UNIVERSITY OF BIRMINGHAM

The Response of Human Spermatozoa to Chemoattractants

By,

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ABSTRACT

The effect of the chemoattractant bourgeonal on $[Ca^{2+}]_i$ and chemotaxis in human sperm was investigated. Burgeonal induced a dose-dependent, slowly-developing tonic elevation in $[Ca^{2+}]_i$. The response was dependent on capacitation. In low-Ca^{2+} or EGTA-buffered saline the response to bourgeonal was inhibited. Pretreating spermatozoa with bis-phenol (20μM) to release stored Ca^{2+} did not alter the response. Thus bourgeonal acts primarily by inducing Ca^{2+} influx. Treatment of sperm with bourgeonal caused an increase in [cAMP].

When cells were pretreted with bourgeonal in low-Ca^{2+} saline, subsequent introduction of Ca^{2+} resulted in a single, large $[Ca^{2+}]_i$ transient in >75% of the cells, indicating that sudden influx of Ca^{2+} caused closure of the bourgeonal-sensitive Ca^{2+}-channel. This negative feedback was not modulated by IBMX (1mM) or dbcAMP (1mM), indicating that cAMP was not involved and that a direct action Ca^{2+} was more likely. Both Ni^{2+} (10μM) and La^{3+} (100μM) inhibited the action of bourgeonal on $[Ca^{2+}]_i$, suggesting a possible role of CNG channels. Exposing sperm to a temporal bourgeonal gradient caused a series of transient $[Ca^{2+}]_i$ elevations in >20% of the cells. A gradient of progesterone (another characterised chemoattractant for human sperm) induced similar Ca^{2+} oscillations (in >20% of the cells), which increased in amplitude and frequency in response to the increasing progesterone concentration.

Human spermatozoa responded chemotactically to a 1nM bourgeonal gradient, Chemotaxis was dependent on capacitation. The response was inhibited in low $[Ca^{2+}]_o$ but was unaltered by TMB-8 (an inhibitor of stored Ca^{2+} store release), thus showing a dependence on Ca^{2+} influx similar to the $[Ca^{2+}]_i$ signal.

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Thank You, for reading this!

For those that make my life a never ending dream,

El tesoro más valioso de la vida es el amor incondicional de la familia,

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LIST OF ABBREVIATIONS

8-bromo cGMP - 8-bromoguanosine-3'-5'-cyclophosphate sodium salt

BSA - Bovine serum albumin

Bourgeonal- 4-t-Butylbenzenepropionaldehyde

3,4CPE- 3-(4'-Carboxyphenyl)-Propionaldehyde Ethyl Ester

Ca^{2+} - Calcium ions

cADPR - Cyclic adenosine diphosphate-ribose

$[Ca^{2+}]_i$ - Intracellular calcium concentration

$[Ca^{2+}]_e$ - Extracellular calcium concentration

$[Ca^{2+}]_o$ - Extracellular calcium concentration

CaM - Calmodulin

cAMP - Cyclic adenosine monophosphate

CASA - Computer-assisted semen analysis

NcF-sEBSS - Ca^{2+}-free or low-Ca2+ supplemented Earle's balanced salt solution

cGMP - Cyclic guanosine monophosphate

CICR - Ca^{2+}-induced Ca2+ release

Cl- - Chloride ions

CNG - Cyclic nucleotide-gated

COC - Cumulus oocyte complex

cAMP -3'-5'-cyclic adenosine monophosphate

cGMP- Cyclic guanosine monophosphate

DAG – Diacylglycerol

(d) cAMP - N^6,2'-O-Dibutyryladenosine-3',5'-cyclic monophosphate

DMSO - Dimethyl sulfoxide

EGTA - Ethylene glycol-bis (_-amino-ethylether)-N,N,N'N'-tetraacetic acid

FAD - Flavin adenine dinucleotide

FMN - Flavin mononucleotide

FS - Fibrous sheath

FSH - Follicle stimulating hormone

GABAA - Gamma-aminobutyric acid type A

GlyR - Glycine receptor/Cl- channel

GnRH - Gonadotropin releasing hormone

GSH - Glutathione

GSNO - S-Nitrosoglutathione

H^+ - Hydrogen ions

hCG - Human chorionic gonadotrophin

Hepes - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFEA - Human Fertilization and Embriology Authority

H_2O_2 - Hydrogen peroxide

HSA- Human Serum Albumin

Hyp - Hyperactive

ICSI - Intracytoplasmic sperm injection

$InsP_3$ - Inositol-1,4,5-triphosphate

$InsP_3R$ - Inositol-1,4,5-triphosphate receptor

IVF - *In vitro* fertilization

K^+-Potassium Ions

LH - Luteinizing hormone

mAC - Membrane-associated adenylyl cyclase

MAPK - Mitogen-activated protein kinases

mPR - Membrane progesterone receptor

MS - Mitochondrial sheath

Na^+ - Sodium ions

NADPH - Nicotinamide adenine dinucleotide phosphate

NO - Nitric oxide

O2•- - Superoxide anion

ODFs - Outer dense fibers

OGB-1AM - Oregon green 488 BAPTA 1-acetoxymethyl

OR17-4 and **OR23** - Olfactory receptors

P- Progesterone

PBS - Phosphate buffered saline

PDL - Poly-D-lysine

PGCs- Primordial germ cells

PGRMC1 - Progesterone membrane receptor component 1

PGRMC2 - Progesterone membrane receptor component 2

PIP2 - Phosphatidylinositol 4,5-biphosphate

PK_A - Protein kinase A

PK_C - Protein kinase C

PK_G - Protein kinase G

PL_C - Phospholipase C

PMCA - Plasma membrane Ca^{2+}-ATPase

R_2 - Coefficient of determination

RNE - Redundant nuclear envelope

ROS - Reactive oxygen species

RyRs - Ryanodine receptors

sAC - Soluble adenylyl cyclase

sEBSS - Supplemented Earle's balanced salt solution

SERCA - Sarcoplasmic-endoplasmic Ca^{2+}-ATPase

sGC - Soluble guanylyl cyclase

SNARE - Soluble N-ethylmaleimide-sensitive factor-attachment protein receptor

SOC - Store-operated Ca^{2+} channel

SPCA - Secretory pathway Ca^{2+}-ATPase

tmACs - Transmembrane adenylyl cyclases

TRP - Transient receptor potential

TRPC - Transient receptor potential-canonical

VAP - Average path velocity

VCL - Curvilinear velocity

VOCCs - Voltage-operated Ca^{2+} channels

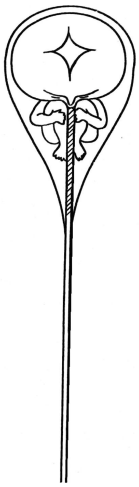
VSL - Straight-line velocity

WHO - World Health Organization

ZP- Zona Pellucida

($^{+}$ ve)-Control-Positive Experimental control

($^{-}$ ve)-Control- Negative Experimental control



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I

CHAPTER ~ONE~

Overcome anything with your everything! Aduén 2009.

Chapter One

Signal Transduction Pathway in Human Spermatozoa

FOREWORD TO CHAPTER ONE

The role of this chapter is to clarify and summarise the recent progress that has been made in understanding the mechanism that control sperm capacitation, AR, sperm motility (specially in chemotaxis) and fertilization.

Emphasis is placed on role of ORs and its role in mammalian sperm chemotaxis in response to cues of chemoattractants and regulatory action of calcium ions (Ca^{2+}) in this process.

1.0 The sperm during mammalian fertilization

The formation of new life by sexual reproduction is dependent on fusion of the haploid gametes. This process normally requires appropriate and often sophisticated communication between mature and competent male and female gametes (Darszon et al., 2005). Fusion of haploid (N) gametes results in the formation of the normal diploid (2N) embryo (Darszon et al., 2005).

The mammalian male gamete (sperm) is a highly polarised cell, which, after biochemical and functional 'maturation' is stored in an inactive state (immotile, unable to fuse with the oocyte) in the cauda epididymis **[Figure.2.]** (Wassarman et. al., 1997). During coitus the sperm are ejaculated as semen, composed of a mixture of spermatozoa suspended in secretions from the testis and epididymis, which are mixed at the time of ejaculation, together with secretions from the prostate seminal vesicles and bulbourethral gland and Cowper's gland (Bar-Chama et al., 1994; WHO, 1999) **[Figure.2.]** The ejaculate is a viscous fluid with a typical volume of 2-6 ml (Griffin et al., 2000) and pH of 7.0-8.3 (WHO, 1999). Semen contains fructose (oxidative substrate for spermatozoa) and prostaglandins, which may be involved in the stimulation of smooth muscle contraction of the female reproductive organs, required for sperm transportation *in vivo* (Berne et al., 2000). In the ejaculate >60% of spermatozoa show forward progressive motility and >60% should have normal morphology (Griffin et al., 2000). The sperm count is normally >20 million/ml with a total sperm per ejaculate of more than 60 million (Griffin et al, 2000). Sperm density below normal values

(20million/ml) can be considered as subfertile; however fertilization is still possible (Ademnan & Cahill, 1989).

Once ejaculated into the female reproductive tract in order to fertilise the egg it must travel a long and difficult journey (from vagina to ovum). With a relative small size (60 µm), sperm must cross the uterus (70-80mm); here they induce a host reaction (Publicover et al., 2007). Through the uterus, leukocytes eliminate normal and abnormal spermatozoa (Publicover et al., 2007). Once passed through the uterus they reach the opening of the fallopian tube (diameter 0.2-0.5mm) and 1 out of 10^5 sperm successfully reach this site (Harper et al., 1982; Williams et al., 1993; Eisenbach et al., 1999; Jaiswal et al., 2002) (Bartram et al., 2003). After this, sperm must travel another 50-80 mm to reach the fertilisation site (Eisenbach et al., 1999; 2004).

During residence in the female tract, in addition to migrating to the right location, the sperm must undergo further physical and biochemical maturation (termed capacitation – Section 1.5) in order to be able to fertilize the oocyte (Darszon et al., 1996, Visconti and Kopf, 1998). Capacitated spermatozoa are capable of acquiring hyperactivated motility. This is necessary both to penetrate viscous fluids such as mucus (enabling sperm to reach the egg) and also to penetrate through the cumulus oophorus and zona pellucida, which surround the oocyte **[Figure.1.]** (Ho and Suarez, 2001), reducing still further the proportion of sperm that may fertilise the oocyte. Within the oviduct, mammalian sperm may locate the oocyte by 'following' a chemical gradient (chemotaxis) generated by the cumulus cells and/or the oocyte (Sun et al., 2005; Eisenbach and Giojalas, 2006) (**Section 1.8 & 1.8.1**). It has been proposed that sperm may also encounter and follow a

temperature gradient (thermotaxis) (Bahat et al., 2003; Eisenbach & Giojalas, 2006). After penetrating the cumulus, sperm bind to the extracellular coat of the oocyte and undergo acrosome reaction. Acrosome reaction and hyperactivated motility enable penetration of the ZP. Once in the perivitelline space, spermatozoa bind and fuse with the oocyte plasma membrane, which activates oocyte mechnisms to prevent polyspermy (fertilization by more than one sperm) (Wassarman *et al.*, 2001).

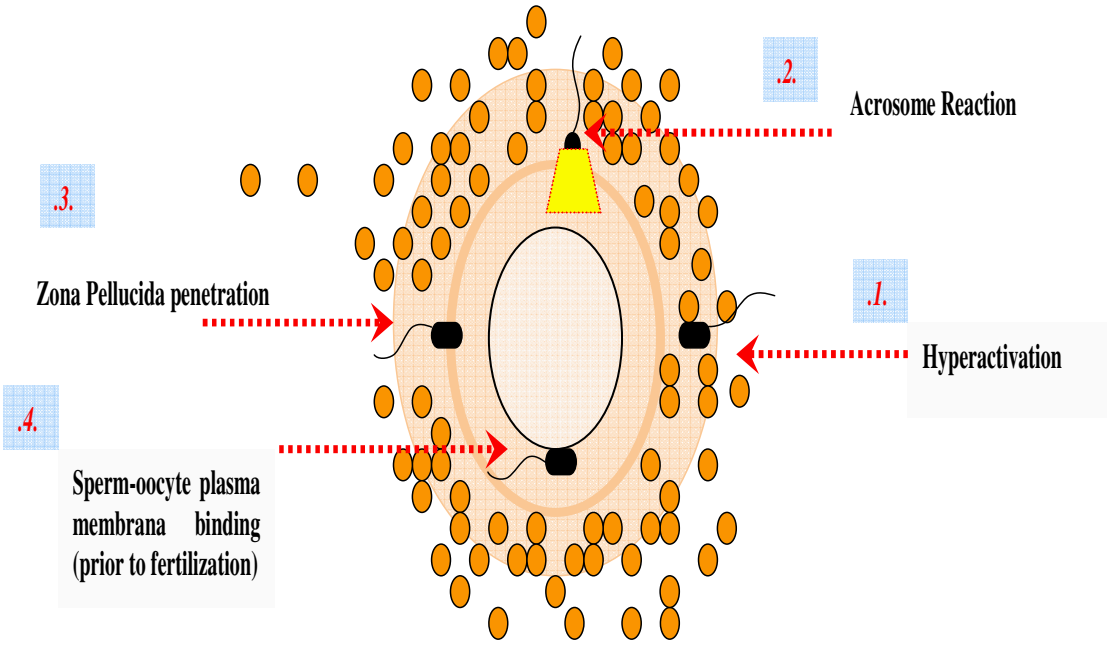

Figure.1. Diagramatic representation of events that led to the formation of a new individual (fertilization).

1.1 The male reproductive system

In humans and in most mammalian species, the male external reproductive organs are the scrotum and the penis (Campbell & Reece, 2002). The male testes are composed of many highly coiled tubes where spermatozoa are formed, the seminiferous tubules, and these are surrounded by several layers of connective tissue (Campbell & Reece, 2002). Within the seminiferous tubule, the Leydig cells are responsible for the production of testosterone and other androgens (Campbell & Reece, 2002) **[Figure.2.].** Once through the seminiferous tubules of a testis, spematozoa reach the coiled tubules of the epididymis (6 m long tubules, in human male), during the sperm transportation through this tubules they become motile. Following this, during ejaculation, spermatozoa travel from the epididymis through the vas deferens (Campbell & Reece, 2002). The pair of vasa deferentia (from each epididymis) run from the scrotum around and the posterior section of the bladder, where these joins a duct from the seminal vesicle, forming a ejaculatory duct (Campbell & Reece, 2002), and openining into the urethra **[Figure.2.].** This tube acts both as excretory system and reproductive system, passing through the penis where it reaches the exterior at the tip of the penis (Campbell and Reece, 2002).

Spermatogenesis is influenced by hormones such as gonadotrophins and testosterone. In addition to hormonal control, spermatogenesis is also influenced by temperature. If the testes reach core body (37˚C) temperature, spermatogenesis is impaired. The temperature of the gonads (the scrotum) is kept at 2 ºC lower than the standard body

temperature, due to the fact that the lie outside the body, moving freely in the scrotal sac, **[Figure.2].**

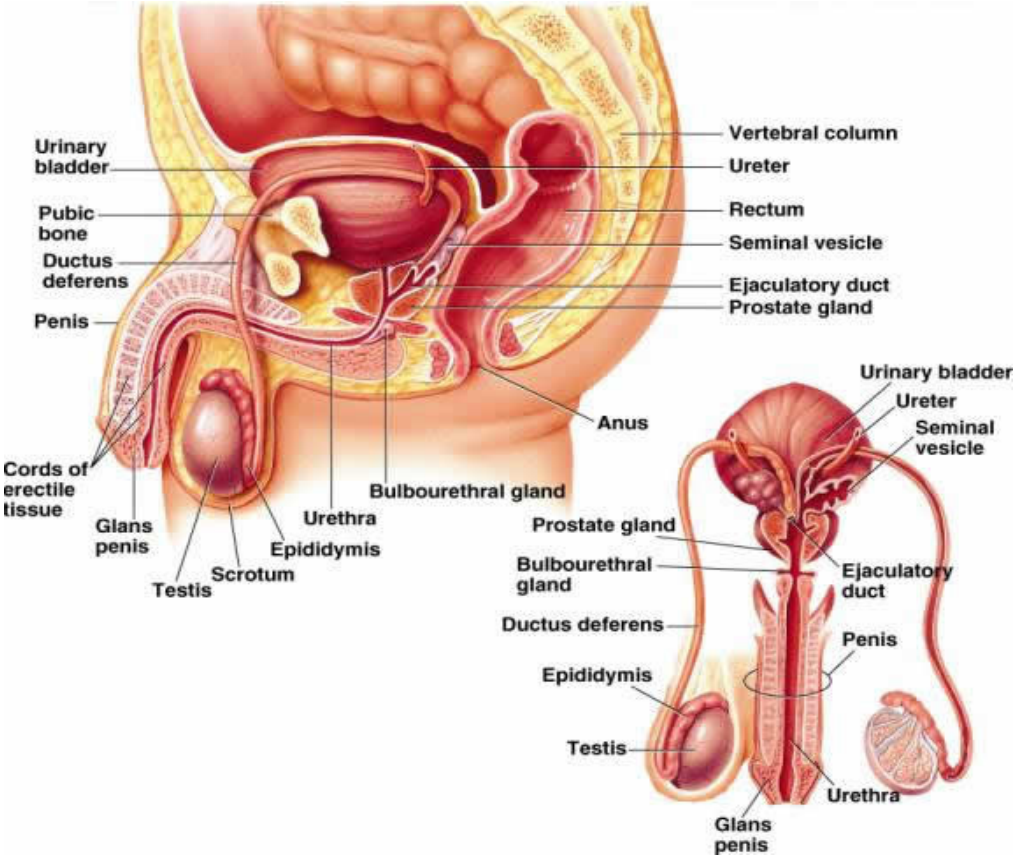

Figure.2. Anatomical structure of male reproductive organ and all the components that form it. Image from www.getceusnow.com.

1.2 Human Spermatogenesis

Spermatogenesis, the production of spermatozoa from male germ cells, is initiated after puberty (Adolf-Friedrich et al., 2003). It consists of mitotic proliferation, meiotic division and extensive cell modelling (Nussey & Whitehead, 2001; Ergün et al., 1994). Meiotic division is regulated differently, and involves different processes in male and female gameteogenesis (Handel & Eppig 1998). Differences in the process and in regulation of gamete production between male and female mammals are summarised in **[Table.1]**

Oogenesis	Spermatogenesis
• Meiosis initiated once in a finite population of cells	• Meiosis initiated continuously in a mitotically dividing stem cell population
• One gamete produced per meiosis	• Four gametes produced per meiosis
• Completion of meiosis delayed for months or years	• Meiosis completed in days or weeks
• Meiosis arrested at first meiotic prophase and reinitiated in a smaller population of cells	• Meiosis and differentiation proceed continuously without cell cycle arrest
• Differentiation of gamete occurs while diploid, in first meiotic prophase	• Differentiation of gamete occurs while haploid, after meiosis ends
• All chromosomes exhibit equivalent transcription and recombination during meiotic prophase	• Sex chromosomes excluded from recombination and transcription during first meiotic prophase

Table.1 Outlined differences between female and male production of gametes. Table from: Handel & Eppig 1998.

Spermatogenesis takes place within the germinal epithelium of the seminiferous tubules. When primordial germ cells (PGCs) (Handel & Eppig 1998), reach the genital ridge of the male embryo, they are introduced into the sex cords (Handel & Eppig 1998). Here they are stored until maturity is reached, then the sex cords hollow out forming the seminiferous tubules, and the epithelium of the tubules differentiates into the Sertoli cells (Handel & Eppig 1998), **[Figure.3].**

The PGCs divide to form spermatogonia; which are smaller in size and are composed of an ovoid shaped nucleus containing chromatin associated within the nuclear membrane (Handel & Eppig 1998). The spermatogonia are stem cells capable of regenerating themselves or producing different type of cells (Handel & Eppig 1998). In effect the spermatogonium has three possible fates: it can divide further to form new spermatogonia, it may undergo apoptosis (cell death) or it may differentiate into the first committed stem cell type, the intermediate spermatogonium (Handel & Eppig 1998). These cells undergo mitotic divisions to develop other forms of spermatogonia. Then they will further divide producing the primary spermatocytes before undergoing meiosis (Dym et. al., 1994). At this stage of development, the cytoplasm of neighbouring germ cells is connected via cytoplasmic bridges of ≈1 μm in diameter (Dym & Fawcet, 1971). Each primary spermatocyte goes through the initial meiotic division to produce two secondary spermatocytes, which then complete the second division of meiosis. The resulting haploid cells, called spermatids, are still able to communicate with their neighbours via the cytoplasmic bridges (Dym & Fawcett 1971). During the series of cell divisions from spermatogonium to spermatid, the cells move further away from the

basement membrane of the seminiferous tubule and close to its lumen. Different cells, at different stages can be identified and localized in different areas of the tubule (Handel & Eppig 1998); **[Figure.3.].**

As spermatids approach the border of the lumen they lose their cytoplasmic connections and differentiate into spermatozoa (Dym & Fawcett 1971) **[Figure.3].** The entire process is dependent on the endogenous environment provided by the somatic cells of the testis (Sertoli & Leydig cells) and it is also dependent on endocrine and para-autocrine regulation as well as well as direct cell to cell interaction (Dym & Fawcett 1971). The spermatogenic germ cells are linked to the Sertoli cells by N-cadherin molecules on the surfaces of both cells and by galactosyltransferase molecules on the spermatogenic cells that bind a carbohydrate receptor on the Sertoli cells (Newton et al. 1993; Pratt et al. 1993). The Sertoli cells provide the developing sperm cells with the nutrients and protection they require (spermatogenesis occurs in the recesses of the Sertoli cells).

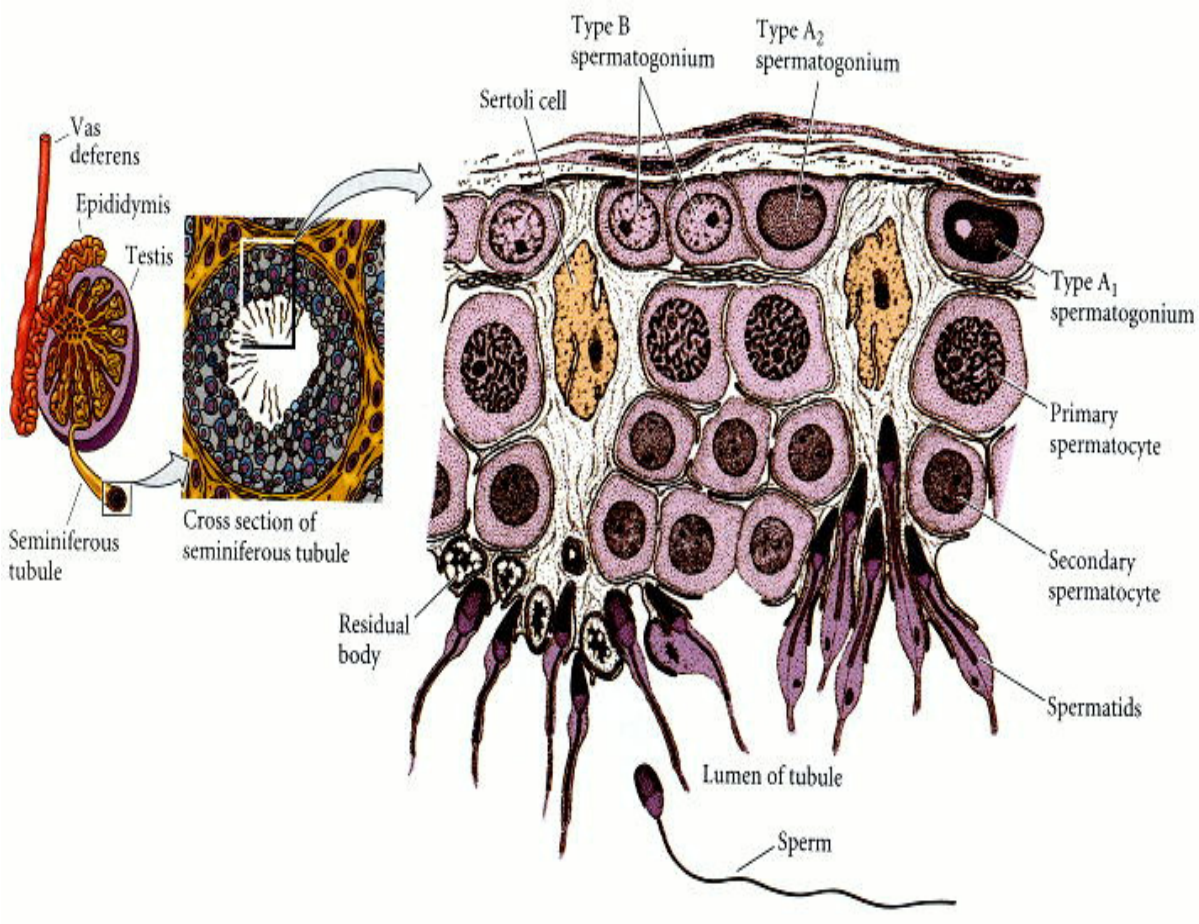

Figure.3. Diagrammatic representation of the physiological environment in the testis; where sperm formation and maturation take place. Furthermore exhibits the relationship with the cells present in the physiological environment where sperm formation and maturation take place (section of the seminiferous tubule). As cells mature, the germ cells migrate toward the lumen of the seminiferous tubule. Sinauer Associates, 2000. Diagram from: *The Male Reproductive System*, Dym, 1977.

After release into the lumen of the spermatogenic tubule, **[Figure.3.]**, the sperm passes to the epididymis, which is made up of a single convoluted tubule divided into three regions: the proximal (caput), the medial (corpus), and the distal (cauda) epididymis (Handel & Eppig 1998). During the sperm's journey through epididymal duct, it undergoes significant changes, including modification of many intra-acrosomal and

sperm plasma membrane molecules (Handel & Eppig 1998), a process known as epididymal maturation (Handel & Eppig 1998). These modifications are essential for the maturation process; leading to the production of a self-propelled functionally competent spermatozoon **[Figure.4.].** The region in the epididymis where spermatozoa become functionally mature may vary between species, but the distal region of the corpus appears to play this role in a number of species (Handel & Eppig 1998).

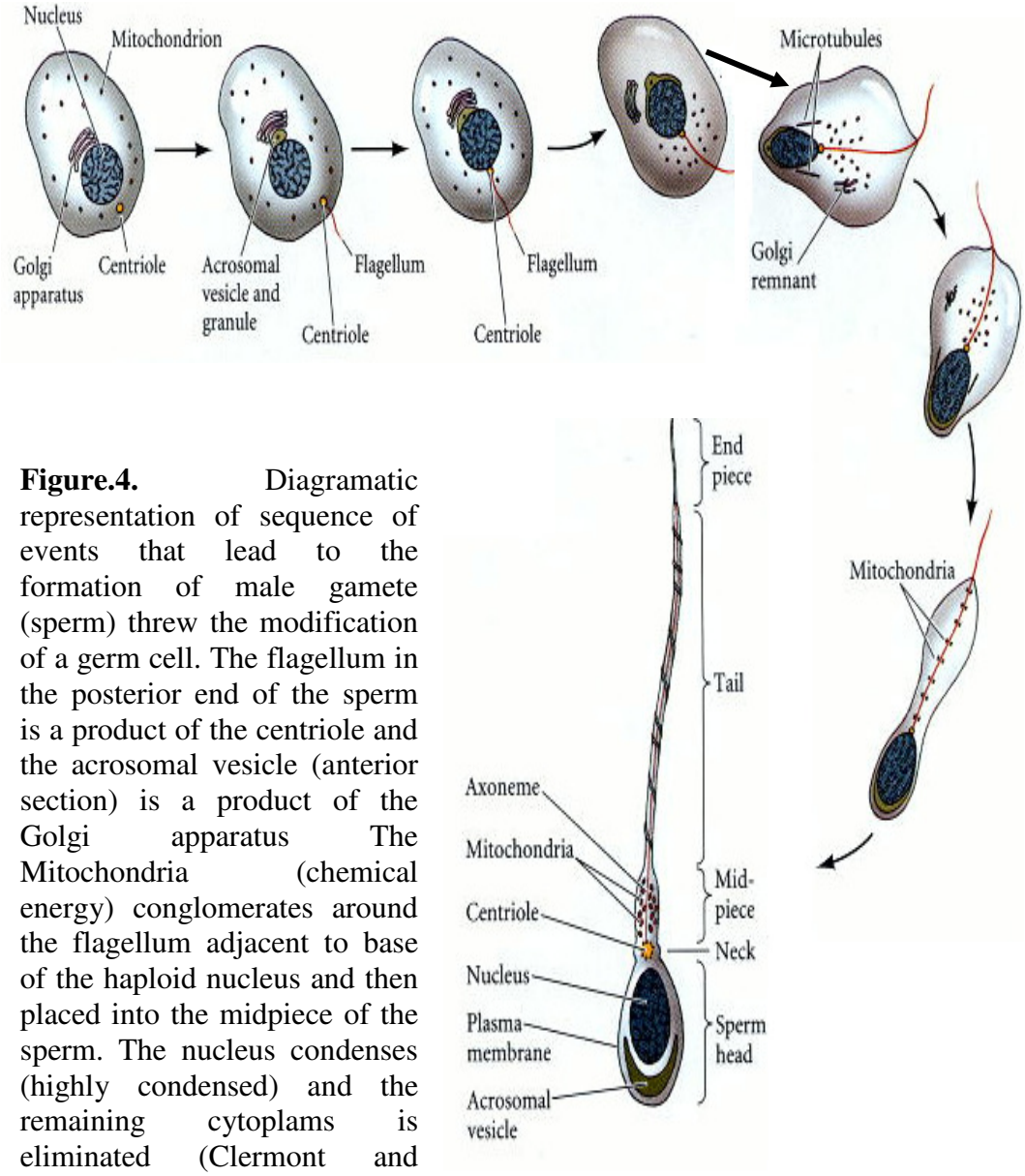

Figure.4. Diagramatic representation of sequence of events that lead to the formation of male gamete (sperm) threw the modification of a germ cell. The flagellum in the posterior end of the sperm is a product of the centriole and the acrosomal vesicle (anterior section) is a product of the Golgi apparatus The Mitochondria (chemical energy) conglomerates around the flagellum adjacent to base of the haploid nucleus and then placed into the midpiece of the sperm. The nucleus condenses (highly condensed) and the remaining cytoplams is eliminated (Clermont and Leblond 1955). Image sequence from: Dym, 1977.

1.3 Sperm Structure

It is only within the past century that the sperm's role in fertilization has been known (Gilbert, 2000) In 1678 a Dutch microscopist, Anton van Leeuwenhoek (sperm's co-discoverer) identified sperm in semen and initially believed that these were parasitic animals living within the semen (spermatozoa, meaning "sperm animals") (Gilbert, 2000).

The end product of sperm biogenenesis (spermatogenesis and spermiogenesis) is a highly polarized cell, which is stored within the cauda epididymis, **[Figure.3.]** (Wassarman et al., 1997). Most mammalian spermatozoa share the same basic structure, comprising a head and a tail (propulsion system) composed of a midpiece, principal piece and end piece (Mortimer et al., 1997), **[Figure.5.].** These components are surrounded by a continuous plasma membrane (Mortimer et al., 1997). Sperm lack cytoplasmic organelles such as ER, ribosomes, Golgi apparatus, which seem to be unnecessary for sperm's task (Handel & Eppig 1998). However, mitochondria are present, located in sperm's midpiece, to supply the sperm with chemical energy, a key requirement being supply of ATP to the flagellum to enable transit though the upper regions of the female reproductive tract and penetration of the zona pellucida (ZP).

The sperm head contains the nucleus (≥65% of the sperm head) and a single large secretory vesicle, the acrosome (Evans, 2004; 2005), **[Figure.5.].** The DNA present in the nucleus is tightly packed in order to facilitate its transport. The chromosomes of

sperm are packed with highly positively charged proteins called protamine; rich in arginine and cysteine (Alberts et. al., 2001). The nucleus is covered by a reduced nuclear envelope. Nuclear pore complexes (NPC) are removed during spermiogenesis (De Jonge & Barratt, 2006) and accumulate in the redundant nuclear envelopes (RNE) at the base of the sperm nucleus in the region of the sperm neck (Ho and Suarez, 2003). The sperm nucleus is protected by the perinuclear theca (PT), also reffered as the "perinuclear matrix", a rigid shell composed of disulfide bond-stabilized structural proteins united with various other proteins (Oko, 1995).

The anterior portion of the sperm head includes the acrosome, an exocytotic vesicle derived from the Golgi during spermatogenesis. This forms a cap which lies just beneath the plasmalemma and tightly encloses the anterior portion of the nucleus **[Figure.5.].** The inner and the outer acrosomal membrane encloses a dense acrosomal matrix, including enzymes (proteases) involved in the digestion and formation of a opening in the ZP (reviewed by Gerton, 2002; Yoshinaga and Toshimori, 2003) and receptors required for the sperm interaction and penetration of the ZP (De Jonge & Barratt, 2006; **Section 1.6**). The subacrosomal layer of the PT, underlying the acrosomal, functions to anchor this vesicle. The equatorial segment is a folded-over complex of the perinuclear, inner and outer acosomal membranes, which carries receptors involved in the initial binding of the sperm to the egg plasma membrane, once fertilizing sperm cells penetrate through the ZP and reaches the perivitelline space, **[Figure.2.]** (De Jonge & Barratt, 2006). The postacrosomal part of the PT is believed to include a complex of signalling proteins all combinedly referred as SOAF, or sperm

borne, oocyte acting factor that are released by PT dissolution into the oocyte cytoplasm at fertilization (reviewed by Sutovsky et al., 2003).

The mechanism of sperm motility varies according to how the species has adapted to the environmental conditions (Gilbert, 2000). In some parasitic species such as the roundworm, Ascaris, the sperm move by the amoeboid motion of lamelliopdial extensions of the cell membrane (Gilbert, 2000). In most species, sperm is capable of travelling long distances by beating of a flagellum (Gilbert, 2000). The energy source (chemical energy, ATP) for this process is provided, at least in part, by the ring of mitochondria located in the midpiece of the sperm, **[Figure.5.],** (Gilbert, 2000). In mammals, a layer of dense fibers has interposed itself between the mitochondria sheath and the axoneme.

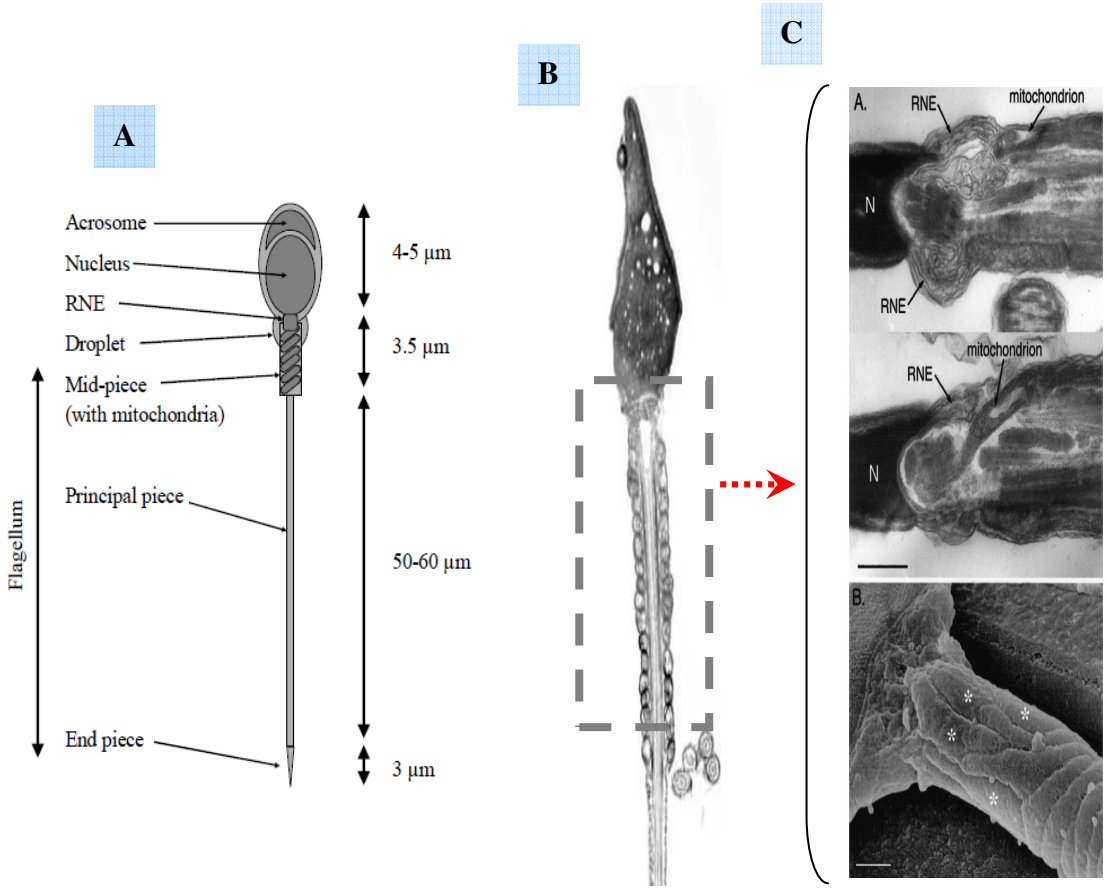

Figure.5. **(A)** Diagrammatic view of human mature spermatozoa structural components (image from: Machado-Oliveira, 2008), **(B)** Transmission electron microscopy (TEM) side image of mammalian spermatozoa head and midpiece (image from: Costello et al., 2008) **(C)** TEM images of spermatozoon midpiece and neck region composed of mitochondria (chemical energy in the form of ATP for motility)and the RNE. Images from: Han-Chen Ho & Suarez, 2001.

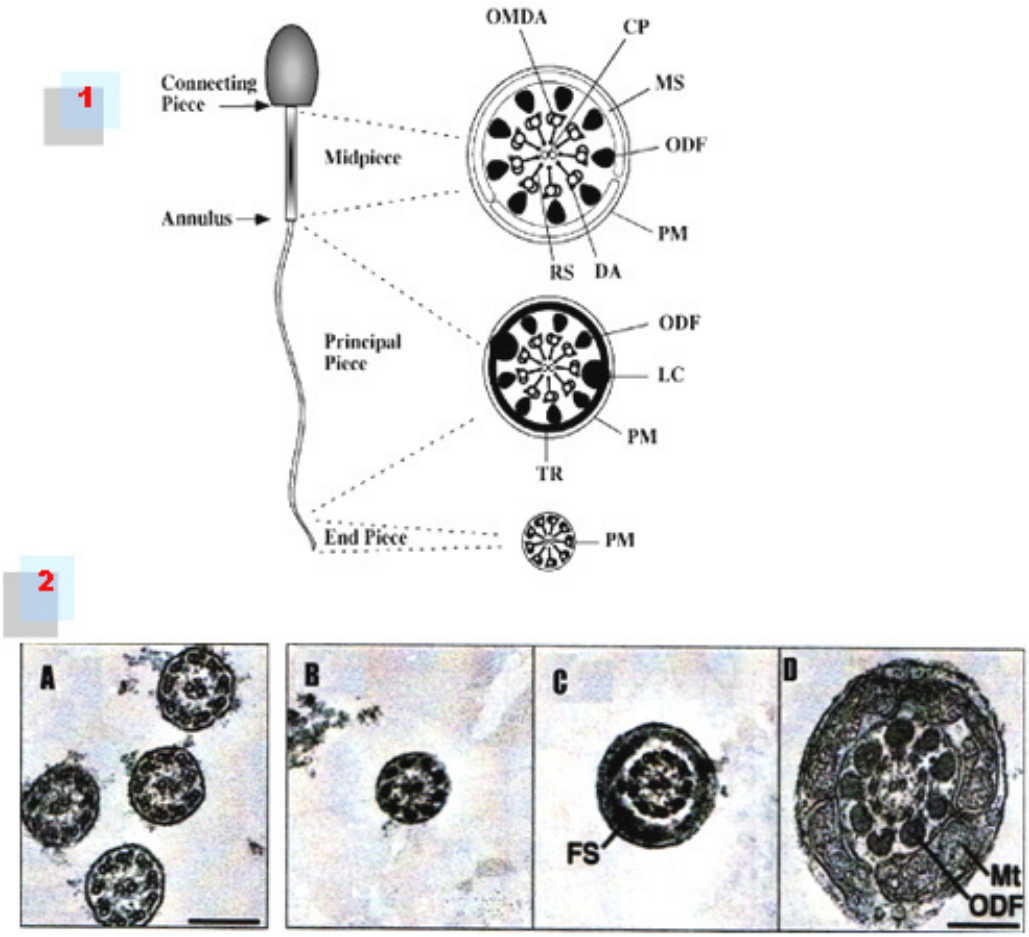

Figure.6. **(1)** Diagrammatic representation of components that form the mammalian sperm flagellum and the division of the flagellum into three areas (midpiece, principal piece and end piece). Additionally it shows the cross-section of the four components (Turner et al., 2003). **The midpiece** shows the plasma membrane and the mitochondrial sheath (MS) surrounding the 9 outer dense fibers (ODFs). In the ODFs are the components of the axoneme: the 9 outer microtubule doublets of the axoneme (OMDA) with associated dynein arms (DA) and radial spokes (RS) and the central pair of microtubule doublets (CP). **The principal piece** show the PM surrounding 7 ODFs, whilst the ODFs 3 and 8 are replaced by two longitudinal columns of the fibrous sheath (LC), connected by transverse ribs (TR). Here the axonemal components are not modified. **The end piece** show that the ODFs and the FS gradually lessens when the principal piece ends and they are not present in the end piece, leaving the PM surrounding the axoneme (Turner et al., 2003) **(2)** **(A)** Electron microscopic image of the cross-section of *Ciona flagellum*; **(B→D)** Electron microscopic images of the components that form the human mature sperm flagellum (B: end piece, C: principal piece & D: mid piece).

1.3.1 The sperm flagellum

Structure of the flagellum. *T*he ultrastructure within the mammalian flagellum is highly conserved and is composed of the contractile axoneme, surrounded by the outer dense fibres (ODF). The axoneme is composed of central two microtubules, connected by linkages (Pedersen, 1970), surrounded by nine microtubule doublets (in a 9 + 2 pattern) (Fawcett, 1965), **[Figure.6.].**

The two central microtubules are surrounded by a central sheath composed of a spiral of two fibres (Pedersen, 1970). The outer doublets are made of an A subunit creating a complete microtubule, and a B subunit (C-shaped) with an attached end to the A subunit. Adhered to each of the A subunits are the dynein arms (Afzelius, 1956; Gibbons & Grimstone, 1960; Gibbons, 1961). These multi-subunit ATPase complexes are responsible for the transformation of chemical energy (ATP) into kinetic energy. This is possible by permitting adjacent microtubule doublets to slide relative to one another, leading to axonemal bending and creating flagellar movement. Nexin links connect the microtubule doublets (Gibbons, 1965; Stephens, 1970); between the A and the B subunits (Baccetti et al., 1985). It has been suggested that nexin links may have elastic properties that allow the control and elastic retention of shear forces during doublet sliding. It has also been hypothesised that these may also be involved in the retention of flagellar bending and axonemal symmetry during sliding (Linck, 1979; Brokaw, 1980).

In organism that are involved in internal fertilization, the 9 + 2 axonemal structure is further surrounded by auxiliary dense fibres (ODFs) which are attached to the distal end of the segmented columns in the connecting piece (Fawcett, 1975; Baccetti et al., 1976) and a fibrous sheath (Pedersen, 1970). Each ODF is associated with the axonemal microtubule doublet (AMD), and numbered according to the doublet with which is associated. These fibres each contain a cortex and a medulla and are composed of a keratin-like protein (Baccetti et al, 1973).

Regions of the flagellum. The connecting piece links the flagellum to the sperm head. This is made of segmented columns possibly articulated, which would enable the neck region to bend without straining the link between the tail and the sperm head (between the capitellum and the basal plate) (Curry & Watson, 1995). The sperm midpiece extends from the distal end of the connecting piece to the annulus (Mortimer, 1997). The midpiece is the area where mitochondria are located (arranged in a helical-like form around the proximal portion of the axoneme), these are involved in production of ATP; required for flagellar movement (Curry & Watson, 1995). The flagellar principal piece extends from the annulus to the terminal piece and contains the fibrous sheath. This is a cytoskeletalcomponent that surrounds the axoneme and the ODFs. It is composed of two peripheral longitudinal columns in the plane of the central pair of microtubules, linked by semicircular circumferential ribs; that form a tubular-like connection (anastomose) (Fawcett, 1965). The two columns of the fibrous sheath have been shown to overlie, and be fused with, the two shortest ODFs and continue an attachment with their associated microtubule doubles following the distal termination of these ODFs

(Mortimer, 1997).The constituent proteins of the fibrous sheath are linked by disulphide bonds, making the structure very stable (Oko, 1988; Brito et al., 1989), suggesting that this structure may assist the flagellar motility by providing support of the flagellar beat. The terminal piece (or end piece) of sperm tail extends beyond the fibrous sheath. This section of the sperm tail is uniquely composed of the 9 + 2 axoneme covered by plasma membrane. The components forming the flagellar axonemal are terminated successively with the disappearance of the dynein arms, then the central pair of the microtubule subunits (Wolley & Nickels, 1985).

Flagellar bending. The creating of flagellar movement is through an attachment-detachment cycle between the dynein arms and the adjacent doublet (Marchese-Ragona & Johnson, 1990). The exact nature of the interaction between the dynein and tubulin is still unclear, however the dynein-tubulin binding enables active sliding, which involves the B subunits of the nearby MTD, and is regulated by ATP (Gagnon, 1995). Projecting from the A subunit microtubule doublet and towards the central sheath (As-MTD) are the so-called "Radial Spokes" (RS) (Afzelius, 1959; Gibbons & Grimstone, 1960; Hopkins, 1970). These are composed of 17 proteins, in the stalk region (adhered to the subfibre A of the microtubule doublet) and 5 on the globular head region, which projects towards the central pair (Gagnon, 1995). During the microtubule doublet sliding cycle, the RS attach and detach (Mortimer, 1997) **[Figure.7.].**

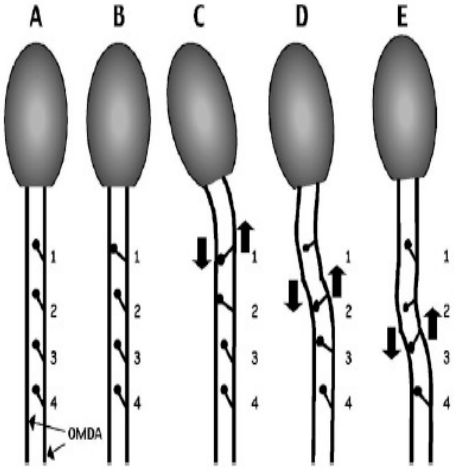

Figure.7. Diagrammatic representation of the mechanism of microtubule sliding under the action of dynein arms, in order to provide the sperm flagellar beat (Gagnon & Lamirande, 2006). For simplicity purposes in this diagram, only two outer microtube doublets of the axoneme are present and each of the numbered arms represents a pair of inner and outer arms (Gagnon & Lamirande, 2006). There is no flagellar bend when the dynein arms are inactive state. **(A)** Here the first dynein arm encounter the adjacent microtube **(B)** creating a downward stroke, the sliding force is turned into a bend in the axoneme **(C)** and then the second dynein arm encounters the adjacent microtubule doublet. The flagellar beat generated is spread with the relaxation of the first dynein arm, the second generating the downward stroke **(D)** and the third engaging into action **(E)**. A similar sequence is constantly repeated and occurs at the same time on the nine microtubule doublets in an asynchronous but coordinated manner all along the flagellum and around the circumference enabling for swimming in three dimensions (Gagnon & Lamirande, 2006) Diagram from: Turner, 2003.

1.4 Sperm Motility

1.4.1 Hyperactivation

Motility is perhaps the most easily observed function of sperm, and is yet arguably the least well understood (Darszon et al., 2006). Mammalian sperm show two forms of motility, activated motility, when they are initially exposed to the female reproductive tract and hyperactivated motility, which is observed in capacitated sperm in the vicinity of the oocyte. It has been well established, that Ca^{2+} contributes to the regulation of sperm activated motility (Tash & Means, 1987), and it may also be the primary factor

that leads to hyperactivated motility (Suarez et al., 1987; Lindemann & Goltz, 1988; White & Aitken, 1989; Brokaw, 1991; Yanagimachi, 1994; Ho et al., 2002).

The two forms of sperm motility can be identified by the difference in amplitude of the flagellar beat. When sperm is introduced in the female reproductive tract, they show a relatively low low-amplitude flagellar beat. Capacitated motility is stimulated by Ser/Thr and Tyr phosphorylation of the flagellar proteins (Turner, 2006). This phosphorylation cascade is influenced by: cAMP and a soluble form of adenylyl cyclase (sAC). The cAMP regulates this process by its activation on protein kinase A (PKA), and this may be activated by the stimulation of sAC. The activity of this enzyme is influenced by the presence of bicarbonate ions (HCO^{-3}) and Ca^{2+} (Livin et al., 2003, Esposito et al., 2004). In hyperactivated sperm, in the upper regions of the female reproductive tract, the sperm flagellar beat is characterised by large amplitude. This high amplitude causes sperm to swim in a "figure-of-eight" formation, in areas of low viscosity. On the other hand, in areas of high viscosity, hyperactivated motility is more progressive (Suarez & Ho, 2003), allowing the sperm to ascend the oviduct and penetrate the egg cumulus (Suarez & Ho, 2003). Calcium plays a key role in the initiation and maintenance of hyperactivated motility, **[Figure.8.],** by directly influencing the components of the axoneme (Darszon et al., 2006; Suarez ,2001).

Recently a novel class of Ca^{2+} channels known as the "CatSper" have been identified and localized in the testes. Four members of this family have been identified; two of them have been localized to the principal piece of the flagellum, CatSper 1 & CatSper 2 (Quill et al., 2001, Ren et al., 2001, Lobley et al., 2003, Jin et al., 2005). Sperm from

mice null for any of the four CatSper genes result in a dysfunction of the CatSper channels and consequently the loss of hyperactivated motility (Publicover et al., 2007; Qi et al., 2007). The plasma membrane Ca^{2+} ATPase 4 (PMCA4) is also restricted to the flagellum. Furthermore, sperm of PMCA4-null mice are immotile under conditions that lead to hyperactivated motility, consequently leading to an inappropriate Ca^{2+} regulation in the flagellum (Publicover et al., 2007; Okude et al., 2004; Schuh et al., 2004; Wennemuth et al., 2003).

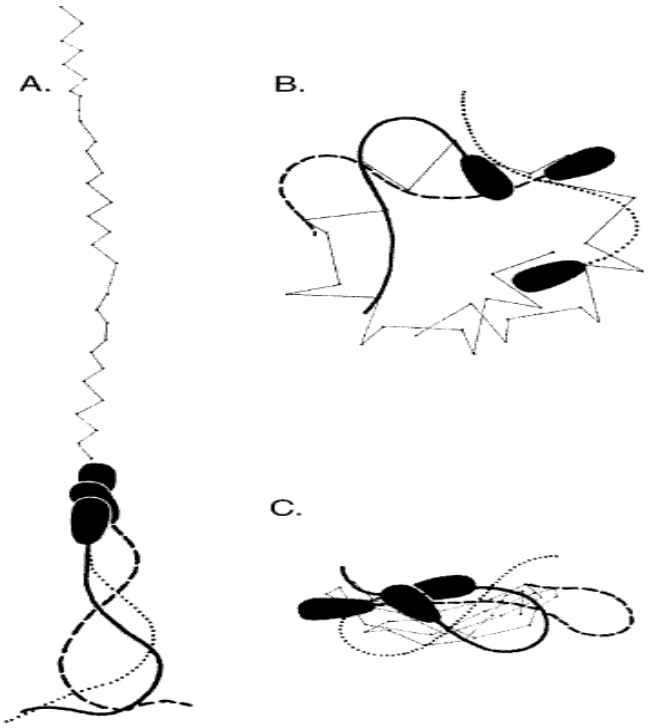

Figure.8. **(A)** Mammalian active motility with symmetrical flagellar bend and **(B)** & **(C)** Hyperactivated motility. Diagram from: Han-Chen Ho & Suarez, 2001.

1.4.2 Chemotaxis

Echinoderms. Sperm chemotaxis may occur throughout the Metazoans, from marine species such as sea urchins and corals, to humans (Miller, 1985; Cosson, 1990; Eisenbach & Tur-Kaspa, 1994; Eisenbach et al., 1999, 2004). The occurrence of sperm chemotaxis in externally fertilising organsisms (mostly marine) is well established. Echinoderms have been particularly useful for the study of mechanisms underlying sperm chemotaxis since these cells tend to swim with a planar circular trajectory (Christofer et al., 2005). In the presence of a concentration gradient of chemoattractant sperm undergo a series of sharp turns, each followed by a period of straighter swimming, that direct them towards the source of the stimulus (Kaupp et al., 2003, 2008), **[Figure.9.].** These brief turns are promoted by concise increases in flagellar asymmetry, enabled by an elevation in the acute angle between the long axes of the head and of the flagellum (Miller & Brokaw, 1970).

Ca^{2+} is a key factor in sperm chemotaxis; this process of sperm guidance is dependent on an external supply of Ca^{2+}; suggesting that Ca^{2+}-permeant pathways maybe involved in this process (Darszon et al., 2006). The initial response to stimulus is a short increase in cyclic guanosine monophosphate (cGMP) (Kaupp et al., 2003, 2008). Changes in cAMP lead to a biphasic elevation of $[Ca^{2+}]i$, with an initial fast component followed by a slow decaying plateau phase (Christopher et al., 2005). Sperm flagellar asymmetry is regulated via the entrance of an external source of Ca^{2+} (Ishikawa et al., 2004). In demembranated sea urchin sperm the Ca^{2+} concentration is proportionally related to the

degree of flagellar asymmetry (Brokaw et al., 1979); although this has not been demonstrated with intact flagella (Christopher et al., 2005).

