported that in astrocytes and neurons, 4-AP induced dose dependent, reversible $[Ca^{2+}]$i responses that were independent of

extracellular calcium $[Ca^{2+}]_o$ (Grimaldi et al., 2001). (Grimaldi et al., 2001)(Grimaldi et al., 2001) Assessment of IP_3 levels indicated that 4AP treatment activated the phospholipase C pathway (PLC) (Grimaldi et al., 2001). (Alasmari et al., 2013b) showed that in human sperm 4AP raised $[Ca^{2+}]_i$ independently of $[Ca^{2+}]_o$.

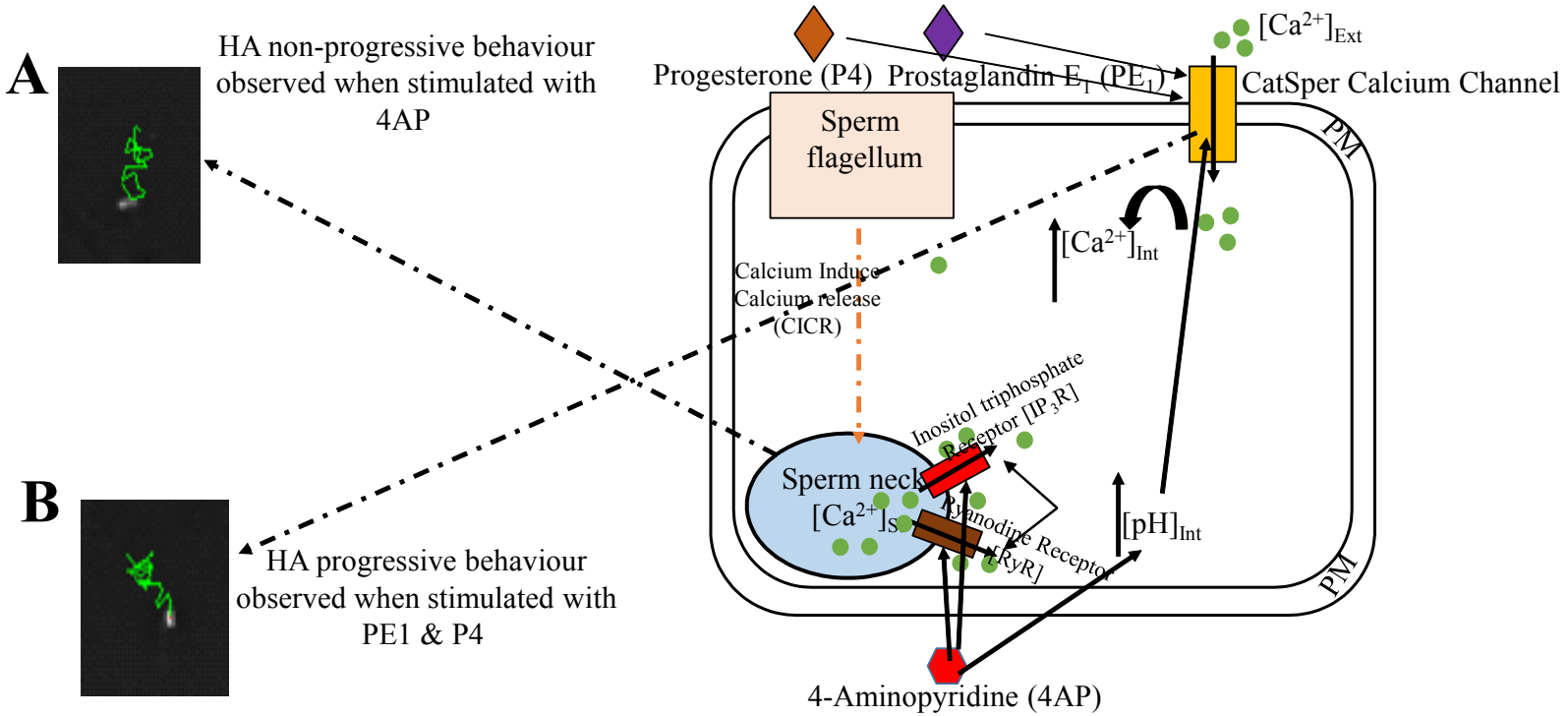


Figure 19: Calcium is responsible for initiating different behaviours in human sperm. A. Explains that the stimulation of human sperm with 4 aminopyridine (4AP) activates intracellular calcium signalling $[Ca^{2+}]_i$ by mobilising calcium from the sperm neck region. Activation of $[Ca^{2+}]_i$ signalling by 4AP triggers hyperactivated non-progressive behaviour. Hyperactivated non-progressive behaviour is associated with deep flagellar bend with an increase in curvilinear velocity (VCL) and a decrease in progressive velocity (VSL) and linearity (LIN). **B.** Explains that stimulating human sperm with prostaglandin (PE_1) and progesterone (P4) activates $[Ca^{2+}]_i$ signalling by mobilising calcium from the sperm principle piece (CatSper). Activation of $[Ca^{2+}]_i$ signalling by PE_1 and P4 triggers hyperactivated progressive behaviour, which is ideal for sperm to penetrate viscous and viscoelastic environments.

Aims and Objectives

Aims

During ascent of the female tract, sperm must navigate various physically and anatomically complex environments. Sperm can adopt a number of different behaviour types and the ability to select different behaviours as required may be crucial to achieving successful fertilisation.

The aim of this research study is to: **(i)** investigate the effects of different preparation techniques (SU & DG) on sperm motility characteristics (VCL, ALH and %LIN), on their penetration of artificial mucus; and on their response sot manipulation of Ca^{2+} signalling and **(ii)** study the significance of human sperm behaviours and their regulation by $[Ca^{2+}]_i$ in the penetration of artificial mucus.

Objectives

To achieve the aims, the following objectives were designed:

- To investigate the effect of sperm preparation methods (density gradient centrifugation and direct swim-up) on human sperm motility and on the penetration of artificial mucus (methylcellulose) – **Chapter 3**.

- To investigate the effects of different behaviours, induced by manipulation of Ca^{2+} stores and CatSper channels, on the penetration through artificial viscous (methylcellulose) and viscoelastic (polyacrylamide) media of human sperm prepared using density gradient (DG) centrifugation and direct swim-up (SU) – **Chapter 4.**

- To assess whether any differences in motility between swim-up and density-gradient prepared sperm are associated with a difference in sperm maturation (capacitation) – **Chapter 5.**

- To investigate possible differences in CatSper channel expression and function, assessed using progesterone-induced $[Ca^{2+}]_i$ responses and immunofluorescent assessment of CatSper expression, between swim-up and density-gradient prepared cells – **Chapter-6.**

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were cell culture tested (for suppliers see Appendix 9.1). Media were prepared in the laboratory using chemicals obtained from Sigma-Aldrich Ltd (for a list of chemicals used in preparing media, please see Appendix 9.2). They were supplemented immediately before use with 0.3% (w/v) fatty-acid-free bovine serum albumin (BSA) acquired from United States Biological (Swampscott, MA, USA) and distributed by Stratech Scientific Limited (Oaks Drive, Suffolk, UK), unless otherwise stated. Osmotic strength was maintained by adjusting NaCl content. Human sperm cells were separated from semen by using direct swim-up and the Percoll-density gradient method. Percoll was purchased from Sigma-Aldrich, UK.

For the intracellular calcium imaging experiments, Pluronic F-127 (1 ml in 20% solution in Dimethyl sulfoxide (DMSO)) and Fluo-4 AM were obtained from Invitrogen Molecular Probes, and poly –D-lysine from BD-Biosciences, UK.

Anti-phosphotyrosine antibody for Western blotting was obtained from New England Biolabs Ltd (Hitchin, Hertfordshire, UK) and Goat anti-Mouse IgG secondary antibody was purchased from LI-COR Biosciences, Ltd. UK.

For the immunofluorescence experiments, 4% formaldehyde was used as a cell fixing agent (37% stock solution), purchased from Merck Chemicals, UK. Rabbit anti-CatSper four polyclonal antibody (used at 1:50 and 1:100) and control antigen (used at 1:50) was obtained

from Alomone Labs (Jerusalem, Israel). Fluorescein isothiocyanate (FITC)-conjugated affini pure goat anti-rabbit immunoglobulin (IgG) was obtained from Jackson Immuno Research Laboratories, West Baltimore Pike, West Grove, PA, USA. For blocking non-specific sites, normal goat serum (NGS) (used at 1:10) was procured from Sigma-Aldrich Company Ltd., Dorset, UK. Rabbit serum was used as a negative control (used at 1:100) and was purchased from Santa Cruz Biotechnology, Dallas, TX, USA. PetmaFluor aqueous mounting medium was used to mount cell smears in preparation for viewing by fluorescence microscopy; this was obtained from Thermo Scientific. Phosphate buffer saline (PBS) was obtained from Sigma-Aldrich Company Ltd., Dorset, UK. For sperm cell permeabilisation, 0.2% Triton X-100 was purchased from Sigma-Aldrich Company Ltd., Dorset, UK.

All other chemicals, including 4-aminopyridine (4AP), prostaglandin E_1 (PE_1), progesterone (P4) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPEs), were bought from Sigma-Aldrich Company Ltd., Dorset, UK. Agonists 4AP and P4 were dissolved as a concentrated stock solution in dimethyl sulfoxide (DMSO), and PE_1 was dissolved as a concentrated stock solution in ethanol where necessary, and diluted in supplemented Earle's balanced salt solution (sEBSS) containing 0.3% BSA, 15 mM HEPES at pH 7.4 and osmolarity 292 mOsm before application.

2.1.2 Laboratory Instruments and Consumables

Samples were collected in 100 ml specimen pots from Alpha Laboratories (Hampshire) and transferred to 5ml round bottom swim-up tubes supplied by Starlabs UK Ltd (Milton Keynes, UK). Computer-assisted sperm analysis (CASA) was performed using a Hamilton Throne CASA system running CEROS v.12 (Massachusetts, USA). For the human in vitro sperm penetration assessment, 5cm flattened capillary tubes were used (0.4x4.0mm section, 0.2 mm inner depth; C M Scientific). Phosphotyrosine proteins were separated by sodium dodecyl

sulphate – polyacrylamide gel (10%) electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane by using the western blot/immunoblot method.

Human sperm cell imaging was performed using a perfusion chamber manufactured by Biosciences Workshop (University of Birmingham, UK), mounted on a Nikon TE300 inverted fluorescence microscope, fitted with a Cairn Opto LED light source using either a Rolera-XR cooled CCD camera or an Andor Ixon 897 EMCCD camera controlled by a PC running iQ v.3 software (Andor Technology, Belfast, UK).

2.2 Donor Recruitment

Human research sample donors were recruited at the Department of Biosciences at the University of Birmingham (UK) in accordance with the Human Fertilisation and Embryology Authority (HEFA) code of practice. An ethical review of the research self-assessment form was submitted online, and ethical approval was obtained from the University of Birmingham's Department of Biosciences Ethical Committee; all donors gave informed written consent to the research.

2.3 Experimental Work flow

The semen sample was split and different sperm preparation techniques (Direct Swim up (SU) and Density gradient (DG) centrifugation) were applied to the two portions of the sample. See sections 2.4.1 and 2.4.2. for technical details. After sperm preparation the DG & SU cells were left to capacitate (sperm cell maturation) for 4 h 30 min in an incubator (37^{o}C, 5% CO_2). After the completion of capacitation, concentration (both DG & SU) was adjusted to $3x10^6$ sperm cells/ml and cells were then aliquoted into multiple tubes (aliquot volume of 100µl).

To assess the hyperactivation (HA) in DG and SU cells, both untreated (control) and cells treated with different agonists (4AP, P4 and PE_1) were analysed and compared for different parameters (VCL, ALH and %LIN) that define human sperm hyperactivated motility, using computer assisted semen analysis (CASA). After the completion of CASA analysis, the Kremer Penetration Test was performed (section 2.6; figure 2.5). Cells (DG & SU) were left to incubate for 1 hr in the incubator at 37°C and penetration into and through viscous/viscoelastic column was assessed by counting their numbers at 0, 1 and 2cm from the base of the capillary tube. In vitro penetration was compared between untreated (control) cells and cells stimulated with different agonists (4AP, PE_1 and P4) at different distances (0,1, & 2cm) (section 2.6).

After this step, the Kremer tubes were placed on the CASA microscope stage and the hyperactivation parameters (VCL, ALH & %LIN) were analysed and compared in both untreated (Control) and cells treated with different agonists (4AP, P4 &PE1) at different distances (0, 1 & 2cm). The same experimental design was carried out for different donors.

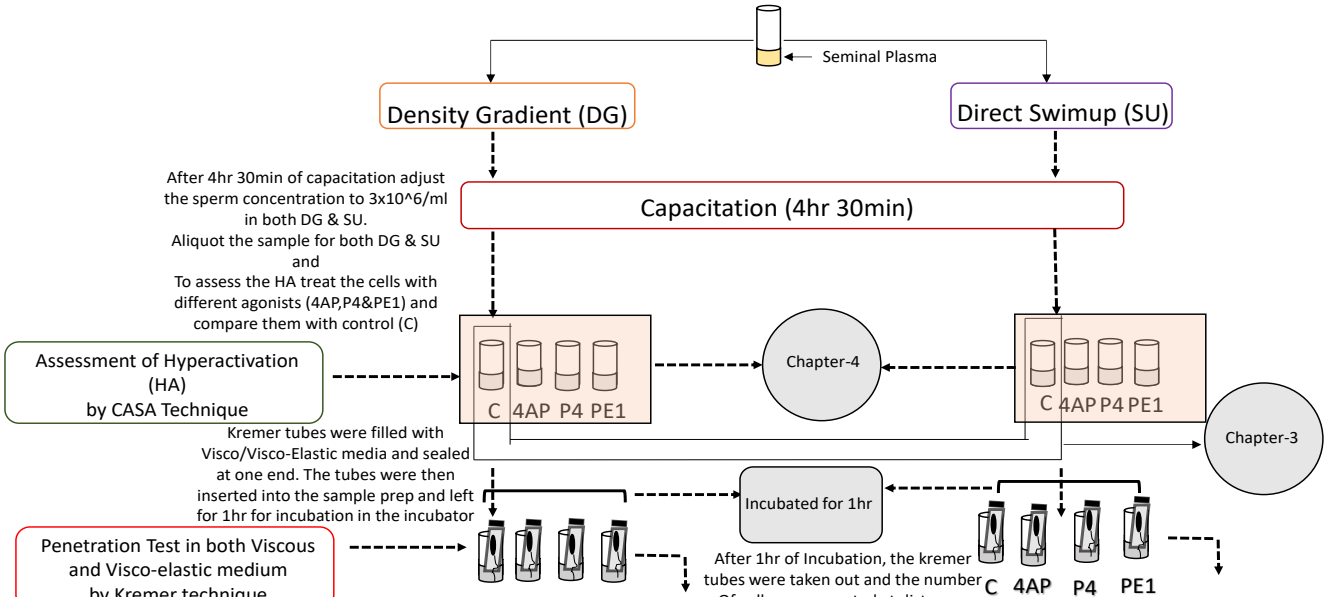


Figure 2.0: Flow chart showing the experimental design. Human sperm cells are separated from the liquefied semen sample using the density gradient (DG) and Direct swim-up technique (SU). After sperm cells were from separated from the liquefied semen sample both DG and SU cells were left to capacitate for 4hr 30min at 37°C in the incubator. After capacitation the cell concentration was adjusted to 3x10^6/ml in both DG and SU preparations and then the cells were aliquoted. To assess the hyperactivation (HA) in DG and SU cells, both untreated (Control) and cells treated with different agonists (4AP,P4 &PE1) were analysed and compared for their hyperactivation parameters (VCL,ALH &%LIN) using Computer assisted semen analysis (CASA). After CASA analysis, kremer's penetration technique was performed where 5cm glass capillary tubes were filled with either 1% (w/v) viscous (methylcellulose) or viscoelastic (polyacrylamide) medium as an in vitro penetration medium. One end of the tube is sealed with cristal seal wax and the other end is placed in contact with the sperm reservoir. Cells (DG & SU) were left to incubate for 1hr in the incubator at 37°C, and after the incubation penetration of motile human sperm into and along the viscous/viscoelastic column is assessed by counting their numbers at 0, 1 and 2cm from the base of the capillary tube. In vitro penetration is compared between untreated (control) cells and cells stimulated with different agonists (4AP, PE_1 and P4). After this step, the kremer tubes were then placed on the CASA stage and the hyperactivation parameters (VCL,ALH & %LIN) was analysed and compared in both untreated (Control) and cells treated with different agonists (4AP,P4 &PE1). The same experimental design was carried out for different donors.

2.4 Human Sperm Preparation

2.4.1 Direct Swim-Up (SU)

Highly motile spermatozoa were harvested by direct swim-up (~60 min) (Figure 2.1) into supplemented Earle's balanced salt solution (sEBSS) containing 0.3% BSA. Cell concentration was determined using an improved Neubauer haemocytometer and adjusted to $6x10^6$/ml. Cells were left to capacitate for further 4.5 hr in an incubator (37ºC, 5% CO_2). Afterwards, the cell density was adjusted to $3x10^6$/ml and used for the experimental study.

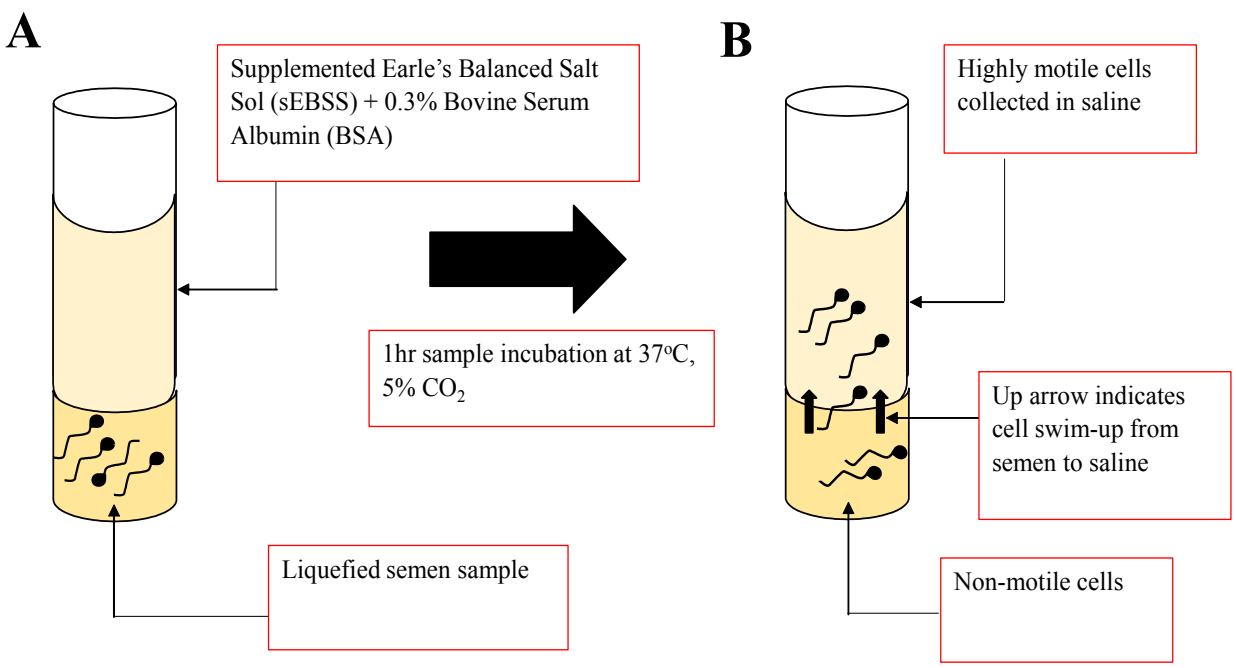


Figure 2.1: Sperm cell preparation technique – direct swim-up. Human sperm cells are separated from the liquefied semen sample using the direct swim-up technique. **A**. The liquefied semen sample is placed at the bottom of the swim-up tube under Supplemented Earle's Balanced Salt Solution (sEBSS) containing 0.3% Bovine Serum Albumin (BSA). **B**. After 1 hour of incubation at 37ºC, 5% CO_2, highly motile cells are collected from the sEBSS. The upward arrows indicate highly motile sperm cells swimming up from semen into saline during the sample incubation.

2.4.2 Density Gradient (DG) Centrifugation

1ml of semen was gently added to the top layer of the density gradient (1ml of 80% Percoll overlaid with 1ml of 40% Percoll). Percoll gradients made isotonic with M medium. (For a list of chemicals used in preparing M medium, please see Appendix 9.2.) Cells were centrifuged at 300 g for 20 min. The pellet was washed in phosphate buffer saline (PBS) (500 g, 10 min) and then resuspended in sEBSS (0.3% BSA). Cell concentration was determined using an improved Neubauer haemocytometer and adjusted to $6x10^6$/ml. Cells were left to capacitate for 4.5 hr in an incubator (37ºC, 5% CO_2). Afterwards, the cell density was adjusted to $3x10^6$/ml and used for the experimental study (Figure 2.2)

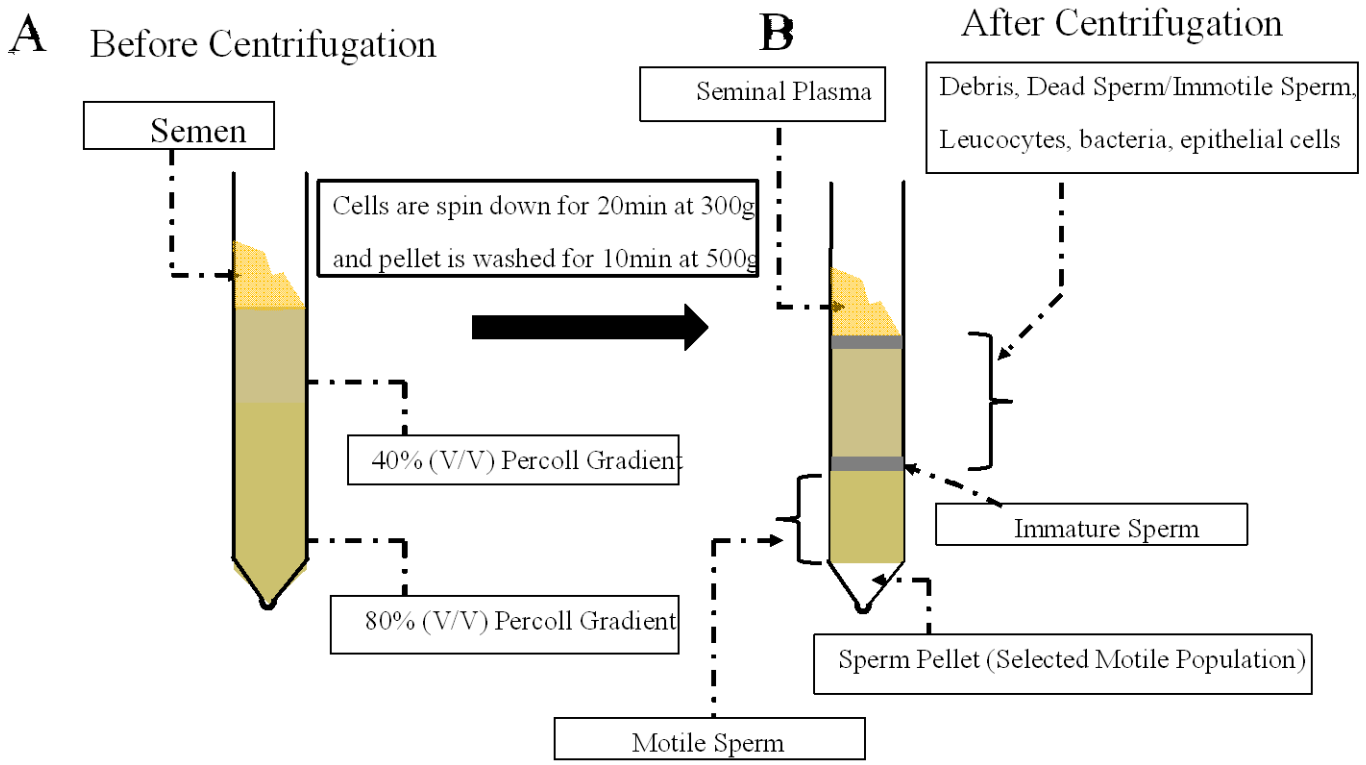


Figure 2.2: Sperm cell preparation technique – density gradient centrifugation. Human sperm cells are separated from the liquefied semen sample using density gradient centrifugation. **A**. 1ml of 80% Percoll (V/V) is overlaid with 1ml of 40% Percoll (V/V), and 1ml of the liquefied semen sample is placed at the top of this gradient solution (both 40% and 80%). **B**. This solution (liquefied semen sample + 40% Percoll + 80% Percoll) is centrifuged for 20min at 300g. After 20min, the sperm pellet (motile sperm population) is collected at the bottom and washed with PBS for 10min at 500g. Cells are then resuspended in sEBSS containing 0.3% BSA for further experiments. For western blot experiments, cells are resuspended in non-capacitating HEPES-buffered medium adopted from sEBSS lacking both albumin and bicarbonate.

2.5 Assessment of motility by Computer Assisted Semen Analysis (CASA)

CASA was performed using a Hamilton Thorne CEROS system (HTM-CEROS; Hamilton Thorne, Inc. Beverly, MA, USA) connected to an Olympus CX41 microscope with heated stage (37^{o}C) using a 10x negative phase contrast objective. Motility of a was analysed at 60Hz

100µl aliquots of sperm suspension (3x10^{6}cells/ml) prepared by direct SU and DG were treated with agonists (4AP (5mM), PE_1 (2µM) and P4 (3µM) as required and a 5µl aliquot was immediately added to either side of the pre-warmed CASA 2X-CEL chamber to a depth of 20µm.

At least 20 frames, collected at 60 Hz, were used for analysis of motility, with a minimum of 100 cells in each sample. The playback option (which replays the acquired video overlaid with the sperm tracks derived by the software) was used to check successful identification and discrimination of cells. Cells departing the area during analysis are identified as a blue track (not counted by CASA), motile cells entering the area are identified as green or cyan (light blue), red dots show immotile/static cells, white dots show cells that collided during their movement (leads to miscounting - not counted by CASA).

Motility characteristics (kinematics) were downloaded from CASA system for offline analysis. Cells were considered to be hyperactivated when they satisfied the criteria of curvilinear velocity (VCL) $\geq$ 50µ m/s, linearity (LIN) < 50%, increased amplitude of lateral head displacement (ALH) $\geq$ 7µm (Mortimer, 2000c).

Paired t-tests were used to compare motility of agonist-treated cells with parallel controls treated with vehicle. If $P < 0.05$, then the effects were considered statistically significant.

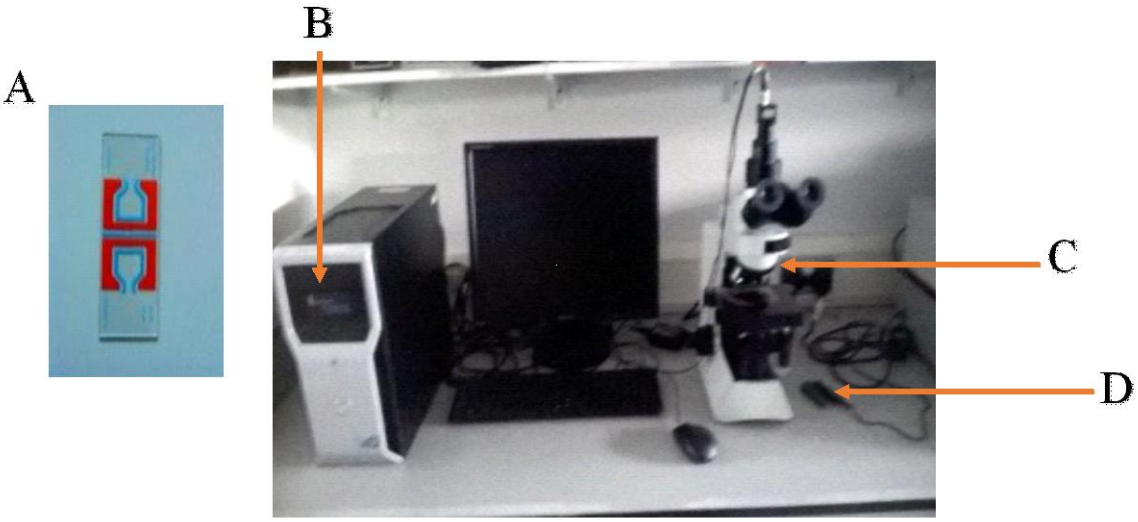


Figure 2.3: Showing different sections of a CASA. Assessment of human sperm hyperactivated motility (in vitro) is performed using CASA. **A** A CASA 2X-CEL chamber (Hamilton Thorne Biosciences) with a depth of 20µm. **B** Human sperm motility software (HTM-CEROS) obtained from Hamilton Thorne, Inc. Beverly, MA, USA. **C** An Olympus CX41 microscope connected to a computer. **D** The thermo-slider of an Olympus CX41 microscope, part of the CASA unit.

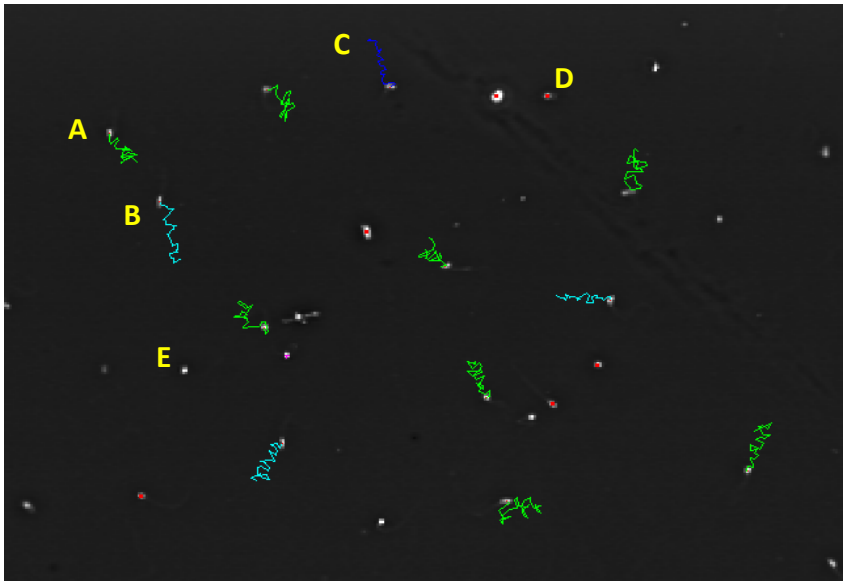


Figure 2.4: Showing different motility tracks analysed by the CASA. Image showing an output of video sequences of 20 data fields analysed by the CASA. **A & B** Motile cells entering the area, identified by a green or cyan (light blue) overlay of the actual track the cell followed. **C** The blue track shows cells departing the area during the analysis. These cells are not considered by the CASA. **D** Immotile/static cells, assigned a red track. **E** Cells displayed as white dots have collided with other cells during their movement. These cells lead to a miscounting of total sperm numbers, and hence they are excluded during analysis by the CASA.

2.6 Kremer's Penetration/Migration Test

2.6.1 Establishment of Artificial Penetration Test

Sperm penetration into viscous (methylcellulose) (4,000 centipoise (cp), artificial viscous medium, 1% w/v) and viscoelastic (polyacrylamide) media (2.4 – 2.7 centipoise (cp), artificial visco-elastic medium, 1% w/v) was assessed using 5 cm flattened capillary tubes (0.4x4.0mm section, 0.2mm inner depth; C M Scientific) (Figure 2.5).

For viscous medium methylcellulose was created using a 1% w/v methylcellulose in sEBSS medium supplemented with 0.3% BSA. After this, the methylcellulose solution was incubated O/N (with mild shaking for proper mix) in a cold room and next day the solution was left in the incubator at (37ºC, 5% CO_2). Prior to the start of the penetration experiment methylcellulose solution (~200µl) was then aliquoted into 1.5ml eppendrof tubes and then glass capillary tubes were inserted into the eppendrof tubes and left it for ~2min. During this time (~2min) methylcellulose solution was transferred into glass capillary tubes by capillary action, once the glass capillary tubes were filled with methylcellulose solution the glass capillary tubes were taken out from eppendrof tubes, and they were wiped with a soft tissue to remove excess of methylcellulose. After this, one end of the glass capillary tubes were sealed with cristal seal wax.

Human sperm cells were prepared by direct SU and DG methods (as explained in sections 2.4.1 & 2.4.2). Following this, 1µl of agonist was added to 99µl of sperm suspension, giving final concentrations of 5mM 4-aminopyridine and 2µM prostaglandin E_1.

Now the open end of the glass capillary tube were placed into the sperm preparation and then incubated for 1 hr (37ºC, 5% CO_2). Afterwards, the tubes were then removed, wiped and viewed (using a 20x phase contrast objective lens).

For visco-elastic medium polyacrylamide was created using 1% w/v polyacrylamide in sEBSS medium supplemented with 0.3% BSA. The same procedure was followed (as explained in the case of viscous medium) in preparing visco-elastic medium and filling the glass capillary tubes.

2.6.2 Data Acquisition

At 0, 1 and 2cm from the base of the capillary tube, three microscope fields were selected at a depth of 0.1mm (100microns (μ)) and the average cells/field was calculated. In experiments where the effect of drug treatment was investigated cell counts were normalised to parallel, untreated controls to allow comparison between different experiments. Different motility characteristics were also analysed at 0, 1 and 2cm using CASA.

2.6.3 DG Data Normalisation

Though the cells prepared by SU and DG were adjusted to the same cell density (see section 2.4.1 & 2.4.2), CASA recordings showed that the proportion of progressively motile cells was lower in DG samples than in samples prepared by the direct SU technique. It was thus not possible to make a direct comparison of the number of penetrating DG and SU cells.

To overcome this, the data were normalised by calculating the ratio of motile cell counts in direct SU and DG samples and scaling the observed number of cells counted in the Kremer tests with DG cells.

Mathematically, it is described as:

$$Z = \frac{\%\ \text{progressive cells (from direct SU)}}{\%\ \text{progressive cells (from DG)}}$$

Normalised cell count in DG experiments = Z * no. of cells observed

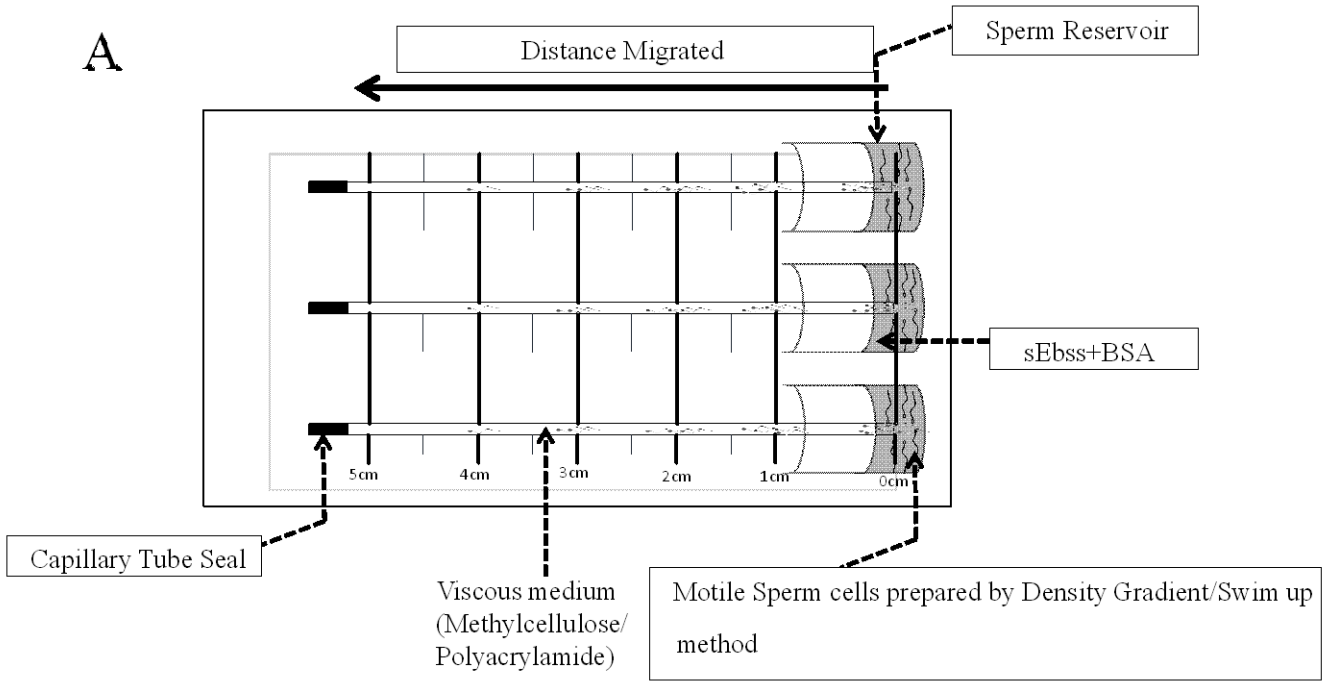


Figure 2.5: Kremer penetration test – viscous/viscoelastic medium. Assessment of human sperm penetration (in vitro) is performed by a Kremer penetration test. **A.** Showing the construction of the Kremer penetration test. Kremer tubes are 5cm glass capillary tubes filled with either 1% (w/v) viscous (methylcellulose) or viscoelastic (polyacrylamide) medium as an in vitro penetration medium. One end of the tube is sealed with cristal seal wax and the other end is placed in contact with the sperm reservoir. Penetration of motile human sperm into and along the viscous/viscoelastic column is assessed by counting their numbers at 0, 1 and 2cm from the base of the capillary tube. In vitro penetration is compared between untreated (control) cells and cells stimulated with different agonists (4AP, PE_1 and P4).

2.7 Western Blot/Immunoblot

2.7.1 Sample Preparation

Donors recruitment is same as explained in section 2.2. Semen was collected from healthy donors by masturbation after 2-3 days of sexual abstinence and allowed to liquefy for 30min at 37ºC, 5% CO_2. Cells were prepared using DG (For DG technique see figure 2.2) in a non-capacitating HEPES-buffered medium adopted from sEBSS lacking both albumin and bicarbonate. (For a list of chemicals used in preparing non-capacitating medium (NCM), please see Appendix II)

For density gradient technique (For DG technique see figure 2.2) and gradient preparation is same as explained in section 2.4.2.

Cells were centrifuged at 300g for 20min. The pellet was washed in PBS (500g, 10min) and then resuspended in non-capacitating HEPES-buffered medium adopted from sEBSS lacking both albumin and bicarbonate. (For a list of chemicals used in preparing NCM, please see Appendix II) To initiate capacitation, bicarbonate and BSA were added ($1/10^{th}$ of the final obtained volume from DG). Cell concentration was determined using an improved Neubauer haemocytometer and adjusted to $1x10^6$/ml. In direct SU (for technique, please see Figure 2.1), motile spermatozoa were harvested (~60min) into the non-capacitating HEPES-buffered medium adopted from sEBSS lacking both albumin and bicarbonate. (For a list of chemicals used in preparing NCM, please see Appendix II). To initiate capacitation, bicarbonate and BSA were added ($1/10^{th}$ of the final obtained volume from direct SU). Cell concentration was determined using an improved Neubauer haemocytometer and adjusted to $1x10^6$/ml.

At 0, 30, 90 and 180 min an aliquot containing $1x10^6$ was taken (the T0 sample was taken immediately following resuspension in the medium with a maximum of 1 min incubation). Aliquots were immediately centrifuged at 2,000 rpm at 25ºC for 5 min, and the sperm pellet was then washed in PBS for 5min, at 2,000 rpm and 25ºC. The supernatant was discarded (without disturbing the sperm pellet), 5µl of 5X sample buffer containing 5X vanadate (tyrosine phosphatase inhibitor) was added to the pellet, and the sample was frozen at -20ºC. The same experimental procedure was carried out using sperm samples from 5 different donors.

2.7.2 SDS-PAGE and Western/Immunoblot Setup

Frozen samples were boiled at 100ºC for 5min, sonicated (10sec) and centrifuged at maximum relative centrifugal force (rcf) between 22ºC and 23ºC for 5min. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (10% gels) was used to separate the proteins. For detailed SDS-PAGE procedure, please see Appendix III.

After completion of electrophoresis, the proteins were electrotransferred onto nitrocellulose membrane at 100V for 1hr. 5% skimmed milk was used to block non-specific binding sites on nitrocellulose membrane and washed with TTBS (Tris-buffered saline (TBS) (0.9% NaCl, 20mM tris-HCl, pH 7.8) supplemented with 0.1% Tween 20).

The nitrocellulose membrane was incubated overnight at 4ºC with anti-phosphotyrosine antibody (pAb) (1/10,000). After this step, the membrane was washed with TTBS three times (10 min) and incubated with the corresponding secondary antibody (sAb), conjugated with horseradish peroxidase for 1hr and washed three times with TTBS (10min).

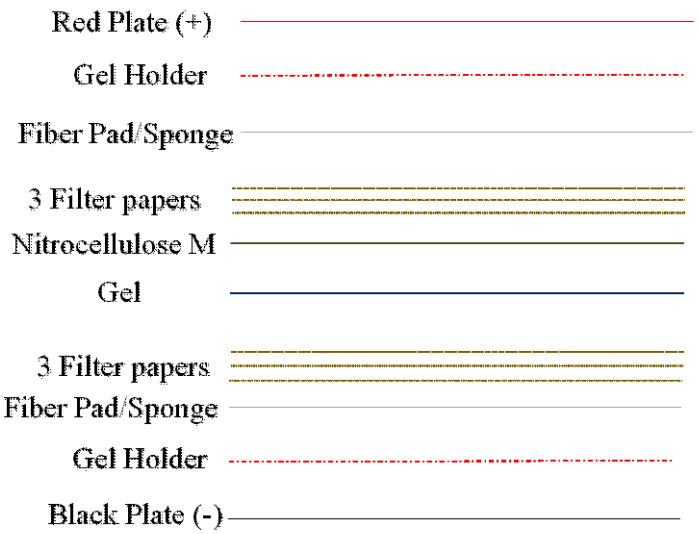


Figure 2.6: Showing the assembly of a sandwich in western blot/immunoblot. The figure shows the construction of a sandwich preparation containing nitrocellulose membrane and gel. Filter papers and fiber pads are placed on both sides of the assembly to ensure maximum protection for the gel and nitrocellulose membrane. The membrane is placed between the gel and positive electrode (Red plate (+)). During transfer, an electric field is directed perpendicular to the surface of the gel, which results in proteins moving out of the gel and attaching to the nitrocellulose membrane.

Immunoreactive bands were detected using an Odyssey Infrared Imaging System. Silver staining was performed to confirm equal protein loading for all samples.

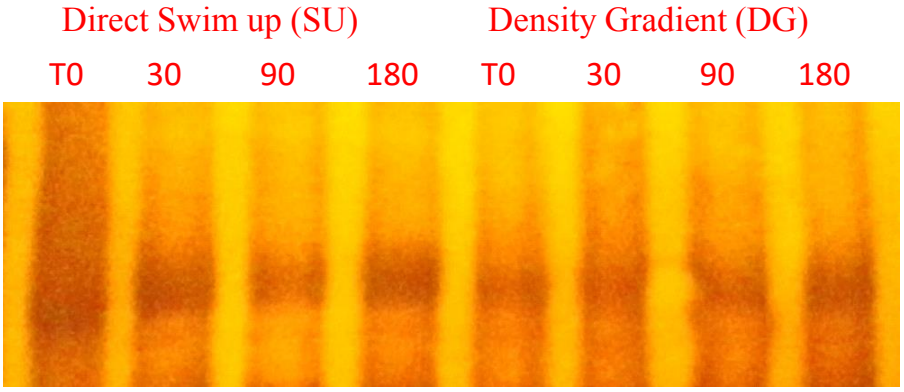


Figure 2.7: Showing silver staining image of a nitrocellulose membrane. Silver staining image of proteins transferred onto a nitrocellulose membrane. The image shows equal protein loading for all samples during various time intervals (T0, 30, 90 and 180min) of capacitation (sperm maturation) when cells were prepared using direct SU and DG. Protein tyrosine phosphorylation was used as a marker to evaluate capacitation.

Chapter 3: Effect of Sperm preparation techniques (DG & SU) on human sperm motility & penetration of artificial mucus

3.1 Objective:

The objective of this chapter is to investigate the effect of sperm preparation methods (density gradient centrifugation and direct swim up) on human sperm motility and on the penetration of artificial mucus (methylcellulose).

3.2 Introduction

Motility is crucial to the sperm's ability to interact with an oocyte and achieve successful fertilisation. Testicular spermatozoa of humans, as well as the sperm of all other eutherian mammals, are either motionless or poorly motile, but become progressively motile upon ejaculation, rapidly leaving the seminal plasma. Exposure of sperm to seminal plasma for long periods (>30min) could affect sperm function, leading to a decrease in fertilising potential (Rogers et al., 1983). Therefore, it is important to use sperm preparation methods that can separate sperm from seminal plasma, eliminate decapacitation factors and support capacitation when suspended in a culture medium under in vitro conditions.

When selecting the sperm preparation techniques, four basic approaches were used to establish a safe method (Mortimer and Mortimer, 1992). The four basic approaches were: **A.** Simple dilution and washing to separate human sperm from seminal plasma; **B**. Methods based on sperm migration to acquire highly motile sperm (direct from liquefied semen, a washed sperm pellet or from a suspension of washed sperm) **C.** Selective washing procedures (density gradients) to separate motile sperm; and **D**. Adherence-based methods to remove dead sperm and debris. Of these approaches B (swim-up) and C (selective washing/density gradient centrifugation) are the most widely used.

The normal swim-up technique is an example of migration-based techniques, and involves a double wash with a centrifugation step at a speed of 220g for 10min, followed by upward migration of the sperm, separating a highly motile sub-population (Lopes et al., 1998, Younglai et al., 2001). Inclusion of a centrifugation step may remove the antioxidant properties of seminal plasma (Aitken, 1999) resulting in DNA damage in sperm (Twigg et al., 1998, Mortimer, 1991). Direct swim-up avoids this problem by allowing sperm to swim directly from semen into prepared medium, separating the highly motile sub-population and excluding ROS-generating cells (Mortimer, 2000a).