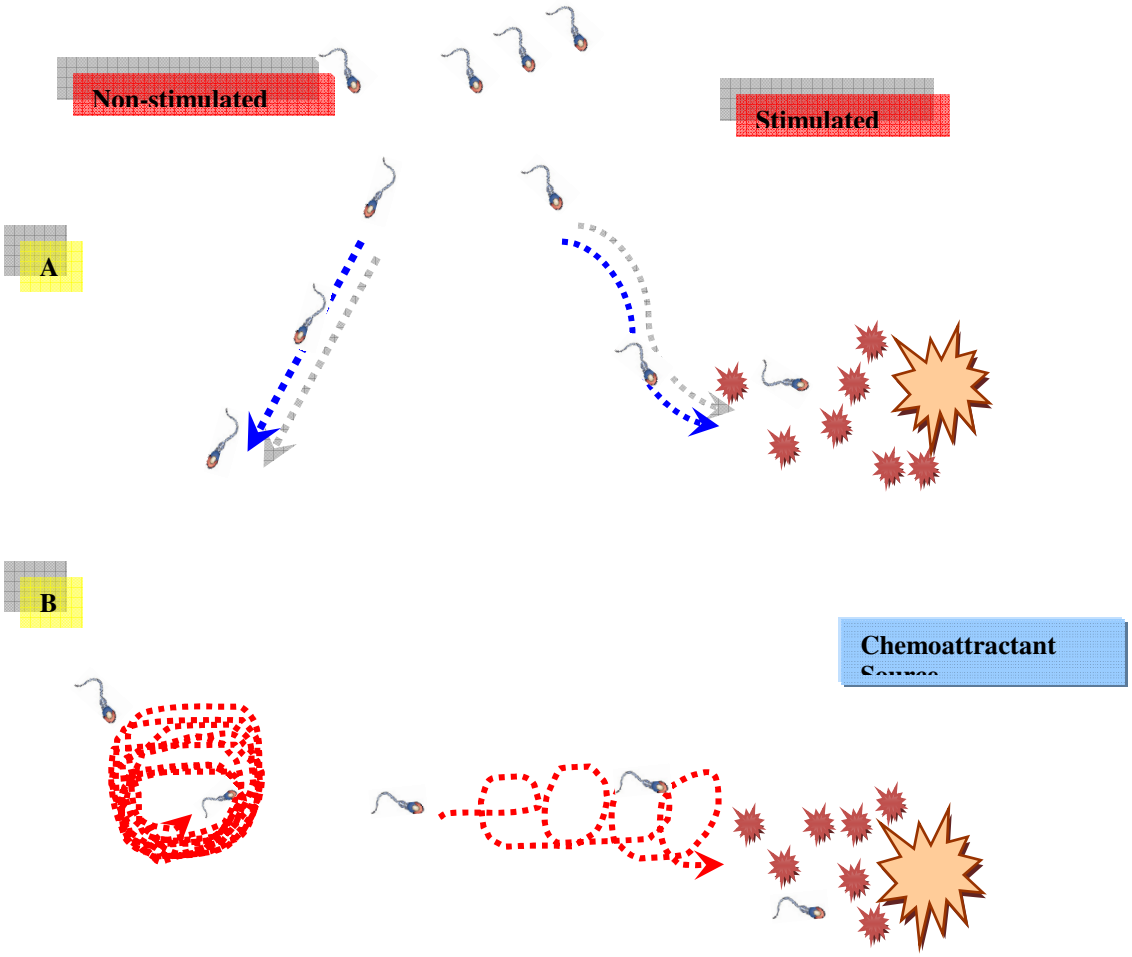

Figure.9. Diagrammatic view of the two different kinds of spermatozoa responses to a chemoattractant source. The lines with arrows represent the spermatozoa's swimming pattern with/out the presence of a chemoattractant source. **(A)** Shows non-stimulated spermatozoa (no chemoattractant) swimming pattern, linear or semi-linear swimming. In the presence of a chemoattractant gradient the direction of the swimming patterns changes dramatically towards the chemoattractant source (Based on Human spermatozoa, (Ralt et al., 1994; Jaiswal et al., 1999). **(B)** Shows non-stimulated spermatozoa (no chemoattractant) swimming pattern, circular motion. In the presence of a chemoattractant gradient the swimming pattern changes into loop forms towards the chemoattractant source (Based on ascidian's & sea-urchin spermatozoa, (Kaupp et al., 2003; Solzin et al., 2004). Diagram Modified from: Eisenbach & Giojalas, 2006.

The swimming patterns observed in mammalian species, either 'straight' or curved, are dependent on flagellar movement (Ishijima et al., 1990). It has been demonstrated that when human spermatozoa are swimming towards an ascending chemical gradient their flagellar beat is symmetrical and they appear to reach the chemoattractant source without changes in their swimming direction (Spehr et al., 2003, 2004). In addition there is an increase in speed, this effect is known as chemokinesis (Ralt et al., 1994). This increase is possible due an elevation in frequency of the flagellar beat (↑ beats = ↑ speed) [Spehr et al., 2003, 2004]. Additionally, when human spermatozoa are swimming away from the chemoattractant source (e.g. bourgeonal) the cells are reported to turn and swim towards the source. This change in direction is due to an asymmetrical flagellar beat (Spehr et al., 2003, 2004)**.**

Mammals. The occurrence of sperm chemotaxis in mammals was initially doubted due to the fact that a very large quantity of sperm (10^7-10^9) is ejaculated directly into the female reproductive tract**,** where many reach the egg by chance (Eisenbach et al., 1999, 2004). However, only small numbers of sperm may reach the fertilisation site (Section 1.81) and the physical complexity of the female tract is such that the probability of mammalian sperm reaching the egg without chemical guidance (possibly thermal guidance too, thermotaxis) is probably very low (Eisenbach et al., 1999, 2004; Eisenbach & Giojalas, 2006).

However, establishing the occurrence of mammalian sperm chemotaxis has proved very difficult. An important difference between internal and external fertilisers is that in marine invertebrates most spermatozoa are capable of responding to a chemoattractant but in mammals only a small percentage of capacitated spermatozoa (10-15%) are responsive (Cohen-Dayag et al., 1994, 1995; Fabro et al., 2002; Villanueva et al., 1998; Coheng-Dayang et al., 1994; Ralt et al., 1991 Giojalas et al., 2004). This was first identified by the finding that both capacitation (see below) and chemotactically responsive sperm had similar life spans (equally short) and they are continuously replaced (Eisenbach & Giojalas, 2006). In addition, depletion of capacitated spermatozoa results in the loss of the cells that respond to a chemoattractant and the depletion of chemotactic spermatozoa leads to loss of the capacitated spermatozoa (Coheng-Dayag et al., 1995; Giojalas et al., 2004; Jaiswal et al., 2002; Oliveira et al., 1999). The restriction of capacitation (and chemosensitivity) to a small, changing, proportion of cells is thought to ensure the presence of capacitated and chemotactically responsive spermatozoa in the female reproductive tract over an extended period (Coheng-Dayag et al., 1995). This is important in species such as humans, where ovulation is periodic (Eisenbach & Giojalas, 2006). Thus assays of mammalian sperm chemotaxis must be carried out in the context of a low signal-to-noise ratio (Eisenbach et al., 1999; 2004). However, good evidence for the occurrence of mammalian sperm chemotaxis became apparent when the behaviour of mammalian sperm was analysed according to parameters that distinguish chemotaxis from chemokinesis (Eisenbach et al., 1999, 2004).

The molecular mechanism involved in mammalian sperm chemotaxis is still an enigma; we still lack the knowledge completely to understand the molecular events (Eisenbach & Giojalas, 2006). What we do know about the molecular mechanism of sperm chemotaxis in mammals is the identity of some of the receptors involved and the chemoattractant-induced $[Ca^{2+}]_i$ changes (Eisenbach & Giojalas, 2006, Kaupp et al., 2008; see below section 1.8). The identification and localization of the G-protein-coupled olfactory receptors (Parmentier et al., 1992; Branscomb et al., 2000; Vanderhaeghen et al, 1993; Vanderhaeghen et al., 1997; Walensky et al., 1995; Walesky et al., 1998; Defer et al., 1998) in the midpiece of the tail of mature mammalian spermatozoa (Walensky et al., 1995; Spehr et al., 2003, 2004, 2006) suggest that these proteins could play a physiological role as receptors in mature sperm in mammalian sperm chemotaxis (Parmentier et al., 1992;Vanderhaeghen et al., 1997).

1.5 Spermatozoa Capacitation

Mammalian spermatozoa once fully differentiated then acquire their motile ability when these reside and mature in the epididymis (Darszon et al., 2005, 2006). When a sperm is initially introduced into the female reproductive tract it is unable to fertilize the oocyte. Here, as consequence of interaction with epithelial tubual cells and actions of the microenvironment within the female tract, it undergoes further maturation and acquires the ability to fertilize the egg during the sperms journey to reach the fertilization site (Yanagimachi R., 1994). The functional biochemical and biophysical changes that occur, which include a series of plasma membrane changes and changes in intracellular metabolism (Brucker et al., 1995), are known collectively as "Capacitation" (Austin, 1952; Chang, 1951; Yanagimachi R., 1994). Spermatozoa are exposed to significant changes in ion concentrations, osmolarity and different environment during its journey in the female reproductive tract (Darszon et al., 2005, 2006). Functional coupling of the signal transduction pathways that regulate the initiation of the acrosome reaction (see below, Section 1.6) is known to be a key outcome of capacitation. An increased response of mammalian sperm to ZP3 (Evans et al., 2004; Florman et al., 1994), progesterone (Baldi et al., 1998) and other inducers of the acrosomal reaction (AR) is a commonly used indicator that capacitation has occurred (Baldi et al., 2000). Many aspects of capacitation will contribute to this change, one of which is membrane potential hyperpolarisation, which occurs during capacitation, partly due to activation of potassium (K^+) channels (Zeng et al., 1995). Hyperpolarisation releases T-type calcium channels from voltage dependent inactivation, allowing them to participate in ZP3 signal transduction (Baldi et al, 2000). Hyperpolarisation is caused, at least in part, by

an increased contribution of potassium channels (K-channels) in setting membrane potential.

Spermatozoa can be capacitated *in vitro* when spermatozoa are incubated in a defined medium containing all the components at the correct concentrations in order for this process to take place (similar to oviductal fluid). This defined medium must contain three key elements: Ca^{2+} , HCO_3^- ,and serum albumin (BSA, HSA) (Visconti et al., 1995; Yanagimachi, 1994). In mouse spermatozoa extracellular calcium must be at a range of 100-200μM in order for these cells to capacitate (Fraser, 1987; Marin-Biggiler et al., 2003; Darszon et al., 2005). Furthermore, *in vitro* studies have demonstrated that spermatozoa plasma membrane fluidity is modified as a consequence of cholesterol removal by albumin present in the female reproductive tract (Cross, 2003; Travis and Kopf, 2002; Visconti et al., 2002). Moreover, the coapplication of cholesterol with albumin consequently will unable spermatozoa to undergo *in vitro* capacitation (Osheroff et al, 1999; Visconti et al., 1999).

When spermatozoa contact seminal fluid at ejaculation they aquire decapitating factors (DF), which bind the sperm surface and suppress capacitation. These are gradually released from the surface of spermatozoa as they capacitate (Baldi et al., 2000). It has been suggested that the DFs activate an intracellular Ca^{2+}-ATPase keeping the intracellular calcium level low $[Ca^{2+}]_i$ (Luconi et al., 2000) and release from the sperm surface permits an increase in intracellular calcium $[Ca^{2+}]_i$ (Baldi et al., 2000). Two potential DF candidates are uteroglobin and transglutaminase, inhibitors of sperm capacitation and motility; additionally these are shown to be present within the seminal

plasma (Luconi et al., 2000). Another inhibitory component found in the seminal plasma is cholesterol (Cross et al., 1996; Khorasani et al., 2000). Within the female tract cholesterol efflux from sperm membrane is mediated by sterol-binding proteins (high-density lipoproteins) and initiates many aspects of capacitation. Sperm membrane reorganization is one of the initial steps in the process of sperm capacitation, after cholesterol removal from the plasma membrane of spermatozoa (Cross, 2004; Travis and Kopf, 2002, Visconti et al., 2002) **[Figure.10.].** On the other hand there are also stimulatory molecules, which bind to sperm within the female tract and stimulate the fertilising ability of sperm. These are known as fertilization-promoting peptide (FPP) These small peptides stimulate sperm capacitation (Funahashi et al, 2000) and inhibit spontaneous loss of the acrosome before the spermatozoa reach the fertilization site (Baldi et al., 2000).

In addition to acquiring competence to undergo AR, another key marker of capacitation is the tyrosine phosphorylation of proteins through a cAMP-dependent mechanism. This phosphorylation event may be controlled by a bicarbonate-sensitive, soluble form of adenylyl cyclase (sAC); which reflects the strong requirement for extracellular bicarbonate (HCO^{-3}) in the capacitation process. Bicarbonate is present at low levels in epididymis and at high concentrations in seminal plasma and in the oviduct (Brooks et al., 1988) and is thus apparently critical in the inhibition of capacitation in the epididymis and the stimulation of capacitation in the female reproductive tract (Purohit et al., 2004). The mechanism by which activation of sAC and generation of cAMP leads to tyrosine phosphorylation is still not fully resolved. Elevation of intracellular pH and

bicarbonate (HCO^{-3}) levels, with the associated stimulation of cAMP production, may activate the cyclic nucleotide-gated and pH-gated channels that are present in sperm flagella and are linked to the control of flagellar motility (Navarro et al, 2008; Baldi et al., 2000).

In summary, capacitation is a complex 'suite' of changes that is believed to influence metabolism, membrane biophysical characteristics, protein phosphorylation state, intracellular pH (pH_i) (Uguz et al., 1994; Cross et al., 1997; Neill et al., 1987), Ca^{2+} levels, hyperpolarisation of membrane potential (Baldi et al., 2000) and probably other aspects of the sperm's biochemistru and physiology **[Figure.10.].**

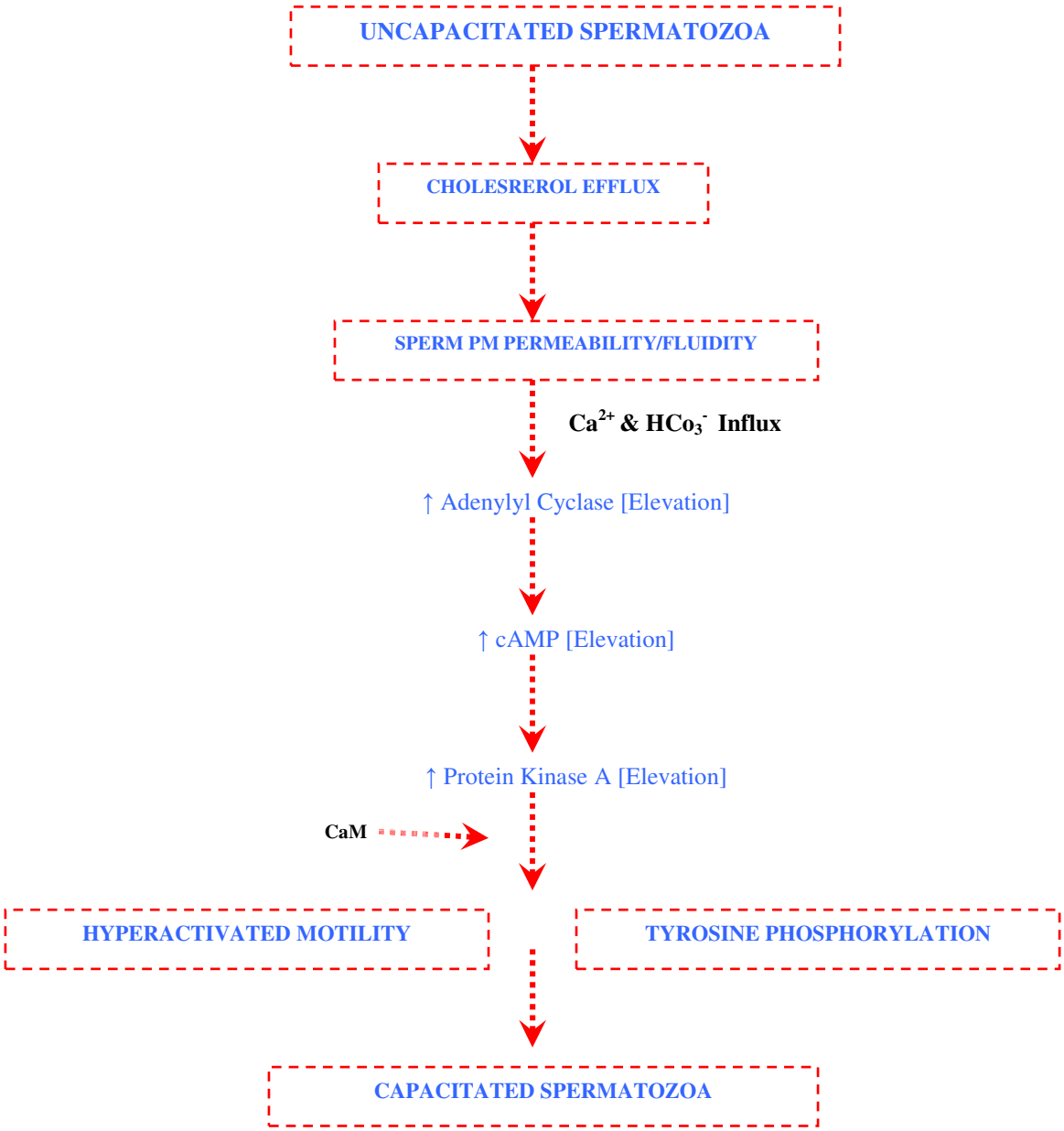

Figure.10. Diagram representing the possible chemical and physical changes that result in the capaciation of mammalian spermatozoa (Diagram and literature from: Abou-Haila et al., 2009 and from literature reporting experimental research from other groups).

1.6 Acrosome Reaction (AR)

The Acrosome reaction (AR) has been studied in great detail in many mammalian species (including humans); although our understanding of the events that take place during this process is greater in mice (Jimenez-Gonzalez et al., 2006).

The mammalian oocyte is surrounded by a thick glycoprotein coat, the zona pellucida (ZP) and at the time of ovulation the ZP is surrounded by the granulosa cells (Yanagimachi, 1994). Prior to the egg fertilisation, a spermatozoon must penetrate the ZP, and before this takes place, the spermatozoon must undergo the acrosome reaction. Binding to the ZP triggers extrusion fusion, at multiple points, between the outer acrosomal membrane and the overlying plasma membrane, often referred to as "acrosomal exocytosis" (Gerton et al., 2002). As a result of this membrane fusion, the acrosomal content including a great array of hydrolysing enzymes, is exposed **[Figure.11].** This, in combination with hyperactivated motility, permits the penetration of the zona pellucida (Baldi et al., 2002; Breitbart and Spungin, 1997; Cross et al., 1988; Flesch and Gadella, 2000; Wassarman et al., 2004; Yanagimachi & Usuf, 1974).

The sperm, with its nucleus covered by the inner acrosomal membrane, passes through the ZP and across the perivitelline space to fuse with the oolemma of the oocyte (Baldi et al., 2000).

In the mouse the main biological promoter of sperm AR is the zona pellucida (ZP). Experimental studies, in mouse have led to the conclusion that ZP contains proteins (ZP1, ZP2, and ZP3) [Lefièvre et al., 2004]. Thus alternative nomenclature was proposed based on the gene size, ZPA (ZP2), ZPB (ZP1) and ZPC (ZP3) (Harris et al.,

2004; Lefièvre et al., 2004). However it was later shown that ZP1 and ZPB genes in human are paralogues (Hughes and Barratt, 1999), suggesting that the human contained four ZP genes and not three (Lefièvre et al., 2004), correlating with the identification of both ZP1 and ZPB genes in chicken (Bausek et al., 2000) and rat (Lefièvre et al., 2004). Additionally, Lefièvre et al. (2004) were the first to experimentally demonstrate the existence of the four glycoproteins in human (ZP1, ZP2, ZP3 and ZPB), also identified in rats. The ZPB gene identified in mouse spermatozoa, has acquired a series of modifications resulting in its unlikely expression (Lefièvre et al., 2004). ZP3 (83kDa) exhibits most of the sperm binding and AR-inducing activity (Florman et al., 1989). Furthermore, ZP2 is a secondary ligand that binds to the spermatozoa that have undergone AR and stimulates a cascade of events that result in the prevention of polyspermy (Bleil and Wassarman, 1980; Bleil et al., 1981). The remaining glycoprotein ZP1, has been proposed to play a role in maintaining the structural intergrity of the ZP matrix (Greve & Wassarman, 1985; Green, 1997; Wassarman, 1997; Lefièvre et al., 2004) and does not have a direct effect in spermatozoa binding (Lefièvre et al., 2004; Rankin et al., 1999).

There are other agonists that may act *in vivo* that could potential induce AR: Serum album, epidermal growth factor, ANP, platelet-activating factor, progesterone (17βOH-progesterone), ATP and prostaglandin E1 (Yanagimachi et al., 1994; Baldi et al., 1998, 2000). ZP3 is able to bind readily to sperm and it is also capable of functioning as a competitive inhibitor of adhesion (Evans et al., 2004). The nature of the adhesion to the zona pellucida is based on the protein-carbohydrate recognition process, through the association of O-linked oligosaccharides (carbohydrate chains attached to polypeptide

by serine/threoninyl: N-acetylgalactosaminyl linkages) from ZP3 with a cognate receptor on sperm (Florman et al., 1985; Wassarman et al., 2001).

ZP3 binding of mouse sperm leads to a cascade of events which ultimately cause an increase in $[Ca^{2+}]_i$, which triggers AR. Initial events in the response of mouse sperm to ZP3 include the opening of T-type, low voltage-activated calcium channels, that results in a transient calcium influx and the activation of the heterotrimetic G proteins, G_{i1} and G_{i2} (Florman et al., 1998; Arnoult et al., 1996). These initial responses are believed to activate phospholipase C (PLC) (Tomes et al., 1996; Fukami et al., 2001; Roldan et al., 1994) and cause an increase in intracellular pH (Arnoult et al., 1996; Florman et al., 1989), resulting in a sustained calcium influx that directly drives exocytosis (Florman et al., 1989; Florman et al., 1994). A variety of studies have tried to identify the channel(s) that lead to the sustained phase of ZP3-evoked Ca^{2+} entry (Evans et al., 2004). The main candidate subunit of the PLC-dependent calcium entry channels are canonical TRP family (Jimenez-Gonzalez et al., 2006; Minke et al., 2002). The gating mechanism that links PLC action to the opening of TRPC channels is still unknown and there are hypotheses based both on lipid products or PLC hydrolysis and also on the generation of inositol-1,4,5-trisphosphate (InsP3) and the activation of a calcium-store depletion-operated pathway (Mike et al., 2002). Various TRPC genes are expressed in the mammalian germ lineage (Wissenbach et al., 1998; Jungnickel et al., 2001) and TRPC2 has been shown to be a subunit of the sustained calcium entry channel in mouse sperm that is activated by ZP3 (Jungnickel et al., 2001). Furthermore, $InsP_3$ receptors are present in the sperm acrosome (Walensky et al., 1995), acting as an IP_3-mobilised Ca^{2+}

store (Jimenez-Gonzalez et al., 2006) and may play a role in the stimulation of the TRPC2 channels by the action of ZP3 (Jimenez-Gonzalez et al., 2006). Additionally it has been recently identified that the TRPC channels can be stimulated by a variety of mechanisms (Padinjat & Andrews, 2004) and the mechanism that lead to the sustained Ca^{2+}-influx is still uncertain (Evans & Florman, 2002).

In humans TRPC2 is apparently a psueudogene (Wes et al., 1995; Vannier et al., 1994) and possibly in bovine systems too (Wissenbach et al., 1998). Thus in other species the identity of the channels involved, and possibly the nature of their activation, may be different. Our knowledge of the ZP-induced intracellular calcium signal in humans cells is relatively limited (Serres et al., 2000), possibly another member of the TRPC family plays the role that TRPC2 plays on mouse sperm (Jimenez-Gonzalez et al., 2006).

Various SNARE proteins (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) have been identified in the acrosomal region of mammalian and sea urchin sperm and these have been propose to play a role in membrane fusion resulting in exocytosis (Darszon et al., 2005).

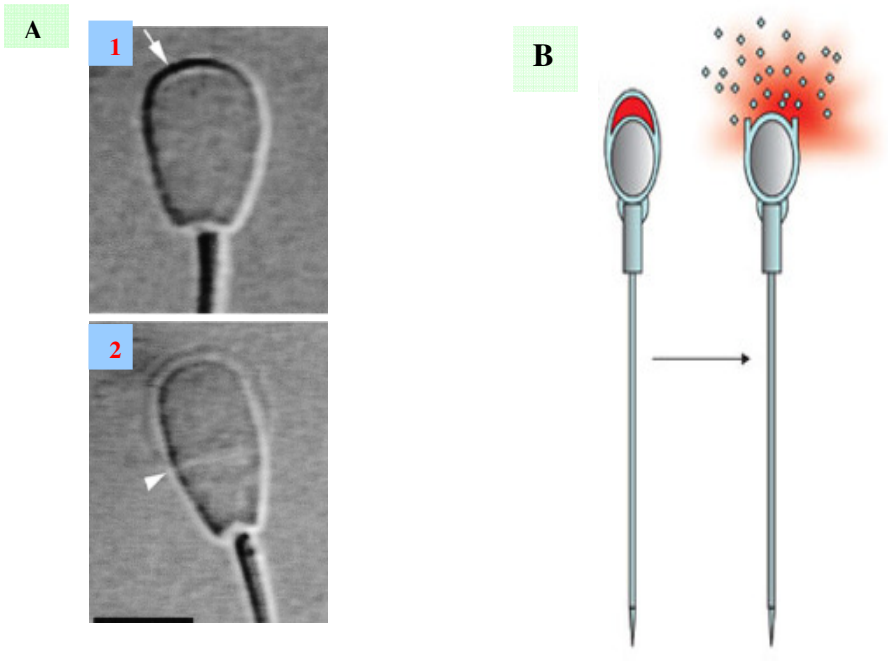

Figure.11. (A) & (B) Image and diagrammatic representation of acrosome reacted and acrosome intact spermatozoa, resulting in the release of hydrolytic enzymes (acrosomal content). **(1)** Acrosome intact sperm with a distinct apical ridge, **(2)** An acrosome-reacted sperm has a prominent equatorial segment (white arrowhead). Image (A) modified from Han-Chen Ho & Suarez, 2001; diagram (B) from Publicover et al., 2007.

In many species, spermatozoa that undergo acrosome reaction prematurely display reduced fertilization potential due to a failure to penetrate the cumulus oophorus, an increased propensity for binding to the cells comprising the cumulus oophorus (thus excluding them from interaction with the ZP), and an inability to adhere to the ZP

(Kopf, 2002). This premature response AR response, which is normally not a common process (Yanagimachi et al., 1994); may occur as a result of self-aggregation of the sperm receptor for the zona pellucida (Sailing et al., 1989). Additionally this could also be as a result of a reduction the pumping efficiency of the Na^+ and/or Ca^{2+} pumps, consequently leading to a increase in intracellular Ca^{2+} and pH (Yanagimachi et al., 1994).

1.7 Calcium [Ca^{2+}] ions and Cellular Regulation

Calcium (Ca^{2+}) signal transduction regulates a great array of cellular functions in the eukaryotic kingdom (Quill et al, 2001). Unlike many other second messenger molecules, Ca^{2+} is vital, yet the prolonged high intracellular Ca^{2+} levels lead to cell death. Calcium (Ca^{2+}) cannot be metabolized like other second messenger molecules so cells tightly regulate intracellular levels through a great array of binding and specialized extrusion proteins (Cheek & Hazon, 1993), **[Figure.12.].** The standard intracellular calcium $[Ca^{2+}]_i$ levels are at ~100 nM; which is approximately 20,000 fold lower than the 2mM extracellular concentration. Scores of cellular proteins have been adapted to tightly bind calcium (Ca^{2+}), in some cases just to buffer or lower free Ca^{2+} levels, and in others to trigger second messenger pathways. In ejaculated spermatozoa $[Ca^{2+}]_i$ plays an important role in many physiological processes. In spermatozoa, intracellular calcium, $[Ca^{2+}]_i$ modulates a great array of physiological process, which include the acrosome reaction (Publicover et al., 2007; Evans et al., 2002, Kirkman-Brown et al., 2002) flagellar beat mode; including hyperactivated motility (Publicover et al., 2007; Harper et al., 2004; Suarez and Ho, 2003), chemotaxis (Kaupp et al., 2006; Eisenbach, 1999; Spehr et al., 2003), and is an important component in the spermatozoa's capacitation (Publicover et al., 2007; Breitbart 2002). Furthermore all the above processes are necessary in order to successfully achieve spermatozoon's goal, which is to successfully reach the fertilization site and fuse with the oocyte. Consequently sperm must strictly ensure that all of these physiological processes are initiated at their required times (Jimenez-Gonzalez et al., 2006).

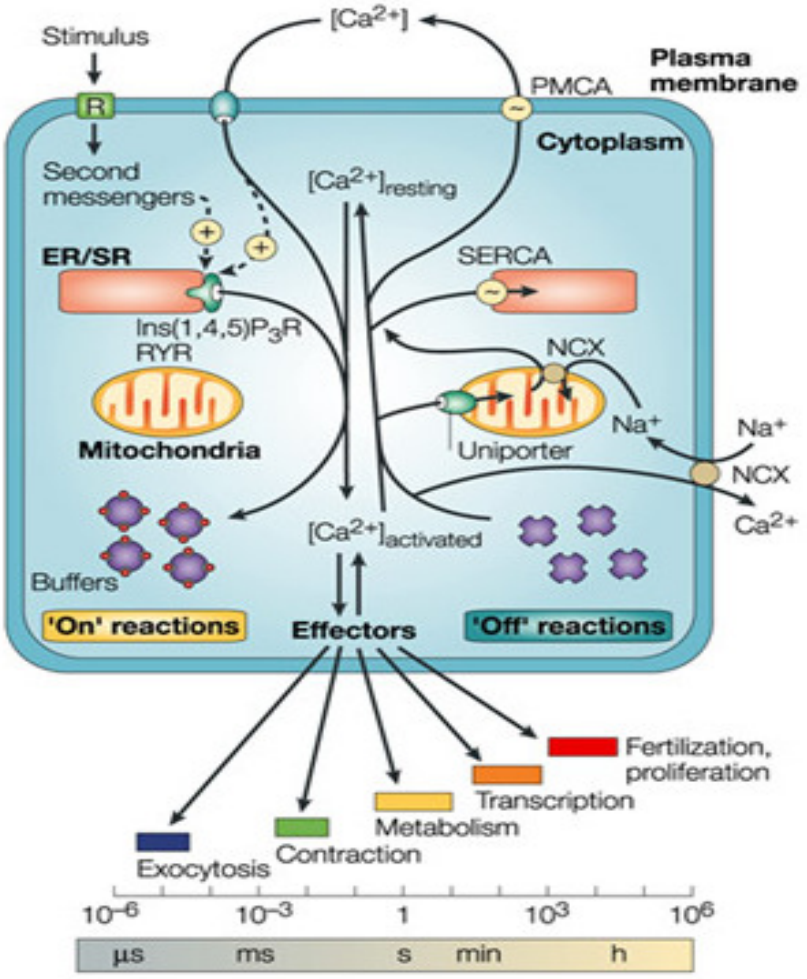

Figure. 12. Diagrammatic representation of calcium-signalling dynamics and calcium homeostasis. Diagram from: Berridge et al., 2003.

1.7.1 Calcium Signalling in Mammalian Spermatozoa

During spermiogenesis much of the cytoplasm is shed from the male germ cell, such that the structure of the mammalian sperm, compared to somatic cells, appears very simple. However, the remaining plasma and intracellular membranes are rich in Ca^{2+} channels and pumps, allowing the mature sperm to regulate $[Ca^{2+}]_i$ and to generate complex and (apparently) localized $[Ca^{2+}]_i$ signals **[Figure.13.].** In this section the Ca^{2+}-signalling apparatus of mammalian sperm is reviewed.

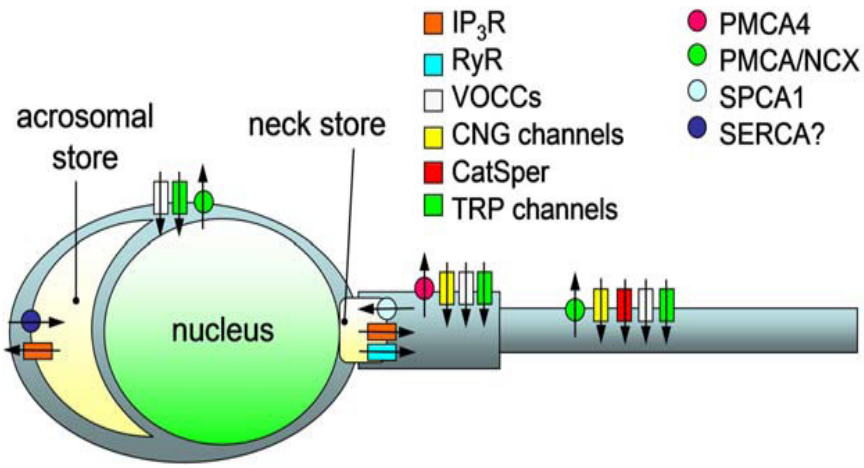

Figure.13. Diagrammatic illustration of the classes and location of pumps, channels and intracellular storage organelles located in mammalian sperm, potentially involved in the regulation of intracellular calcium (Publicover et al., 2008). Channels are illustrated as rectangles (▮) and the pumps as circles (●). The arrows illustrate the normal direction of calcium. The question mark next to SERCAs illustrates the presence and/or its functional significance is under dispute (Publicover et al., 2008). Mitochondria located in the sperm midpiece of mammalian sperm, are involved in the accumulation of calcium (into matrix space) via a uniporter on the inner membrane (driven by e-transport), hence resulting in intracellular calcium buffering (not illustrated) (Publicover et al., 2008). Diagram from: Publicover et al., 2008.

1.7.2 Ca^{2+} channels at the Plasmalemma

1.7.2.1 Voltage-Operated Ca^{2+} Channels

Voltage operated Ca^{2+} channels (VOCCs) are a family of trans-membrane, channel-forming proteins, with strong structural similarities to each other and to the voltage operated Na^{+} channels (Catterall, 2000 and Ertel et al., 2000). The VOCC pore-forming unit is the α1 subunit, which comprises four homologous domains (I-IV) linked via cytoplasmic linker regions (Jimenez-Gonzales et al., 2006). Ten α1 genes have been identified, α1A- α1I and α1S. Each domain of the α1 subunit is composed of (6) six transmembrane helices (S1-S6). Between S5 and S6 segments there is a non-helical region (P-loop) (Jimenez-Gonzalez et al., 2006). These components (S5-P loop-S6) line the channel pore and are important in the determination of ion conductance and selectivity (Jimenez-Gonzalez et al-. 2006; Catterall et al., 2003).

The VOCCs include 3 or 4 other subunits, in addition to the α1 subunit, which contribute to the channel's characterization, regulation and location (Jimenez-Gonzales et al., 2006). The VOCCs are a "family" of great diversity in biophysical characteristics such as voltage dependence, kinetics of activation and inactivation and also in pharmacological sensitivity (Jimenez-Gonzales et al., 2006). Recent nomenclature identifies 3 sub-families (Cav1, Cav2 and Cav3). Cav1 and Cav2 types form high voltage activated channels. Cav3 (a1G(Cav3.1), a1H(Cav3.2) and a1I(Cav3.3)) all encode T-type channels which activate in response to low levels of depolarisation (Caterall et al., 2003).

VOCCs have been identifies in both mature and immature sperm cells (Arnoult et al., 1996a, 1996b) **[Figure.13.].** Patch clamping of the VOCCS in immature male germ cells (human and rodent) has demonstrated that these cells express low-voltage activated (LVA), fast inactivating (T-type) currents (Hagiwara and Kawa, 1984; Arnoult et al., 1996a; Lievano et al., 1996). However, patch clamping has been unable to identify high-voltage-activated currents (Jimenez-Gonzales et al., 2006). Electrophysiological recording from immature male germ cells of α1G (Cav3.1 newer nomenclature) knockout mice demonstrated that the absence of the T-type channel had no considerable effects on the currents. The results obtained suggested that the α1H (Cav3.2) is the main functional VOCC in wild-type germ cells (Stamboulian et al., 2004). Immunolocalisation suggests that the distribution of the three different T-type channels varies within human sperm. α1H(Cav3.2) is located in the principal piece of the tail and in the back of the sperm head and α1I(Cav3.3) is present in the sperm midpiece (Serrano et al., 2004). A number of groups have attempted detection and quantification of different mRNA for CaV subunits in preparations of motile sperm (Park et al., 2003). mRNAs for α1c(Cav1.2) and α1I(Cav3.3) were present, consistent with the hypothesis that T-type (especially α1H(Cav3.2) and α1G(Cav3.1)) and possibly non-L-type (α1E(Cav2.3) and α1B(Cav2.2)) Ca^{2+} channels are involved in the acrosome reaction as the primary Ca^{2+} entry pathways (Jimenez-Gonzales et al., 2006).

1.7.2.2 Calcium (Ca^{2+}) Store-operated Channels

Calcium (Ca^{2+}) efflux from intracellular stores is believed to activate Ca^{2+}-permeable ion channels (store-operated channels-SOCs) in the plasmalemma, **[Figure.13.],** a process called capacitative Ca^{2+} entry (CCE) [Putney, 1990]. This physiological process is believed to occur in both non-excitable (Parekh and Penner, 1998) and excitable cells (Zhu et al., 1996; Garcia and Schilling, 1997; Philipps et al., 1998; Fomina and Nowycky, 1999; Li et al., 1999; Liman et al., 1999). It has been hypothesised that the canonical transient receptor potential channel (TRPC), **[Figure.13.],** are involved in the formation of SOCs (Padinjat and Andrews, 2004). Castellano et al. (2003) demonstrated that the distribution of these (TRPCs) are not only located in the sperm head but also in the flagellum, suggesting a potential role in sperm motility, **[Figure.14.],** (Jimenez-Gonzales et al., 2006).

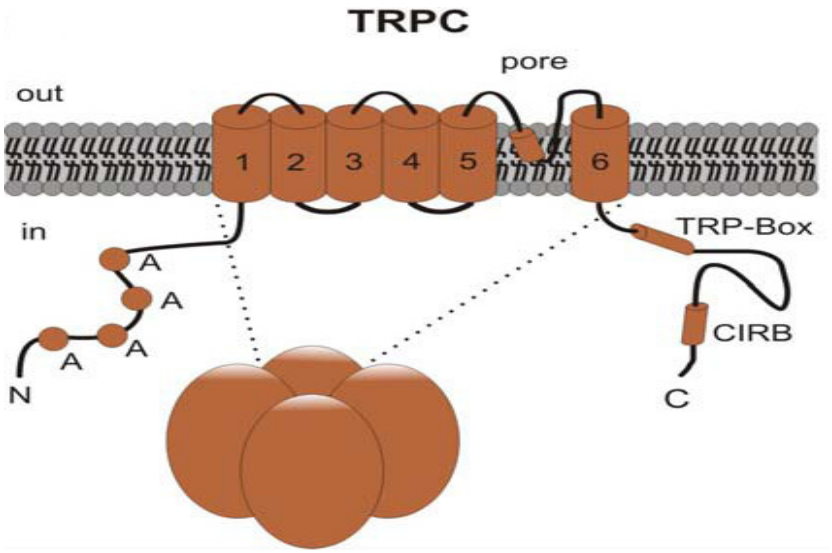

Figure.14. Diagramatic illustration TRP channel structure; each TRP channel subunit are composed of six transmembrane-spanning segments (1-6). The pore region of the channel is located between the 5 and 6 segments (Krannich, 2008). The TRP N- and C-terminus are located in the cytoplasmic side of the membrane, ankryrin-like repeats (A) are present in the N-terminus. The C-terminus contains a region (CIRB) that binds CaM and IP_3R, as well as a conserved region of unknown function called the TRP-box. Diagram from Krannich, 2008.

A variety studies involving the mobilisation of stored Ca^{2+} capacitative Ca^{2+} influx have demonstrated the occurrence of CCE in non-capacitated human sperm (Blackmore, 1993), spermatogenic and sperm cells of mouse, bull and ram (Santi et al., 1998; Dragileva et al., 1999; O´Toole et al., 2000; Rossato et al., 2001). The signal (gating signal) that leads to the opening of the SOC in the plasma membrane, is achieved by the stimulation of calcium (Ca^{2+}) efflux from the intracellular stores. Ca^{2+} entry through SOCs, producing a sustained elevation in intracellular calcium $[Ca^{2+}]_{i,}$ may be responsible for the stimulation of the acrosome reaction of spermatozoa in mammalian sperm and non-mammalian sperm (Section 1.6) (O´Toole et al., 2000; Gonzalez-

Martinez et al., 2001; Hirohashi and Vacquier, 2003). CCE has also been implicated in the regulation of the chemotactic behaviour of ascidian sperm (Yoshida et al., 2003).

1.7.2.3 Cyclic Nucleotide-gated Channels (CNG)

Cyclic nucleotide signalling plays a critical role in the physiological mechanisms in mammalian and invertebrate spermatozoa (Jimenez-Gonzalez et al., 2006; Kaupp et al., 2003, 2008) **[Figure.13.].** The cyclic nucleotide-gated channels are composed of two subunits (α and β), or subunit A and B; these assemble and form a heteroligomeric complex, where the α-subunit forms the channel (Darszon et al., 1999,2005; Molday, 1996). CNG channels show poor selectivity between sodium (Na^+) and potassium (K^+), are blocked by magnesium (Mg^{2+}), and show permeability to calcium (Ca^{2+}). The CNG channels are more sensitive to cyclic guanosine monophosphate (cGMP) than to cyclic adenosine monophosphate (cAMP) (Darszon et al., 1999). **[Figure.15.].** The activity of the CNG channels is modulated by CaM (calmodulin), a Ca^{2+}-binding protein; with half-maximum modulatory action occuring at calcium concentrations of ≈4μM (Molday, 1996). In olfactory CNG channels, the affinity of the CNG for cAMP decreases ˜20 fold in the presence of Ca^{2+}/CaM (Chen et al., 1994). In human sperm an olfactory receptor (hOR17-4) is believed to activate a cyclic nucleotide-medicated Ca^{2+} influx and control sperm chemotaxis (Spehr et al., 2003, 2004, 2006; Jimenez-Gonzales et al., 2006).

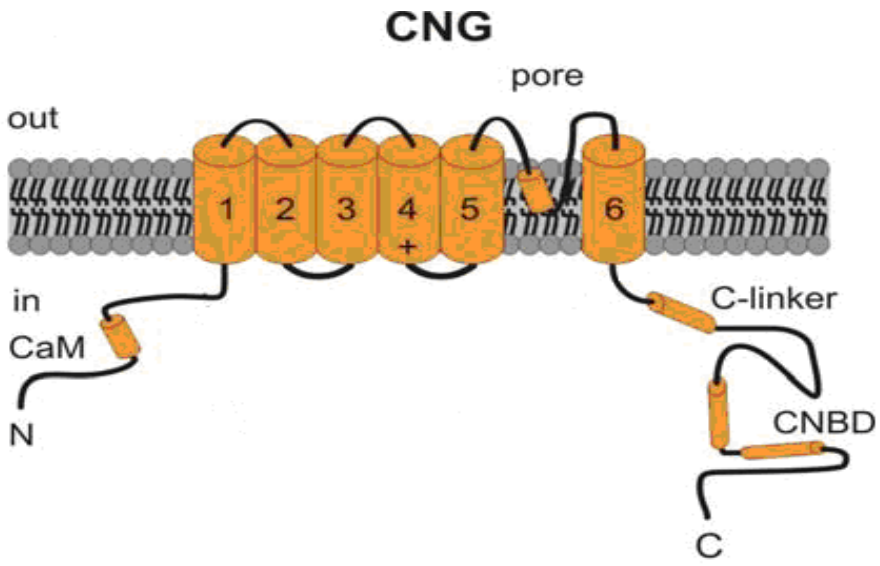

Figure.15. Diagramatic structure of CNG channels located in the plasma membrane of mammalian spermatozoa (Krannich, 2008). **CaM** in the diagram represents the calmodulin modulatory binding domain, the **CNBD** represents the cylic nucleotide binding domain. Diagram obtained and modified from: Krannich, 2008.

1.7.2.4 CatSper ion Channels

The CatSpers proteins CatSper1 (Ren et al., 2001), CatSper2 (Quill et al., 2001) and CatSper3 and 4 (Lobley et al., 2003) are subunits that combine to form sperm-specific ion channels **[Figure.13.].** They are subunits, each subunit including 6 transmembrane segments, such that the complete channel has a structure similar to a VOCC. Each subunit has a putative voltage-sensor in the S4 domain (Jimenez-Gonzales et al., 2006). The pore region of these channels is Ca^{2+}-permeable and its transmembrane sequence resembles that of voltage-gated calcium and sodium channels (Jimenez-Gonzales et al., 2006). It is still uncertain if the CatSpers are homo- or heteroterrameric, however the expression of only one subunit does not result in the formation of functional channels

(Quill et al., 2001; Ren et al., 2001). It has been proposed that in order to obtained a functional tetrameric channel, the CatSper proteins require further subunits (or factors) (Qill & Ren, 2001; Jimenez-Gonzalez et al., 2006).

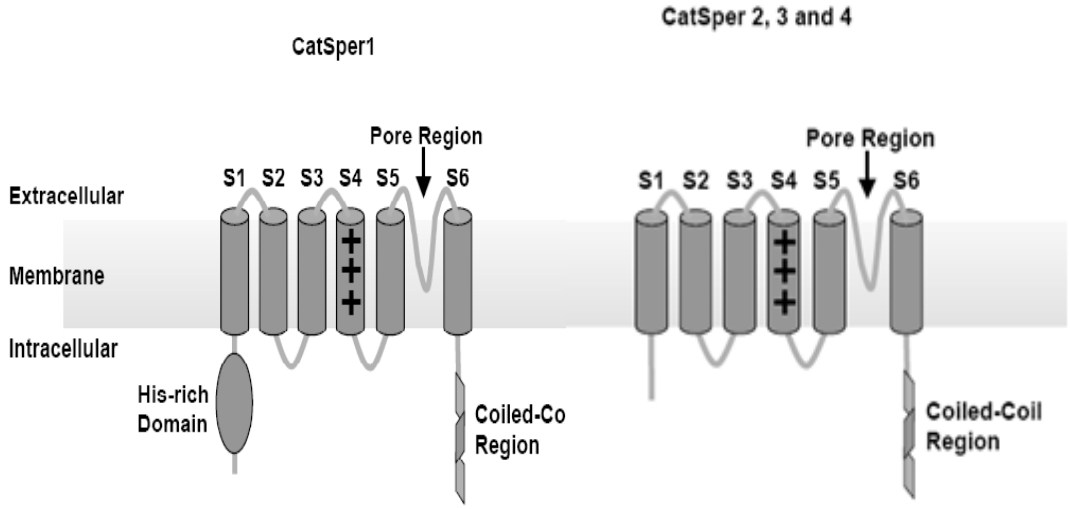

Figure.16. Diagrammatic representation of the CatSper ion channel ultrastructure. Diagram from: Lobley et al. Reproductive Biology and Endocrinology 2003.

CatSper were initially identified and localised in the testis during spermatogenesis, when round spermatids are formed during this process (Ren et al., 2001; Nikpoor et al., 2004). CatSper2 proteins are localized in the flagellum of mature sperm, **[Figure.17.]**, (Quill et al., 2001) and CatSper1 to the principal piece of the tail (Ren et al., 2001), suggesting that CatSper channels may be involved in regulation of sperm motility (Jimenez-Gonzales et al., 2006). CatSper expression is apparently reduced in some cases of human sperm that lack motility (Nikpoor et al., 2004).

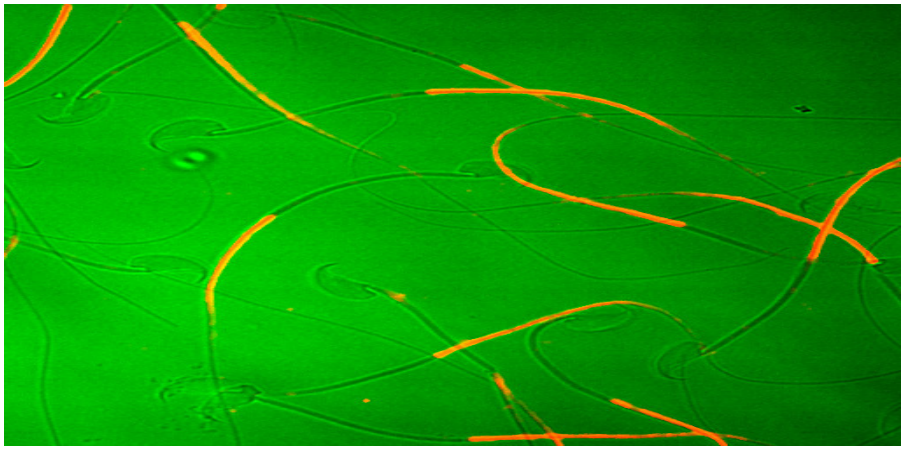

Figure.17. The image represents the immunostaining of mature mouse sperm. This enabling to localize and identify the CatSper ion channels in the principal piece of the flagellum.

CatSper knockout mice were unable to fertilize the egg, due to a decrease in sperm motility (Ren et al., 2001). CatSper1 and CatSper2 play a critical role in sperm hyperactivated motility (involved in zona penetration) (Carlson et al., 2003; Quill et al., 2003). Furthermore all four CatSper proteins are required for the alkalinization-activated $I_{CatSper}$ necessary for the stimulation of hyperativation in spermatozoa (Qi et al., 2006). When spermatozoa travel from the vagina, experiencing a pH of 5 to the cervical mucus, to a pH of 8, during this journey spermatozoa experience intracellular alkalinization (Qi et al., 2006). In normal spermatozoa when the internal pH is acidic and the resting membrane potential is of -40mV, the $I_{CatSper}$ show little activity, hence little Ca^{2+} is introduced into the cell. Consequently the conductance of the CatSper is drastically elevated when alkalinization takes place, hence stimulating Ca^{2+}-influx mediated by $I_{CatSper}$ (Kirichok et al., 2006), this drastic Ca^{2+} entry results in an increase

in flagellar bending (Qi et al., 2006). Although, little is known on the mechanism by which intracellular Ca^{2+} modifies the flagellar bend in spermatozoa (Qi et al., 2006).

1.7.3 Calcium (Ca^{2+}) Clearance Mechanisms in sperm

In most of the cells Ca^{2+}clearance is performed mainly by ATP Ca^{2+}pumps (Ca^{2+}-ATPase) or by a Na^{+}-Ca^{2+} exchanger (NCX) **[Figure.13.].** These exclude Ca^{2+} from the cell or into intracellular membranous Ca^{2+} stores A variety of experiments carried out on mouse sperm emphasise the importance of the Ca^{2+} pumps and Ca^{2+} exchangers in Ca^{2+} clearance in mammalian sperm (Wennemuth et al; 2000; Jimenez-Gonzales et al., 2006). Membrane Ca^{2+} ATPase pumps are the fastest Ca^{2+} extrusion mechanism in sperm, while Na^{+}-Ca^{2+} exchanger and MCU (mitochondrial Ca^{2+} uniporter) are about a third as fast (Wennemuth et al; 2000; Jimenez-Gonzales et al., 2006)

1.7.3.1 Ca^{2+} pumps in Spermatozoa

There are three types of ATP-utilising Ca^{2+} pumps: the plasma membrane Ca^{2+} ATPase (PMCA); the sarcoplasmic-endoplasmic Ca^{2+} ATPase (SERCA) and the secretory pathway Ca^{2+} ATPase (SPCA) (Michelangeli et al., 2005) **[Figure.13.].** All of the three types demonstrate approximately 30% sequence similarity to each other (Guteski-Hamblin et al., 1992) suggesting that these have similar structures and similar mechanism of action (Jimenez-Gonzales et al., 2006). The Ca^{2+} pumps in sperm are all

part of the P-type family of ATPase, which become temporarily phosphorylated enabling the transportation of the ions across the membrane via an E1 to E2 conformational change, as proposed by De Meis and Vianna (1979).

The SERCA 1a isoforms and all other types of Ca^{2+} ATPase, are composed of three large cytoplasmic domains: ATP binding; phosphorylation and an actuator domain which contributes to the rearrangement of the transmembrane helices, allowing Ca^{2+} to move from one side of the membrane to the other (Toyoshima and Inesi, 2004).

1.7.3.2 Plasma Membrane Calcium (Ca^{2+}) ATPase (PMCA)

With a molecular weight of 130-140kD, PMCAs represent the largest of the three types of Ca^{2+} ATPase. Their large size correlates with the presence of an additional calmodulin-binding region located at the C-terminus of the protein; involved in the regulation of ATPase activity (Carafoli and Brini, 2000). PMCA has four isoforms (PMCA 1 – 4) and around a dozen splice variants (Carafoli and Brini, 2000). Isoforms PMCA1 and PMCA4 are identified in most mammalian tissues, suggesting a role in cell Ca^{2+} homeostasis.

PMCA proteins have been identified in germ cells, in rat spermatids and mouse spermatozoa (Berrios et al., 1998; Wennemuth et al., 2003) **[Figure.13.].** PMCA in mouse sperm has been localized and identified in the principal piece of sperm flagellum and PMCA4 located in the principal piece (Okunade et al., 2004; Schuh et al., 2004). Absence of the sperm's PMCA (in flagellum) (PMCA4-Null mice) resulted in

inmotility in spermatozoa incubated under conditions that would normally lead to hyperactivated motility. This apparently reflects failure of Ca^{2+} regulation in the sperm flagellum (Publicover et al., 2007; Schuh et al., 2004; Okunade et al., 2004).

1.7.3.3 Sarcoplasmic-endoplasmic Ca^{2+} ATPase (SERCA)

The presence and role of SERCA in mature sperm is still a matter of controversy. Quantitative studies suggest that Ca^{2+} clearance in mature mouse sperm is unlikely to involve activity of SERCA (Wennenmuth et al., 2003). SERCA has been identified/localized (involving BODIPY-FL-thapsigargin, fluorescent analogue) in the acrosome and midpiece of spermatozoa (Rossato et al., 2001), **[Figure.13.].** However, attempts to detect SERCA in sperm by Western blotting have produced varying results (Harper et al., 2005; Lawson et al, 2008).

Rossato et al. (2001) reported that, ion mature human sperm, both Ca^{2+} mobilization and acrosome reaction could be induced by a SERCA-specific inhibitor thapsigargin (10-100nM). However, application of very high (non-specific conc. to inhibit SERCA) concentrations (1-10µM) of thapsigargin were required to induced Ca^{2+}-mobilization and disruption of Ca^{2+}-signaling in sperm (Wictome et al., 1992, Brown et al., 1994; Harper et al., 2005) lower (more specific) concentrations having no effect (Jimenez-Gonzales et al., 2006).

mRNA from two SERCA isoforms (SERCA 2 & SERCA 3) has been demonstrated to be expressed in mouse spermatids (Hughes et al., 2000). Additionally in rat spermatids it was able to identify a specific SERCA interaction (applying thapsigargin <100nM) (Berrios et al., 1998). These findings suggest that sperm SERCA may only be required during spermatogenesis and once the sperm matures this is not further expressed or degraded (still unclear why this occurs) (Jimenez-Gonzales et al., 2006).

1.7.3.4 Secretory pathway Ca^{2+} ATPase

In somatic cells the SPCAs are located on the Golgi apparatus or secretory vesicles (Wuytack et al., 2003; Wootton et al., 2004). Within the Golgi the SPCAs might control the levels of Ca^{2+} and Mn^{2+} in order to regulate its physiological functions (Missiaen et al., 2004; Michelangeli et al., 2005). Two isoforms, SPCA 1 and SPCA 2, have been identified. These show 60% sequence similarity to each other (Gunteski-Hamblin et al., 1992). The mRNA for SPCA1 has been identified in rat spermatids (Wootton et al., 2004) and the protein has been detected in mature human sperm (Harper et al, 2005). Immunolocalisation showed the protein in the anterior midpiece and the back of the sperm head, **[Figure.18.],** (Harper et al., 2005), possibly suggesting expression in the putative Ca^{2+} store of the redundant nuclear envelope (RNE) (Ho and Suarez, 2003).

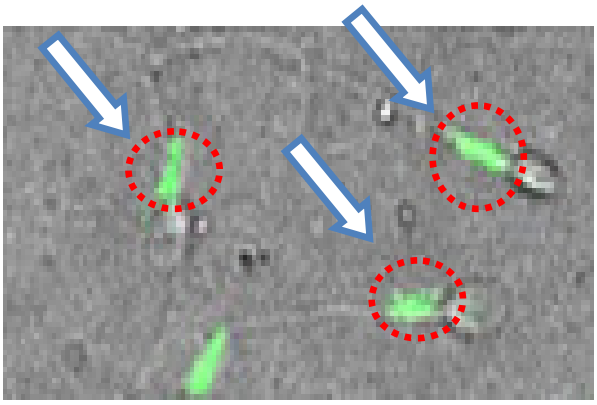

Figure.18. Image representation of the localization of the SPCAs in mature human sperm, present in the anterior region in the anterior midpiece and further extending to the back of the head, the neck region (Jimenez-Gonzalez et al., 2006). Image from: Jimenez-Gonzalez et al., 2006.

1.7.3.5 The Sodium/Calcium (Na^+-Ca^{2+}) Exchanger

The Sodium/Calcium (Na^+-Ca^{2+}) exchanger exports calcium ions (Ca^{2+}) using the energy from the sodium gradient (Na^+) at the cell membrane **[Figure.13.].** These exchangers have been located in various tissues, such as the cardiac, smooth and skeletal muscle, nervous system and the retina rod cells strongly suggesting their crucial role in homeostasis of calcium (Shiba et al., 2006; Blaustein and Lederer, 1999). Confocal microscopic studies by Krasznai et al. (2006), showed a heterogeneous distribution of the NCX, where binding of antibody was more remarkable in the acrosomal region and midpiece of spermatozoa (Krasznai et al., 2006) **[Figure.19.].** The ATP in these exchanger, is used indirectly through activity of the Na^+, K^+-ATPase (Blaustein and lederer, 1999; Philipson and Nicoll, 2000). NCX is capable of working in

reverse mode, exporting Na^+ and importing Ca^{2+}, the direction of the transport is dependent on the electrochemical gradients of the substrate ions (Philipson et al., 2002: Iwamoto, 2004). There are two groups of Na/Ca exchanger, the Na/Ca (NCX) exchanger and K^+-dependent Na/Ca exchanger (NCKX) (Jimenez-Gonzales et al., 2006). The Na^+/Ca^{2+} exchanger present in the plasma membrane of mammalian spermatozoa are thought to play a crucial role in the tight regulation of Ca^{2+} homeostasis (Reddy et al., 2001; Su and Vacquier, 2002). Krasznai et al. (2006) showed that an elevation in intracellular Ca^{2+} activates NCX, resulting in a decrease in intracellular Ca^{2+}, simultaneously elevating intracellular Na^+, later removed (the excessive sodium) by other energy-required active transport mechanisms (Márián et al., 2005). Furthermore the inhibition of NCX with various blockers of sperm motility was also inhibited, suggesting that NCX might participate in human sperm motility (Krasznai et al., 2006). Vines et al. (2001) showed that the motility initiation of herring spermatozoa is controlled by a reverse NCX (Krasznai et al., 2006) In spermatozoa of invertebrates, sea urchin, a flagellar K^+-dependent NCX maintains low calcium levels, demonstrated by Su and Vacquier (2002).

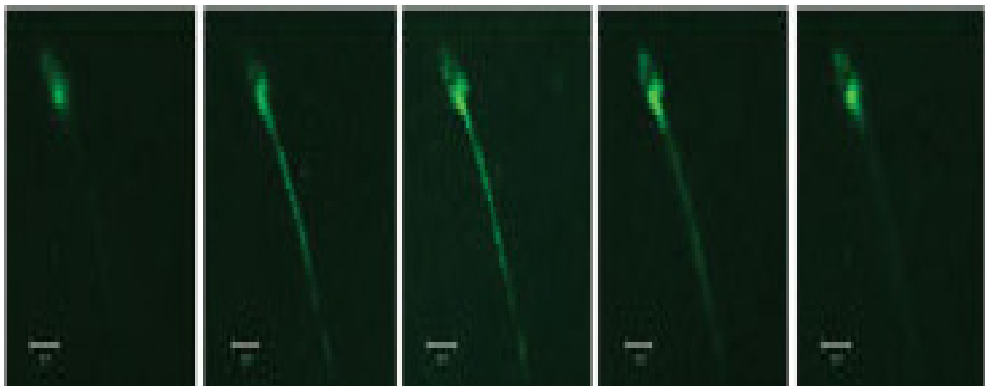

Figure.19. Image of human spermatozoa labeled with NCX anti-body and visualized with Alexa 488 conjugated secondary antibody (Kraszanai et al., 2006). Image sequence from: Kraszanai et al., 2006.

1.7.3.6 Mitochondrial Calcium (Ca^{2+}) uptake

Calcium (Ca^{2+}) uptake by mitochondria is a well recognized and studied physiological process in somatic cells; acting as a regulator of mitochondrial function and in the majority of the cells contributing to the generation and the shaping of the calcium $[Ca^{2+}]_i$ signals (Bianchi et al., 2004). In spermatozoa the mitochondria are only located in the midpiece and potentially have an important Ca^{2+}-buffering effect within this location.

Various studies have shown that the mitochondria area able to accumulate calcium (Ca^{2+}) *in situ* (Storey & Keyhani, 1973; 1974; Babcock et al., 1976). Additional experimentation involving sperm of various mammalian species (at various stages of maturation) suggest that the nature of mitochondrial accumulation may vary and may be regulated (Jimenez-Gonzalez et al., 2006). Furthermore it has been proposed that in

human spermatozoa, mitochondria might be involved in the maintenance of stable $[Ca^{2+}]_i$ levels (Jimenez-Gonzales et al., 2006).

1.7.4 Mobilization of stored Ca^{2+} in spermatozoa

1.7.4.1 IP_3 Receptors (IP_3R)

The physiological role and nature of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channels (IP_3 receptor or IP_3R) has been studied extensively. These are present in somatic cell as well as germ cells (Vermassen et al., 2004). These channels are activated by the binding of IP_3 (inositol triphosphate) second messenger, resulting in an increase in intracellular calcium concentration $[Ca^{2+}]_i$ (Michelangeli et al., 1995). IP_3 is produced by the activation of phospholipase C which leads to hydrolysis of phospatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and IP_3. In mammalian organisms three isoforms of these channels have been identified (identified as $IP_3R1,2$ and 3) and in humans these show more than 74% sequence similarity with each other (Taylor et al., 1999).

The IP3R genes encode a single polypeptide composed of approximately 2500 amino acid residues, which can be subdivided into three major domains. IP3 binding domain is the region closest to the amino-terminus; the location of the membrane-spanning spanning channel domain is the region closest to the C-terminus (Jimenez-Gonzales et al., 2006). In between these regions there is a great array of phosphorylation sites, ATP

binding sites and other regulatory sites, referred to as modulatory or coupling domains (Bultynck et al., 2003). The IP_3R has a tetramer conformation in its native state (Da Fonseca et al., 2003). It has been demonstrated that mammalian sperm express the G-protein Gq and PLCβ and are therefore capable of agonist-stimulated production of IP_3 (Walensky & Snyder 1995; Kuroda et al., 1999).

IP3Rs were identified in the acrosomal region within the sperm head, suggesting that the acrosome may contain stored Ca^{2+} ready to be mobilized (Walensky & Snyder 1995). Furthermore, the type 1 isoform has been located in the same region (isoform-specific IP_3R antibodies) in human and bovine sperm (Kuroda et al., 1999; Ho & Suarez 2003). The IP_3R labeling in this site was reduced or lost when sperm acrosome reacted (AR), suggesting their presence in the outer acrosomal membrane (Walensky & Snyder 1995 and Kuroda et al., 1999). Labeling of human sperm with IP3R3-specific antibodies was also detected in the sperm neck and midpiece. IP_3R2 was not detected (Jimenez-Gonzalez et al., 2006). In other mammalian sperm such as bull sperm, labeling using IP3R1-specific antibodies showed that the region at the back of the head, identified as the redundant nuclear envelope (RNE), was labeled (Ho & Suarez, 2003),**[Figure.20.].** In human spermatozoa anti-IP3R also localizes to the nuclear envelope in 50% of spermatozoa, although it was more intense in the acrosomal region (>90% of the cells) (Naaby-Hansen et al., 2001).

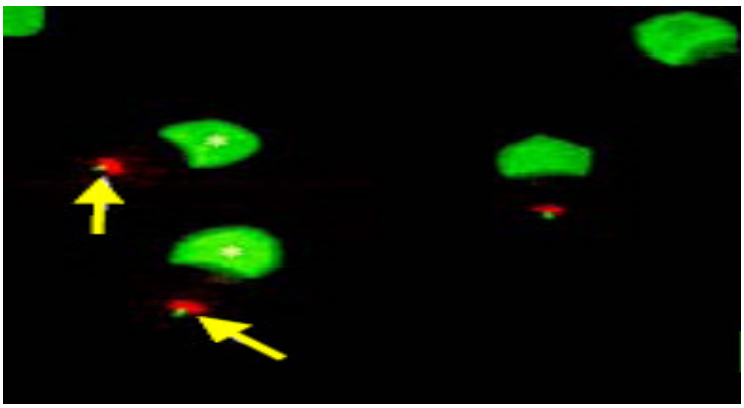

Figure.20. Images of bovine spermatozoa representing immunolocalisation of InsP3Rs, in green, and the nuclear pore complex proteins, in red, indicating the location of the RNE (Costello et al., 2008). The InsP3 receptors are located over the acrosomal region and the neck of the spermatozoa (yellow arrow indicating location). Image from Constello et al.., 2008.

Two classes of binding sites have been identified in IP_3R isoforms located within sperm, a high affinity (Kd of 20-30nM) and a lower affinity binding site (Kd of 1-2 uM) (Walensky & Snyder 1995; Kurida et al 1999). The difference in binding site is possible due to the presence of the two IP_3R isoforms present in sperm, IP3R1 and IP_3R3; which have been shown to have different IP3R binding affinities (Wojcikiewicz & Luo 1998) and different IP_3 sensitivities for Ca^{2+} release (Dyer and Michelangeli, 200) (Jimenez-Gonzalez et al., 2006). The physiological role of IP_3R has been demonstrated in sperm (Herrick et al., 2005), by the stimulation of the AR with thimerosal (IP3R activator) (assessor of Ca^{2+} mobilization from IP_3R containing Ca^{2+} stores in sperm) (Bootman et al 1992; Sayers et al 1993). Calreticulum (a low affinity, high capacity Ca^{2+} buffering protein), which is associated with IP_3R containing Ca^{2+} stores in somatic cells, is also present in the acrosomal and sperm neck, consistent with the presence of Ca^{2+} stores in

these regions (Naaby-Hansen et al., 2001, Ho & Suarez , 2003). In human spermatozoa the Ca^{2+} stored in the RNE was shown to be mobilized upon progesterone stimulation by Ca^{2+}-induced Ca^{2+} release, in an IP_3-independent manner (Harper et al., 2004).

1.7.4.2 Ryanodine Receptor (RyR) in Sperm

Ryanodine receptors (RyRs) were initially identified in the sarcoplasmic reticulum membrane of skeletal muscle (Jimenez-Gonzales et al., 2006). RyRs in the skeletal muscle sarcoplasmic reticulum function as a Ca^{2+}-induced Ca^{2+} release channel, which play an important role in excitation-contraction coupling of striated muscle (Fill & Copello, 2002). The genes for the three mammalian isoforms of RyR code a large protein composed of 500 amino acids and show a high degree of sequence homology with each other (approx. 70% overall) (Brini, 2004). The different RyR isoforms are located in different areas, RyR1 located in skeletal muscle, RyR2 located in cardiac muscle and RyR3 in the brain, although this is further distributed (Brini, 2004). The RyRs are composed of two major domains: the amino-terminal region, which forms a large cytoplasmic structure (possibly containing ligand and modulatory protein binding sites), and the C-terminal region forming the transmembrane channel domain (possibly composed of 4 membrane helices) (Brini, 2004). In addition to variations in $[Ca^{2+}]_i$, RyRs are potentially activated by changes in cyclic adenosine diphosphate-ribose (cADPR) (a putative second messenger), and via conformational-coupling with other associated proteins (Zucchi & Ronca-Testoni, 1997; Jimenez-Gonzalez et al., 2006).

In mouse sperm the developing spermatocytes and spermatids express RyR1 and RyR3 (Trevino et al., 1998 & Chiarella et al., 2004). Only the RyR3 isoform was detected in mature sperm and was present in both intact and acrosome-reacted sperm (Trevino et al., 1998) **[Figure.13.].** Human sperm, show progesterone-induced intracellular (Ca^{2+}) oscillations that are IP3-independent but are influenced by ryanodine with low doses increasing the frequency of the intracellular (Ca^{2+}) oscillation and higher doses reducing the frequency (Harper et al., 2004). Human sperm specifically labeled with fluorescent analogue of ryanodine (BODIPY-FL-X-ryanodine) showed staining mainly focused around the sperm neck., co-localising with SPCA1 and with oscillations of $[Ca^{2+}]_i$ that occur in response to progesterone stimulation (Jimenez-Gonzales et al., 2006). Additionally low levels of labeling were observed in the acrosome, **[Figure.21.]** (Harper et al., 2004).

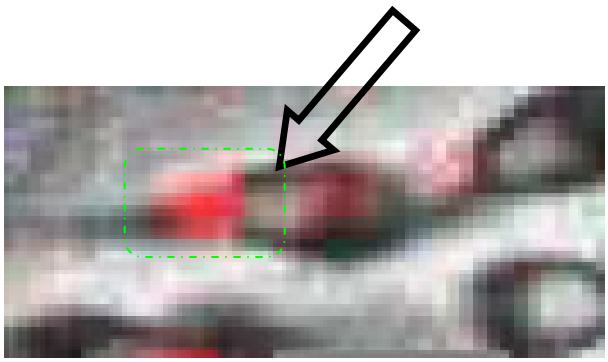

Figure.21. Image showing the localization of the RyR in human spermatozoa by fluorescent analogue of ryanodine (BIDIPY-FL-X-ryanodine). Image modified from: Jimenez-Gonzalez et al., 2006.

1.8 Guidance mechanisms for mammalian sperm *in vivo*

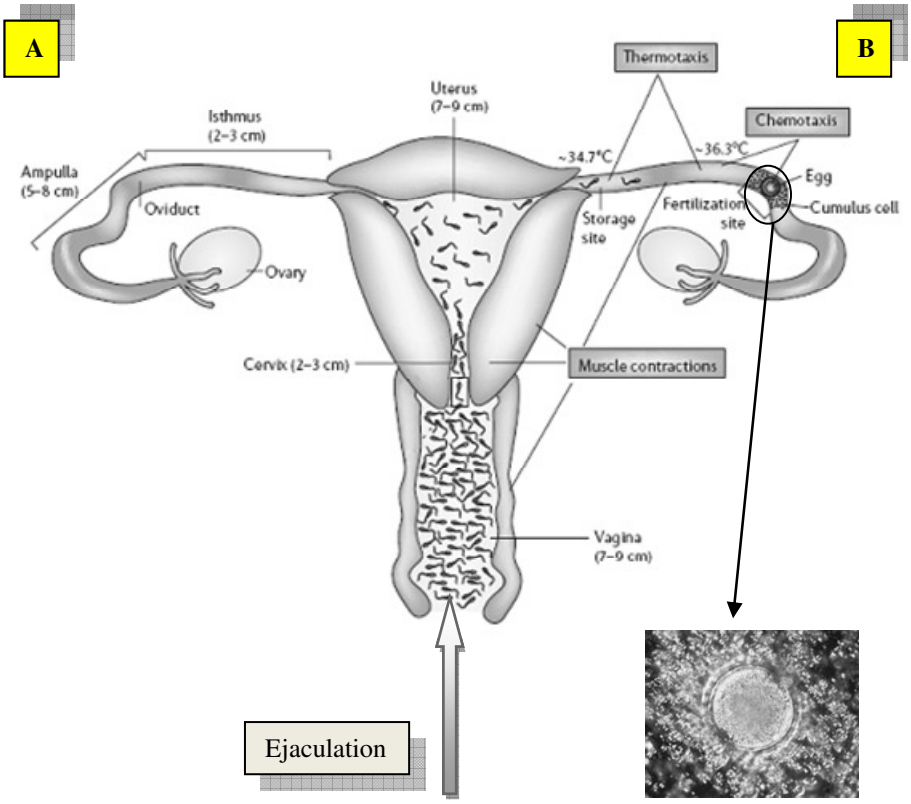

Figure.22. (A) Diagrammatic representation of physiological route human sperm must travel in order to reach and successfully fertilise the oocyte. Additionally exposing the potential mechanisms involved in order to facilitate the sperm's transportation through the female genital tract, to increase the possibilities of a successful fertilization of the egg **(B)** Image representation of the egg covered by hundreds of cumulus cells (Eisenbach & Giojalas, 2006). Diagram modified from Eisenbach & Giojalas, 2006.

During their passage through the female tract (Section 1.8.1 &1.8.2) sperm, at least in some mammals, are 'stored' in the region of the isthmus. At the time of ovulation a temperature gradient occurs between this storage site and the fertilization site (Smith & Yanagimachi, 1991; Lefebvre & Suarez, 1996; Eisenbach & Ralt, 1999; Suarez, 1998)

which may act to guide the sperm to the oocyte by thermotaxis (Bahat et al., 2005; David et al., 1972; Hunter et al.,1986; Eisenbach et al., 2006) **[Figure.22.]**. Sperm at the storage site are maintained with low $[Ca^{2+}]_i$, which maintains longevity and function; detachment is associated with an increase in $[Ca^{2+}]_i$ leading to vigorous motility (Publicover et al., 2007). Spermatozoa may also encounter chemoattractants secreted along the oviduct (Eisenbach & Giojalas, 2006), potentially providing a series of short-range chemotactic cues which guide the sperm to the fertilisation site (Eisenbach et al., 1999; 2004;Eisenbach & Giojalas, 2006). As sperm approach the vicinity of the fertilization site they may sense a chemoattractant gradient originating from the cumulus cells (Sun et al., 2005), which guides them to the egg-cumulus complex (Sun et al., 2005; Eisenbach & Giojalas, 2006). Finally, a chemoattractant gradient established within the cumulus matrix, originating at the egg may guide spermatozoa to the egg (Sun et al., 2005) **[Figure.22.]**.

1.8.1 *In vivo* Chemoattractant Source(s)

Follicular fluid (FF), which is composed of pre-ovulatory secretions of the egg and its surrounding cells, was the first physiological substance to be investigated (for chemotaxis) and hypothesised to be a chemoattractant source *in vivo* (Eisenbach, 1999, 2004, 2006). Follicular fluid and oviductal fluids are mixtures of different molecule types (peptides, steroids, heparin, adrenaline, oxytocin, calcitonin and acetylcholine) (Tevez et al., 2006). In mammalian spermatozoa follicular fluid has been shown to

have a chemotactic effect (Ralt et al., 1994) and in human spermatozoa this correlates with the chance of fertilizing an egg (Ralt et al., 1994) (Eisenbach et al., 1999, 2004, 2006). However, sperm chemotaxis to FF in vivo is very unlikely (Sun et al., 2005) since follicular fluid is only released as a single event at ovulation (Eisenbach & Giojalas, 2006), whereas the chemoattractant gradient in the oviduct should be maintained throughout the period of residence of the oocyte in the female tract, in humans this would be approximately 24 hours post-ovulation (Harper et al., 1982). Thus the chemoattractants must be secreted not only prior to ovulation (into the fluid within the follicle) but also after egg maturation outside the follicle (Sun et al., 2005; Eisenbach & Giojalas, 2006). This was demonstrated with chemotactic responsiveness of sperm towards media containing mature eggs (human) and the cumulus cells (Sun et al., 2005). These observations suggest that oocyte and the cells of the surrounding cumulus oophorus independently secrete sperm chemoattractants (Sun et al., 2005).

1.8.2 Human sperm chemoattractants

Spermatozoa accumulation in response to a chemical gradient may be due to processes other than chemotaxis, such as chemokinesis and sperm trapping (Eisenbach et al., 1999, 2004; Eisenbach & Giojalas, 2006). Sperm trapping is the net effect on spermatozoa that result in cell accumulation in a particular location due to a reduction in sperm speed. This is possibly due to a negative effect of a stimulus on motility, from a gradient-independent change in swimming behavior in response to a specific concentration, as a result of mechanical effects such as sperm interaction with glass or

capillary, or possibly due to a combination of all of these (Eisenbach et al., 2004, Eisenbach & Giojalas, 2006). On the other hand, chemokinesis is an increase in sperm cell motility in response to a stimulus (Eisenbach et al., 1999, 2004; Eisenbach & Giojalas, 2006). Studies involving the identification of putative chemoattractants should have a clear-cut criterion for distinguishing between these processes (Eisenbach, 1999, 2004, 2006) To date, only a small number of putative chemoattractants satisfy the criteria for acceptance as true chemoattractants. Their physiological significance, if any, is still unclear (Eisenbach et al., 1999, 2006).

Identified sperm chemoattractants in non-mammalian animals are primarily peptides or proteins with a low molecular mass (MM) (1-20kDa), heat stable and sensitive to proteases (Miller, 1985; Cosson, 1990). Exceptions to the above include sperm chemoattractants of corals, lipid-based substances (140-250 Da) (Coll and Miller, 1992), and the attractants of ascidians ciona, which are nonproteinaceous small molecules (Yoshida et al., 1993). Identified sperm attractants for plants, such as ferns are aremalic acids (partially ionized) and a great array of unsaturated four-carbon cis-dicarboxylic acids (Cosson et al., 1990). Sperm chemoattractants for algae are pheromones of low molecular mass (Maier & Müller, 1986; Cosson, 1990).

In mammals, the identity of the chemoattractant produced by the egg is still unknown, but there are a number of candidates including progesterone, which has been reported to be a chemoattractant secreted by the cumulus cells (Eisenbach et al., 2004, 2006; Teves et al., 2006, 2009).

Progesterone is the main steroid hormone present in the egg and has been assayed for human sperm chemotaxis by different groups, giving rise to contradictory results (Sliwa et al., 1995; Villanueva-Diaz et al., 1995; Wang et al., 2001; Jeon et al., 2001; Jaiswal et al., 1999). Initially it was demonstrated that at nM to mM concentration progesterone lead to sperm accumulation and this was inhibited by a specific progesterone receptor antagonist (Sliwa et al., 1995; Villanueva-Diaz et al., 1995; Wang et al., 2000). However, it was later shown that this was due to sperm trapping as a result of hyperactivated motility induced by progesterone (Jaiswal et al., 1999).

Teves 2006, demonstrated that at pM concentration range progesterone is a true chemoattractant for human and rabbit spermatozoa (Teves et al., 2006). Futhermore, it has also has been demonstrated that within the cumulus cell mass there is a gradient of progesterone production which will result in a progesterone concentration gradient from the centre to the periphery of the cumulus cellular mass (Teves et al., 2006).

Post-ovulation, the cumulus cells synthesise and secrete progesterone (Yamashita et al., 2003) and its carrier proteins (Baltes et al., 1998). Secreted progesterone becomes soluble (Sun et al., 2005). It has been proposed that the role of progesterone in vivo could be the following: In the vicinity of the cumulus the low concentration of progesterone could activate the high-affinity progesterone receptors, resulting in the attraction of sperm toward the cumulus mass via chemotaxis (Teves et al., 2006). When the sperm reaches the cumulus cells the levels of progesterone are much higher, leading to the activation of the low-affinity progesterone receptors and the stimulation of hyperactivated motility, enabling them to pass across the cumulus mass and the zona

pellucida (ZP) (Teves et al., 2006). Furthermore, the secretion of an unknown chemoattractant by the egg would result in the sperm guidance to the egg surface (Sun et al., 2005; Teves et al., 2006). Teves et al. (2009) propose that when progesterone binds to a surface receptor, it stimulates tmAC elevating cAMP and possibly activating PKA. This might result in the mediation of protein phosphorylation in sperm equatorial segment and tail region **[Figure.23.]**. They also propose a cascade of other signals including activation of PLC, producing DAG and IP3, release of stored Ca^{2+} and CCE (Teves et al., 2009).

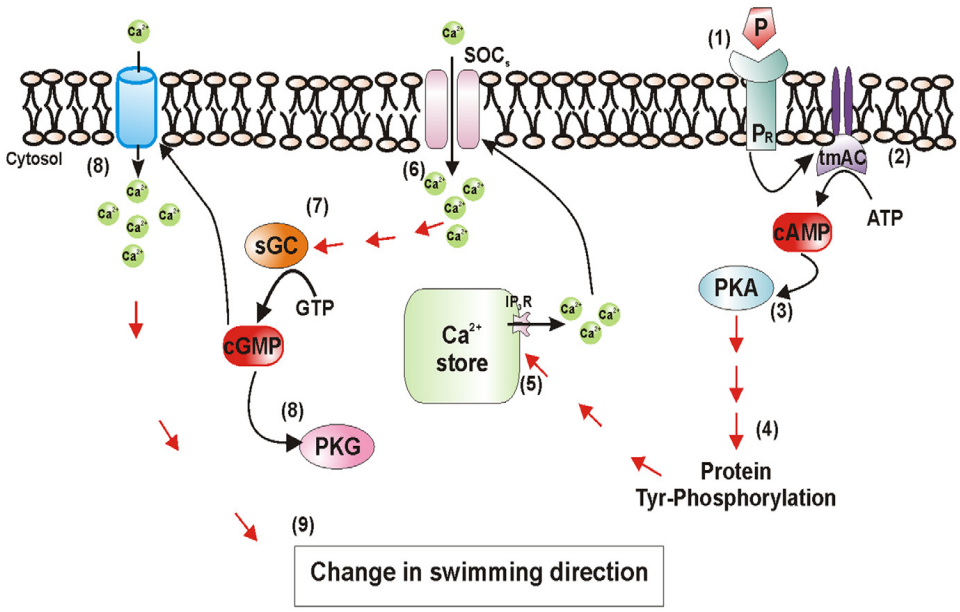

Figure.23. Proposed model of chemotactic siganaling when human spermatozoa sense a concentration gradient (ascending) of progesterone. Diagram from: Teves et al., 2009.

Nitric oxide (NO) a highly reactive free radical, synthesized from L-arginine by NADPH-dependent NO synthases (Wink et al., 1998; Miraglia et al., 2007), plays an important role in various biological processes, such as vasodilatation, neurotransmission, immune response, and apoptosis and recently in mammalian sperm chemotaxis (Miraglia et al., 2007). The exposure of spermatozoa to low concentrations of NO has shown to enhance the motility of mouse, hamster, and human spermatozoa, and the ability of mouse, bull and human spermatozoa to acrosome react, together with the stimulation of the binding ability of human spermatozoa to ZP (Herrero et al., 2001; Revelli et al., 2002). Moreover, high concentration of this free radical impairs the motility of human spermatozoa and has negative effects on the viability (cytotoxic), and metabolism of human spermatozoa in vitro (Revelli et al., 2002; Rosselli et al., 1995). Miraglia et al. (2007) demonstrated that human sperm chemotaxis by NO, using a NO donor GSNO (100nmol/L), may involve a NO/cGMP-signalling pathways, with an experimental assay capable of discriminating between chemotaxis and other processes that result in sperm accumulation. Furthermore, with different NO donor (sodium nitroprusside) it has been reported that mouse spermatozoa show a chemotactic response to NO at a lower concentration, 50nmol/L (Sliwa et al., 2000). These difference in concentration response may be attributed to a difference is NO donors, time of incubation and/or the different sample species investigated (Miraglia et al., 2007). Furthermore mammalian spermatozoa encounter cues of NO concentrations whilst approaching the oocyte (Machado-Oliveira et al., 2008) **[Figure.24.].** Like progesterone (Teves et al., 2006, 2009) synthesized by occyte and cummulus cells (Sun

et al., 2005), NO might represent another important candidate for *in vivo* sperm chemotaxis (Miraglia et al., 2007).

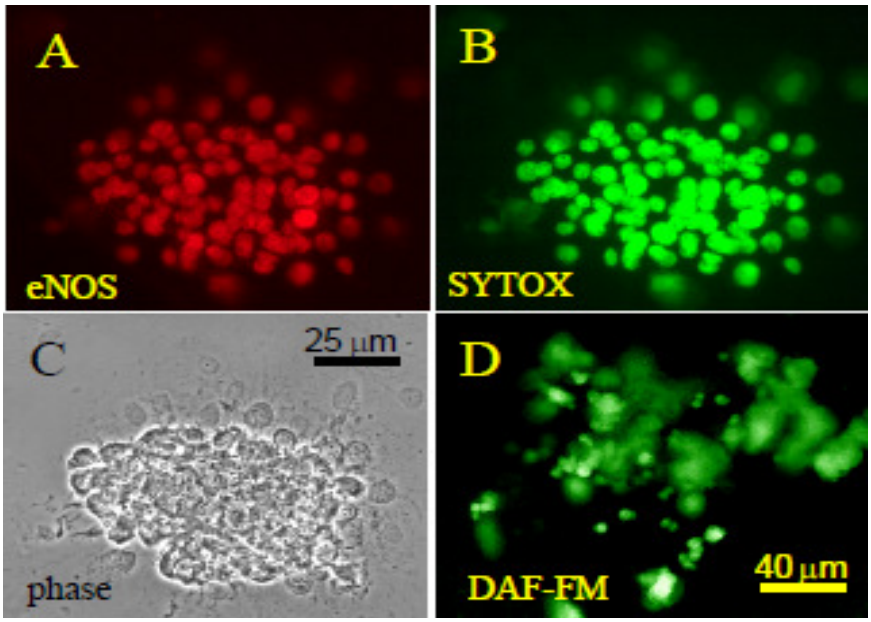

Figure.24. Images demonstrating the detection of NOS and NO synthesis in the cummulus cells of human female. **(A)** Human cumulus fragment sample stained for the detection of eNOS, (Machado-Oliveira et al., 2008), **(B)** Cumulus cells (same sample) stained with SYTOX Grenn to identify and locate all the cells **(C)** Phase image of cumulus cells. **(D)** DAF-FM diacetate staining (green fluorescence) of the human cumulus cells. Images from: Machado-Oliveira et al., 2008.

The chemokine **RANTES** is a potent chemoattractant for eosinophils, monocytes and T lymphocytes (Fukuda et al., 2004; Alam et al., 1993). RANTES is a 68-amino acid peptide of 8kDa (Nelson et al., 1993; Schall et al., 1990) is a member of the CC subfamily of chemokines (Wells et al., 1999). Human spermatozoa are exposed to this in the female and male genital tract before they reach the fertilization site (Naz & Leislie, 2000). RANTES is present in seminal plasma (Naz & Leslie, 2000), uterine

fluid (Hornung et al., 1997) and peritoneal fluid (Khorram et al., 1993; Hornung et al., 2001) as well as FF. The mRNA for its receptor has also been identified in human spermatozoa (Isobe et al., 2002). This chemokine is produced in the follicle in the ovaries by the granulosa cells, prior to ovulation (Eisenbach et al., 2006). Furthermore the production of RANTES is upregulated in some diseases that are associated with infertility, such as endometriosis (Khorram et al., 1993; Hormung et al., 2001) and male genital tract infection (Naz & Leslie, 2000). However the human chemotactic role of RANTES *in vivo* has not been demonstrated, it is also unknown if RANTES is also secreted in the female reproductive tract after ovulatory process (Eisenbach et al., 2006). Isobe et al. (2000) demonstrated that RANTES had a dose-dependent chemotactic effect on human spermatozoa. In FF the average concentration of RANTES in a "healthy" woman is 174 pg/ml (Machelon et al., 2000), in a patient affected by endometriosis this concentration was elevated to 530.2 pg/ml (Khorram et al., 1993). Isobe et al. (2000) examined the chemotactic effects of RANTES using a physiological concentration 234 pg/ml, demonstrating its chemotactic effect on human spermatozoa. Furtheremore, the same group demonstrated that the chemotactic effect of RANTES was neutralised by anti-RANTES rabbit IgG, simultaneously the anti-RANTES antibody inhibited the chemotactic effect observed with FF (Isobe et al., 2000). Further demonstrating the *in vitro* effect of RANTES on human spermatozoa chemotaxis.

ANP (polypeptide hormone) is secreted in large quantities by the atrial section of the heart; although it can also be secreted by a variety of mammalian cell types. The effect of ANP is dependent on the activation of particulate cyclase (Brenner et al., 1990;

Ruskoaho, 1992). ANP has been identified as a component of human follicular fluid (Sundfjord et al., 1989) and specific ANP receptors have been identified on human spermatozoa (Silvestroni et al., 1992). Sperm accumulation as a result of chemotaxis was demonstrated in vitro with ANP, in capillaries with ascending (Anderson et al., 1995) and descending (Zamir et al, 1993) gradient and by choice assays (Zamir et al., 1993). Chemotaxis to ANP at physiological concentrations is only possible with the presence of a neutral endopeptidase inhibitor such as phosphoramidon (Anderson et al., 1995; Zamir et al., 1993). ANP hormone has been demonstrated to be able to activate particulate guanylyl cyclase; the same way to the physiological attractant in vivo (Brenner et al., 1990; Rukohoaho et al., 1992; Anderson et al., 1995). However is uncertain whether ANP plays a physiological role in sperm chemotaxis in vivo (Zamir et al., 1993).

Heparin is present in follicular fluid and induces capacitation of bull spermatozoa (Eisenbach et al., 1999). In human spermatozoa heparin induces the acrosome reaction (AR) (Silwa et al, 1993). Heparin leads to leads to sperm accumulation via the stimulation of hyperactivated motility which leads to sperm trapping; chemotaxis to this substance has not yet been reported (Eisenbach, 1999, 2006). In vitro studies have demonstrated that **hyaluronic acid** (HA) a glycoaminoglycan present in human oviductal fluid has an influence in sperm motility and causes accumulation in the wells containing HA (Sliwa et al., 1999). Although, the absence of FF post-ovulation would consequently suggest that HA does not play a chemotactic role post-ovulation.

Antithrombin III, a component of follicular fluid that is not synthesised in the follicles, has been demonstrated to accumulate boar sperm (Lee et al., 1994). Further assays to distinguish the accumulation from other processes other than chemotaxis have not been carried out. Anitithrombin III has been demonstrated to enhance sperm motility, suggesting that chemokinesis is the process that leads to the accumulation of sperm.

Small synthetic N-formulated peptides, such as N-formyl-Met-Leu-Phe (fMLP) are derived from bacteria (Ralt et al., 1994) and are attractants for neutrophils and macrophages (Schiffmann et al., 1975). The binding of these peptides to specific sites on human spermatozoa (Gnessi et al., 1986; Ballesteros et al., 1988) result in accumulation of human sperm (Gnessi et al., 1985). This effect has also has also been observed in bull sperm (Iqbal et al., 1980). However, further assays investigation lead to the conclusion that the accumulation was not due to sperm chemotaxis (Miller et al., 1982).

Other potential sperm chemoattractants include heparin and hyaluronic acid for human sperm (Sliwa et al, 1993, 1995 & 1999) and mouse sperm accumulation occurs with adrenalin, heparin, oxytocin, calcitonin and acetylcholine (Eisenbach, 1999). The importance of some of these observations with respect to chemotaxis is still an enigma, due to the fact that no distinction was made between the other processes that might cause sperm accumulation (Eisenbach, 1999; **see above**).

Bourgeonal (4-t-Butylbenzenepropionaldehyde) is colourless oil-like substance, **[Figure.25.],** an aromatic aldehyde used in fragrances to mimic the aroma of the Lily of the Valley (Spehr et al., 2004, 2006). Spehr at al. (2003) showed that spermatozoa accumulate at the tip of the microcapillary pipette (the chemoattractant source), in an ascending bourgeonal gradient.

The expression of the α7 nicotinic acetylcholine receptors (AChR) subunit in the midpiece of human spermatozoa (Meizel et al.; 2005; Bray et al., 2005) and stimulation of these by **acetylcholine** leads to a stimulation initiated in the midpiece and later spread to the sperm head; similar to the sperm response to bourgeonal (agonist of hOR17-4) (Spehr et al., 2003). Suggests that acetylcholine might be involved in the regulation of sperm motility. Furthermore mice lacking the α7 nicotinic acetylcholine receptors (AChR) subunit show some deficiencies in their motility and poor hyperactivated motility (Bray et al., 2005). However, no distinction has been made to identify if acetylcholine as a chemoattractant effect on human spermatozoa or its physiological significance, if any, in the female reproductive tract.

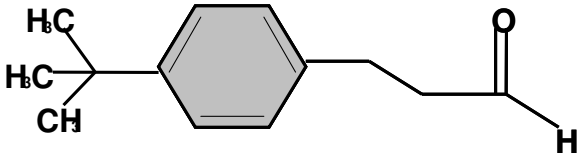

Figure.25. Structural image of bourgeonal ($C_{13}H_{18}O$, Molecular Mass: 190.28) an aromatic aldehyde, a potent agonist of the hOR17-4 located in the midpiece of human spermatozoa (Spehr et al., 2003).

1.9 Sperm olfactory receptor proteins (ORs)

A small number of substances have been identified that apparently act as sperm chemoattractants but are probably not produced or present in vivo. However, the identification of receptors for these compounds and the downstream signalling events might lead to the identification of the natural ligand and understanding of the signalling mechanisms involved in mammalian sperm chemotaxis (Eisenbach et al., 2006). Bourgeonal and its derivative (4-t-Butylbenzenepropionaldehyde) have been identified to cause human sperm accumulation by chemotaxis (Spehr et al., 2003,2004). The action of these agents on sperm is apparently through olfactory receptors, of the type expressed in the olfactory epithelium. Various receptors, enzymes, and ion channel proteins initially thought to be neuron-specific have been identified in sperm (Darszon et al., 1999; Meizel, 2004; Spehr et al., 2006). These include members of both visual and olfactory GPCR-mediated (G-Proteins) signal transduction pathways (Baxendele and Fraser, 2003a), G protein receptor kinase 3 (GRK3) and β-arrestin2 (Walensky and Snyder, 1995), cone photoreceptor CNG channels (Weyand et al., 1994; Wiesber et al., 1998), particulate adelylate cyclases (Defer et al., 1998; Gautier-Courteille et al., 1998; Baxendale and Fraser, 2003b; Spehr et al., 2006).

1.9.1 Olfactory receptors (ORs)

The members of the odorant receptors family are usually found on cilliary membranes of nasal olfactory sensory neurons (OSNs), these are coupled to complex signal transduction pathways (Spehr et al., 2004, 2006). The binding of a ligand to the ORs leads to a modification of the heptahelical OR conformation consequently activating a membrane-bound type III adenylate cyclase (mAC III) via Alpha olf ($G_{\alpha olf}$) (Spehr et al., 2006). cAMP-dependent opening of cyclic nucleotide-gated (CNG) channels followed by the activation of Ca^{2+}-gated Cl^- channels, results in an increase of the intracellular calcium (Ca^{2+}) and sodium (Na^+) concentration and membrane depolarisation (Parmentier et al., 1992; Spehr et al., 2004, 2006).

The odor receptors present in vertebrate organisms are members of the class I G protein-coupled receptors (GPCRs); which share many features with other G protein-coupled receptors (GPCRs) (Mashukova, 2006). These features include a coding region that lacks introns, structural features that predicts seven α-helical membrane-spanning domains linked by intracellular and extracellular loops of variable lengths, and various conserved short sequences (Mashukova, 2006). However, the ORs also have further specific, characteristic features including a long second extracellular loop which contains an additional set of cysteines and also other short characteristic sequences (Mombaerts, 1999).

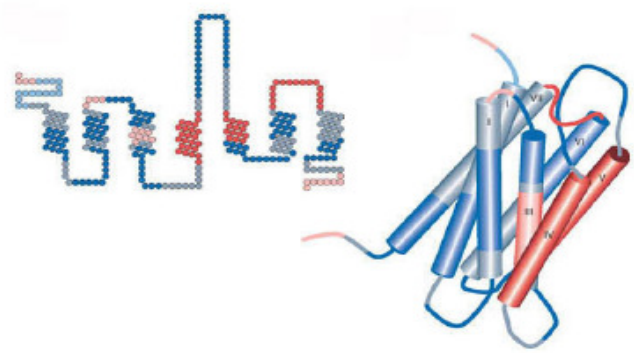

Figure.26. Diagrammatic representation of the three-dimensional (3D) structure of the G-protein coupled receptor (Palczewski et al., 2000; Firestein, 2001). Diagram from: Mashukova, 2006.

Sequence similarity within the members of the ORs family ranges from <40% to over 90% identity (Mashukova, 2006). However, the third, fourth and fifth transmembrane region shows strong divergence (Mashukova, 2006). Three-dimensional (3D) studies of the GPCRs have demonstrated that the three α-helical barrels are parallel to one another and form a pocket, which extends approximately one third of the way into the membrane (Pipel & Lancet, 1999; Mashukova, 2006). Various studies involving other class I GPCRs, have lead to the conclusion that the pocket-forming region represents the binding site of the ligands (Mashukova et al., 2006) **[Figure.26.].** The variability observed among the ORs in this region provides the first molecular basis for understanding the range, diversity and large number of olfactory ligands that can be detected and discriminated (Firestein et al.,2001; Mashukova, 2006).

1.9.2 Olfactory receptor ligands and sperm motility

The presence and the function of the olfactory receptors in sperm have been known for some time (Spehr et al., 2003). Some of the human ORs are mainly expressed in or restricted to the spermatozoon (Parmentier et al., 1992; Vanderhaeghern et al., 1993; Vanderhaeghern et al., 1997). The identification together with the characterization of ORs in human (Spehr et al, 2003) and mouse sperm (Fukuda et al., 2004) has provided a major template for the understanding of the role of these receptors in mammalian sperm (Spehr et al., 2006). The hOR17-4 sperm receptors (protein receptors) reside in the flagellar midpiece (Spehr et al, 2003). hOR17-4 has the capability of accommodating aldehydes of relatively small size such as bourgeonal, demonstrated to be a potent ligand in these receptors (Spehr et al., 2006). Bourgeonal is a synthetic additive used in perfumes in order to mimic the scent of lilies of the valley (Spehr et al., 2003). The effects of bourgeonal can be inhibited by undecanal (antagonist), an aliphatic aldehyde (Spehr et al., 2003). Undecanal binds to the receptor active site without activation of the hOR17-4 (Spehr et al., 2006).

Both of the olfactory receptors, hOR17-4, in human spermatozoa (OR1D2) and mOR23 in mice (mOR267-13) mediate robust calcium (Ca^{2+}) signals in mature spermatozoa and modulation of motility (Spehr et al., 2006). Single cell Ca^{2+} imaging recordings and radiofluorometric population screening showed that the effect of bourgeonal is dose-dependent and leads to Ca^{2+} flux over the plasma membrane (Spehr et al., 2003, 2004, 2006). Elevation of $[Ca^{2+}]_i$ is theory initiated at the spermatozoa midpiece, where the

hOR17-4 is located (Neuhaus et al., 2006). This propagates to the sperm head (with an average latency of >2S) (Jimenez-Gonzalez et al., 2006; Spehr et al., 2004).The changes in intracellular calcium (Ca^{2+}) and spermatozoa swimming behaviour as a result of the activation of the hOR17-4 (in human sperm) seems to be dependent on a cAMP-regulated pathway (Spehr et al., 2003). Bourgeonal probably lead to the activation of membrane adenylate cyclase (mACIII), **[Figure.28.]**, activated via G_{alpha} olfactory ($G_{\alpha olf}$) (identified in sperm flagellum & midpiece), **[Figure.28.]**, resulting in a Ca^{2+} influx through cAMP regulated channels (Spehr et al., 2003, 2004, 2006), **[Figure.27.].** However, the identities and properties of the AC(s) in human sperm is still matter of controversy (Spehr et al., 2004).

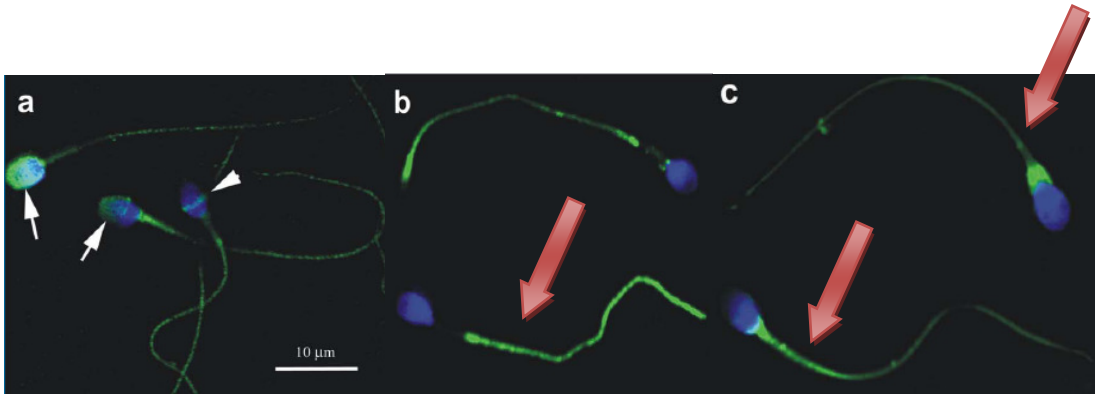

Figure.27. Images representing the identification and location of potential components of the sperm's olfactory signal transduction (Spehr et al., 2004). (**A**) Identification and localization (anti-mACIII) of mACIII in mature spermatozoa identified in the head and midpiece. (**B**) Identification and localization (anti-mACVIII) of mACVIII in mature spermatozoa identified in the flagellum. (**C**) Identification and localization of G-olfactory receptor protein (G_{olf}) in mature spermatozoa identified in the midpiece and the flagellum (Spehr et al., 2004). Image from Spehr et al., 2004.

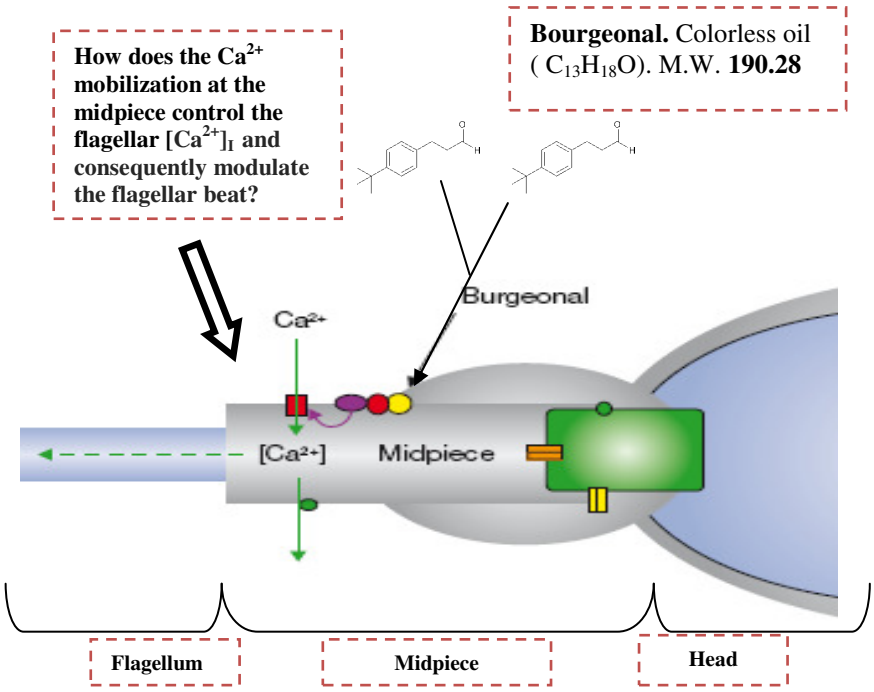

Figure.28. Diagrammatic representation of the proposed mechanism in response to bourgeonal. Binding to the receptor (hOR17.4; yellow circle) stimulates a membrane adenylate cyclase (probably mAC III, purple oval) through Golf (red circle). The consequent rise in cAMP concentration opens a Ca^{2+}-permeable channel, either directly or by activation of PKA allowing influx of Ca^{2+} across the plasmalemma (Publicover et al, 2007). Image modified from: Publicover et al., 2007.

When spermatozoa are placed in an ascending gradient of bourgeonal, this leads to chemotaxis, chemokinesis and hyperactivation in a dose-dependent manner and inhibited by undecanal (Spehr et al., 2006). In the ascending gradient the spermatozoa tend to have a direct swimming patters, towards the chemoattractant source (Spehr et al., 2003, 2004). This further supports the hypothesis that the hOR17-4 might play a role in the sperm-egg communication in vivo (Spehr et al., 2003, 2004, 2006).

RESEARCH AIMS

To:

Study the effect of bourgeonal on $[Ca^{2+}]_i$ and AR in capacitated and non-capacitated mammalian sperm and to compared the effects of bourgeonal with the response to a structural homolog (3,4,CPEE).

Examine the roles of Ca^{2+} and cAMP in determining the kinetics of the $[Ca^{2+}]_i$ response to bourgeonal. Additionally to investigating generation of cAMP in capacitated human spermatozoa exposed to bourgeonal.

Investigate the chemotactic effect of bourgeonal in both capacitated and non-capacitated human spermatozoa. Furthermore, to attempt to demonstrate the crucial role of $[Ca^{2+}]_o$ in response to bourgeonal and if the intracellular stores play a role in the response to the ligand.

Study the response of capacitated spermatozoa to application of a temporal bourgeonal gradient (fM to mM) and progesterone gradient (fm to μM) in order to attempt to study the $[Ca^{2+}]_i$ response.

II

CHAPTER ~TWO~

El amor de mi familia es el fuerza que mueve mi vida. Aduén 2009.

Chapter Two

MATERIALS & METHODS

2.1 Materials

Bourgeonal (4-t-Butylbenzenepropionaldehyde), (>99% pure, 5 M stock), acquired from BIOMOL, UK.

3,4,CPEE; (3-(4'-Carboxyphenyl)-Propionaldehyde Ethyl Ester), (>99% pure, 5 M stock), acquired from Fluorochem, UK. DMSO (Dimethyl Sulfoxide) Percoll, Formaldehyde, Progesterone were acquired from SIGMA-Aldrich (USA). IBMX IBMX (3-isobutyl-1-methylxanthine), dbcAMP (dibutyryl cyclic adenosine monophosphate) and Trifluoperazine (2HCL, Stelazone) acquired from ENZO Life Sciences (UK). Oregon Green 488 BAPTA 1-acetoxymethyl (OGB-1AM) and 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate were obtained from Invitrogen Molecular Probes (Paisley, UK). Poly-D-lysine (PDL) was acquired from BD Biosciences (Oxford, UK). Earls Balanced Salt Solution (sEBSS), HEPES-buffered media, Low Calcium Earls Balance Salt Solution (referred as Calcium Free media), all were made in the lab (Apendix.1, for composition). HAM F-10 medium (Apendix.1, for composition) with glutamine were acquired from Invitrogen, USA. BSA (Bovine Serum Albumin) was acquired form SAFC, UK. HSA (Human Serum Albumin) was acquired from UNC, Argentina. All chemicals were cell culture-tested grade where available.

2.2 Spermatozoa preparation, Capacitated and Non-Capacitated

2.2.1 Swim-up

Donors were recruited at the Birmingham Women's Hospital (HFEA Centre 0119), in accordance with the Human Embryology Authority Code of Practice. All donors gave informed consent. Semen was donated by healthy donors by masturbation. After semen liquefaction for approximately 30 minutes, motile sperm were harvested by swim-up (Mortimer, 1994). 1 ml of sEBSS + 0.3% BSA, pH adjusted to 7.3-7.4, was underlayered with 0.3 ml of liquefied sample in polystyrene Falcon round-bottom tubes (Becton Dickinson, USA). After 1 hour incubation at 37°C, 5% CO_2 and at an angle of 45° (to maximize yield), the top layer of each tube, containing the motile cells, was collected into a 15 ml polystyrene Falcon tube (Becton Dickinson, USA) **[Figure.1.]**. Sperm concentration was determined using a Neubauer counting chamber, in accordance with the World Health Organization methods (WHO, 1999) and adjusted to 6 million cells/ ml with sEBSS + 0.3% BSA (Kirkman-Brown *et al.,* 2000). Sperm suspension was incubated for approximately 4-6 hours, at 37°C and 5% CO_2, for imaging and AR experiments. To reduce or suppress spermatozoa capacitation (non-capacitated spermatozoa) the albumin (BSA or HSA) was suppressed from the media and the incubation period is drastically reduced (5 hours to <1hour). Once swumup, spermatozoa are not allowed to undergo the capacitation time period (4-6 hours), these are directly used in any experimental technique.