The selective washing procedure involves separation of cells based density, cells being distributed throughout a gradient column according to their individual density. During the early days of this procedure, colloidal silica was used as a gradient medium to allow separation of sperm, but these cells show fertility problems (Mortimer, 2000a). Later, this technique was upgraded with the introduction of modified colloidal silica as a gradient medium. In 1970, polyvinylpyrrolidone (PVP) coated silica particles were introduced by (Pertoft et al., 1978) and later, in 1980, this was commercialised as Percoll. Normal (morphologically) and mature sperm have a density (specific gravity) above 1.12g/ml, whereas immotile and abnormal sperm have a lower density between 1.06 and 1.09g/ml (Oshio et al., 1987). Colloidal silica (Percoll) prepared at 40% and 80% has densities of 1.06 and 1.10g/ml, respectively. Therefore, in a discontinuous gradient (Arcidiacono et al., 1983, Lessley and Garner, 1983, Dravland and Mortimer, 1985, Mortimer, 2000a), composed of layers of 80% and 40% Percoll, most mature and normal spermatozoa can penetrate through the lower gradient (80%) whereas abnormal cells will tend to be retained at the boundary.

Both direct swim-up and discontinuous density gradient centrifugation techniques are effective, providing a sperm population with high levels of motility, fewer DNA nicks, low levels of abnormal sperm and better chromatin stability than raw semen (Sakkas et al., 2000, Tomlinson et al., 2001, Mortimer, 2000a). However, yields with DG are much higher than with SU. This may reflect relative inefficiency of the SU technique, or may indicate that the population selected by these two techniques differ in their characteristics. I therefore compared motility and penetration of artificial mucus (methylcellulose) of cells prepared by DG and SU.

In this study computer-assisted sperm analysis was used for the evaluation of motility. Hyperactivated and activated motilities have characteristic features which can be distinguished by their different kinematic parameters. These kinematic parameters include

velocity of movement, the lateral movement of the sperm head and frequency of side-side movement of the sperm head (Mortimer, 2000b). Several kinematic parameters are defined below (Mortimer, 2000c).

VSL is defined as the straight-line distance between the beginning and end of the track divided by the time elapsed (μm/sec). VCL is defined as the total distance between each centre of brightness (CB) of sperm head position for a given cell during acquisition divided by the time elapsed (μm/sec). It is always higher than VAP and VSL. Average path velocity is the distance the spermatozoon has travelled in the regular direction of movement during the observation period. In some cases, where the sperm head trajectory is very regular and linear and there is low lateral movement, the VAP is almost the same as VSL. In the case of irregular trajectories (low linearity and high lateral deviation), the VAP is much higher than VSL. ALH is the width of the lateral movement of the sperm head. It is calculated as the total width of the head trajectory and is expressed in micrometres (μm).

There are two ways of reporting amplitude of lateral head displacement: ALH mean or ALH maximum. According to Mortimer, ALH mean is the mean of a set number of ALH readings along the trajectory. ALH maximum is explained as the maximum ALH value analysed for a trajectory segment. The CASA instrument from Hamilton Thorne uses ALH maximum (Mortimer, 2000c). Linearity measures the departure of the cell track from a straight line. It is the ratio of VSL/VCL. Straightness measures the departure of the cell path from a straight line. It is the ratio of VSL/VAP. Human sperm with curvilinear velocity (VCL) ≥ 50 μm/s, linearity (LIN) <50%, high amplitude of lateral head displacement (ALH) ≥ 7 μm are considered to be hyperactivated (Mortimer 2000).

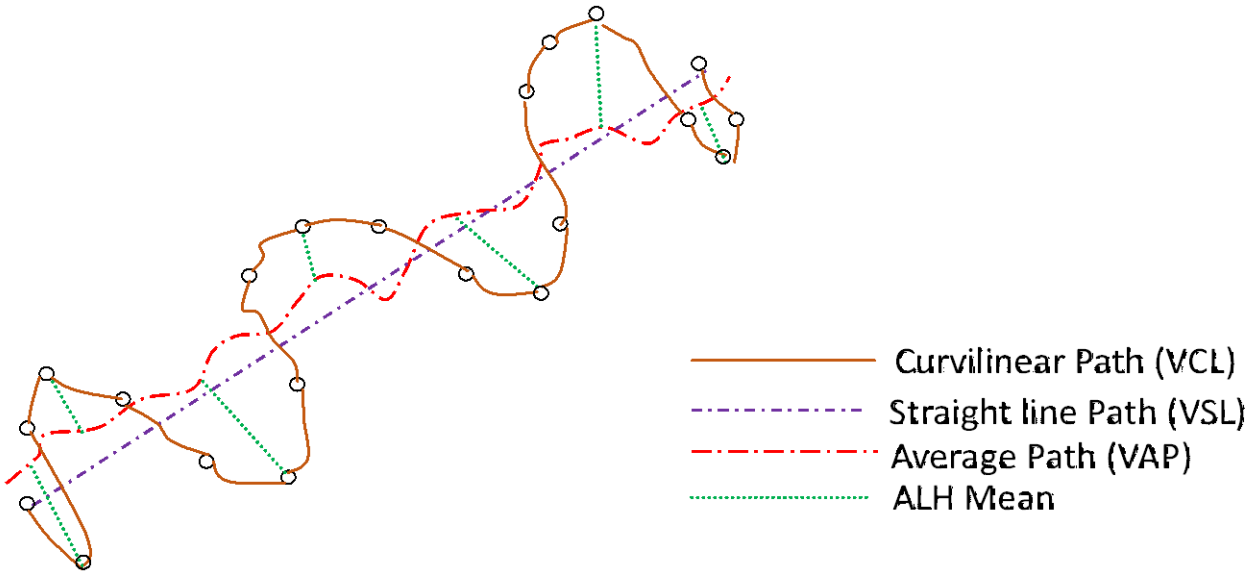


Figure 3.0: Different types of motility tracks in human sperm: Figure analysing different types of motility by tracking the sperm head. **Brown** = Showing curvilinear path (VCL), **Violet** = Showing straight line path (VSL), **Red** = Average path velocity (VAP), **Green** = Showing average value (ALH mean) of individual amplitude of lateral head measurements made over the length of the track.

3.3 Materials and Methods

3.3.1 Materials

For the Materials section, see Chapter 2.1.1. and 2.1.2

Agonists like 4-Aminopyridine (4AP), Prostaglandin E_1 (PE_1) & Progesterone (P4) were not used in this experiment.

3.3.2 Methods

3.3.2.1 Donor Recruitment

Donor recruitment was conducted as described in Chapter 2.2.

3.3.2.2 Sperm cell preparation

Human seminal fluid was collected from healthy donors by masturbation after 2-3 days of sexual abstinence and then semen sample was allowed to liquefy for 30min at 37^{o}C (5% CO_2). After liquification the semen sample was split into two halves with one-half was used to harvest highly motile sperm using direct swim up (SU) procedure.

In direct swim up (SU) method (Figure 2.1) 1ml of sEBSS medium (contains 0.3% BSA and 15mM HEPES) were underlaid with 0.2ml of liquefied semen. After this tubes were then incubated at a 45^{o} angle for 1hr at 37^{o}C and 5% CO_2. After 1hr of incubation the top layer (containing highly motile cells) was removed from each tube and pooled together. Cell concentration was determined using an improved Neubauer haemocytometer and adjusted to $6x10^6$/ml. Cells were left to capacitate for further 4.5 hr in an incubator (37^{o}C, 5% CO_2). Afterwards, the cell density was adjusted to $3x10^6$/ml and used for the experimental study.

The other half (1ml) of the liquefied semen was gently added to the top layer of the density gradient (1ml of 80% Percoll overlaid with 1ml of 40% Percoll) (Figure 2.2). Percoll gradients made isotonic with M medium. (For a list of chemicals used in preparing M medium, please see Appendix 12.2.) Cells were centrifuged at 300 g for 20 min. The pellet was washed in PBS (500 g, 10 min) and then resuspended in sEBSS (0.3% BSA and 15mM HEPES). Cell concentration was determined using an improved Neubauer haemocytometer and adjusted to $6x10^6$/ml. Cells were left to capacitate for 4.5 hr in an incubator (37^{o}C, 5% CO_2). Afterwards, the cell density was adjusted to $3x10^6$/ml and used for the experimental study.

3.3.2.3 Computer-assisted Semen Analysis (CASA)

The effect of sperm preparation methods (SU & DG) on human sperm motility was assessed in this experiment by using CASA (Section 2.5). Cells were considered to be hyperactivated when they satisfied the criteria of curvilinear velocity (VCL) ≥ 50μ m/s, linearity (LIN) < 50%, amplitude of lateral head displacement (ALH) ≥ 7μm (Mortimer, 2000c).

3.3.2.4 Kremer's Penetration Test

For Kremer's Penetration Test, See Chapter 2.6.

The effect of sperm preparation methods (SU & DG) on the penetration of artificial mucus (methylcellulose) in vitro was observed in this experiment. No agonists were used for this experiment.

3.4 Results

CASA was used to assess the effect of sperm preparation techniques (DG and SU) on motility characteristics in relation to hyperactivated motility. Human sperm cells prepared by DG showed greater levels of hyperactivated motility (HA) **(DG 6.25%)** (as measured by CASA) compared to cells prepared by direct swim-up **(SU 3.75%)** ($p = 0.004$; $n = 20$; paired t-test) (Figure 3.1, **A**).

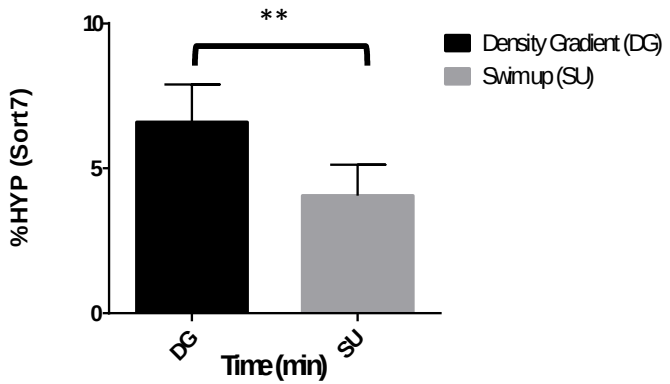
Specific parameters ALH, VCL & VSL (Figure 3.1 **B**, **C** & **D**) and VAP, %LIN and %STR (Figure 3.1 **E**, **F** & **G**) were also assessed. Cells prepared by DG and SU showed a significant difference in VAP, %LIN ($p < 0.001$; $n = 20$; paired t-test) (Figure 3.1, **E (DG 54.3% Vs SU 63.13)** and **F (DG 54.1% Vs SU 60.3)**) and %STR ($p < 0.05$; $n = 20$; paired t-test) (Figure 3.1, **G (DG 81.85% Vs SU 87.25)**). Cells prepared with DG shown reduced forward motility (VSL) (Figure 3.1, **D (DG 45.585% Vs SU 55.89)**) when compared with SU cells. There was no significant difference in ALH and VCL between cells prepared by DG and SU (Figure 3.1, **B (DG 3.795% Vs SU 4.05%)** and **C (DG 91.345% Vs SU 94.16%)**).

Table 3.1 below showing the effect of different sperm preparation techniques (DG and SU) on different motility parameters (HYP – Sort7%, ALH, VCL, VAP, %LIN and %STR).

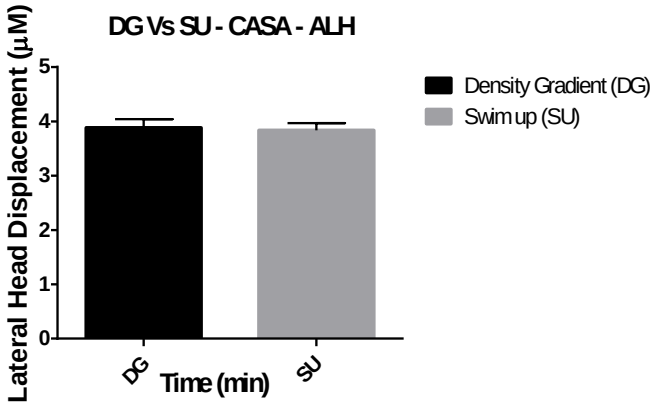
Density Gradient Centrifugation (DG)			Direct Swim up (SU)	
PARAMETER	Mean	S.E.M	Mean	S.E.M
Hyperactivation (% Sort 7)	6.25**	0.695	3.75	0.742
Lateral amplitude (ALH) (µm)	3.795	0.099	4.05	0.129
Curvilinear velocity (VCL) (µm/s)	91.345	3.134	94.16	2.192
Straight line/Prog. Or Forward motility (VSL) (µm/s)	45.585****	1.423	55.89	1.722
Average path velocity (VAP) (µm/s)	54.3***	1.585	63.13	1.573
Linearity (LIN) (% LIN)	54.1***	1.309	60.3	1.675
Straightness (STR) (% STR)	81.85*	1.809	87.25	0.891

Table 3.1: Effect of different sperm preparation techniques density gradient centrifugation (DG) and direct swim up (SU) on motility parameters. Experiments were carried out in pairs, where cells from the same semen sample were analysed by CASA. Cells were prepared in sEBSS (15mM HEPES, pH=7.4) without any treatment. For each sperm preparation technique (DG and SU), hyperactivation (sort 7%), lateral head amplitude (ALH), curvilinear velocity (VCL), straight-line/progressive or forward motility (VSL), Average path velocity (VAP), Straightness (%STR) and linearity (% LIN) were determined. $P = 0.004$; with DG compared to SU (sort 7%), $P < 0.001$with DG compared to SU (VSL, VAP and % LIN) and $P < 0.05$ with DG compared to SU (%STR) paired t-test; n=20.

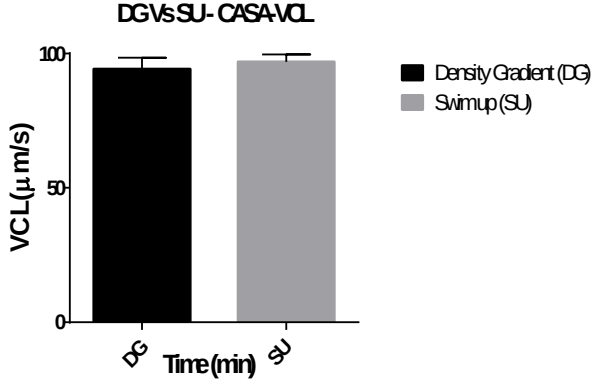
A


DG Vs SU - CASA- %HYP7
**
%HYP (Sort7)
10
5
0
DG
SU
Time (min)
Density Gradient (DG)
Swim up (SU)

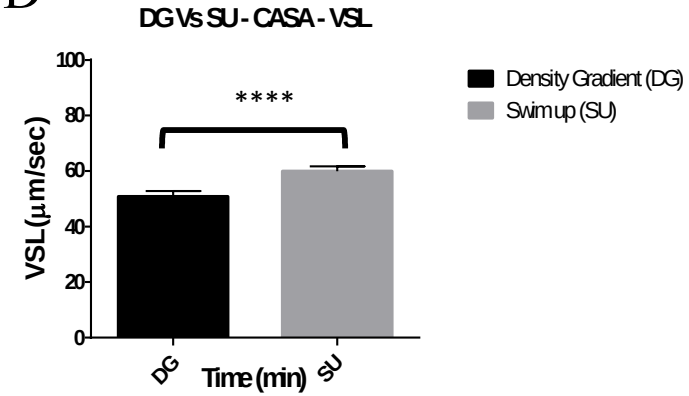
B


DG Vs SU - CASA - ALH
Lateral Head Displacement (µM)
5
4
3
2
1
0
DG
SU
Time (min)
Density Gradient (DG)
Swim up (SU)

C


DG Vs SU - CASA-VCL
VCL(µm/s)
100
50
0
DG
SU
Time (min)
Density Gradient (DG)
Swim up (SU)

D


DG Vs SU - CASA - VSL
****
VSL(µm/sec)
100
80
60
40
20
0
DG
SU
Time (min)
Density Gradient (DG)
Swim up (SU)

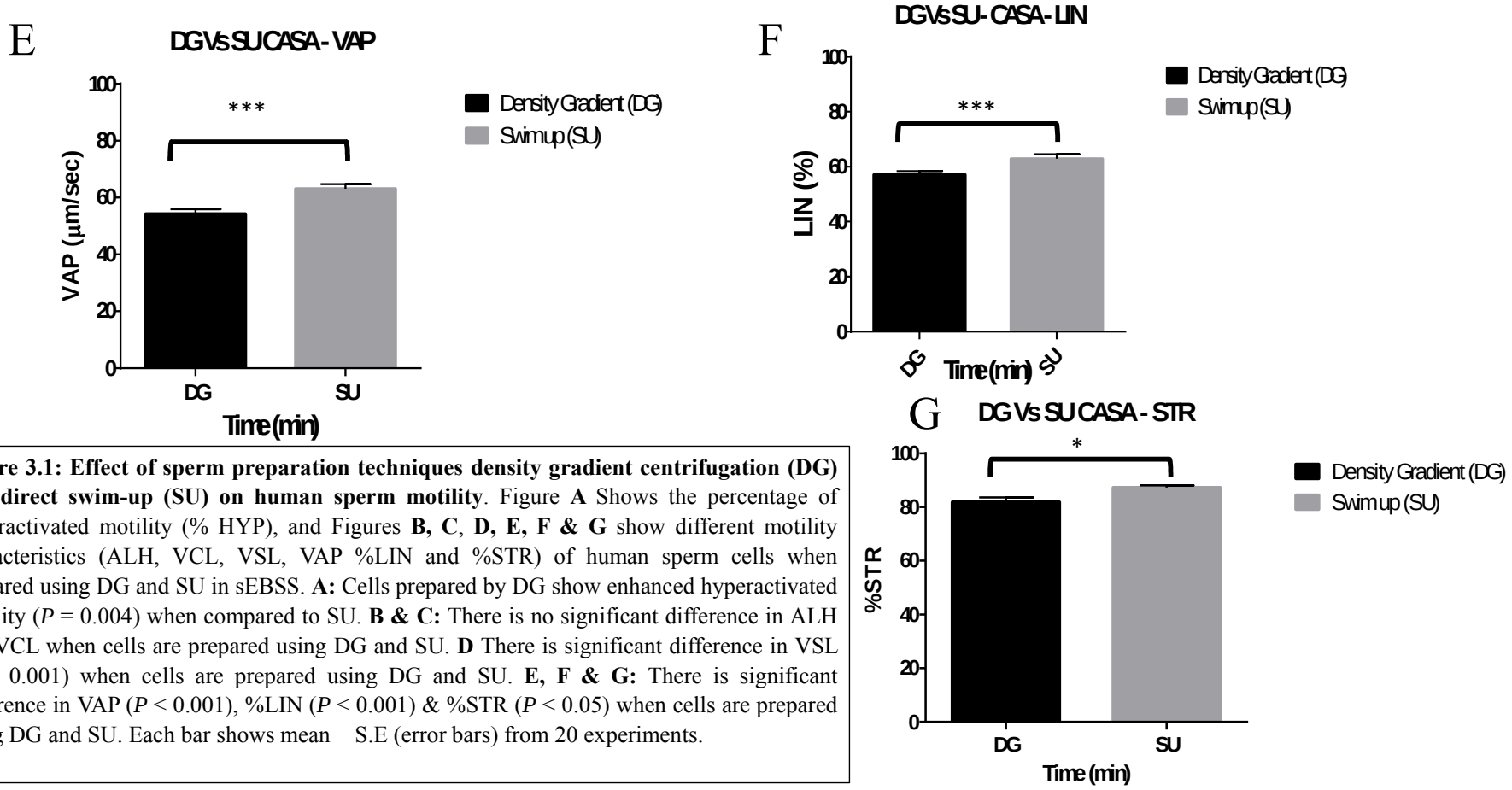
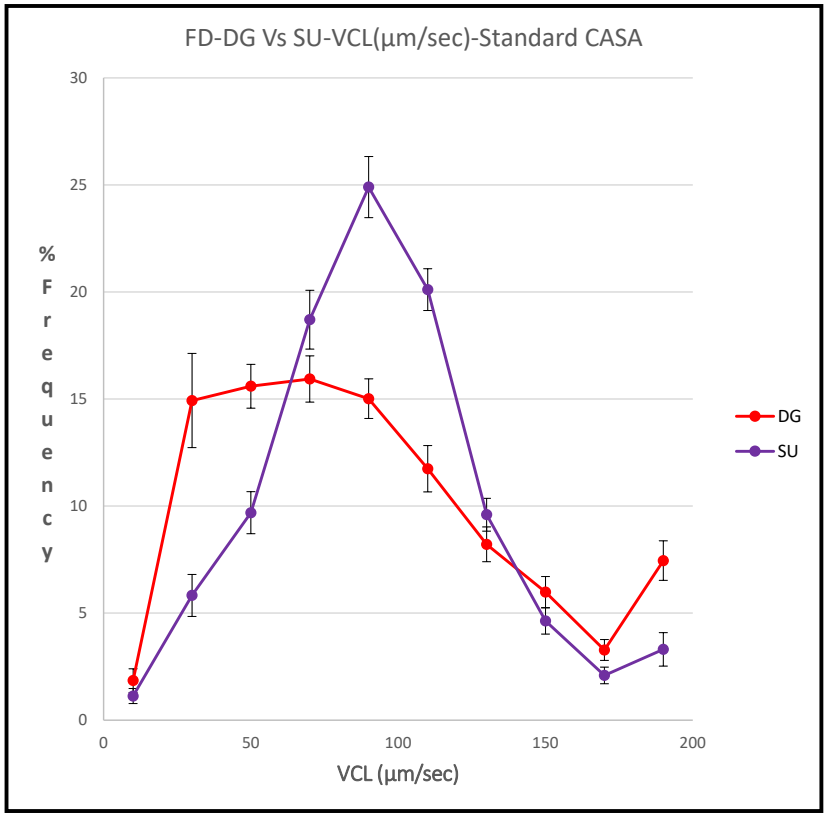


Figure 3.1: Effect of sperm preparation techniques density gradient centrifugation (DG) and direct swim-up (SU) on human sperm motility. Figure **A** Shows the percentage of hyperactivated motility (% HYP), and Figures **B, C**, **D, E, F & G** show different motility characteristics (ALH, VCL, VSL, VAP %LIN and %STR) of human sperm cells when prepared using DG and SU in sEBSS. **A:** Cells prepared by DG show enhanced hyperactivated motility ($P = 0.004$) when compared to SU. **B & C:** There is no significant difference in ALH and VCL when cells are prepared using DG and SU. **D** There is significant difference in VSL ($P < 0.001$) when cells are prepared using DG and SU. **E, F & G:** There is significant difference in VAP ($P < 0.001$), %LIN ($P < 0.001$) & %STR ($P < 0.05$) when cells are prepared using DG and SU. Each bar shows mean S.E (error bars) from 20 experiments.

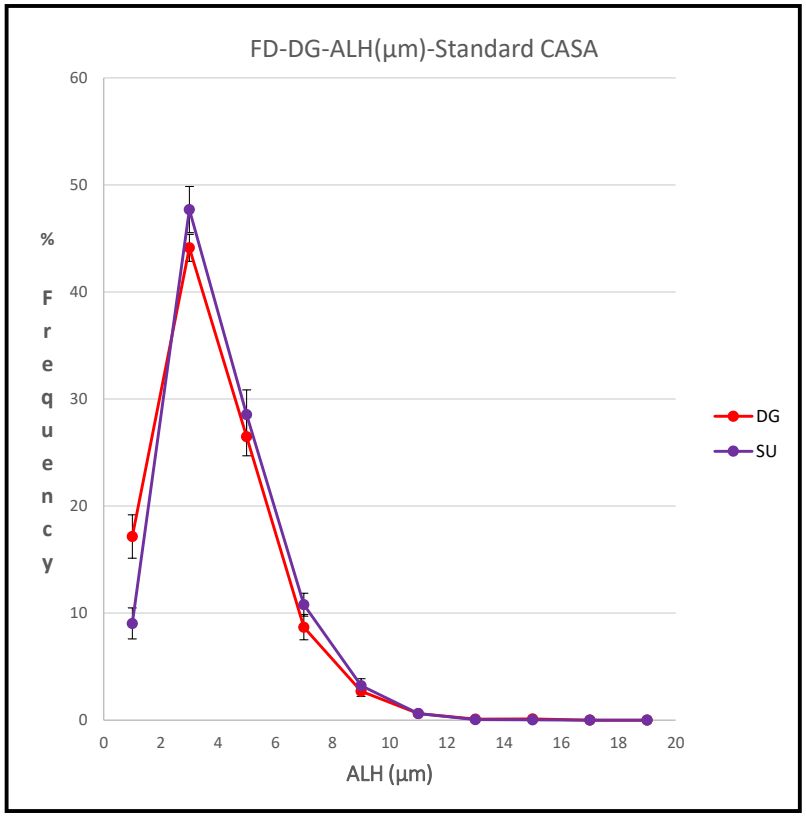
Frequency distributions (FD) were collected from the raw CASA output and were analysed in Excel. FD plots for DG and SU cells in standard CASA is shown in figure 3.2 **A**, **B** & **C**).

The peak of the FD for VCL of cells prepared by DG swimming in saline (standard CASA) is clearly shift to the left (lower VCL) compared to SU cells but in DG cells there was also a greater sub-population of cells at the extreme right of the distribution (very high VCL) compared to the SU (Figure 3.2 **A**). There was no difference in the FD for ALH (Figure 3.2 **B**) but LIN was lower in the DG cells (Figure 3.2 **C**) compared to the SU cells.

A


FD-DG Vs SU-VCL(µm/sec)-Standard CASA
% Frequency
VCL (µm/sec)
0
5
10
15
20
25
30
0
50
100
150
200
DG
SU

B


FD-DG-ALH(µm)-Standard CASA
% Frequency
ALH (µm)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
20
DG
SU

C

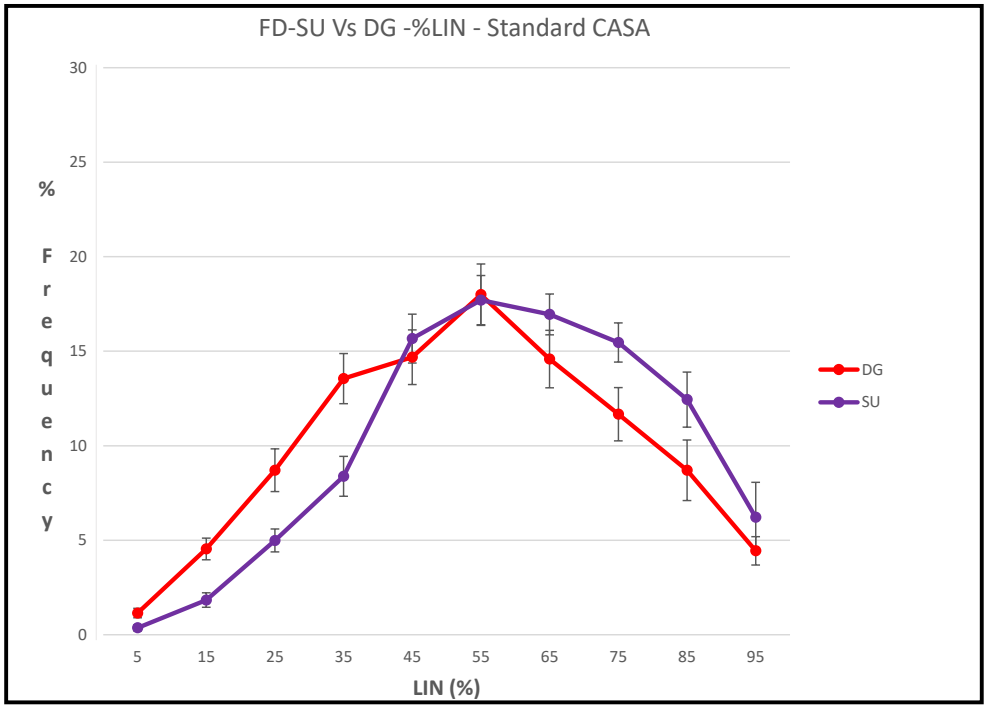


Figure 3.2 Showing the frequency distributions (FD) between direct SU and DG cells using CASA analyser. A, B, and C Showing the frequency distributions (FD) of Hyperactivated (HA) motility determinants (VCL (µm/sec), ALH (µm) and %LIN) when cells were prepared with direct SU (Purple) and density gradient techniques (DG) (Red) and analysed using Computer assisted semen analyser (CASA). When the frequency distributions were analysed there was a increase in **(A)** VCL and **(B)** ALH with a decrease in **(C)** %LIN in both DG & SU cells. When **(A)** VCL where analysed between DG & SU cells, the VCL response peak for the DG cells shift left compared to the SU cells and at the end of the distribution there is an increase in VCL in DG cells compared to SU. This increase in the VCL in the DG cells contributed to a decrease in the **(C)** %LIN compared to the SU cells with no difference in ALH between the cells prepared using DG & SU techniques. Each plot shows mean S.E (error bars) of 20 experiments between direct SU and DG techniques.

Kremer's in vitro penetration test (Figure 3.3 **A** & **B**) was used to investigate how differences in the motility characteristics between cells prepared by DG and SU may affect their ability to penetrate artificial mucous (methylcellulose). Because the % progressive motility of cells prepared by DG was consistently lower than that of cells prepared in parallel SU, cell counts in the DG Kremer experiments were normalised, as described in Chapter 2.6 cells were prepared by DG and SU.

Though cells prepared by DG showed increased levels of hyperactivated motility **(**Figure 3.1, **A**), when cells from the same aliquot were tested for their ability to penetrate artificial mucous (methylcellulose), they were clearly performed less well than SU cells. Without normalisation this difference was significant at distances of 0cm ($P < 0.005$), 1cm ($P < 0.001$), and 2cm ($P < 0.0001$) **(Figure 3.3, A,** n = 12; multiple t-tests). When data for DG cells were normalised to take account of the lower numbers of progressively motile cells, there was no significant difference 0cm and the small difference at 1cm was not significant. At 2cm, however, penetration by DG cells was significantly lower than that of SU cells even after normalisation ($P < 0.005$; n = 12; multiple t-tests). **Figure 3.3**, **B).**

FDs were also analysed for the cells penetrated at 0, 1, and 2cm into viscous medium (methylcellulose). As cells penetrated further in to methylcellulose VCL and LIN of both DG and SU cells had very similar characteristics though there appeared to be fewer SU cells with high ALH at 1 or 2 cm into the gel (compare figs 3.4B, 3.5B and 3.6B). At 2 cm the FDs for VCL and ALH of DG cells were shifted to the right compared to SU cell but LIN was clearly higher in SU.

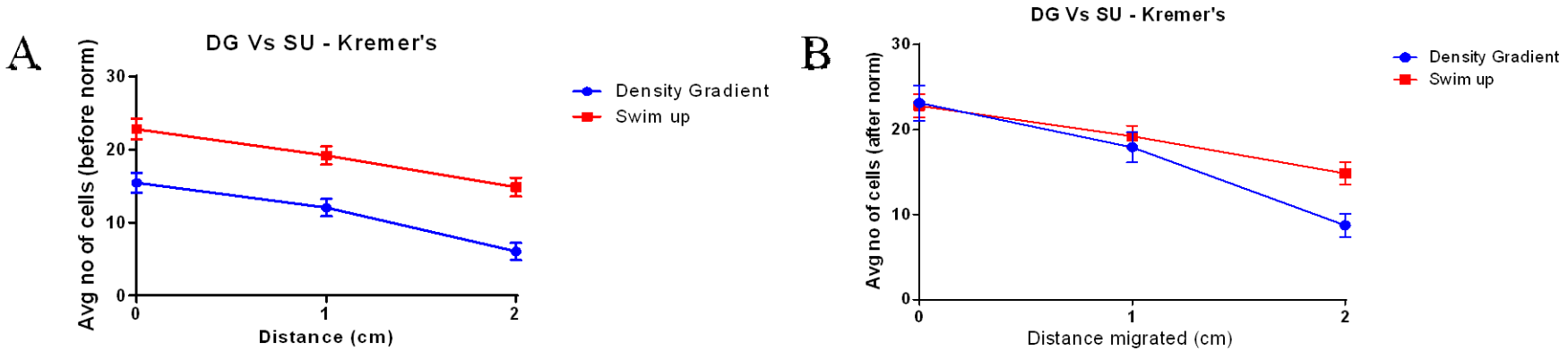
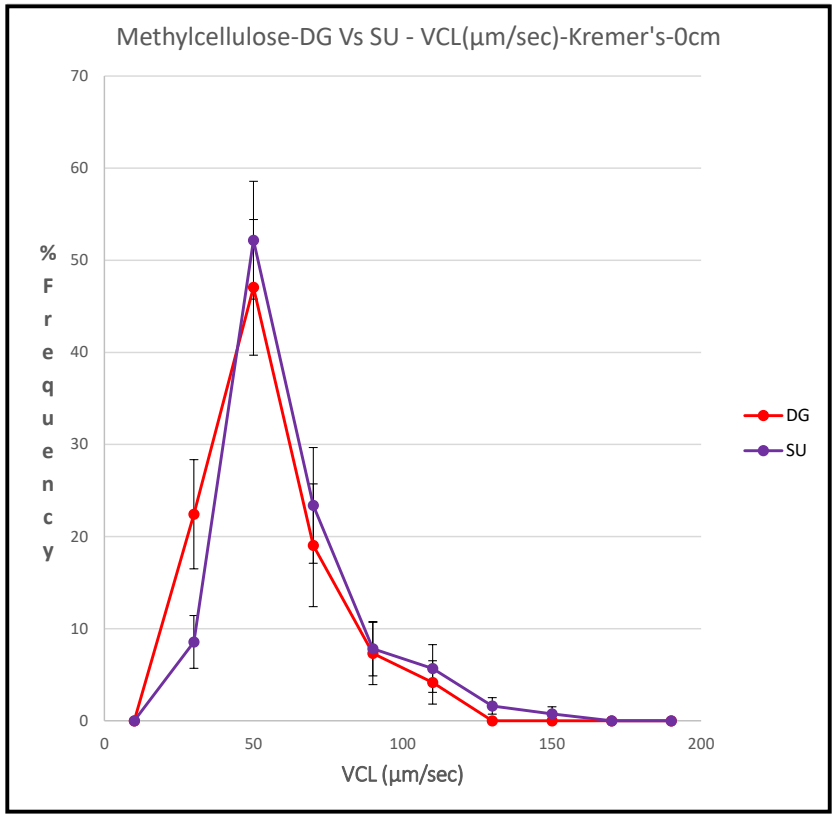


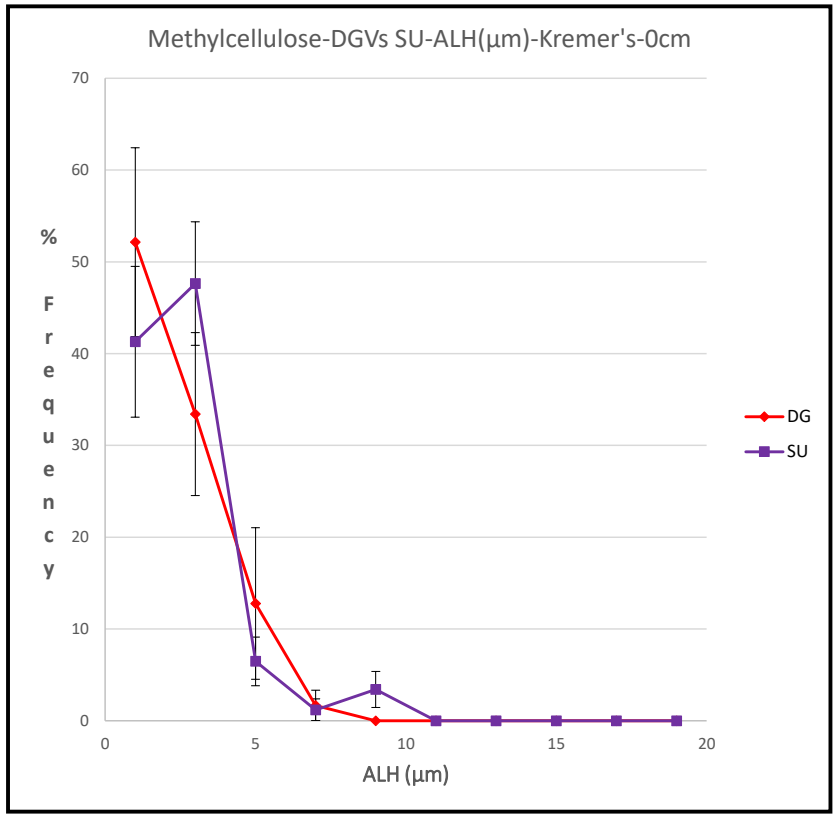
Figure 3.3: Effect of sperm preparation techniques density gradient centrifugation (DG) and direct swim-up (SU) on penetration of artificial mucus (methylcellulose) (in vitro).

Figures **A & B** show sperm enhancement in an artificial viscous medium when prepared using DG and SU before and after normalisation. **A:** Cells prepared by SU show better penetration at 0cm ($P < 0.005$), 1cm ($P < 0.001$), and 2cm ($P < 0.0001$) when compared to cells prepared by DG (before normalisation). **B:** At 2cm ($P < 0.005$), cells prepared by SU show better penetration when compared to cells prepared by DG (after normalisation). Each bar shows the mean S.E (error bars) from 12 experiments.

A


Methylcellulose-DG Vs SU - VCL(µm/sec)-Kremer's-0cm
% Frequency
VCL (µm/sec)
0
10
20
30
40
50
60
70
0
50
100
150
200
DG
SU

B


Methylcellulose-DGVs SU-ALH(µm)-Kremer's-0cm
% Frequency
ALH (µm)
0
10
20
30
40
50
60
70
0
5
10
15
20
DG
SU

C

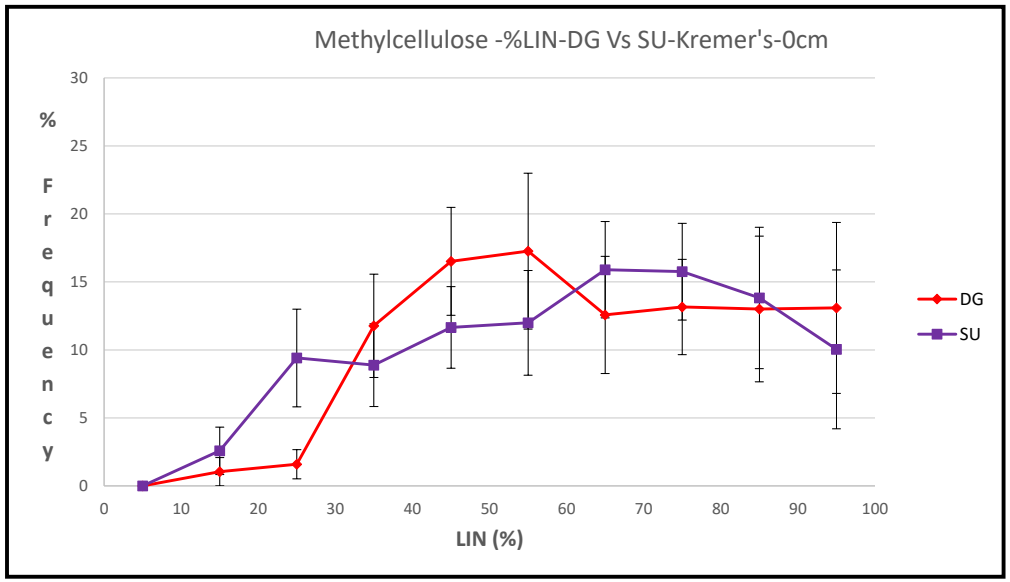
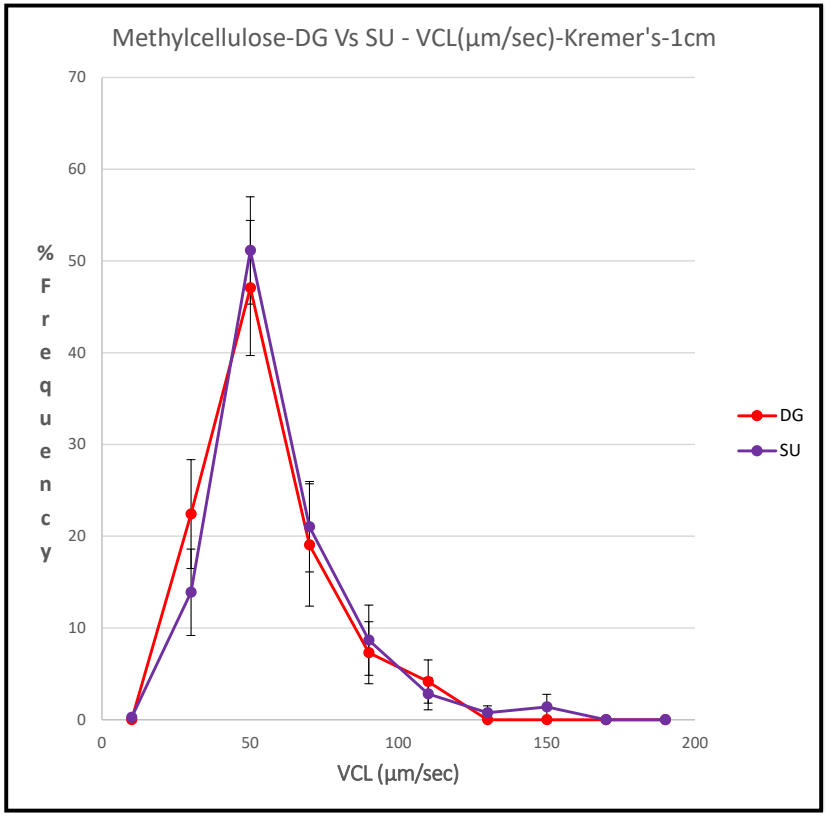
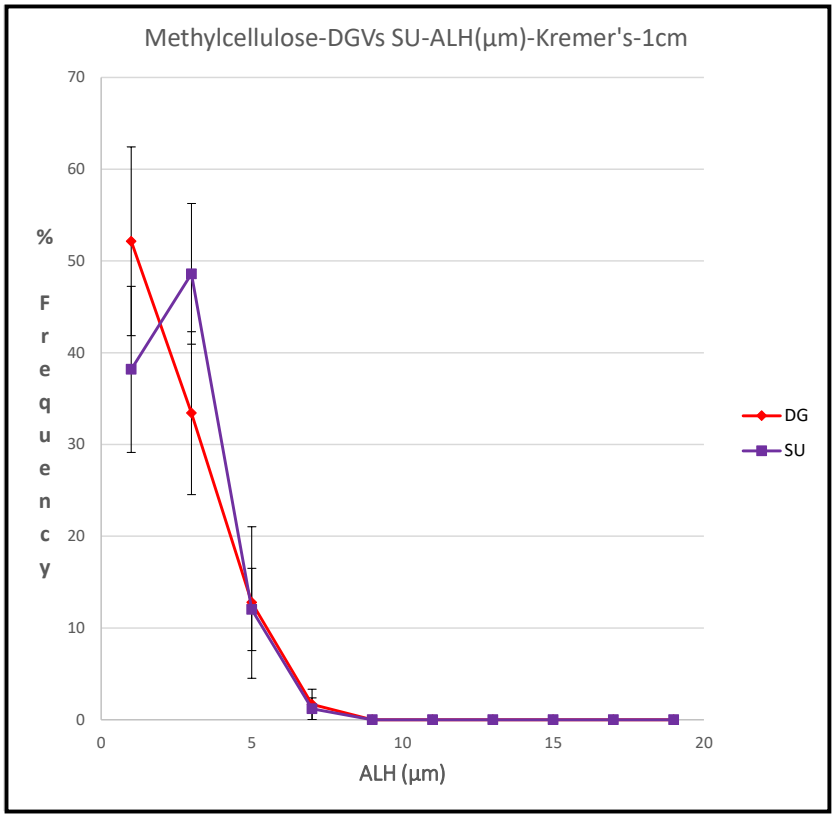


Figure 3.4 Showing the frequency distributions (FD) between direct SU and DG cells in kremer's (In Viscous medium) at 0cm using CASA analyser. A, B, and C Showing the frequency distributions (FD) of Hyperactivated (HA) motility determinants VCL (µm/sec), ALH (µm) and %LIN when cells were prepared with direct Swimup (SU) (Purple) and density gradient techniques (DG) (Red). The cells were measured at 0cm in the viscous medium prepared using methylcellulose and the frequency distributions were analysed using CASA analyser. Cells prepared with SU shown an slight increase in the **(A)** VCL, with a decrease in the **(B)** ALH (observed at the beginning) and a decrease in the **(C)** LIN suggesting SU cells were slightly more hyperactivated which helps them (SU cells) them to enter into the kremer tube (tube filled with the viscous medium) in an effective way compare to the DG cells. Each plot shows mean S.E (error bars) of 12 experiments between direct SU and DG techniques.

A


Methylcellulose-DG Vs SU - VCL(µm/sec)-Kremer's-1cm
% Frequency
VCL (µm/sec)
0
10
20
30
40
50
60
70
0
50
100
150
200
DG
SU

B


Methylcellulose-DGVs SU-ALH(µm)-Kremer's-1cm
% Frequency
ALH (µm)
0
10
20
30
40
50
60
70
0
5
10
15
20
DG
SU

C

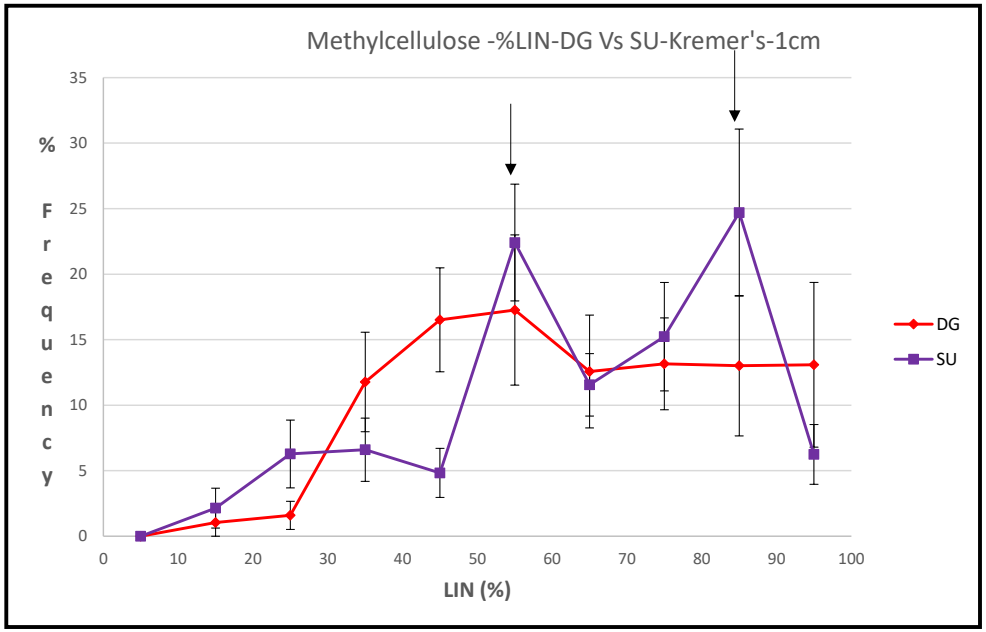
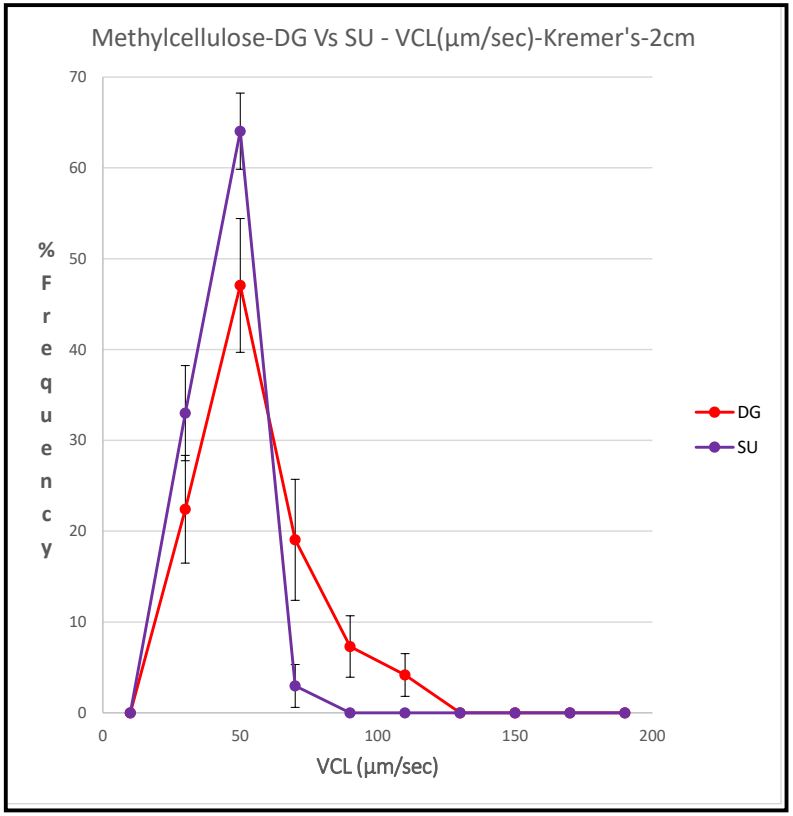
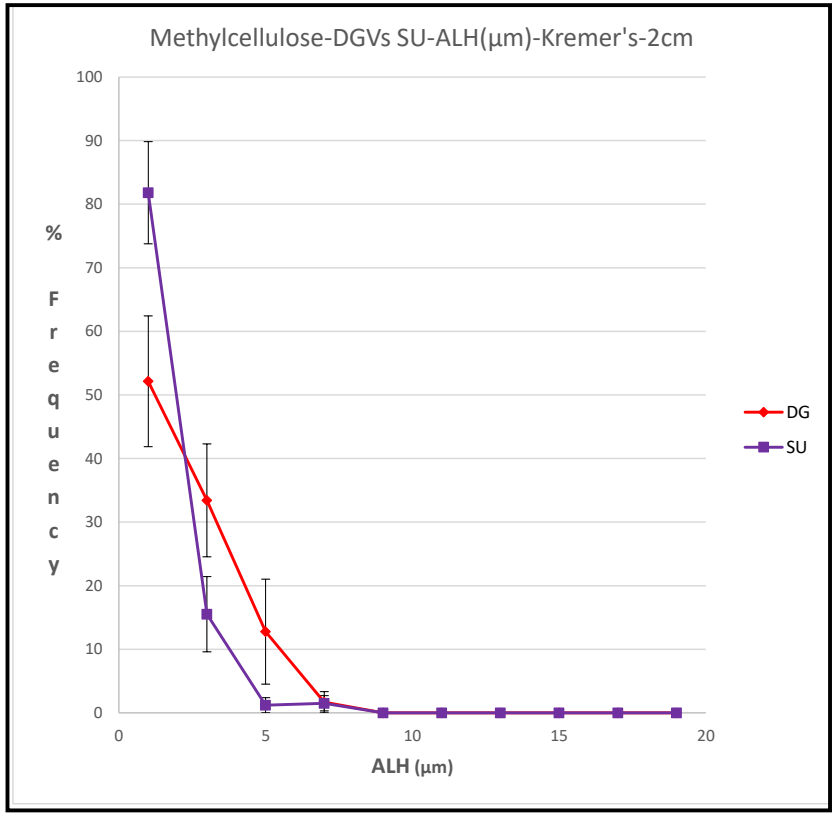


Figure 3.5 Showing the frequency distributions (FD) between direct SU and DG cells in kremer's (In Viscous medium) at 1cm using CASA analyser. A, B, and C Showing the frequency distributions (FD) of Hyperactivated (HA) motility determinants VCL (µm/sec), ALH (µm) and %LIN when cells were prepared with direct Swimup (SU) (Purple) and density gradient techniques (DG) (Red). The cells were measured at 1cm in the viscous medium prepared using methylcellulose and the frequency distributions were analysed using CASA analyser. Cells prepared with SU shown an slight increase in the **(A)** VCL, with a decrease in the **(B)** ALH (observed at the beginning) and even though there is a decrease in the **(C)** LIN (at the end), there was a increase in the LIN in SU cells which were higher (**See black arrows**) in the parts of the distribution compared to the DG cells suggesting this increase in the LIN assist SU cells to progress further in the viscous medium than in DG cells. Each plot shows mean S.E (error bars) of 12 experiments between direct SU and DG techniques.

A


Methylcellulose-DG Vs SU - VCL(µm/sec)-Kremer's-2cm
% Frequency
VCL (µm/sec)
0
10
20
30
40
50
60
70
0
50
100
150
200
DG
SU

B


Methylcellulose-DGVs SU-ALH(µm)-Kremer's-2cm
% Frequency
ALH (µm)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
DG
SU

C

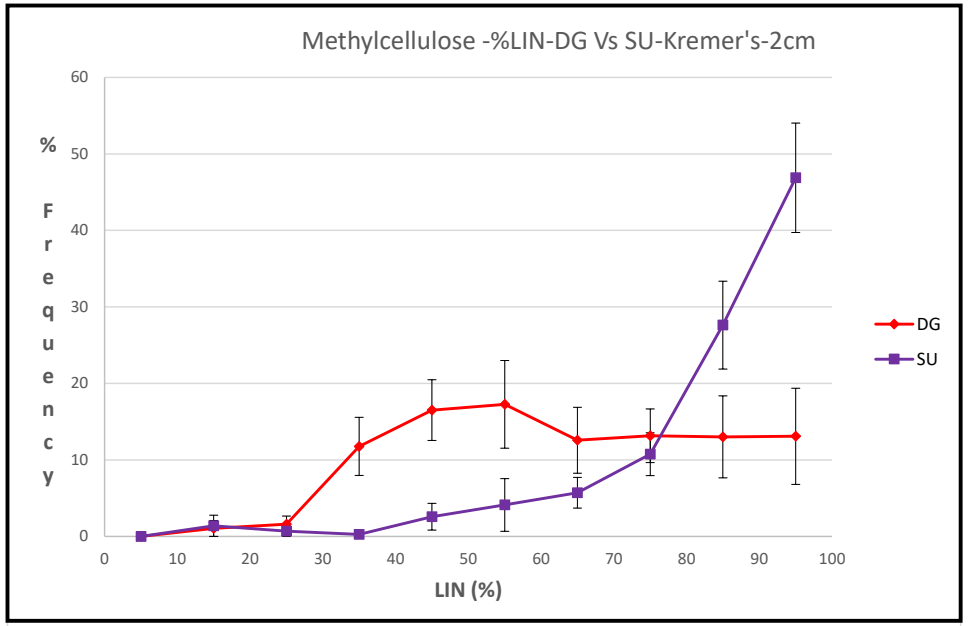


Figure 3.6 Showing the frequency distributions (FD) between direct SU and DG cells in kremer's (In Viscous medium) at 2cm using CASA analyser. A, B, and C Showing frequency distributions (FD) of Hyperactivated (HA) motility determinants VCL (µm/sec), ALH (µm) and %LIN when cells were prepared with direct Swimup (SU) (Purple) and density gradient techniques (DG) (Red). The cells were measured at 2cm in the viscous medium prepared using methylcellulose and the frequency distributions were analysed using CASA analyser. At 2cm SU cells show an increase in the **(A)** VCL (a slight shift to the left side), **(B)** ALH and **(C)** LIN suggesting they are hyperactivated and with a increase in LIN makes them to be better progressive (compared to DG) in the viscous medium, i.e they (SU cells) switch to **hyperactivated progressive behaviour** which enables them to perform better compare to the DG cells in the viscous medium. This also suggesting that the hyperactivation is a very important mechanism that sperm cells adopts to face the penetration challenge that they encounter in the viscous medium or mucus. Each plot shows mean S.E (error bars) of 12 experiments between direct SU and DG techniques.

3.5 Discussion

In this chapter, I investigated the effects of two different sperm preparation techniques (density gradient centrifugation (DG) and direct swim-up (SU)) on human sperm motility and the ability to penetrate into artificial mucus.

Density gradient centrifugation (DG) cells show better hyperactivated motility (%HA) in comparison to direct swim-up cells (SU).

When human sperm motility characteristics was analysed by CASA (See section 2.4), DG cells shown a lower progressive motility compared to SU cells (Figure 3.1 **D**). DG cells show a significantly higher rate of hyperactivation when compared to SU cells (Figure 3.1 **A**). Hyperactivation was defined according to Mortimer (Mortimer, 2000c) and included cells where curvilinear velocity (VCL) ≥ 50μ m/s, linearity (LIN) < 50%, amplitude of lateral head displacement (ALH) ≥ 7μm.g.

Although DG cells show a slightly higher ALH and VCL (Figure 3.1 **B** & **C**), these differences were not significant, but LIN of DG cells was significantly lower (Figure 3.1 **F**) and this appears to be the primary reason for the difference between the levels of HA in cells prepared by the two techniques (Figure 3.1 **A**). LIN is VSL/VCL: the ratio of straight line velocity (calculated using the linear distance between first and last points of the sperm track) to the curvilinear velocity (calculated using the total distance of the sperm head track). Lateral movement of the sperm head (ALH) was not increased in DG cells (Figure 3.1**B**) therefore the reduction in LIN must reflect a more curved path in the DG cells.

Frequency Distributions (FD) from Standard CASA and in methylcellulose

Comparison of FDs for SU and DG cells showed clear differences. In standard CASA (cells swimming in saline) the peak of the FD for VCL in SU cells was clearly higher than in DG. (fig 3.2A). This is surprising since DG cells have significantly higher levels of hyperactivation (fig 3.1A). However, in DG there was a sub-population (approx. 7.5% of cells) with extremely high VCL. In combination with the lower LIN of DG cells (fig 3.2C) this is sufficient to account for the higher proportion of cells in DG-prepared samples that are classified by CASA as hyperactivated. FDs for DG and SU cells in methylcellulose were similar at 0 and 1 cm but at 2 cm the DG cell distributions showed higher VCL and ALH and lower LIN. These kinematics, characteristic of hyperactivated motility, may explain, in part, the poorer performance of DG cells in Kremer assays (see below).

Direct swim-up cells (SU) show better penetration into artificial mucus when compared to density gradient centrifugation (DG) cells.

An in vitro penetration assessment was performed using Kremer's penetration test. Figure 3.3 **A** and **B** show the effect of sperm preparation techniques on the penetration into artificial mucus (methylcellulose). SU cells clearly have a better penetration ability, even after adjustment (normalisation) to take account of the lower proportion of progressive cells in these preparations (Figure 3.3 **B**). The reason for this difference is not clear, but one possibility is that the increase in HA motility impairs the ability of DG sperm to penetrate through the methylcellulose. To investigate this possibility, the effect of artificial induction of HA on penetration was investigated (see chapter 4).

I can conclude that different sperm preparation techniques (DG and SU) affect motility and the penetration of artificial mucus in vitro. Cells prepared by the SU method show better functional (penetration) ability when compared to DG cells, suggesting that these cells (SU cells) might have a better fertilising potential.

Chapter 4: Effect of different behaviours on penetration – artificial viscous & viscoelastic media (Met/Poly) – DG & SU

4.1 Objective:

The objective of this chapter is to investigate the effects of different behaviours on the penetration of human sperm through artificial viscous (methylcellulose) and viscoelastic (polyacrylamide) media when prepared using different sperm preparation methods (density gradient centrifugation and direct swim-up). These behaviours are artificially induced *in vitro* by mobilising calcium (the primary regulator of sperm motility) from stores or by CatSper (calcium-specific for sperm cell signalling).

4.2 Introduction

For successful fertilisation, mammalian sperm must be able to enter, progress through the female reproductive tract, and successfully bind to the oocyte. During mammalian sperm migration in the female reproductive tract, they encounter various physically and anatomically complex environments (viscous and viscoelastic environments in cervical mucus) that can hinder sperm interaction with the oocyte, which plays a crucial role in determining successful fertilisation.

To successfully pass through these barriers (viscous and viscoelastic), human sperm are believed to adopt an appropriate flagellar beat pattern that results in a characteristic behaviour that is essential for sperm transportation and fertilisation. Incubation of sperm under capacitating conditions (capacitation was defined as the mammalian sperm maturation event that happens in the female reproductive tract and enables sperm to achieve fertilising potential) (Ikawa et al., 2010) is necessary to initiate a more vigorous type of motility, which is termed as "hyperactivated motility".

In 1970 Yanagimachi (YANAGIMACHI, 1970) reported that movement of hamster spermatozoa is extremely active after completing capacitation. Later, several researchers considered hyperactivation to be an important marker for capacitation and its motion characteristics were examined in humans (Mortimer and Mortimer, 1990, Burkman, 1984, Mortimer et al., 1997, Aitken et al., 1985). A typical hyperactivated sperm cell showed motility characteristics of an asymmetrical beat pattern, increased lateral head movement, deep flagellar bends, and increased bending of the midpiece/proximal flagellum (Ho and Suarez, 2001). To assess human sperm motility (in vitro) in real time we use computer-assisted sperm analyser (CASA) that can distinguish different sorts of behaviours (from a hyperactivated behaviour pattern to a linear behaviour pattern) by tracking sperm head motion, which cannot be measured or observed manually.

4.2.1 *In vitro* penetration test – artificial viscous (methylcellulose) & viscoelastic (polyacrylamide) media

Computer-assisted sperm analysis provides information about motility, morphology, and concentration of a sperm sample. Although CASA plays a vital role in understanding different aspects of sperm, its role in providing accurate prognostic and diagnostic information is limited. Given this scenario, a technique called the ***in vitro* sperm penetration test into human cervical mucus (HCM)** has been developed to provide valuable information about sperm function (Ivic et al., 2002) that is important in understanding the fertility outcome.

4.2.1.1 *In vitro* penetration test (Kremer test)

Under *in vivo* conditions, before sperm interacts with the oocyte for successful fertilisation, sperm needs to penetrate through cervical mucus, and the fertilisation success depends on the ability of the sperm to transit through the female tract and reach the fertilisation site in the ampulla of the oviduct. Therefore, *in vitro* assessment of human sperm penetration provides valuable information about the fertilising capacity of a sperm sample. Under *in vitro* conditions, the human sperm penetration test is performed by using glass capillary tubes filled with penetration medium and placed with one end in contact with the sperm sample. Penetration is assessed by counting motile sperm cells at various distances after establishing contact (sperm – penetration medium).

Dr Jan Kremer developed this sperm penetration test in 1965 (Kremer, 1965). Initially, the method used circular cross-section capillaries, which have now been replaced by rectangular glass capillary tubes for easier examination under a microscope.

Use of this technique is limited when considering the problems that were associated with human cervical mucus (HCM) (limited availability, instability and storage problems) (Ivic et al., 2002). Due to this reason, there is a need for using an alternative artificial penetration medium to assess human sperm penetration under *in vitro* conditions.

4.2.1.2 Methylcellulose (viscous) – artificial penetration medium (in vitro)

Methylcellulose is used as an alternative medium to human cervical mucus for the sperm penetration test under *in vitro* conditions (Ivic et al., 2002). Methylcellulose is a long-chain substituted cellulose with 30% of its hydroxyl groups in the form of methyl ether. Different grades of methylcellulose have viscosities in the range of 10-10,000 centipoise (cp) for a 2% solution, which are similar to the range of human cervical mucus, where viscosities are 2800–10,000 cp (Karni et al., 1971) (Ivic et al., 2002). The advantages of using methylcellulose comprise its stability over extended periods of time, uniform quality and consistency, and that it is easy to obtain and significantly cheaper than hyaluronic acid. As it is non-toxic, it is widely used in pharmaceutical and food industries. It is also used to dilute sperm in intra-cytoplasmic sperm injection procedures (ICSI) (Ray et al., 1995) (Ivic et al., 2002).

4.2.1.3 Polyacrylamide (viscoelastic) – artificial penetration medium (in vitro)

Polyacrylamide has also been used as an artificial sperm penetration medium since polyacrylamide gels are visco-elastic and thus resemble more closely the characteristics of mucus. Polyacrylamide chains have a molecular mass of 6000 kDa (Berg and Turner, 1979) (Suarez and Dai, 1992). Previous experimental results have shown that viscoelastic fluid response can increase the speed and efficiency of a simple moving swimmer, although the work is carried out by immersing a flexible sheet in a fluid rather than working on a sperm model (Teran et al., 2010).

Work done in the laboratory of Susan. Suarez on mouse sperm showed that the motility of hyperactivated sperm becomes more progressive when the viscosity of the medium is increased. It was proposed that under *in vivo* conditions, when mouse sperm encounter the viscoelastic environment present in the oviductal region the development of hyperactivated motility will enable sperm to progress more effectively (Suarez and Dai, 1992).

Average path length (VAP) indicates that the translation of the flagellar beat pattern into directional movement of a sperm cell is lower in the case of both fresh and hyperactivated sperm in viscous medium, but the hyperactivated flagellar beat is superior for sperm in polyacrylamide.

4.2.1.4 Comparison of HCM (human cervical mucus), methylcellulose and polyacrylamide as human sperm penetration media

Parameters	Human cervical mucus (HCM)	Methylcellulose	Polyacrylamide
Toxicity	Not toxic	Not toxic	Toxic
Nature	Viscous	Viscous	Viscoelastic
Stability	Instability	Stable	Stable
Availability	Difficulty in collecting	Easy to obtain	Easy to obtain
Storage	Storage problem	Can easily store	Can easily store
Cost	Expensive	Not expensive	Expensive

Table 4.1: Comparison of HCM, methylcellulose and polyacrylamide as human sperm penetration media.

Different parameters, e.g. toxicity, nature of the medium, stability, availability, storage and cost, are compared between human cervical mucus (HCM), methylcellulose and polyacrylamide to use as human sperm penetration media. Methylcellulose seems to be a better alternative than HCM and polyacrylamide as it is non-toxic, stable for longer periods, easy to obtain, and not expensive.

4.3 Materials and Methods

4.3.1 Materials

Supplemented Earle's balanced salt solution (sEBSS) contains 1.0167 mMNaH_2PO_4, 5.4 mM KCl, 0.81mM $MgSO_4 \cdot 7H_2O$, 5.5 mM $C_6H_{12}O_6$, 2.5 mM$C_3H_3NaO_3$, 19.0 mM $CH_3CH(OH)COONa$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 25.0 mM $NaHCO_3$, 118.4 mM NaCl, and 15 mM HEPES (pH 7.4, 292 mosM), supplemented with 0.3% (w/v) fatty acid-free BSA. Osmotic strength was maintained by adjusting NaCl.

Chemicals used in the preparation of media were from Sigma-Aldrich (Poole, Dorset, UK). Fatty acid-free Bovine Serum Albumin (BSA) was from United States Biological (Swampscott, MA, USA). Osmotic strength was maintained by adjusting NaCl. Drugs (4-Aminopyridine (4AP, 5 mM), Progesterone (P4, 3 μM) and Prostaglandin E_1 (PE_1, 2 μM)) used in the experiments were from Sigma-Aldrich (Poole, Dorset, UK).

4.3.1.1 Materials – viscous medium

1% w/v methylcellulose (4000cp viscosity in a 2% solution at 20°C, Sigma-Aldrich, UK) made up in sEBSS, 50 x 4 x 0.4mm glass capillary tubes (CM Scientific, UK), Cristaseal wax (Hawksley, UK) to seal one end of the glass capillary tube, and 1.5ml Eppendorf tubes.

4.3.1.2 Materials – viscoelastic medium

1% w/v polyacrylamide (2.4-2.7cp viscosity in a 0.1% solution 25°C, Sigma-Aldrich, UK) made up in sEBSS, 50 x 4 x 0.4mm glass capillary tubes (CM Scientific, UK), Cristaseal wax (Hawksley, UK) to seal one end of the glass capillary tube, and 1.5ml Eppendorf tubes.

4.3.2 Methods

4.3.2.1 Donor Recruitment

Donor recruitment was conducted as described in Section 2.2.

4.3.2.2 Human Sperm Preparation

For human sperm preparation by direct swim up method (SU) and density gradient centrifugation methods please see Section 2.4

4.3.2.3 Computer-assisted Semen Analysis (CASA)

For CASA, See Chapter 2.5.

After 4 hours and 30 min of capacitation, 100µl aliquots of sperm suspension were treated with or without different agonists (4AP, PE_1 & P4). From each of these stimulants, 5 µl of sperm suspension were immediately added to either side of the pre-warmed CASA 2X-CEL chamber (Hamilton Thorne Biosciences) with a depth of 20 µm, and temperature is

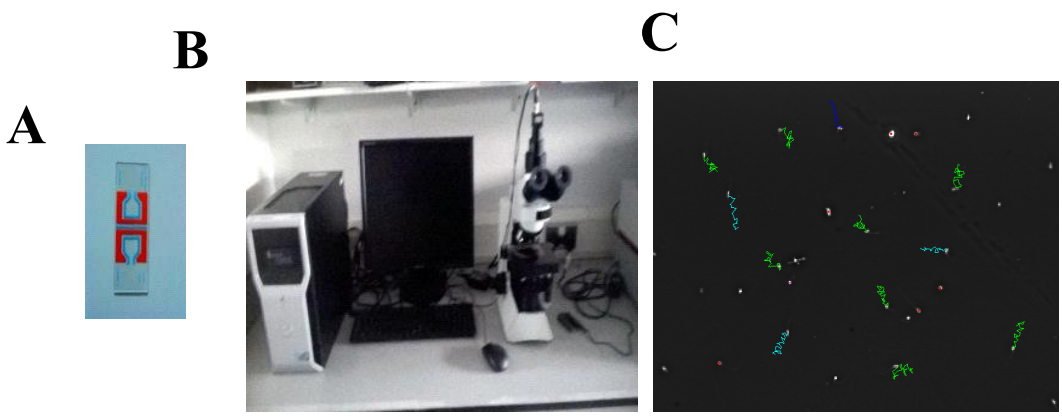


Figure 4.0: Computer assisted sperm analysis (CASA). Assessment of human sperm hyperactivated motility (*in vitro*) is performed by a CASA. **A** Showing CASA 2X-CEL chamber (Hamilton Thorne Biosciences) with a depth of 20 µm. **B** Showing computer assisted semen analyser. **C** Showing sperm motility tracks. **Green** = Hyperactivated non-progressive motility, **Light Blue** = Hyperactivated progressive motility, **Dark Blue** = Activated motility, **Red** = Immotile/Dead sperm.

At least 20 data points were selected for analysis of each sample, with a minimum of 100 cells observed for each sample. Motility of a hyperactivated human sperm was analysed at 60 Hz using a 10x objective. According to Mortimer (Mortimer, 2000c), hyperactivated human sperm is defined as curvilinear velocity (VCL) ≥ 50 μm/s, linearity (LIN) <50%, increased amplitude of lateral head displacement (ALH) ≥7 μm (Mortimer, 2000b).

Different motility behaviours were observed as cells were treated with different stimulants (4AP, PE_1 & P4). Treated cells were compared with parallel untreated cells (control) and analysed using statistical software. A paired t-test is performed to test statistical significance between stimulants and control. If $P<0.05$, then the results were considered statistically significant.

4.3.2.4 Kremer's Penetration Test

For Kremer's Penetration Test, See Chapter 2.6.

The effect of different behaviours (established artificially by stimulating human sperm with different agonists 4AP, PE_1 & P4) on the penetration of human sperm through artificial viscous (methylcellulose) and viscoelastic (polyacrylamide) media in both direct SU and DG cells where analysed using kremer's penetration test.

4.3.2.5 Work flow and control data.

In Table 4.2 and 4.3 effects of agonists (4AP, P4 and PE1) on motility parameters are compared to the untreated parallel control in both DG and SU cells. The control data shown in Table 4.2 (DG Cells) and Table 4.3 (SU Cells) are the same as those listed in Table 3.1 in Chapter 3. This reflects analysis of different aspects of the data generated during the same

experimental protocol. A detailed experimental work flow is given in Section 2.3 and Figure 2.0, explaining the use of same control in both Chapters 3 and 4.

4.4 Results

4.4.1 Assessment of human sperm hyperactivated motility by 4-Aminopyridine (4AP), Prostaglandin E_1 (PE_1) and progesterone (P4) agonists by two different sperm preparation techniques – DG & SU

Stimulation with 5 mM 4AP enhanced hyperactivated motility compared to parallel controls, both in cells prepared by direct swim-up (($p< 0.0001$; **** n=20; paired t-test) and density gradient centrifugation (($p< 0.0001$; **** n=20; paired t-test) (Figure 4.1). Consistent with this increase in hyperactivation, 4AP-treated showed significantly increased lateral head displacement (ALH) and curvilinear velocity (VCL) and significantly decreased (VSL) and linearity in both swim-up and density gradient-prepared cells (figs 4.2-4.5)

Statistical *F-TEST* was performed to test if there is any equal variability between two sperm preparation techniques (DG & SU) when cells are stimulated with 4AP. Since F_{value} (0.30722) is smaller than $F_{Critical}$ (0.46120) there was no significant difference in the variability of two sperm preparation techniques (DG & SU) when cells are stimulated with 4AP (SU Vs DG).

2μM Prostaglandin (PE_1) or 3μM Progesterone (P4) were much less effective in inducing hyperactivation. Only treatment of DG cells with PE_1 caused a significant increase in hyperactivation compared to the parallel controls ($p=0.0142$; Figure 4.1).

FDs of key kinematic parameters (generated by CASA) were analysed using Microsoft Excel. The FD of DG cells stimulated with PE1 and P4 were similar to the control but 4-AP induced clear differences compared to the control and to PE1 and P4-treated cells. In Figure 4.6 (A, B) it can be seen that the FDs for VCL and ALH of 4AP-treated cells each have a peak similar to that of the control but also a ‘tail’ of cells with much higher values.

There was also a marked decrease in LIN of 4AP-treated cells with the entire curve shifted to the left (Figure 4.6 **C**). When FD data were analysed for the SU sperm a similar pattern was observed, though in these cells, whereas 4AP caused a shift of the LIN distribution to the left, PE1 and P4 also shifted the LIN curve but to the right (Figure 4.7 **A, B** & **C**).

Density Gradient Centrifugation (DG)								
PARAMETER	**Control**	S.E.M.	**5mM 4-Amino pyridine (4AP)**	S.E.M.	**2µM Prostaglandin (PE_1)**	S.E.M.	**3µM Progesterone (P4)**	S.E.M.
Hyperactivation (% Sort 7)	6.25	0.695	19.45****	1.669	8.8**	0.972	6.4	1.037
Lateral amplitude (ALH) (µm)	3.795	0.099	5.16****	0.208	3.93	0.124	3.97	0.131
Curvilinear velocity (VCL) (µm/s)	91.345	3.134	123.87****	6.045	94.82	3.277	92.17	3.502
Straight-line / Prog. velocity (VSL) (µm/s)	45.585	1.423	34.435****	1.297	45.28	1.752	44.31	1.679
Linearity (LIN) (% LIN)	54.1	1.309	35.2****	1.634	53	1.374	51.7	1.682

Table 4.2: Effects of 4-Aminopyridine (4AP), Prostaglandin (PE_1) and Progesterone (P4) on motility parameters, as determined by CASA when cells are prepared by density gradient centrifugation (DG). Experiments were carried out in pairs, where cells from the same semen sample were analysed by CASA with and without treatment (different agonists). Cells without treatment were prepared in sEBSS (15mM HEPES, pH=7.4), and no treatment in control (no colour). Cells were stimulated with 5mM 4AP (4AP, Brown), 2µM PE_1 (PE_1, Green) and 3 µM (P4, Blue). For each condition, hyperactivation (sort 7%), lateral head amplitude (ALH), curvilinear velocity (VCL), straight-line/progressive velocity (VSL), and linearity (% LIN) were determined. **** $P<0.0001$; with 4AP compared to control (sort 7%, ALH, VCL, VSL & % LIN); paired t-test; n=20. ** $P<0.0142$; with PE_1 compared to control (sort 7%); paired t-test; n=20.

Direct Swim-up (SU)								
PARAMETER	Control	S.E.M.	**5mM 4-Amino pyridine (4AP)**	S.E.M.	**2µM Prostaglandin (PE_1)**	S.E.M.	**3µM Progesterone (P4)**	S.E.M.
Hyperactivation (% Sort 7)	3.75	0.742	26.4****	3.011	4.6	0.877	4.8	0.869
Lateral amplitude (ALH) (µm)	4.05	0.129	6.54****	0.319	3.995	0.127	4.77****	0.159
Track speed (VCL) (µm/s)	94.16	2.192	130.47****	6.717	94.185	2.944	95.61	2.566
Straight-line / Prog. velocity (VSL) (µm/s)	55.89	1.722	37.89****	1.595	55.365	1.907	45.965****	1.517
Linearity (LIN) (% LIN)	60.3	1.675	34****	1.676	59.9	1.465	50.05****	1.595

Table 4.3: Effects of 4-Aminopyridine (4AP), Prostaglandin (PE_1) and Progesterone (P4) on motility parameters, as determined by CASA when cells are prepared by direct swim-up (SU). Experiments were carried out in pairs, where cells from the same semen sample were analysed by CASA with and without treatment (different agonists). Cells without treatment were prepared in sEBSS (15mM HEPES, pH=7.4), with no treatment in control (no colour). Cells were stimulated with 5mM 4AP (4AP, Brown), 2µM PE_1 (PE_1, Green) and 3 µM (P4, Blue). For each condition, hyperactivation (sort 7%), lateral head amplitude (ALH), curvilinear velocity (VCL), straight-line/progressive velocity (VSL), and linearity (% LIN) were determined. **** $P<0.0001$; with 4AP compared to control (sort 7%, ALH, VCL, VSL & % LIN); paired t-test; n=20. **** $P<0.0001$; with P4 compared to control (ALH, VSL & LIN); paired t-test; n=20.

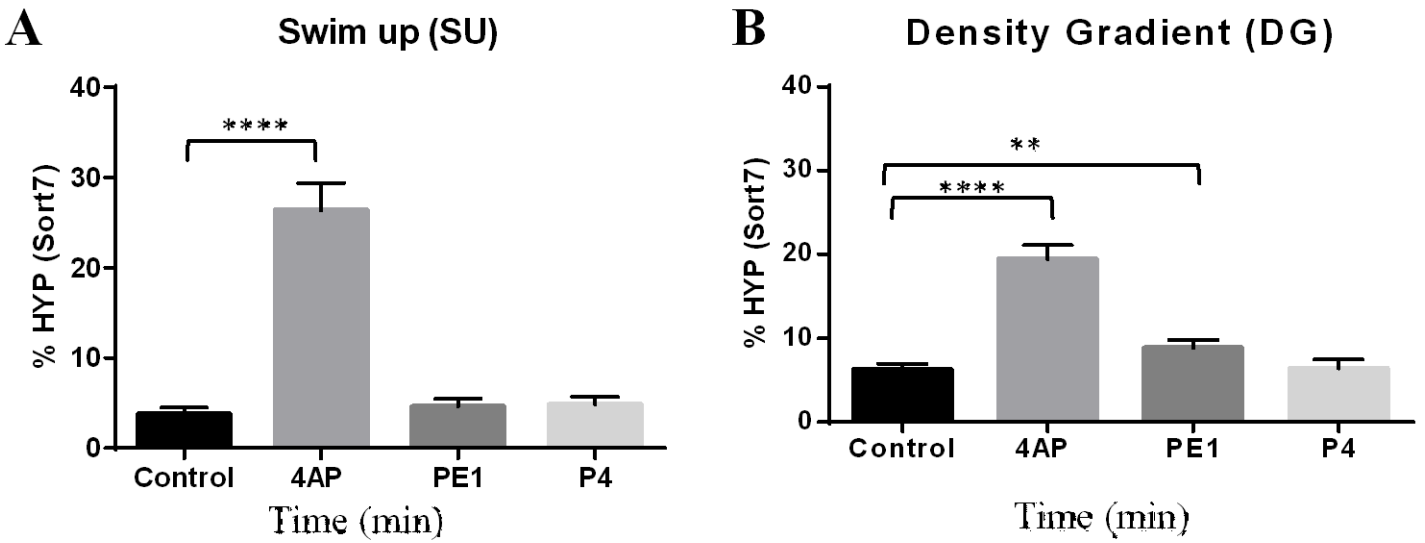


Figure 4.1: 4-Aminopyridine is a potent inducer of hyperactivation. Figures A & B show the percentage of hyperactivated cells induced by 5mM 4-Aminopyridine (4AP), 2µM Prostaglandin (PE1), and 3µM Progesterone (P4). The cells are prepared by direct swim-up into sEBSS; **B.** cells prepared by density gradient centrifugation into sEBSS. **A.** 4-AP shows enhanced hyperactivated motility when prepared using the direct swim-up technique ($p<0.0001$); **** (paired *T-test*). **B.** Density gradient method ($p<0.0001$); *** (paired *T-test*) compared with control. Statistical *F-TEST* of 4AP (DG&SU) [$F_{Value} = 0.30722$ & $F_{critical} = 0.46120$]. Each bar shows the mean S.E. (error bars) of 20 experiments.

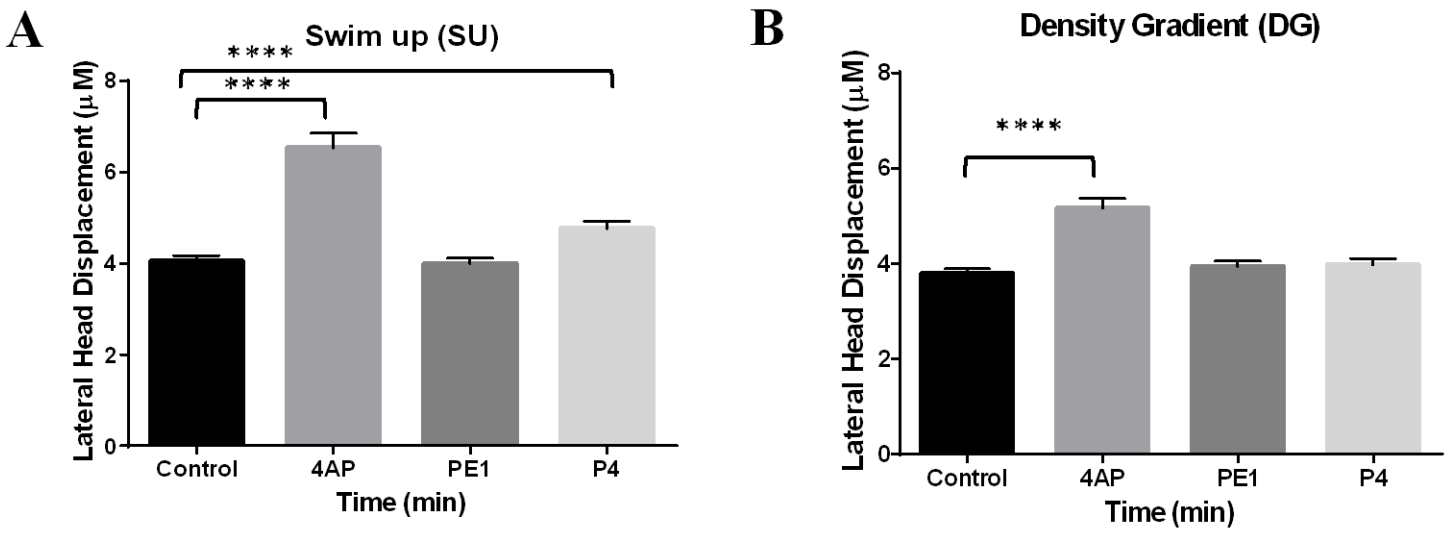


Figure 4.2: Motility characteristics (Lateral Head Displacement, ALH) of human sperm. Figures A & B show the percentage of lateral head displacement of human sperm cells when induced by 5mM 4-Aminopyridine (4AP), 2μM Prostaglandin (PE1) and 3μM Progesterone (P4). The cells are prepared by direct swim-up into sEBSS; **B.** cells prepared by density gradient centrifugation into sEBSS. **A.** When compared with control (4-AP), $p<0.0001$; **** and (P4) $p<0.0001$; **** shows a significant difference in lateral head displacement of human sperm cells when prepared using the direct swim-up technique. **B.** When compared with control (4-AP), $p<0.0001$; **** shows a significant difference in lateral head displacement of human sperm cells when prepared using the density gradient method. Each bar shows the mean S.E. (error bars) of 20 experiments.

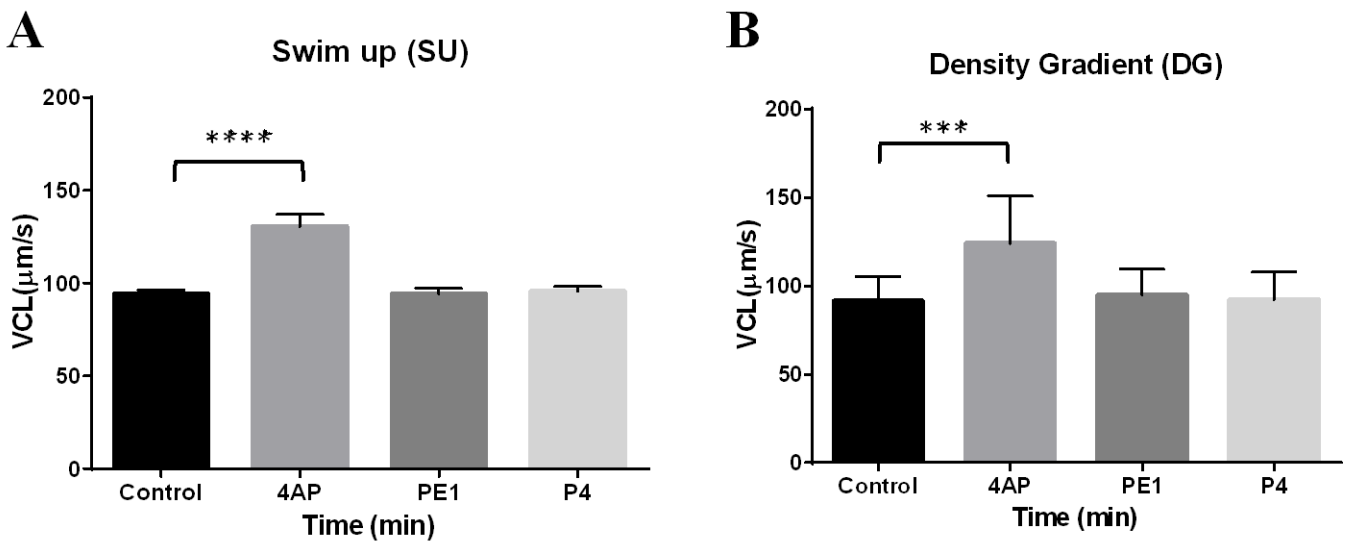


Figure 4.3: Motility characteristics (Curvilinear Velocity, VCL) of human sperm. Figures A & B show the VCL percentage of human sperm cells when induced by 5mM 4-Aminopyridine (4AP), 2μM Prostaglandin (PE1), and 3μM Progesterone (P4). The cells are prepared by direct swim-up into sEBSS; **B.** cells prepared by density gradient centrifugation into sEBSS. **A.** When compared with control (4-AP), $p<0.0001$; **** shows a significant difference in curvilinear velocity (VCL) of human sperm cells when prepared using the direct swim-up technique. **B.** When compared with control (4-AP), $p=0.0001$; ** shows a significant difference in curvilinear velocity of human sperm cells when prepared using the density gradient method. Each bar shows the mean S.E. (error bars) of 20 experiments.

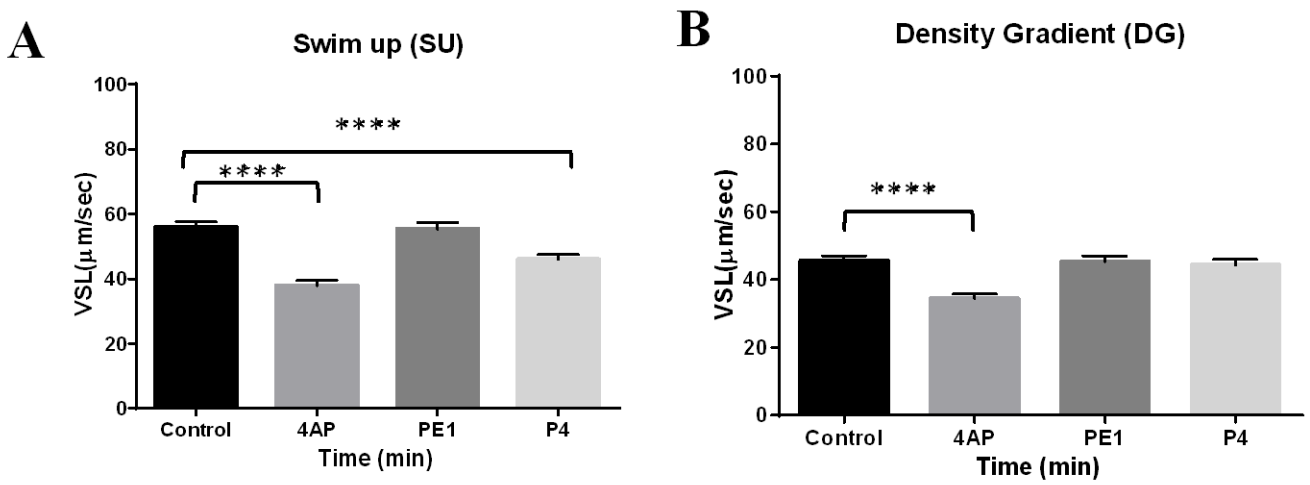


Figure 4.4: Motility characteristics (Straight-Line Velocity, VSL) of human sperm. Figures A & B show the VSL percentage of human sperm cells when induced by 5mM 4-Aminopyridine (4AP), 2µM Prostaglandin (PE1), and 3µM Progesterone (P4). The cells are prepared by direct swim-up into sEBSS; **B.** cells prepared by density gradient centrifugation into sEBSS. **A.** When compared with control (4-AP), $p<0.0001$; **** and (P4) $p<0.0001$; **** shows a significant difference in straight-line velocity (VSL) of human sperm cells when prepared using the direct swim-up technique. **B.** When compared with control (4-AP), $p<0.0001$; **** shows a significant difference in straight-line velocity of human sperm cells when prepared using the density gradient method. Each bar shows the mean S.E. (error bars) of 20 experiments.

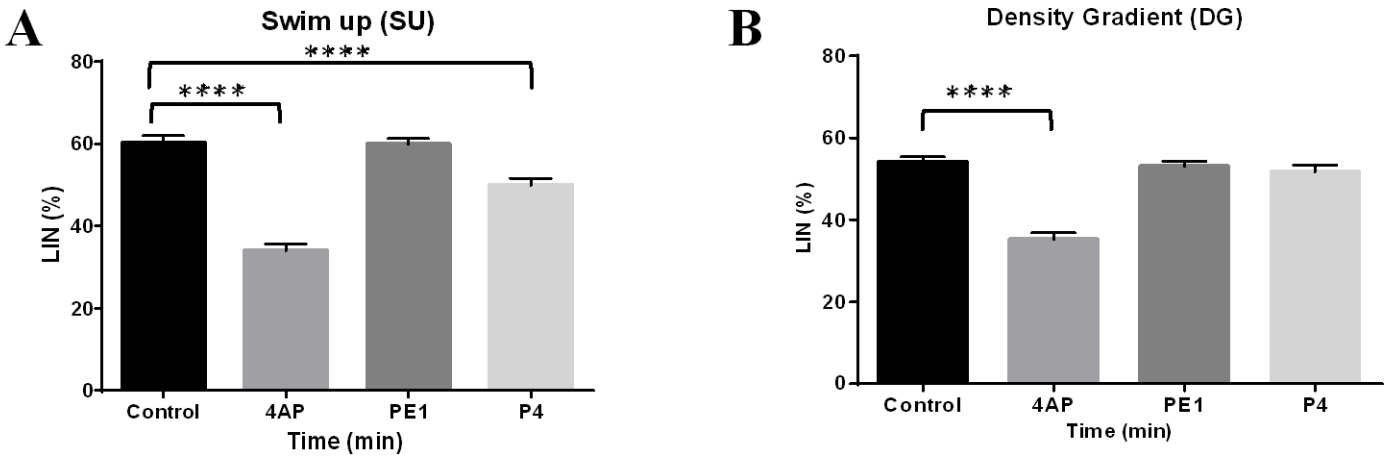
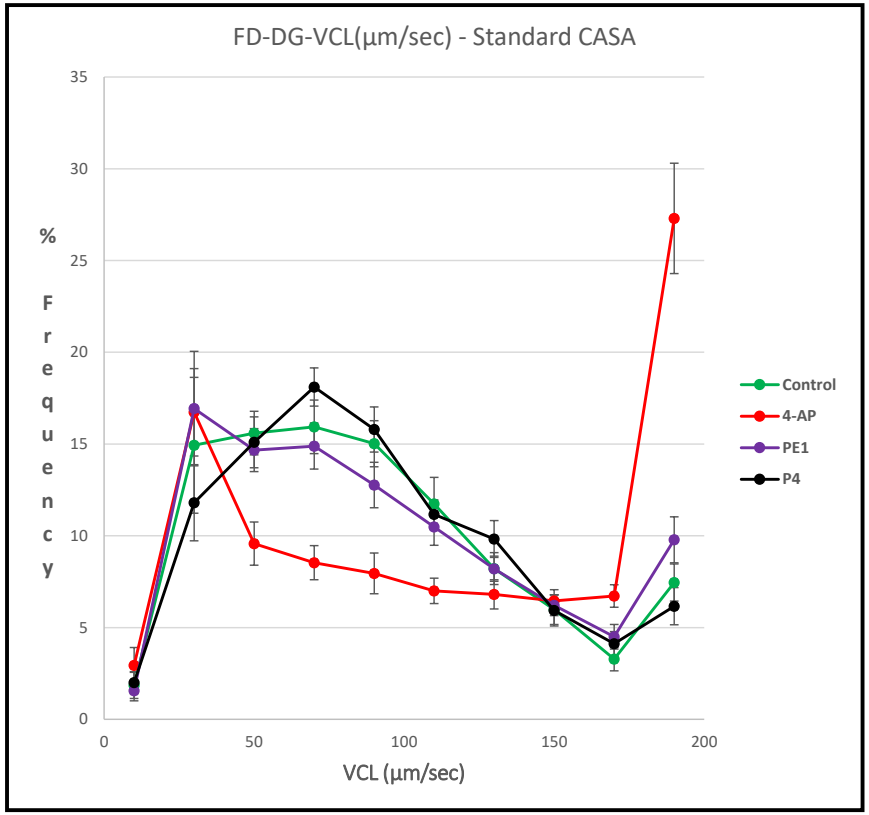
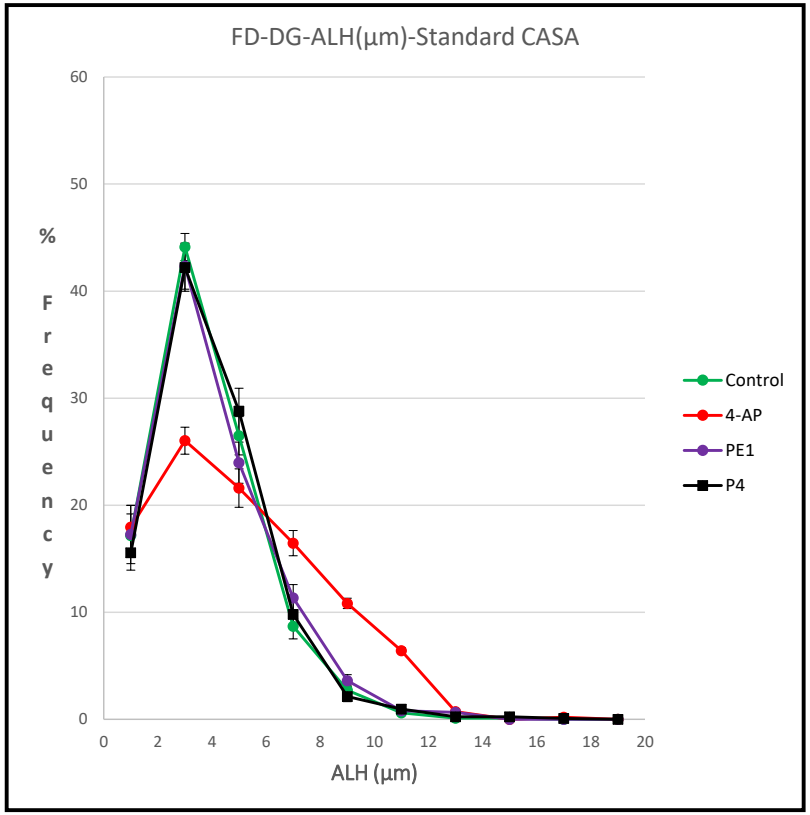


Figure 4.5: Motility characteristics (Linearity, LIN) of human sperm. Figures A & B show the %LIN percentage of human sperm cells when induced by 5mM 4-Aminopyridine (4AP), 2µM Prostaglandin (PE1), and 3µM Progesterone (P4). The cells are prepared by direct swim-up into sEBSS; **B.** cells prepared by density gradient centrifugation into sEBSS. **A.** When compared with control (4-AP), $p<0.0001$; **** and (P4) $p<0.0001$; **** shows a significant difference in linearity (LIN) of human sperm cells when prepared using the direct swim-up technique. **B.** When compared with control (4-AP), $p<0.0001$; **** shows a significant difference in straight-line velocity of human sperm cells when prepared using the density gradient method. Each bar shows the mean S.E. (error bars) of 20 experiments.