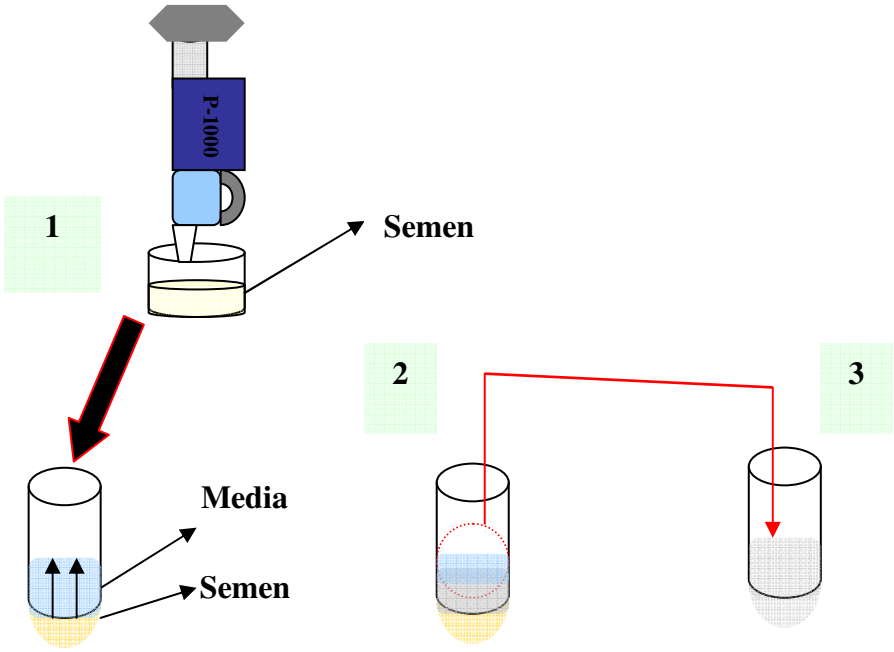

Figure.1. Schematic illustration of swimup technique, **(1)** 250-300µL of semen pipetted into the bottom of a 15 mL Falcon (polystyrene) containing 1mL medium, **(2)** consecutively the Falcons containing the semen and medium are incubated for 1hour at 37ºC, 5% CO2, **(3)** spermatozoa swimup and the top layer (cloudy equatorial region) containing highly motile spermatozoa are introduced into 15 mL Falcon and concentration is adjusted to 6 million cells/mL to capacitate in BSA.

2.2.2 Percoll

Spermatozoa from healthy donors were separated from the seminal plasma using (95%,47%) Percoll gradient in HAM F-10 culture medium (Teves et al., 2009; Guidobaldi et al., 2008). Consecutively spermatozoa exhibiting high motility were adjusted to $7x10^6$ cells/ml in HAM F-10 supplemented with 1% HSA (for capacitation) or without the albumin; in assays with non-capaciated spermatozoa; followed by incubation at 37°C in 5% CO_2 on air for 4-6hrs (Chen et al., 2000). All the experiments

were carried out with cells incubated under capacitating conditions unless otherwise specified.

2.3 Acrosome Reaction

Following spermatozoa separation and capacitation as described above (Section 2.2.2) , 32µL aliquots of spermatozoa (35µL for quantification of spontaneous AR) were treated with bourgeonal (20µM), DMSO (0.02%) and Ca^{2+} ionophore (8µM) and incubated for 30 minutes at 37°C in 5% CO_2. Consecutively each tube containing cells and testing substances were treated with formaldehyde (200 µL at 2%) and later incubated for 20 minutes at room temperature. Following this, 300µL of ammonium acetate (100mM) was added to each tube containing cells and then centrifuged for 7 minutes at 1800 rpms. Once concluded, the supernatant was removed and the whole process repeated. Following the centrifugation process the cells were vortexed for resuspension and 15µL of each tube was spread on each slide (with surface previously marked with permanent marker) and left to air dry. Once dry, the cells were stained with freshly made Coomassie stain (0.22% Coomassie Blue G-250, 50% methanol, 10% glacial acetic acid, 40% water) for 7-10 minutes, followed by wash with abundant distilled water H_2O and left to air dry. With 7µL glycerol (90% in PBS) 18x18 mm coverslips placed on slides, covering area with stained spermatozoa and sealed with nail varnish. Once sealed and dry, a total of two hundred cells were scored for each treatment under light microscope (x100 mag.) and % AR was calculated (equation in following page).

Spontaneous and induced acrosome reaction was calculated using the following equations:

$$\frac{\textbf{Nº AR Sperm.} \quad \textbf{x 100}}{\textbf{Nº Counted sperm.}} = \textbf{\% AR}$$

* **Nº AR Sperm=** Is the number of acrosome reacted spermatozoa (both induncef and spontaneous).

* **Nº Counted sperm=** The number of counted sperm.

2.4 Single cell imaging

Sperm density was reduced to 3.5-4 million cells/ ml, using the same medium, immediately before cell labeling and chamber preparation. 200 µl aliquots of cells were then loaded with 1.2 µL of Oregon Green BAPTA-1 AM ester (OGB, 488), with a K_d (Ca^{2+}) of 0.17µM, (0.6% DMSO, 0.12% pluronic F-127) for 20 minutes. This resulted in the removal of any potential residues e.g. vacuum grease, excessive poly-d lysine or

unknowns. Following this, the entire aliquot was transferred to a perfusable imaging chamber (200 µl volume) for 40 minutes, at 37°C and 5% CO_2 . The chamber lower surface was a 0.01% PDL coated coverslip, allowing cells to adhere, **[Figure.5.]**. The imaging chamber was connected to the imaging system and fresh medium (25°C) was washed through to eliminate excess dye and unattached spermatozoa. All experiments were performed at 25±0.5°C, in a constant flow of medium, with a perfusion rate of approximately 0.4 ml/minute. Cells were imaged with a Nikon TE200, **[Figure.3. (A)]**, inverted fluorescence microscope, fitted with a Cairn 75W xenon source and an epifluorescence accessory (excitation=485 DF 15, emission=535 DF 35). Images were captured every 10 seconds using a X 40 objective and a Rolera XR cooled CCD camera controlled by iQ software (Andor Technology, Belfast, UK).

In all experiments, OGB-1AM loaded sperm were superfused with sEBSS (or NCFsEBSS) +0.3% BSA for an initial control period before application of agonists.

Control experiments consisted in cell superfusion either with sEBSS + 0.3% BSA or NCFsEBSS + 0.3% BSA. DMSO controls were performed with spermatozoa bathed in sEBSS supplemented with solvent. This did not stimulate a significant elevation in OGB fluorescence above control levels (P>0.05%) **[Figure.2.].**

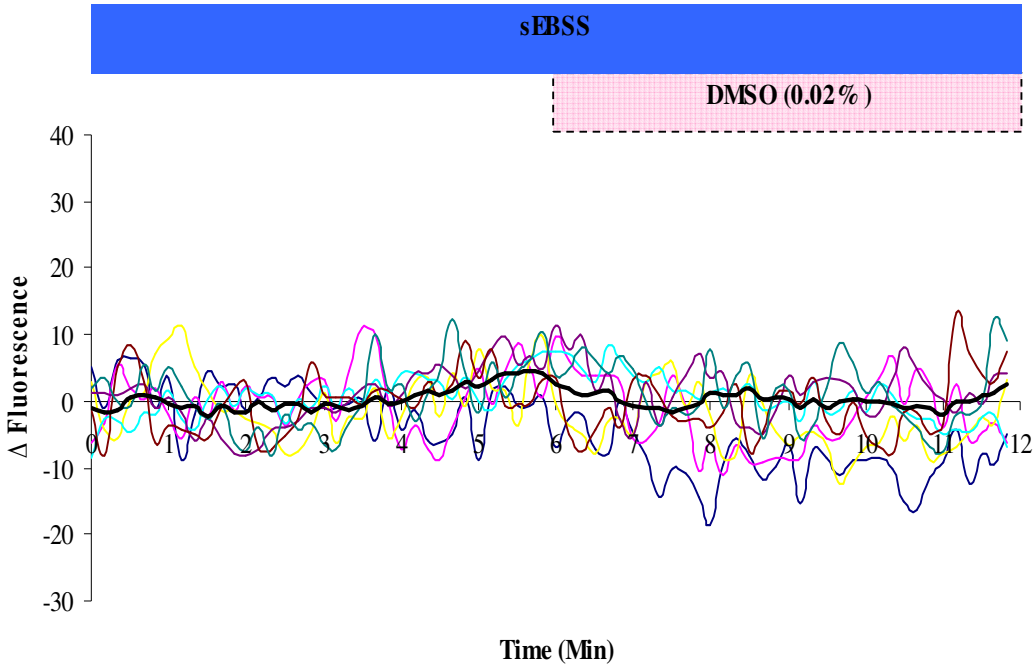

Figure.2. Capacitated human spermatozoa exposed to solvent vehicle, DSMO (0.02%) in sEBSS; resulting in no significant elevation in OGB fluorescence. Human spermatozoa were incubated for >5 hours in sEBSS, followed superfusion with sEBSS (Dark-blue bar) for 6 minutes and introduced to DMSO (Light-pink bar) in the same bathing media (sEBSS). Traces show 7 single cell responses and thicker black trace indicate the $\mathbf{R}_{tot}$ of one experiment [>100 cells].

2.4.1 Imaging Data Processing

Data were processed offline using iQ software**[Figure.6.]**, a lasso was drawn around the posterior region of the head of each cell in the selected field, considering as many cells as possible. Each cell was directly observed to ensure that only cells, where the region of interest remained inside the lasso, were used in the analysis. Cells that moved excessively and showed lack of adhesion to the glass surface were excluded from the

analysis (head is impossible to select, due to their excessive movement, due to bad adhesion). Dying cells or dead cells were not included in the analysis, these were easy to identify because they do not retain dye and gradual loss of fluorescence was visible during the control period (Tesarik et al., 1996). The majority of cells showed vigorous flagellar motility. A small subpopulation of cells showed a clear upward drift of fluorescence, during control period or, in some cells, spontaneous oscillation of $[Ca^{2+}]_i$. This may reflect loss of $[Ca^{2+}]_i$ homeostasis or 'over-capacitation'**.** These cells were also excluded from the analysis.

The average fluorescence intensity within the selected area in each spermatozoon was acquired for every image.

Raw intensity values were imported into Microsoft Excel and normalized to pre-stimulus values with the equation:

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

R is normalized fluorescence intensity, *F* is fluorescence intensity at a time *t* and F_{rest} is the mean of at least 10 determinations of *F* acquired during the control period. The mean value of R for all cells in the experiment (R_{tot}) was calculated for each time point and the total series of $\mathbf{R}_{tot}$ were plotted to give the mean normalized response of head fluorescent intensity for that experiment. Cell responses were observed from time-fluorescence intensity plots. Consequently the cells were visually sorted into those

showing increase, decrease or no change in fluorescence after treatment. Data from series of experiments were meaned to calculate the frequency of each type of response, which is stated in the text as mean ± SEM.

2.4.2 Imaging Equipment & Software

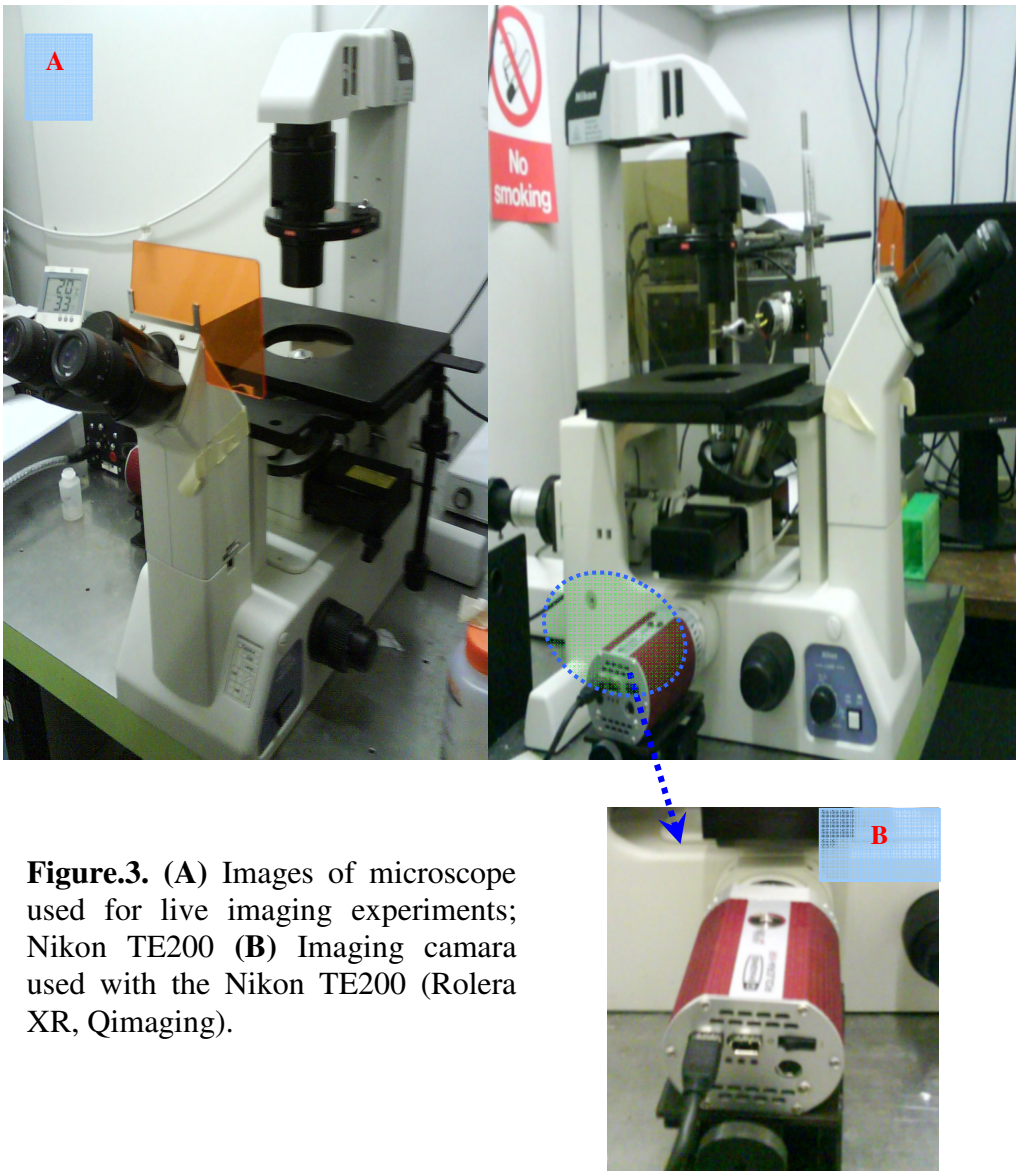

Figure.3. **(A)** Images of microscope used for live imaging experiments; Nikon TE200 **(B)** Imaging camara used with the Nikon TE200 (Rolera XR, Qimaging).

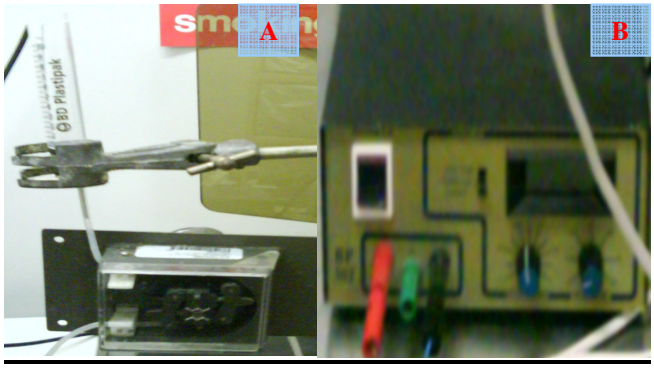

Figure.4. (A) Perfusion system used for the introduction of bathing media (control) and/or media supplemented with agonist(s), **(B)** Voltage controler of motor involved in perffusion system (standard voltage, 1.1 V, 0.8-1 amps)

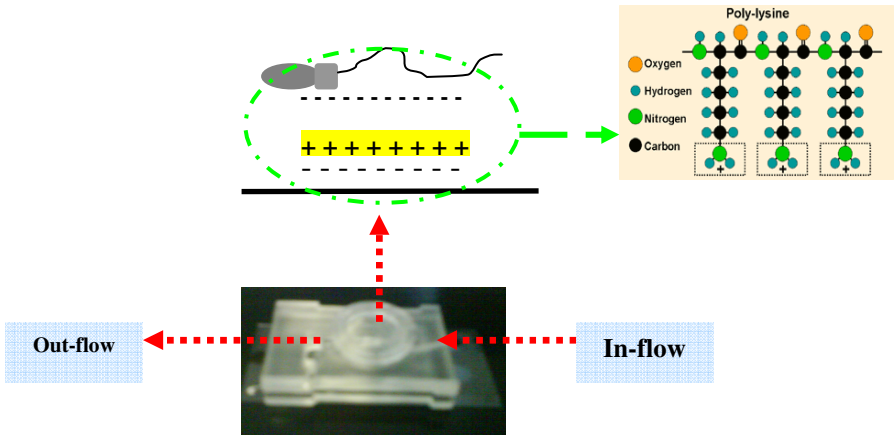

Figure.5. Image of imaging chamber used during the experiments, where the cells are adhered to slide with poly-d-lysine. The cells are introduced into the chambers and are attached to the slide, with poly-d-lysine. A net positive charge in culture media is formed at the end of the lysine, created by the amino group, making it hydrophylic, which makes it good for cell attachment (Poly-lysine structure & literature from http://www.corning.com).

2.4.3 Imaging Software

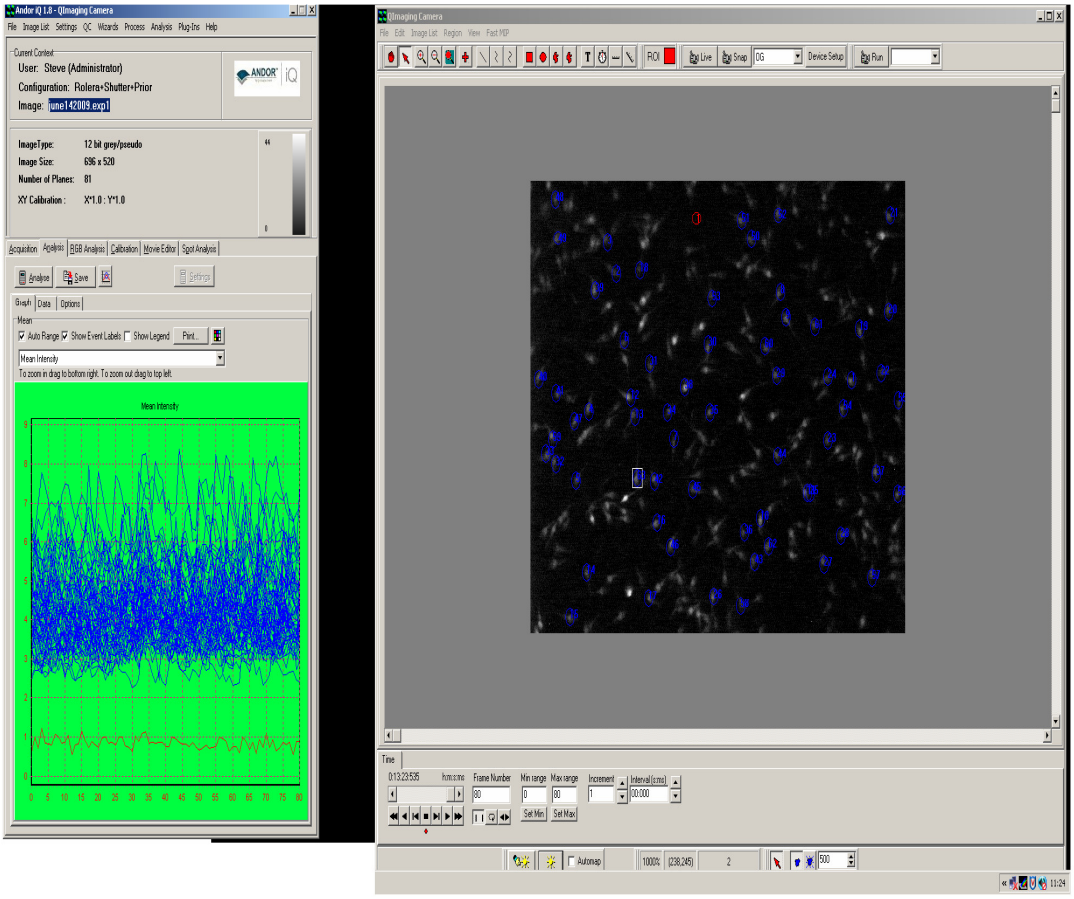

Figure.6. Imaging software (Andor iQ, advance imaging, updated version 2007), cell are selected, enabling to investigate cell responses, raw values are imported into Excel (Microsoft Office 2003), these are analyzed and appropriately interpreted.

2.5 Sperm chemotaxis and motility determination

Chemotaxis assays were performed in a chemotaxis chamber **[Figure.7. (A)]** composed of two wells separated by a 2 mm wall, one of the cells filled with media (HAM F-10) with or without attractants and the other well composed of spermatozoa (Teves et al., 2006, 2009). The chemotaxis chamber was sealed with a glass coverslip, forming a capillary space (called bridge) formed between both wells and over the wall separating well with cells and well with tested substance. Across the bridge, a one dimension attractant concentration gradient was formed in the direction of the well containing spermatozoa, which in turn, swamup over the bridge (Teves et al., 2006, 2009). Consecutively following the sealing of the chamber, the cells were incubated for fifteen minute (≈15 minutes) at 37ºC in order to stabilize the distribution of spermatozoa and the chemoattractant gradient. Subsequently after the incubation period spermatozoa movement was recorded along the fields in the middle of the bridge **[Figure.7. (A)].**

Following this the sperm tracks were analyzed by video-microscopy and computer image analysis to evaluate the percentage (%) chemotactic responding cells. For each of the sperm tracks the distance travelled along the X axes, (representing the attractant gradient; DX) and the Y axes (representing the absence of the attractant gradien, DY) were calculated (Teves et al., 2006, 2009). Assuming that a chemotactic responsive spermatozoon travel a longer distance along the X axes than in the Y axes, the directionality of spermatozoa was calculated by the quotient DX/|DY| **[Figure.7. (B)].** When this values was >1, the spermatozoon was considered oriented towards the cell containing the attractant under investigation. As a negative control, the well containing

the attractant was replaced by culture medium (HAM F-10); removing the attractant from the well, where ≈25% of spermatozoa swimming at random are expected to be oriented towards the well containing the experimental media (HAM F-10), in replacement of the attractant. The chemotactic responding subpopulation was considered as the difference in the percentage of "oriented spermatozoa" between the attractant solution and the negative control (well with attractant replaced with media, HAM F-10). Spermatozoa chemotactic response is strongly dependent on the attractant concentration, several doses of the attractant tested solution are assayed. Thus, a bell-shaped curve, typical of any chemotactive cell is observed, where at low concentration there is not sufficient receptor stimulation, although the receptors are saturated at higher attractant concentrations (Teves et al., 2006, 2008; Gidobaldi et al., 2008) **[Figure.7. (C)]**. Hence as a consequence in both extremes the chemotaxis response is abolished and the levels of "oriented spermatozoa" show no significantly difference to the basal negative control (≈25%). On the other hand, at a optimum attractant concentration the cells are able to detect the gradient and respond with a chemotactic movement orientation, resulting in a level of "oriented spermatozoa" statistically higher than basal negative control (>25%) (Teves et al., 2006, 2008; Gidobaldi et al., 2008). In mammalian spermatozoa such difference is ≈10%, a higher number of spermatozoa per treatment must be analyzed (minimum 150 cells), in at least 2 experiments.

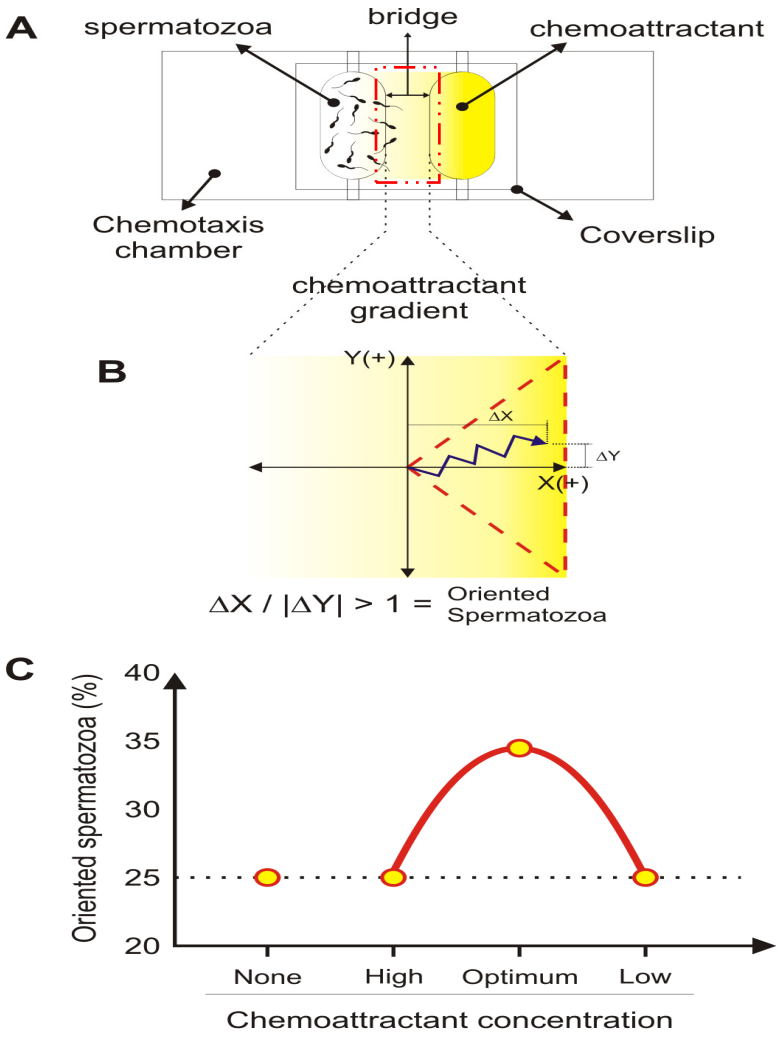

Figure.7. Detection system for spermatozoa chemotaxis. **(A)** Plane view of zigmoond chamber composed of two wells separated by a 1 mm wall, (well on right-side) filled with medium (HAM F-10) supplemented with or without attractants and the other well (well on left-side) with capacitated or non-capaciated mammalian spermatozoa (Teves et al., 2006, 2008; Gidobaldi et al., 2008). **(B)** The tracks of spermatozoa are analyzed by computer imaging to calculate chemotatixis, sperm velocity and pattern of movement (Teves et al., 2006, 2008; Gidobaldi et al., 2008).The distance traveled along the X axes (representing the attractant gradient; ΔX) and the Y axes (representing the absence of attractant gradient; ΔY) are calculated for each sperm track. Assuming that a chemotactic spermatozoon travel a longer distance along the X axes than in the Y axes, sperm directionality is calculated by the quotient ΔX/|ΔY| (Teves et al., 2006, 2008; Gidobaldi et al., 2008). **(C)** Spermatozoa chemotactic response is dependent on the concentration of the attractant; therefore, an array of doses of tested attractant solution is assayed. Hence resulting in a typical bell-shaped curve in response to the array of concentrations of chemoattractant.

Human spermatozoa were diluted at $4x10^6$ cells/ml for the chemotaxis assay and exposed to HAM F-10 culture medium (negative control), or medium supplemented with an array of doses of bourgeonal; for positive control progesterone was used at 10pM in all of the experiments. Chemotaxis assays involving inhibitors involved incubation with these for 1-15 minutes, depending on inhibitor; prior to chemotaxis assays.

Images were recorded at 6Hz with the VirtualDub software (ver. 1.6.16, Avery Lee; http://www.virtualdub.org/).

The sperm directionality and motility were analyzed with the ImageJ software (ver. 1.38, NIH, USA) and the MtrackJ plugin (ver.1.1.0, Eric Meijering) **[Figure.8.].** The percentage of "oriented spermatozoa" was calculated with the SpermTrack software (ver. 4.0, UNC, Argentina); designed by Dr A. Gidobaldy **[Figure.9.].**

2.5.1 Chemotaxis Software

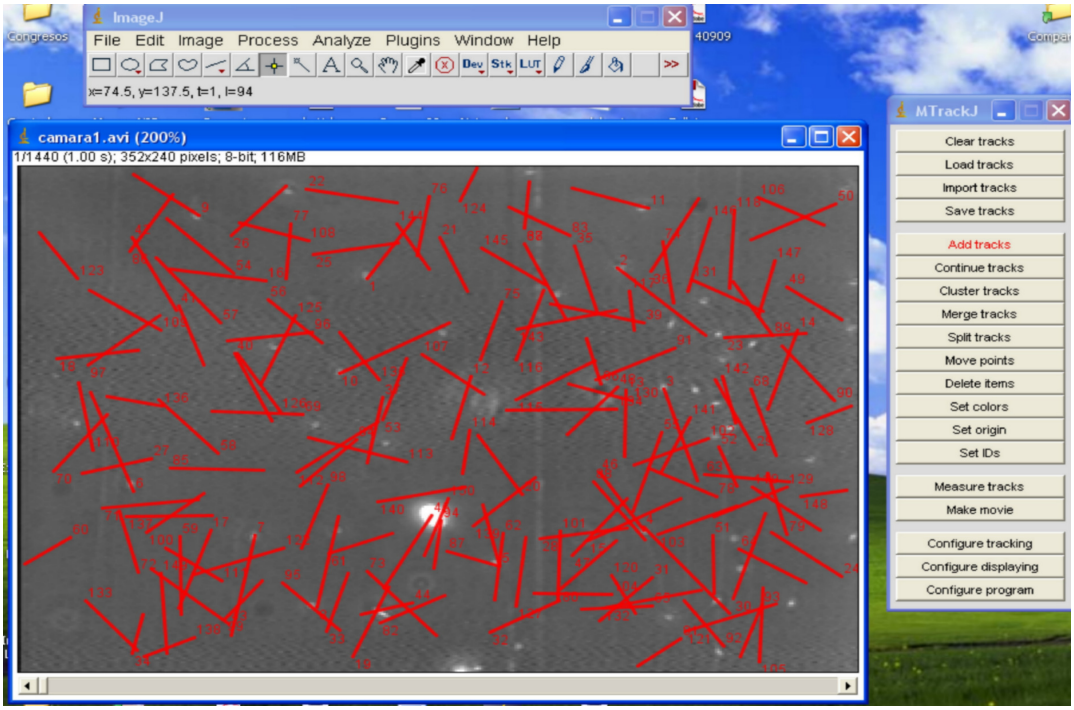

Figure.8. Image illustration of ImageJ software (ver.1.38, NIH, USA) with MtrackJ plugin (ver.1.1.0, Eric Meijering) designed to analyse sperm directionality and motility. The coordinates obtained here are imported into SpermTrack software (By Dr A. Gidobaldy, Argentina).

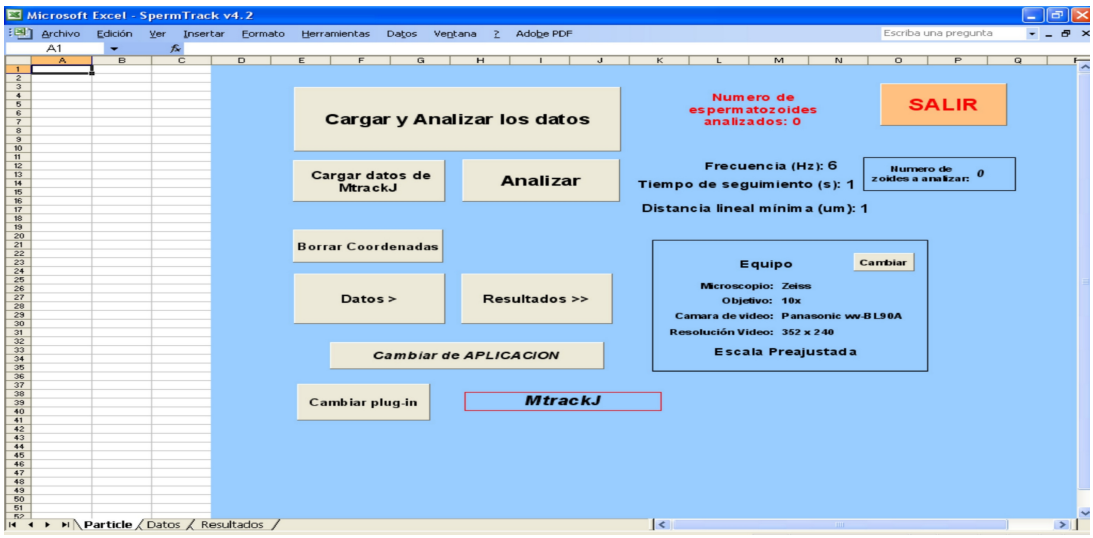

Figure.9. Image illustration of SpermTrack software (ver. 4.0, UNC, designed by Dr A. Gidobaldy, Argentina) designed to calculate the percentage “oriented spermatozoa”.

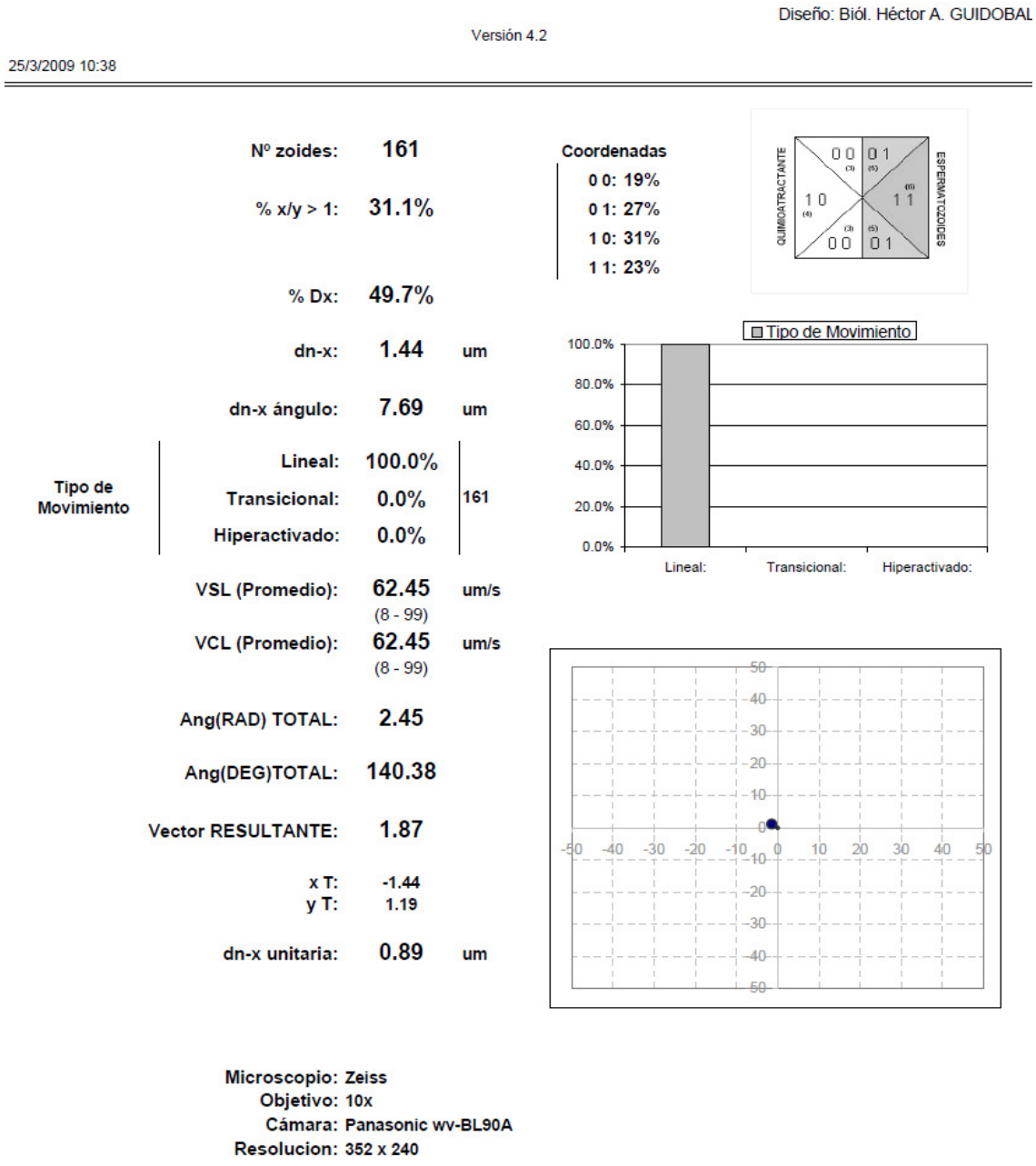

Figure.10. Image illustration of document obtained from SpermTrack software (PDF format), illustrating an array of parameters obtained from the software designed by Dr A. Gidobaldi (Argentina).

2.6 cAMP measurement

Aliquots of human capaciated spermatozoa in HAM F-10 at 15 x 10^6 cells/ml, were incubated for 20 minutes at 37ºC with 100 µM IBMX (phosphodiesterase inhibitor), in the absence or presence of bourgeonal (20µM and 1nM) or progesterone (10pM). Cells were then fixed with 1% formaldehyde for 20 minutes and washed twice with PBS. Subsequently the cells were centrifuged at 2000xg for 15 minutes at 4ºC. (pellets are stored at -20ºC. When required the pellet were resuspended in 300µL lysis reagent (from cAMP or cGMP EIA Kit) and sonicated at a frequency of 70Hz (two times, 30 seconds), the tubes were kept in ice until the end of the sonication process (critical that all the reagents are kept at room temperature and used within 1hour of preparation). When the multiwell were marked and loaded with reagents, these were mixed (shaken gently) for 2 hrs at 3-5ºC (critical that temperature does not exceed 5ºC). cAMP-peroxidase conjugate is added to all of the wells except the blank well, then mixed and incubated for 1h at 3-5ºC. Subsequently the solutions in the wells are removed and washed (4 times) with buffering solution (washing buffer) and dried removing (lab tissue) all residues. Immediately after substrate enzyme was added to all the wells, which must be kept at room temperature (mix gently for 60 minutes at room temperature 15-30ºC). Following the mixing period, 1M sulphuric acid (H_2SO_4) was introduced into all of the wells and mixed gently. The reactions were read at 450nm within 30 minutes after the application of the acid. Once reading were obtained, cAMP was quantified following manufacturer's instructions (cAMP Biotrak Enzymeimmunoassay System, Amersham Biosciences).

2.7 Statistical Analysis

Microsoft Excel (2003) and SigmaStat software (SPSS, Inc, USA) was used to perform *F*-test (analysis or variance), *t*-tests (equal/unequal variance) for unpaired data (paired if necessary). Statistical significance was set at $P<0.05$ (*) (not significant, $P>0.05$). Statistical differences between treatments were determined by means of one-way ANOVA and the Tukey-Kramer tests with the SigmaStat software (SPSS, Inc, USA).

III

CHAPTER ~THREE~

Chapter Three

HUMAN SPERMATOZOA Ca^{2+} SIGNAL RESPONSE TO BOURGEONAL & HOMOLOG 3,4,CPEE

3.1 Introduction

Ca^{2+} and Ca^{2+} binding proteins are crucial for a great array of biochemical processes in all living cells, as such, calcium homeostasis is a prerequisite for the effective functioning of all living organisms (Cheek et al., 1993). Calcium's high versatility enables it to regulate many cellular functions (Berridge, 2003). An incresase in intracellular calcium ions from a resting level of approximately 0.1 μmol l^{-1} to a stimulated level of 1-10 μmol l^{-1} initiates many physiological processes such as fertilization, exocystosis secretion, muscle contraction and cell division (in many animals) (Cheek et al., 1993). In spermatozoa calcium plays a critical role in all the major processes preceding fertilization (Publicover et al., 2008). Sperm possess the equipment (membrane calcium channels and pumps, stores, calcium binding proteins) to generate, control and respond to changes in Ca^{2+} concentration and Ca^{2+} signalling plays a vital role in the functioning of male gametes (Publicover et al., 2008; Darszon et al., 1999, Publicover et al., 1999). Ca^{2+} plays a vital role in capacitation (cellular adaptation) (Darszon et al., 2006), hyperactivated motility (high amplitude flagellar beat), active motility (low amplitude flagellar beat) (Suarez et al., 1993, Carlson et al., 2003), AR (exocytotic process) (Yanagimachi et al., 1974) and sperm chemotaxis (mechanism of guidance; Teves et al., 2009).

Mammalian spermatozoa cannot fertilize eggs immediately after ejaculation (Darszon et al., 2006) but must undergo a series of intra- and extra-sperm modifications that will enable sperm to become functionally competent "capacitated" (Abou-Haila & Tulsiani,

2009). It is now clear that sperm becomes "competent" or capacitated after residing for a time period in the female reproductive tract (*in vivo*). During this period albumin present in the female environment removes cholesterol from the mammalian sperm altering its plasma membrane organization and permeability (Darszon et al, 2006). Capacitation can be induced *in vitro* with the appropriate medium (Abou-Haila & Tulsiani, 2009), which must contain energy substances (pyruvate, glucose) electrolytes (including Ca^{2+}), bicarbonate ions, and a cholesterol acceptor.

Cholesterol located in the sperm membrane has been demonstrated to limit ion permeability, protein insertion and mobility in phospholipid biolayers (Beddu-Addo, 2006), as well as to rigidity and stabilize membranes, consequently unabling some of the events associated with sperm capacitation (de Lamirande et al., 1997). The incubation of sperm in a supplemented medium with a cholesterol remover (BSA, HSA), for a period of 3-5 hours (WHO, 1999), 37°C, 5% CO_2 will provide the means for sperm capacitation *in vitro* (WHO, 1999). It has been postulated that cholesterol efflux consequently leads to the changes in the membrane structure and fluidity that results in sperm capacitation. All of these intra and extra- cellular changes may be reversible (Laglais & Roberts, 1985; Bedu-Addo et al, 2006). This "maturation process" is inhibited by the application of cholesterol and/or cholesterol analogues to the capacitating media (Visconti et al., 1999). An array of studies have shown that cholesterol influx may reduce the rate of spontaneous AR (Davis, 1980; Flemming and Yanagimachi, 1981) and may also inhibit or delay capacitation in an array of species including human spermatozoa (Moubasher and Wolf, 1986).

Mammalian sperm chemotaxis is a well established process, though the signalling involved has not been elucidated (Muciaccia et al., 2005). After incubation in capacitating medium, approximately 10-15% of cells would be capacitated at any time point (Eisenbach & Giojalas, 2006). This subpopulation of cells is capable of responding to an ascending gradient of any attractant molecule i.e. 10 pM progesterone (Teves et al., 2006, 2009) or even to the artificial odorant Bourgeonal (Spehr et al., 2003, 2004; see below). This has been demonstrated by the suppression of the capacitated cells, *in vitro*, resulting in the loss of the chemotactively responsive cells and vice versa (Eisenbach et al., 1999).

Olfactory receptor proteins (ORs) are usually expressed in the cilliary compartments of nasal olfactory sensory neurons. The stimulation of these results in the stimulation of mAC III and finally resulting in an increase in Ca^{2+} and Na^{+} concentration, resulting in membrane depolarization (Firestein, 2001). Several olfactory receptor protein (ORs) are expressed primarily or exclusively in mammalian sperm cells (Firestein, 2001; Vanderhaeghen et al., 1997). The G-protein coupled) receptors (hOR17-4), present in the flagellar midpiece of human sperm, is believed to act as chemosensory receptor that mediates chemotaxis (Spehr et al. 2003). Bourgeonal a synthetic odorant, used in perfumes to mimic the scent of lily of the valley was identified as potent agonist of hOR17-4 and in human spermatozoa, exerts a strong chemoattractant effect (Spehr et al., 2003, 2004). It has recently been reported that the bathing medium must contain more than 10^{-4}M Ca^{2+} in order to obtain a significant chemotactic response to

bourgeonal (Gakamsky et al., 2009). Exposure of human sperm to bourgeonal, results in a results in a dose-pendent elevation in $[Ca^{2+}]_i$ which is dependent on $[Ca^{2+}]_{e.}$ (Spehr et al, 2003, 2004). The response initiates in the midpiece, where the receptor is located, and spreads to the head (Spehr et al, 2003, 2004). Binding of hOR17-4 is believed to stimulate membrane adenylate cyclase (mACIII) through G_{olf}, the resulting rise in cAMP concentration and activation of a Ca^{2+} -permeable channel, either directly or by the activation of PKA enabling the influx of Ca^{2+} across the plasmalemma (Publicover et al., 2007). However, it's still unclear how the Ca^{2+} mobilisation in the midpiece regulates flagellar $[Ca^{2+}]_i$, and the consequent beat and/or probable change in direction (towards source, in ascending gradient).

Chapter Aims

The aim of this chapter was to examine the effect of bourgeonal on $[Ca^{2+}]_i$ and AR in capacitated (incubated with sEBSS media +0.3% BSA, >5hours incubation) and non-capacitated human sperm. Additionally, the effect of bourgeonal was compared with the response to a structural homolog (3,4,CPEE), both in Ca^{2+} and in the absence of Ca^{2+}.

3.2 Results

3.2.1 Capacitated human spermatozoa respond to Bourgeonal

Following the incubation for >5hours in sEBSS, (0.3% BSA), the capacitated human spermatozoa were initially bathed in sEBSS for 3 minutes (control period), following this, the cells were superfused in sEBSS supplemented with bourgeonal (20μM). A significant $[Ca^{2+}]_i$ elevation (P<0.05%, contol levels against agonist levels) was detected in the $\mathbf{R}_{tot}$ of the experiments, elevating OGB fluorescence 14.87% (±1.35% S.E.M), **[Figure.1. (D)],** above control levels, within 2-5 minutes **[Figure.1. (A)].** Visual examination of individual cells showed that 46% (±6.2 S.E.M) of the cells responded with a distictive rise in OGB fluorescence (fifteen experiments, >1200 cells examined) **[Figure.1. (B)]**. Within the individually examined spermatozoa, 38.10% (±3.87 S.E.M) showed a clear significant sustained elevation in OGB fluorescence 21% above control levels, and 8.9% (±2.3% S.E.M.) generating a significant single transient elevation, peaking within ≈0.66 minutes **[Figure.1. (C).]**. A subpopulation of responding cells showed a gradual and modest decrease in $[Ca^{2+}]_i$ during exposure to bourgeonal.

Visual examination of individual cells showed that a small subgroup of cells within each experiment showed high resting OGB fluorescent levels. This might be due to over-staining of the cells or elevated resting levels of $[Ca^{2+}]_i$,. When superfused with bourgeonal (20μM) these cells did not show a significant $[Ca^{2+}]_i$ elevation. The small

proportion of cells showing spontaneous activity [see methods **section 2.2.4.1**] were excluded from analysis. In these cells application of bourgeonal either had no effect on frequency of the oscillations but appeared to increase their magnitude in some cells.

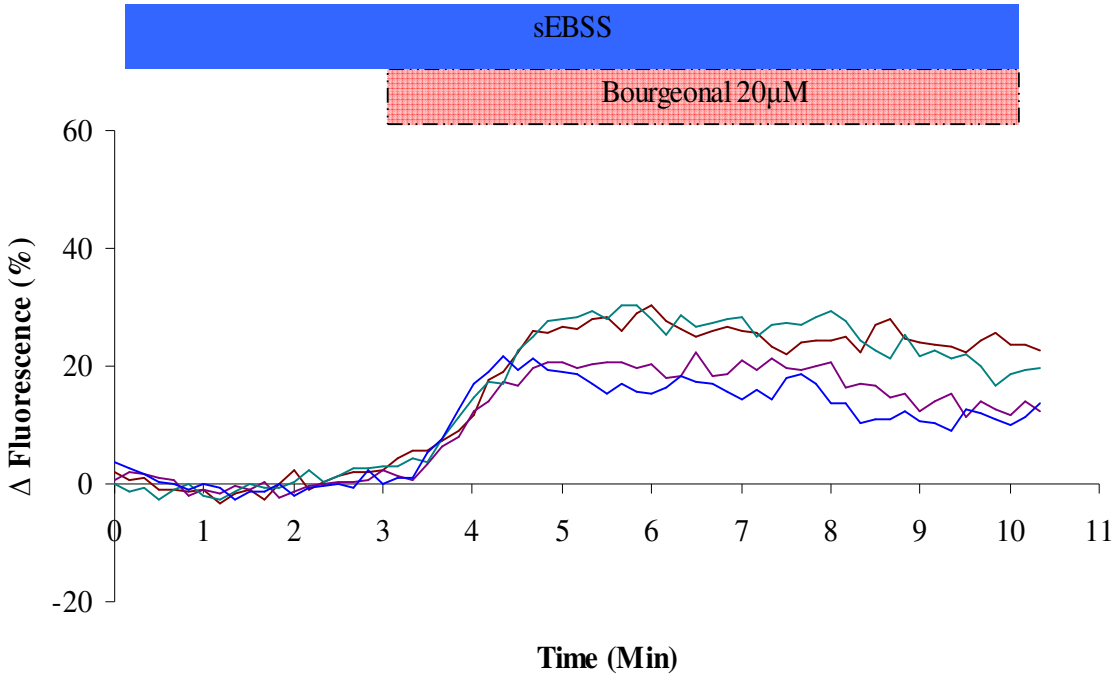

Figure.1. (A) Effect of bourgeonal (Light-red bar)on capacitated human spermatozoa bathed in sEBSS (Dark-blue bar) (incubated for >5hours in sEBSS). Capacitated human spermatozoa were bathed in sEBSS for 3 minutes (control period), followed by the superfusion of the cells with bourgeonal (20µM) in the same medium.Traces showing individual cell responses of 4 cells.

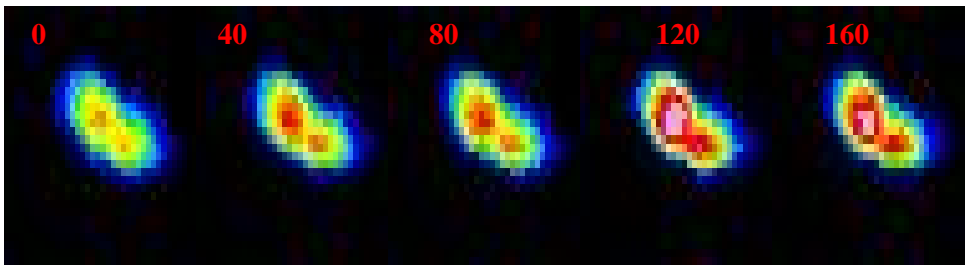

Figure.1. (B). Pseudocolour image series (warm colours indicate high $[Ca^{2+}]_i$) of capacitated human spermatozoa response to bourgeonal, with an sustained elevation in OGB fluorescence. Numbers in red (top left) indicates time course in seconds. Human spermatozoa incubated under capacitating conditions for >5hours were bathed in sEBSS (+BSA) (post labeling with OREGON GREEN*488 BAPTA-1, AM, see Methods, Chapter 2) during control period, and later exposed to bourgeonal (20µM) stimulating an elevation in OGB fluorescence.

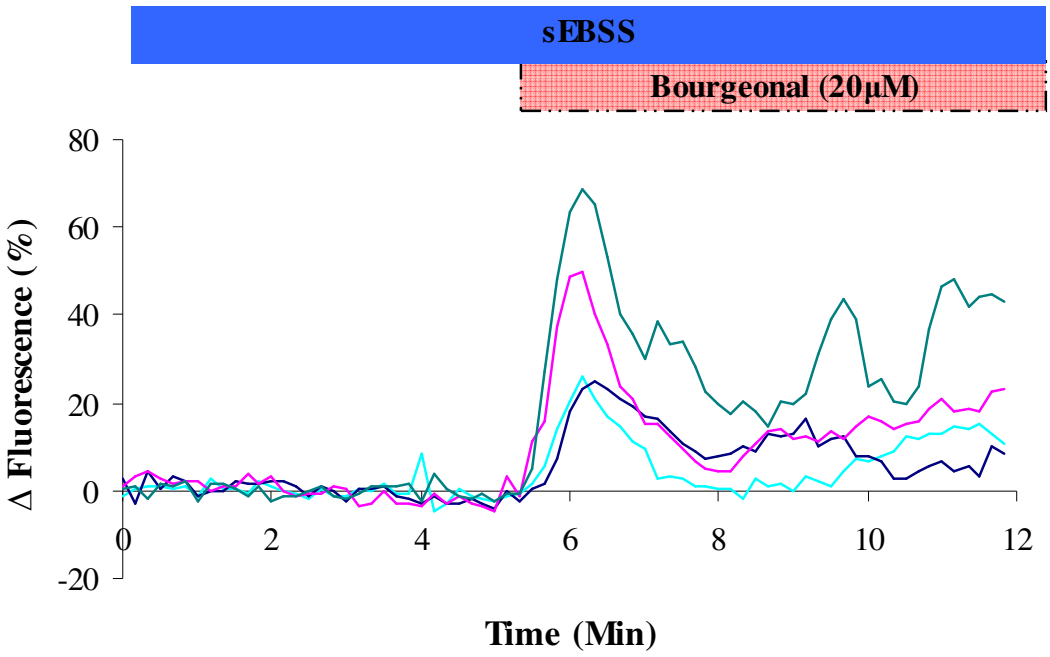

Figure.1. (C). Transient elevation in OGB fluorescence identified in subpopulation (≈8%) of capaciated human spermatozoa stimulated with bourgeonal (20µM) in sEBSS (cells incubated in sEBSS for >5hours). Traces show 4 single cell responses.