A


FD-DG-VCL(µm/sec) - Standard CASA
% Frequency
VCL (µm/sec)
0
5
10
15
20
25
30
35
0
50
100
150
200
Control
4-AP
PE1
P4

B


FD-DG-ALH(µm)-Standard CASA
% Frequency
ALH (µm)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
20
Control
4-AP
PE1
P4

C

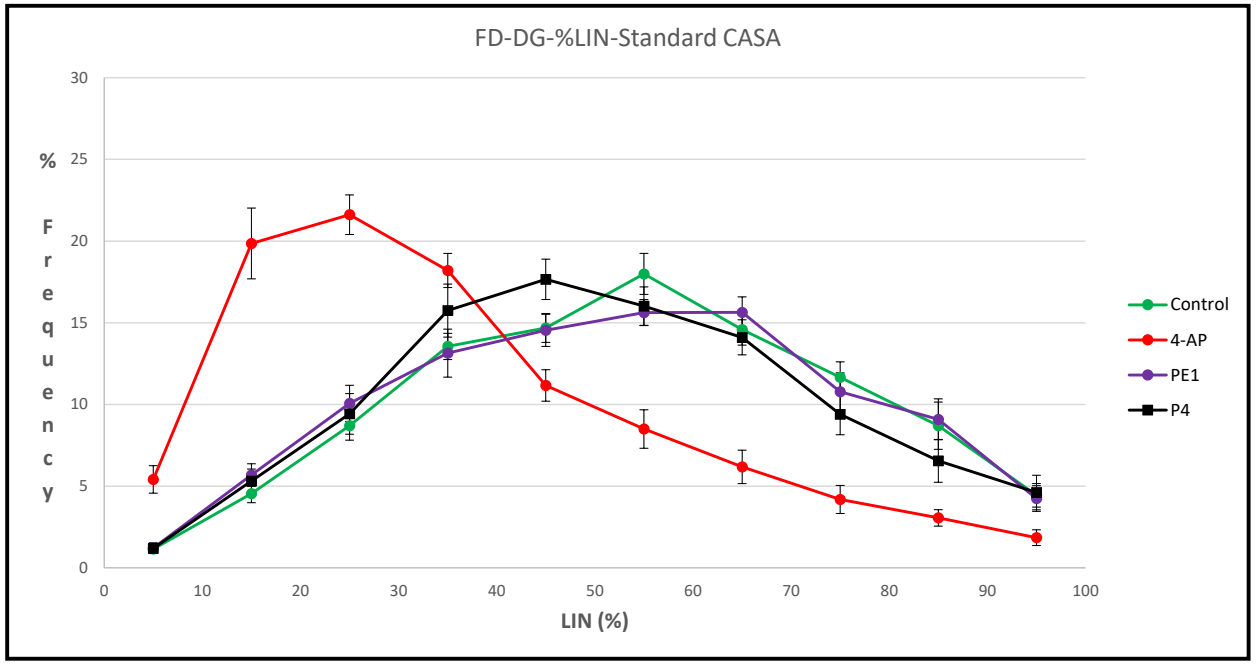
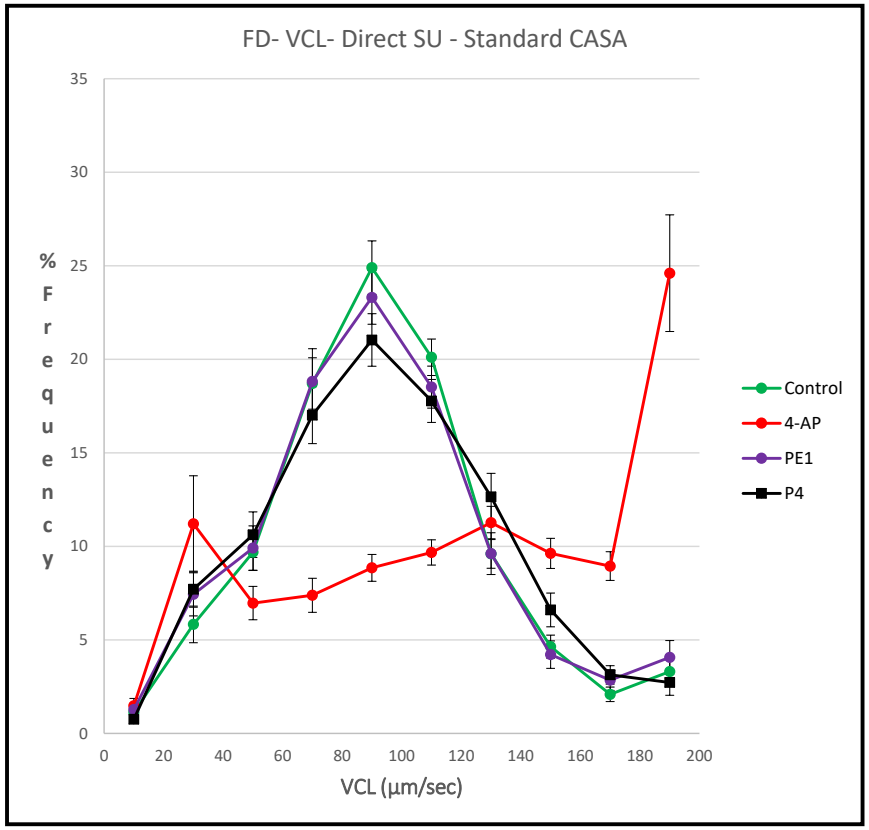
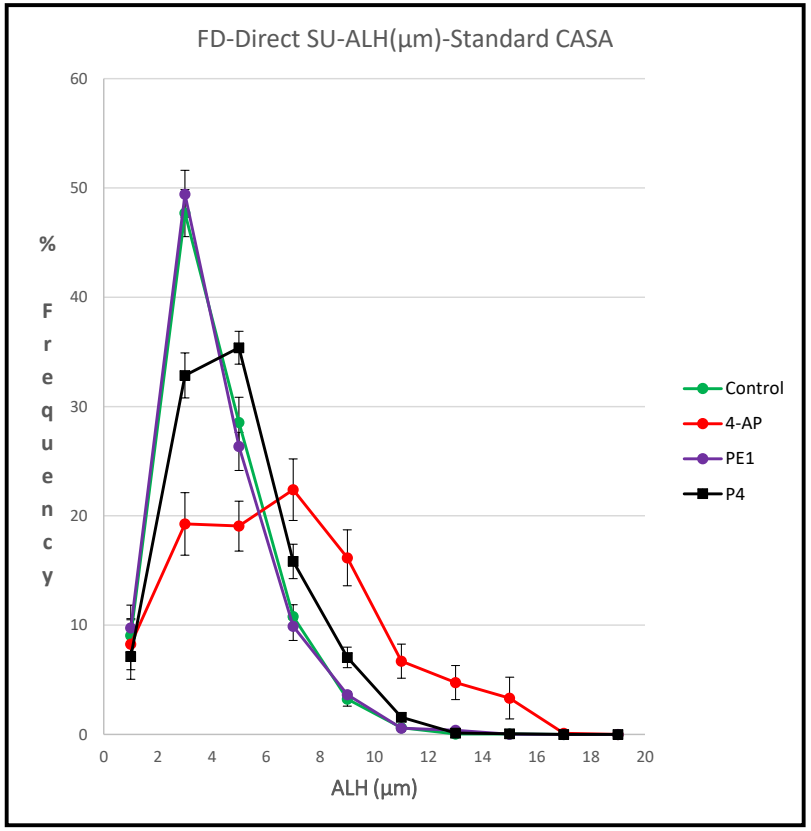


Figure 4.6 Showing the frequency distribution (FD) data for the DG cells. Showing the FD of **A** Curvilinear velocity (VCL) (µm/sec), **B** Amplitude of lateral head displacement (ALH) (µm) and **C** Linearity (%LIN) for the cells prepared using DG technique under the control conditions **(Green)** and after the stimulation of human sperm cells with 5mM 4-Aminopyridine (4-AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black)** in the standard CASA**.** Compared to the control, DG cells stimulated with 4-AP shown an increase in the **A** VCL with a decrease in the **C** linearity (LIN) and also there is a shift in the linearity curve towards the left side. At the beginning of the distribution the 4-AP stimulated cells show a decrease in **B** ALH compared to the control. Cell stimulated with PE1 **(Purple)** and P4 **(Black)** shown similar characteristics (ALH & LIN) compared to the control, where as in the case of **A** VCL the P4 peak shift to right compared to the control. The data suggests that cells treated with 4-AP behave differently in their motility characteristics (VCL, ALH and LIN) compared to the Control, PE1 and P4. Each plot shows mean ± S.E (standard error) of 20 experiments when human sperm cells were prepared using density gradient (DG) technique.

A


FD- VCL- Direct SU - Standard CASA
% Frequency
VCL (µm/sec)
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
140
160
180
200
Control
4-AP
PE1
P4

B


FD-Direct SU-ALH(µm)-Standard CASA
% Frequency
ALH (µm)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
20
Control
4-AP
PE1
P4

C

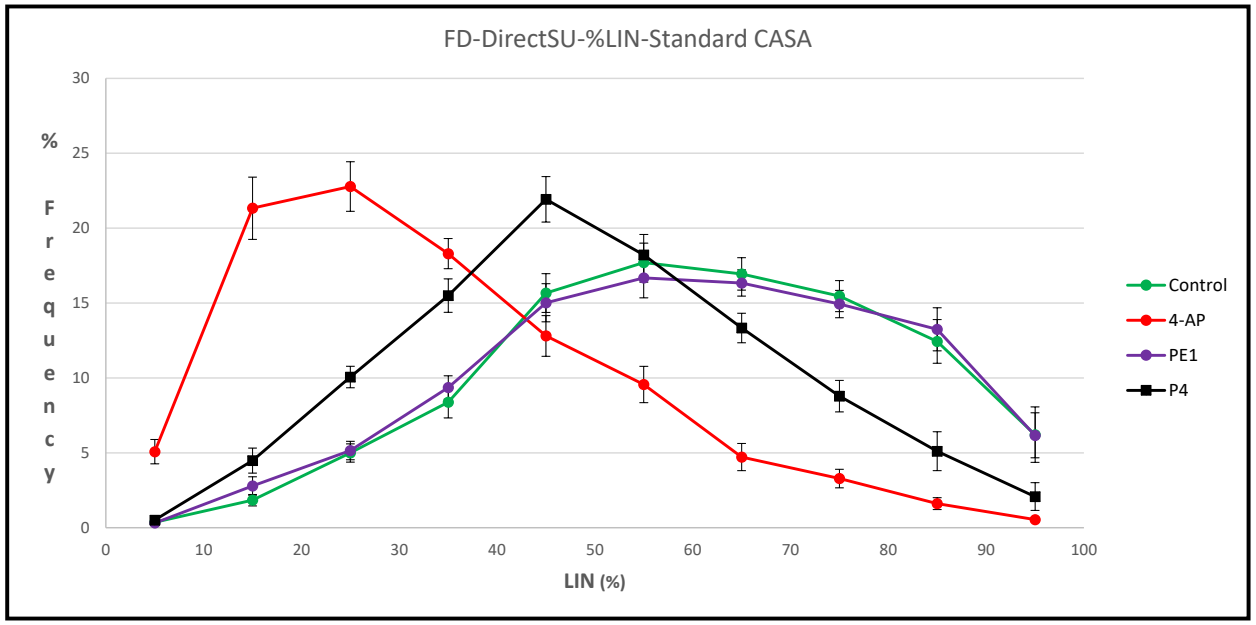


Figure 4.7 Showing the frequency distribution (FD) data for the SU cells. Showing the FD of **A** Curvilinear velocity (VCL) (µm/sec), **B** Amplitude of lateral head displacement (ALH) (µm) and **C** Linearity (%LIN) for the cells prepared using SU technique under the control conditions **(Green)** and after the stimulation of human sperm cells with 5mM 4-Aminopyridine (4-AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black)** in the standard CASA**.** Compared to the control, DG cells stimulated with 4-AP shown an increase in the **A** VCL with a decrease in the **C** linearity (LIN) and also there is a shift in the linearity curve towards the left side. 4-AP **(Red)** stimulated cells show a decrease in **B** ALH compared to the control. Cells stimulated P4 **(Black)** shown a **B** ALH response that is lower than control but higher than 4-AP and the **C** P4 **(Black)** LIN peak shift to the left side but stands in-between control and 4-AP. Cells stimulated with PE1 **(Purple)** show similar motility characteristics (ALH and LIN) and **A** VCL was slightly lower to the control. The data suggests that 4-AP cells behave differently from other treated (PE1 and P4) and untreated (control) ones, with increase in hyperactivation contributing to the decrease in linearity. Each plot shows mean ± S.E (standard error) of 20 experiments when human sperm cells were prepared using density gradient (DG) technique.

4.4.2 Comparative study of penetration into methylcellulose of sperm prepared by the two different sperm preparation techniques when stimulated with 4-Aminopyridine (4AP), Prostaglandin E_1 (PE_1) and progesterone (P4)

Kremer's *in vitro* penetration test was used to investigate how stimulation of different sperm behaviours, as measured by a CASA, affected the ability of cells to enter viscous medium. Treatment with 4-AP potently increased hyperactivated motility (as described above, 4.4.1) but when cells were tested for their ability to penetrate into viscous medium (methylcellulose), both density gradient and swim-up cells were clearly inhibited at all distances into the methylcellulose ($p=0.004$; $n=12$, Figure 4.8 c & Figure 4.8 a). Treatment of DG cells with PE_1 (to activate CatSper channel) had no effect in curvilinear velocity (VCL) (Figure 4.3 b) but enhanced sperm entry in the viscous medium ($p=0.019$; $n=12$ compared to control; paired t-test; Figure 4.8 c). There was no significant enhancement of penetration in cells prepared by swim up ($p=0.0964$; $n=12$ compared to control; paired t-test; Figure 4.8 a).

FDs were analysed for cells penetrated 0, 1, and 2 cm into viscous medium (methylcellulose). In cells that had penetrated further into methylcellulose FDs for VCL and LIN of both DG and SU cells maintained similar characteristics to those at 0 cm, but it was clear that there were fewer cells with high ALH (compare 4.13B and 4.14B with 4.9B and 4.10B), consistent with poor progress of hyperactivated cells through viscous medium. Stimulation with P4 or PE1 had no consistent effects on the FDs of cells inside methylcellulose.

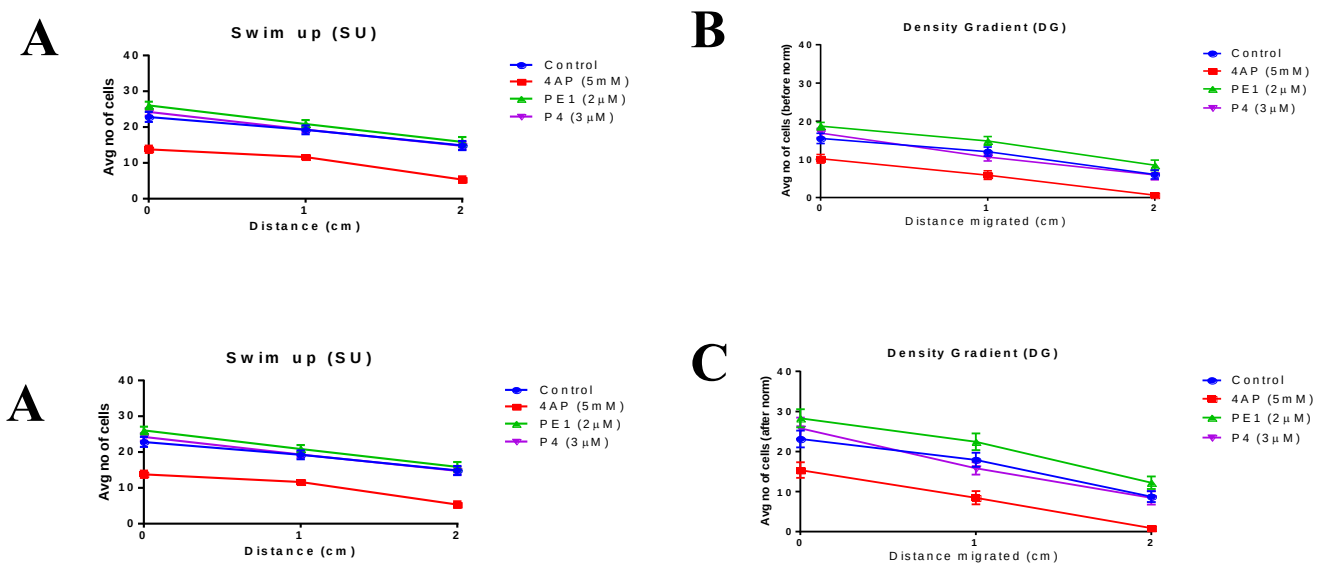
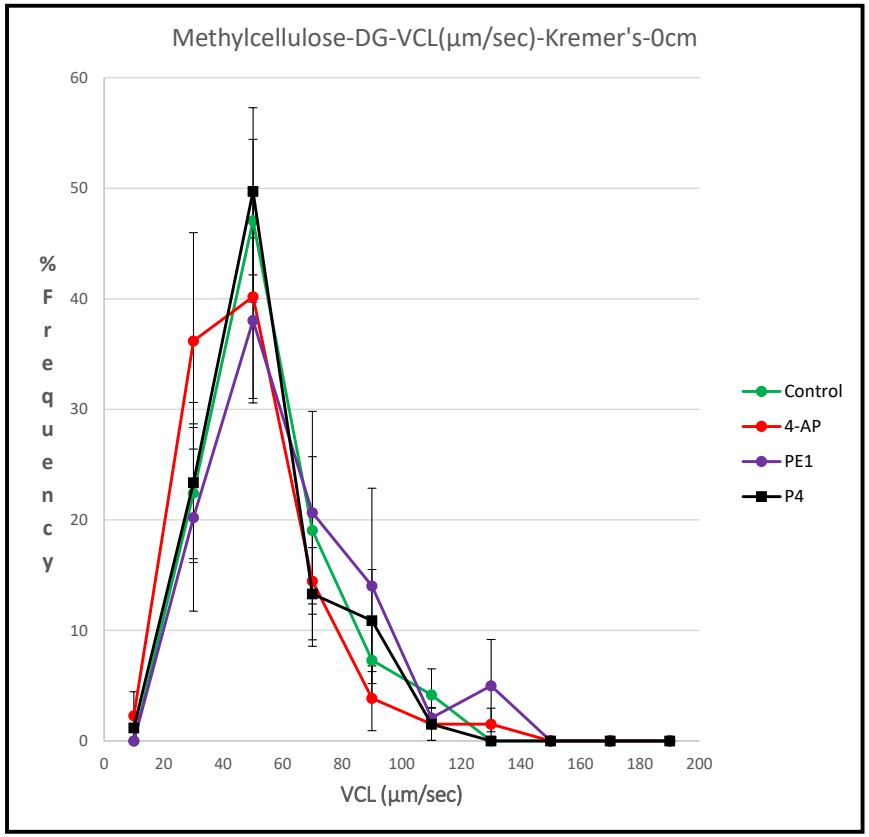
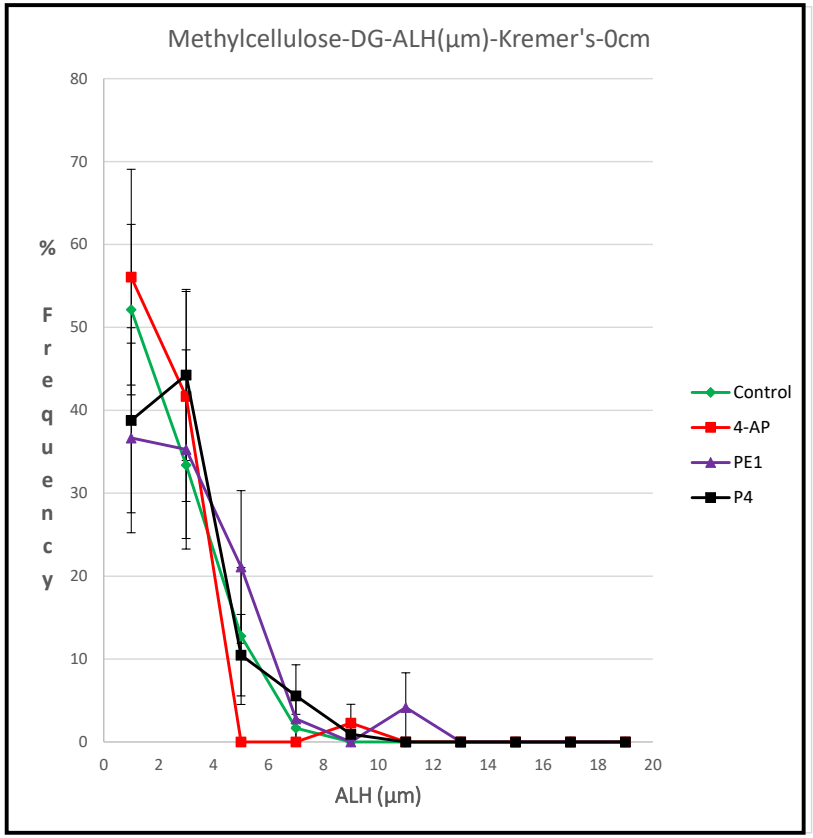


Figure 4.8: 4AP strongly inhibits penetration into the viscous medium (methylcellulose) (*in vitro*). Figures A, B & C show an average number of human sperm cells penetrated into the viscous medium (*in vitro*) when induced by 5mM 4-Aminopyridine (4AP), 2µM Prostaglandin (PE1), and 3µM Progesterone (P4). **A.** Cells are prepared by the direct swim-up technique; **B & C.** cells prepared by density gradient centrifugation (before and after normalisation). **A.** When compared with control (4-AP), p=0.004; ** significantly inhibits penetration at 0, 1 and 2 cm when prepared using the direct swim-up technique. **B & C.** When compared with control (4-AP), p=0.0023; ** (before normalisation) and p=0.004; ** (after normalisation) significantly inhibits penetration at 0, 1, and 2 cm when prepared using the density gradient method. **A & C.** Treatment of cells prepared by direct swim-up and density gradient with PE_1 enhanced performance in a viscous medium. When compared with control (PE_1), p=0.019; * significantly enhances penetration in a viscous medium when prepared using the density gradient method, other than the direct swim-up method (PE_1), p=0.0964; each bar shows the mean S.E. (error bars) of 12 experiments.

A


Methylcellulose-DG-VCL(µm/sec)-Kremer's-0cm
% Frequency
VCL (µm/sec)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
140
160
180
200
Control
4-AP
PE1
P4

B


Methylcellulose-DG-ALH(µm)-Kremer's-0cm
% Frequency
ALH (µm)
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12
14
16
18
20
Control
4-AP
PE1
P4

C

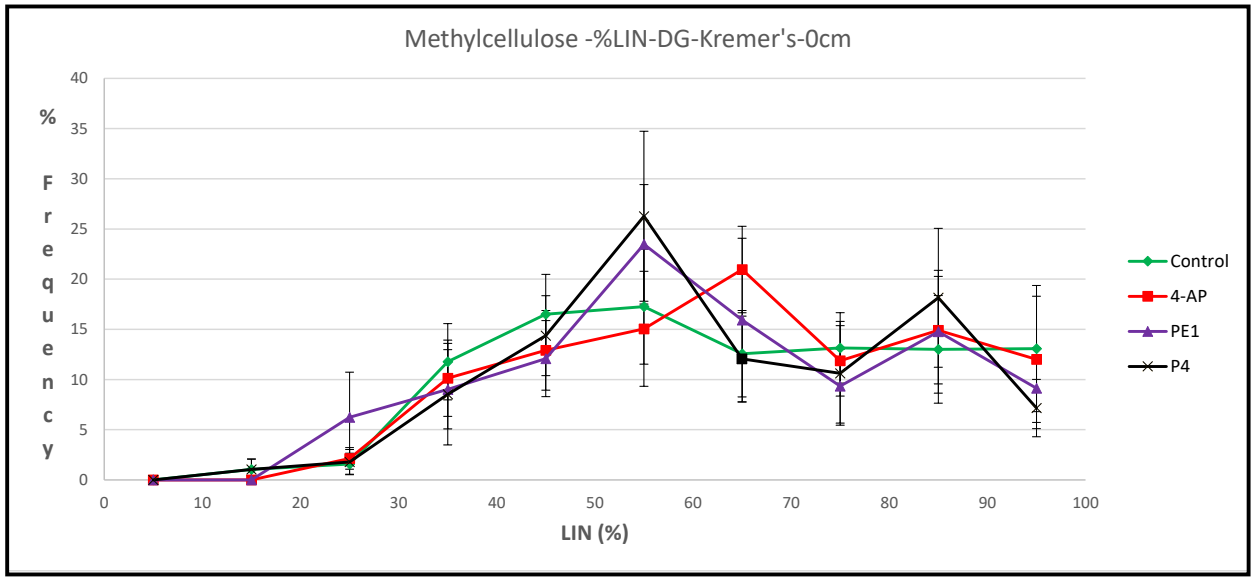
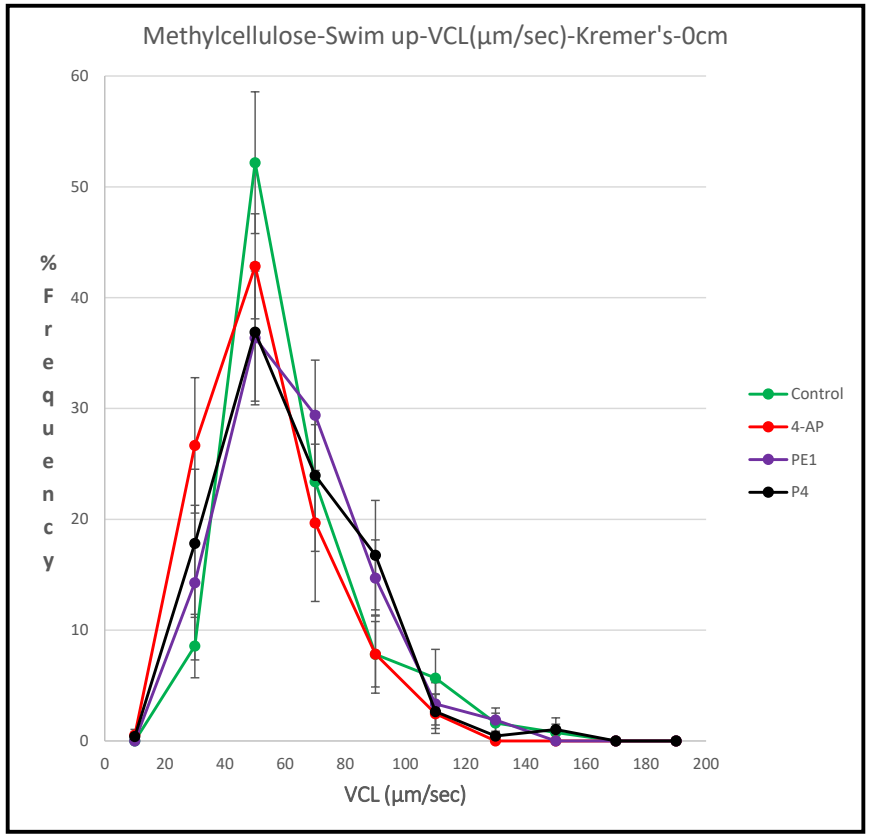
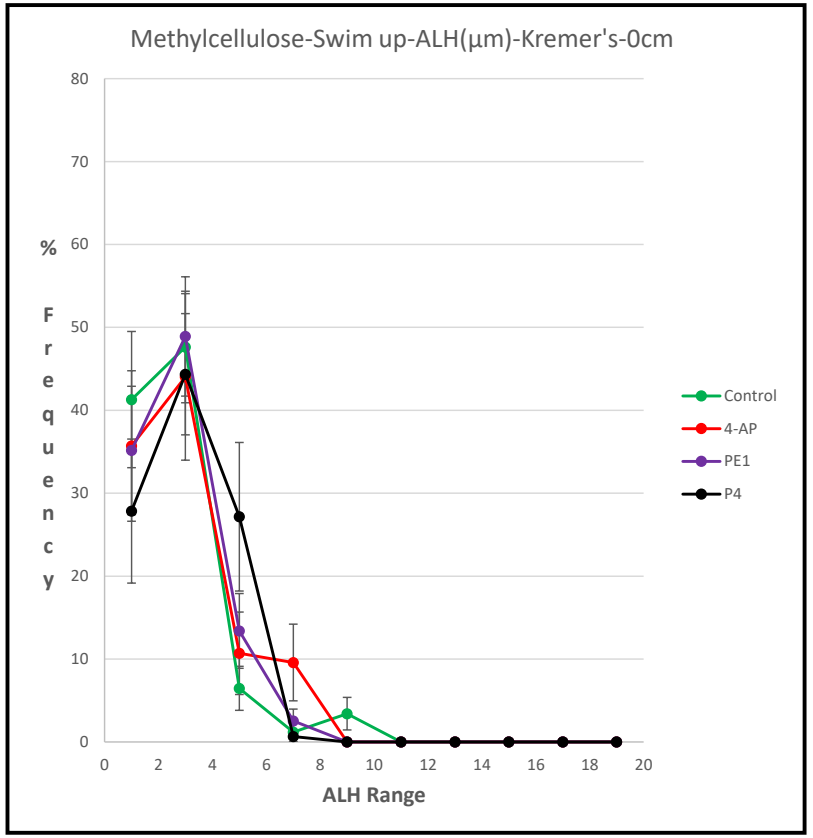


Figure 4.9 Showing the frequency distributions (FD) for the DG cells in response to different agonists in an artificial viscous penetration medium (methylcellulose). A,B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial viscous medium (methylcellulose) at **0cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the viscous medium). Compared to the control **(Green)** cells stimulated with 3µM P4 **(Black)** shown an slight increase in **A** VCL (greater than 4-AP **(Red)**) with a decrease in **C** Lin. This suggesting that the P4 stimulated cells were hyperactivated (more than 4-AP) and this was necessary for the P4 cells to make a entry into the viscous medium. This was similar to the control cells although the **C** LIN for P4 cells were lower than control (at the beginning) but later the LIN increases suggesting they would progress better for the further distances in the viscous medium. Each plot shows mean ± S.E (standard error) of 12 experiments prepared using density gradient (DG) technique.

A


Methylcellulose-Swim up-VCL(µm/sec)-Kremer's-0cm
% Frequency
VCL (µm/sec)
Control
4-AP
PE1
P4

B


Methylcellulose-Swim up-ALH(µm)-Kremer's-0cm
% Frequency
ALH Range
Control
4-AP
PE1
P4

C

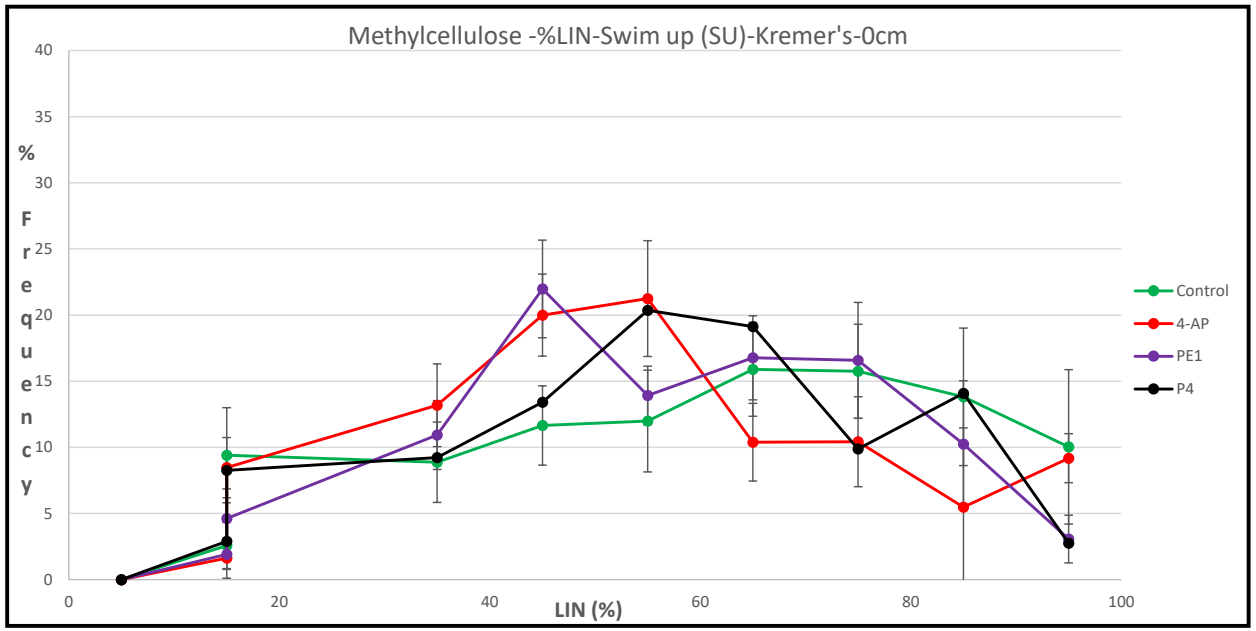
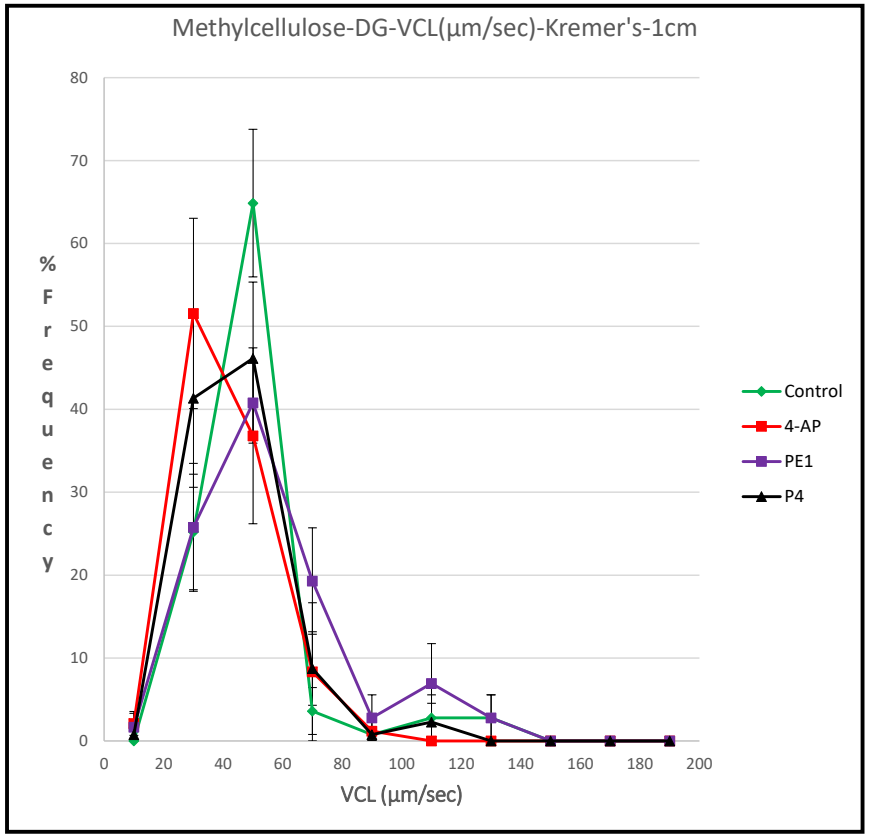
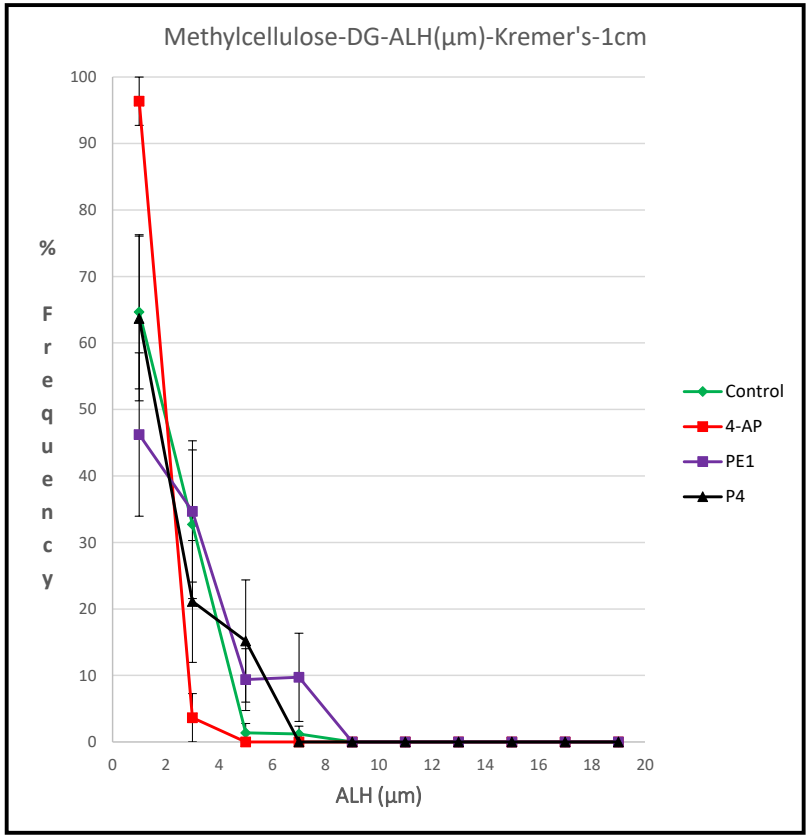


Figure 4.10 Showing the frequency distributions (FD) for the SU cells in response to different agonists in an artificial viscous penetration medium (methylcellulose). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial viscous medium (methylcellulose) at **0cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the viscous medium). Compared to the control **(Green)** the cells stimulated with P4 **(Black)** show lower **A** VCL, **B** ALH but increase in **C** LIN, this suggesting even though the hyperactivation is lower because of high LIN the P4 stimulated cells would make a better entry compare to the control. Each plot shows mean ± S.E (standard error) of 12 experiments prepared using direct swim up (SU) technique.

A


Methylcellulose-DG-VCL(µm/sec)-Kremer's-1cm
% Frequency
VCL (µm/sec)
Control
4-AP
PE1
P4

B


Methylcellulose-DG-ALH(µm)-Kremer's-1cm
% Frequency
ALH (µm)
Control
4-AP
PE1
P4

C

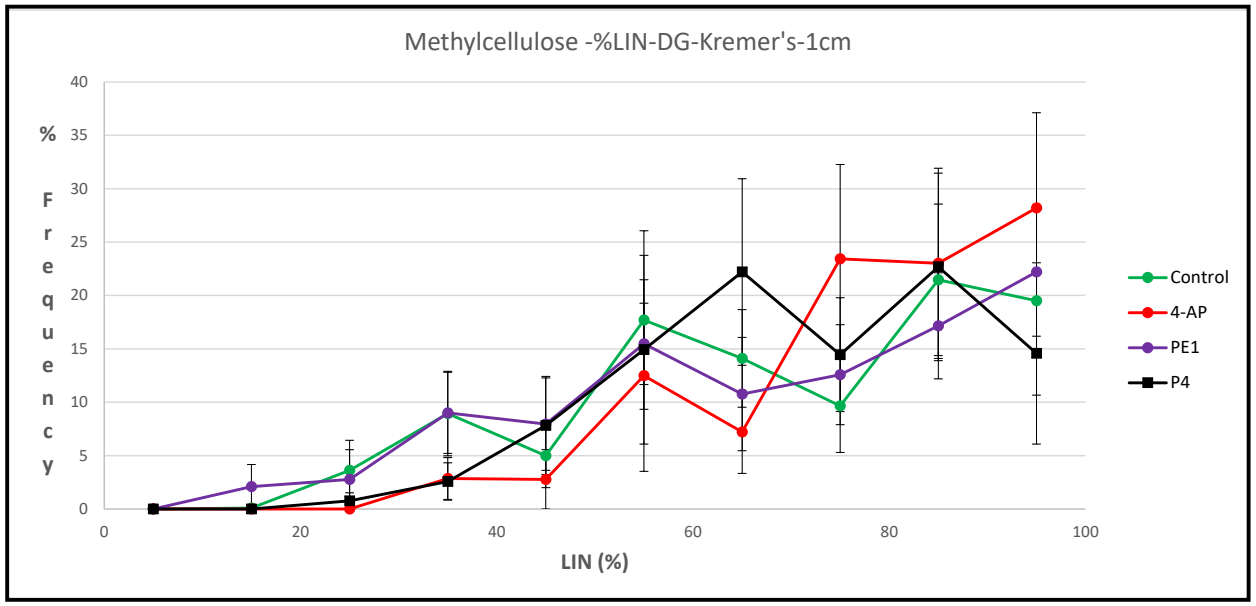
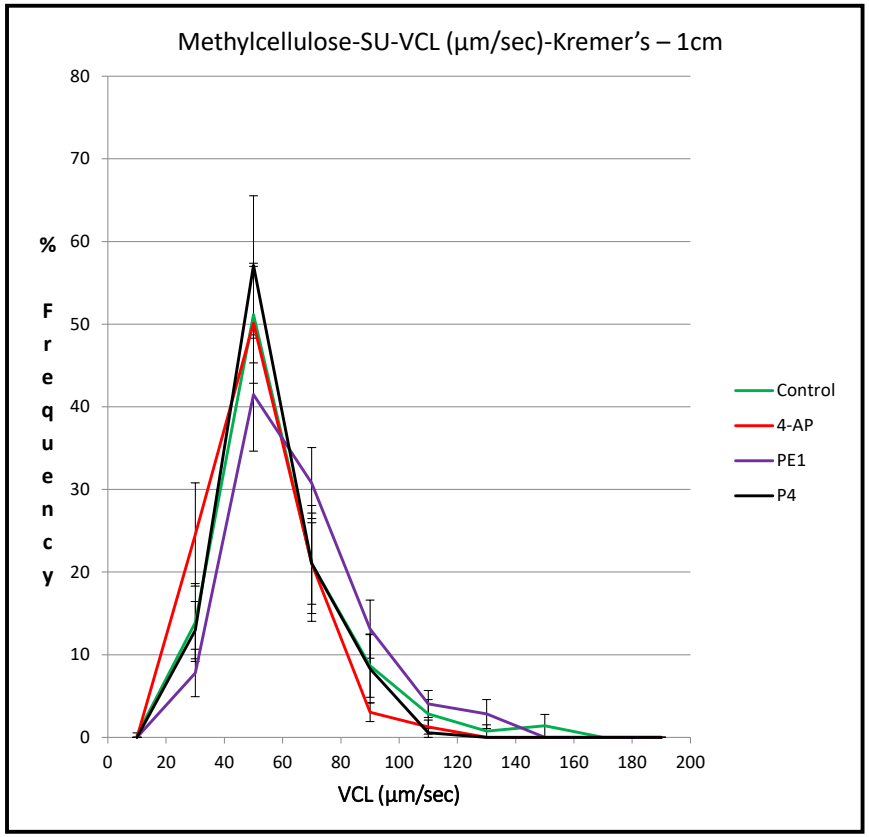
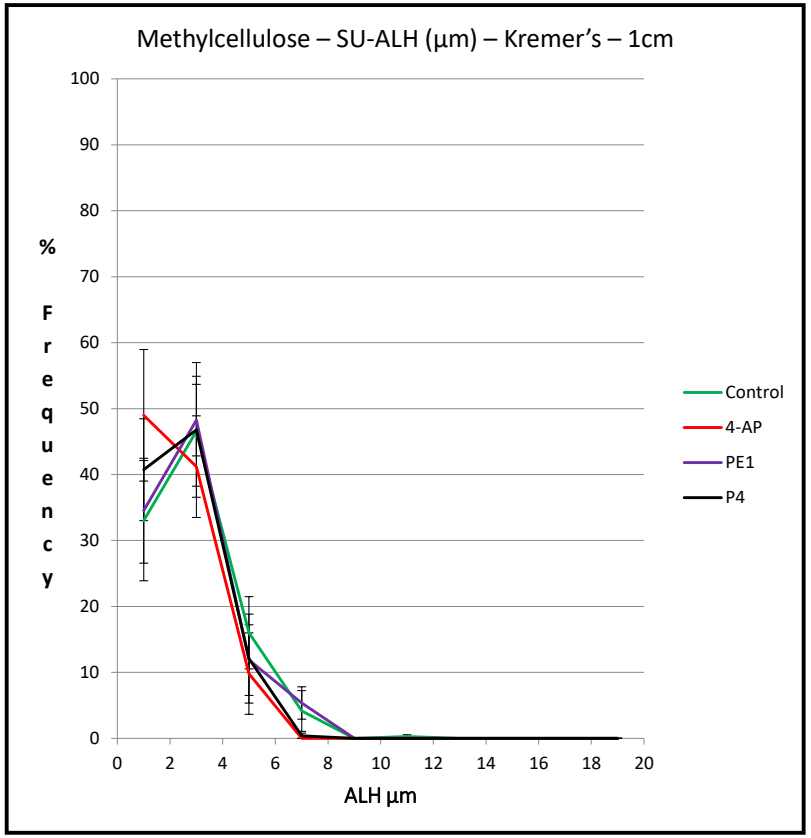


Figure 4.11 Showing the frequency distributions (FD) for the DG cells in response to different agonists in an artificial viscous penetration medium (methylcellulose). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial viscous medium (methylcellulose) at **1cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the viscous medium). Compared to the control **(Green)** the cells stimulated with P4 **(Black)** show lower **A** VCL with **B** ALH response similar to the control but there is a increase in **C** LIN compared to the control. This data suggests P4 stimulated cells with an increased LIN capable of progressing further distance in the tube. Cells stimulated with 4-AP **(Red)** although shown less hyperactivation (lower VCL) compared to the control, because of their (4-AP) lower LIN (compared to the control) they would progress at a lower pace compare to the control. Each plot shows mean ± S.E (standard error) of 12 experiments prepared using the density gradient (DG) technique.

A


Methylcellulose-SU-VCL (µm/sec)-Kremer's – 1cm
% Frequency
VCL (µm/sec)
Control
4-AP
PE1
P4

B


Methylcellulose – SU-ALH (µm) – Kremer's – 1cm
% Frequency
ALH µm
Control
4-AP
PE1
P4

C

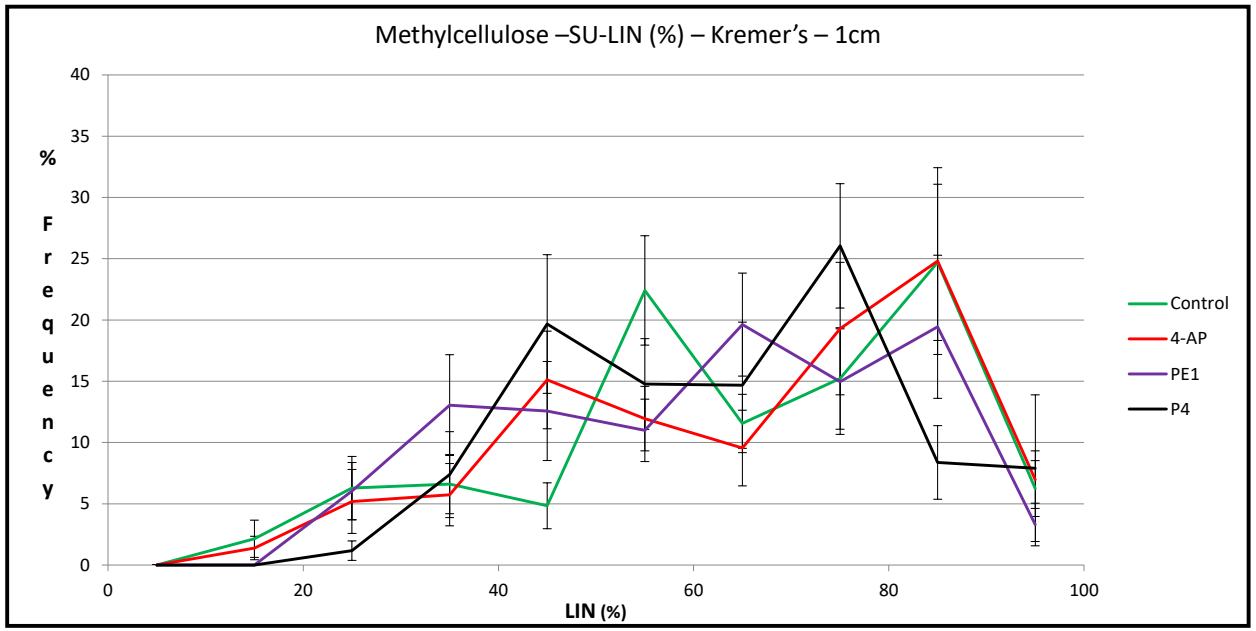
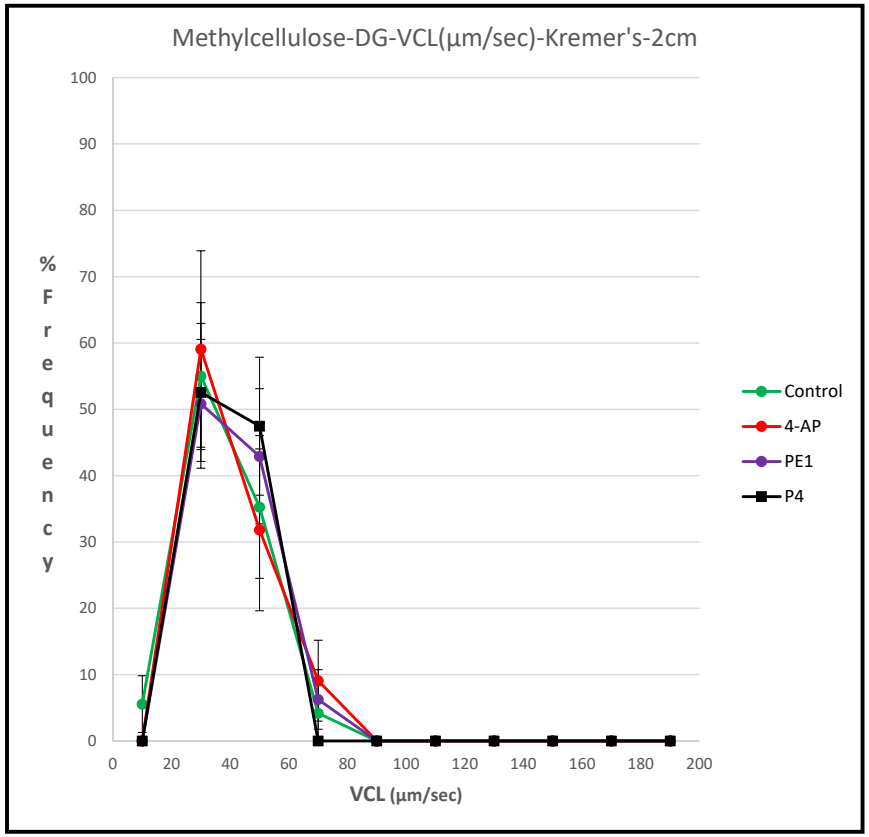
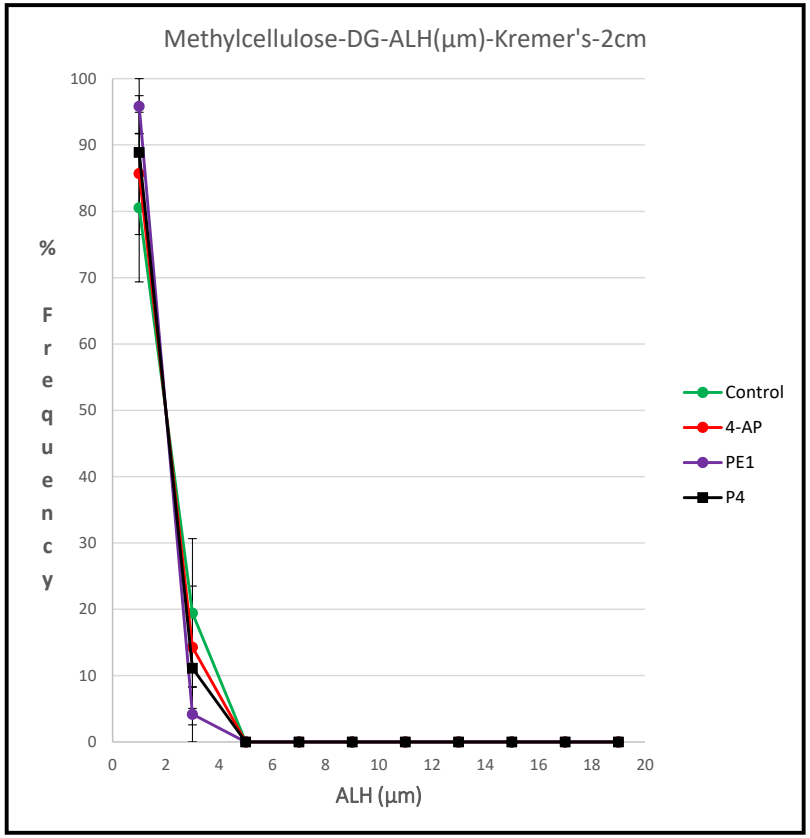


Figure 4.12 Showing the frequency distributions (FD) for the SU cells in response to different agonists in an artificial viscous penetration medium (methylcellulose). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial viscous medium (methylcellulose) at **1cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the viscous medium). Compared to the control **(Green)** the cells stimulated with P4 **(Black)** show slightly higher **A** VCL compared to the control, **B** ALH was similar to that of control, but with a higher **C** LIN suggesting their better progressive ability in the viscous medium. Each plot shows mean ± S.E (standard error) of 12 experiments prepared using direct swim up (SU) technique.

A


Methylcellulose-DG-VCL(µm/sec)-Kremer's-2cm
% Frequency
VCL (µm/sec)
Control
4-AP
PE1
P4

B


Methylcellulose-DG-ALH(µm)-Kremer's-2cm
% Frequency
ALH (µm)
Control
4-AP
PE1
P4

C

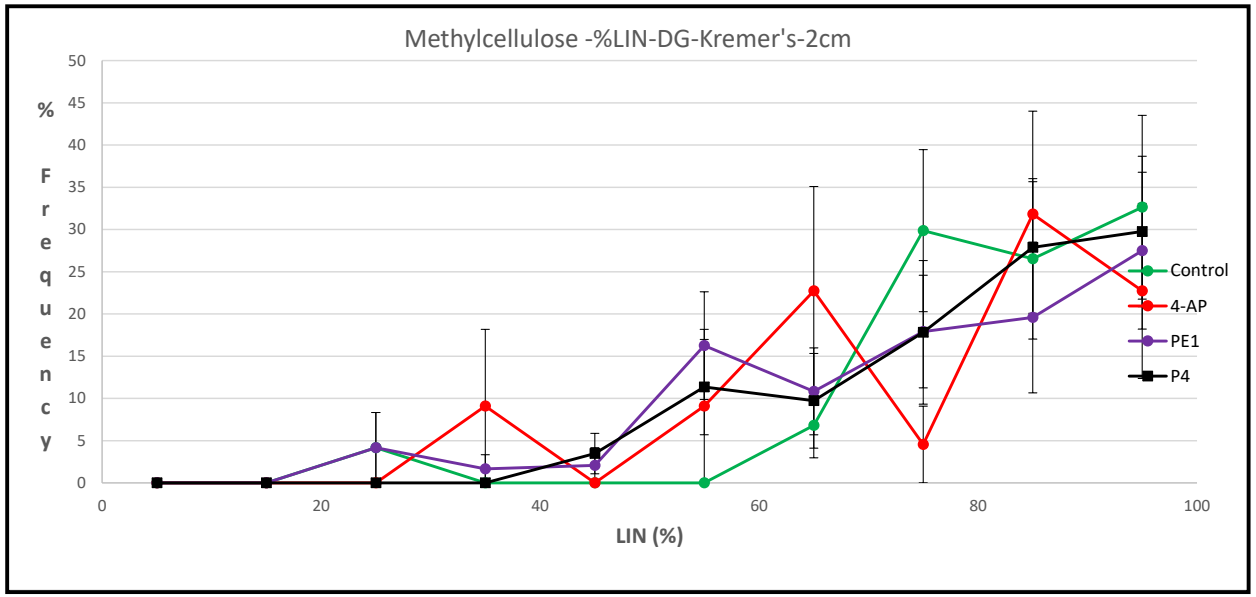
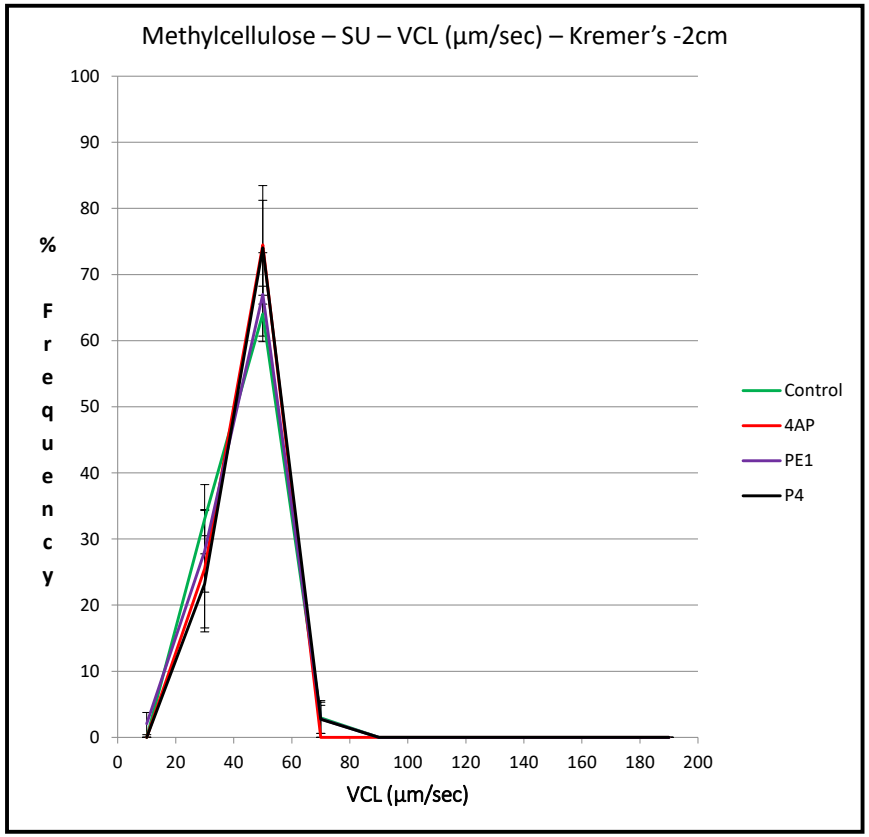
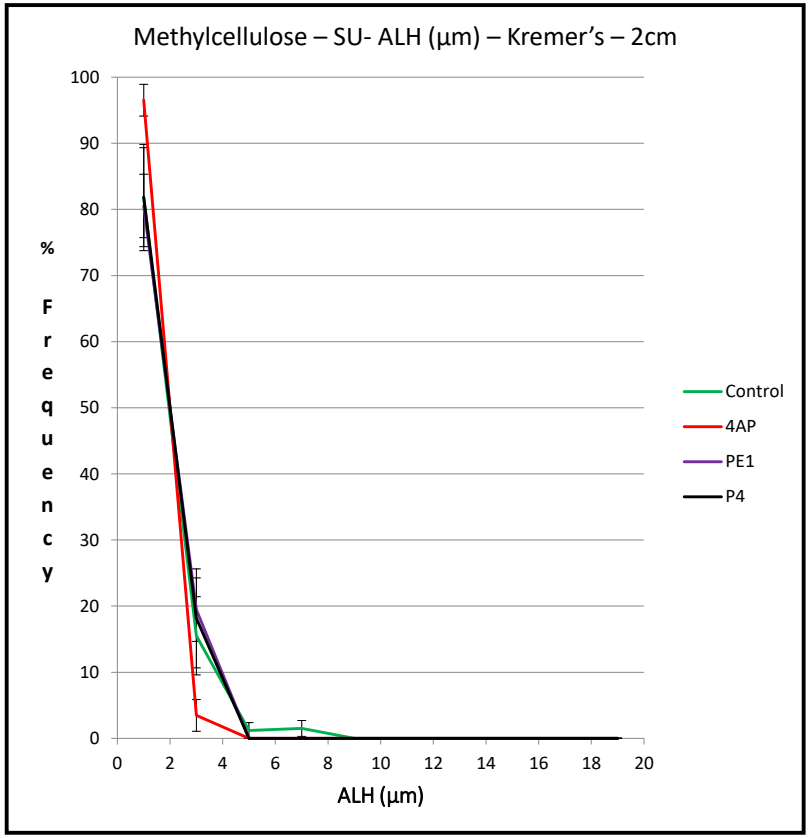


Figure 4.13 Showing the frequency distributions (FD) for the DG cells in response to different agonists in an artificial viscous penetration medium (methylcellulose). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial viscous medium (methylcellulose) at **2cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the viscous medium). Compared to the control **(Green)** the cells stimulated with PE1 **(Purple)** show lower **A** VCL compared to the control, **B** ALH was higher to that of control, but with a higher **C** LIN suggesting their progressive behaviour in the viscous medium. In the case of cells stimulated with 4-AP show higher **A** VCL and **B**ALH with low **C** LIN (when compared to the control) suggesting they (4-AP) were associated with hyperactive non-progressive behaviour which is restricting them perform better in the viscous medium. A behaviour that is contrast to PE1. Each plot shows mean ± S.E (standard error) of 12 experiments prepared using the density gradient (DG) technique.

A


Methylcellulose – SU – VCL (µm/sec) – Kremer's -2cm
% Frequency
VCL (µm/sec)
Control
4AP
PE1
P4

B


Methylcellulose – SU- ALH (µm) – Kremer's – 2cm
% Frequency
ALH (µm)
Control
4AP
PE1
P4

C

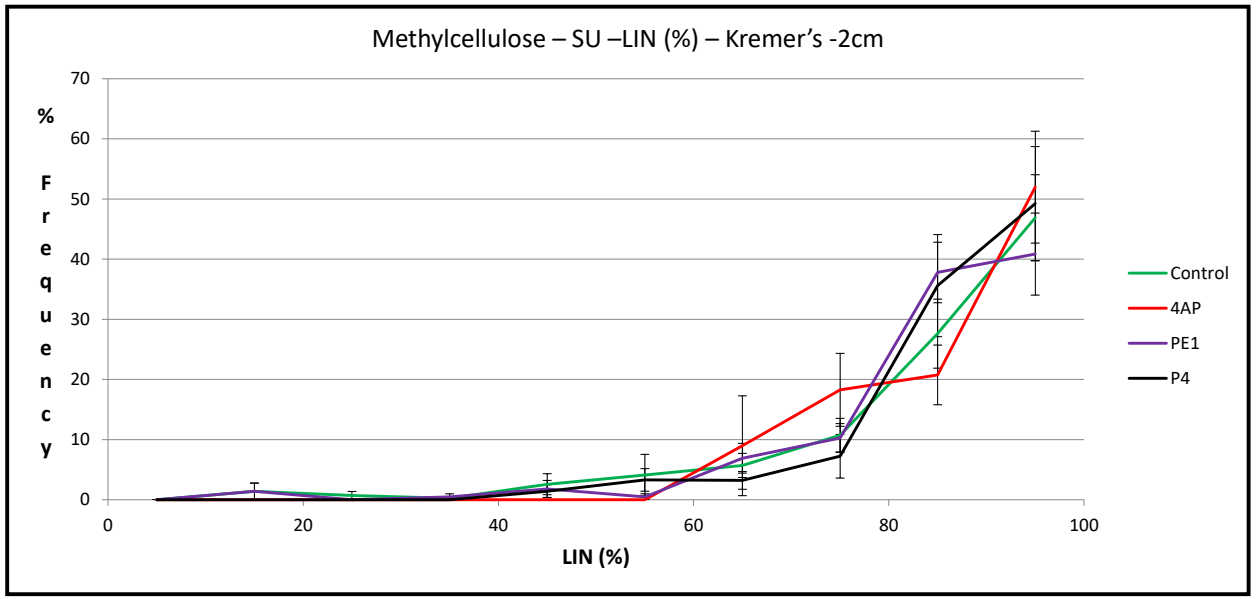


Figure 4.14 Showing the frequency distributions (FD) for the SU cells in response to different agonists in an artificial viscous penetration medium (methylcellulose). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial viscous medium (methylcellulose) at **2cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the viscous medium). Compared to the control **(Green)** the cells stimulated with PE1 **(Purple)** show a slightly higher **A** VCL compared to the control **B** ALH was similar to that of control and with higher **C** LIN. 4-AP stimulated cells show high **A &B** VCL and ALH with low **C** LIN. This data suggests even though 4-AP cells were hyperactivated, but because of low LIN they struggle to penetrate in the viscous medium and therefore compare to control, PE1 and P4, 4-AP stimulated cells has a higher inhibition in the viscous medium. Also suggesting hyperactive non-progressive behaviour of 4-AP cells is what contributing to their inhibition in the viscous medium. Each plot shows mean ± S.E (standard error) of 12 experiments prepared using the direct swim up (SU) technique.

4.4.3 Comparative study of penetration behaviour (polyacrylamide) in relation to hyperactivated motility when stimulated with 4-Aminopyridine (4AP), Prostaglandin E_1 (PE_1) and progesterone (P4) agonists by two different sperm preparation techniques – DG & SU

Kremer's *in vitro* penetration test was used to investigate how stimulation of different sperm behaviours, as measured by a CASA, affected the ability of cells to enter visco-elastic medium. Treatment with 4-AP potently increased hyperactivated motility (as mentioned in the previous section, 4.4.1) but when cells from the same aliquot were tested for their ability to penetrate into viscoelastic medium (polyacrylamide) both density gradient and swim-up cells were clearly inhibited at all distances into the medium ($p<0.006$; n=8, Figure 4.15 a & Figure 4.15 c). Treatment of cells prepared by density gradient with PE_1 (to activate the CatSper channel) has no effect in curvilinear velocity (Figure 4.3 b) but enhanced sperm entry into viscoelastic medium although this was not statistically significant when compared to control ($p=0.2215$; n=8 compared to control; paired t-test; Figure 4.15 c).

Treatment of cells prepared by swim-up with PE_1 had similar effects but again these were smaller than in density gradient cells and again not statistically significant when compared to control ($p=0.2877$; n=8 compared to control; paired t-test; Figure 4.15 a).

FDs were analysed for cells penetrated 0, 1, and 2cm into viscoelastic medium (polyacrylamide). As cells penetrated further in to polyacrylamide, FDs for VCL and LIN of both DG and SU cells had very similar characteristics, though the peak of the VCL distribution was shifted slightly to the left in some assays. However, as with analysis of cells in methycellulose, there were very few sperm with high ALH (≥5 μm) at 1 or 2 cm into the gel (compare 4.20B and 4.21B with 4.16B and 4.17B). 4AP clearly shifted the peak VCL of both DG and SU cells at 0 cm (figs 4.16A, 4.17A) and SU cells at 1 cm (fig 4.19A) to lower

values (Figure 4.16A, 4.17A). Stimulation P4 or PE1 had no consistent effects on the FDs of cells inside polyacrylamide.

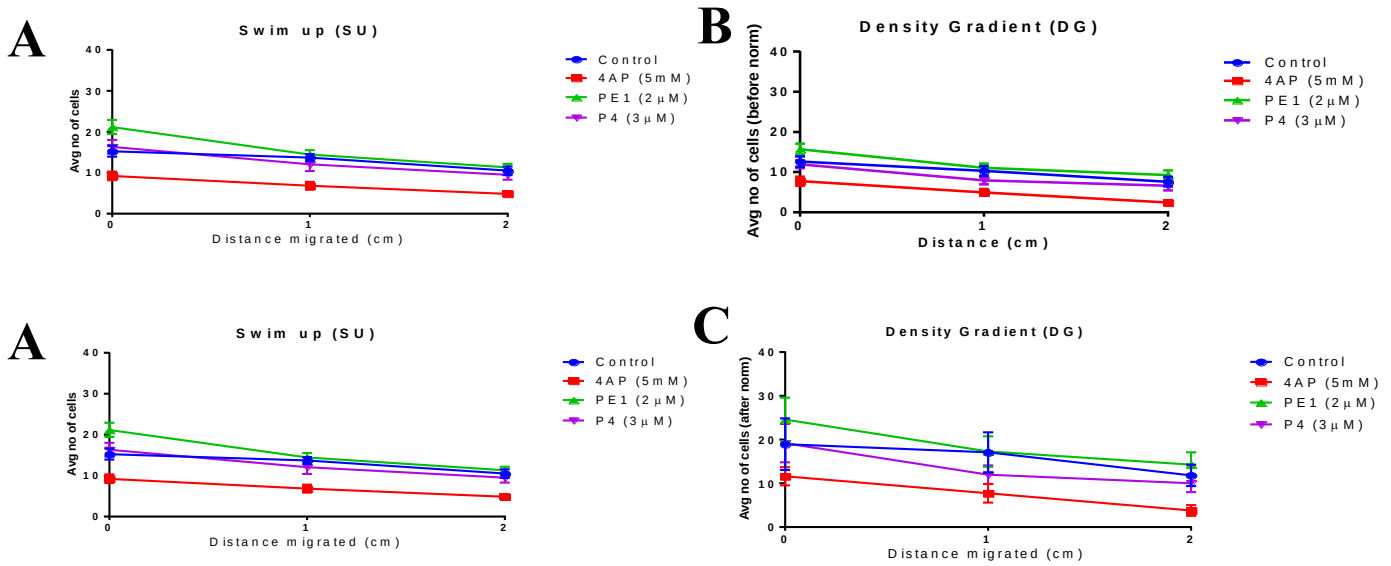
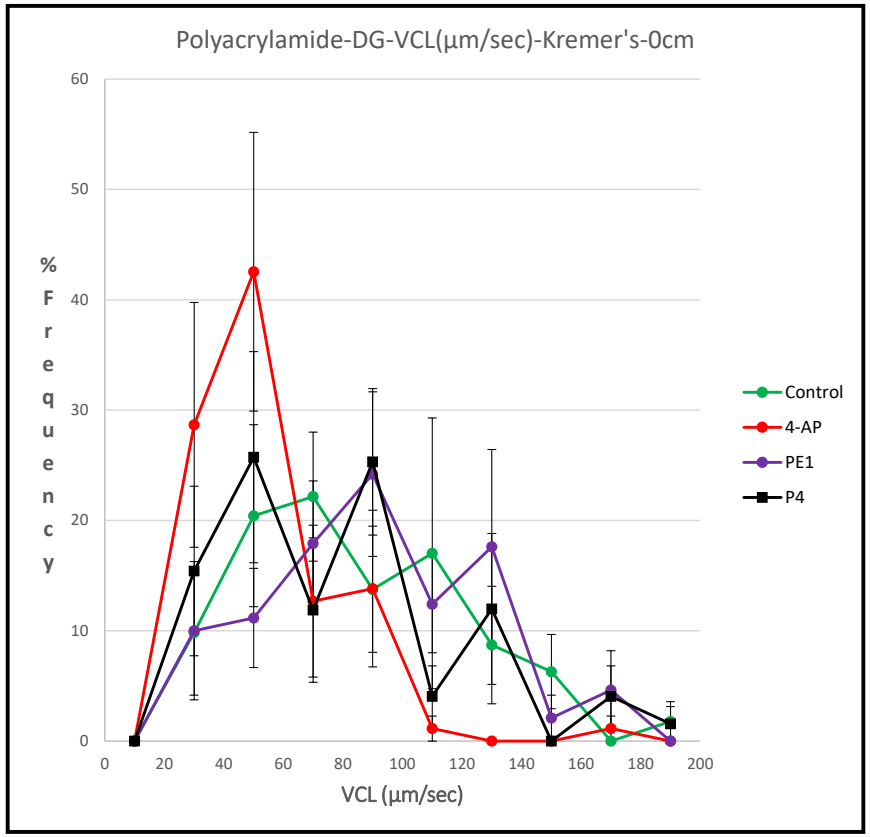
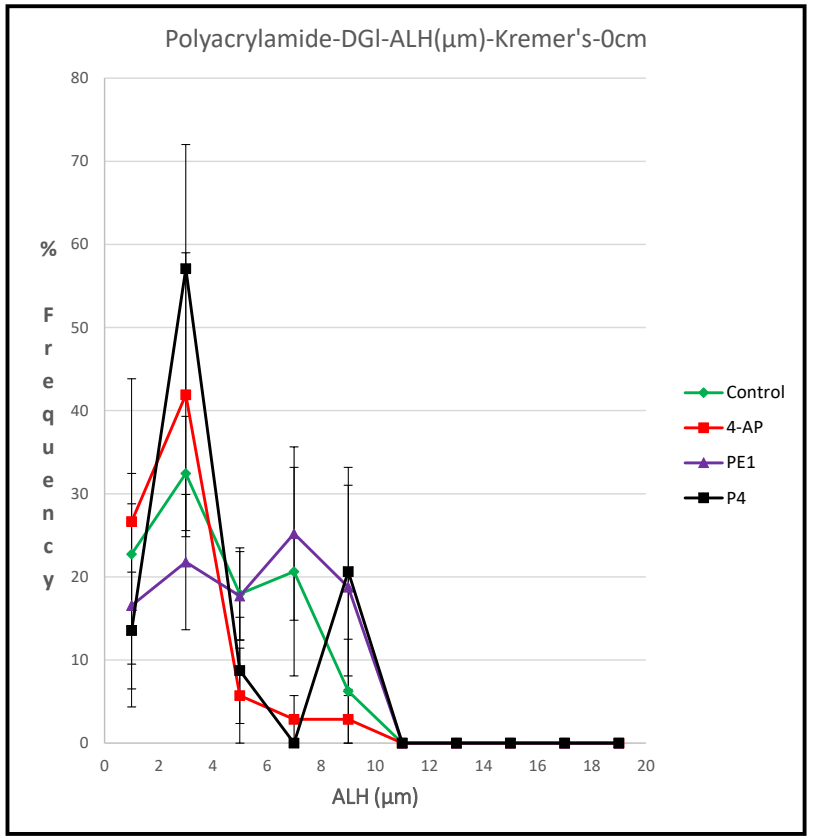


Figure 4.15: 4AP strongly inhibits penetration into the viscoelastic medium (polyacrylamide) (*in vitro*). Figures A, B & C show an average number of human sperm cells penetrated into the viscoelastic medium (*in vitro*) when induced by 5mM 4-Aminopyridine (4AP), 2μM Prostaglandin (PE1), and 3μM Progesterone (P4). **A.** Cells are prepared by the direct swim-up technique; **B & C.** cells prepared by density gradient centrifugation (before and after normalisation). **A.** When compared with control (4-AP), p=0.003; ** significantly inhibits penetration at 0, 1 and 2 cm when prepared using the direct swim-up technique. **B & C.** When compared with control (4-AP), p=0.0007; *** (before normalisation) and p=0.005; ** (after normalisation) significantly inhibits penetration at 0, 1, and 2 cm when prepared using the density gradient method. **C.** Treatment of cells prepared by density gradient with PE_1 enhanced performance in a viscoelastic medium when compared with control, although they are not statistically significant (after normalisation); each bar shows the mean S.E. (error bars) of 8 experiments.

A


Polyacrylamide-DG-VCL(µm/sec)-Kremer's-0cm
% Frequency
VCL (µm/sec)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
140
160
180
200
Control
4-AP
PE1
P4

B


Polyacrylamide-DGl-ALH(µm)-Kremer's-0cm
% Frequency
ALH (µm)
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12
14
16
18
20
Control
4-AP
PE1
P4

C

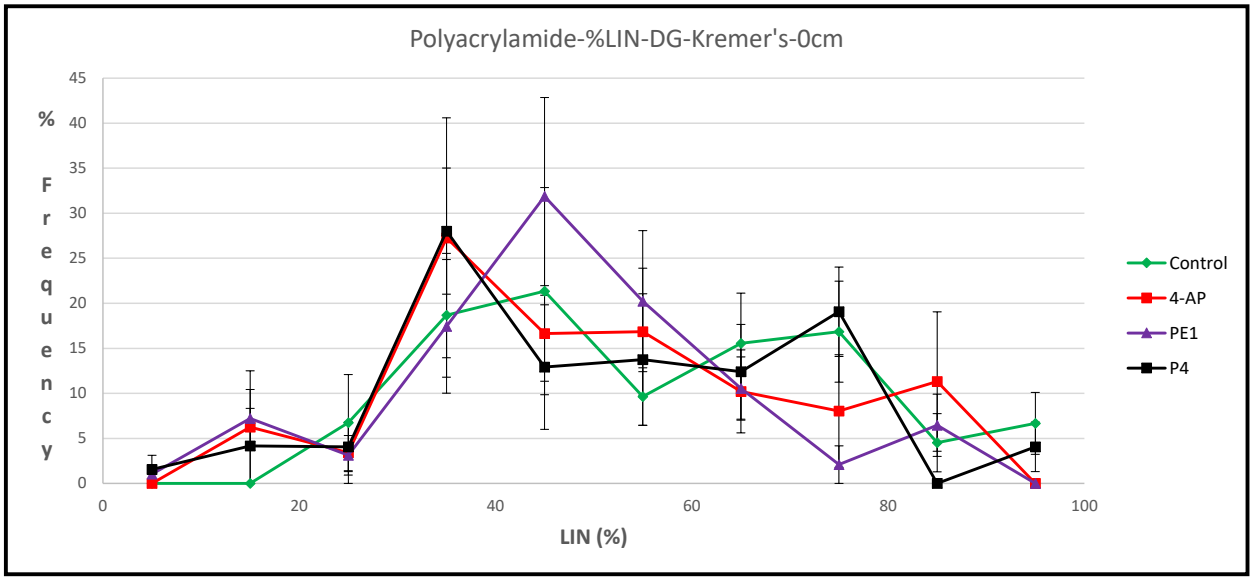
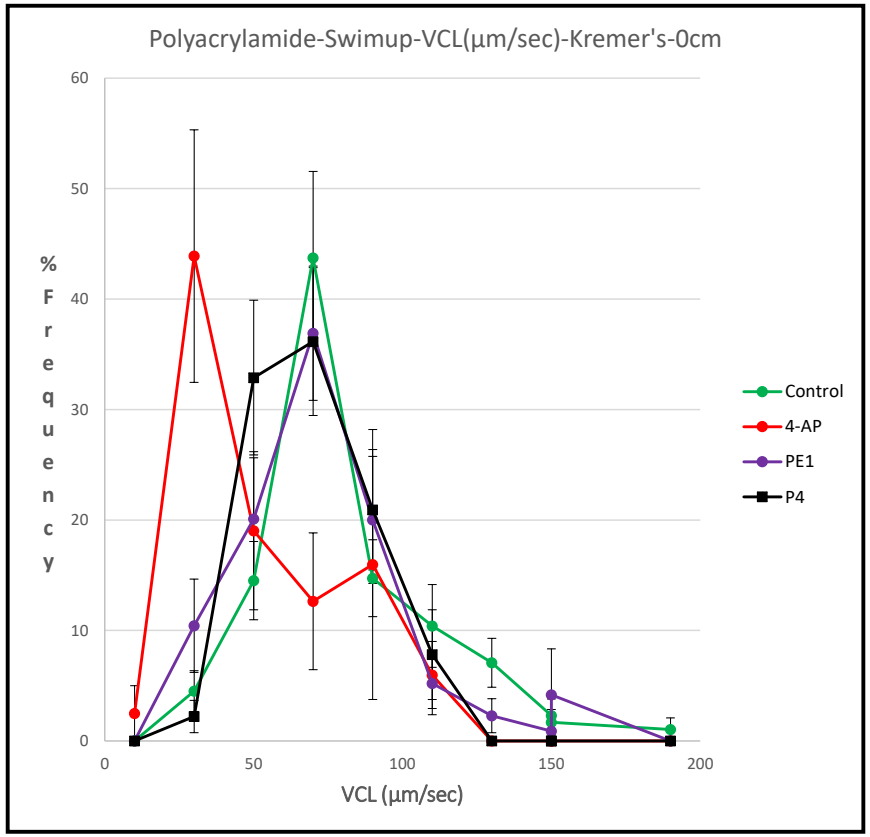
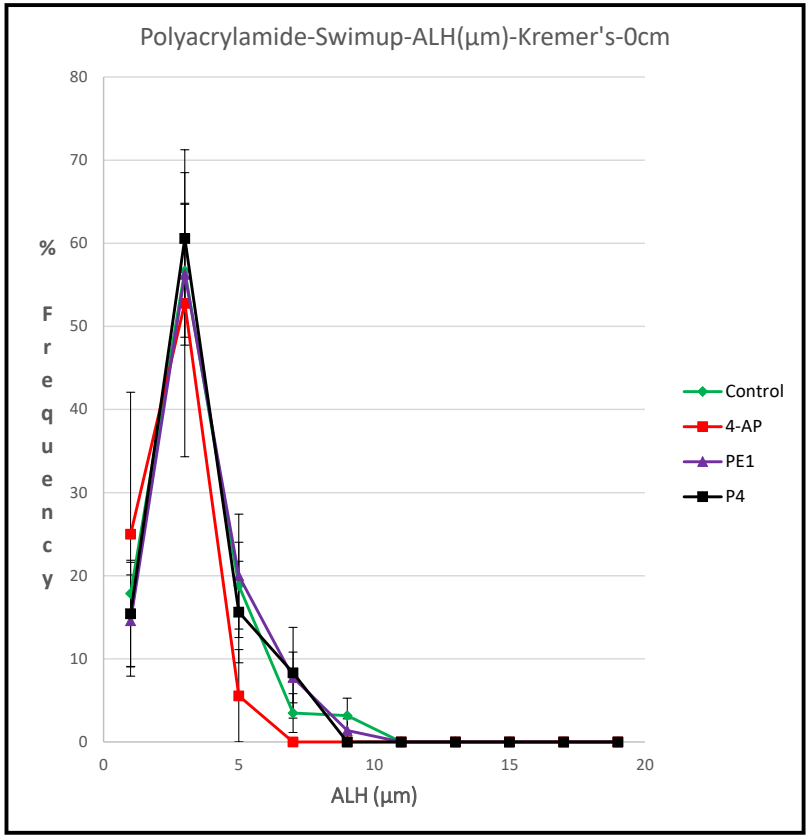


Figure 4.16 Showing the frequency distributions (FD) for the DG cells in response to different agonists in an artificial visco-elastic penetration medium (polyacrylamide). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial visco-elastic medium (polyacrylamide) at **0cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the visco-elastic medium). Cells stimulated with PE1 show slightly higher **A** VCL, **B** lower ALH and higher **C** LIN. This data suggests with high linearity PE1 stimulated cells make an better entry into the visco-elastic medium. Each plot shows mean ± S.E (standard error) of 8 experiments prepared using density gradient (DG) technique.

A


Polyacrylamide-Swimup-VCL(µm/sec)-Kremer's-0cm
% Frequency
VCL (µm/sec)
0
10
20
30
40
50
60
0
50
100
150
200
Control
4-AP
PE1
P4

B


Polyacrylamide-Swimup-ALH(µm)-Kremer's-0cm
% Frequency
ALH (µm)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
Control
4-AP
PE1
P4

C

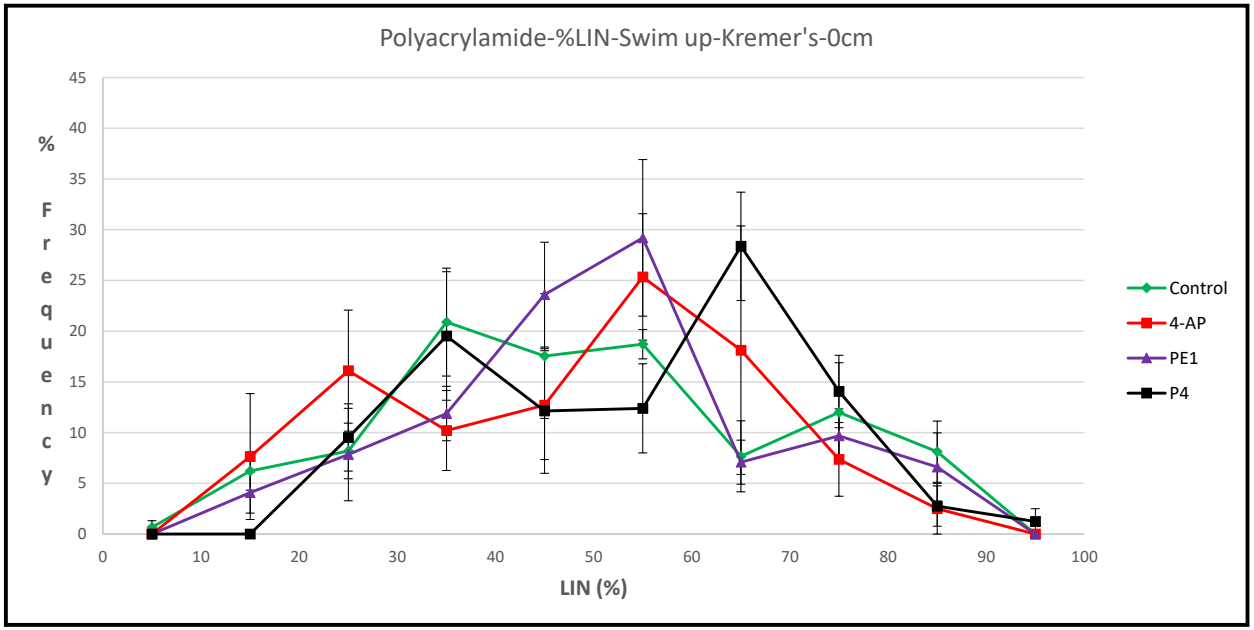
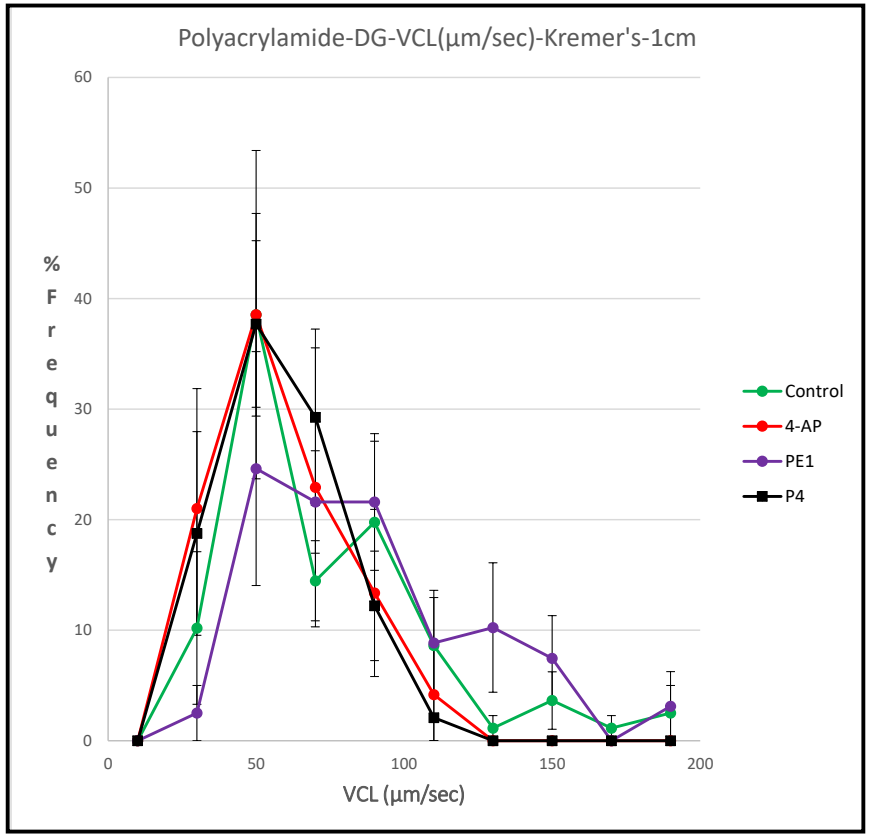
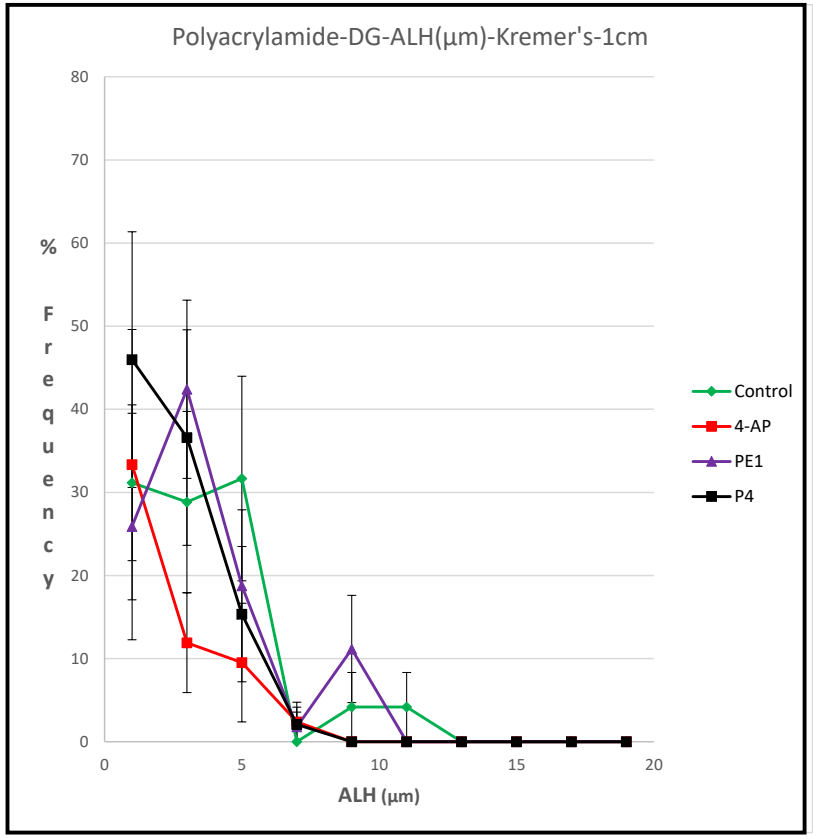


Figure 4.17 Showing the frequency distributions (FD) for the SU cells in response to different agonists in an artificial visco-elastic penetration medium (polyacrylamide). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial visco-elastic medium (polyacrylamide) at **0cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the visco-elastic medium). Cells stimulated with PE1 show A low VCL (compared to the control) with a similar **B** ALH and a higher **C** LIN. With higher **C** LIN and lower VCL, PE1 cells make a better entry in to the visco-elastic medium. Contrast to PE1, 4-AP cells when compared to control show low **C** LIN and high **A** VCL (peak shifts to left) suggesting they (4-AP treated cells) were more hyperactivated and fall behind (to PE1 treated cells) in entering in to the visco-elastic medium. Each plot shows mean ± S.E (standard error) of 8 experiments prepared using direct swim up (SU) technique.