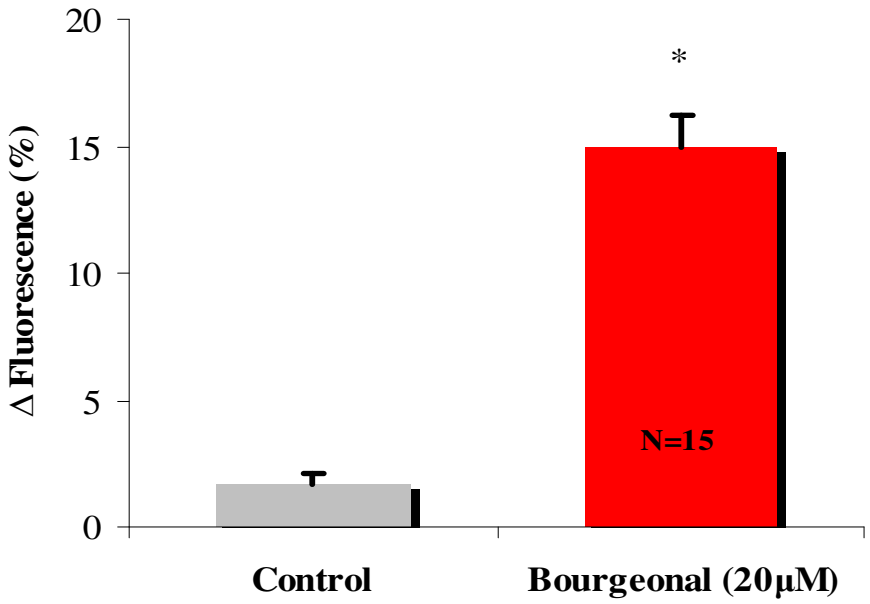

Figure.1. (D). Bar graph illustrating the mean normalized increase in fluorescence post stimulation of capacitated human spermatozoa with 20μM in sEBSS and over control period (cells bathed in sEBSS media prior to stimulation with bourgeonal). The error bars indicates the standard error of the mean (S.E.M.) and the number of experimental replicates is indicated within each of the bars (N). Asterisk (*) indicates the significance difference ($P<0.05\%$).

3.2.2 The effect of bourgeonal is reversible

Following the identification and quantification of human sperm response to bourgeonal, the next step was to investigate whether the response to bourgeonal was reversible. Spermatozoa were incubated in capacitating media for >5 hours (sEBSS), later bathed in the same medium for 5 minutes (control period). Consequently the cells were exposed to sEBSS supplemented with bourgeonal (20 μM), followed by a wash-off period, were there was a clear reversal of the effect of bourgeonal on $[Ca^{2+}]_i$ **[Figure.2.].** Upon washout fluorescence decreased to below stimulating levels. When bourgeonal (20μM) is reintroduced into the bathing media, the cells were capable of once again responding to the stimulus, with an elevation in OGB in fluorescence >15% above control levels, significantly higher than resting levels and higher than washout levels.

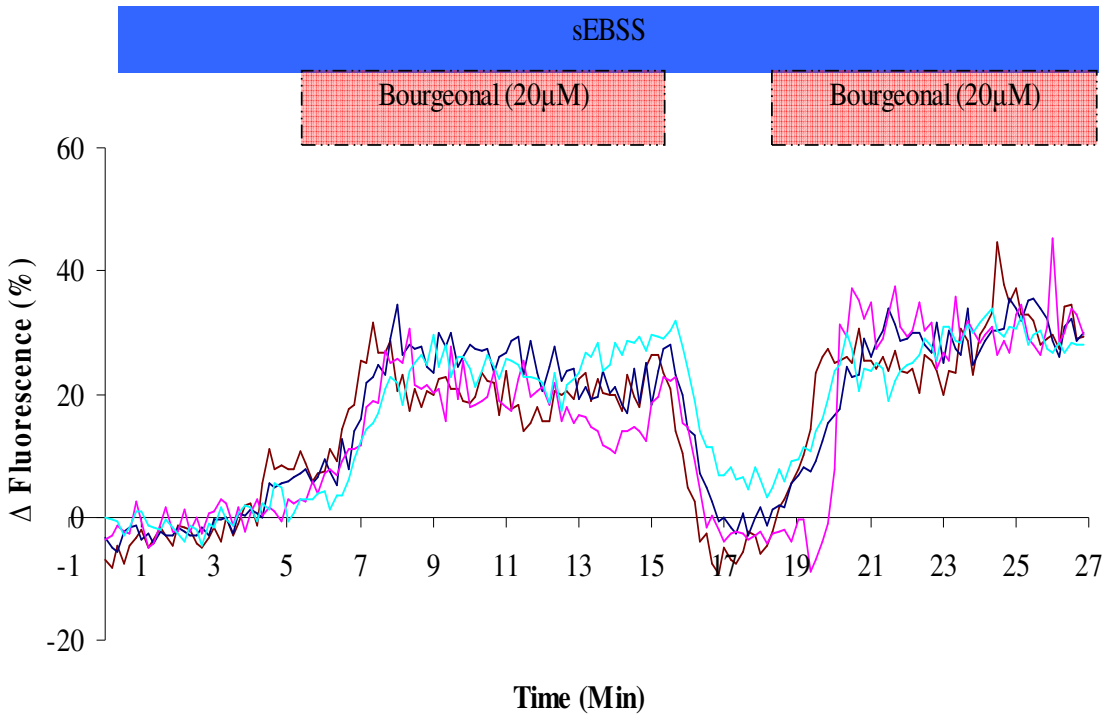

Figure.2. Effect of bourgeonal washout on capacitated human sperm bathed in sEBSS (were incubated in sEBSS for >5 hours). Human spermatozoa were superfused with sEBSS for 5 minutes (Dark-blue bar), and subsequently bathed with 20 µM bourgeonal (Light-red bar) in sEBSS for 10 minutes. This was followed by the superfusion of the cells with sEBSS; finally bourgeonal was reintroduced in the same media and at the same dose. Traces show 6 single cell responses.

3.2.3 Dose-dependence of the effect of bourgeonal

Previous research suggest that human spermatozoa respond to bourgeonal in a dose-response manner (Spehr et al., 2004, 2006). After control superfusion with sEBSS for 4 minutes, capacitated spermatozoa were exposed to 2 μM bourgeonal, then 20 μM and finally 200 μM bourgeonal. Bourgeonal (2 μM) induced a significant sustained elevation in $[Ca^{2+}]_i$, in <30% of the cells, maximum elevation reaches 9.23 (±0.3% S.E.M) above control levels (one experiment, >20 cells examined), significantly greater than basal control levels (P<0.05%). Subsequent exposure to 20 μM bourgeonal caused a significant sustained elevation in OGB fluorescence. In >30% of the cells, amplitude of response was 21.76 (±1.3% S.E.M) above control levels, within 2-5 minutes (P<0.05% compared to 2 μM and to control period) **[Figure.3.].** Furtheremore, 200 μM bourgeonal then induced an additional significant single transient elevation in $[Ca^{2+}]_{i,}$ in >65% of the cells, maximum amplitude >34.13 (±0.2% S.E.M) (P<0.05% compared to control and other doses) above control levels, which peaked at ≈1.5 minutes.

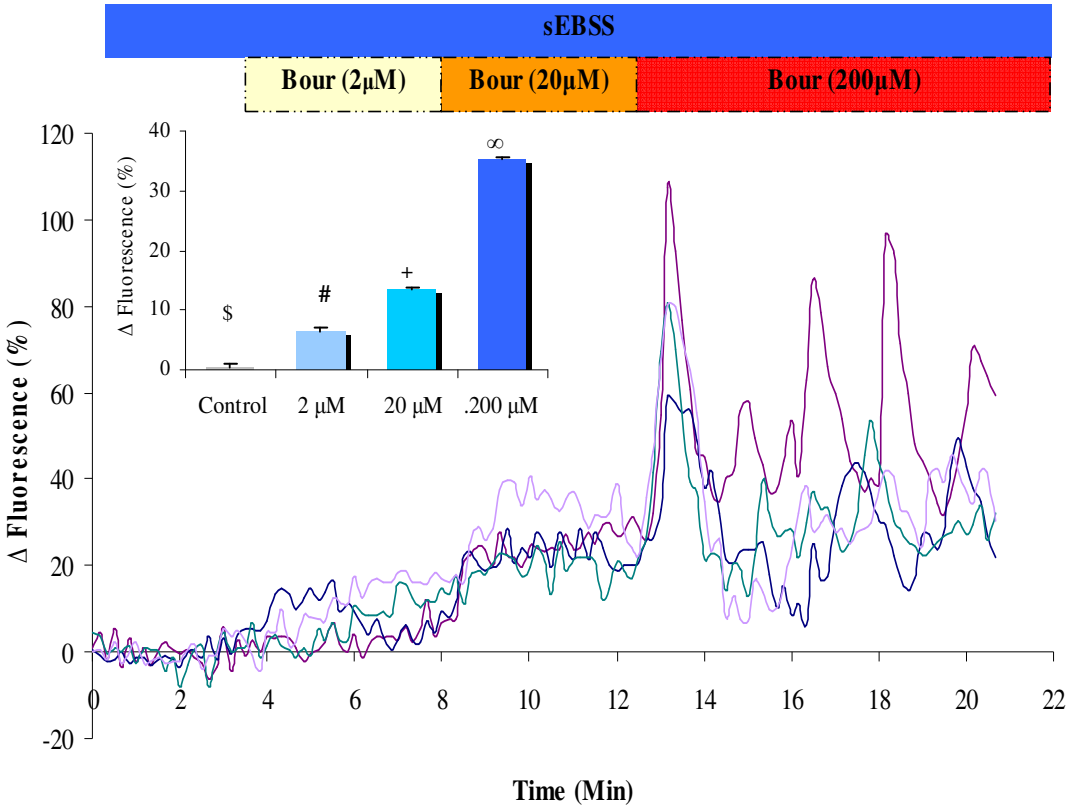

Figure.3. (A). Effect of stepped increase in bourgeonal concentration (2µM, 20µM, 200µM) on capacitated human spermatozoa bathed in sEBSS (incubated for >5hours in sEBSS). Cells were bathed in sEBSS (Dark-blue bar) for 4 minutes (control period), followed by the introduction of 2µM bourgeonal (Yellow bar) for 4 minutes, subsequently 20µM bourgeonal (Orange bar) was introduced for 4.5 minutes and finally 200µM (Red bar) was applied to the cells in the same media. Traces show 4 single cell responses. **Insert:** Bar chart indicates the mean normalized increase in fluorescence in one experiment (N=1, >20cells); illustrating the elevation in OGB fluorescence post stimulation with 2µM, 20µM, 200µM bourgeonal in sEBSS and over control period (cells bathed in sEBSS media prior to stimulation with agonist). The error bars indicates the standard error of the mean (S.E.M.) and different symbols in each bar ($,∞,+, #) indicate the significance between samples tested (P<0.05%).

3.2.4 Dependence of the action of bourgeonal on $[Ca^{2+}]_o$

To investigate the importance of extracellular Ca^{2+} in the response of human sperm to bourgeonal, cells were incubated for >5hours in capacitating media (sEBSS, +0.3% BSA, pH 7.25-7.4) and later bathed in NCFsEBSS (<5μM/L Ca^{2+}) (+0.3% BSA) for 3 minutes. Consecutively the cells were superfused in NCFsEBSS supplemented with bourgeonal (20μM) were no significant change in OGB fluorescence was detected in the $\mathbf{R}_{tot}$ of the experiments (six experiments; P>0.05%), **[Figure.4. (A)].** Strongly suggesting the crucial role of Ca^{2+} in the response to bourgeonal **[Figure.4. (B)].** Individual cell examination revealed that 11% (±2% S.E.M) showed a clear significant sustained elevation in OGB fluorescence, with a maximum elevation 10.4% (0.9% S.E.M) above control levels, after a period of 2-5 minutes (six experiments, >300 cells examined).

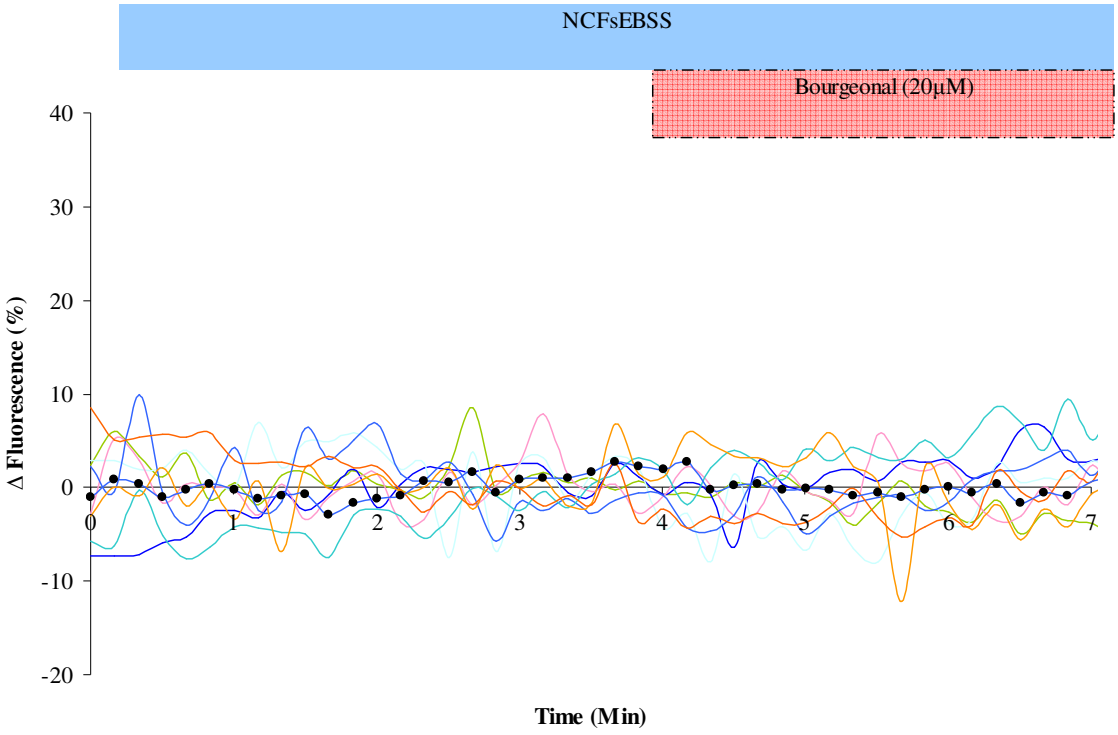

Figure.4. (A). Effect of bourgeonal in capacitated human spermatozoa bathed in low-Ca^{2+} (incubated for >5hours in sEBSS). Capacitated human spermatozoa were bathed in NCFsEBSS (Light-blue bar) for 3 minutes (control period), followed by introduction of bourgeonal (20µM) in the same media. Trances of 10 cells showing no significant response to bourgeonal (Light-red bar) in NCFsEBSS. Dotted (●) line in graph indicates $\mathbf{R}_{tot}$ of experiments (average response of >30 cells).

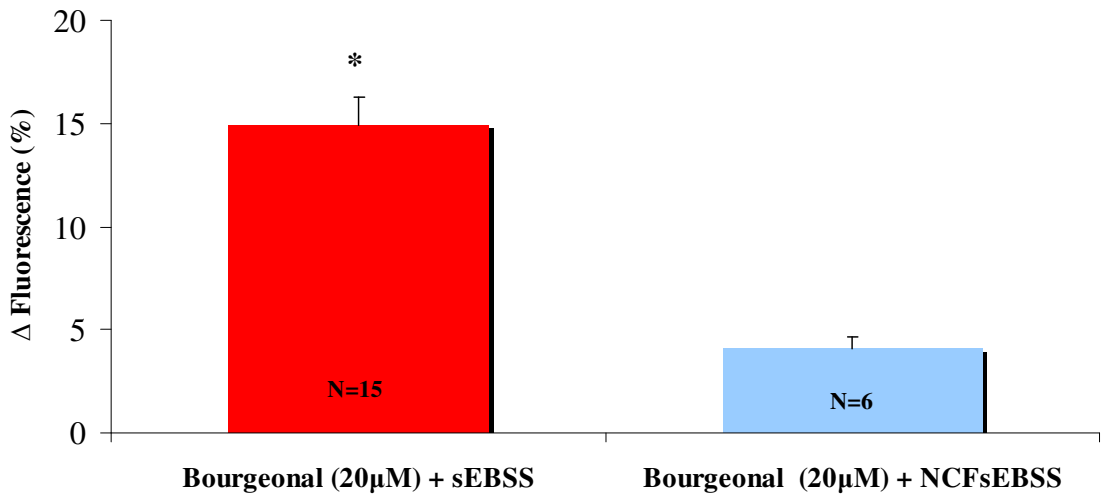

Figure.4. (B). Bar chart indicates the mean normalized increase in fluorescence post stimulation of human capacitated spermatozoa with bourgeonal (20µM) in sEBSS; against capacitated spermatozoa bathed in NCFsEBSS supplemented with bourgeonal at the same dose (20µM). The error bars indicates the standard error of the mean (S.E.M.) and the number of experimental replicates is indicated within each of the bars (N). Asterisk (*) indicates the significance ($P<0.05\%$).

In order to further reduce $[Ca^{2+}]_o$; EGTA-buffered NCFsEBSS was used (3mM EGTA). Capacitated human spermatozoa were first superfused with NCFsEBSS then exposed to EGTA-buffered NCFsEBSS for 5 min. This caused a decrease in OGB fluorescence of >20%; bellow resting control levels. Subsequently the application of bourgeonal (20µM) under these conditions caused no discernible response (two experiments, >100 cells examined) **[Figure.5.].**

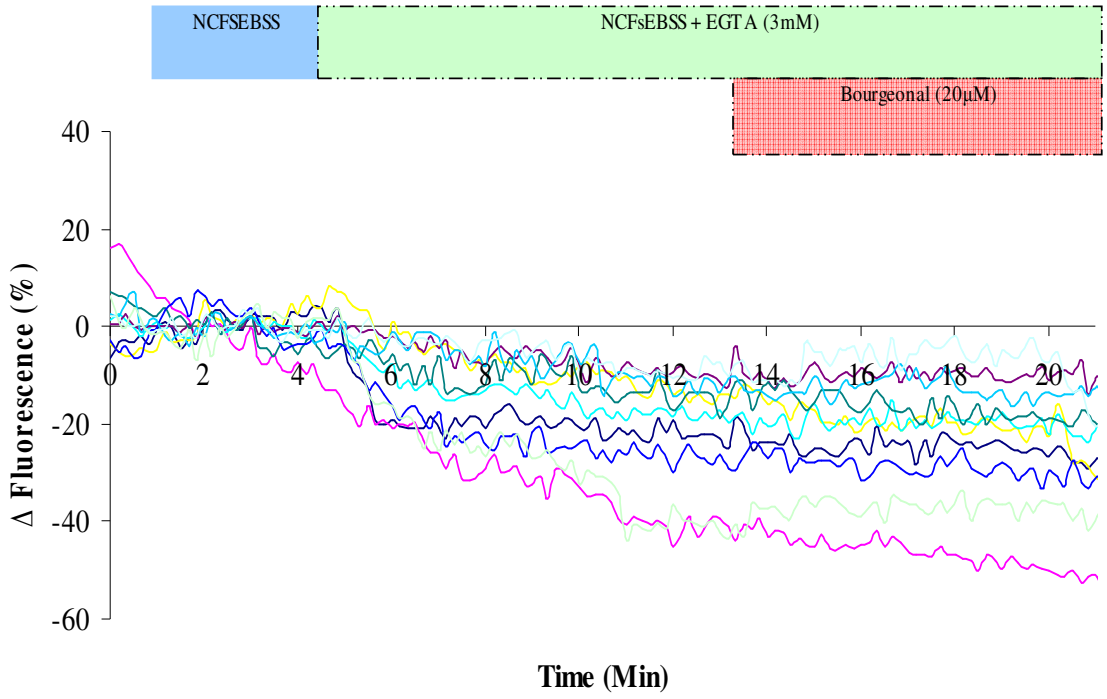

Figure.5. (A). Response of EGTA (low-Ca^{2+}) pretreated capacitated human spermatozoa in NCFsEBSS to bourgeonal (20µM) in EGTA-buffered NCFsEBSS (incubated for >5hours in sEBSS). Human spermatozoa were bathed for 5 minutes in NCFsEBSS (light-blue bar), followed by superfusion of the cells with EGTA-buffered NCFsEBSS for 8 minutes (Light-green bar), and finally bathed with bourgeonal (light-red bar) in the same EGTA-buffered media. Traces show 10 single cell responses.

3.2.5 Ca^{2+} stores and the action of bourgeonal

Though the response to bourgeonal was completely inhibited by EGTA, it is possible that strong buffering of $[Ca^{2+}]_o$ was acting to deplete Ca^{2+} stores before application of bourgeonal. In order to distinguish whether stored Ca^{2+} was involved in the response to bourgeonal, the cells were pre-treatmented with bis-phenol (20μM) **[Figure.7. (A)]**. Bis-phenol inhibits both SERCAs and SPCA Ca^{2+}-pumps at doses ranging from 10 to 40μM and evacuates Ca^{2+} stores (Brown et al., 2004). Superfusion of cells bathed in sEBSS with bis-phenol (20μM) resulted in a rapid elevation in $[Ca^{2+}]_i$ in ≥85% of the cells, (mean increase in OGB fluorescence = 28.0±3.60%; six experiments, >500 cells examined), a significant increase above control levels (P<0.05%). Subsequent application of bourgeonal (20μM) caused a significant elevation in OGB fluorescence 15% above non-treated levels (P<0.05%), **[Figure.7. (B)]**, within 2-5 minutes. Visual examination showed that 43.3±6.5 of the bis-phenol pretreated cells showed a clear rise in OGB fluorescence upon application of bourgeonal. Within these cells 37%(±3.7%S.E.M) responded with a sustained elevation in $[Ca^{2+}]_{i,}$, and a small proportion (≈6%) of the responding cells showed a single transient response in $[Ca^{2+}]_i$ (as previously reported with cells bathed in sEBBS supplemented with bourgeonal) and the remaining cells ≥57% showed no significant response when stimulated with bourgeonal, hence no elevation in $[Ca^{2+}]_i$ was detected. Additionally when the above experiment performed in NCFsEBSS, under these low-Ca^{2+} too, spermatozoa did not respond to bourgeonal.

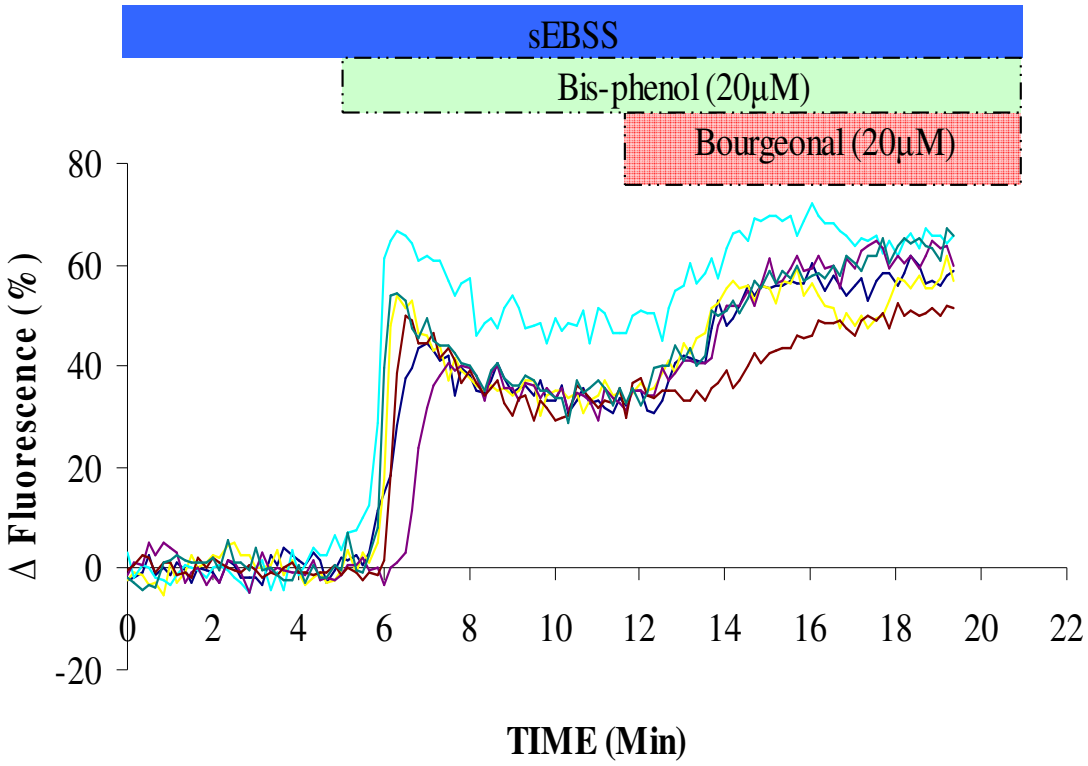

Figure.7. (A). Effect of bourgeonal (20µM) in bis-phenol (20µM) pretreated capacitated human spermatozoa bathed in sEBSS (incubated for >5hours in sEBSS). Spermatozoa were bathed in sEBSS (Dark-blue bar) for 5 minutes (control period), followed by the superfusion of bis-phenol (20µM) in the same media (light-green bar) for 7 minutes, and finally bathed in sEBSS supplemented with bis-phenol and (20µM) bourgeonal (Light-red bar) (20µM). Traces show 5 single cell responses.

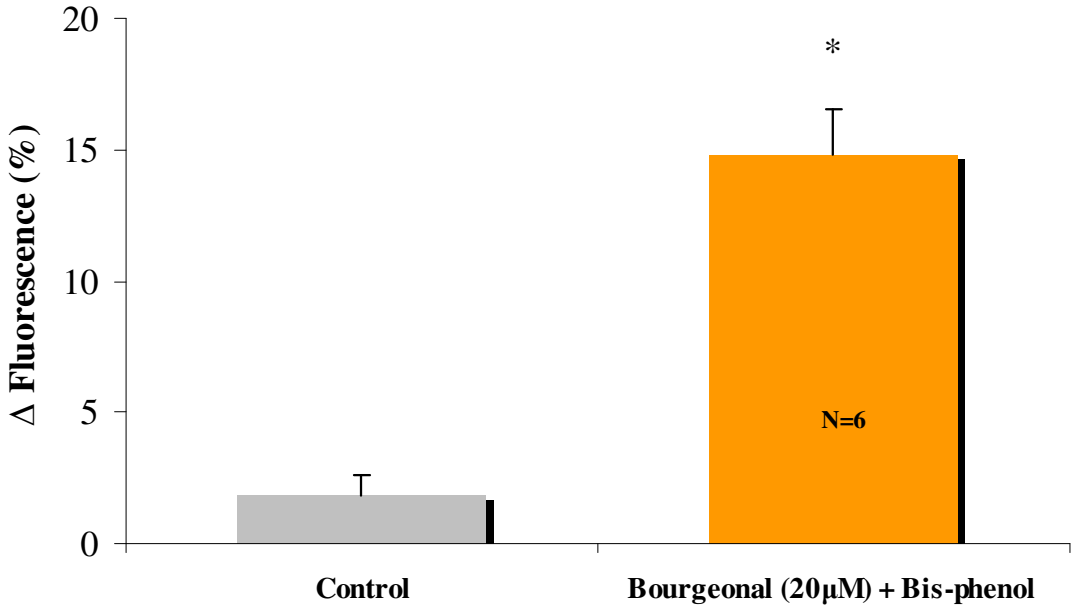

Figure.7. (B). Bar chart indicates the mean normalized increase in fluorescence post stimulation of bis-phenol pretreated cells with bourgeonal (20µM) in sEBSS; and over control period (non-treated cells), cells bathed in sEBSS media prior to the application of any stimulation. The error bars indicates the standard error of the mean (S.E.M.) and the number of experimental replicates is indicated within each of the bars (N). Asterisk (*) indicates the significance between samples tested (P<0.05%).[Bourgeonal + Bis-phenol = Bis-phenol (20µM) pretreated cells bathed in sEBSS supplemented with bourgeonal (20µM) and bis-phenol].

3.2.6 Action of bourgeonal in non-capacitated human sperm

The next step was to examine if non-capacitated spermatozoa respond to bourgeonal in sEBSS. For these experiments the spermatozoa incubation period was significantly reduced, (from > 5 hours to ≤1.5hours) and the incubation medium albumin was not added, a vital factor for capacitation (see **Introduction section 3.1**). After swim-up into sEBSS lacking albumin, cells were stained straight away (see **Methods section 2.2.1**). The uncapacitated spermatozoa were initially superfused with albumin-free sEBSS during the control imaging period, then exposed to bourgeonal (20μM). The uncapacitated spermatozoa failed to show a significant increase in OGB fluorescence (P>0.05%, against resting control period), in response to bourgeonal (two experiments, >100 cells examined), **[Figure.8. (A)].** In parallel experiments (same day with same donor/s) with cells that were swum up in sEBSS in the presence of albumin (BSA) and incubated under capacitating conditions (sEBSS + 0.3% BSA, pH 7.25-7.4, 5% CO_2, >5hour Incubation), bourgeonal reinduced a significant elevation in $[Ca^{2+}]_i$ (OGB fluorescence) in >35% of the cells (as previously reported) **[Figure.8. (C)]**. In three further experiments (with no cell controls, with capacitated spermatozoa) with uncapacitated spermatozoa no significant elevation in OGB fluorescence was detected in the $\mathbf{R}_{tot}$ of the experiments (control levels compared to treated levels, P>0.05%).

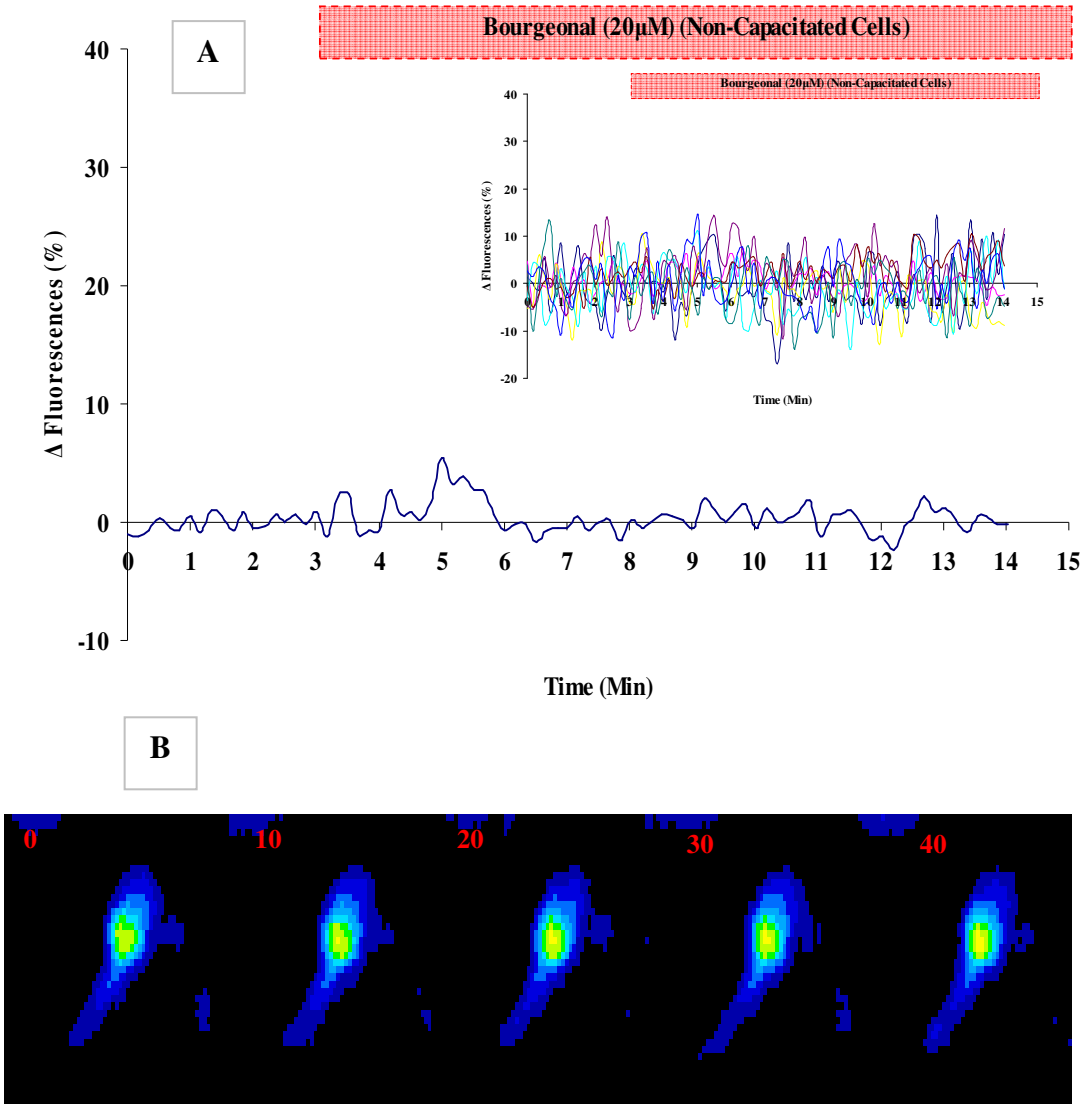

Figure.8. (A). Bourgeonal effect in non-capacitated human spermatozoa bathed in sEBSS. (incubated for ≤1.5hours in sEBSS, no albumin). Human spermatozoa incubated under non-capacitating conditions were bathed in sEBSS for 3 minutes followed by the superfusion with sEBSS supplemented with 20µM bourgeonal. Single trace illustrates the $\mathbf{R}_{tot}$ of one experiment (for 60 cells).**Insert:** 10 superimposed single cell records of non-capacitated human spermatozoa. **(B)** Pseudocolour image series (light colours show low $[Ca^{2+}]_i$) of non-capacitated spermatozoa showing no response to bourgeonal in sEBSS. Numbers in red (top left) indicate time course in seconds.

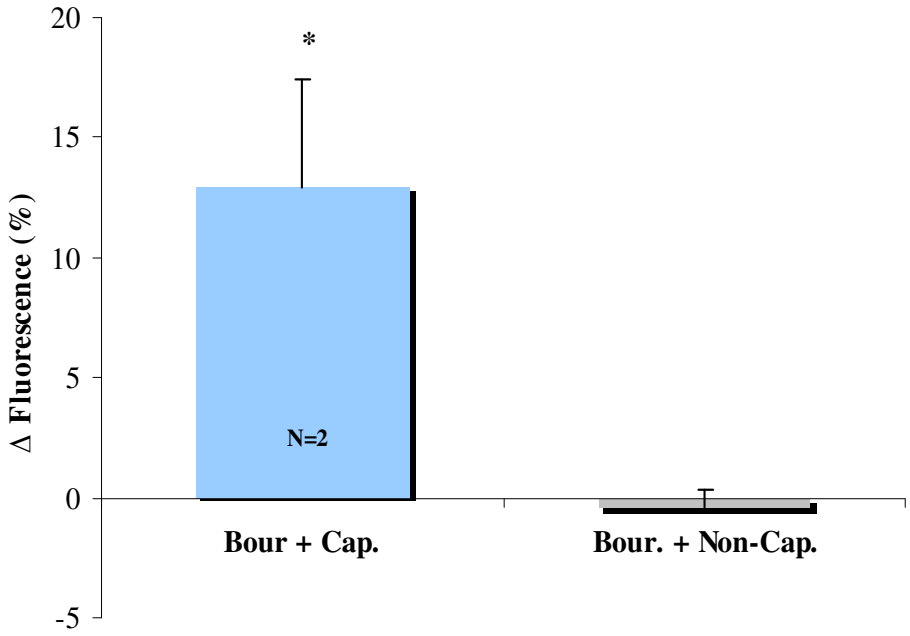

Figure.8. (C). Effect of bourgeonal in capaciated and non-capacitated human sperm. Bar chart indicates the mean normalized increase in fluorescence post stimulation of capaciated spermatozoa and non-capacitated spermatozoa with bourgeonal (20μM) in sEBSS. Capacitated spermatozoa were incubated for >5hours in sEBSS supplemented with BSA (0.3%) and non-capacitated were in sEBSS (during swimup process with no BSA, see Methods) for ±1.5 hour with no albumin. The error bars indicates the standard error of the mean (S.E.M.) and the number of experimental replicates is indicated within each of the bars (N). Asterisk (*) indicates the significance between samples tested ($P<0.05\%$). [Bour+Cap.= Capaciated spermatozoa treated with bourgeonal in sEBSS; Bour+No-Cap.= Non-capacitated spermatozoa treated with bourgeonal, in the same media].

3.2.7 Acrosomal Reaction and Bourgeonal

Capacitation is apparently required for cells to respond to bourgeonal with an elevation of $[Ca^{2+}]_i$ (see above, Section 3.3.3). AR, which is induced by elevation of $[Ca^{2+}]_e$ (Publicover et al., 2007, 2008), is also dependent upon capacitation. To investigate whether treatment of capacitated human spermatozoa with bourgeonal (20μM) leads to AR, the cells were incubated with Ca^{2+} ionophore (8μM $^{+}$ve control), bourgeonal (20 μM) and DMSO (0.02%, in the absence of bourgeonal) for 30 minutes, then processed for assessment of acrosomal status (see **Methods section 2.2.3**). When the cells were incubated with Ca^{2+} ionphore (8μM) for 30minutes in HAM F-10 media, 13.93% (±1.46 S.E.M) of cells were stained as acrosomal reacted **[Figure.9. (B)]**, (positive control), this was significantly higher than the % spontaneous acrosome reacted spermatozoa (-ve control, cells incubate in HAM F-10 alone; **Appendix One** for composition) ($P<0.05\%$; positive control against spontaneous AR) **[Figure.9.]**. When capacitated spermatozoa were incubated with HAM F-10 supplemented with bourgeonal (20μM) no percentage (%) increase in AR above control levels was detected (5.33±1.63% S.E.M) [N=3, >600 cells examined]. The % AR values were significantly lower than Ca^{2+} ionophore (Positive control, $P<0.05\%$); and showed no significant difference compared to solvent vehicle DMSO ($P>0.05\%$). The results clearly indicate that capacitated human spermatozoa incubated with 20μM bourgeonal (in HAM F-10) does not significantly induce AR.

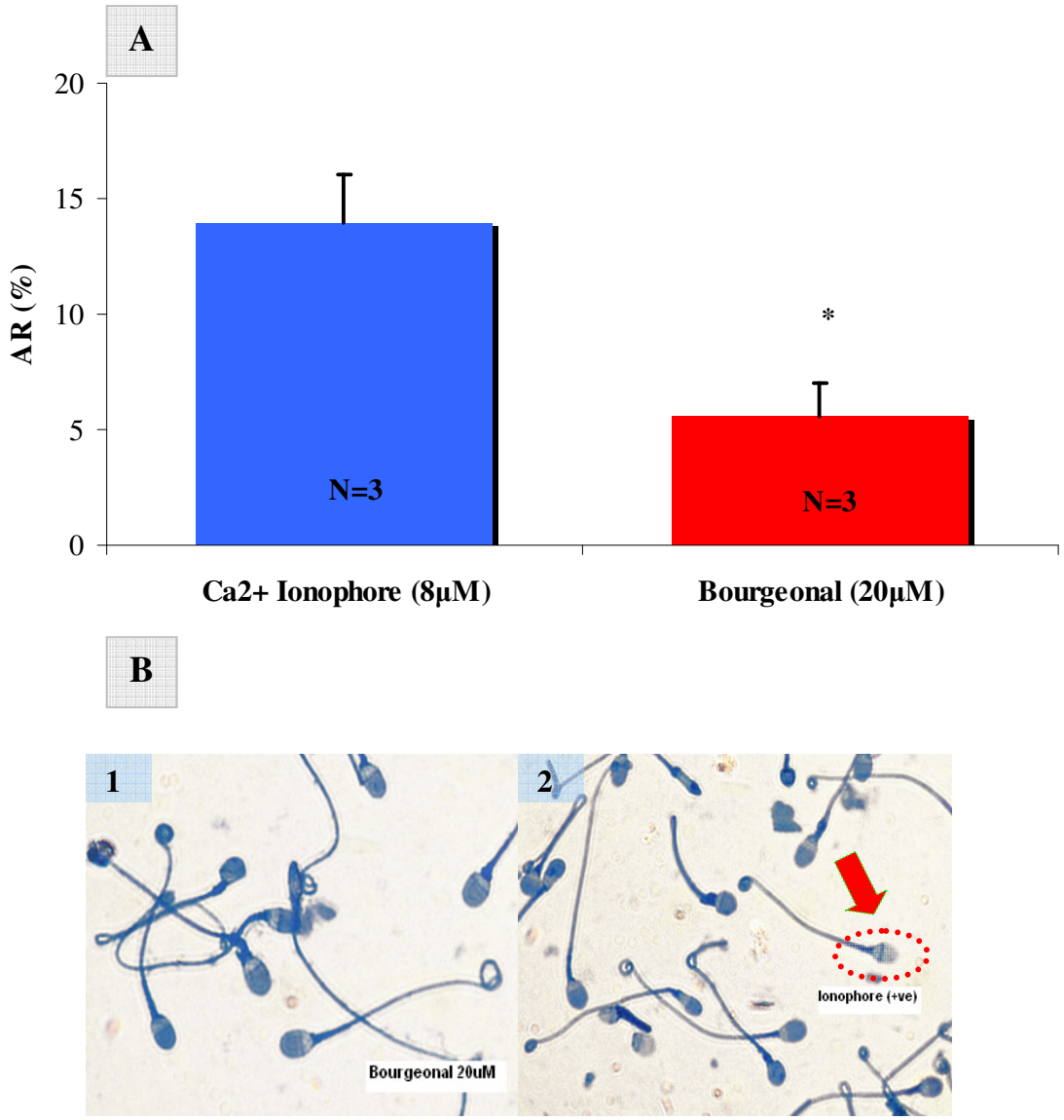

Figure.9. Effect of bourgeonal on the acrosomal status of capacitated human spermatozoa. **(A)** Bar chart indicates the mean percentage (%) induced AR, in spermatozoa incubated with Ca^{2+}-ionophore (8µM) and cells incubated with bourgeonal (20µM) in Ca^{2+}-containing media (HAM-F10). Asterisk (*) indicates the significant difference (P<0.05%). **(B)** Images of capacitated human spermatozoa incubated in HAM-F10 supplemented with ionophore (8µM) and bourgeonal (20µM). **(B.1.)** Capacitated spermatozoa incubated HAM-F10 (+HSA) supplemented with bourgeonal (20µM). **(B.2.)** Capacitated human spermatozoa incubated with HAM-F10 supplemented with Ca^{2+} Ionophore (8µM), (positive control). Red (→) arrow indicates a acrosome reacted spermatozoa.

3.2.8 Human sperm response to 3,4,CPEE

When capacitated cells, bathed in sEBSS for >5hours, were superfused with 3,4,CPEE (20μM), there was a clear and significant elevation of OGB fluorescence. Similarly to the effect of bourgeonal, 3,4,CPEE led to a elevation in OGB fluorescence 14.57 (±0.85% S.E.M) above control levels, within 2-5 minutes **[Figure.10. (A)]**. Visual examination of individual cells showed that, like bourgeonal, **[Figure.10. (C)],** 39.4±5.0% (S.E.M) of cells responded with a significant sustained elevation in OGB fluorescence, 22% above resting levels (four experiments, >200 cells examined) and ≈14% of cells responded with a significant single transient elevation in $[Ca^{2+}]_i$, peaking within ≈0.66 minutes **[Figure.10. (B)]**, and >50% of the cells showed no distictive elevation.

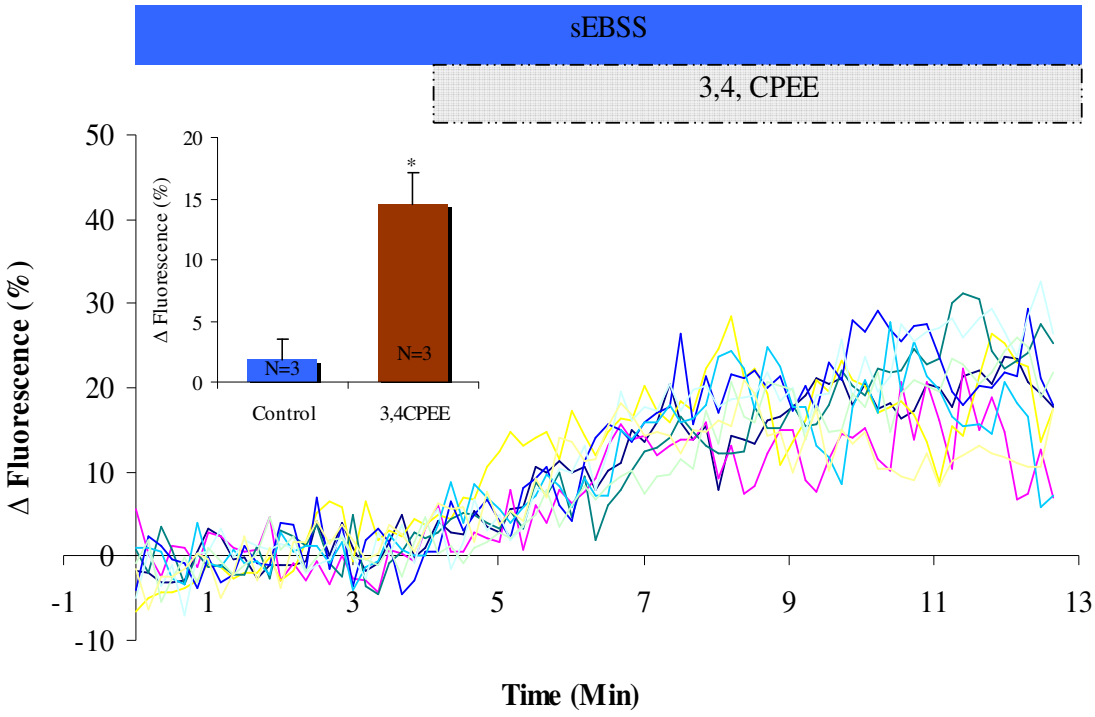

Figure.10. (A). Effect of 3,4,CPEE (20µM) in capacitated human spermatozoa bathed in sEBSS (incubated for >5hours in sEBSS). The cells were bathed with sEBSS (Dark-blue bar) for 4 minutes (control period), followed by the superfusion with sEBSS supplemented with 3,4 CPEE (20µM), for 8 minutes (Light-grey). Traces show 6 single cell responses. **Insert:** Mean normalized increase in $[Ca^{2+}]_i$ (fluorescence) after exposure to 3,4,CPEE (20µM) in sEBSS. The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar.). Asterisk (*) indicates the signifincance between samples tested (P<0.05%).

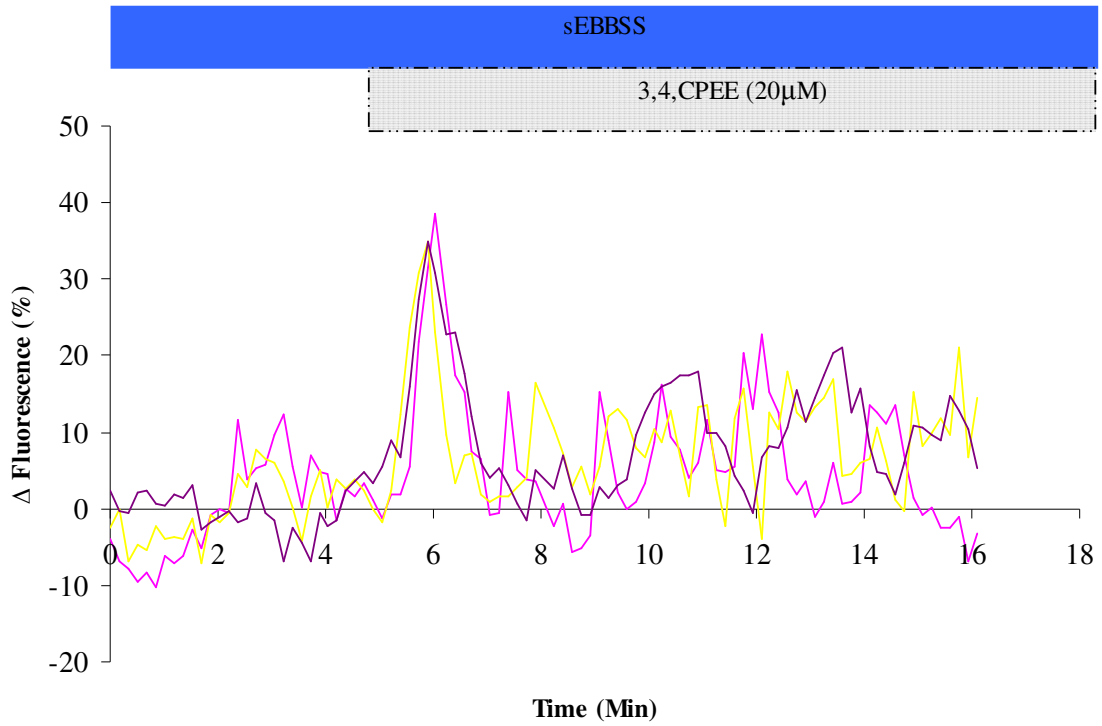

Figure.10. (B). Transient response to 3,4,CPEE in sEBSS obtained in ≈14% of capacitated spermatozoa. Following >5hour incubation, spermatozoa were bathed in sEBSS (Dark-blue bar) for 5 minutes and subsequently superfused with sEBSS supplemented with 3,4,CPEE (Light-grey bar). Traces show 3 single cell responses.

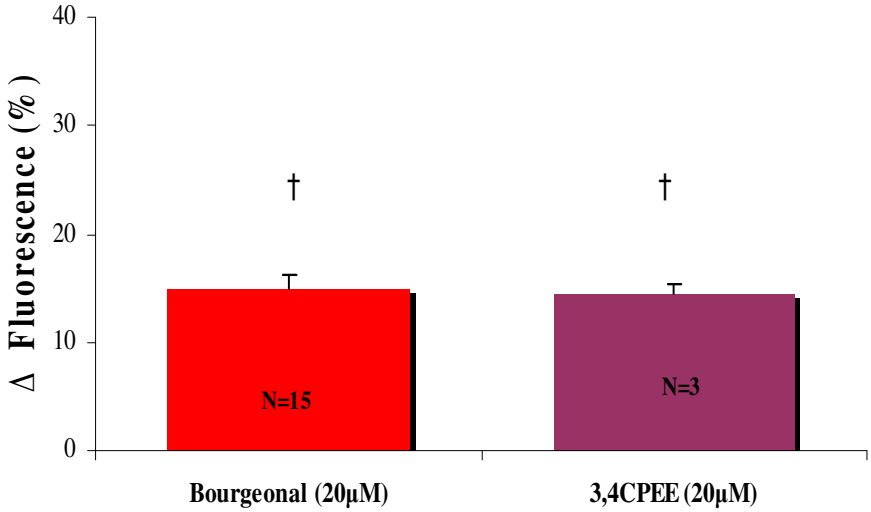

Figure.10. (C). Mean normalised increase in $[Ca^{2+}]_i$ (fluorescence) to bourgeonal (20µM) in sEBSS and 3,4,CPEE (20µM) in the same media. The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. The use of identical symbols above graphs (†) indicates no significant difference (P>0.05%).

Similarly to experiments with bourgeonal, the response of capacitated human spermatozoa bathed in NCFsEBSS (nominal calcium free media) was examined (<5µM Ca^{2+}; Harper et al., 2004) to 3,4,CPEE. Capacitated cells were superfused with NCFsEBSS (0.3% BSA) for >2 minutes, then exposed to 3,4,CPEE (20µM) in the same media. This did not elicit a significant elevation in OGB fluorescence above resting levels in the $\mathbf{R}_{tot}$ of the experiments (P>0.05%, control levels against treated levels) (three experiments, >300 cells examined) **[Figure.11.]**. Visual examination of individual cells showed that a sustained increase in OGB fluorescence occurred in 11.6±1.88% (S.E.M) of the cells, reaching maximum elevation of 12.0±0.55% (S.E.M) above control levels (three experiments, ≥ 300 cells examined). The remaining 88.45% (±1.88% S.E.M) of the cells showed no distinctive elevation in $[Ca^{2+}]_i$. The peak

amplitude of response was similar to that for cells bathed in NCFsEBSS exposed to bourgeonal (20µM) (P>0.05 %, t test, Bourgeonal vs. 3,4,CPEE in sEBSS) **[Figure.11. (B)**].

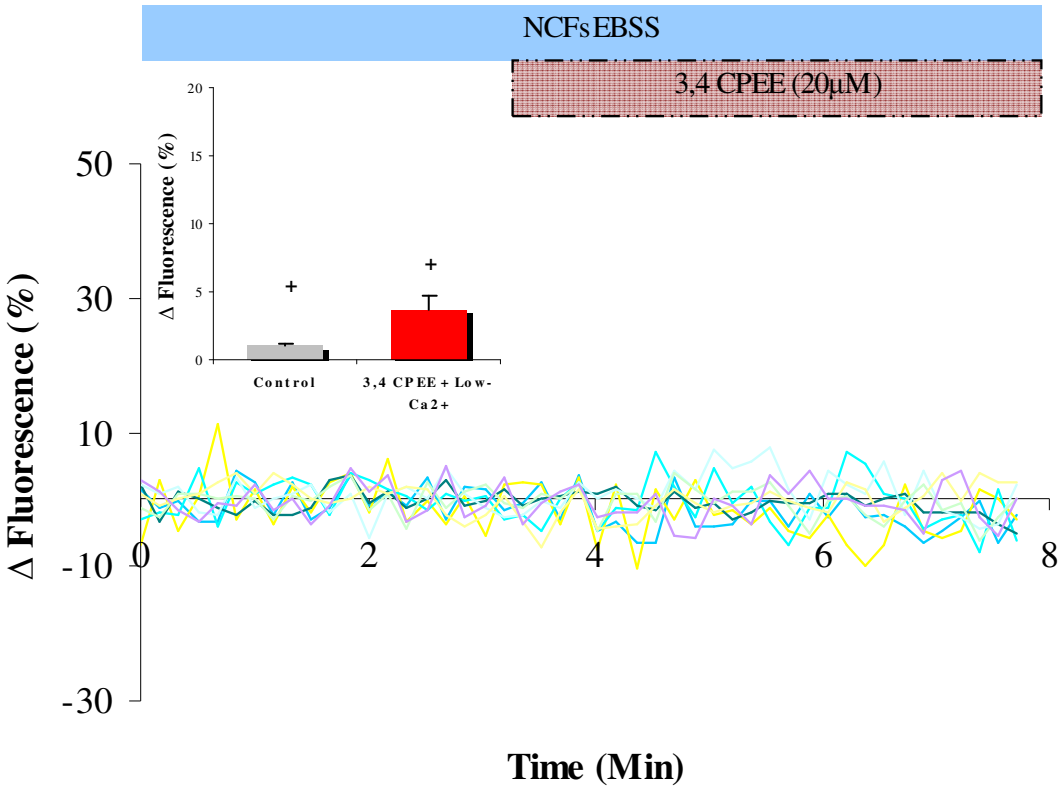

Figure.11. (A). Effect of 3,4,CPEE (20µM) in capacitated spermatozoa bathed in NCFsEBSS (incubated for >5hours in sEBSS). Capacitated human spermatozoa were bathed for 4 minutes in NCFsEBSS (Light-blue bar) (control period). Traces showing 8 single cell responses. **Insert:** Bar graph illustrating the mean normalized increase in fluorescence response to 3,4,CPEE (20µM) in NCFsEBSS. The error bars represent the SEM, and the number (N=3) of experimental replicates is indicated in each bar. The same symbol above graph (†) indicates no significant difference (P>0.05%).