A


Polyacrylamide-DG-VCL(µm/sec)-Kremer's-1cm
% Frequency
VCL (µm/sec)
0
10
20
30
40
50
60
0
50
100
150
200
Control
4-AP
PE1
P4

B


Polyacrylamide-DG-ALH(µm)-Kremer's-1cm
% Frequency
ALH (µm)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
Control
4-AP
PE1
P4

C

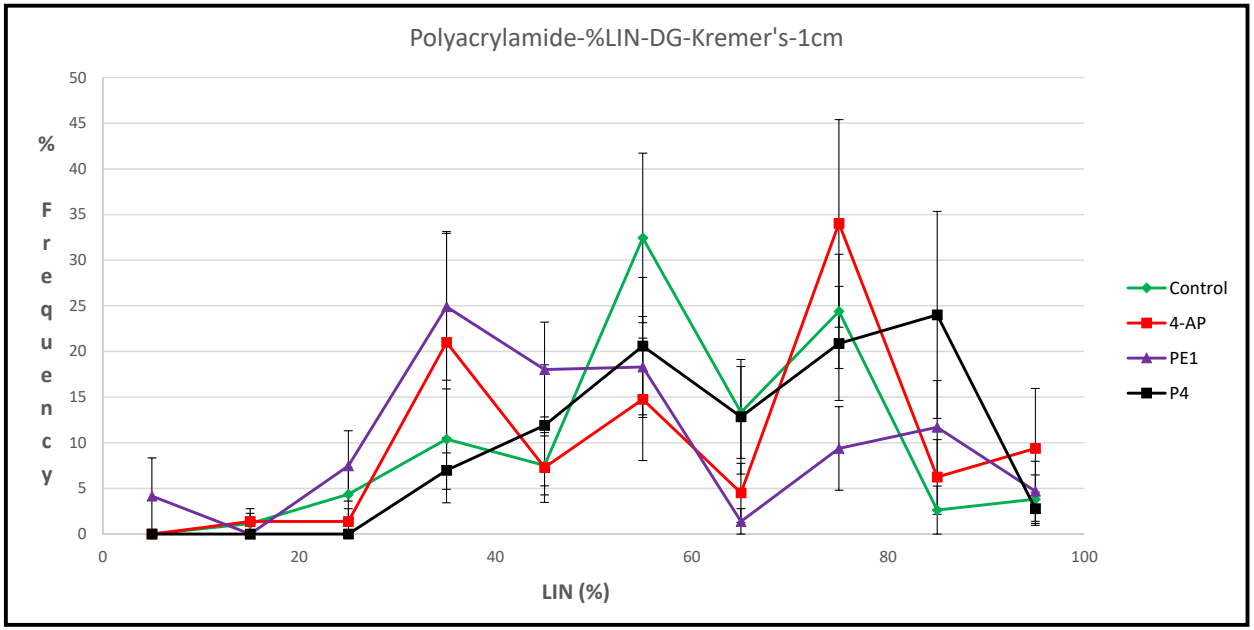
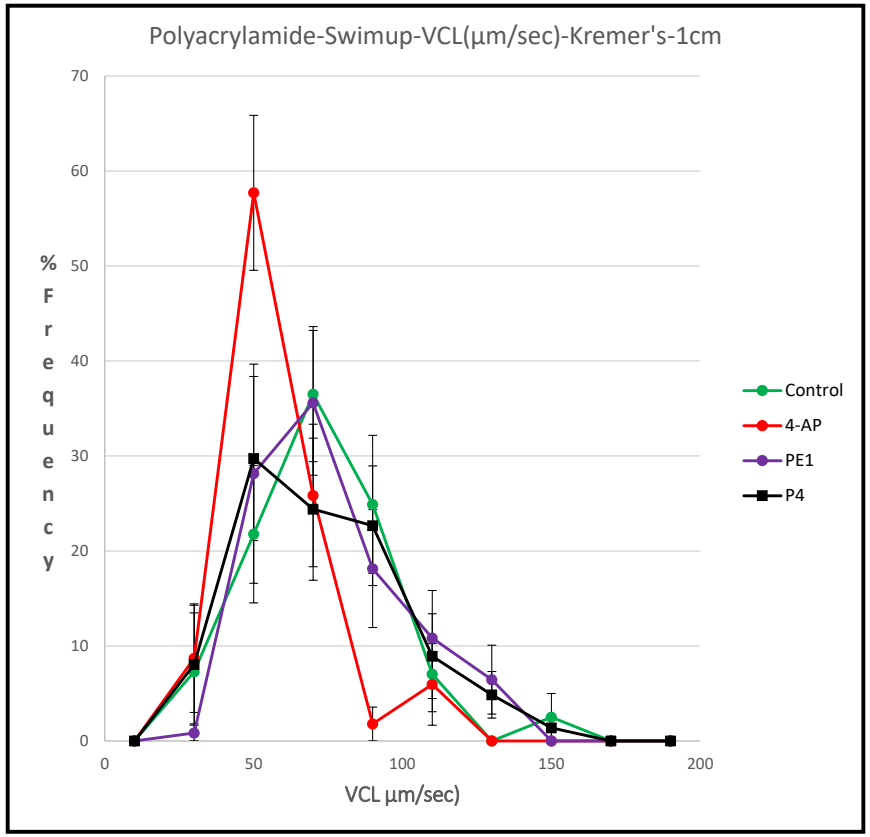
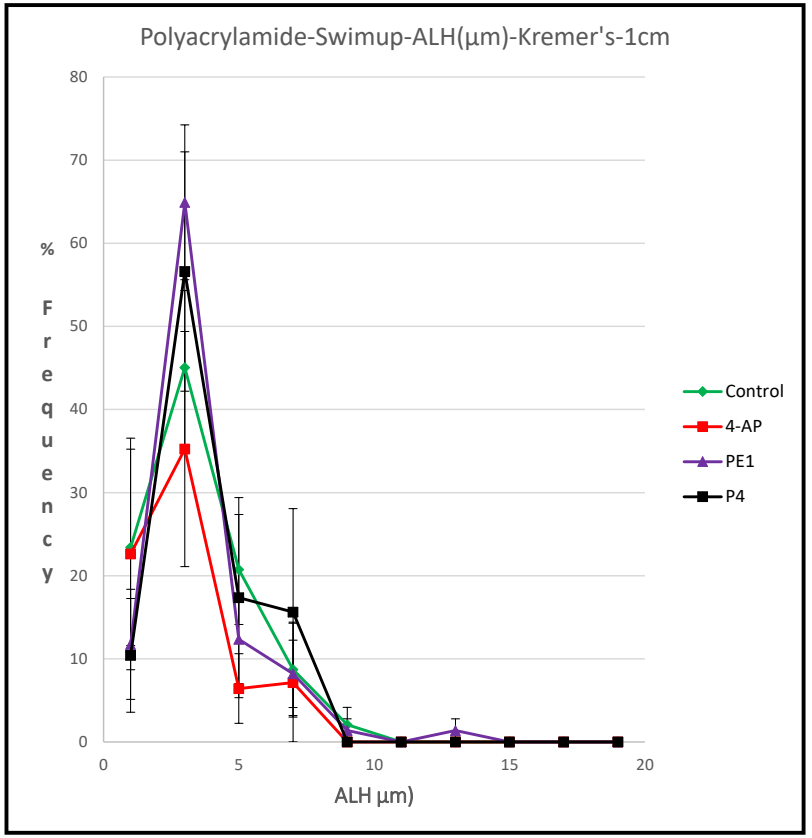


Figure 4.18 Showing the frequency distributions (FD) for the DG cells in response to different agonists in an artificial visco-elastic penetration medium (polyacrylamide). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial visco-elastic medium (polyacrylamide) at **1cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the visco-elastic medium). When compared to the control cells stimulated with PE1 show low **A** VCL, Higher **B** ALH and a higher **C** LIN (both at the beginning and at the end of the peak). This data suggests with high linearity and low VCL PE1 stimulated cells progress into the visco-elastic medium. Each plot shows mean ± S.E (standard error) of 8 experiments prepared using density gradient (DG) technique.

A


Polyacrylamide-Swimup-VCL(µm/sec)-Kremer's-1cm
% Frequency
VCL µm/sec)
0
10
20
30
40
50
60
70
0
50
100
150
200
Control
4-AP
PE1
P4

B


Polyacrylamide-Swimup-ALH(µm)-Kremer's-1cm
% Frequency
ALH µm)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
Control
4-AP
PE1
P4

C

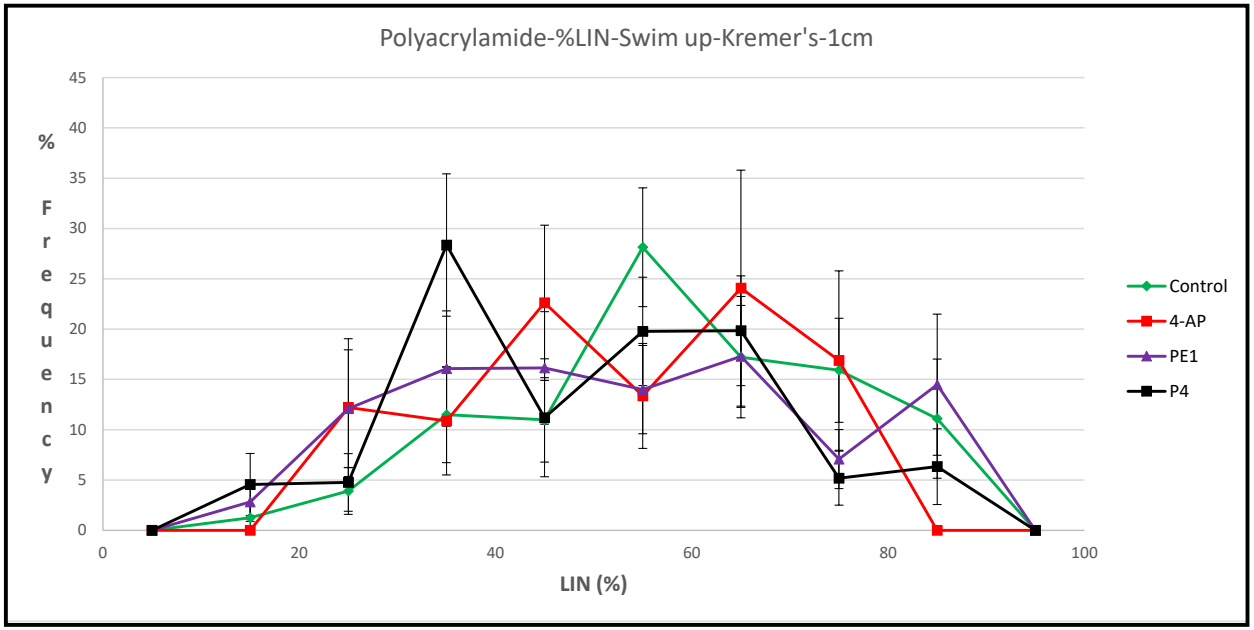
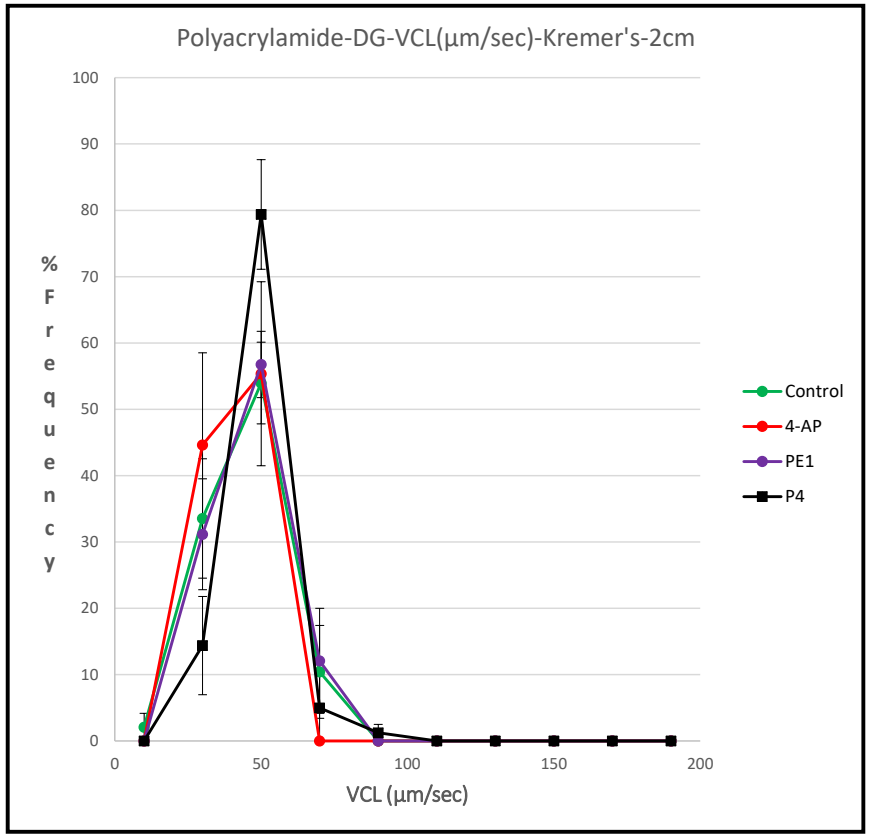
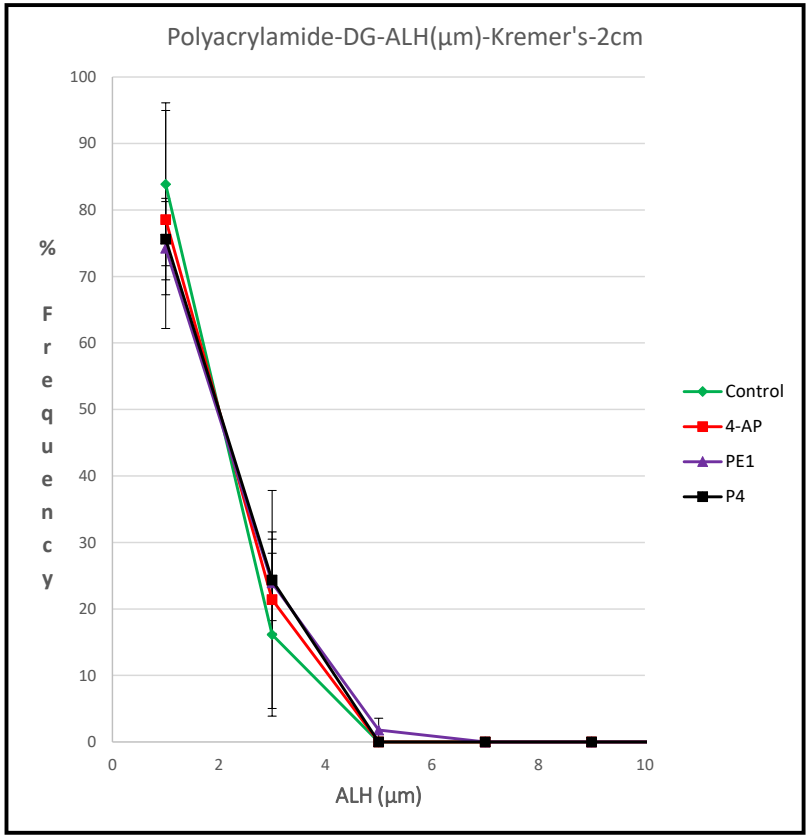


Figure 4.19 Showing the frequency distributions (FD) for the SU cells in response to different agonists in an artificial visco-elastic penetration medium (polyacrylamide). A,B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial visco-elastic medium (polyacrylamide) at **1cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the visco-elastic medium). Cells stimulated with 4-AP show very high **A** VCL and **C** LIN which is contrast to control and P4. This suggests 4-AP stimulated cells makes less progression further into the visco-elastic medium. With respect to 4-AP same behaviour was observed even in DG (Fig 4.11), but in DG 4-AP stimulated cells show low VCL suggesting they were less hyperactivated in DG than in SU. Each plot shows mean ± S.E (standard error) of 8 experiments prepared using direct swim up (SU) technique.

A


Polyacrylamide-DG-VCL(µm/sec)-Kremer's-2cm
% Frequency
VCL (µm/sec)
Control
4-AP
PE1
P4

B


Polyacrylamide-DG-ALH(µm)-Kremer's-2cm
% Frequency
ALH (µm)
Control
4-AP
PE1
P4

C

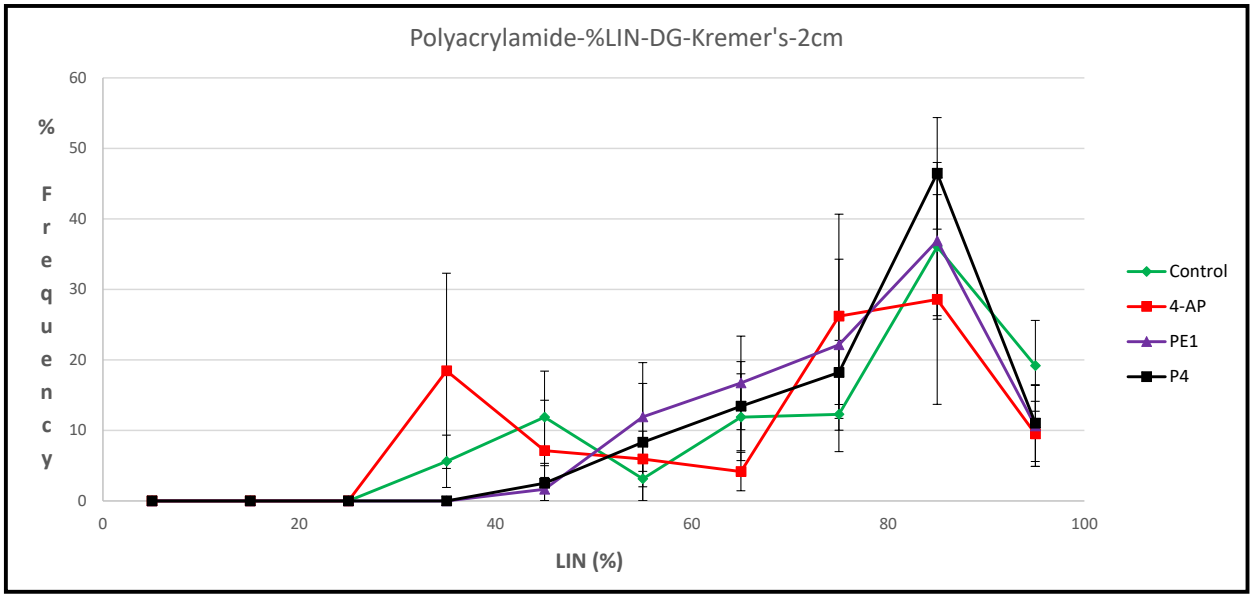
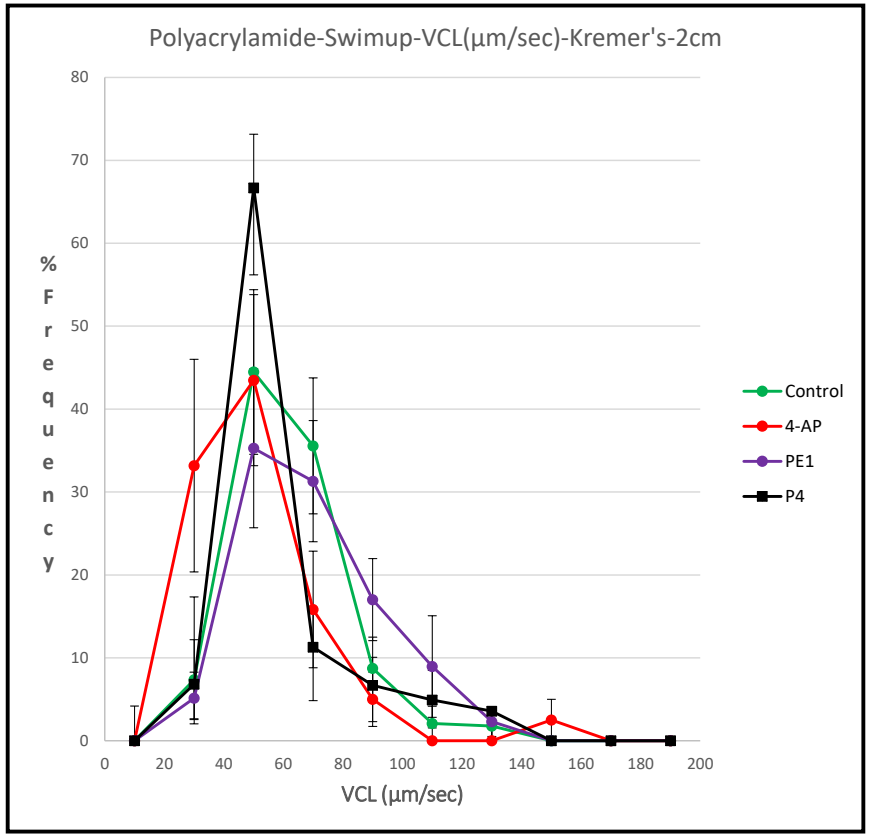
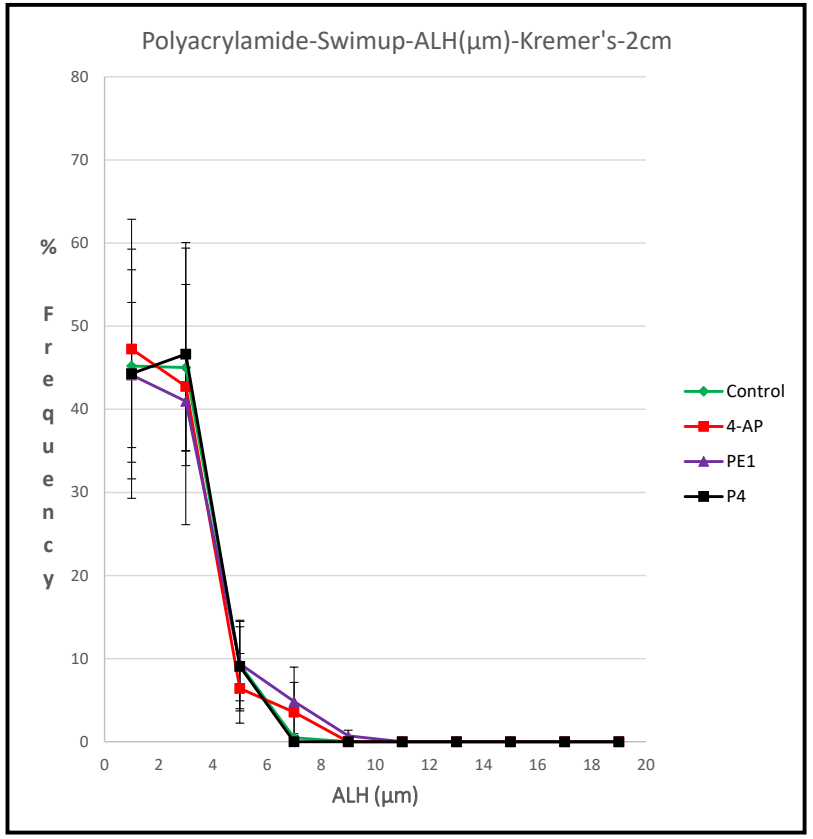


Figure 4.20 Showing the frequency distributions (FD) for the DG cells in response to different agonists in an artificial visco-elastic penetration medium (polyacrylamide). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial visco-elastic medium (polyacrylamide) at **2cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the visco-elastic medium). When compared to the control cells stimulated with PE1 show low **A** VCL, Low **B** ALH and a higher **C** LIN. This data suggests with high linearity and low VCL PE1 stimulated cells progress further (2cm) into the visco-elastic medium. Each plot shows mean ± S.E (standard error) of 8 experiments prepared using density gradient (DG) technique.

A


Polyacrylamide-Swimup-VCL(µm/sec)-Kremer's-2cm
% Frequency
VCL (µm/sec)
Control
4-AP
PE1
P4

B


Polyacrylamide-Swimup-ALH(µm)-Kremer's-2cm
% Frequency
ALH (µm)
Control
4-AP
PE1
P4

C

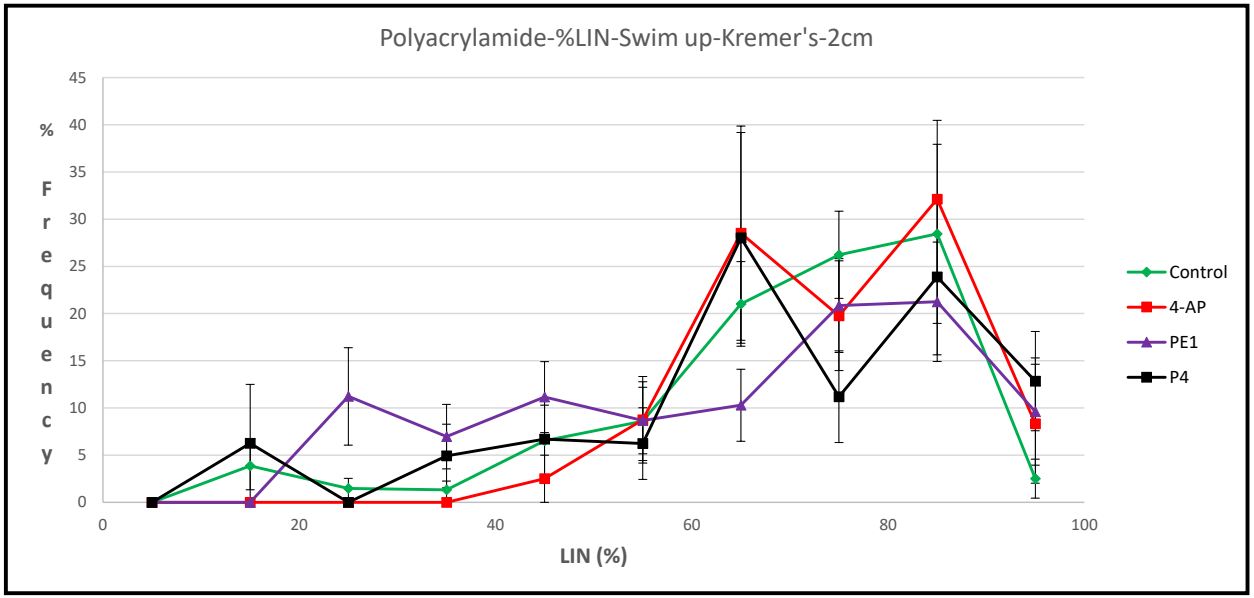


Figure 4.21 Showing the frequency distributions (FD) for the SU cells in response to different agonists in an artificial visco-elastic penetration medium (polyacrylamide). A, B and C showing the frequency distributions (FD) **of curvilinear velocity (VCL (µm/sec)), amplitude of lateral head displacement (ALH (µm)) and Linearity (%LIN)** during human sperm cells enhancement into the artificial visco-elastic medium (polyacrylamide) at **2cm** under control conditions **(Green)** and after stimulation of human sperm cells with 5mM 4-Aminopyridine (4AP) **(Red),** 2µM Prostaglandin E_1 (PE_1) **(Purple)** and 3µM Progesterone (P4) **(Black).** The FD data was recorded using the CASA analyser when the cells were in the kremer's (in the visco-elastic medium). Contrast to DG in SU cells stimulated with 4-AP show lower **A** VCL, slightly higher **B** ALH and a high **C** LIN. This suggests 4-AP cells would perform better in the SU preparation compared to 4-AP stimulated cells in DG preparation. Each plot shows mean ± S.E (standard error) of 8 experiments prepared using direct swim up (SU) technique.

4.5 Discussion

It is crucial to understand the sperm journey into the female tract. Research advances in this area help us to address infertility problems, improve diagnosis, and develop effective contraceptives. During sperm migration in the female reproductive tract, it will encounter different environments, e.g. viscous and viscoelastic environments. To reach the oocyte and be able to fertilise, the sperm must select and switch appropriate behaviours that will enable successful migration through distinct environments (viscous and viscoelastic), detach from the oviductal epithelium, penetrate through the cumulus matrix and zona pellucida and, finally, fertilise with the oocyte. $[Ca^{2+}]_i$ signalling plays a key role in regulating these events and influx of extracellular Ca^{2+} and/or mobilisation of calcium from intracellular organelles is therefore essential. Absence or malfunction of these calcium sources in human sperm can lead to infertility.

In this chapter I used manipulation of Ca^{2+} release from stores in the posterior head and neck region (RNE (redundant nuclear envelope)) and activation of Ca^{2+} influx in the flagellar principal piece (CatSper) to induce different behaviours. 4-Aminopyridine (4AP) was used to mobilise calcium from **stores** in the posterior head and neck region, inducing hyperactivation (Alasmari et al, 2011) and prostaglandin E_1 (PE_1) and progesterone (P4), which increase Ca^{2+} influx into the flagellar principal piece by activating the CatSper channel, were used to enhance activated motility (Alasmari et al 2011). Using human sperm prepared by direct swim-up and density gradient methods, the effects of sperm behaviours induced by these manipulations of $[Ca^{2+}]_i$ signalling on penetration of viscous (methylcellulose) and viscoelastic (polyacrylamide) media was assessed.

4.5.1 Effects of 4AP, PE1 and P4 on hyperactivated motility in swim-up compared to density gradient

4-Aminopyridine (4AP) significantly increased hyperactivated motility (measured by % sort 7) in both swim-up and density gradient cells but this effect was significantly greater in swim-up sperm (Figure 4.1). An increase in hyperactivation corresponds to an increase in different motility characteristics, e.g. ALH (Figure 4.2), VCL (Figure 4.3), VSL (Figure 4.4), and a decreased LIN (Figure 4.5). This data suggests that cells prepared with swim-up and stimulated 4AP show enhanced hyperactivated motility when compared to the density gradient method.

In contrast to the above result, agonists PE_1 show significant increase in hyperactivated motility (measured by % sort 7) when cells are prepared by density gradient compared to swim-up (DG; Table 4.2).

4.5.2 Effects of 4AP, PE1 and P4 on penetration in viscous and viscoelastic environments

Data from standard CASA suggests that 4AP is a potent inducer of hyperactivation (Figure 4.1) in both swim-up and density gradient, but when analysing Kremer penetration results of DG and SU cells, 4AP shows an inhibitory effect in both viscous (Figure 4.8) and viscoelastic (Figure 4.15) environments. When compared to 4AP, both PE_1 and P4 show less hyperactivation in standard CASA data (Figure 4.1) but show greater penetration (Figures 4.8 & 4.15). Although penetration of PE1 and P4 treated cells is similar, DG-prepared cells when treated with PE_1 show slightly higher curvilinear velocity (VCL) (Figure 4.3) when compared to swim-up (Figure 4.3), which may assist in enhancing sperm entry into the viscous and viscoelastic media.

Frequency distribution (FD) data from both DG and SU cells inside viscous (methylcellulose) and visco-elastic (polyacrylamide) media showed little variation in the distribution of VCL or LIN as cells progressed up the tube. However, it was noticeable that at 0 cm ALH was variable in both viscous and viscoelastic environments. Some of these cells had 'high' ALH (> 5 μm). In cells that had progressed 1 and 2 cm into the tube the FD was narrower and more consistent between the different treatments (e.g. see figs 4.12B, 4.13B, 4.20B, 4.21B). This suggests that cells with low ALH progress better within the viscous and visco-elastic environments, which is consistent with the poor performance of 4AP treated cells in the Kremer assay. PE_1-stimulated cells, in contrast, maintain a low lateral head movement (ALH) and had better progressive ability in both viscous and viscoelastic media.

Previous research done by Suarez (Suarez and Dai, 1992) explained that increase in hyperactivated motility enhance sperm penetration in to viscous and visco-elastic medium. (though this experimental work was carried out in mouse model). But from this chapter, it suggests that CatSper (PE_1 & P4) plays a crucial role in enhancing sperm penetration through viscous and viscoelastic environments, whereas store-mediated hyperactivation (4AP), significantly reduces the ability of sperm to enter viscous or visco-elastic environments.

Chapter 5: Assessment of Capacitation using Protein Tyrosine Phosphorylation – direct SU & DG

5.1 Objective:

From Chapter 3, I observed that there is a difference in human sperm motility when prepared using direct SU and DG. As capacitation is a prerequisite to acquire functional competence (motility) to achieve successful fertilisation, it would be of great interest to investigate whether the difference in motility that is observed between these two techniques is associated with a difference in sperm maturation (capacitation). In this chapter, I use protein tyrosine phosphorylation as a marker to evaluate sperm capacitation in both direct SU and DG.

5.2 Introduction

5.2.1 Capacitation

For successful fertilisation sperm must undergo a maturation process called “capacitation” (Chang, 1951a, Baldi et al., 2000, Bedu-Addo et al., 2005, Battistone et al., 2013), which regulates sperm motility, zona pellucid binding, acrosome reaction and a number of other key aspects of sperm function and therfore it is (Capcitation) absolutley important (Baldi et al., 1996, de Lamirande et al., 1997, Visconti et al., 1999, Baldi et al., 2000, Bedu-Addo et al., 2005, Tateno et al., 2013, Aitken and Nixon, 2013). Capacitation normally happens in the female reproductive tract, but can also be initiated in in vitro conditions by incubation in medium containing appropriate amounts of glucose, sodium bicarbonate ($NaHCO_3^-$), calcium and bovine serum albumin (BSA), which has allowed study of the events that occur.

During capacitation, the sperm undergoes set of modifications that include cholesterol removal from the plasma membrane and increased fluidity, increased ionic membrane permeability (Cross, 1998) and increased intracellular levels of $[Ca^{2+}]_i$ and cyclic adenosine monophosphate (cAMP). Hyperactivated motility also becomes more vigorous with the start of capacitation, but hyperactivated motility and capacitation are believed to be two different events which differ in their regulation (Ho and Suarez, 2001)

It has been shown that increased levels of tyrosine phosphorylation (TyrP) is associated with capacitation (Visconti, 2009). I have therefore used TyrP as a marker for sperm maturation, to investigate whether differences in motility of SU and DG may be due to differences in capacitation.

5.2.2 The Role of Protein TyrP in Capacitation

It has been shown that an increased level of sperm tyrosine phosphorylation (TyrP) is associated with in vitro capacitation of mammalian sperm (including human) induced by HCO_3^-, BSA and Ca^{2+} (Leclerc et al., 1996a) (Visconti, 2009). Increased TyrP was absent in the non–capacitation medium (Visconti and Kopf, 1998, Visconti, 2009). Also, during capacitation, the association between TyrP and sperm motility was observed (Leclerc et al., 1996a, Leclerc and Goupil, 2002). I have therefore used TyrP as a marker for sperm maturation, to investigate whether differences in motility of SU and DG may be due to differences in capacitation.

For signalling and biochemical events associated with capacitation, please see fig.11 in section 1.6.1

5.3 Materials and Methods

5.3.1 Sample Preparation – direct SU and DG

For sample preparation, please see section 2.7.1

5.3.2 SDS and Western/Immunoblot setup

For SDS and western/immunoblot setup, please see section 2.7.2

5.4 Results

5.4.1 Comparison of Human Sperm Capacitation in both direct SU and DG using TyrP

Capacitation was performed with cells prepared using direct SU and DG, and samples were assessed for TyrP immediately upon suspension in capacitating medium (with a delay of not more than 2min; T0) and following incubation in capacitating medium for 30 min (T30), 90 (T90) and 180 min (T180).

Figure 5.1 shows the expression of TyrP of two major proteins of 105 and 81KDa extracted from sperm prepared using direct SU and DG. There was a clear difference in intensity of TyrP as capacitation developed (compare T0 and T180) but no difference in TyrP was observed between direct SU and DG cells.

Silver staining of the proteins transferred to the nitrocellulose membranes shows equal protein loading in all wells (Figure 5.1).

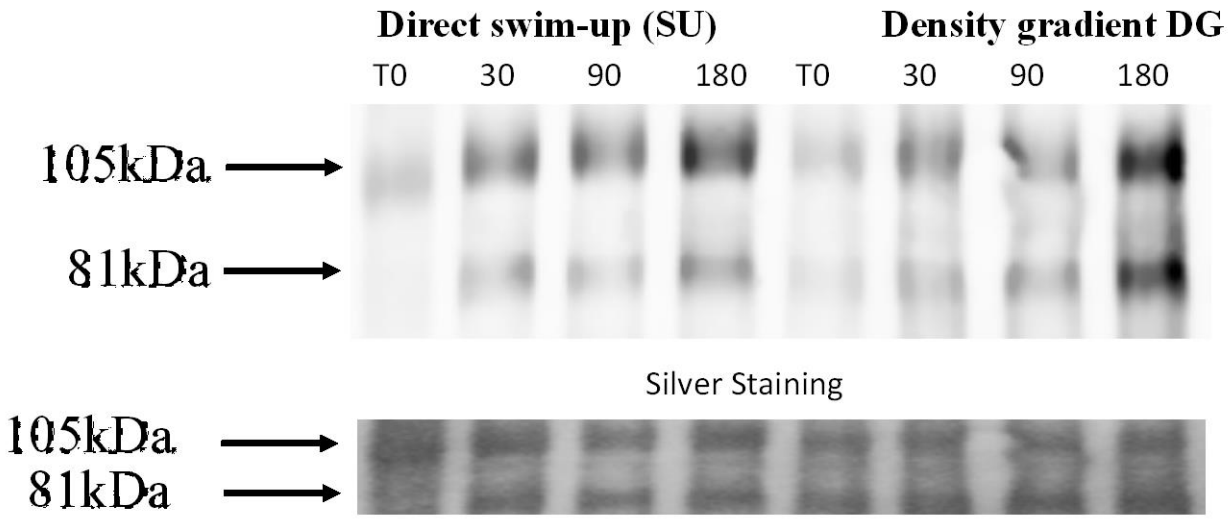


Figure 5.1: Using protein Typr as a marker to evaluate human sperm capacitation. The figure shows western blot analysis of protein Typr in human sperm prepared by direct SU and DG methods. No difference in intensity or time course was observed in any of the five experiments. Image from sliver staining shows equal protein loading in all wells.

5.5 Discussion

There is a significant difference in human sperm motility (hyperactivated motility and specific kinematic components) between sperm prepared by direct SU and by DG (see Chapter 3). As hyperactivated motility is normally associated with capacitation, it was of interest to investigate whether the difference in hyperactivated motility between SU and DG cells was associated with a difference in capacitation status of sperm prepared by the two techniques.

From Figure 5.1, it appears that there was no major difference in the level of tyrosine phosphorylation, between direct SU and DG sperm. Furthermore, the time course of increased protein TyrP was similar irrespective of how the cells were prepared. These data thus show no differences in the occurrence or time-dependence of capacitation (as assessed by TyrP) between direct SU and DG cells, suggesting that the observed differences in motility (chapter 3) are unlikely to reflect differences in capacitation. However, this conclusion must be interpreted with caution. The use of TyrP as a marker of capacitation has recently been questioned since fertilisation competence in mouse sperm can be achieved by temporary elevation of $[Ca^{2+}]_i$ (using the ionophore A23187), in the absence of TyrP (Tateno et al., 2013). TyrP is therefore not a requirement for achieving capacitated status though it is not yet clear whether TyrP is part of the processes leading to 'natural' capacitation, perhaps by regulation of Ca^{2+} signalling.

Chapter 6: Intracellular calcium signalling $[Ca^{2+}]_i$ & Assessment of calcium channel [CatSper] activity – direct SU and DG

6.1 Objective:

Differences were observed between direct SU and DG cells in their motility and penetration of viscous media (chapter 3). In this chapter the possible significance of CatSper channel function in these differences (HA motility & penetration) is investigated. I use progesterone (P4) as a potent activator of CatSper channel under *in-vitro* conditions and compare progesterone induced intracellular calcium $[Ca^{2+}]_i$ responses in both direct SU and DG cells.

Differences in CatSper channel expression between DG (40/80% boundary and 80% Percoll pellet) and direct SU cells was investigated using Immunofluorescence (IF) technique.

6.2 Introduction

In somatic cells, regulation of many cellular activities is controlled by gene expression, through control of transcription and translation of proteins. However, human sperm are transcriptionally and translationally silent and therefore rely on post-translational modification to regulate various sperm functions which are required for fertilisation. For hyperactivation, chemotaxis and acrosome reaction this is achieved primarily through $[Ca^{2+}]_i$ signalling (Costello et al., 2009, Darszon et al., 2011).

Human sperm possess different calcium signalling mechanisms which are now well established (Publicover et al., 2007). These signalling mechanisms involve the entry of calcium from extracellular space into cytoplasm and the mobilisation of calcium from intracellular stores (Acrosome & Redundant nuclear envelope (RNE)) (Costello et al., 2009, Alasmari et al., 2013b).

A central feature of calcium signalling in human sperm is the CatSper channel, a calcium-permeable channel present on the plasma membrane of the flagellum (Ren et al., 2001, Kirichok et al., 2006, Qi et al., 2007, Kirichok and Lishko, 2011, Singh and Rajender, 2015).

Results from use of Catsper knock-out models and clinical studies show that CatSper channels are necessary for male fertility, and mutations of CatSper genes in human's lead to male infertility (Hildebrand et al., 2010, Singh and Rajender, 2015, Williams et al., 2015).

From earlier research observations and from my research findings (Chapter 4) it can be concluded that calcium signalling regulates human sperm motility (Publicover et al., 2007). The activation of a CatSper channel (see figure 6.1 **A**), and calcium mobilisation from stores through the calcium induce calcium release (CICR) mechanism (see figure 6.1 **B**), induces functionally different behaviours; therefore, the sperm motility pattern is determined, at least in part, by the site of calcium mobilisation (Alasmari et al., 2013b).

For clinical studies, the most widely used sperm preparation method is the density gradient technique (Kim et al., 2015), because of its high yield (Henkel and Schill, 2003) compared to the direct swim up technique (in lab method). Although DG offers high yields, its effects on sperm motility are often ignored. From my research findings (from Chapter 3), it was observed that sperm prepared by DG and by direct SU differed in their functional abilities (HA and penetration of viscous medium), with direct SU cells offering better penetration and lower levels of HA, as compared to DG cells.

Considering these functional differences as observed with respect to DG and direct SU cells, it is of great interest to investigate the characteristics of $[Ca^{2+}]_i$ signalling (by studying the activity of CatSper) and important to evaluate Catsper protein expression using immunofluorescence method (IF) in relation to the functional differences observed between DG and direct SU cells. I have used progesterone (P4), a potent activator of CatSper channel (Lishko et al., 2011, Strunker et al., 2011, Smith et al., 2013, Alasmari et al., 2013b), in order to observe $[Ca^{2+}]_i$ responses in both DG and direct SU cells. To observe the effect of intracellular calcium $[Ca^{2+}]_i$ responses in both DG and direct SU cells under *in vitro* conditions, a fluorescent calcium indicator, Fluo4, was used. When bound to calcium, Fluo4 shows an increase in fluorescence. The Fluo4 calcium indicator is a cell impermeant so the cell permeant Fluo4 acetoxymethyl (AM) derivative (Tsien, 1981, Takahashi et al., 1999) was used. Unlike its original form (Fluo4), Fluo4 AM can passively penetrate across the lipid membrane. Once the Fluo4 AM is inside the cell, esterases cleave the acetoxymethyl group, trapping Fluo4 inside the cell (see figure 6.1 **C**).

In addition to $[Ca^{2+}]_i$ signalling & evaluating Catsper protein expression in direct SU and DG cells, I have demonstrated, here, the existence of the CatSper protein in two different patterns (Continuous & Punctuated signal) in human sperm.

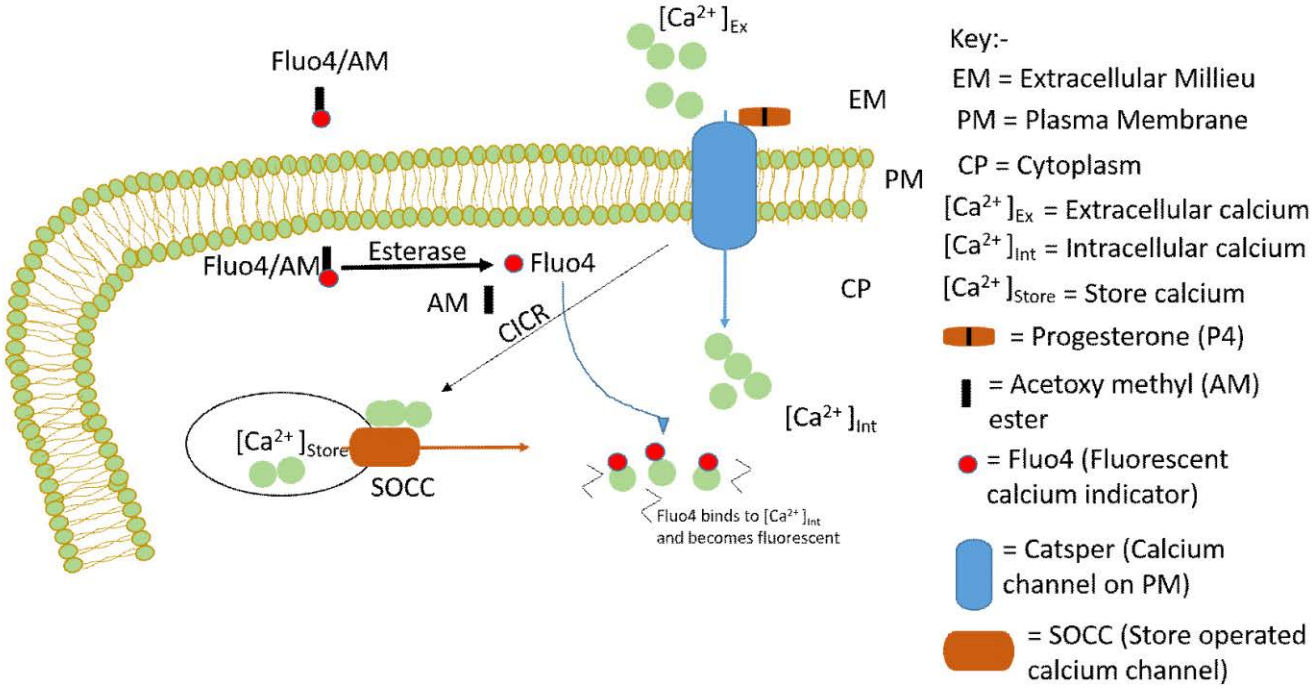


Figure 6.1 Showing Calcium mobilisation and Fluo-4 calcium indicator working model. A shows an influx of calcium from extracellular to intracellular space through a CatSper channel located on the plasma membrane. Progesterone (P4) is used to activate the CatSper channel. **B** This shows calcium mobilisation from intracellular store through the calcium induced calcium release (CICR) mechanism associated with P4. **C** This shows an acetoxymethyl (AM) ester derivative of the fluorescent indicator (Fluo4/AM). Protection of carboxylic groups as AM esters makes the Fluo4 neutral so that it can penetrate the cell membrane. After entering into the cell, AM ester groups are cleaved by the esterase and Fluo4 with a charge (-), becomes cell impermeant (Fluo4$^-$ cannot leave the cell). When Fluo4$^-$ binds to intracellular calcium $[Ca^{2+}]_i$ it becomes more fluorescent.

6.3 Materials and Methods

6.3.1 Single Cell Imaging

6.3.1.1 Sample Preparation

Sample preparation was performed as mentioned in sections 2.4.1 and 2.4.2.

After 4.5hr of capacitation, the cell concentration was adjusted to $3x10^6$ cells/ml with sEBSS containing 0.3% BSA prior to imaging experiments.

6.3.1.2 Experimental setup

A perfusable, polycarbonate imaging chamber was used for imaging. The base of the chamber, through which the cells were viewed, was a coverslip (22x32mm) coated with 0.5% Poly-D-lysine, attached using high vacuum grease (Dow Corning). A 12mm circular glass coverslip formed the upper surface of the chamber, allowing transillumination.

Fluo-4 AM was used for labelling cells. 50µl of Pluronic F-127 (1 ml in 20% DMSO) was added to 50µg of Fluo-4 AM and left at room temperature for 2~3 min. From this mixture 1µl was added to 200µl aliquots of capacitated sperm (prepared using direct SU and DG methods) and incubated for 25 min at 37ºC and 5% CO_2. Following this incubation, an aliquot volume of 125 µl was transferred to the imaging chamber. A further 15min incubation of the imaging chamber at 37ºC and 5% CO_2 allowed the labelled cells to attach to the Poly-D-lysine-coated coverslip.

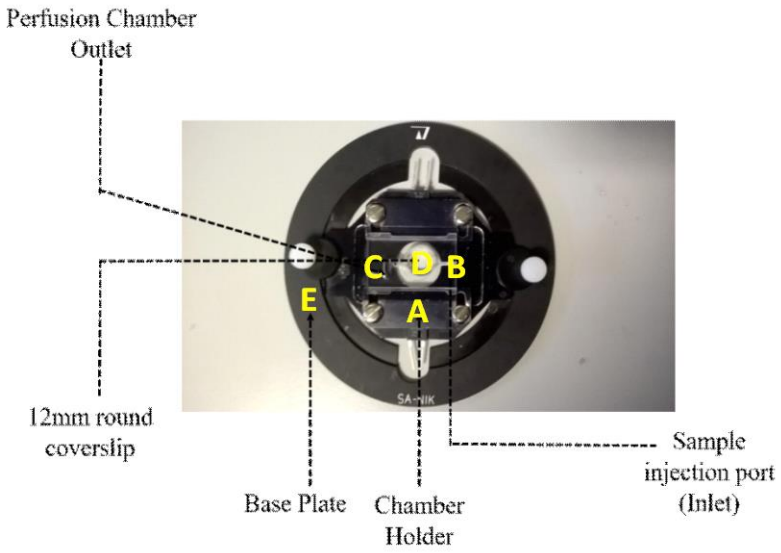


Figure 6.2: Showing the construction of an imaging perfusion chamber. A An imaging chamber was placed on a holding frame purchased from Warner Instruments. **B & C** A sample inlet and outlet of a perfusion chamber. **D** A 12mm round coverslip attached to the centre of the chamber using high vacuum grease. Cells adhere to the poly D-lysine coated coverslip which is placed at the bottom of the chamber (not shown in the figure). **E** The base plate (holding frame) to hold the chamber.

After incubation, the chamber was mounted above a 40x oil objective on a Nikon TE300 inverted fluorescence microscope that was fitted with a Cairn Opto LED light source and filters for excitation 494nm and emission 523nm (Cairn Research, Kent, UK). The imaging chamber was connected to a perfusion system consisting of a peristaltic pump with a perfusion rate of approximately 0.66 ml/min.

Before the start of the experiment, at least 10ml of fresh sEBSS medium was washed through the chamber to remove excess dye and unattached cells. Following a 2min recorded control period, the sEBSS was removed and agonists, such as progesterone (P4), were added directly to the perfusion header. A 38.4 s (0.64min) delay was taken into account (the time takes for saline to travel from the perfusion tube to the inlet of the imaging chamber) when assessing response kinetics.

Imaging experiments were carried out at 25ºC. Continuous perfusion of the imaging chamber at a constant and regulated temperature was achieved by maintaining the imaging room and equipment at the working temperature. Working at higher temperatures using a microscope incubator and/or heated stage in combination with perfusion caused temperature fluctuations and consequent effects on focus. Experiments carried out at 31ºC and 37ºC (achieved by maintaining the room at these temperatures) have established that responses to progesterone (including generation of $[Ca^{2+}]$ oscillations) at 25ºC are functionally similar (though slower) than at higher temperatures (Harper et al., 2004).

6.3.1.3 Image Acquisition and analysis

Cells were illuminated and fluorescence images were normally captured at 0.2Hz (Frame rate 5s) using a 40x oil objective and images were obtained using an Andor Ixon EMCCD camera controlled by a PC running Andor IQ acquisition and analysis software. For offline analysis regions of interest (ROI) were drawn around the posterior head of each of at least 20 cells. Fluorescence was background corrected and the raw data was imported into Microsoft Excel and normalised using:

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

Where R is the normalised fluorescence intensity

F is called the fluorescence intensity at time t

F_{rest} is the mean of at least 20 determinations of F taken during the control period before stimulation

The normalised fluorescence intensity values (R) at each time point were compiled to generate a mean value for normalised head fluorescence from all cells in the experiment

(R_{tot}). The resulting values were then plotted on a time-fluorescence intensity graph (see Figure 6.3).

For calculating transient responses (peak amplitude), the five highest consecutive points on the peak (the highest point and two points on the either side of the peak) were selected and averaged. Transient responses were recorded within ~ 38.4sec (7.68 frames) of agonist (progesterone) addition. The amplitude of sustained calcium response was recorded for five consecutive points after ~3min of agonist (progesterone) addition, and the data was averaged. To compare responses in SU and DG cells derived from the same sample paired t-tests were performed using Graph Pad Prism, and $P < 0.05$ was considered as statistically significant.

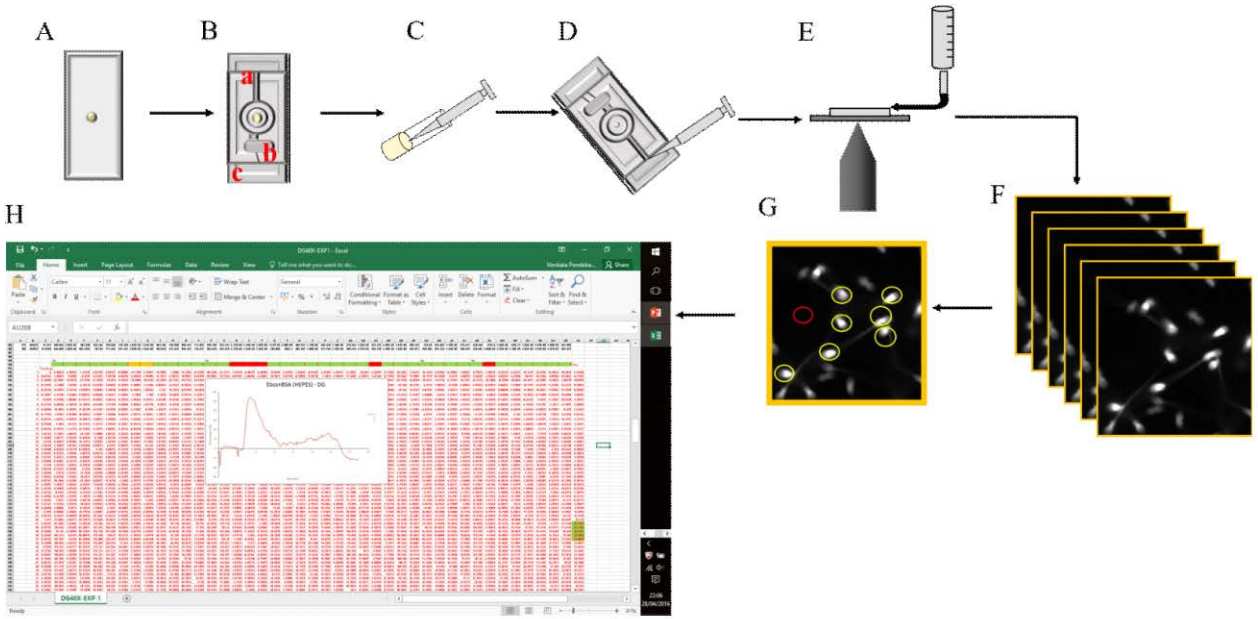


Figure 6.3: Showing experimental construction of single cell imaging. **A** 0.5% poly-D-lysine coated on a coverslip (22x32mm). **B** The polycarbonate imaging chamber with sample inlet (**a**) and outlet (**b**). (**c**) The imaging chamber was attached to a poly-D-lysine coated slide coverslip in such a way that the top position of the coverslip (containing the poly-D-lysine) faced the bottom section of the imaging chamber. **C** Cells prepared by DG were left to capacitate for 4.5hr. Afterwards, 200µl of human sperm cells were incubated with fluorescent dye (Fluo-4 AM) for 25min. **D** After 25min of incubation, the cells were loaded in the imaging chamber through the sample inlet and incubated for a further 15min. **E** The imaging chamber was then mounted above a 40x oil objective on an inverted fluorescence microscope and perfused with sEBSS that contained the agonist (progesterone (P4)). **F** A sequence of time lapse images were acquired. **G** The **background** and **region of interests (ROI)** were selected. **H** The acquired data was then analysed offline in Microsoft Excel. The same procedure was repeated with cells prepared by direct SU.

6.3.2 Immunofluorescence (IF)

6.3.2.1 Sample preparation – Direct SU and DG

SU sample preparation was same as explained in section 2.4.1. After adjusting concentration to6x10^6/ml., SU cells were used straight away for the IF technique.

DG sample preparation was as in section 2.4.2 except that cells were collected from both 40/80% boundary and the 80% Percoll pellet. Cells were then washed in PBS (500g for 10min) and then resuspended in sEBSS (0.3% BSA). Cell concentration was determined using an improved Neubauer haemocytometer and adjusted to 50x10^6/ml. After adjusting concentration, the cells were used straight away for the IF technique.

6.3.2.2 Experimental Procedure

Frost slides were numbered 1 to 15 (for slide layout, please see Table 6.1) and coated with Poly-L-lysine (1:10 dilution with deionized water) for 5min then left to dry overnight. The next day, 70µl of 6x10^6/ml cells from direct SU and 20µl of 50x10^6/ml cells obtained from 40/80% Percoll and 80% Percoll gradients were added onto each frost slide and smeared and then left it to dry at room temperature (RT). 10ml of 4% formaldehyde (diluted from a 37% stock solution using PBS) was used to fix the cells (6 min at RT) and then the slides were washed three times with 100% PBS for 5 min each.

Cells were permeabilised with 0.2% Triton-X 100 (TX-100) in PBS for 20 min and washed once with 0.1% TPBS (0.1% TX-100 in PBS) for 5 min. After this step, 10% normal goat serum in TPBS was used to block non-specific sites for 1 hr at RT and slides were then washed once for 5 min using 0.1 % TPBS. Based on slide layout (please see Table 6.1), cells were incubated overnight at 4°C with 0.8 mg/ml anti-CatSper-4-antibody at two different dilutions (1:50 and 1:100), both with and without blocking peptide), 4µg/ml rabbit serum or

0.1% TPBS alone was used as negative control. Slides were then washed 3-4 times with 0.1% TPBS for 5min each then incubated in the dark for 60 min with fluorescein-conjugated Affini Pure Goat Anti – Rabbit IgG (dilution 1:100 in 0.1% TPBS). After further washing (3-4 times with 0.1% TPBS for 5 min each in the dark) the cells were mounted with 15µl Perma Fluor (mounting medium).

6.3.2.3 Data Acquisition

Frost slides were examined using a Nikon Eclipse E600 fluorescence microscope equipped with FITC filter set, excitation max 490 and emission max 525 and an oil immersion x100 objective. CatSper 4 expression was evaluated in 2,000 sperm cells prepared using 40/80% gradient and 80% gradient, and ~1000 sperm cells prepared using direct SU.

A chi-square test (in Graph Pad Prism) was used to assess differences in the proportion of cells expressing CatSper 4 in the three different groups (40/80%, 80% and direct SU).

6.3.2.4 Slide Layout

	Formaldehyde														
	Percoll 80% (DG)					Percoll 40/80% (DG)					Direct SU				
Slide #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A CatSper 4 0.8mg/ml (1/50)	X					X					X				
B CatSper 4 0.8mg/ml (1/100)		X					X					X			
C CatSper 4+ Blocking Peptide 1:1 (1/50)			X					X					X		
D Normal Goat Serum	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
E Rabbit Serum 4µg/ml (1/100)				X					X					X	
F 0.1% TPBS					X					X					X
G 2nd Anti-Rabbit - FITC	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100

Table 6.1: Showing slides layout for IF technique. Human sperm cells were prepared using direct swim up (SU) and density gradient centrifugation (DG) using Percoll gradient. In DG cells were collected from two different layers (Percoll 40/80% and Percoll 80%). **A** Slides 1,6 and 11 were incubated with 0.8mg/ml anti-catsper 4 antibody (dilluted 1:50) (test sample) and **B** Slides 2,7 and 12 were incubated with 0.8mg/ml anti-catsper 4 antibody (dilluted 1:100) (test sample). **C** Slides 3,8 and 13 were incubated with equal parts of anti-catsper-4 antibody + blocking peptide 1:1 (dilluted 1:50) (Positive control). **D** In order to block non-specific sites all slides were treated with 10% Normal goat serum (NGS). **E** Slides 4,9 and 14 were incubated with 4µg/ml Rabbit Serum (1:100) & **F** slides 5,10 and 15 were treated with 0.1% TPBS alone (Negative control). **G** All slides were incubated with Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG (dilluted 1:100).

6.3.2.5 IF Experiment Construction

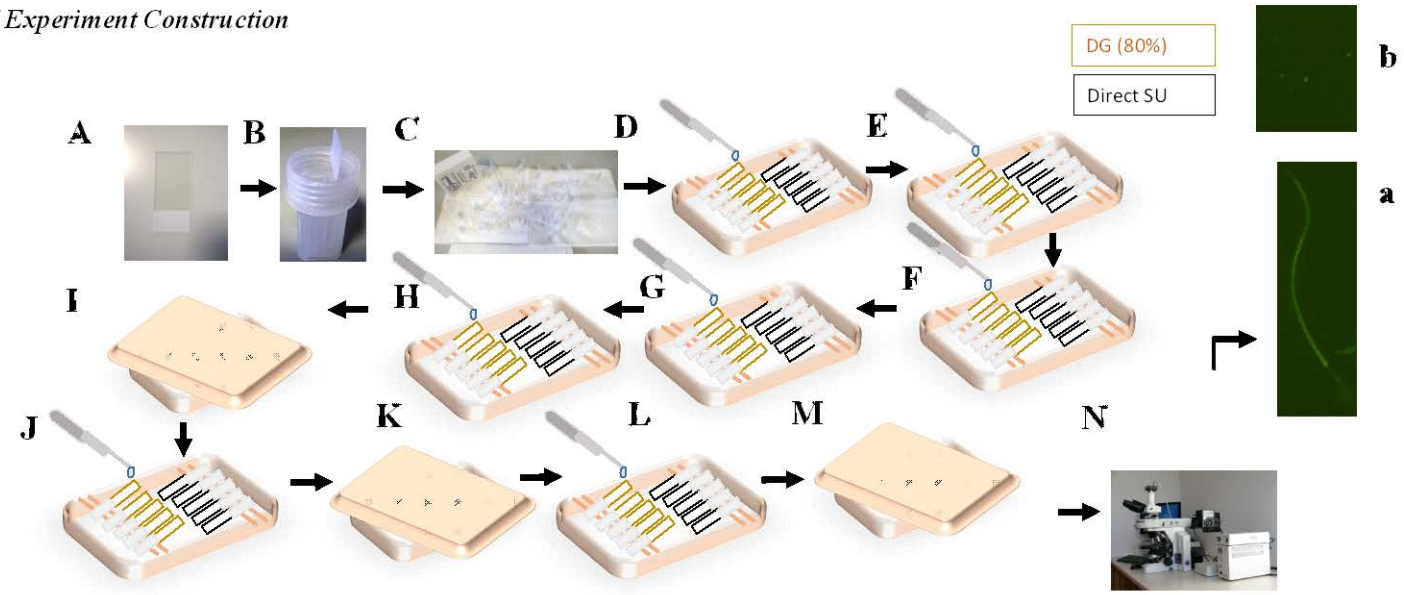


Figure 6.4: Showing the construction of the IF Experiment. A A typical frost slide used in IF experiments. **B** Slides were treated using poly L-lysine for 5min. **C** Poly-L-lysine coated slides were left to dry overnight (O/N) at room temperature (RT). **D** Cells prepared using direct SU and DG were fixed on the frost slide using 4% formaldehyde for 6min at RT. **E** After washing with PBS, cells were permeabilised with 0.2% Triton-X100. **F** Cells were washed once using TPBS (0.1% TX-100 in PBS) for 5min. **G** 10% normal goat serum (NGS in TPBS) was used to block non-specific sites for 1hr at RT. **H** After washing for 5min in TPBS, cells were treated with different antibodies (except the secondary antibody) (see Table 2.1 for details of antibody addition), **I** and left to incubate O/N at 4°C. **J** After O/N incubation, cells were washed 4-5 times (5min each) with TPBS. **K** Incubation with fluorescein conjugated secondary antibody (for sAb details, see Table 2.1) for 1hr at RT. **L** After sAb incubation, slides were washed 3-4 times (5min each) and **M** incubated with 15µl mounting medium in dark for 1hr. **N** Images were captured using fluorescence microscopy. **a** Fluorescence images of CatSper protein (continuous signal associated in principal piece of a human sperm). Image **b** shows punctuated signal of CatSper protein in human sperm. Same procedure was carried out with human sperm cells prepared using DG (40/80) gradient.

6.4 Results

6.4.1 Progesterone (P4) induced intracellular calcium response – direct SU and DG

Progesterone induced $[Ca^{2+}]_i$ responses were evaluated in single sperm cells that had been prepared from the same ejaculate using either direct SU and DG. Figure 6.5 and Figure 6.6 shows the Intracellular calcium traces of the individual cells prepared using both direct swimup (SU) and density gradient (DG) techniques. When SU and DG cells were treated with 3µM progesterone (P4) sperm cells show a transient (T) calcium response and subsequent calcium oscillations. Sequential intracellular calcium image series of SU and DG cells treated with 3µM P4 are shown in the Fig 6.5 B (SU cells) and Fig 6.6 B (DG cells). In both SU and DG cells a $[Ca^{2+}]_i$ transient response followed by $[Ca^{2+}]_i$ oscillations were also observed. Table 6.2 summarises different parameters (Response (Transient only (T only)) – cells that give a transient response but no oscillation, Oscillations (O) – cells that give a transient response with oscillations, Total responsive cells - responsive cells as a proportion of all cells (TS); Reactive Live cells - responsive cells as a proportion of live cells (RL); No response (NS) and Die (D)) between SU and DG cells. When the statistical significance of each parameter was looked at between SU and DG cells, O, TS, RL, NS & D was shown to be statistically significant ($P<0.05$) between SU & DG techniques (Table 6.2). In contrast to this there was no statistical significance ($P>0.05$) with respect to the cells that show response (T only) up on 3µM P4 stimulation.

Figure 6.7 shows the time course of average normalised fluorescence responses in both direct SU and in DG cells. Stimulation with 3µM progesterone, P4, (a saturating dose for calcium channel activation, Alasmari et al., 2013) shows a biphasic $[Ca^{2+}]_i$ elevation (consisting of an early transient peak (T) followed by a sustained response (S)) in both direct SU and DG cells.

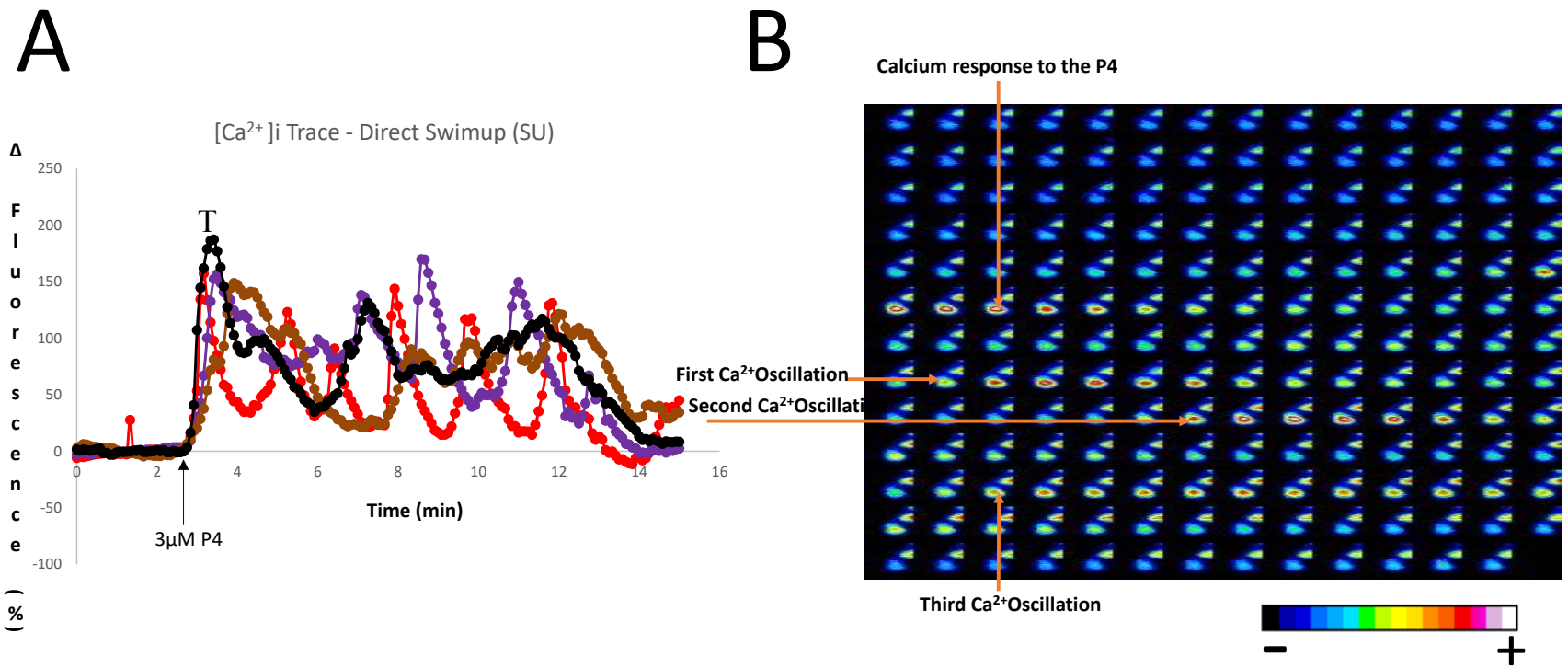


Figure 6.5: Intracellular Calcium response and Oscillations of SU cell. A Showing traces of Intracellular Calcium transient (T) response and Oscillations of the individual cells for a single experiment when treated with 3µM Progesterone (P4) and prepared using direct swimup (SU) technique. **B** Pictorial representation of the sequential image series of the SU cells showing the intracellular calcium transient (T) response to P4 and the subsequent calcium oscillations. Time interval between the acquired images is 5sec. Calibration bar with different colours representing the areas of low to high intracellular calcium levels. − Sign representing the area with the low intracellular calcium levels (Black Colour) and + representing the area with the high intracellular calcium levels (White Colour).

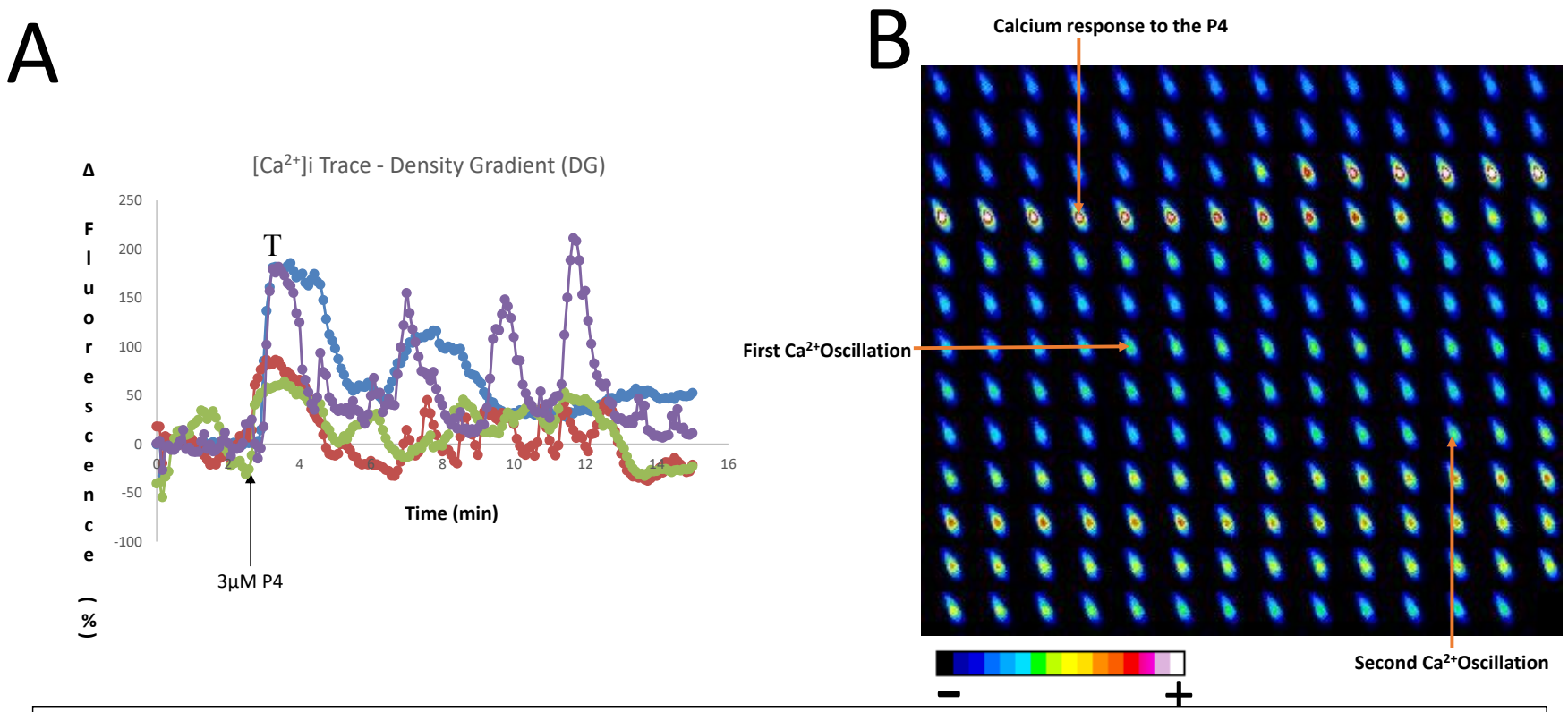


Figure 6.6: Intracellular Calcium response and Oscillations of DG cell. A Showing traces of Intracellular Calcium transient (T) response and Oscillations of the individual cells for a single experiment when treated with 3µM Progesterone (P4) and prepared using the density gradient (DG) technique. **B** Pictorial representation of the sequential image series of the DG cells showing the intracellular calcium transient (T) response to P4 and the subsequent calcium oscillations. Time interval between the acquired images is 5sec. Calibration bar with different colours representing the areas of low to high intracellular calcium levels. − Sign representing the area with the low intracellular calcium levels (Black Colour) and + representing the area with the high intracellular calcium levels (White Colour).

Direct Swimup (SU)			Density Gradient (DG) Centrifugation	
PARAMETER	Mean	S.E.M	Mean	S.E.M
% Response (T only)	64.487	3.023	53.922	5.784
% Oscillations (O)	25.189****	3.142	5.346	5.198
% Total Response (TS) (TS=T only + O)	89.676*****	3.195	59.268	5.784
% Reactive Live (RL) RL= [TS/(TS+NS)*100]	98.9****	0.624	70.370	5.670
% No Response (NS)	0.980***	0.549	23.650	4.563
% Die (D)	9.343*	3.118	17.080	3.399

Table 6.2: Table showing the data of different parameters (T only, O, TS, RL, NS & D) obtained from the Calcium Imaging analysis when cells were prepared using both Direct Swimup (SU) and density gradient (DG) techniques. For Calcium Imaging experiments human sperm cells were treated with 3µM Progesterone (P) and the obtained imaging data was analysed with each different parameter. When each parameter **[(Response (Transient (T)) only, Oscillations (O), Total Response (TS), Reactive –Live (RL), No Response (NS) and Die (D)]** was analysed between SU and DG, cells prepared using Direct Swimup (SU) perform significantly ($P < 0.05$) better compare to Density Gradient (DG) technique. Contrast to this no significant difference ($P > 0.05$) was observed with respect to the cells showing calcium response (S) when treated using 3µM Progesterone (P) and prepared using both SU and DG techniques. Paired t-test; n=17.

From figure 6.7, in direct SU cells both transient (T) $[Ca^{2+}]_i$ elevation and the sustain (S) $[Ca^{2+}]_i$ plateau were higher compared to those of DG cells. After 23 frames (F) of the control period, Progesterone (P4) was added at 2 min (24 frames), the $[Ca^{2+}]_i$ response of both DG & direct SU cells was observed at 2.64 min (~32 frames) with a delay of ~ 38.4 s (0.64min). This delay refers to the time taken for progesterone (P4) to travel from the perfusion tube to the inlet of the imaging chamber. In direct SU and DG cells the peak transient (T) $[Ca^{2+}]_i$ response was observed at 3.36min (40.32 frames). Sustain (S) response was observed at ~5min (60 frames) in direct SU cells compared to ~6.5min (78 frames) in DG cells (See fig. **6.7**).

From figure 6.8 amplitudes from both transient (T) and sustain (S) $[Ca^{2+}]_i$ responses were compared in P4 stimulated sperm prepared using direct SU and DG techniques. Cells prepared using DG and direct SU shown a transient responses (T) of **51.08%** (DG) compared to **74.7%** in direct SU.

The observed differences in transient responses (T) was not significant (*P>0.05*) between DG and direct SU. When sustain (S) $[Ca^{2+}]_i$ responses was measured between DG & direct SU cells, DG cells shown a sustain (S) response of **12.87%** (DG) compared to **44.40%** in direct SU cells. The observed differences in sustain responses (S) between DG and direct SU was statistically significant (*P=0.0002*).

Figure 6.9 shows a pseudo-coloured image series of responding cells prepared using direct SU and DG methods. Both higher and lower levels of $[Ca^{2+}]_i$ are shown, and the image series were compared – i.e. between the direct SU and DG methods.

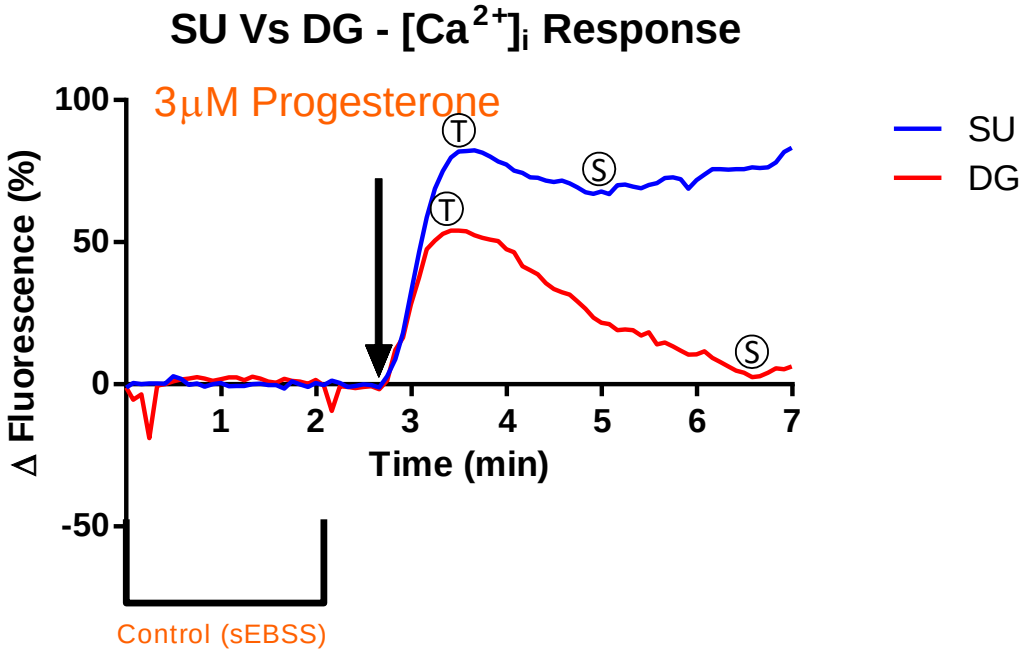


Figure 6.7 Intracellular calcium signalling in human sperm. The figure represents average fluorescence trace vs time from one experiment. It shows progesterone (3µM) induced $[Ca^{2+}]_i$ responses in Fluo4-AM loaded cells prepared by density gradient (DG) and by direct swim-up (SU). After a control period, sperm cells (both direct SU and DG) were treated with P4 (3µM) which entered the perfusion chamber at 2 min 40 s (arrow). P4 induced Bi-phasic (both Transient (T) and Sustained (S)) intracellular calcium responses.

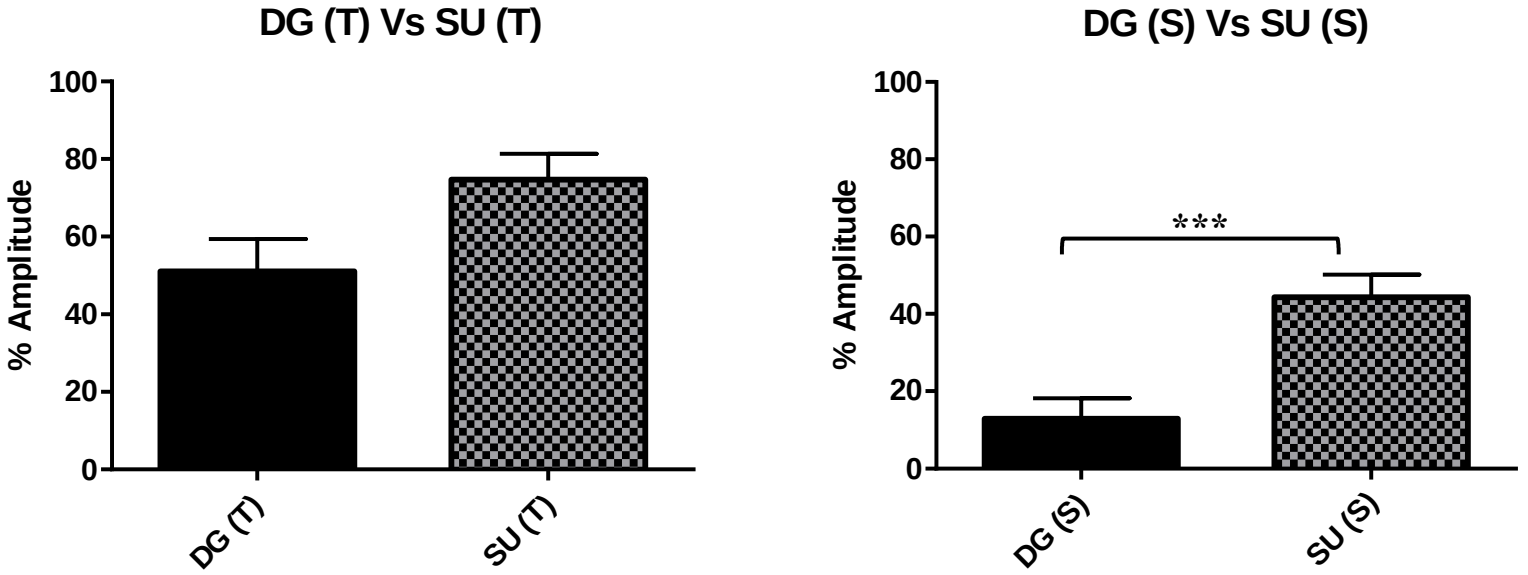


Figure 6.8 Transient (T) and Sustain (S) Intracellular calcium $[Ca^{2+}]_i$ responses in DG and direct SU cells. A&B showing amplitudes of transient (T) and sustain (S)$[Ca^{2+}]_i$ responses in progesterone (P4) stimulated human sperm cells prepared using DG & direct SU respectively. Sustained $[Ca^{2+}]_i$ response are significantly smaller in DG prepared cells (*P=0.0002*). Each bar shows mean ± S.E. (error bars) of 17 experiments.

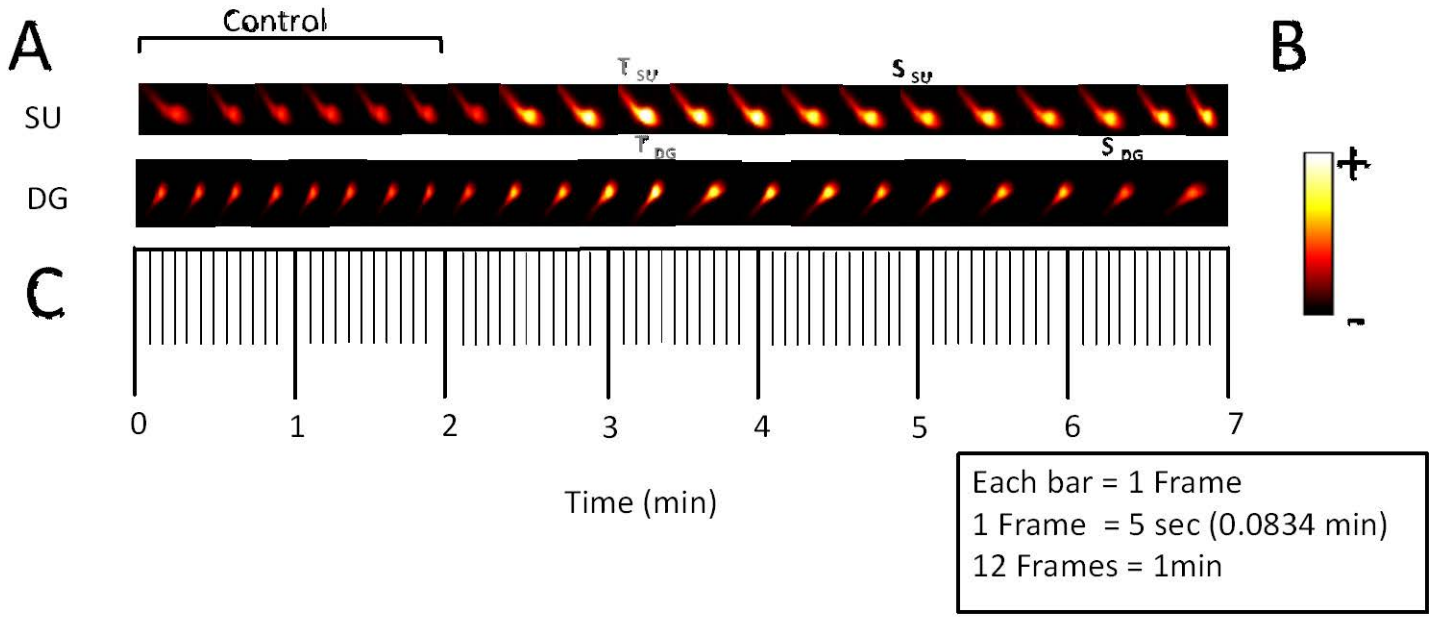


Figure 6.9 Showing $[Ca^{2+}]_i$ in direct SU & DG cells. A Shows a pseudocolour image series of responding cells when loaded with Fluo4-AM and prepared using direct SU & DG respectively. Time interval between 0 to 2min was considered as control period (no drug is added during this time). T_{SU} **&** T_{DG} (observed at 3.36min) represent peak transient intracellular calcium $[Ca^{2+}]_i$ response in both direct SU and DG cells. $\mathbf{S_{SU}}$ **&** $\mathbf{S_{DG}}$ ($\mathbf{S_{SU}}$ observed at ~5min & $\mathbf{S_{DG}}$ observed at ~6.5min) represent sustain intracellular calcium $[Ca^{2+}]_i$ response in both direct SU and DG cells. **B** Shows a fluorescence intensity bar with "+" indicating maximum fluorescence intensity (in white) and "- " indicating minimum fluorescence intensity (in black). **C** Represents a scale bar showing an experimental time period of 7min. Each bar represents 1 Frame = 5sec (0.0834min).

6.4.2 Evaluation of CatSper protein expression using IF

Since $[Ca^{2+}]$ i responses to stimulation with progesterone were markedly different in SU and DG cells, evaluation of CatSper expression (CatSper4 protein) was performed using immunofluorescence (IF). DG cells (from both the 40/80% boundary and the 80% Percoll pellet) and SU cells were examined. IF images in Figures **6.10, 6.11 & 6.12** show comparisons of CatSper4 expression (in the presence of anti-CatSper4 antibody at two different dilutions - 1:50 and 1:100) to their respective controls (absence of anti-CatSper4 antibody - **D** & **E** in Figures **6.10, 6.11 & 6.12**) and to the situation pertaining in the presence of CatSper4 peptide (**C** in Figures **6.10, 6.11 & 6.12**).

CatSper4 protein expression is observed in the sperm principal piece in Figures **6.10, 6.11 & 6.12 A** & **B** whereas the IF images from Figures **6.10, 6.11 & 6.12 C**, **D** & **E** show negligible staining in the principal piece. Many cells showed staining for most of the length of the principal piece but in some cells a punctate signal was observed as described by (Tamburrino et al., 2014). For different types of CatSper4 expression patterns see Figure **6.14 A** (continuous signal) and **B** (punctate signal).

The percentage of cells expressing CatSper4 varied significantly between the three preparation methods (40/80%, 80% and direct SU) **64%** of SU cells expressed CatSper4, compared to **36%** in DG 80% Percoll pellet and **8%** in DG 40/80% boundary ($p< 0.0001$; Chi-square). (Figure **6.13**)

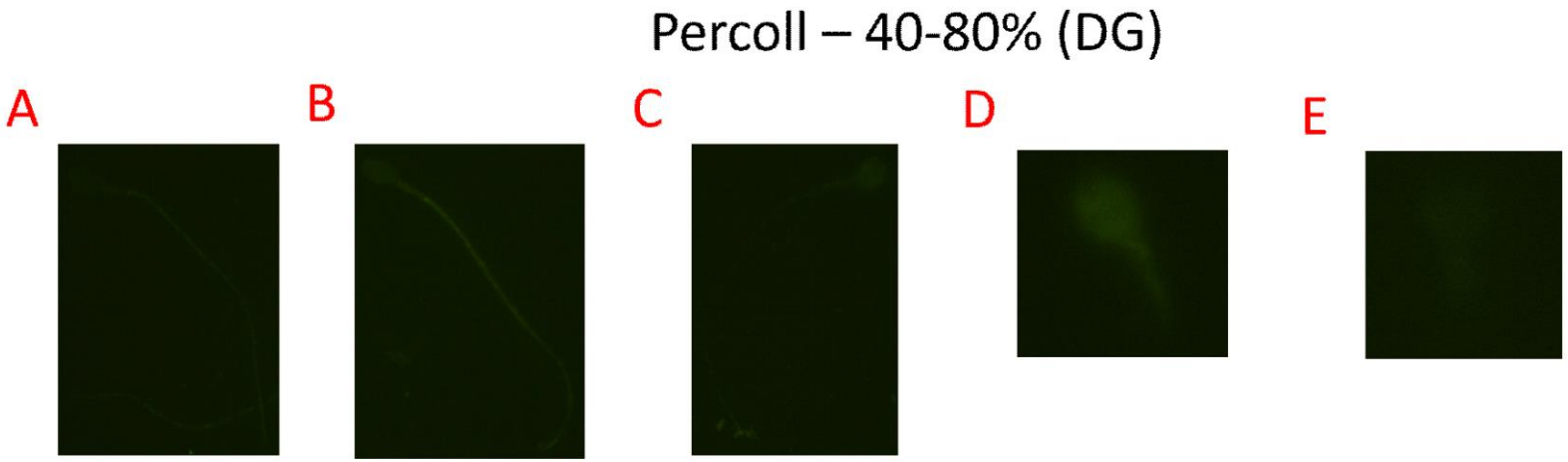


Figure 6.10 Comparison of CatSper 4 protein expression in Human sperm when prepared using density gradient method (DG). Figures A, B, C, D & E show human sperm cells which were collected from a **40/80% boundary** of Percoll gradient and where CatSper 4 expression was evaluated using the Immunofluorescence technique (IF). Figures **A & B** represent human sperm treated with different concentrations of CatSper 4 antibodies (**A** 1:50, **B** 1:100). In both figures **A & B,** Catsper 4 protein was expressed (in the form of a continuous signal) exclusively in the principal piece of human sperm. Figure **C** showing the CatSper 4 signal was knocked off, in the principal piece, when human sperm cells were treated with CatSper peptide (+VE Control). Figure **D** shows the absence of Catsper 4 expression in the presence of rabbit serum (1:100), substituting CatSper 4 antibody, using as a negative control (-VE Control). Figure **E** shows an IF image obtained in the absence of CatSper 4 primary antibodies (1:50 & 1:100), used as a –VE Control.