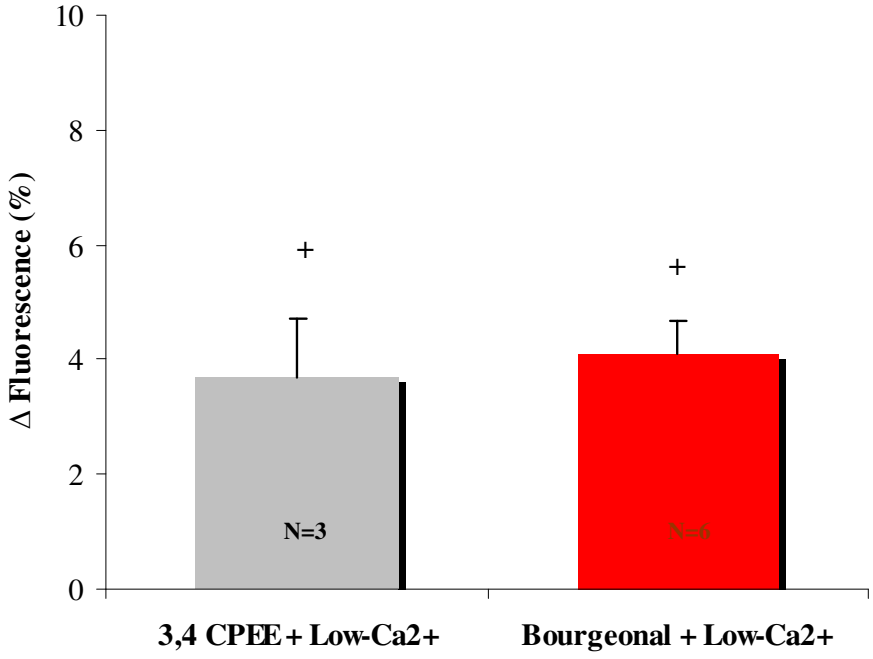

Figure.11. (B). Mean normalized increase in fluorescence response to 3,4,CPEE (20µM) in NCFsEBSS (Low-Ca^{2+}); against response of human capaciated spermatozoa to bourgeonal (20µM) in the same media (Low-Ca^{2+}). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. The same symbol above graph (**+**) indicates no significant difference (P>0.05%).

3.3 Discussion

Intracellular calcium $[Ca^{2+}]_i$ signalling is crucial to sperm function (Jimenez-Gonzalez et al., 2006). Calcium signalling is achieved by enabling Ca^{2+} entry to the cytoplasm (here concentration is kept very low) from the extracellular space and/or from intracellular organelles (stores), where Ca^{2+} concentration is up to four orders of magnitude higher (Costello et al., 2009). The presence and crucial role of Ca^{2+} channels in mammalian spermatozoa plasma membrane is well established in many physiological processes including capacitation, hyperactivation, chemotaxis and acrosome reaction (Costello et al., 2009). Identification of the steroid hormone, progesterone (together with the chemotactic dose 10pM) as a chemoattractant compound produced *in vivo* by oocyte and cumulus cells emphasized that chemotaxis and its associated signalling cascade in mammalian sperm is a puzzle that must be solved (Teves et al., 2006, 2009; Sun et al., 2005). The identification of the hOR17-4 in the flagellar midpiece (G-protein olfactory receptor), and its respective agonist (bourgeonal being the most potent) also shed light on the importance of human sperm chemotaxis (Spehr et al., 2003, 2004, 2006). Ca^{2+} signalling induced by bourgeonal, a potent agonist of hOR-17.4 (Spehr et al., 2003) was examined in this chapter - specifically:

(1) Do human spermatozoa respond to bourgeonal with elevation of $[Ca^{2+}]_i$?

(2) Is the response dependent on extra- or intra- cellular calcium ?

(3) Is the response reversible ?

(4) Does bourgeonal stimulate AR like progesterone (Publicover et al., 2007) ?

(5) Is there any response in uncapacitated spermatozoa ?

When capacitated human spermatozoa were exposed to bourgeonal (20μM) 38% responded with a sustained elevation in $[Ca^{2+}]_i$, and ≈8% responded with a single transient elevation $[Ca^{2+}]_i$. When a series of 'stepped' concentrations were introduced (2, 20, 200μM), there was, at each dose, a significant elevation both of the amplitude of the Ca^{2+} signal and of the percentage responding cells in $[Ca^{2+}]_i$. This is in agreement with previous studies that the response of human sperm to bourgeonal shows dose-dependence (Spehr et al., 2003, 2004). The response to bourgeonal was dependent on $[Ca^{2+}]_{e.}$. In NCFsEBSS (<5 μM Ca^{2+}; Harper et al., 2004) no significant response was detected in the $\mathbf{R}_{tot}$ of the experiments, as previously reported, (Spehr et al., 2003, 2004, 2006; Gakamsky et al., 2009). However, visual examination showed a significantly modest elevation in $[Ca^{2+}]_i$ occurred in <10% of the cells and when NCFsEBSS media was buffered with EGTA (3 mM) the response to bourgeonal was abolished. Though data obtained using EGTA must be treated cautiously due to the effects on intracellular stored – these results suggest that the response to bourgeonal involved Ca^{2+}-influx. Consistent with this conclusion, pretreatment with bis-phenol (20μM), an inhibitor for both SERCA and SPCA Ca^{2+}-pumps to mobilise Ca^{2+} stores (Brown et al., 2004; Harper et al, 2005), did not prevent cells responding to bourgeonal. More than 35% of the cells showed a clear response to, similar to that seen in non-pretreated cells. Therefore it's possible to conclude that Ca^{2+}-influx is the primary sources of the elevation of $[Ca^{2+}]_i$ induced by bourgenal.

The sustained elevation of $[Ca^{2+}]_i$ seen in bourgeonal-treated human sperm clearly suggests that, when the receptor (hOR17-4) is occupied by bourgeonal, the downstream signal (possibly stimulation of mACIII; Spehr et al, 2004) is tonically activated causes prolonged opening of a membrane Ca^{2+}-permeable channel (unknown). When bourgeonal was applied to capacitated cells and then removed (washed-off) the level of $[Ca^{2+}]_i$ returned to pre-stimulus values within 2-3 min, indicating that, upon removal of the ligand, the signalling events that cause opening of the Ca^{2+} permeable channel are switched off and the channel closes. However, a small subpopulation (<8%), respond with a transient response that terminated despite the continued application of bourgeonal. With 200μM bourgeonal >60% of the responsive cells showed this pattern of response – peaking rapidly and then 'switching off''. Thus the cells are apparently capable of 'inactivating' their response to bourgeonal a feature that may be crucial for chemotactic responses, which will require the ability to discriminate temporal differences in chemoattractant concentration. This is addressed in subsequent chapters (see Chapter 3).

Additionally, in every experiment a small subpopulation of cells showed showed high resting OGB fluorescent levels; possibly due to over-staining of the cells or elevated resting levels of $[Ca^{2+}]_i$,. When these were bathed in bourgeonal no response was detected. The fluorescence from these bright cells showed little (or no) variance over the course of the experiment. These cells may be spermatozoa that have lost their capacity to regulate $[Ca^{2+}]_i$, maybe through an abnormal interaction with the polylysine-treated

glass coverslip as has been proposed for polylysine bound echinoderm spermatozoa (Wood et al., 2003).

Suspension of human sperm in albumin-free medium results in a significant reduction in capacitation (>15% to <3%) and a significant reduction in swiming behaviour (Gakamsky et al., 2009). When the cells were exposed to 20 μM bourgeonal without capacitation (No BSA, no incubation time <1.5 hours), no significant elevation in $[Ca^{2+}]_{i,,}$ occurred. Parallel experiments with capacitated spermatozoa (sEBSS, +0.3% BSA), showed the normal significant elevation in $[Ca^{2+}]_i$ in >35% of the cells. These results strongly suggest that human sperm response to bourgeonal is dependent on capacitation (incubation of cells under capacitating conditions). This is in contrast to the work of Spehr et al (2003) who observed Ca^{2+} responses in uncapacitated cells. However the higher doses used in previous studies (200 μM – 500 μM, used by other - the signalling pathway involved in uncapacitated spermatozoa may differ from the responses of capacitated mammalian spermatozoa to lower doses of bourgeonal.. Significantly, progesterone acts as a chemoattractant for human sperm at pM concentrations (Teves et al., 2006, 2009), but when spermatozoa are exposed to progesterone (nM to μM) there is an immediate elevation in $[Ca^{2+}]_i$ in both capacitated and non-capacitated spermatozoa (Thomas and Meizel, 1989, Blackmore et al., 1990, 1991; Baldi et al., 1991; Bedu-Addo et al., 2005).

Following the study of the intracellular cell response to bourgeonal, it was then examined if the ligand stimulated AR. Once capacitation of mammalian spermatozoa is

completed, spermatozoa are able to acrosome react in response to a physiological stimulus, such as progesterone (Marin-Briggiler et al., 1999). This steriod hormone is capable of stimulating mammalian AR both *in vitro* and *in vivo*, in addition to stimulation of sperm chemotaxis at lower doses (pM; Teves et al., 2006, 2009), both dependent in Ca^{2+} (Publicover et al., 2007, 2008). Bourgeonal (20μM) failed to increase the rate of acrosome reaction above control level. Spehr (2004, 2006), showed that higher doses of bourgeonal (>50μM) also failed to stimulate AR.

A given olfactory receptor (OR) in theory responds to an extensive range of stimulus (Spehr et al., 2003). The molecular receptive field of hOR17-4 was determined using cyclamal as a template (Spehr et al., 2003). Spehr et al. (2003) concluded that stimulating compounds included an aldehyde group connected to an aromatic ring via carbon chain of a defined length (2-4 carbons) in order to effectively stimulate the hOR17-4 receptor (Spehr et al., 2003). Here the $[Ca^{2+}]_i$ response of capacitated human spermatozoa to both bourgeonal [$C_{13}H_{18}O$] and 3,4,CPEE [$C_{12}H_{14}O_3$] were examined and compared, these two are aromatic compounds of similar structure.

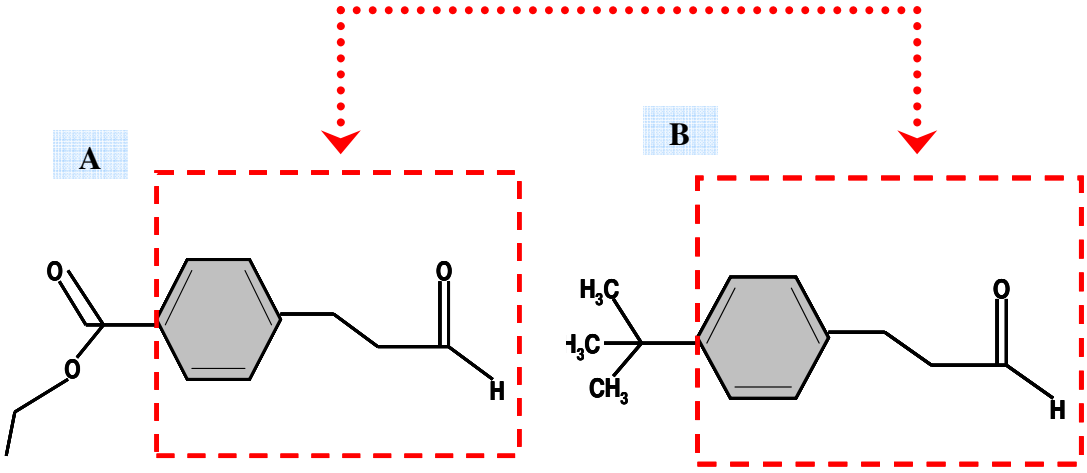

Figure.12. Structural homology between the ligands. **(A)** Structure of bourgeonal (4-t Butylbenzenepropionaldehyde), [$C_{13}H_{18}O$] **(B)** 3,4,CPEE, 3-(4´-Carboxyphenyl)-Propionaldehyde Ethyl Ester) [$C_{12}H_{14}O_3$]. Red arrows indicate the structural similarities between both compounds.

The nature (kinetics and amplitude) of the Ca^{2+} responses and the proportion of the responsive cells were similar for the two compounds, as was the modification of the stimulatory action by omission of Ca^{2+} from the bathing medium (NCFsEBSS). All of the above suggests that human spermatozoa respond to the two both bourgeonal and 3,4,CPEE through the activation of the same receptor (hOR17-4 ?) and signal transduction pathway. 3,4,CPEE [$C_{12}H_{14}O3$], includes all of the components that are thought necessary for the stimulation of the hOR17-04, an aldehyde group connected to an aromatic ring via a carbon chain of >4 carbons (key determinant for an effective stimulation of the OR (Spehr et al., 2003).

A small number of experiments in this chapter were performed in laboratory collaboration with **Dr Teves**, **Dr Giojalas**, **Dr Gidobaldi** and **Dr Uñates** from the Cebicem, University of Cordoba, Argentina and another proportion in collaboration with the Department of Chemistry, University of Birmingham, UK. All of these experiments, both in Argentina and Birmingham were performed by Aduén Andrés Morales García, author of this thesis (U. Birmingham, 2009). Most of the data provided will be used for grant applications and for an upcoming paper(s), in collaboration with **Dr Giojalas, Dr Uñates, Dr Teves and Dr Publicover** as head of the research group (University of Birmingham).

IV

CHAPTER ~FOUR~

"If you believe, you will be a step closer to achieve your goal". Aduén, 2009

Chapter Four

HUMAN SPERMATOZOA $[Ca^{2+}]_i$ ELEVATION AS A RESULT OF AN INCREASE IN cAMP (3'-5'-CYCLIC ADENOSINE MONOPHOSPHATE)

4.1 Introduction

Ca^{2+} is a universal intracellular messenger encoding information by temporal and spatial patterns of concentration (Jimenez-Gonzalez et al., 2006; **see introduction**). In mammalian spermatozoa $[Ca^{2+}]_i$ plays a pivotal role in capacitation (Jimenez-Gonzalez et al., 2006) and after capacitation, controls acrosome reaction (Kirkman-Brown et al., 2002) motility and hyperactivation (Carlson et al., 2003, Suarez & Ho, 2003) and chemotaxis (Eisenbach and Giojalas, 2006; Spehr et al., 2003; Teves et al., 2006, 2009). The $[Ca^{2+}]_i$ response of human sperm exposed to bourgeonal is small and prolonged (**Chapter 3**). However, in some cells the $[Ca^{2+}]_i$ response is transient or decays slowly, despite the continued presence of the agonist. Thus some cells may have the ability to terminate or down-regulate the response to the agonist, which is likely to be important in chemotactic signaling pathway (Kaupp et al., 2003, 2008; Eisenbach & Giojalas, 2006).

Like Ca^{2+}, cAMP plays an important role in the regulation of many physiological processes in sperm and it is likely that these two second messengers interact. It has been suggested that activation of ORs in mammalian spermatozoa leads to elevation of $[Ca^{2+}]_i$ through a pathway involving G_{olf}, membrane AC and generation of cAMP (Spehr et al., 2004, 2006). Cyclic nucleotide-gated channels (CNG) have been identified and localized in mammalian spermatozoa (Publicover et al., 2008).

Chapter Aims

This chapter attempts to examine and understand the role of Ca^{2+} and cAMP in determining the kinetics of the $[Ca^{2+}]_i$ response to bourgeonal, particularly the termination of the Ca^{2+} signal, which may be crucial for regulation of chemotaxis (Kaupp et al., 2003). This has been addressed by i) examining the response of spermatozoa exposed to bourgeonal in low-Ca^{2+} saline followed by readmission of Ca^{2+}, where any feedback effects of elevated $[Ca^{2+}]_i$ will be 'delayed' (prior to Ca^{2+}-readmission), ii) investigating the effects of manipulation of [cAMP] on the Ca^{2+} response and iii) investigating generation of cAMP in capacitated human spermatozoa exposed to bourgeonal in Ca^{2+}.

4.2 Results

4.2.1 Response to bourgeonal is enhanced by 'pretreatment' under low Ca^{2+} conditions

After incubation in capacitating medium (sEBSS, +0.3% BSA, for >5 hrs) spermatozoa were superfused in NCFsEBSS for 4 minutes, followed by the superfusion with NCFsEBSS supplemented with bourgeonal, conditions under which the agonist has little effect on $[Ca^{2+}]_i$ (see **Chapter 3**). After 3 minutes, Ca^{2+} was returned to the medium (sEBSS supplemented with 20μM bourgeonal), upon which a single rapid $[Ca^{2+}]_i$ transient occurred detected in the $\mathbf{R}_{tot}$ of the experiment, individual cell examination showed that >70% of the cells (four experiments, > 300 cells examined) responded in this way. Mean increase in fluorescence of OGB was 41.45% (±2.7% S.E.M), significantly higher than control levels (P<0.05%, against control levels), reaching maximum elevation ($\mathbf{T}_{max;}$) within ±0.66 minutes (six experiments, >200 cells examined) **[Figure.1. (A)]**. This response to readmission of Ca^{2+} in the presence of bourgeonal could be repeated if Ca^{2+} and bourgeonal were washed out of the chamber and then reapplied **[Figure.2.].** Here more than >75% of the cells responded with a transient elevation in OGB fluorescence, and out of the responding cells >96.5% of bourgeonal pretreated cells in NCFsEBSS responded to both first Ca^{2+}-readmission and post washout to the second readmission. The rest of the cells, 3.4%, responded exclusively to the second Ca^{2+} readmission.

The response was dose-dependent **[Figure.4.]**. When Ca^{2+}-readmission was carried out but in the presence of 100μM bourgeonal a significant single transient elevation in OGB fluorescence of 89.8% (±5.82% S.E.M) above control (<0.05%– significantly bigger than with 20 μM), **[Figure.4.],** reaching maximum elevation within ±0.6 minutes, was detected in >90% of cells (two experiments, >140 cells examined) **[Figure.3.].**

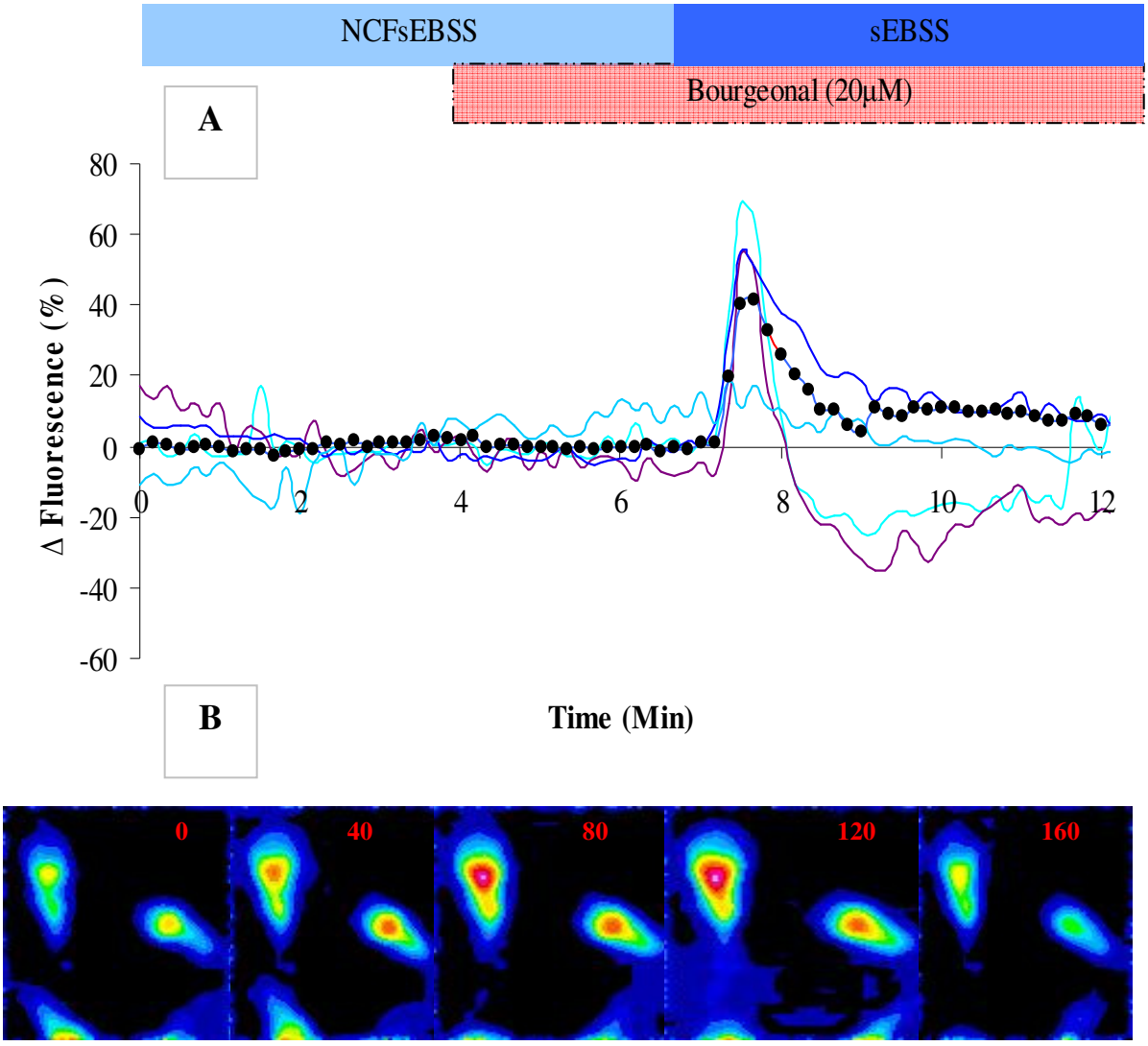

Figure.1. (A) Response of capacitated human spermatozoa pretreated with bourgeonal (20µM) in low-Ca^{2+} (NCFsEBSS) to the introduction of sEBSS (Ca^{2+}-high) supplemented with bourgeonal [cells incubated in sEBSS for >5hours]. Capacitated human spermatozoa were bathed in NCFsEBSS (Light-blue bar) for 4 minutes, followed by the superfusion with bourgeonal (Light-red bar) in the same media for 3 minutes, consecutively the capacitated human spermatozoa were bathed in sEBSS (Dark-blue bar) supplemented with bourgeonal (20µM). Traces showing 4 single cell responses and black-dotted single trace (●) indicating the $\mathbf{R}_{tot}$ of one experiment (>30 cells). **(B).** Pseudocolour image series (warm colours show high $[Ca^{2+}]_i$) of capacitated human spermatozoa responding to Ca^{2+} readmission with single transient elevation in OGB fluorescence. Numbers in red indicates time course in seconds.

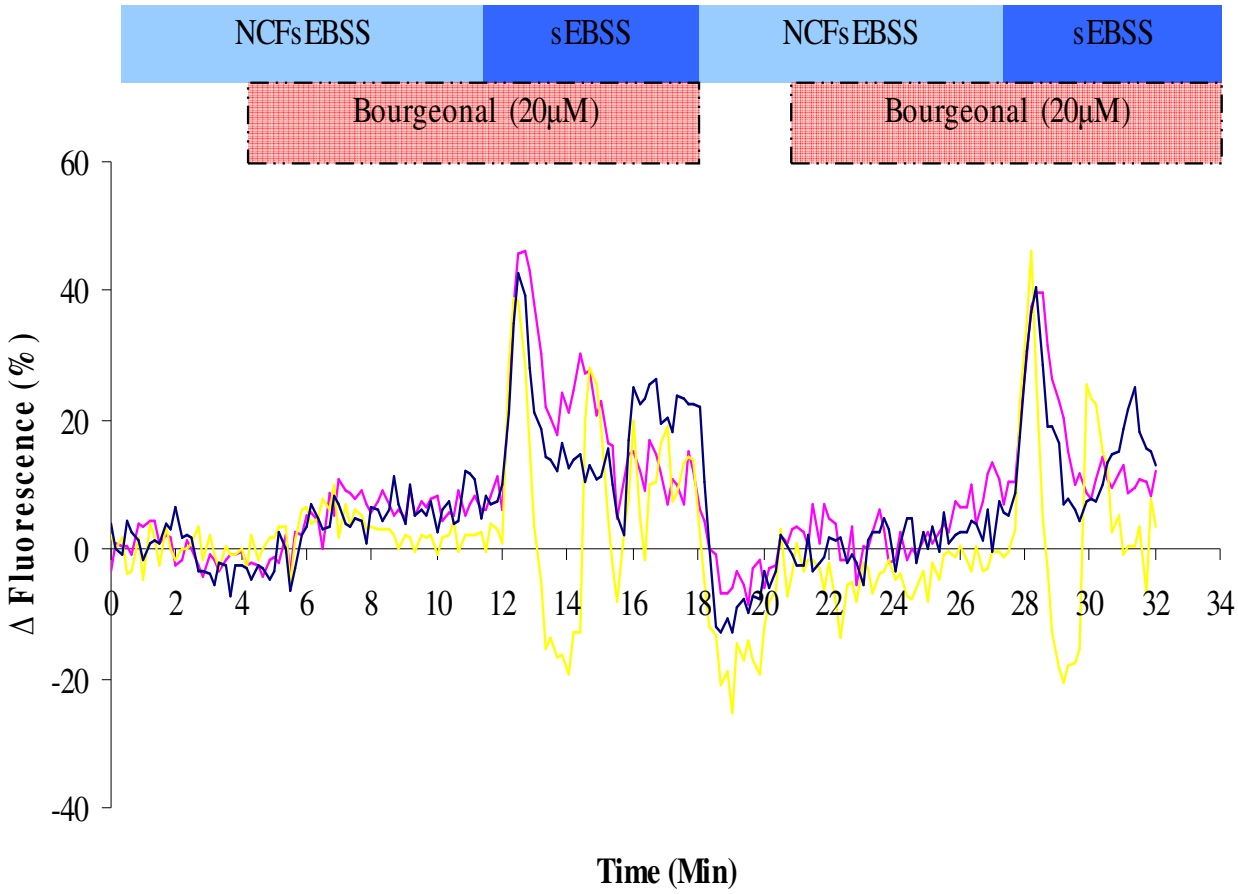

Figure.2. Response to readmission of Ca^{2+} in the presence of bourgeonal (20µM) could be repeated if Ca^{2+} and bourgeonal washed out of the chamber and then reapplied [cells previously incubated in sEBSS for >5hours]. Capacitated spermatozoa were bathed in NCFsEBSS (Light-blue bar) for 4 minutes, followed by the superfusion with bourgeonal (Light-red bar) in the same media for 8 minutes, and subsequently bathed in sEBSS (Dark-blue bar) supplemented with bourgeonal (20µM). Bourgeonal and Ca^{2+} were washed out with NCFsEBSS and were reintroduced at minute 27.7, to the cells in the imaging chamber. Traces of 3 single cell responses.

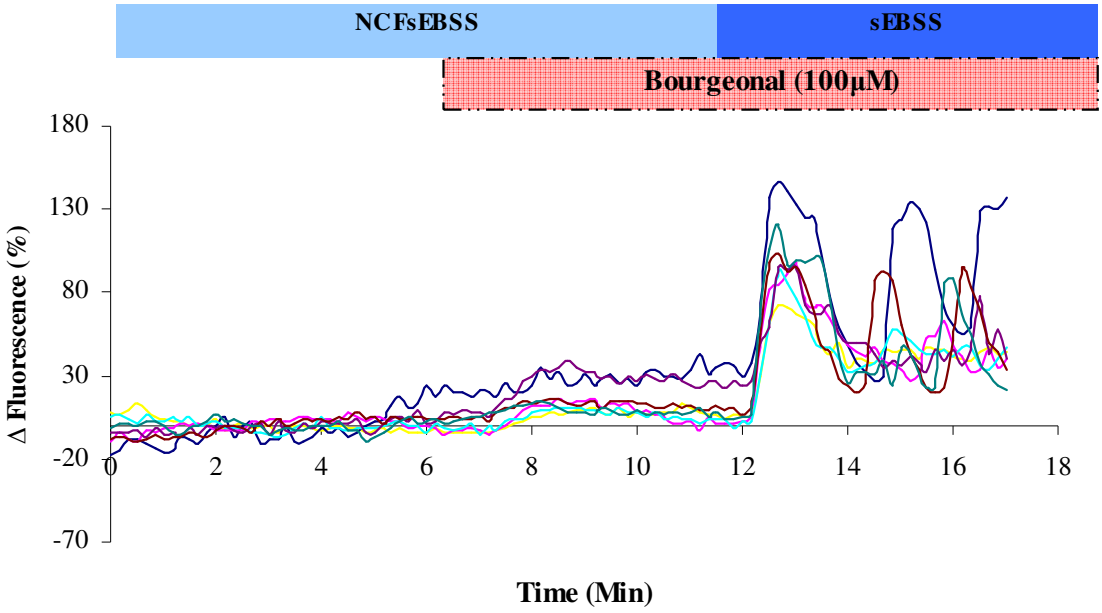

Figure.3. Response of capacitated human spermatozoa pretreated with bourgeonal (100µM) in low-Ca^{2+} (NCFsEBSS) to the introduction sEBSS supplemented with 100µM bourgeonal (Ca^{2+}-high) [cells incubated in sEBSS for >5hours]. Capacitated human spermatozoa were bathed in NCFsEBSS (Light-blue bar) for 7 minutes (Control period), followed by the superfusion with 100µ M bourgeonal (Light-red bar) in the same media for 6 minutes, and finally bathed in sEBSS (Dark-blue bar) supplemented with bourgeonal (100µM). Traces showing 7 single cell responses.

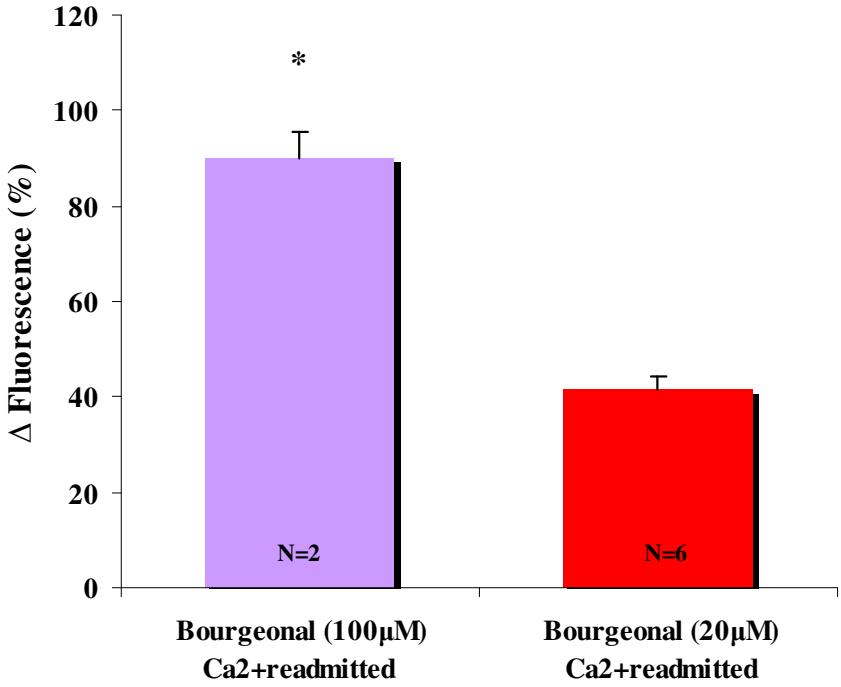

Figure.4. Response to bourgeonal is enhanced by 'pretreatment' under low Ca^{2+} conditions**.** Bar graph of the mean normalised fluorescence upon Ca^{2+} introduction to the bourgeonal pretreated cells at 20µM; against cells pretreated with 100µM bourgeonal under the same experimental conditions. The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Asterisks (*) show differences in significance (P<0.05%).

To assess the significance of the action of bourgeonal in the observations described above (response to readmission of Ca^{2+} to the incubation chamber), the response of the same cells to readmission of Ca^{2+} (switch from NCFsEBSS to sEBSS) both under control conditions and in the presence of bourgeonal (20µM), was then examined.

When capacitated spermatozoa were bathed in NCFsEBSS for 4 minutes, followed by the superfusion with sEBSS there was a sustained elevation in OGB fluorescence in >75% of the cells, which stabilized ≈20 % above control levels (P<0.05%, against

control levels), within >2 minutes. Consecutively NCFsEBSS was re-applied into imaging chamber (4 minute wash), significantly decreasing OGB fluorescence to resting levels, followed by bathing of the cells with NCFsEBSS supplemented with bourgeonal (20μM), for 4 minutes. Subsequent application of sEBSS supplemented with bourgeonal (20 μM) resulting in a single transient elevation in OGB fluorescence, >40% above control levels (as previously described), significantly greater than the response of non-bourgeonal-pretreated cells ($P<0.05$%, bourgeonal response against non-bourgeonal pretreated) **[Figure.5.].** In addition, both the kinetics of the response in bourgeonal-pretreated cells (time to peak fluorescence) and the nature of the response (>75% transient response with bourgeonal, >75% sustained response in controls) were significantly different. These data strongly suggest that the transient response seen upon readmission of Ca^{2+} to the saline is due to the presence of bourgeonal. Plasma membrane channels opened by bourgeonal in NCFsEBSS remain open, Ca^{2+} is readmitted to the saline and $[Ca^{2+}]_i$ rises, at which point they are apparently 'switched off'.

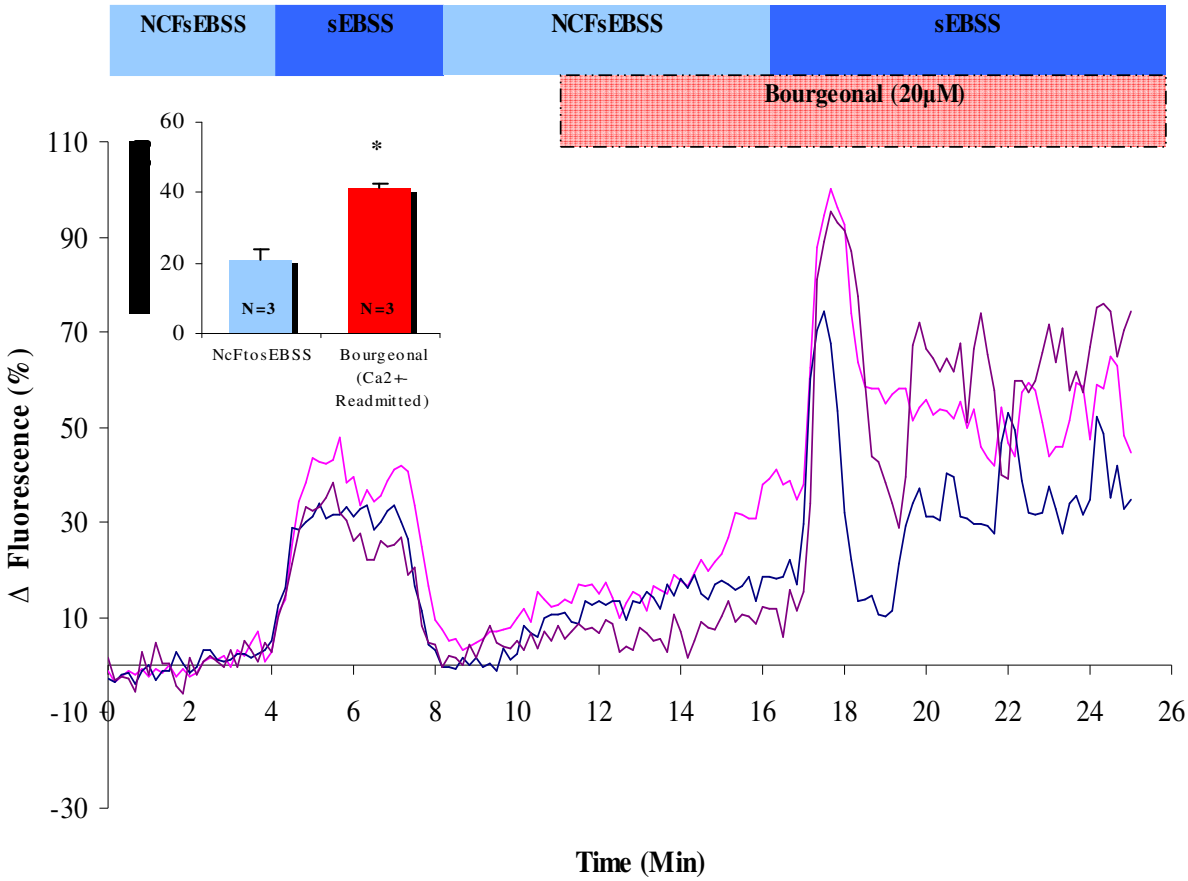

Figure.5. Effect of non-treated-bourgeonal cells, bathed in NCFsEBSS and exposed to sEBSS (NCFsEBSS→sEBSS); compared to the response of bourgeonal-treated spermatozoa in NCFsEBSS introduced to sEBSS (NCFsEBSS + bourgeonal → sEBSS + bourgeonal), in one experiment. Capacitated human spermatozoa were bathed in NCFsEBSS (Light-blue bar) for 4 minutes, followed by the superfusion with sEBSS (Dark-blue bar) for 3 minutes, subsequently the cells were bathed in NCFsEBSS for 4 minutes (Ca^{2+} wash out). The cells were then superfused with 20µM bourgeonal in the same media (NCFsEBSS) for 6 minutes with the introduction of sEBSS supplemented with 20µM bourgeonal (Light-red bar). **Insert:** Effect of non-treated cells, bathed in NCFsEBSS and exposed to sEBSS (NCFsEBSS→sEBSS); compared to the response of bourgeonal-treated spermatozoa in NCFsEBSS introduced to sEBSS supplemented with bourgeonal (NCFsEBSS + bourgeonal → sEBSS + bourgeonal). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Asterisks (*) show differences in significance (P<0.05%). [Light-blue vertical bar on graph represent cells bathed in NCFsEBSS introduced to sEBSS; and dark-red vertical bar bourgeonal pretreated cells exposed to bourgeonal in sEBSS].

4.2.2 - 3,4,CPEE induces a transient Ca^{2+} response similarly to bourgeonal

Capacitated cells (incubated for >5 hours in sEBSS (0.3% BSA)) were bathed in NCFsEBSS for 3 minutes, followed by 3,4,CPEE (20μM) in NCFsEBSS. When Ca^{2+} (sEBSS supplemented with 20μM 3,4,CPEE) was readmitted to the chamber there was a significant transient elevation in OGB fluorescence detected in the $\mathbf{R}_{tot}$ of the experiments (peaking within ±0.90 minutes), with a mean elevation in OGB fluorescence 44.8 ±2.9% S.E.M above control levels (P<0.05%, against resting levels; three experiments, >200 cells examined), as previously reported with bourgeonal at the same dose **[Figure.6.].** Visual examination of individual cells revealed that >75% of the cells responded with a transient response**.** No significant difference was detected between the effects of readmission of Ca^{2+} in the presence of 3,4,CPEE and in the presence of bourgeonal (mean normalized fluorescence of both drugs at 20 μM; P>0.05%) **[Figure.7.].**

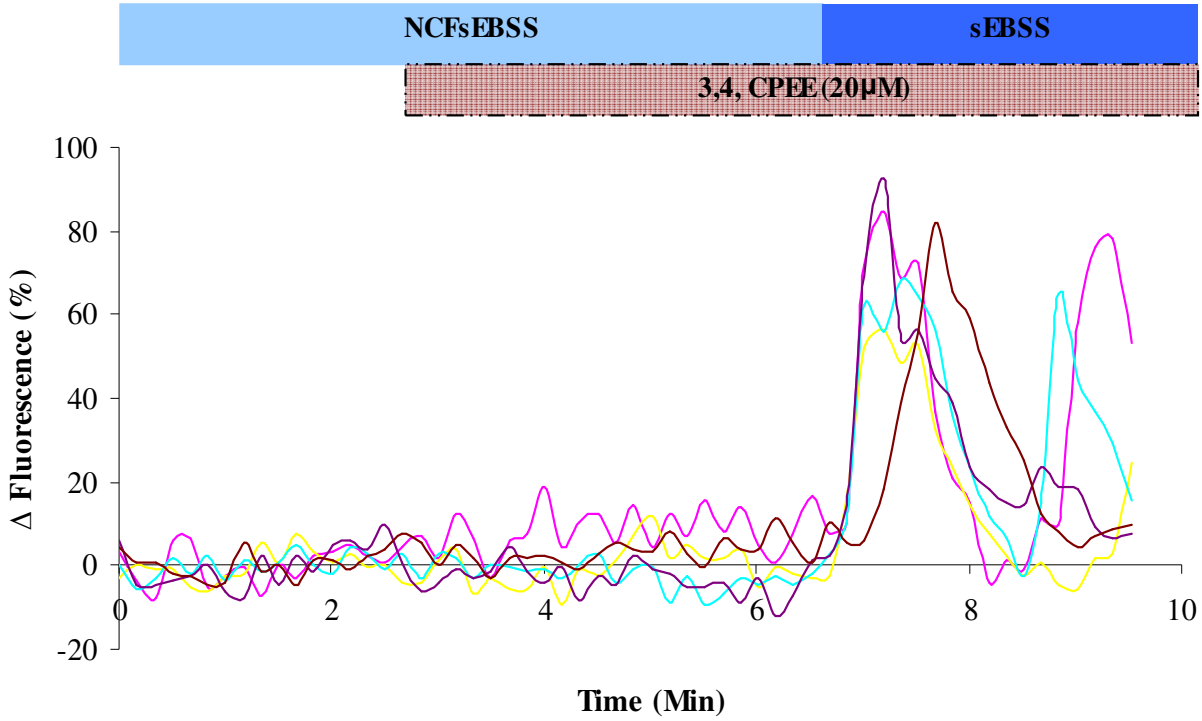

Figure.6. Response of capacitated human spermatozoa pretreated with 3,4,CPEE (20µM) in low-Ca^{2+} (NCFsEBSS) to the introduction of Ca^{2+}. [cells incubated in sEBSS for >5hours]. Capacitated human spermatozoa were bathed in NCFsEBSS (Light-blue bar) for 3 minutes, followed by the superfusion with 3,4,CPEE (Light-grey bar) in the same media for 4 minutes, and finally bathed in sEBSS (Dark-blue bar) supplemented with 3,4,CPEE (20µM). Traces showing 5 single cell responses.

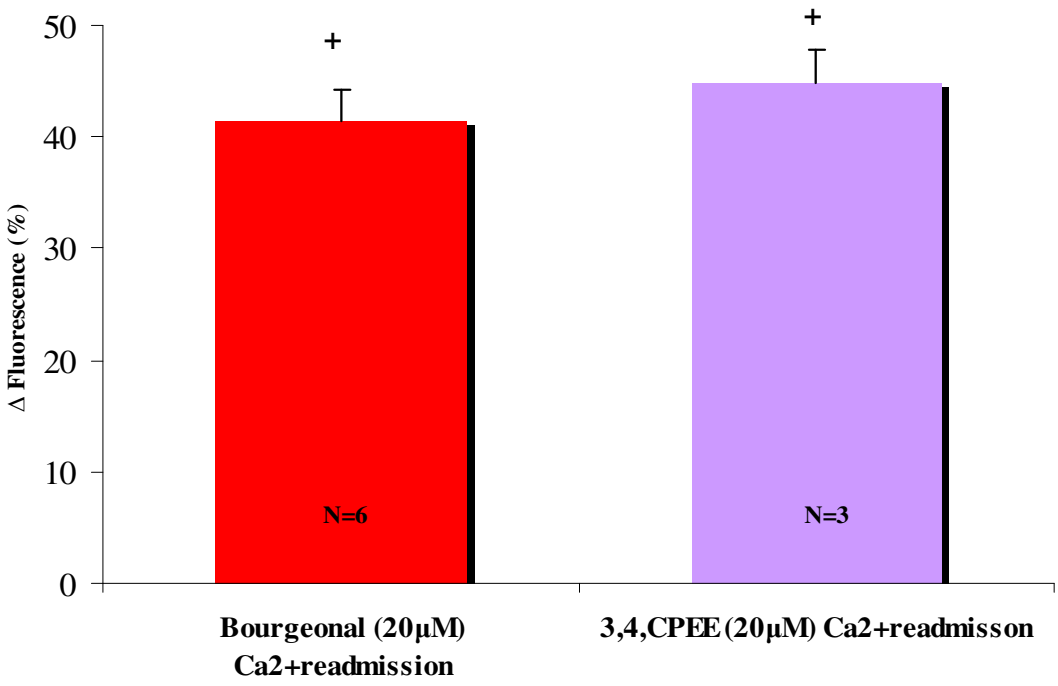

Figure.7. Response to bourgeonal and 3,4,CPEE is enhanced by 'pretreatment' under low Ca^{2+} conditions**.** Bar chart represents the mean normalised fluorescence response to 3,4,CPEE upon reintroduction of Ca^{2+} in 3,4,CPEE (20µM) pretreated cells bathed in NCFsEBSS; against bourgeonal (20µM) under the same experimental conditions (NCFsEBSS + 3,4,CPEE → 3,4,CPEE + sEBSS Vs. NCFsEBSS + Bourgeonal→ sEBSS + Bourgeonal). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. The use of the identical symbols (+) to show no significant differences (P>0.05%).

4.2.3 Does cAMP shape the bourgeonal-induced Ca^{2+} transient?

In the experiments described above (see **section 4.2.2**) it was shown that, if bourgeonal is applied to human sperm in NCFsEBSS, readmission of Ca^{2+} causes a large increase in $[Ca^{2+}]_i$, This response is transient, suggesting that when $[Ca^{2+}]_i$ rises significantly (and/or rapidly), the bourgeonal-activated channels switched off. Spehr et al., (2003, 2004) hypothesized that the opening of the membrane Ca^{2+}-permeable channel by bourgeonal is due to an elevation of cAMP and/or activation of PKA. In this section the potential role of cAMP was examined, if any, in shaping the transient nature of the response that occurs when Ca^{2+} is readmitted.

The cells were exposed to the same protocol as described above (see **Sections 4.2.1, 4.2.2**) but in this case an inhibitor of cyclic nucleotide phosphodiesterases (PDE), IBMX 3-isobutyl-1-methylxanthine (1mM) was included in the NCFsEBSS to potentiate the cGMP/cAMP signaling pathways (Wood et al., 2003). Upon application of IBMX there was a rapid elevation in OGB fluorescence in >90% of cells (maximum increase ≈15% above control levels). After a further 2 minutes bourgeonal (20μM) was applied to the cells. When Ca^{2+} was readmitted to the imaging chamber there was a single transient elevation in OGB fluorescence detected in the $\mathbf{R}_{tot}$ of the experiments, (40.8 ±3.16% S.E.M; P<0.05%) which peaked within ±0.64 minutes (six experiments, >300 cells examined **[Figure.8.].** Furthermore, the magnitude and kinetics of the response showed no significant difference compared with cells under the same bathing conditions in the absence of IBMX (1mM) **[Figure.10.].**

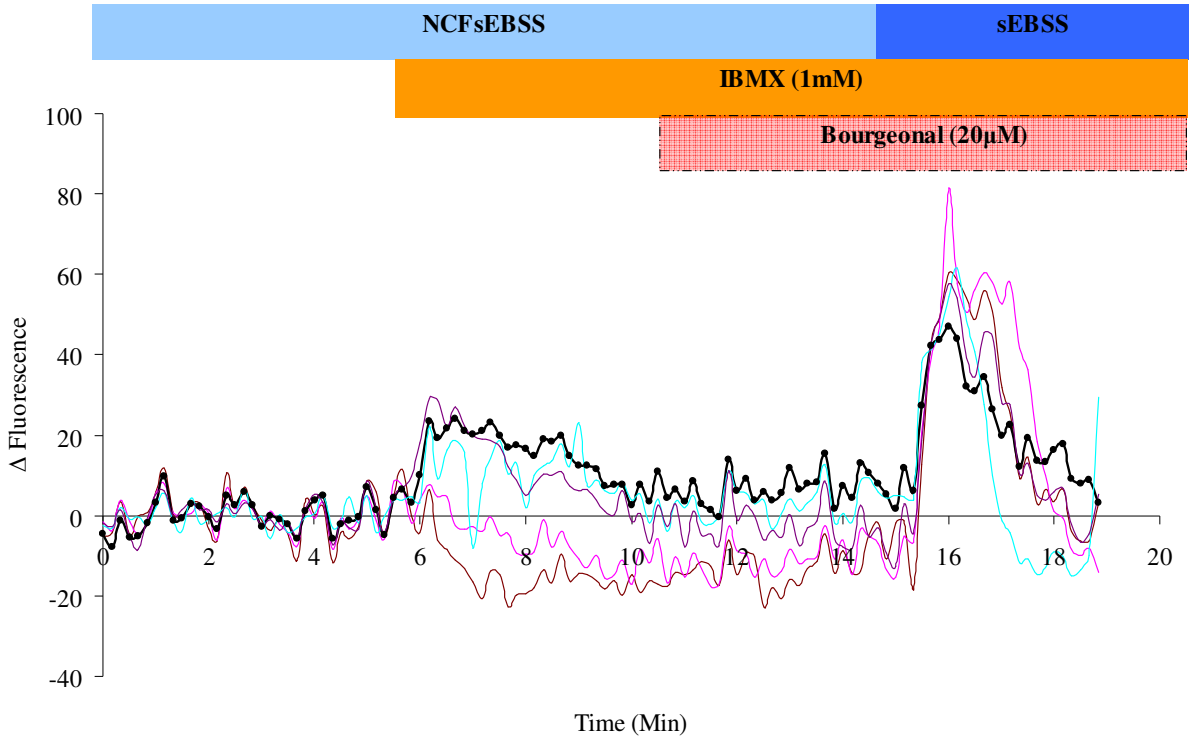

Figure.8. OGB fluorescence response of capacitated human spermatozoa pretreated with bourgeonal (20µM) and IBMX (1mM) in low-Ca^{2+} (NCFsEBSS) introduced to sEBSS (Ca^{2+}-high) supplemented with IBMX (1mM) and bourgeonal (20µM) [cells incubated in sEBSS for >5hours]. Capacitated spermatozoa were bathed in NCFsEBSS (Light-blue bar) for 5 minutes (Control period), followed by the superfusion with IBMX (1mM) (Orange bar) in the same media for 5 minutes. Subsequently the cells were bathed in NCFsEBSS supplemented with bourgeonal (Light-red bar) and IBMX (1mM) (Orange bar). This was finally followed by the bathing of the cells in sEBSS (Dark-blue bar) supplemented with bourgeonal (20µM) and IBMX (1mM). Traces showing 5 single cell responses and black-dotted single trace (●) indicating the $\mathbf{R}_{tot}$ of one experiment (100 cells).

The failure of IBMX to modify the transient response to Ca^{2+} indicates that kinetics of (cAMP) do not determine kinetics of the Ca^{2+} response. Another possibility is that cAMP is responsible only for the activation of the response, termination being regulated separately. To investigate this, 1mM N^6,2'-O-Dibutyryladenosine-3',5'-cyclic

monophosphate (dbcAMP) was used, the membrane permeable form of cyclic AMP (Meyer and Miller, 1974; Heit et al., 1991). Following >5 hours incubation in capacitating media, spermatozoa were bathed in a low-Ca^{2+} media (NCFsEBSS, <5µM Ca^{2+}) for >2 minutes. Subsequently the cells were bathed in NCFsEBSS with dbcAMP (1mM), followed by superfusion with sEBSS supplemented with dbcAMP (1mM), resulting in a significant single transient elevation in $[Ca^{2+}]_i$, in >77% of the cells, reaching maximum increase in OGB fluorescence of 38.8±1.32% (SEM; P<0.05%) within ±0.70 minutes (four experiments, >240 cells examined) **[Figure.9.].** Showing no significant difference in magnitude **[Figure.10.]** and $\mathbf{T}_{max.}$ (time to reach maximum elevation in OGB fluorescence) **[Figure.11.],** compared with the response obtained with IBMX (under the same conditions) and bourgeonal pretreated cells introduced to Ca^{2+} .

When capacitated human spermatozoa pretreated with dbcAMP (1mM) and 20µM bourgeonal in NCFsEBSS, were introduced to Ca^{2+}, under these conditions too the cells responded with a single transient elevation in OGB fluorescence. However this data is not significant, due to the excessive variables (treatments) in the experiment.

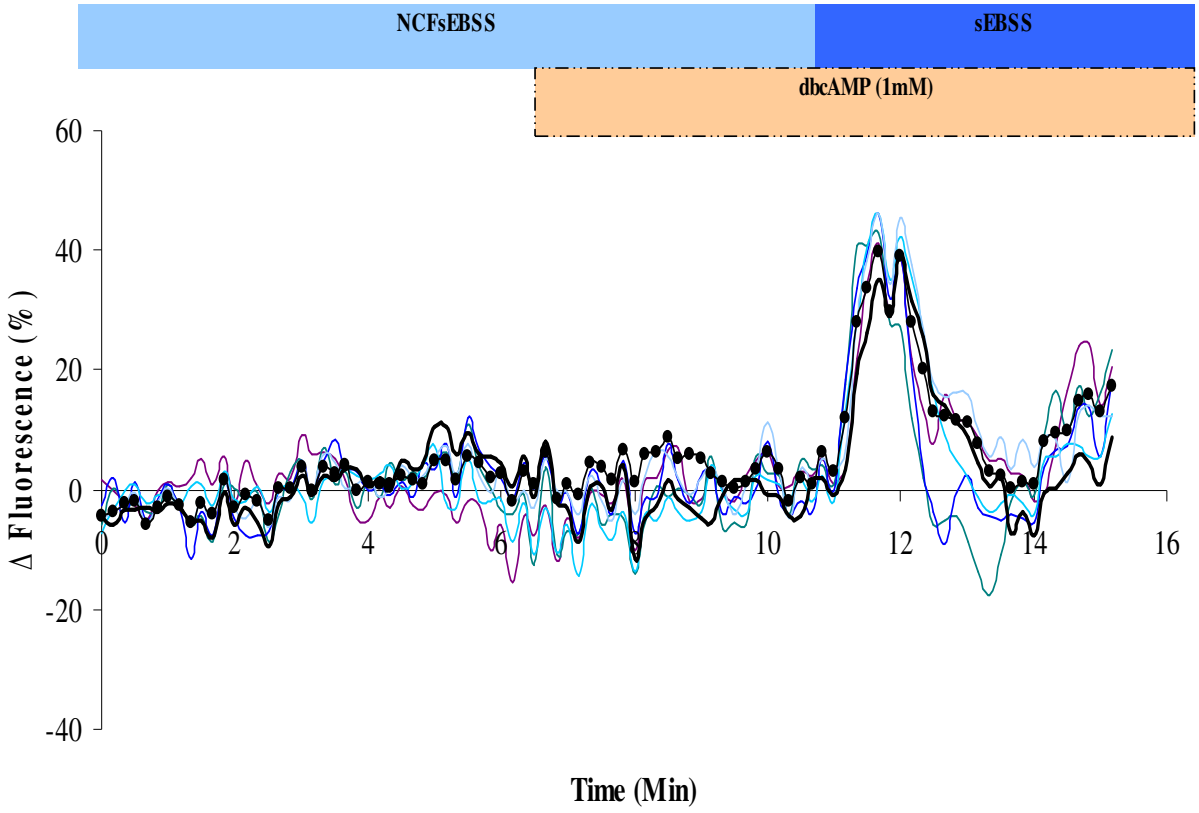

Figure.9. Response of capacitated human spermatozoa pretreated with dbcAMP (1mM) in low-Ca^{2+} (NCFsEBSS) to the introduction Ca^{2+}. [cells incubated in sEBSS for >5hours]. Capacitated human spermatozoa were bathed in NCFsEBSS (Light-blue bar) for 7 minutes, followed by the superfusion with dbcAMP (Light-beige bar) in the same medium for 4 minutes, and finally bathed in sEBSS (Dark-blue bar) supplemented with dbcAMP (1mM). Traces showing 7 single cells response and black dotted single trace (●) indicating the $\mathbf{R}_{tot}$ of one experiment (70 cells).

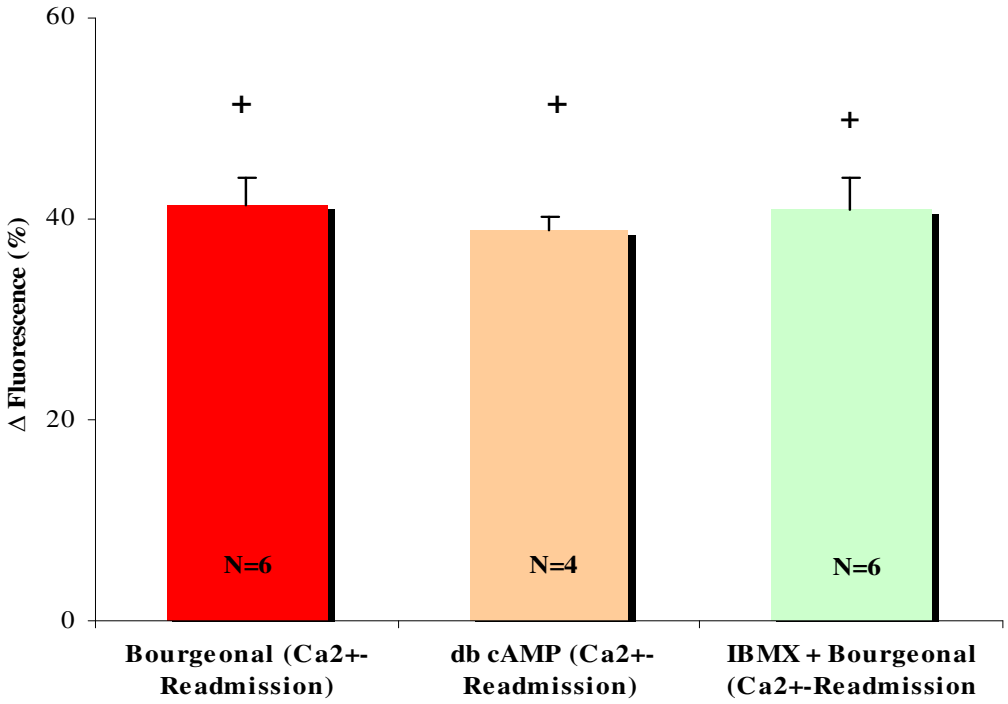

Figure.10. Mean normalized increase OGB fluorescence response to Ca^{2+} readmission in capacitated human spermatozoa pretreated in NCFsEBSS supplemented bourgeonal, dbcAMP (1mM) and (1mM) IBMX coapplied with bourgeonal (20µM). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. The use of the same symbols (+) above bar graph indicates no significant differences (P>0.05%).

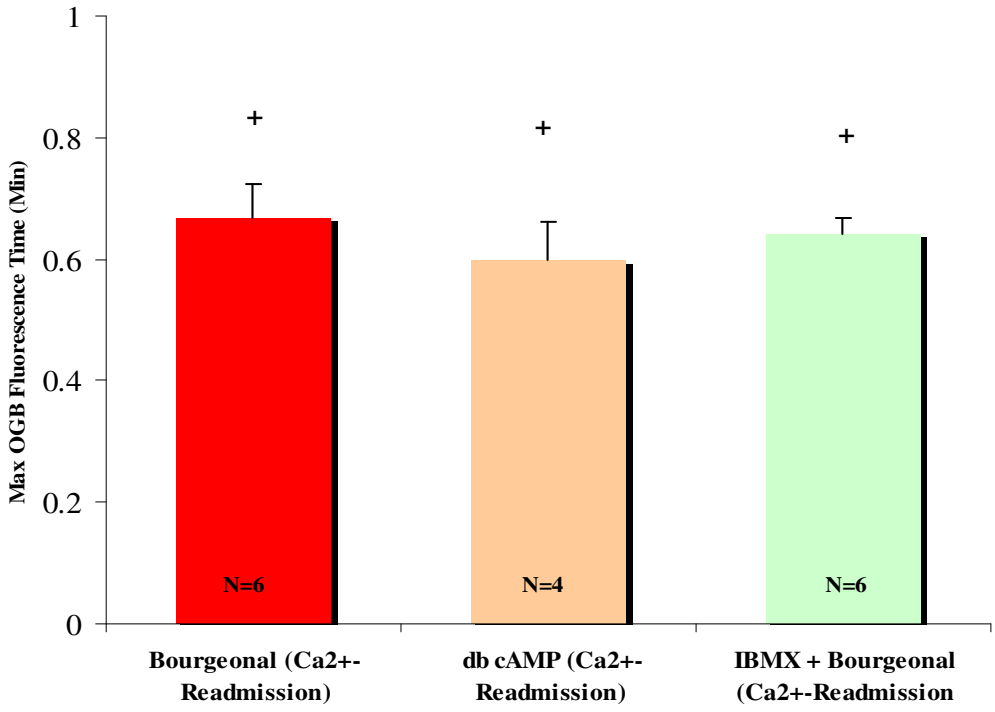

Figure.11. Bar graph illustrating the mean latency time for the response to peak (minutes) for each treatment. The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Identical symbols indicate (+) no significant differences (>P0.05%).

4.2.4 dbcAMP induces elevation of $[Ca^{2+}]_i$

Since exposure to dbcAMP was able to induce a $[Ca^{2+}]_i$ transient (upon readmission of Ca^{2+} to the imaging chamber) indistinguishable from that seen with bourgeonal, the response to dbcAMP of cells superfused with sEBSS was investigated, to allow comparison with the effect of bourgeonal. Spermatozoa were incubated in capacitating medium (sEBSS +0.3% BSA) for >5hours (WHO, 1999). After imaging OGB fluorescence in SEBSS for >2 minutes (control period), cells were exposed to dbcAMP (1mM) in the same medium and resulting in a significant, sustained elevation in OGB fluorescence 15.22% (±1.16% S.E.M) above control levels (P<0.05%, against resting control levels), within ≈3 minutes **[Figure.12.].** Individual cell examination revealed that 41.6% of the cells responded with clear elevation in OGB fluorescence (mean =20.1±2.6% S.E.M) above control levels (five experiments, >300 cells examined). Additionally as previously reported with bourgeonal (20μM) in sEBSS, a small proportion of cells (<7%) responded with a significant single transient response in OGB fluorescence, peaking within 0.6-0.8 minutes at ≈20% above control levels (P<0.05%, against resting control levels). The remaining cells showed no significant response.

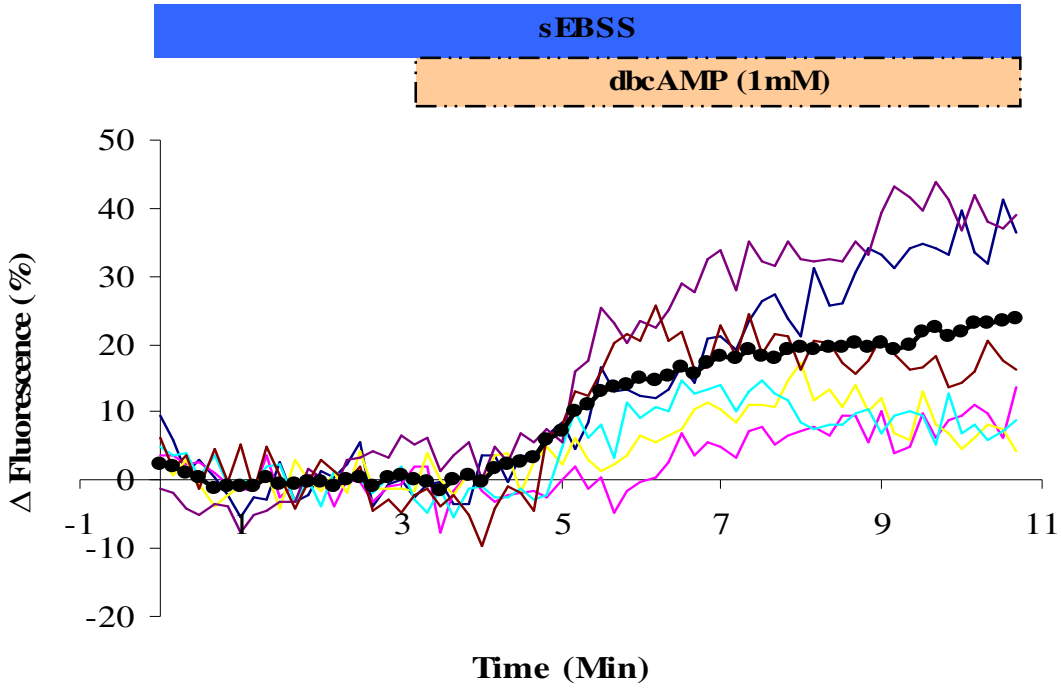

Figure.12. Effect of dbcAMP (1mM) on OGB fluorescence in capacitated spermatozoa bathed in sEBSS (cells incubated for >5 hours in sEBSS). The cells were initially bathed in sEBSS (Dark-blue bar) for 4 minutes, and subsequently superfused with dbcAMP (Light-beige bar) in the same media (sEBSS). Traces show 6 single cell responses; black dotted (●) single trace indicating $\mathbf{R}_{tot}$ of experiment (>30 cells).

When capacitated human spermatozoa were superfused with NCFsEBSS for >2minutes (control period), followed by the superfusion with dbcAMP in NCFsEBSS. Human spermatozoa failed to show a significant increase in OGB fluorescence (P>0.05%, against resting control period), in the $\mathbf{R}_{tot}$ of the experiment, in response to dbcAMP in low-Ca^{2+} (four experiments, >200 cells examined) **[Figure.13.].** Visual examination of individual cells showed that 10.5% showed a clear sustained elevation in OGB fluorescence ≈12% above control levels (P<0.05%, against resting control levels), the remaining cells showing no distinctive elevation in OGB fluorescence. As previously

reported with bourgeonal in NCFsEBSS (low-Ca^{2+}), no transient response were observed in cells exposed to dbcAMP in low-Ca^{2+} containing.

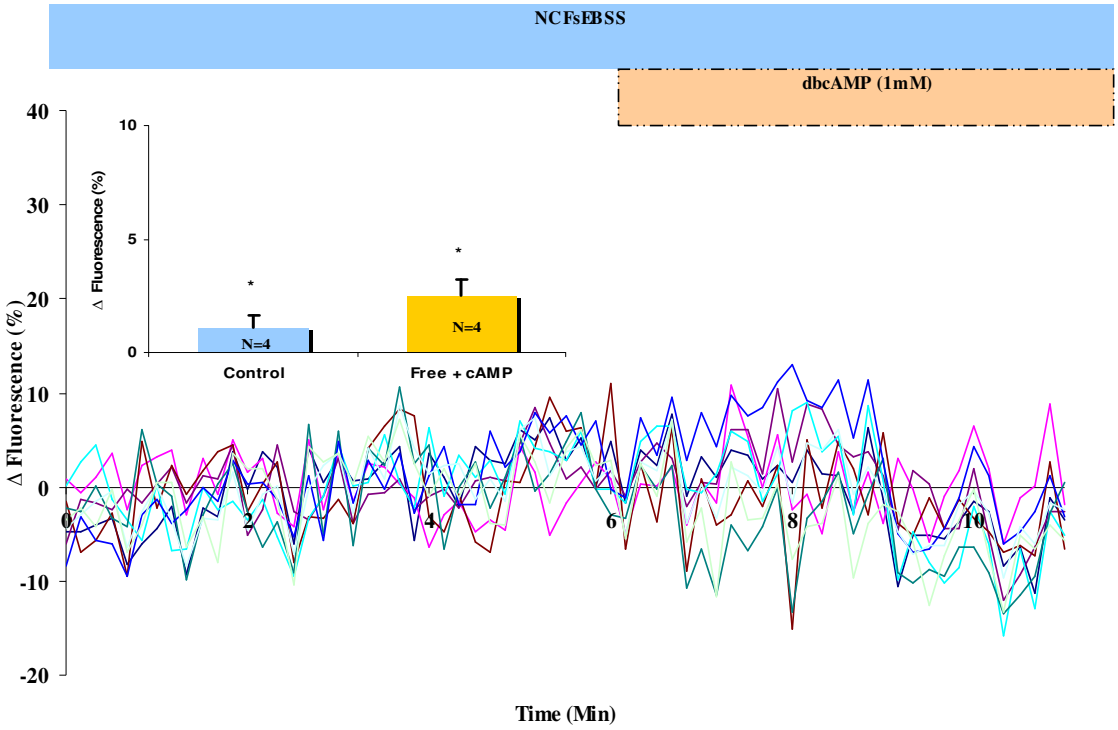

Figure.13. Effects of dbcAMP (1mM) on OGB fluorescence in capacitated human spermatozoa bathed in NCFsEBSS (incubated in sEBSS for >5 hours). Capacitated spermatozoa where bathed in NCFsEBSS for 6 minutes (light-blue bar) and then introduced to dbcAMP (Light-beige bar) in the same media. Trances of 6 single cell responses. **Insert:** Bar graph illustrating mean normalized fluorescence post stimulation of 1mM dbcAMP in NCFsEBSS. The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Same symbols (*) above graphs indicates no significant differneces (P>0.05%). In bar graph Free + cAMP = dbcAMP in Low-Ca^{2+} containing media.

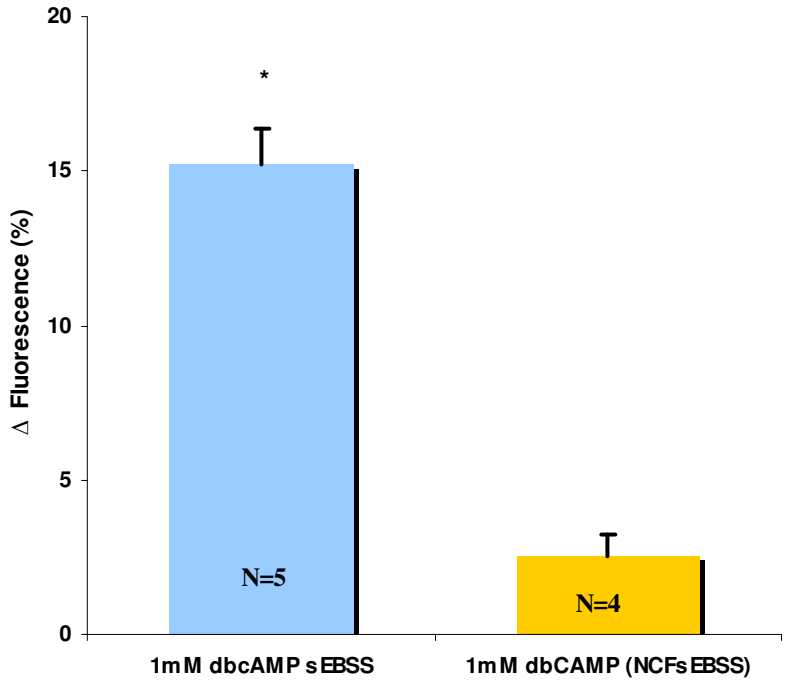

Figure.14. Response of human capacitated spermatozoa exposed to 1mM dbcAMP in sEBSS; against cells treated with 1mM dbcAMP in NCFsEBSS. The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Asterisks (*) show differences in significance (P<0.05%).

4.2.5 Pretreatment with dbcAMP occludes the response to bourgeonal

Capacitated cells (>5 hours in sEBSS) superfused with sEBSS were imaged for >2 minutes then exposed to dbcAMP (1mM) for 2 minutes causing a significant elevation in OGB fluorescence . When bourgeonal (20μM) was then applied to the cells in sEBSS (in the continued presence of dbcAMP) no significant elevation in the OGB fluorescence above non-treated levels was detected in the $\mathbf{R}_{tot}$ of experiments. (four

experiments, >300 cells examined); **[Figure.15.].** Examination of individual cell records showed that where spermatozoa responded to dbcAMP (1mM) in sEBSS with a transient response, when subsequent exposure to bourgenal (20µM) caused a further transient elevation in OGB fluorescence.

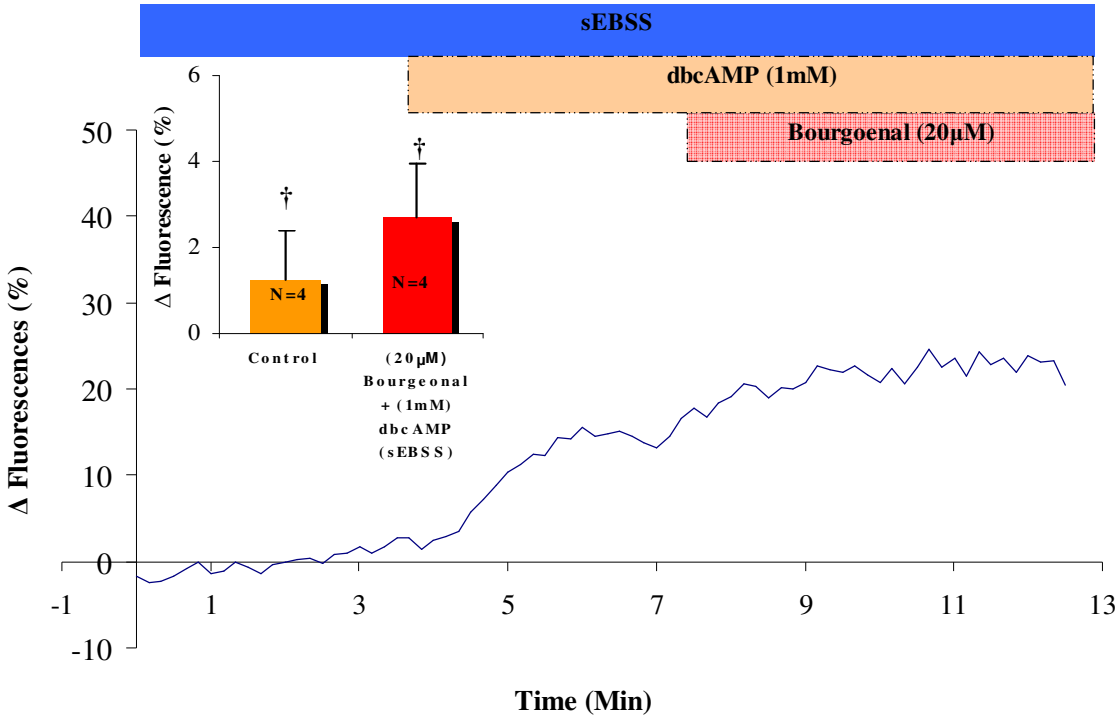

Figure.15. Pretreatment with dbcAMP occludes the response to bourgeonal (Cells incubated in sEBSS for >5 hours). Single trace (Dark-blue line) indicating $\mathbf{R}_{tot}$ of one experiment. Capacitated human spermatozoa were bathed in sEBSS for 4 minutes (Dark-blue bar), followed by superfusion with dbcAMP (Light-beige bar) in the same media. Subsequently the cells were bathed in sEBSS supplemented with 1mM dbcAMP and 20µM bourgeonal (Light red bar). **Insert:** Bar graph illustrating mean normalized fluorescence post stimulation of 1mM dbcAMP pretreated cells with bourgeonal (20µM). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Same symbols (†) above graphs indicates no significant differences (P>0.05%).

4.2.6 Human spermatozoa synthesise cAMP in response to bourgeonal (20μM)

Using a cAMP EIA kit to assess production of cAMP, spermatozoa were incubated for 20 min in HAM F-10 (pH 7.25-7.4, +HSA, >5mM Ca^{2+}) under control conditions, with the phosphodiesterase inhibitor IBMX (100μM) [positive control] and with IBMX + bourgeonal (20μM). In both positive control, cell incubated in HAM F-10 (+HSA) supplemented with IBMX, and in the tested sample, HAM F-10 supplemented with bourgeonal (20μM) and IBMX (100μM), there was an significant elevation in cAMP (both compared to negative control, cells in HAM F-10 medium) ($P<0.05\%$, against negative control). Bourgeonal (20μM) caused a significant elevation in cAMP (1.56±0.16% S.E.M pm/ 10mill) compared both with IBMX alone (1.05±0.04% S.E.M pm/10mill.) ($P<0.05\%$ against positive control) and to untreated control (0.51±0.03% S.E.M pm/ 10mill) (3 experiments; $P<0.05\%$ against untreated control) **[Figure.16.].** Stimulation of capacitated human spermatozoa with bourgeonal increases the production of cAMP *in vitro*.

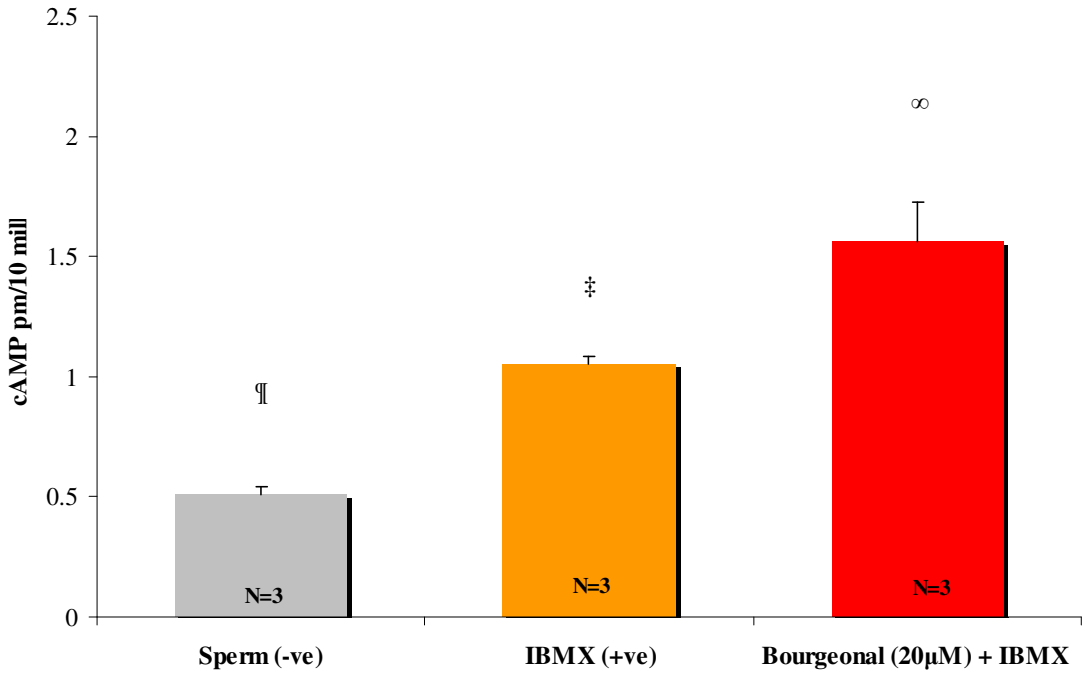

Figure.16. Stimulation of cAMP synthesis in response to bourgeonal in Ca^{2+} in capacitated spermatozoa incubated in HAM F-10 medium. Capacitated human spermatozoa were incubated with HAM F-10 medium supplemented with IBMX (100µM, positive control), bourgeonal (20µM) and IBMX (100µM, tested sample) and HAM F-10 media alone. The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Different symbols (¶,‡,∞) indicate significant differences (P<0.05%).

4.2.7 Ni^{2+} (10μM) and La^{3+} (100μM) inhibit the $[Ca^{2+}]_i$ response to bourgeonal

The effect of La^{3+} (100μM) and Ni^{2+} (10μM) ions on the membrane channel(s) involved in the cell response to bourgeonal was examined. Lanthanum a non-specific inhibitor of Ca^{2+}-permeable cation channels (Krannich et al., 2008) and Ni^{2+} (10 μM) an inhibitor for olfactory cyclic nucleotide channels (Sharona et al., 1995), were applied to capacitated spermatozoa to further understand the nature of the ion channel(s) involved in response to bourgeonal. After capacitation (in sEBSS) the cells were bathed in HEPES-buffered media for 3 minutes (0.3% BSA). Spermatozoa were then superfused with HEPES-buffered media supplemented with La^{3+} (100μM) which caused a significant transient decrease in OGB fluorescence, with a maximum drop of ≈14% below control levels within ±1.5 minutes (<0.05%, against control levels) in >80% of cells. OGB fluorescence recovered to control levels within ±4 minutes. When the cells were superfused with HEPES-buffered media supplemented with La^{3+} (100μM) and bourgeonal (20μM), no significant elevation in OGB fluorescence above control levels was detected $\mathbf{R}_{tot}$ of the experiments (three experiments, >150 cells examined) (P>0.05% against control levels) **[Figure.17.].** Furthermore, at higher doses of La^{3+} (1mM) (one experiments, >100 cells examined) a greater drop was detected in the $\mathbf{R}_{tot}$ and no significant elevation in OGB fluorescence was detected with the application of HEPES-buffered media supplemented with bourgeonal (20μM) and La^{3+} (1mM) (P>0.05%, against control levels).

When sperm were superfused with HEPES-buffered media (0.3% BSA) supplemented with Ni^{2+} (10μM) there again was a transient decrease in OGB fluorescence, with a maximum fall of 15% below control levels within ±1.6 minutes (two experiments, >100 cells examined), followed by a gradual recovery to control levels. Subsequent exposure to bourgeonal (20μM) caused no significant elevation in OGB fluorescence in the $\mathbf{R}_{tot}$ of the experiments (P>0.05% against control levels) **[Figure.18.].** Additionally at higher doses (100μM) Ni^{2+} showed a greater drop in basal level. No recovery of fluorescence occurred at this concentration of Ni^{2+} and no elevation in OGB fluorescence was detected upon exposure to bourgeonal (20μM) (two experiments, >100 cells examined).

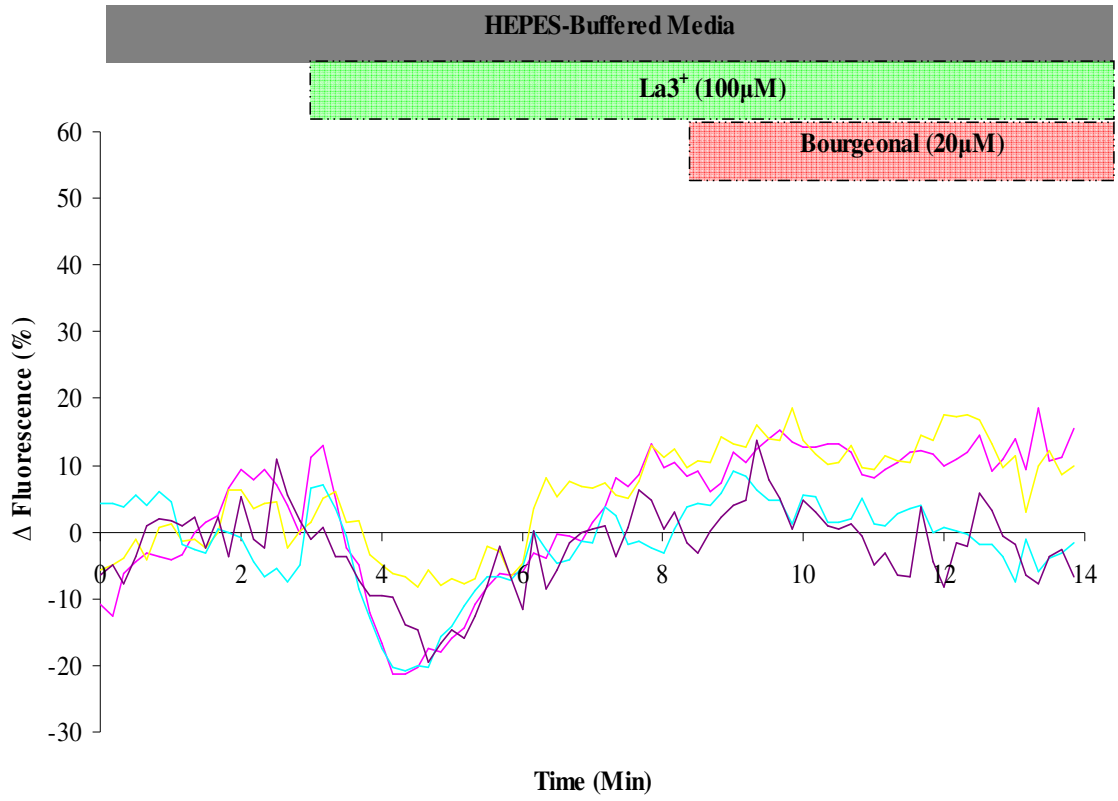

Figure.17. Inhibitory effect of La^{3+} (100µM) on the response of capacitated human spermatozoa to bourgeonal in Ca^{2+}-containing media (HEPES-buffered media). Following the incubation of the cells in sEBSS for >5hours, the cells were bathed in HEPES-buffered media (Dark-grey bar) for 3 minutes. Consecutively the cells were superfused with the same media supplemented with La^{3+} (Bright-green bar) for 5 minutes, followed by superfusion with HEPES-buffered media supplemented with La^{3+} and bourgeonal (Light-red bar) (20µM). Trances show 4 single cell responses.

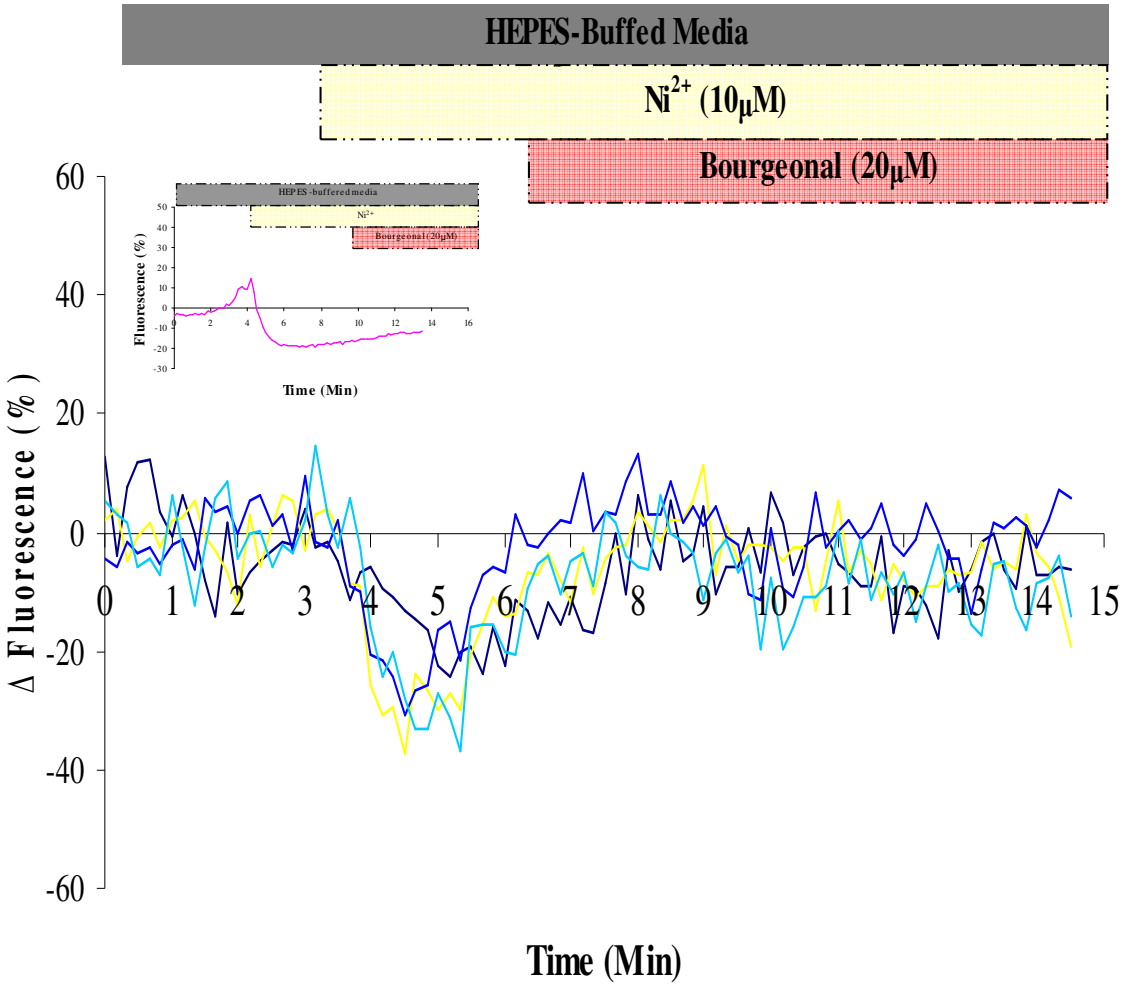

Figure.18. Ni^{2+} inhibit the $[Ca^{2+}]_i$ response to bourgeonal. Following the incubation of the cells in sEBSS for >5hours, the cells were bathed in HEPES-buffered medium (Dark-grey bar) for 3 minutes and consecutively superfused with Ni^{2+} (Light-yellow bar) (10µM) in the same medium for 4 minutes. Subsequently the cells were bathed in same media supplemented with bourgeonal (Light-red bar) (20µM) in with Ni^{2+} (10µM). Traces show 4 single cell responses**. Insert: R_{tot}** of one experiment (50 cells); were capacitated spermatozoa pretreated with 100µM Ni^{2+} introduced to bourgeonal bathed in HEPES-buffered medium containing Ni^{2+}.

4.2.8 Calmodulin inhibition by trifluoperazine (30μM) and the $[Ca^{2+}]_i$ response to bourgeonal

The data described above (see **Section 4.2.7**), are consistent with the model that plasma membrane channels opened by bourgeonal in NCFsEBSS remain open until Ca^{2+} is readmitted to the saline and $[Ca^{2+}]_i$ rises, at which point they are apparently 'switched off'. CNG channels show such sensitivity to Ca^{2+}, which is exerted through Ca^{2+}-calmodulin, which binds the channel (Song et al., 2008, Molday et al., 1996). Trifluoperazine (30μM) a calmodulin antagonist was used to investigate the possible role of calmodulin in shaping the response to bourgeonal. Spermatozoa were capacitated in sEBSS (+0.3% BSA), followed by the superfusion with NCFsEBSS for >2 minutes. Cells were then exposed to trifluoperazine (30μM) for >2 minutes prior to the application of bourgeonal (20μM) and then readmission of Ca^{2+}. Under these conditions the increase in fluorescence was >40% above control levels, followed by a plateau that showed only a moderate and gradual decay in fluorescence (≈8%; 2 experiments, >150 cells examined) **[Figure.19.].** Examination of single cell responses showed that ≈75% of the responsive cells behaved in this way, ≈25% of the cells giving a transient elevation in OGB fluorescence. Thus the effects of pretreatment with trifluoperazine are consistent with a role for calmodulin in termination of the response to bourgeonal.

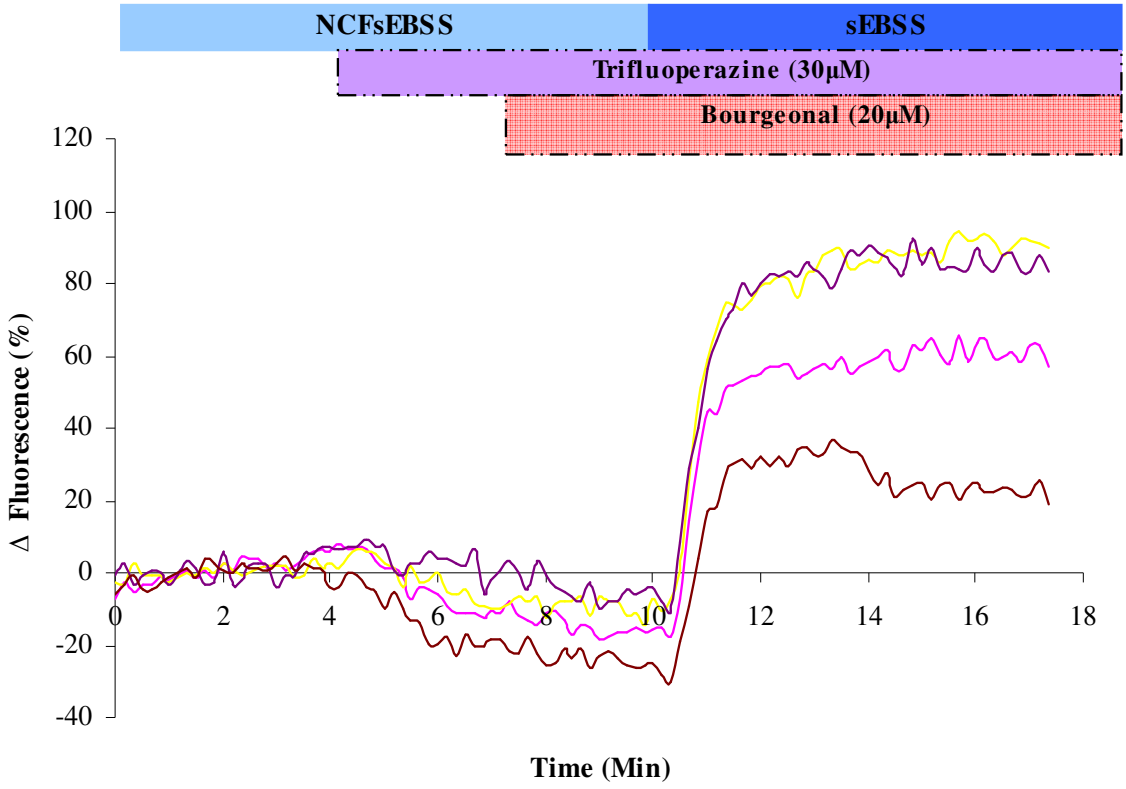

Figure.19. Response of capacitated human spermatozoa pretreated with trifluoperazine to Ca^{2+} readmission when treated with bourgeonal in NCFsEBSS. Spermatozoa were incubated for >5 hours in capacitating media and then bathed in NCFsEBSS for 4 minutes followed by the introduction of trifluoperazine in the same media for 3 minutes. Consecutively the cells were bathed in NCFsEBSS supplemented with bourgeonal (20 µM) and trifluoperazine (30 µM) for 3 minutes and finally bathed in sEBSS supplemented with 20 µM bourgeonal and 30 µM trifluoperazine. Traces show 4 single cell responses.

In addition to possible non-specific effects of trifluoperazine (Luthra et al., 1982), inhibition of calmodulin activity may have effects on $[Ca^{2+}]_i$ unrelated to regulation of CNG channels, such as reduction of ion activity of PMCAs. To assess this, the effect of trifluoperazine was examined on the transient response to progesterone (3µM). Under control conditions progesterone induced a bi-phasic response, a transient elevation in

OGB fluorescence, peaking with 1 minute and dropping with the same time-course, followed by a smaller, sustained elevation in OGB fluorescence (one experiment) **[Figure.20.].** When progesterone was applied to cells pretreated with trifluoperazine there was a rapid elevation in OGB fluorescence in all of the cell (39% above control levels) within ≈1 minutes, but fluorescence then fell only partially (10.5%) over the following 4 minutes (two experiments, >150 cells examined), **[Figure.21.].**

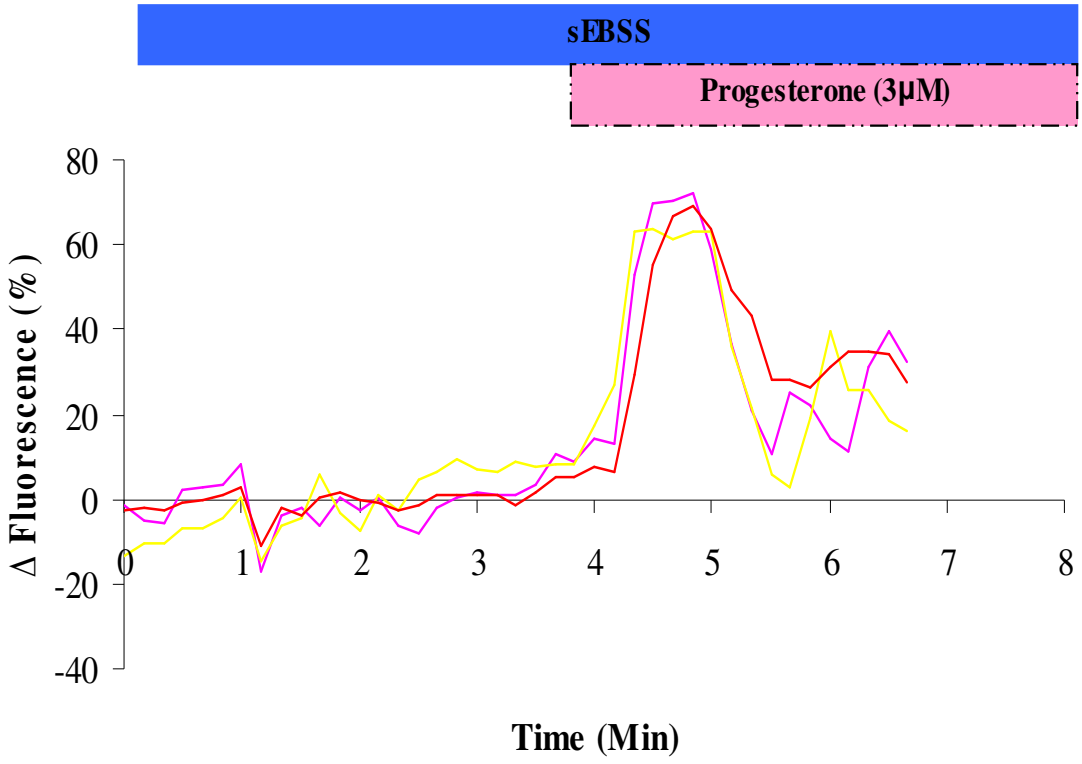

Figure.20. Control experiment**.** Human capacitated spermatozoa response to 3µM progesterone in sEBSS. The cells were bathed in sEBSS (dark-blue bar) for 4 minutes followed by the introduction of P (pink-bar) in the same media. Traces show 3 single cell responses.

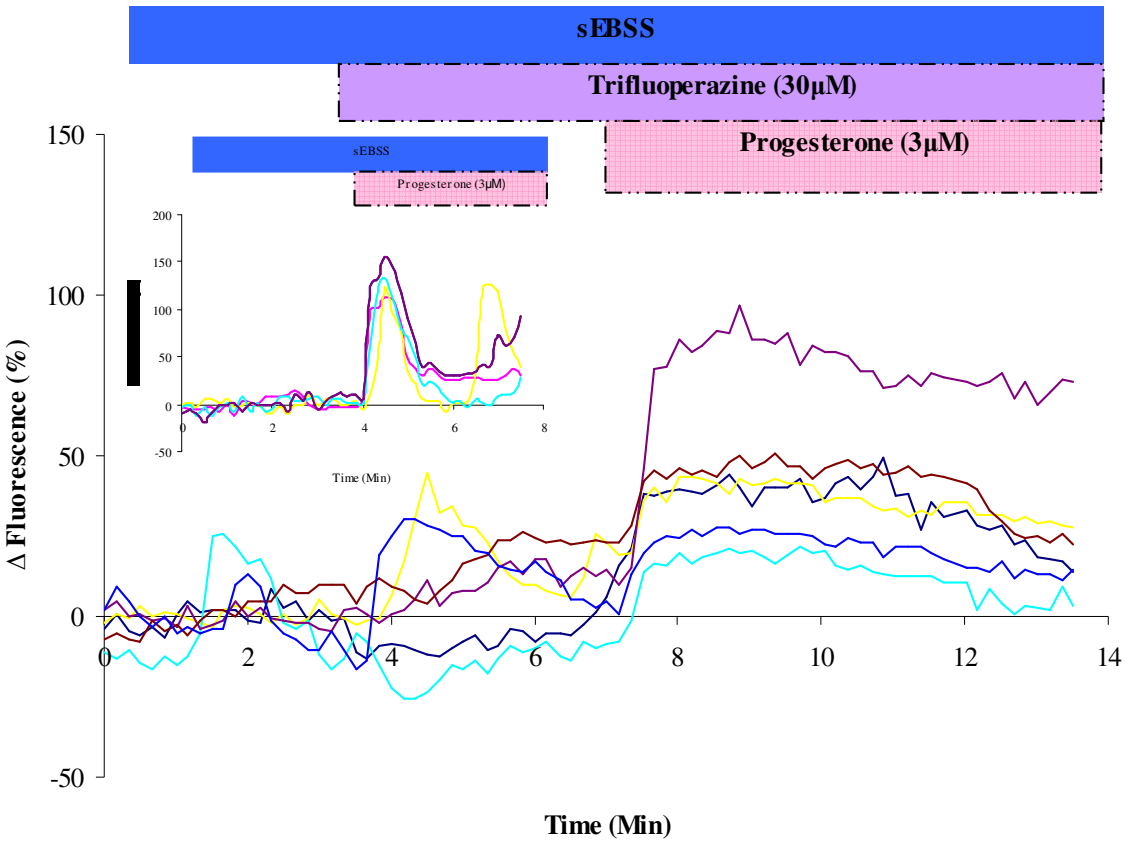

Figure.21. Control experiment with trifluoperazine pretreated cells exposed to 3µM progesterone in sEBSS. Inhibitory effect of trifluoperazine (30µM) (light-purple bar) on response of capacitated spermatozoa to progesterone (3µM) (light-pink bar) in sEBSS. The cells were bathed in sEBSS (dark-blue bar) for 3 minutes and then introduced to trifluoperazine in the same media for 4 minutes; consecutively the cells were superfused with sEBSS supplemented with progesterone (3µM) and 30µM trifluoperazine. Traces show 6 single cell responses. **Insert:** Control experiment with capacitated spermatozoa treated with 3µM progesterone in sEBSS. Showing clear differences in kinetics of response when trifluoperazine is present. Traces of 3 single cell responses.

4.3 Discussion

In this chapter the role of cAMP was examined in the response to bourgeonal and assessed elevation in cAMP in response to the ligand. Due to the nature of spermatozoa, highly differentiated non-transcriptionally active cells (Teves et al., 2009), most of the molecular approach cannot be applied in this cell (Teves et al., 2009). A pharmacological approach was therefore used, exposing sperm to inhibitors (IBMX, Trifluoperazine) and analogues (dbcAMP); widely used in this area of research. Spehr et al. (2003, 2004) proposed a central role for cAMP in the response to bourgeonal; but did not directly assess elevation in cAMP in response to the ligand.

To investigate the possible existence of a feedback mechanism, controlling the elevation of $[Ca^{2+}]_i$ by bourgeonal, a strategy was used in which cells were first exposed to bourgeonal in saline lacking Ca^{2+} (NCFsEBSS), so that transduction mechanisms were activated without elevation of $[Ca^{2+}]_i$, then Ca^{2+} was readmitted to the imaging chamber. This led to the surprising discovery that, in contrast to the slowly developing effect seen when bourgeonal is simply applied to the cells, using this protocol, nearly all spermatozoa responded with a single large transient elevation of $[Ca^{2+}]_i$. When the protocol was followed without application of bourgeonal the effect of Ca^{2+} readmission was minimal and showed very different kinetics. At a higher dose of bourgeonal (100μM) the cells responded once again with a single transient elevation in OGB fluorescence, with a significantly higher elevation in OGB fluorescence compared to that observed with 20μM bourgeonal. Furthermore, identical results were obtained when

3,4, CPEE was applied using this protocol, confirming that the structural homology of 3,4, CPEE is sufficient to exert an effect on the hOR17-4 receptor on human sperm (Spehr et al., 2003, 2004).

These observations, both with bourgeonal and 3,4, CPEE, are consistent with the existence of a negative feedback mechanism that responds to elevation of $[Ca^{2+}]_i$ by inhibition of receptor or signal transduction, truncating the Ca^{2+} signal. When spermatozoa are pretreated with bourgeonal in NCFsEBSS this may 'prime' the cells, activating a transduction cascade and opening of the membrane channels, but in the absence of significant Ca^{2+} influx. When Ca^{2+} is readmitted to the imaging chamber (in the presence of bourgeonal) there is an abrupt influx of Ca^{2+}, followed by the rapid closure of the membrane channel; resulting in a rapid single transient elevation in OGB fluorescence in most cells. This Ca^{2+} spike strongly correlates with the response observed in spermatozoa of invertebrate species when responding to cues of chemoattractant. In the sea urchin *Arbacia punctulata,* chemotactic responses to the peptide resact involve activation of receptor guannylyl cyclase, which induces a rapid and transient elevation in $[Ca^{2+}]_{i,}$ (Böhmer et al., 2005). The Ca^{2+} 'spike' causes a transient increase in flagellar asymmetry and re-orientation of sperm towards the chemoattractant source (Kaupp et al., 2003, 2008). The single transient elevation in Ca^{2+} observed when bourgeonal is applied using the protocol described here, apparently the result of negative feedback may reflect activation of mechanisms by which bourgeonal can cause directed turns in human sperm.

To investigate the involvement of cAMP in the cascade of events that results in this rapid signal response, spermatozoa were pretreated with modulators of cAMP signaling. IBMX did not significantly alter the kinetics, the percentage responding cells nor the amplitude and nature of the response. IBMX will prolong (and probably enhance) any effects of bourgeonal on [cAMP] concentration. Since the rapid termination of the $[Ca^{2+}]_i$ signal was not affected it appears most unlikely that (cAMP) alone gates the Ca^{2+} channel opened by bourgeonal. Furthermore, when bourgeonal was replaced by dbcAMP in the Ca^{2+} readmission protocol (here the [cAMP] will remain elevated regardless of the activities of adenyl cyclase or phosphodiesterase) the $[Ca^{2+}]_i$ response was again a transient elevation. Thus it is clear that while cAMP may be responsible for activation of the bourgeonal-activated Ca^{2+} influx, closing of the channel is regulated by a different mechanism. In agreement with such a conclusion, when spermatozoa were treated with dbcAMP (1mM) in sEBSS there was a sustained elevation of $[Ca^{2+}]_i$, in >35% of the cells and a small subpopulation of cells responded with a transient elevation in OGB fluorescence, as previously reported with cells exposed to high doses of bourgeonal.

The data described above are consistent with the suggestion that the channel involved in response to bourgeonal was activated by cAMP (Spehr et al. 2004, 2006). Assessment of cAMP production demonstrated that cells incubated bourgeonal (20µM) generated cAMP at a significantly higher rate than in parallel controls, though accurate assessment of (cAMP) kinetics (as has been done for cGMP in sea urchin sperm [Kaupp et al., 2003, 2008]) will be necessary to show that chemotactic responses could be regulated in

this way. Closure of the channel occurred only when Ca^{2+} influx was permitted (by readmission of Ca^{2+} to the imaging chamber). Such negative feedback through Ca^{2+} is characteristic of CNG channels, which are modulated by the Ca^{2+}-binding protein calmodulin. To investigate this further two known inhibitors of CNG channels were used, Ni^{2+} (10μM) La^{3+} (100μM). Both significantly inhibited the $[Ca^{2+}]_i$ response of spermatozoa to bourgeonal. To further investigate this trifluoperazine (30μM) was used in the experiments, a calmodulin inhibitor. When cells were treated with the drug, readmission of Ca^{2+} caused a much more sustained elevation in OGB fluorescence, as might be expected if feedback regulation of the channels was prevented. However, a control experiment using progesterone (3μM) showed a similar extension of the Ca^{2+} transient. There is no evidence that progesterone activates a CNG channel and thus it is likely that this effect of trifluoperazine was through another mechanism, such as inhibition of the calmodulin-dependent plasma membrane Ca^{2+} ATPase.

The data presented here and in the previous chapter (see **Chapter 3**) strongly suggest the existence of a feedback mechanism which regulates the response to bourgeonal, terminating the response to the ligand through a Ca^{2+} dependent mechanism. In rod and cone photoreceptors a Ca^{2+} feedback mechanism, involving calmodulin, has been identified involving nucleotide-gated channels (Kaupp et al., 2002). The data reported here are not inconsistent with the existence of a similar mechanism in human sperm, but it is not yet possible to draw conclusions regarding the nature of the channel involved.