Key:-
A = Catsper (1:50)
B = Catsper (1:100)
C = Peptide (1:50)
D = Rabbit Serum (1:100)
E = Normal Goat Serum

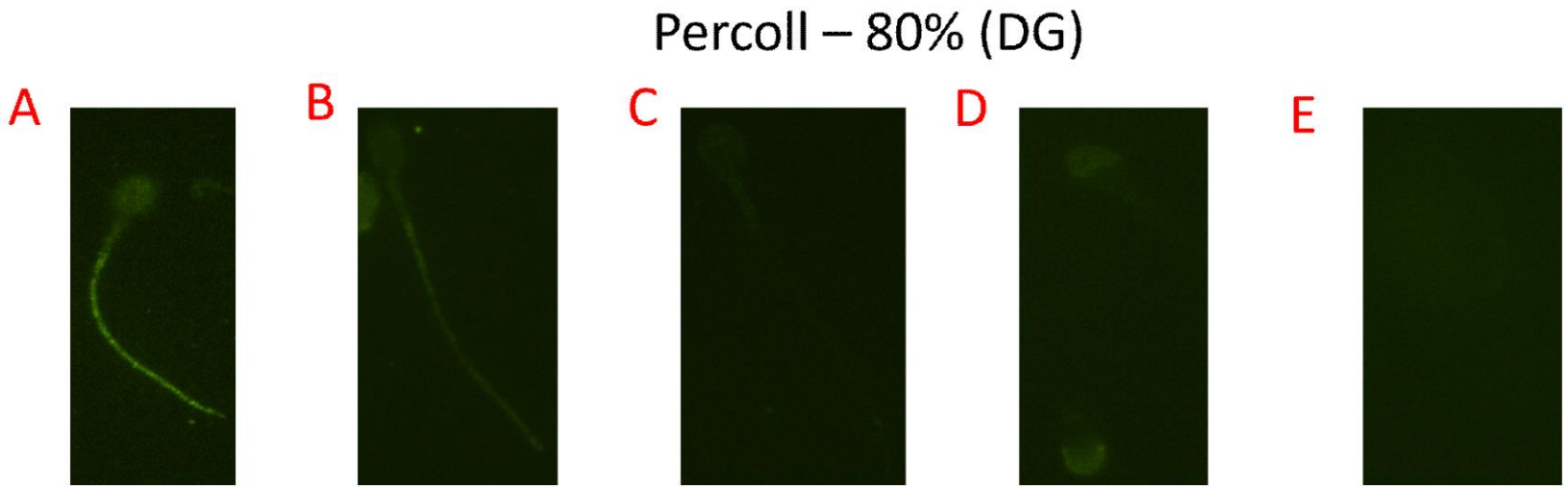


Figure 6.11 Comparison of Catsper 4 protein expression in human sperm when prepared using density gradient method (DG). Figure A, B, C, D & E showing human sperm cells were collected from a **80% Percoll pellet** and Catsper 4 expression was evaluated using Immunofluorescence technique (IF). Figure **A & B** represents human sperm treated with different concentrations of Catsper 4 antibodies (**A** 1:50, **B** 1:100). In both figures **A & B** Catsper 4 protein was expressed (In the form of continuous signal) exclusively in the principal piece of human sperm. Figure **C** showing the Catsper 4 signal was knocked off in the principal piece when human sperm cells were treated with Catsper peptide (+VE Control). Figure **D** showing the absence of flagellar staining in the presence of rabbit serum (1:100) substituting Catsper 4 antibody, using as a negative control (-VE Control). Figure **E** showing IF image in the absence of Catsper 4 primary antibodies (1:50 & 1:100) used as a – VE Control.

Key:-
A = Catsper (1:50)
B = Catsper (1:100)
C = Peptide (1:50)
D = Rabbit Serum (1:100)
E = Normal Goat Serum

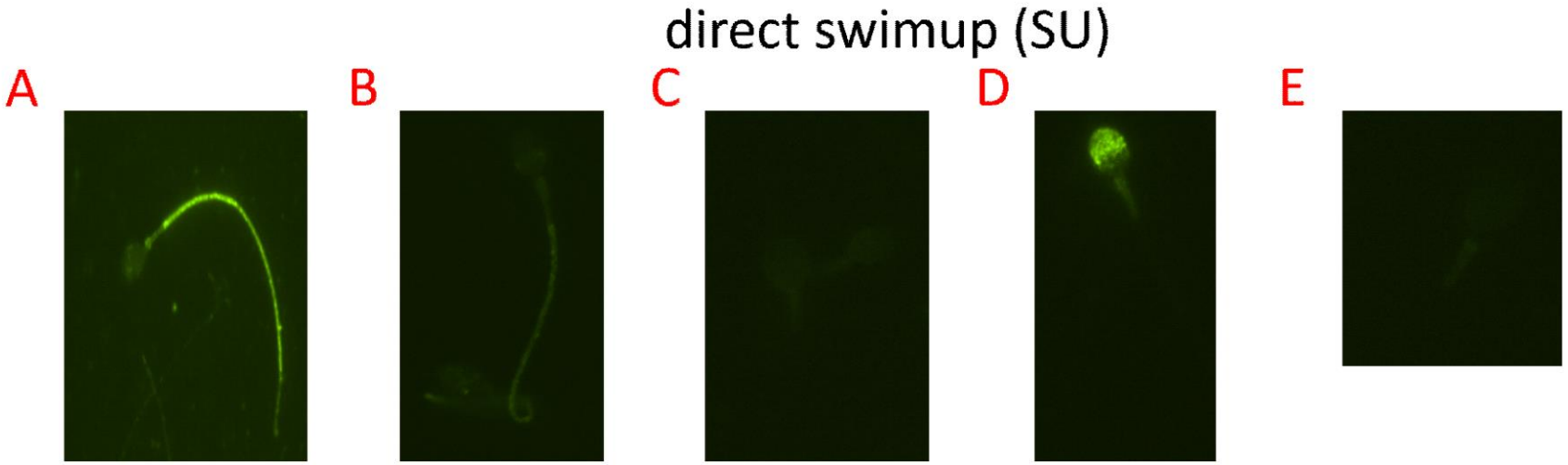


Figure 6.12 Comparison of Catsper 4 protein expression in human sperm when prepared using direct swimup method (SU). Figures A, B, C, D & E show human sperm cells which were allowed to swimup from semen to saline (sEBSS) where CatSper 4 expression was evaluated using the Immunofluorescence technique (IF). Figure **A & B** represents human sperm treated with different concentrations of CatSper 4 antibodies (**A** 1:50, **B** 1:100). In both figures **A & B** CatSper 4 protein was expressed (In the form of continuous signal) exclusively in the principal piece of human sperm. Figure **C** shows the CatSper 4 signal was knocked off in the principal piece when human sperm cells were treated with CatSper peptide (+VE Control). Figure **D** shows the absence of flagellar staining in the presence of rabbit serum (1:100) substituting Catsper 4 antibody, used as a negative control (-VE Control). Figure **E** shows an auto fluorescence signal in mid-piece and the absence of a fluorescence signal in the principal piece of human sperm (absence of Catsper 4 primary antibodies (1:50 & 1:100)) used as a –VE Control.

Key:-
A = Catsper (1:50)
B = Catsper (1:100)
C = Peptide (1:50)
D = Rabbit Serum (1:100)
E = Normal Goat Serum

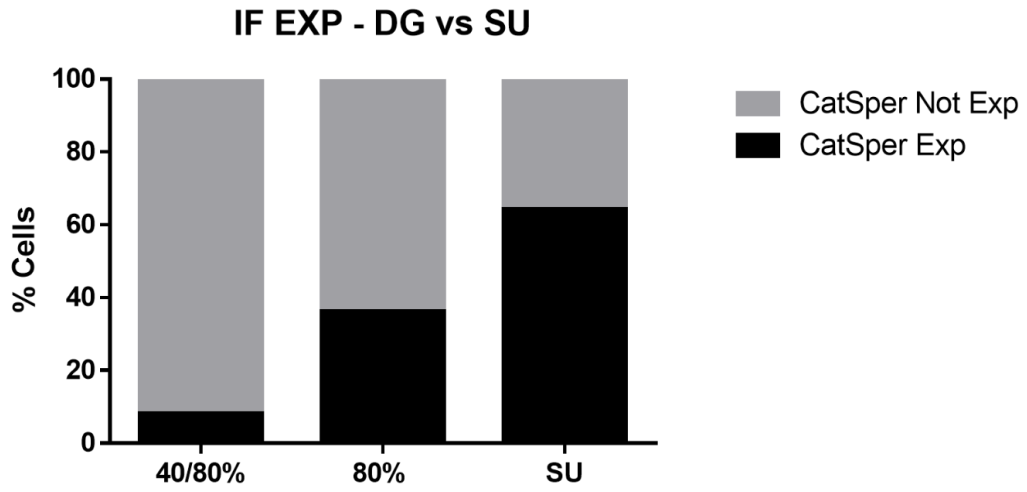


Figure 6.13 Shows the percentage of cells expressing CatSper 4 in all three different groups (40/80%, 80% and direct SU). SU cells show better expression of the CatSper 4 protein **(64%)** as compared to DG (80% Percoll pellet), **36%** & DG (40/80% boundary), **8%,** - zones of Percoll gradient. About **92%** of cells prepared using DG (40/80%) don't express the CatSper 4 protein, as compared to only **36%** of SU cells. The **black bar** shows the % of sperm cells from DG (40/80% & 80%) & SU **expressing** the CatSper 4 protein. The **grey bar** shows the % of cells **not expressing** CatSper 4 in all three different sperm populations. Mean (± SEM) shows both CatSper 4 expressed or not expressed sperm cells when prepared using DG (40/80% & 80%) & SU techniques. The Chi-squared (χ^2) test statistic is 68.06 ($\chi^2 = 68.06$) with an associated probability ($p < 0.0001$).

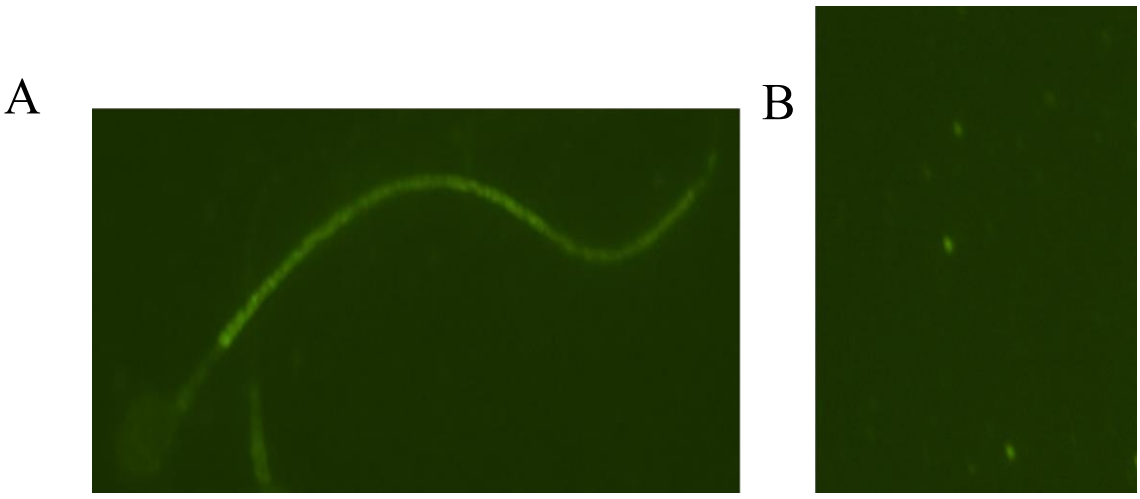


Figure 6.14 Different patterns of Catsper 4 protein expression in human sperm. The CatSper 4 protein is expressed in the principal piece of human sperm either as **(A)** a continuous signal (not observed at the end piece) or as **(B)** a punctuated signal.

6.5 Discussion

6.5.1 Intracellular calcium response – direct SU and DG

From my earlier research work, I have observed that different preparation techniques affect characteristics of sperm motility (Chapter 3), though there is no detectable difference in tyrosine phosphorylation (a marker of capacitation) between direct SU and DG cells (Chapter 5). Previous studies have shown the role of calcium in regulation of human sperm motility and it is therefore of great interest to evaluate intracellular $[Ca^{2+}]_i$ signalling in direct SU and DG cells and use these results to understand better the functional differences (motility and penetration) observed between cells prepared using different sperm preparation techniques (Chapter 3).

In this chapter, I used imaging of direct SU and DG cells (prepared from the same ejaculate) to compare $[Ca^{2+}]_i$ responses induced by progesterone (P4), which activates CatSper channel-mediated calcium influx in human sperm (Lishko et al., 2011). The results presented here (Figure 6.7) show a difference in $[Ca^{2+}]_i$ responses (**T** & **S**) between direct SU and DG cells. In direct SU cells the amplitude of transient (T) $[Ca^{2+}]_i$ response was ~75% (Figure 6.8) as compared to the ~51% in DG cells. In the case of sustained calcium response (S), in direct SU cells show the amplitude was ~45%, significantly greater than the mean of ~12% observed in DG cells.

6.5.2 Evaluation of CatSper protein expression using IF - direct SU and DG

Having observed functional differences between direct SU and DG cells (Chapter 3) and differences observed in characteristics of $[Ca^{2+}]_i$ signalling (fig 6.7) it is important to validate whether these differences have any correlation with the way CatSper channel is being expressed in DG and SU cells. To investigate differences in CatSper expression between DG

and SU cells, I used the immunofluorescence (IF) to compare expression in SU and DG cells and also cells collected from the 40/80% boundary when preparing using the DG method. SU cells showed significantly higher expression of CatSper (% cells) than DG cells, which in turn showed higher expression than 40/80% boundary cells ($P<0.0001$). The fact that DG cells collected from 40/80% boundary show very low CatSper expression may underlie the poor levels of motility seen in these cells.

6.5.3 Potential functional significance

Greater expression of CatSper protein in SU cells (fig.6.13), is consistent with the occurrence of larger and more prolonged intracellular calcium responses – fig **6.7**, better forward progressive motility (as evaluated by CASA in Chapter 3). Furthermore, it has previously been shown that performance in the Kremer (viscous medium penetration) test is inhibited by CatSper blockade (Alasmari et al, 2013) which may explain the observation that SU cells perform better than DG cells in this test (Chapter 3).

General Discussion

Human sperm uses different sets of behaviour to negotiate various aspects of its transit through the female reproductive tract. During this transit, it will encounter different environments (viscous and viscoelastic). To reach the oocyte and be able to fertilise, the sperm must select and switch to appropriate behaviour that will enable successful migration through distinct environments, detach from the oviductal epithelium, penetrate through the cumulus matrix and zona pellucida and, finally, fuse with the oocyte.

In order to successfully complete migration through distinct environments, sperm must undergo capacitation (maturation), be functionally active, not lose its fertilising potential, and must be separated from the seminal plasma as soon as possible in order to avoid the effect of decapacitation factors (DF). Too much exposure to DF could adversely affect sperm function, which potentially decreases its fertilising capacity (Rogers et al., 1983). Therefore, there is a need to use sperm separation techniques that can separate sperm from the seminal plasma and avoid prolonged exposure to DF.

In a clinical setting, density gradient (DG) centrifugation is often used to separate sperm from the seminal plasma (Arcidiacono et al., 1983, Lessley and Garner, 1983, Dravland and Mortimer, 1985, Mortimer, 2000a) because of DG's ability to provide good yield, a clean fraction of highly motile cells, to separate sperm's motile fraction from immature sperm, to separate other debris (e.g. white blood cells) from semen, and to reduce the activity of reactive oxygen species (ROS) (Henkel and Schill, 2003).

Apart from the DG technique, the Direct swim up (SU) method allows sperm to swim directly from the semen into the prepared medium, separating the highly motile sub-population and excluding ROS-generating cells (Mortimer, 2000a). The disadvantage of the SU technique is that it provides low yield in comparison to DG technique (Henkel and Schill,

2003) and this may reflect the relative inefficiency of the SU technique, or may indicate that the sperm population selected by these two techniques differ in their characteristics.

Therefore, I investigated the effects of these two different preparation techniques on human sperm motility and on the penetration of artificial mucus (methylcellulose as artificial viscous medium). When results were analysed, DG cells showed a significantly higher rate of hyperactivation (HA) when compared to SU cells (Figure 3.1 **A**). As explained previously by Mortimer (Mortimer, 2000c), cells showing curvilinear velocity (VCL) ≥ 50µ m/s, linearity (LIN) < 50%, and amplitude of lateral head displacement (ALH) ≥ 7µm are classified as hyperactivated cells.

When the distribution of the motility characteristics (VCL, ALH and LIN) of the hyperactivated cells were looked at, then VCL increases with decreasing of LIN in both DG and SU cells. The VCL distribution curve of DG cells shift left to that of SU cells, contributing to a significantly higher rate of HA when compared to the SU cells (Figure 3.2 **A**). When in vitro penetration was assessed between DG and SU cells, the SU cells show better penetration into the artificial mucus than the DG cells (Figure 3.3 **B**). These observations show that different sperm preparation techniques effect human sperm's functional abilities. The higher hyperactivated motility observed with DG cells is what contributes to a reduced penetration ability of these cells through viscous (methylcellulose) medium (see Chapter 3).

Further research was carried out to investigate the effects of different sperm behaviours on the penetration of human sperm through artificial viscous (methylcellulose) and viscoelastic (polyacrylamide) media when prepared using DG and SU techniques. To distinguish between different kinds of behaviour (from a hyperactivated behaviour pattern to a linear behaviour pattern) in real time, a computer-assisted sperm analyser (CASA) was used. Different types of sperm behaviour were identified by tracking sperm head motion, which cannot be

measured or observed manually. As explained by Alasmari et al (Alasmari et al., 2013b) behaviours are artificially induced in vitro by mobilising calcium (the primary regulator of sperm motility) from stores or by CatSper channels (calcium-specific channels for sperm cell signalling).

From this available evidence, I used different agonists, like 4-aminopyridine (4AP) – a potent activator of hyperactivated motility (Gu et al., 2004, Bedu-Addo et al., 2008, Costello et al., 2009, Alasmari et al., 2013a) which activates the release of stored calcium from the sperm neck region (Alasmari et al., 2013b). Apart from 4AP, I also used prostaglandin E_1 (PE_1) and progesterone (P4). PE_1 and P4 are used to activate CatSper channels in the flagellum (Alasmari et al., 2013b). These agonists (4AP, PE_1 and P4) are used to raise different behaviours artificially (in vitro).

When the results were analysed, it was observed that 4AP is a potent inducer of HA (Figure 4.1) in both SU and DG methods, but when analysing penetration results, it shows an inhibitory effect in both viscous (Figure 4.8) and viscoelastic (Figure 4.15) environments. When compared to 4AP, both PE_1 and P4 show less HA in the standard CASA data (Figure 4.1), but show enhanced penetration in both viscous and viscoelastic environments, by switching to a type of behaviour known as **less hyperactivated with increase in progressive motility** (Figures 4.8 & 4.15).

Previous research done by Suarez and Dai (Suarez and Dai, 1992) explained that an increase in hyperactivated motility enhanced sperm's penetration through viscous and viscoelastic media (although this experimental work was carried out using a mouse model). But from my work it suggests that CatSper (PE_1 & P4) plays a crucial role in enhancing sperm penetration through viscous and viscoelastic environments, whereas store-mediated hyperactivation (4AP), significantly reduces the ability of sperm to enter viscous or visco-elastic environments (see Chapter 4).

As previously explained, for successful fertilisation, sperm must undergo a maturation process called capacitation within the female reproductive tract (Chang, 1951a, Baldi et al., 2000, Bedu-Addo et al., 2005, Battistone et al., 2013), which regulates sperm motility, zona pellucida binding, the acrosome reaction and a number of other key aspects of sperm function. From this evidence, I have investigated whether the observed motility differences between DG and SU (as explained in Chapter 3) are associated with a difference in sperm maturation (capacitation) and not with the way the sperm cells are prepared (using DG and SU). It has been shown that increased levels of tyrosine phosphorylation (TyrP) is associated with capacitation (Visconti, 2009). I have, therefore, used TyrP as a marker for identifying sperm capacitation in both DG and SU cells.

The results explain that there was no difference in the capacitation process, as assessed by the level of TyrP, between DG and SU sperm. Furthermore, the increase in levels of protein TyrP was similarly time-dependent irrespective of how the cells were prepared. This suggests that neither occurrence nor time-dependence of capacitation varies significantly between SU and DG cells, and that the observed differences in motility (in Chapter 3) are unlikely to reflect differences in capacitation (Chapter 5).

As previously explained human sperm possesses different calcium signalling mechanisms **(**entry of calcium from extracellular space into cytoplasm and the mobilisation of Ca^{2+} from intracellular stores (acrosome and redundant nuclear envelope (RNE)) (Costello et al., 2009, Alasmari et al., 2013b) that regulate sperm motility (Publicover et al., 2007).

Understanding the importance of calcium in the regulation of sperm motility and the observed functional differences observed between DG and SU cells (Chapter 3), I investigated the characteristics of $[Ca^{2+}]_i$ signalling (by studying the activity of CatSper), and evaluated CatSper protein expression using immunofluorescence (IF) technique.

I have used P4, a potent activator of CatSper channels (Lishko et al., 2011, Strunker et al., 2011, Smith et al., 2013, Alasmari et al., 2013b), in order to observe $[Ca^{2+}]_i$ responses in both DG and SU cells. Apart from $[Ca^{2+}]_i$ observations and CatSper protein expression evaluation in SU and DG cells, I have also demonstrated the presence of the CatSper protein in two different patterns (continuous (not observed at the end piece) and punctuated signal) in human sperm.

The results showed that there is a difference in $[Ca^{2+}]_i$ (both transient (T) and sustained (**S**)) responses (Figure 6.8), with SU cells performing better in both $[Ca^{2+}]_i$ T and S responses compared to DG cells. When CatSper channel expression was evaluated (using IF) in relation to the functional differences between SU and DG cells (see Chapter 3), differences were observed in characteristics of $[Ca^{2+}]_i$ signalling (Figure 6.7), and SU cells showed significantly higher expression of CatSper (% cells) than DG cells (see Chapter 6). The fact that DG cells show low CatSper expression correlates with the poor levels of motility of these cells.

Better CatSper expression in SU cells than in DG cells (Figure 6.13) is shown to be consistent with the larger and more prolonged intracellular calcium responses (Figure 6.7), better forward progressive motility (Figure 3.1 **D**) and better performance in the Kremer's penetration test (Figure 3.3). The previous research investigations carried by Alasmari et al. (2013) explained that performance in the Kremer (viscous medium penetration) test is inhibited by CatSper blockade.

Previous studies comparing SU and DG techniques have varied in their outcomes and conclusions, suggesting that exact experimental approach and the assessments used may result in differing outcomes. (Facio et al., 2016) compared the effect of SU and DG sperm preparation on normozoospermic human samples, measuring concentration, motility and morphology. They concluded that DG cells show higher concentration than SU cells but there

was no significant difference in the motility between the SU and DG cells. Moohan and Lindsay (1995) (Moohan and Lindsay, 1995) also reported that DG gave a greater yield of motile sperm but also reported that DG cells showed greater hyperactivation with higher levels of VCL and ALH accompanied by a decrease in the linearity (LIN). In comparison observations from my experiments showed that no significant difference in the VCL and ALH between the two techniques (DG & SU) (Figure 3.1 **B** (ALH) and **C** (VCL)) but agreeing to the fact that there was greater hyperactivation (Figure 3.1 **A**) and lower linearity (Figure 3.1 **F**) in DG cells. The higher levels of hyperactivation in DG cells probably reflect the fact that though the peak of the VCL frequency distribution for DG cells was shifted towards the left side (decrease in VCL) there was an increase in the subpopulation of cells with very high VCL (>150 $\mu m.s^{-1}$; Figure 3.2 **A**). Thus, though DG and SU cells have similar VCL (Figure 3.1 **C**), their behaviour is different and the DG cells have a subpopulation that are classified as hyperactivated.

Although agreeing with the Moohan and Lindsay's observation that DG show a greater yield of motile cells compared to the SU, my research findings looking at CatSper expression between these two techniques showed that DG has a lower proportion of CatSper positive cells compared to SU, suggesting even though DG produces a higher yield the cells were of poorer quality. Also, in contrast to Moohan and Lindsay (1995), who reported higher progressive velocity in DG cells at 6 hrs of incubation, I observed significantly higher progressive velocity (VSL) in SU cells ($p<0.05$), although this observation was recorded at a different time interval (4hr 30 min - immediately after completion of capacitation) (Figure 3.1 **D**). I also observed a better functional characteristics (as assessed by the Kremer penetration test in both the visco and visco-elastic media) in SU cells.

The data reported here show that Ca^{2+} signals and CatSper expression are greater in SU than DG cells. Similarly, recent reports have shown that direct swim up (SU) selected cells had

larger P4 induced $[Ca^{2+}]_i$ responses compared to the pure sperm (a density gradient method) (Tamburrino et al., 2014, Luconi et al., 2004). Agreeing with this observation my work also shown that SU selected cells has a better P4 induced $[Ca^{2+}]_i$ responses compared to the density gradient (DG) method, although in my work I have not used pure sperm, instead used percoll as a gradient. CatSper expression was also higher in SU cells in normozoospermic cells compared to the asthenozoospermic samples and was positively correlated with progressive motility (Tamburrino et al., 2014, Luconi et al., 2004). Although in my work I have not compared normozoospermic vs asthenozoospermic, the subjects I have used in my work are of having normal motility (normozoospermic) and the CatSper expression was higher in SU prep cells.

Research investigations by (Morales et al., 1991) were performed in looking at the functional differences (motility and acrosome reaction) and sperm yield between DG and SU cells. Results shown that DG cells show a significantly ($p<0.001$) higher sperm yield compare to the SU cells, this observation agree with my work as DG cells show higher sperm yield compare to the SU cells, apart from the sperm yield there is no significant difference ($p>0.05$) in the percentage of motile cells which is contrast to my work where I see a significant difference ($p<0.05$) in the percentage of motile cells between these two sperm preparation methods (DG & SU).

Other research investigations performed in looking at the differences between these two techniques (DG & SU) in humans show, DG has a better yield (McClure et al., 1989, Ng et al., 1992, Mortimer, 1994, Chen et al., 1995, Shalika et al., 1995, Facio et al., 2016) and better recovery of progressive motile cells (Ng et al., 1992, Shalika et al., 1995, Chen et al., 1995) compare to SU, while SU cells show better progressive motility (Chen et al., 1995) and velocity (Ng et al., 1992), better percentage with normal morphology (Ng et al., 1992, Brandeis and Manuel, 1993) better acrosome intact sperm (Ng et al., 1992). Although I have

not looked at morphology and acrosome intact sperm, I agree with the observation that DG cells has a better yield compare to SU and SU cells show better progressive motility. Although I don't agree with the observation that DG has a better recovery of progressive motile cells, from my work when the penetration (*in vitro*) results were looked at clearly SU cells progress better with more progressive cells observed at 2cm compare to DG and these differences are statistically significant ($p<0.05$).

Clinical Implications

Successful performance of assisted reproductive technologies (ART – In vitro fertilization (IVF), Intrauterine insemination (IUI)) for the patients having infertility problems, requires effective sperm selection procedures. Use of seminal fluid directly for the ART procedures can result in the poor fertilization rates. Apart from the sperm, seminal plasma contains other components such as leukocytes and other cell debris. It was reported that these components present in the seminal plasma produce reactive oxygen species (ROS) that can decrease the fertilizing potential of the normal sperm (Aitken and Clarkson, 1988) and therefore sperm separation procedures are essential. Density gradient (DG) centrifugation and swim up (SU) are the preferred technique for the successful separation of sperm from the seminal plasma prior to ART procedures.

The main factors that must be considered when addressing male infertility is the quality of the sperm that are obtained from the seminal plasma. These cells must be able to penetrate the visco/visco-elastic environments that are encountered in the cervix and the uterus (*in vivo*), bind to zona, undergo the acrosome reaction and fuse with the oocyte. These characteristics will depend on a large number of aspects of the cell's structure and physiology (such as express of ion-channels that are involved in the fertilisation). The analysis of these two different sperm separation procedures (DG and SU) reported here confirms previous reports that the DG technique provides a higher yield of cells than SU. Also previous studies

reported that morphology of SU cells is poorer (Englert et al., 1992, Facio et al., 2016), although this indirect SU technique does involve a centrifugation step. However, this may not be sufficient to conclude that DG is the more suitable technique to use in preparation for ART. Is yield more important than the quality? Although SU isolates fewer cells, my observations, suggest they are of a better quality (progressive motility) and are functionally better (perform better in the Kremer's penetration test (*in vitro*) and have higher levels of CatSper expression and intracellular calcium signalling. Loss or abnormities in CatSper channel function can lead to fertilisation failure at IVF suggesting it's importance in fertilisation success (Williams et al., 2015). Thus SU arguable isolates cells that are physiologically (and therefore functionally) more competent.

Recent study has shown higher levels of denature DNA in sperm with large nuclear vacuolization and are highly subject to DNA damage (Franco et al., 2008, Garolla et al., 2008, Hazout et al., 2006, Monqaut et al., 2011). These DNA abnormalities in sperm affect blastocysts development and are responsible for early abortion (Berkovitz et al., 2005, Berkovitz et al., 2006, Jones et al., 1998, Monqaut et al., 2011). Therefore, for successful ART sperm with low DNA damage and low nuclear vacuolization is very important. Both the DG and SU techniques show low sperm vacuolization and DNA damage compared to whole semen but compared to DG technique, SU is more effective for producing the samples with lower vacuolization and less DNA fragmentation and therefore produce high quality cells. (Monqaut et al., 2011). Similarly, both DG and SU sperm show far fewer necrotic and apoptotic sperm than whole semen but the percentage of viable cells is significantly higher in SU compare to DG (Ricci et al., 2009, Monqaut et al., 2011). Thus other assays confirm that the relatively lower yield of SU is enriched in high quality, competent cell.

Currently DG is widely used for sperm preparation in andrology labs. It is clinically useful with patients having asthenozoospermic (reduced sperm motility) and oligozoospermia (low

sperm counts) conditions but it is almost certainly the high yield factor that drives clinicians to use DG over SU. Optimisation of SU may help to overcome this, for instance by using a 45° tube angle to increasing the semen-medium interface area. Also in certain instances using SU and DG techniques in combination may be beneficial. In the mouse a combination of DG and SU is effective in eliminating DNA damage sperm, although this swim up technique has a centrifugation in it (Ghumman et al., 2011). In my view a combination of these two techniques with no centrifugation step in swim up may be a better approach in getting higher yield with high quality cells that can be used for the ART procedures. However, this suggestion must be taken with caution as they (DG/SU in combination) are not yet validated in the *in vitro* conditions.

Apart from the humans, research investigations on the **bull sperm** was performed to examine the ability of DG (Bovipure (gradient) instead of Percoll) and SU techniques on the sperm separation. Different factors such as motility, concentration, membrane activity, membrane integrity and embryo yield (Samardzija et al., 2006) were evaluated. When the bull sperm progressive motility was analysed there is a significant difference ($p<0.05$) in the progressive motility between DG and SU methods, with DG cells (Bovipure gradient) showing better progressive motility. Contrast to this what I observe from the human samples is sperm cells prepared using the SU method (with no centrifugation step involved) show better progressive motility (straight after 4hr 30min of capacitation) to that of human sperm prepared using the density gradient (percoll) method. The SU method that was used in separating the bull sperm has a centrifugation (two times) step involved in it and therefore this inclusion of centrifugation might have resulted in the low progressive motility that is observed in the SU cells. When the concentration factor was looked at between the raw sample and the final prep they was a significant difference ($p<0.05$) but there was no significant difference ($p>0.05$) observed in the concentration between these two techniques which is contrast to my results.

Functional integrity was evaluated using the HOS test and it revealed that DG technique has a higher number of active sperms (the cells swell (tails up)) compare to the SU method. When acrosome status was looked at DG cells shown a high percentage of acrosome intact sperm compare to SU cells when evaluated using EthD/FITC-PSA test. When looking for the membrane activity more live cells were observed in the DG cells than SU cells when evaluated using SYBR-14/PI test. It was also observed that the bovine embryo development was appeared to be better in the DG (Bovipure method) compare to the SU cells. Both the cleavage (day2) and blastocysts (day 7) was significantly ($p<0.05$) higher in DG Bovipure gradient compare to SU cells but there is no significant ($p>0.05$) in the number of hatched blastocysts (day 9). Overall the results suggest that DG is a better technique in separating the bull sperm compare to SU.

It is of great interest to know whether low functional ability that is observed in the DG cells is because of the effect of DG technique on the sperm DNA. Research investigation was carried out in the **mouse sperm** to see which sperm separation technique (DG, SU or combination of both (DG/SU)) is capable of eliminating DNA damage (Sperm DNA damage effects fertility/reproductive potential and pregnancy (humans)) (Tarozzi et al., 2007, Lewis and Aitken, 2005). In mouse the sperm cells were extracted from the cauda epididymis and the obtained sperm suspension was used to study the DNA integrity (before the use of sperm separation techniques - control) and the remaining sperm suspension was used to look at DNA integrity after the sperm preparation methods were applied. When the results were looked between the sperm prepared using the single wash technique and the unprocessed one they was no significant ($p>0.05$) difference in the level of DNA damage. When the effect of sperm cells prepared using swim up technique (Pellet (centrifugation step is involved) and the fraction (no centrifugation step is involved)) on the DNA damage was analysed there was no significant difference ($p>0.05$) was observed. In the case of DG the sperm DNA damage

observed in the 40% gradient is significantly differing to that of unprocessed (control) ones. With significant number of DNA damaged mouse sperm cells retained at gradient 40 suggests that DG technique might induce nuclear anomalies in the mouse sperm (Mortimer, 1991, Zini et al., 1999, Ghumman et al., 2011) or the technique is effective in retaining the DNA damage sperms. When both the techniques were combined (DG/SU) the sperm DNA damage was significantly ($p<0.05$) reduced, although this has a low sperm yield. It is also explained that the mean percentage of motile sperm observed in both SU and DG was significantly higher than the combined technique (DG/SU). Although I have not worked looking at the sperm DNA integrity, when looking at the motility between these two techniques swim up do better compare to the DG but the yield of motile cells in SU is lower than DG (Although this conclusion needs to be taken with caution as this observation is from the human sperm cells but not from the mouse sperm cells).

Research investigations were carried out to evaluate the effects of swim up on **boar sperm** motility (Holt et al., 2010). Different motility characteristics of the boar sperm were looked at and the obtained data was pooled into three different groups based on the type of motility behaviour that sperm show. In the group 1 the boar sperm shown a moderate VCL with low VSL and LIN and this group represents moderately active sperm. In the group 2 the sperm show High VCL (not extremely high), exceptionally high VSL and high LIN and this group was explained as highly active and progressive.

Comparing with my results I agree with this observation as high VSL and high LIN is responsible for the progressive motility and these cells prepared by using the SU shown better CatSper expression and better performance in the *in-vitro* (kremer's) penetration test suggesting they are functionally active which also agrees with the observation that boar sperm cells (prepared using SU) are active. When looked at another group (group 3) the boar sperm cells shown moderate VCL, with very low VSL and low LIN and this group

represented as sperm showing non-progressive behaviour. In my view, although I agree that low VSL and low LIN contribute to the non-progressive behaviour, from my observation (looking at my results) I do not agree from the VCL observations as my results shown in the case of non-progressive behaviour (when treated with 4-AP) the VCL will not be moderate but it will be very high and it is very high VCL with low VSL and LIN that contributes to the non-progressive behaviour, although this work (my results) is carried out in the humans not in the boar.

Overall I conclude that although SU gives less yield, it has high number of high quality (motility) cells, better functional activity (compare to the DG) - as observed in the kremer's penetration test in both visco/visco-elastic medium, better CatSper expression and high transient and sustain intracellular calcium responses to the progesterone when compare to the DG technique. As previously explained significant loss of this principle calcium channel (CatSper) effects the fertilising potential in the human sperm suggest the importance of this (CatSper) calcium channel and with the observation (from my results) that SU cells do have a better functional ability in comparison to DG cells because they have better CatSper expression explaining the clinical significance of this (CatSper) channel in using it as a potential target for the development of new contraceptives and for treating male patients with infertility problems. With direct swim up (SU) prep cells performing better in all aspects (progressive motility, better functional ability, higher intracellular calcium levels and better CatSper expression) compare to the density gradient (DG), explaining SU as a better sperm preparation technique and the cells obtained from this technique may be used in the ART procedures to get better fertilization rates. Although this conclusion must be accepted with a great caution and that there is a need of further research to be carried out in comparing the effect of the different sperm preparation techniques (DG and SU) on the fertilisation rates in a wider sample study.

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APPENDIX

Appendix I – List of suppliers

Appendix II – Media, Gel and Buffer Preparation

Appendix III – SDS PAGE

Appendix I - List of Suppliers

Alpha Laboratories
40 Parham Drive
Eastleigh
Hampshire
UK

BD Biosciences
Edmund Halley Road
Oxford Science Park
Oxford
UK

Cairn Research Ltd
Graveney Road
Faversham
Kent
UK

Calbiochem
Distributed by Merck Biosciences,
Beeston,
Nottingham,
UK

CM Scientific Ltd
1 Ryefield Court
Rye field way
Silsden
UK

Fisher Scientific UK Ltd
Bishop Meadow Road
Loughborough
UK

Hamilton Thorne, Inc.
100 Cummings Centre
Beverly
MA
USA

Invitrogen Life Technologies Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley
UK

LI-COR Biosciences Ltd
St. John's Innovation Centre
Cowley Road
Cambridge
CB4 0WS
UK

Merck Millipore
Building 6
Croxley Green Business Park
Watford
UK

Nikon Instruments UK
380 Richmond Road
Kingston Upon Thames
Surrey
UK

Sigma- Aldrich Company Ltd
The Old Brickyard
New Road
Gillingham
Dorset
UK

Scientific Laboratory Supplies
Wilford Industrial Estate
Ruddington Lane
Wilford
Nottingham
UK

Starlabs UK Ltd
4 Tannors Drive
Blakelands
Milton Keynes
UK

Stratech Scientific Limited
Oaks Drive
Newmarket
Suffolk
UK

United States Biological
PO Box 261
Swampscott
MA 01907
USA

Warner Instruments from Harvard Apparatus
Firecroft Way
Edenbridge
Kent
UK

Appendix II – Media, Gel and Buffer Preparation

Showing different chemicals involved in preparing supplemented **Earle's Balanced Salt Solution (sEBSS)**

Chemical	Formula	M.W	mM	g/l
Sodium Phosphate Monobasic	NaH_2PO_4	119.98	1.0167	0.1219
Potassium Chloride	KCl	74.55	5.4	0.4025
Magnesium Sulphate Heptahydrate	$MgSO_4.7H_2O$	246.48	0.811	0.1998
Glucose	$C_6H_{12}O_6$	180.16	5.5	1.0
Sodium Pyruvate	$C_3H_3NaO_3$	110	2.5	0.275
Sodium Lactate-LD	$C_3H_5NaO_3$.	112.1	19.0	2.1299
Calcium Chloride	$CaCl_2.2H_2O$	147	1.8	0.2646
Sodium Bicarbonate	$NaHCO_3$	84.01	25	2.100
HEPEs	$C_8H_{18}N_2O_4S$	238.31	15	3.574

sEBSS pH was adjusted to 7.4 and then approximately 5g (118.4mM) NaCl (MW 58.44) was added to achieve osmolarity of 292 mOSM. Media was sterilised and filtered into 100ml aliquots before storing at 4^{o}C for future use.

Media was supplemented with 0.3% (w/v) fatty acid free BSA immediately before use.

Showing different chemicals involved in preparing **M - Medium**

Chemical	Formula	M.W	mM	g/l
Potassium Chloride	KCl	74.55	2.5	0.1863
Glucose	$C_6H_{12}O_6$	180.16	10	1.801
HEPEs	$C_8H_{18}N_2O_4S$	238.31	15	3.574

M-Medium pH was adjusted to 7.4 and then approximately 8g (137mM) NaCl (MW 58.44) was added to achieve osmolarity of 292 mOSM. Media was sterilised and filtered into 100ml aliquots before storing at 4^{o}C for future use. M-medium was used to dissolve 40% and 80% Percoll gradients.

Showing different chemicals involved in preparing **Non Capacitating Medium (NCM)**

Chemical	Formula	M.W	mM	g/l
Sodium Phosphate Monobasic	NaH_2PO_4	119.98	1.0167	0.1219
Potassium Chloride	KCl	74.55	5.4	0.4025
Magnesium Sulphate Heptahydrate	$MgSO_4.7H_2O$	246.48	0.811	0.1998
Glucose	$C_6H_{12}O_6$	180.16	5.5	1.0
Sodium Pyruvate	$C_3H_3NaO_3$	110	2.5	0.275
Sodium Lactate-LD	$C_3H_5NaO_3$.	112.1	19.0	2.1299
Calcium Chloride	$CaCl_2.2H_2O$	147	1.8	0.2646
HEPEs	$C_8H_{18}N_2O_4S$	238.31	15	3.574

NCM pH was adjusted to 7.4 and then approximately 8.3g (143.4mM) NaCl (MW 58.44) was added to achieve osmolarity of 292 mOSM. Media was sterilised and filtered into 100ml aliquots before storing at 4^{o}C for future use. **Media was not supplemented with Sodium Bi carbonate ($NaHCO_3$) and BSA.**

Showing different ingrdients involved in preparing **10% gel** (For 3 gels)

Resolving/Running Gel

Chemical	For 3 gels
Polyacrylamide Bis	12.5ml
Tris 2M pH 8.8	7ml
H_2O	17.75ml
20% SDS	1.875ml
TEMED	0.5ml
10% Ammonium per sulphate (APS)	0.375ml
Tot Volume	40ml

Stacking Gel

Chemical	For 3 gels
Polyacrylamide Bis	2.5ml
Tris 0.5M pH 6.8	6.25ml
H_2O	16.25ml
20% SDS	1.25ml
TEMED	0.375ml
10% Ammonium per sulphate (APS)	1.25ml
Tot Volume	~28ml

MOPS Running Buffer (20X MOPS)

Chemical	Mm
MOPS	50mM
Tris Base	50mM
EDTA	1mM
0.1% SDS	

Prepared in 400ml distilled water and pH = 7.7

For 1X MOPS Prep

25ml of 20X MOPS were added to 500ml H_2O

Transfer Buffer (Western's)

TB Ingredients	g/lit
Tris Base	3.0285
Glycine	14.41344
20% Methanol (pH 8.3)	200ml
Water	500ml

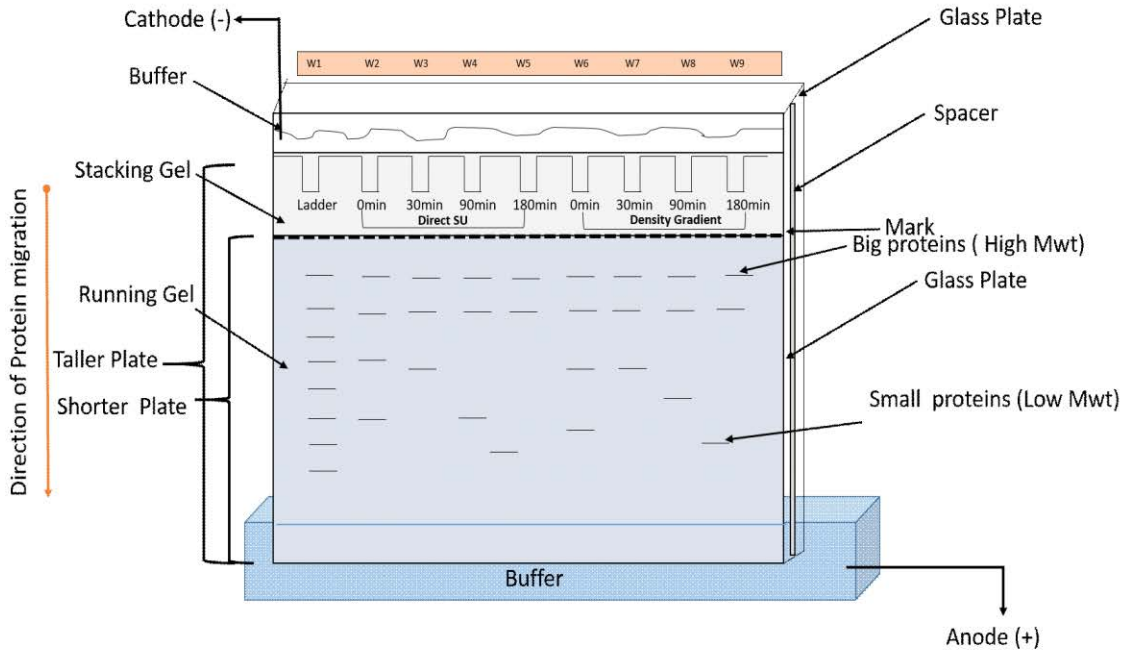
Adjust Vol to 1lit

Appendix III – SDS PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) involves a gel cassette assembly containing two glass plates (one shorter and one taller plate). The two square shaped glass plates are held together by inserting them into the green casting frame and then clamped. After this step, the green casting frame was then placed into the casting stand. Water was added on top of glass plates to make sure there were no leaks in the assembly, and water was then removed by filter paper.

The running gel was prepared (for running gel preparation, please see Appendix II) and immediately (after adding Tetramethylethylenediamine (TEMED) poured (up to the centre level) to cast the gel. The gel was then left for 15min (during this time the running gel solution gets polymerised) and then rest of the plate was filled with stacking gel. (For stacking gel preparation, please see Appendix II) The comb was then placed and left for 10-15min to solidify. After solidification, the casting frame was then placed in the cassette.

Then the whole assembly was then placed in a tank and the inner chamber filled with 3-Morpholinopropane-1-sulfonic acid (MOPS) running buffer. (For buffer preparation, please see Appendix II) The comb was then removed carefully without damaging the wells. Next, 4µl of blue plus2 pre-stained standard was added in first well (Blue Plus2 Pre-stained was purchased from Invitrogen Life Technologies Ltd, Cat no. LC5925) and 5µl of the sample (prepared from direct SU and DG) was loaded from well 2 (W2) to well 9 (W9) (Please see figure below). Electrophoresis was initiated at 200V for 50min.



SDS-PAGE assembly and protein separation. The figure shows the components in the SDS PAGE assembly and electrophoretic protein separation in a polyacrylamide gel. Gel assembly contains one shorter and one taller glass plate. Both glass plates were clamped together by using a casting frame. Both sides of the assembly unit was separated by using a spacer. For gel preparation, initially running gel was added up to the mark (shown in the figure) without having any bubbles and then left it to polymerize. After this step, stacking gel was added and then combs were placed. During this step the gel gets solidified, and then the whole plate is inserted into the cassette. After this, the cassette was placed in the tank and the inner section was filled with running buffer. The combs were removed and samples prepared at different time intervals (0, 30, 90 & 180min) using different techniques (direct SU and DG) were loaded in each well (W2 to W9) along with the ladder. Just enough buffer was added to the tank to submerge the bottom of the gel. Electrophoresis was initiated at 200V for 50min. During this process, the proteins migrate from top to bottom based on their molecular weight. The farthest migrated protein will have the lowest molecular weight.