A small number of these experiments were performed in laboratory collaboration with **Dr Teves**, **Dr Giojalas**, **Dr Gidobaldi** and **Dr Uñates** from the Cebicem (University of Cordoba). Additionally another small proportion of experiments were performed in collaboration for the Department of Chemistry, University of Birmingham, UK. All of these experiments, both in Argentina and Birmingham were done by Aduén Andrés Morales García, author of this thesis. Most of the data provided will be used for grant applications (Chemistry Department; University of Birmingham) and for an upcoming paper(s), in collaboration **with Dr Giojalas, Dr Uñates, Dr Teves and Dr Publicover** as head of the research group (University of Birmingham). Additionally a few sections of this Chapter were about to be published (with Dr Teves as main author).

CHAPTER ~FIVE~

“For those that always believed”

Chapter Five

CHEMOTACTIC RESPONSE TO BOURGEONAL

5.1 Introduction

Out of the millions of spermatozoa ejaculated into the female reproductive tract only ≈1 of every million succeed in the entering of the Fallopian tubes (Harper et al., 1982; William et al., 1993; Eisenbach et al., 1999; Eisenbach & Giojalas, 2006). Out of this subpopulation only capacitated spermatozoa (≈10% in humans) (Cohen-Dayag et al., 1995) are capable of fertilizing the egg. If location of the oocyte by the sperm were left to chance, which was the initially thought to be the case, the probability that such small numbers of spermatozoa would successfully achieve fertilization is very slim (Eisenbach & Giojalas, 2006). It has been recently accepted that the spermatozoa's journey is not just a blind race towards the egg, spermatozoa are guided by chemotaxis.

Sperm chemotaxis is the movement of cells up a concentration gradient of a chemoattractant (Eisenbach, 2004; Eisenbach & Giojalas, 2006). In marine invertebrates, a well studied model, most spermatozoa are chemotactically responsive (Kaupp et al., 2003, 2008). In mammalian spermatozoa a small fraction (≈10%,), which is believed to be the capacitated sub-population, are responsive, (Jaiswal et al., 2002; Coheng-Dayag et al., 1995; Giojalas et al., 2004, Eisenbach & Giojalas, 2006). This was discovered by the observations that:

(i) capacitated and chemotactically responsive spermatozoa had an equally short life span; both being continuously replaced from the rest of the population to ensure the constant presence of these cells *in vivo* (Coheng-Dayag et al., 1995; Eisenbach & Giojalas, 2006); (ii) depletion of capacitated spermatozoa results in the loss of

chemotacticly responsive cells and *vice versa* (Cohen-Dayag et al., 1995; Giojalas et al., 2004; Jaiswal et al., 2002; Oliveira et al., 1999).

Mammalian sperm guidance *in vivo* might involve more than one attractant *including* the role of sperm temperature guidance, known as thermotaxis (Bahat et al., 2003; Kaupp et al., 2008). It seems likely that, after guidance to the fertilization site possibly, the cells sense a chemoattractant gradient origintated from the cumulus cells or the oocyte itself (Sun et al., 2005); guiding spermatozoa to the egg-cumulus complex (Sun et al., 2005; Eisenbach & Giojalas, 2006). This would enable spermatozoa to enter the cumulus and consequently encounter the oocyte (Bedford et al, 1993). However, the timing and location of chemotaxis *in vivo* are still largely uncertain (Eisenbach et al., 1999; Eisenbach & Giojalas, 2006).

Follicular fluid (FF) composed of pre-ovulatory secretions of the egg and surrounding cells (Eisenbach et al., 1999; Eisenbach & Giojalas, 2006) was the first natural source of sperm chemoattractants to be identified (Eisenbach et al., 2004; Eisenbach & Giojalas, 2006). Thus FF might act as an attractant *in vivo*, although it was observed that this was unlikely to be the case (see **Chapter 1**). It has been recently demonstrated by Teves et al. (2006) that the steroid hormone progesterone (P), a product of the cumulus cells, exerts a chemotatic effect on human spermatozoa. Furthermore, it was also demonstrated that a progesterone gradient is likely to exist within the cumulus cell mass due to variation in the density of progesterone-secreting cells (Teves et al., 2006). Additionally when the chemotaxis assay was performed with non-capaciated human spermatozoa no significant response was detected (Teves et al., 2006).

The **ORs** are expressed ectopically in a great array of tissues other than the olfactory epithelium (Kaupp et al., 2008; Spehr et al., 2003, 2004); these were indentified in the midpiece of human spermatozoa (hOR17-4) also located in olfactory epithelium (Kaupp et al., 2008). Spehr et al. (2003, 2004) demonstrated that the odorant bourgeonal acts as an agonist for the G-protein coupled receptor located in the midpiece of human spermatozoa (hOR-17-4) and a chemoattractant of human sperm. Human spermatozoa stimulation with bourgeonal results in a Ca^{2+} response in >35% of the cell population (Spehr et al., 2003, 2004). However, the assay used primarily assessed accumulation of cells at the chemoattractant source rather than directly assessing a directional response of sperm to the gradient.

The mechanism and signal cascade that results in chemotaxis of mammalian spermatozoa is still unclear, although it's beyond doubt that Ca^{2+} signalling plays a key role in this process (Publicover et al., 2008). *In vitro* chemotaxis assays with progesterone (10pM) performed by Teves et al. (2009) demonstrated that the removal of Ca^{2+} from the experimental media significantly suppressed the cell response to the attractant. Furthermore, Gakamsky et al., (2009) and Spehr et al. (2003, 2004) also demonstrated the crucial role of $[Ca^{2+}]_o$ in the cell response to bourgeonal, when the bathing media was buffered with EGTA.

Chapter Aims

This chapter attempts to demonstrate the chemotactic effect of bourgeonal, if any, in both capacitated and non-capacitated human spermatozoa, with the use of an assay capable of discriminating between other processes that result in spermatozoa accumulation. Furtheremore, the potential data acquired in this chapter will try to demonstrate the crucial role of $[Ca^{2+}]_o$ in response to bourgeonal and if the intracellular stores play a role in the response to the ligand. Additionally, by means of fluorescence video microscopy and computer image analysis the response of capaciated spermatozoa to application of a temporal bourgeonal gradient (fM to mM) and progesterone gradient (fm to μM) was examined, in an attempt to study the $[Ca^{2+}]_i$ response.

5.2 Results

5.2.1 Chemotaxis assay with capacitated spermatozoa

Capacitated spermatozoa (incubated for >5 hours in HAM F-10 medium +HSA), were incubated in Zigmond chambers in HAM F-10 medium alone or supplemented with bourgeonal (doses from 10^{-5} to 10^{-14} M). After allowing ≈15 minute for stabilization of chemotactic gradient (Teves et al., 2006, 2009; Gidobaldi et al., 2008), directional responses of the sperm were assessed as described in the methods section. Plotting of the dose-dependence of directional responses gave a bell-shaped response curve; typical of responses in all cellular models (Adler et al., 1973, Teves et al., 2006). With a significant response detected between 10^{-9} and 10^{-10} M bourgeonal. Where the maximum chemotactic response detected at 10^{-9} M bourgeonal (1nM), (32.4±1.5% of cells directed up gradient), significantly higher than negative control 27.4% (±1.8% S.E.M) ($P<0.05\%$) and similar to the 10pM progesterone positive control (31.3±1.1 S.E.M; $P>0.05\%$); **[Figure.1.].**

Directional responses for the bourgeonal analog, 3,4,CPEE in sEBSS were carried out with capacitated human spermatozoa; however no conclusive data has been obtained (Experiment in early stage).

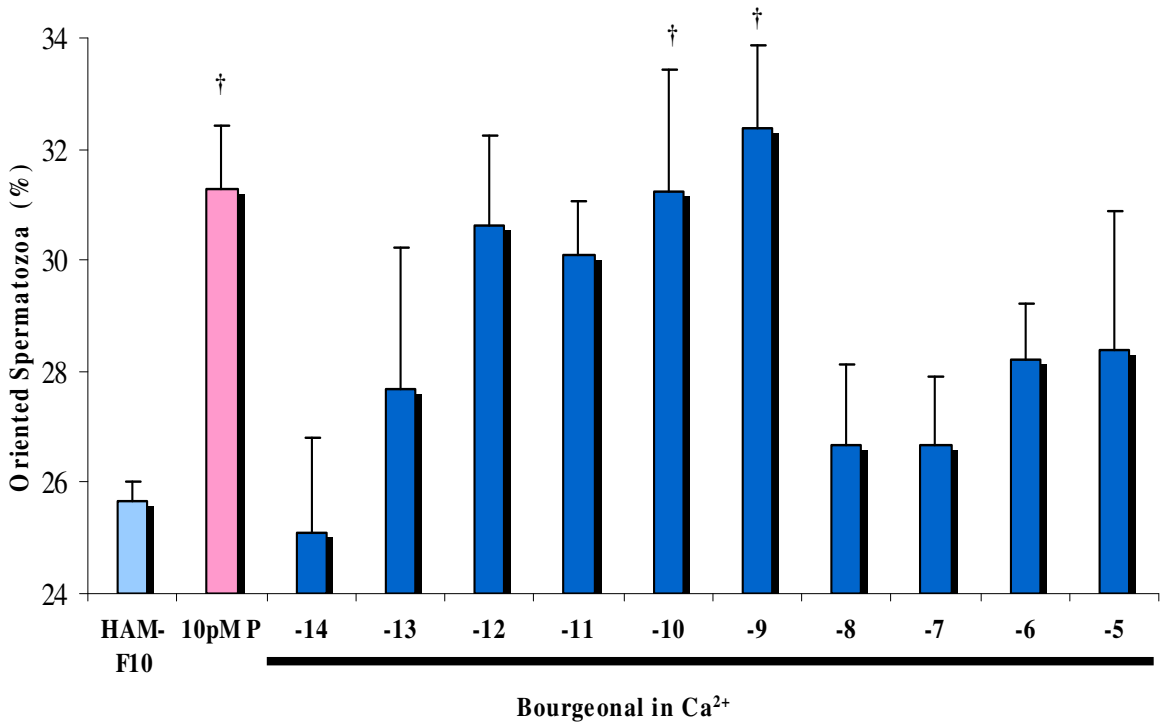

Figure.1. Chemotactic response of capacitated human spermatozoa towards a bourgeonal gradient in Ca^{2+} (HAM F-10 media, +HSA). Bar graph illustrating the percentage oriented spermatozoa (%OS) towards bourgeonal. Capaciated human spermatozoa were confronted with a series of bourgeonal concentration (10^{-5} to 10^{-14} M) in HAM F-10 media containing albumin (+HSA). The error bars indicates the standard error of the mean (S.E.M.). Same symbol on top of bars (†) indicate no significant difference (P>0.05%). [HAM F-10 media, light-blue, negative control; 10pM Progesterone, light-pink, positive control, tested bourgeonal concentrations, dark-blue]. In bar graph (X-axis) values -14 to -5 its equivalent is 10^{-14} to 10^{-5} M bourgeonal respectively. Bourgeonal in Ca^{2+} - These experiments were done in Ca^{2+} containing medium, in this case HAM F-10.

5.2.2 Chemotaxis assay with non-capacitated spermatozoa

In human spermatozoa the capacitated cells (≈10%) are the chemotactically responding cells (Cohen-Dayag et al., 1995). To examine the role of capacitation in the response of sperm to a 1nM bourgeonal gradient; the proportion of capacitated spermatozoa were depleted by significantly reducing the incubation time (>5 hours to <30 minutes), prior to experimentation, and also omitting albumin from the medium (no HSA). Assessment of the chemotactic response in cells prepared in this way showed no orientation up gradient, (25.1%), similar to non-capacitated spermatozoa exposed to HAM F-10 medium alone (negative control; 24.6% P>0.05% t test) **[Figure.2.].** Furthermore, spermatozoa that had been capacitated (>5 hour incubation in HAM F-10, +HSA) showed a clear chemotactic response, 33.1%, which was significantly higher than capacitated spermatozoa exposed to HAM F-10 medium (+HSA) alone (P<0.05%) and significantly higher than non-capacitated spermatozoa exposed to a 1nM bourgeonal gradient (P<0.05%).

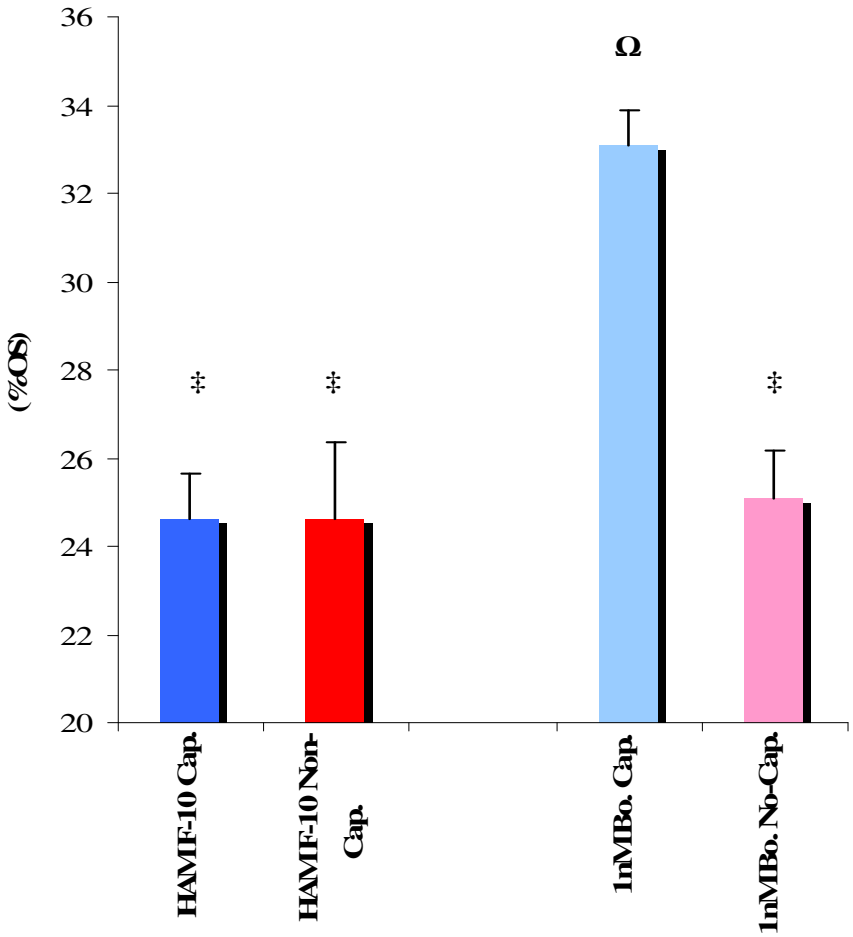

Figure.2. Chemotactic response of non-capacitated human spermatozoa towards a 1nM bourgeonal gradient in Ca^{2+} (HAM F-10 media). Capacitated and non-capacitated spermatozoa were confronted with 1nM bourgeonal gradient in HAM F-10 media. Bar graph illustrating the percentage oriented spermatozoa (%OS) towards bourgeonal in HAM F-10 media (with for capacitated spermatozoa and without albumin for non-capacitated spermatozoa); and the (%OS). The error bars indicates the standard error of the mean (S.E.M.). Different symbols (‡,Ω) indicate significant differences (P<0.05%); the use of identical ones is to indicate no significant differences (P>0.05%). [HAM F10+Cap. =Capacitated spermatozoa in HAM F-10 media alone; HAM F-10+Non-Cap= Non-capacitated spermatozoa in HAM F-10 media; 1nM Bo.+Cap.= capacitated spermatozoa confronted with a 1nM bourgeonal gradient and 1nM Bo.+No-Cap= No-capacitated cells confronted with 1nM bourgeonal bourgeonal gradient].

5.2.3 Chemotaxis in low-Ca^{2+}(<5µM/L Ca^{2+})

Having characterized the dose-response and capacitation dependence of bourgeonal-induced chemotaxis, the role of Ca^{2+} was examined, when reduced from the experimental medium. When spermatozoa swimming in low-Ca^{2+} medium (<5µM/L Ca^{2+}; Harper et al., 2004) were confronted with a 1nM bourgeonal gradient the proportion of cells oriented in the up-gradient quadrant (26.3±0.9% S.E.M) was similar to that in untreated (negative control) cells in low-Ca^{2+} (23.1±1.9%; P>0.05%, treated + 1nM bourgeonal Vs untreated cells). Furthermore, when the cells were exposed to a 1nM bourgeonal gradient in standard sEBSS (Ca^{2+} containing medium) there was a clear chemotactic response (30.6±0.6% of cells oriented up-gradient), significantly higher than in spermatozoa in low-Ca^{2+} alone and spermatozoa exposed to a 1nM bourgeonal gradient in low-Ca^{2+} (P<0.05%, untreated/treated cells in low-Ca^{2+} and untreated cells in high-Ca^{2+} Vs treated cells in high-Ca^{2+}) **[Figure.3.].** When spermatozoa were exposed to a 10 pM progesterone gradient in low-Ca^{2+} this resulted once again in suppression of the chemotactic response. 25.6% (±1.0% S.E.M) were oriented in the up-gradient quadrant, not significantly different to untreated cells in low-Ca^{2+} saline (23.1±1.9%; P>0.05%), whereas cells exposed to a 10pM progesterone gradient in standard sEBSS gave a normal chemotactic response (32.9±0.8% S.E.M), significantly higher than both controls (cells in low-Ca^{2+} , 24.3% and high-Ca^{2+} alone, 23.1%) and cells exposed to a 10pM progesterone gradient in low-Ca^{2+} (P<0.05%). There was no significant difference between the responses to 1 nM bourgeonal and 100 pM progesterone in cells swimming in standard sEBSS (P>0.05; t test).

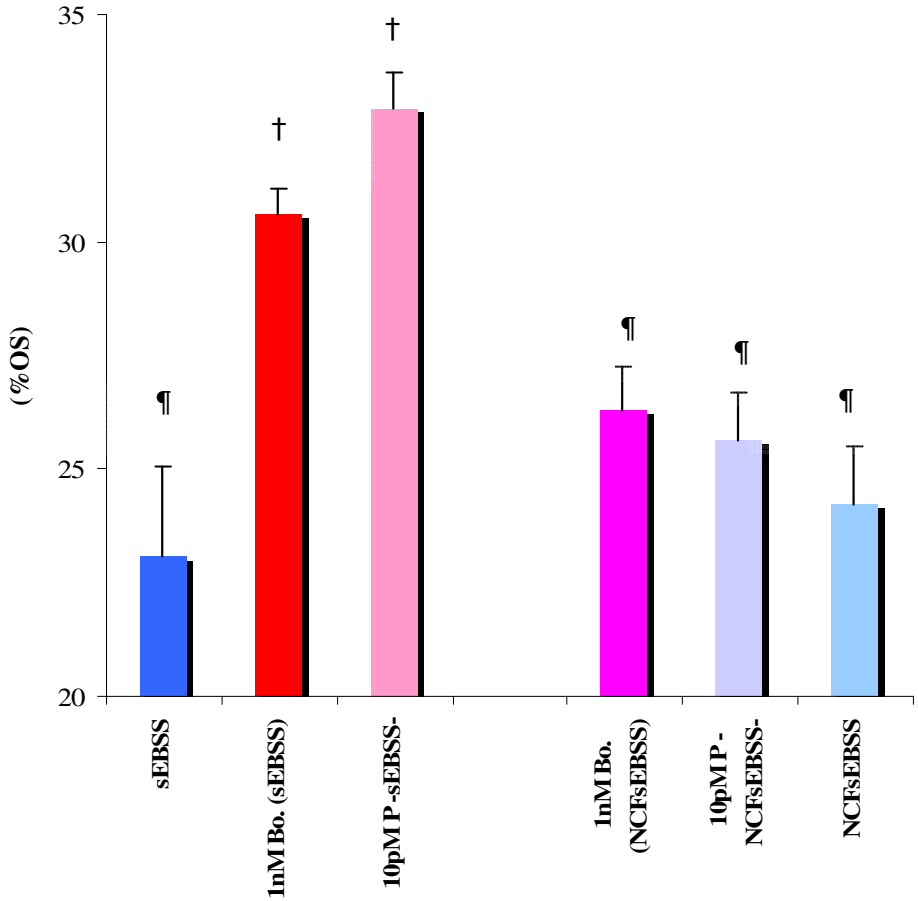

Figure.3. Chemotactic response of capacitated human spermatozoa towards a bourgeonal gradient and 10pM P gradient with or without Ca^{2+} (NCFsEBSS). Bar graph illustrating the percentage oriented spermatozoa (%OS) towards 1nM bourgeonal in NCFsEBSS media (+HSA) and in sEBSS as well as P in both sEBSS and NCFsEBSS. The error bars indicates the standard error of the mean (S.E.M.). Different symbols (†,¶) indicate significant differences (P<0.05%); the use of identical ones indicate no significant differences (P>0.05%) [1nM Bo.(sEBSS)= 1nM bourgeonal in sEBSS; 10pM P (sEBSS)= 10pM progesterone in sEBSS; 1nM Bo.(NCFsEBSS)=1nM Bourgeonal in NCFsEBSS].

5.2.4 Ca^{2+} Store mobilization in bourgeonal-induced chemotaxis

The next step was to examine the role of the intracellular Ca^{2+} stores, if any, in response to a bourgeonal (1nM) gradient in HAM F-10 media. The cells were incubated in HAM F-10 media for >5 hours; and then pretreated with 100 µM TMB-8 (in the same medium), an inhibitor of Ca^{2+} store mobilization in somatic cells (Shimizu et al., 2008). Teves et al. (2009) demonstrated that TMB-8 causes a dose-dependent inhibition of progesterone-induced chemotaxis in human sperm and had no effect on cell motility. Capacitated human spermatozoa were exposed to TMB-8 (100µM) in HAM F-10 medium 30 minutes**.** When chemotaxis was assessed, a clear chemotactic response was detected in the TMB-8 pretreated cells exposed to a 1 nM bourgeonal gradient (31.3%; five experiments). The response was significantly higher than the negative control (untreated cells, HAM F-10 alone; 26.2%; P<0.05%) and was significantly similar to the response obtained with 1nM bourgeonal (31.61%, P>0.05%) and 10pM progesterone in HAM F-10 media (Positive controls, 32.3%, P>0.05%) [**Figure.4. (A)].** In contrast, TMB-8 (100µM) pretreatment resulted in a clear inhibition of the chemotactic response to 10 pM progesterone (P) (26.2%, five experiments). This was significantly different to the response of non-pretreated cells exposed to P in HAM- F10 (32.3%; Positive control; P<0.05%, 10pM P+TMB-8 vs 10pM P in HAM F-10) and was similar to the negative control (HAM F-10 medium alone), 26.20% (P>0.05%; 10pM P+TMB-8 vs HAM F-10 media). Clearly suppressing the chemotactic response of capacitated human spermatozoa to 10 pM P in HAM F-10 media [**Figure.4. (B)].**.

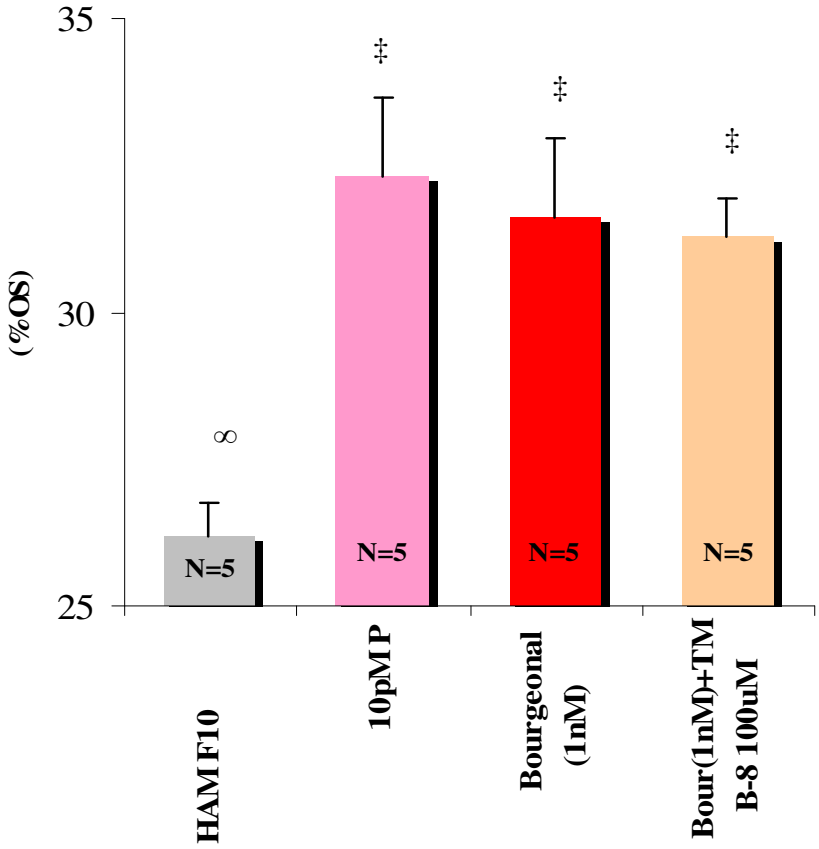

Figure.4. (A). Chemotactic response of TMB-8 pretreated capacitated human spermatozoa towards a 1nM bourgeonal gradient in Ca^{2+} (HAM F-10 media). Bar graph illustrating the percentage oriented spermatozoa (%OS) towards 1nM bourgeonal in HAM F-10 media, HAM F-10 media alone (untreated cells), 10pM P and the response of TMB-8 pretreated cells towards 1nM bourgeonal. The error bars indicates the standard error of the mean (S.E.M.). The use of different symbols (∞, ‡) indicate significant differences between samples (P<0.05%) and the same symbols indicate no significant differences (P>0.05%).

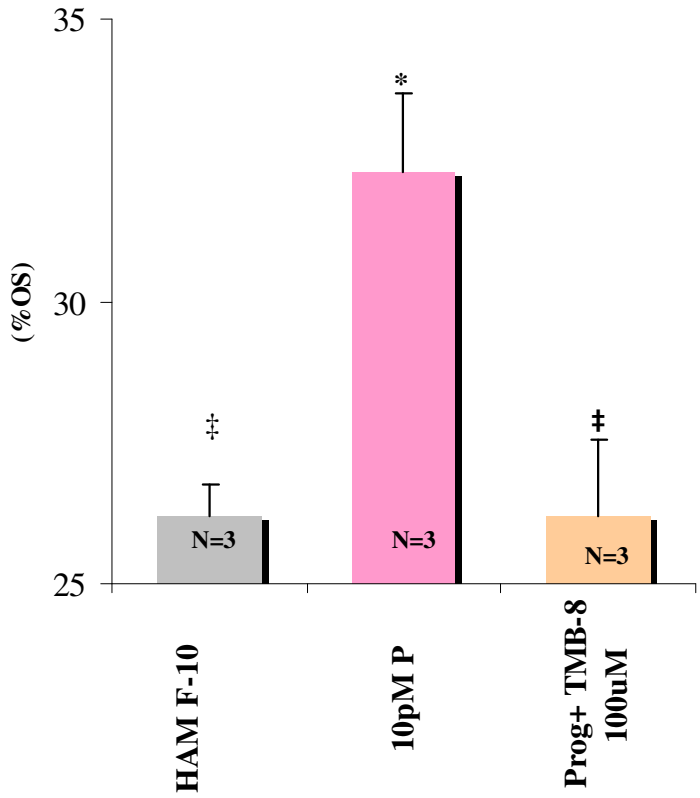

Figure.4. (B). Chemotactic response of TMB-8 pretreated capacitated human spermatozoa towards a 10pM P gradient in Ca^{2+} (HAM F-10 media). Bar graph illustrating the percentage oriented spermatozoa (%OS) towards 10pM P in HAM F-10 media, HAM F-10 media alone and the response of TMB-8 pretreated cells towards 10pM P in HAM F-10. The error bars indicates the standard error of the mean (S.E.M.). The use of different symbols (*,‡) indicate significant difference between samples (P<0.05%) and the same symbol no significant differences (P>0.05%).

5.2.5 Plasma membrane Ca^{2+} channels and chemotaxis

Following the previous procedures examining the role of the Ca^{2+} stores in response to 1nM bourgeonal gradient and 10pM progesterone gradient. Here the role of L-type (Ca_v1) and T-type (Ca_v3) channels were examined, in response to a 1nM bourgeonal gradient. These channels are believed to be expressed in mammalian spermatozoa and might play an important physiological role (Felix et al., 2005; Teves et al., 2009). As previously reported by Teves et al. (2009), these channels play an important role in mammalian chemotaxis in response to a 10pM progesterone gradient. Capacitated cells were treated with an array of doses of nifedipine (1, 10, 100 μM) for 16 minutes. The nifedipine pretreated cells were then exposed to a 1nM bourgeonal gradient. The data showed that at 100μM, the chemotactic response was fully inhibited, 24%, similar to the control value, (cells were exposed to HAM F-10 medium alone, 24.8%) whereas in the positive control, (1nM bourgeonal gradient in HAM F-10 medium) 39% of cells were oriented in the up-gradient quadrant. At lower (more L-channel specific) doses of nifedipine the effect was weaker but still discernible. At 10 μM 27.4% were oriented in the up-gradient quadrant and at 1μM nifedipine the chemotactic response this was 28%. These data suggest that the inhibition of membrane ion channels results in the loss of the chemotactic response.

5.2.6 Acrosomal Reaction and Bourgeonal (1nM)

The chemotactic response to both gradients of 1nM bourgeonal and 10pM progesterone (Teves et al., 2006, 2009) are dependent on capacitation of spermatozoa (Cohen-Dayag et al., 1995). AR, which is induced by elevation of $[Ca^{2+}]_e$ (Publicover et al., 2007), is also dependent upon capacitation. To investigate whether treatment of capacitated cells with bourgeonal (1nM) stimulated AR, capacitated, spermatozoa were incubated with Ca^{2+} ionophore (8μM $^{+}$ve control), bourgeonal (1 nM) and DMSO (0.02%, in the absence of 1nM bourgeonal) for ≈30 minutes, then processed for assessment of acrosomal status (see Methods). Ca^{2+} ionphore (8μM) treatment resulted in 13.9% (±3.1 S.E.M) of cells being stained as acrosomal reacted, (positive control), significantly higher than the % spontaneous acrosome reaction observed in cells bathed in HAM F-10 alone (6.4±2.1% S.E.M; $P<0.05\%$). When capacitated spermatozoa were incubated with HAM F-10 supplemented with bourgeonal (1 nM) no increase in AR above control levels was detected ($P<0.05\%$ t test). Similar values were acquired using treatment with solvent vehicle (0.02% DMSO). The percentage induced AR was significantly lower than positive control (Ca^{2+} ionophore, 8 μM). These results show that bourgeonal (1 nM) does not significantly induce AR in human sperm.

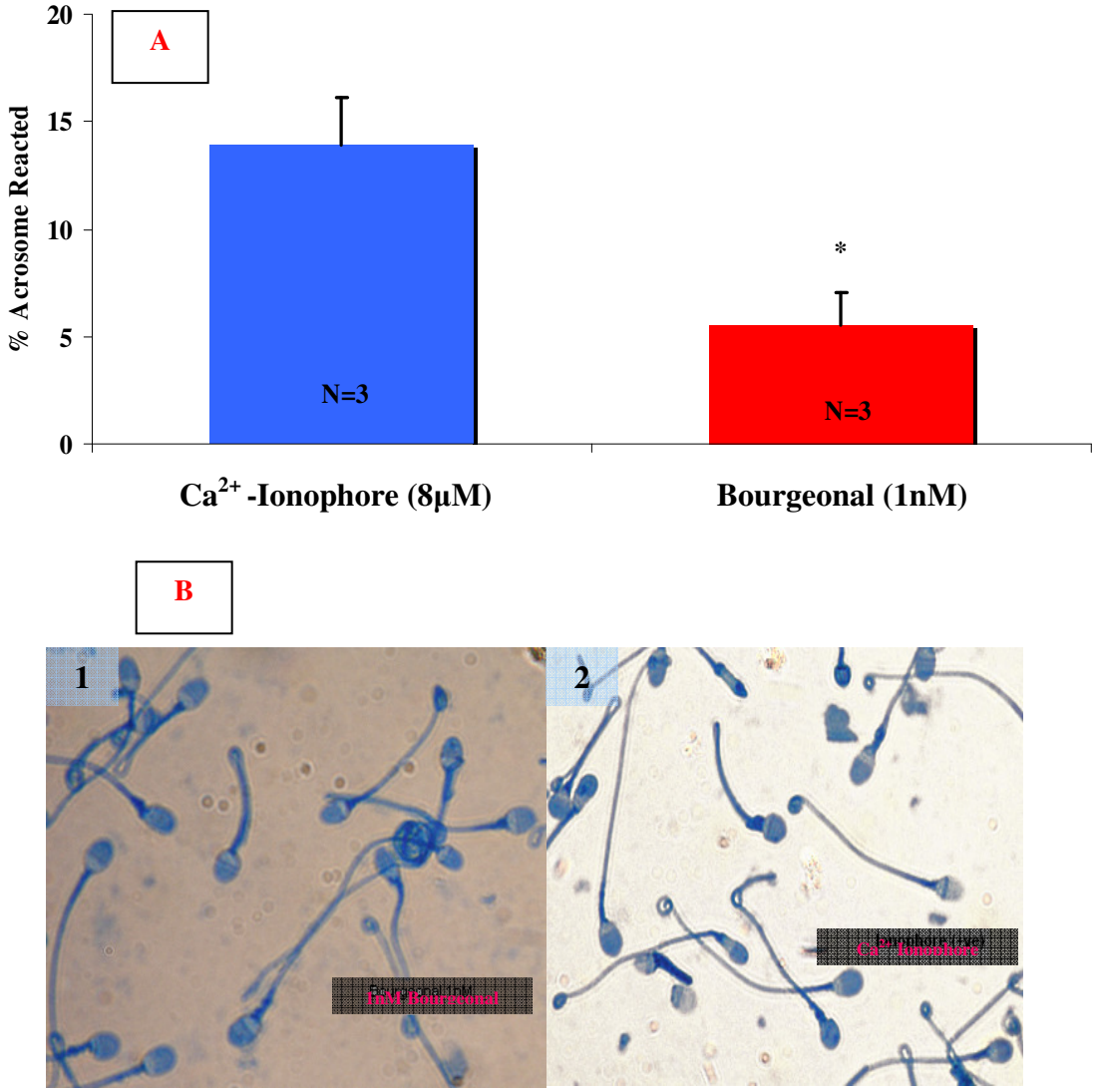

Figure.5. Effect of 1nM bourgeonal on the acrosomal status of capacitated human spermatozoa. **(A)** Bar chart indicates the mean percentage (%) induced AR, in spermatozoa incubated with Ca^{2+}-ionophore (8µM) and cells incubated with bourgeonal (1nM) in Ca^{2+}-containing media (HAM-F10). Asterisk (*) indicates the significant difference between samples (P<0.05%). **(B)** Images of capacitated human spermatozoa stained for the assessment of acromal status. **(B.1)**. Capacitated spermatozoa incubated HAM-F10 (+HSA) supplemented with bourgeonal (1nM). **(B.2.)** Capacitated human spermatozoa incubated with HAM-F10 supplemented with Ca^{2+} Ionophore (8µM), (positive control).

5.2.7 Human spermatozoa cAMP synthesis in response to bourgeonal (1nM) and progesterone (10pM)

As previously demonstrated in **Chapter 4**, the stimulation of capacitated spermatozoa with bourgeonal (20μM) results in the production of cAMP. This section examines whether the bourgeonal (1nM) concentration that results in mammalian sperm chemotaxis of capacitated spermatozoa, stimulates cAMP elevation. Furthermore it was examined whether 10pM progesterone, the peak chemotactic concentration for human spermatozoa (Teves et al., 2006), results in the stimulation of cAMP production. Spermatozoa were incubated in HAM F-10 (+HSA) supplemented with IBMX (positive control), HAM F-10 supplemented with bourgeonal (1nM) and IBMX (100μM) or HAM F-10 supplemented with progesterone (10pM) and IBMX (100μM). Incubation with bourgeonal (1nM) led to significant elevation in cAMP (1.65±0.08% S.E.M pmoles/ 10 million cells), significantly higher with respect to cells incubated with IBMX (100μM) alone (1.05±0.04% S.E.M pmoles/10 million cells; $P<0.05\%$); and significantly greater than the negative control of cells incubated in HAM F-10 media alone (0.51±0.03% S.E.M pmoles/10 million cells; $P<0.05\%$, N=3) **[Figure.6. (A).].** Additionally when capacitated spermatozoa were incubated in HAM F-10 (+HSA) supplemented with progesterone (10pM) and IBMX (100μM) this resulted in a significant elevation in cAMP (1.81±0.25% S.E.M pmoles/10 million cells), significantly greater than HAM F-10 medium ($P<0.05\%$) and cells incubated with IBMX alone **[Figure.6. (B).].**

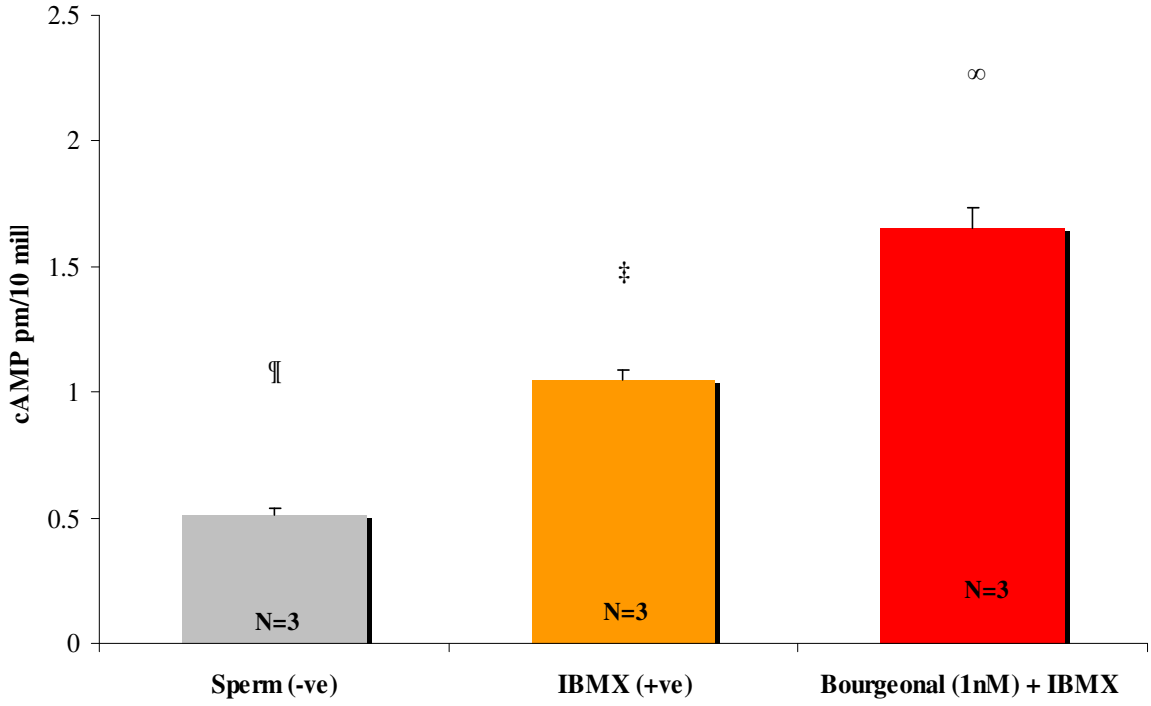

Figure.6. (A) Stimulation of cAMP synthesis in response to incubation of capacitated human spermatozoa with bourgeonal (1nM) in Ca^{2+} (HAM F-10 media). Capacitated human spermatozoa were incubated with HAM F-10 media supplemented with IBMX (100µM, positive control), bourgeonal (1nM) and IBMX (100µM, tested sample) and HAM F-10 media alone (negative control). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar Different symbols (¶,∞,‡)above each bar indicates significant difference between treatments (P<0.05%).

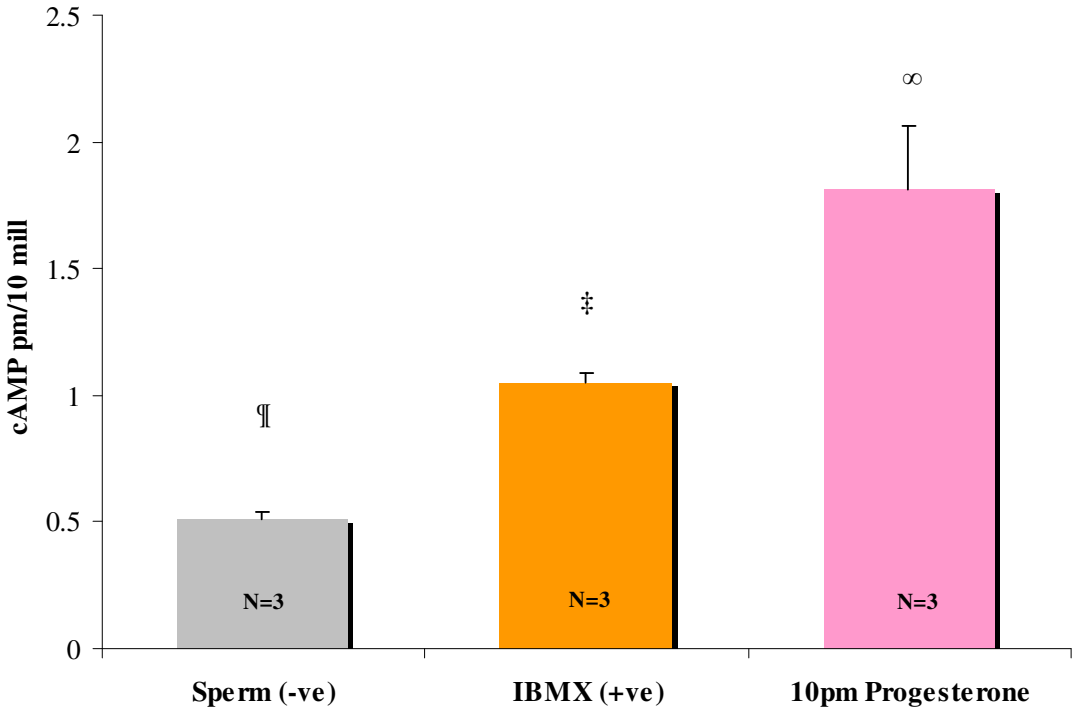

Figure.6. (B). Stimulation of cAMP synthesis in response to incubation of capacitated human spermatozoa with 10pM P in Ca^{2+} (HAM F-10 media). Capacitated human spermatozoa were incubated with HAM F-10 media supplemented with IBMX (100µM, positive control), 10pM P and IBMX (100µM, tested sample) and HAM F-10 media alone (negative control). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Different symbols (¶,∞,‡)above each bar indicates significant difference between treatments (P<0.05%).

5.2.8 $[Ca^{2+}]_i$ responses of cells exposed to a bourgeonal concentration steps (fM – mM)

Sperm loaded with OGB were exposed to a series of step applications of bourgeonal to investigate responses to the type of stimuli that may be of significance *in vivo* when turning within a chemoattractant gradient. Cells were initially capacitated (>5 hours in sEBSS), superfused for >2 minutes in sEBSS, then exposed to a series of bourgeonal concentration (1fM to 1μM). Visual examination of individual cell records demonstrated that >15% of the cells responded with a series of moderate transient elevations in OGB fluorescence and >20% respond with a series of sustained elevations (wave-like elevations), **[Figure.7. (B).],** when exposed to the increasing bourgeonal concentrations. The mean (population) response to 1 fM bourgeonal was a 21.% elevation in OGB fluorescence s which peaked after ≈1.1 minutes**.** Exposure to higher doses of bourgeonal resulted in increases of 18.2% (pM) 22.0%.(nM) and at 34.6% (μM) (one experiment, >50 cells examined). Time to peak response was slow at low concentrations but cells responded faster to the higher doses. In some cells it appeared that desensitization occurred, such that there was no clear response to pM bourgeonal after exposure to fM **[Figure.7. (A).]**

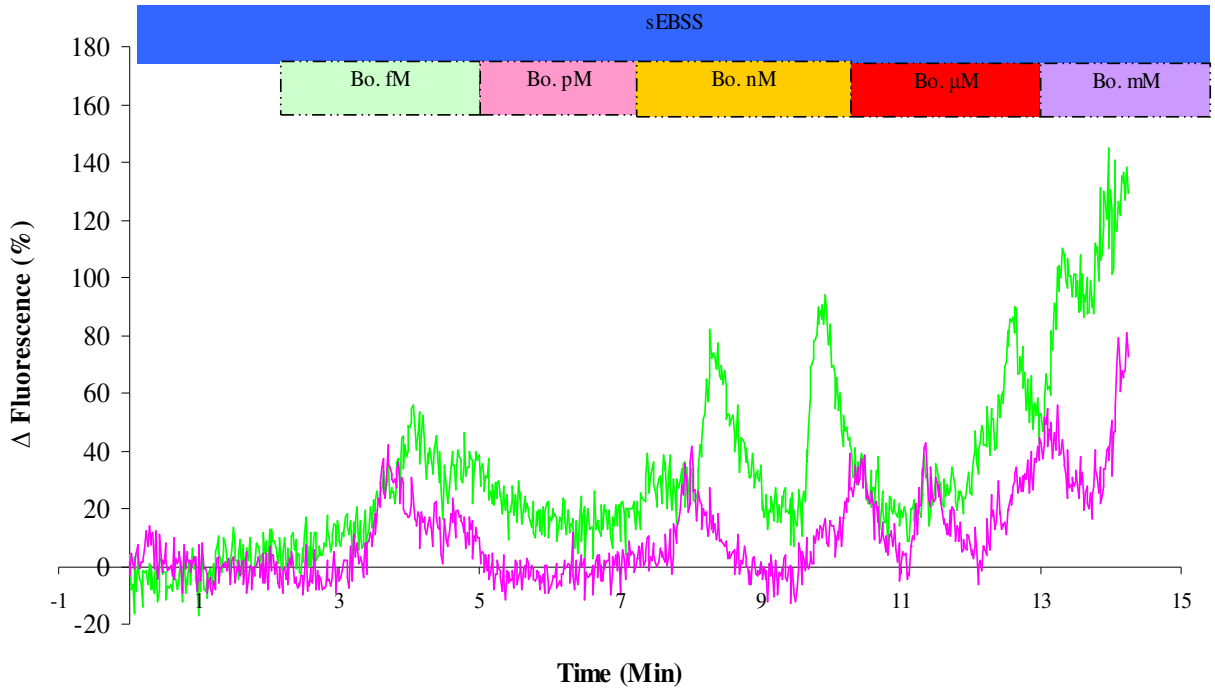

Figure.7. (A) Capacitated human spermatozoa confronted with a bourgeonal gradient in sEBSS (fM to mM), responding with a series of peaks to the increasing bourgeonal. Spermatozoa were incubated in sEBSS for >5hours in sEBSS and bathed in sEBSS (dark-blue box) prior to the stimulation with an ascending bourgeonal concentration gradient. Colored boxes indicate time point and duration that spermatozoa would experience each dose. Light-green = bourgeonal at fM; light-pink at pM; light-orange at nM and dark-red at µM and light-purple= mM). Traces show 2 single cell responses.

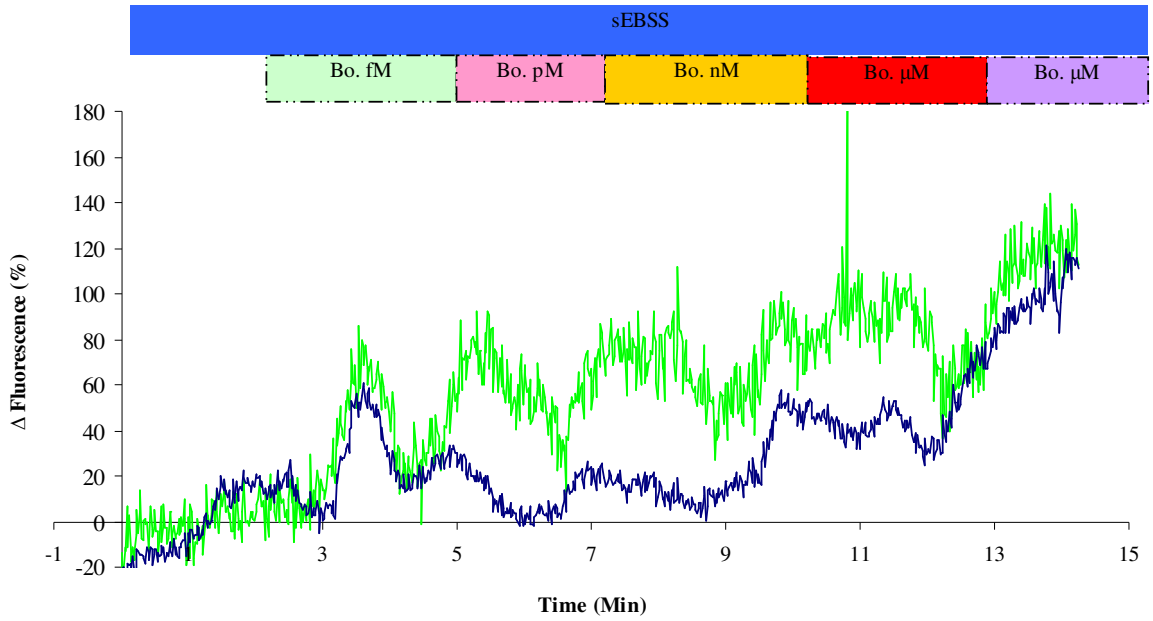

Figure.7. (B) Capacitated human spermatozoa confronted with a bourgeonal gradient in sEBSS (fM to mM). Spermatozoa were incubated in sEBSS for >5hours in sEBSS and bathed in sEBSS (dark-blue box) prior to the introduction of the bourgeonal gradient in the same medium. Colored boxes indicate time point and duration that spermatozoa would experience of each dose. Light-green = bourgeonal at fM; light-pink at pM; light-orange at nM and dark-red at µM and light-purple= mM). Traces show 2 single cell responses.

For comparison, similar experiments were carried out with a series of progesterone concentrations (1fM to 1mM; **Figure.8. (B)**). Progesterone induced repetitive calcium oscillations in 22% (±6% S.E.M) of the cells, which increased in amplitude and frequency as a function of P concentration, starting at the fM progesterone range. When initially exposed to 1 fM progesterone the amplitude of the oscillation were 76.4% above control levels (0.27 oscillations/minute); a subpopulation of spermatozoa responded with a transient transient elevation followed by a decrease in OGB

fluorescence significantly below control levels (indicated with **red arrow** on P graph, **[Figure.8. (A)]**) despite the continued presence of P. At 1 pM progesterone the mean amplitude of the response was 96% (0.40 oscillations/minute); at 1nM 103% (0.57 oscillations/minute) and at 1uM 135.% above control levels (0.89 oscillations/minute) **[Figure.8. (C)].** At μM concentration the amplitude of oscillations were significantly higher than previous doses and the frequency of oscillations too were significantly higher ($P<0.05$% t test) **[Figure.8. (D)].**

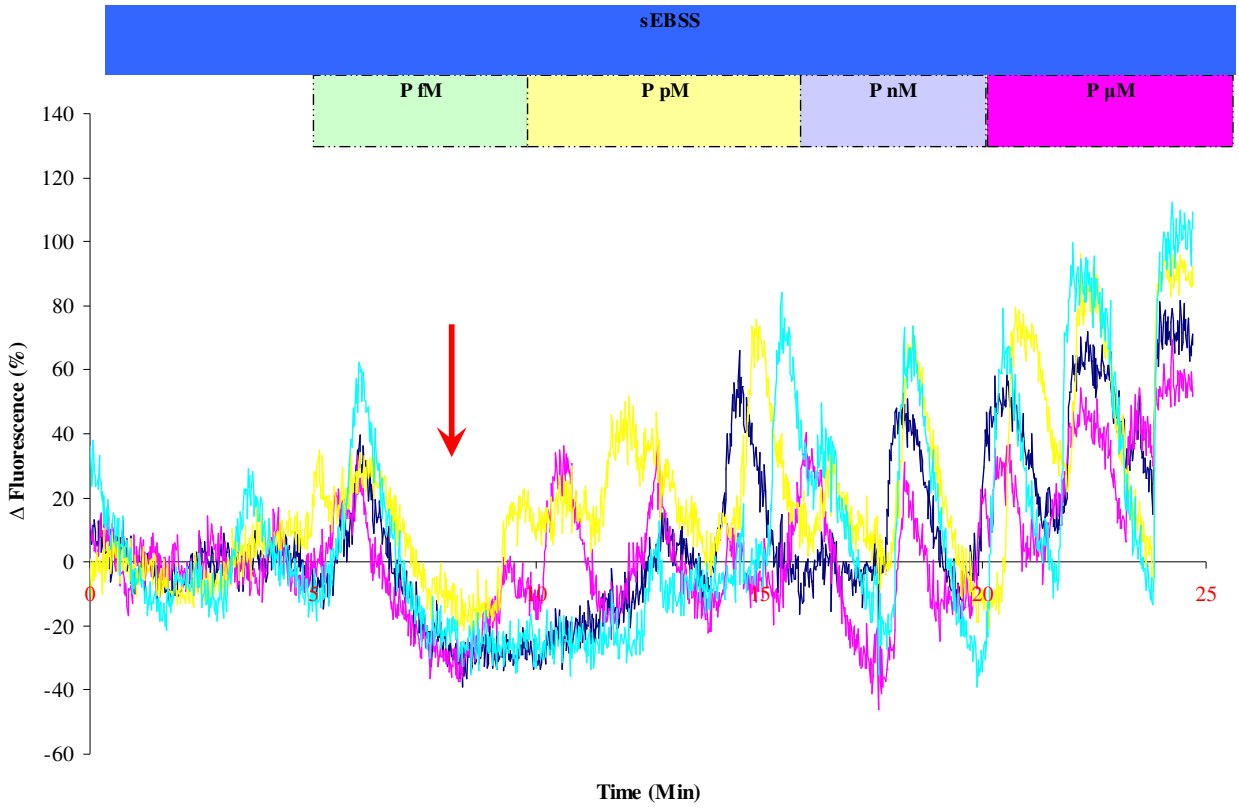

Figure.8. (A). Capacitated human spermatozoa confronted with a P gradient in sEBSS (Dark-blue box) (fM to µM). Prior to the encountering of the cells with a P gradient, capacitated spermatozoa were bathed in sEBSS for >3 minutes. Coloured boxes indicate time point and duration that spermatozoa would experience of each dose. Light-green = Progesterone at fM; Yellow-box at pM; light-purple at nM and bright-pink at µM. Red arrow indicates area of interest (see section 5.28). Traces show 4 single cell responses.

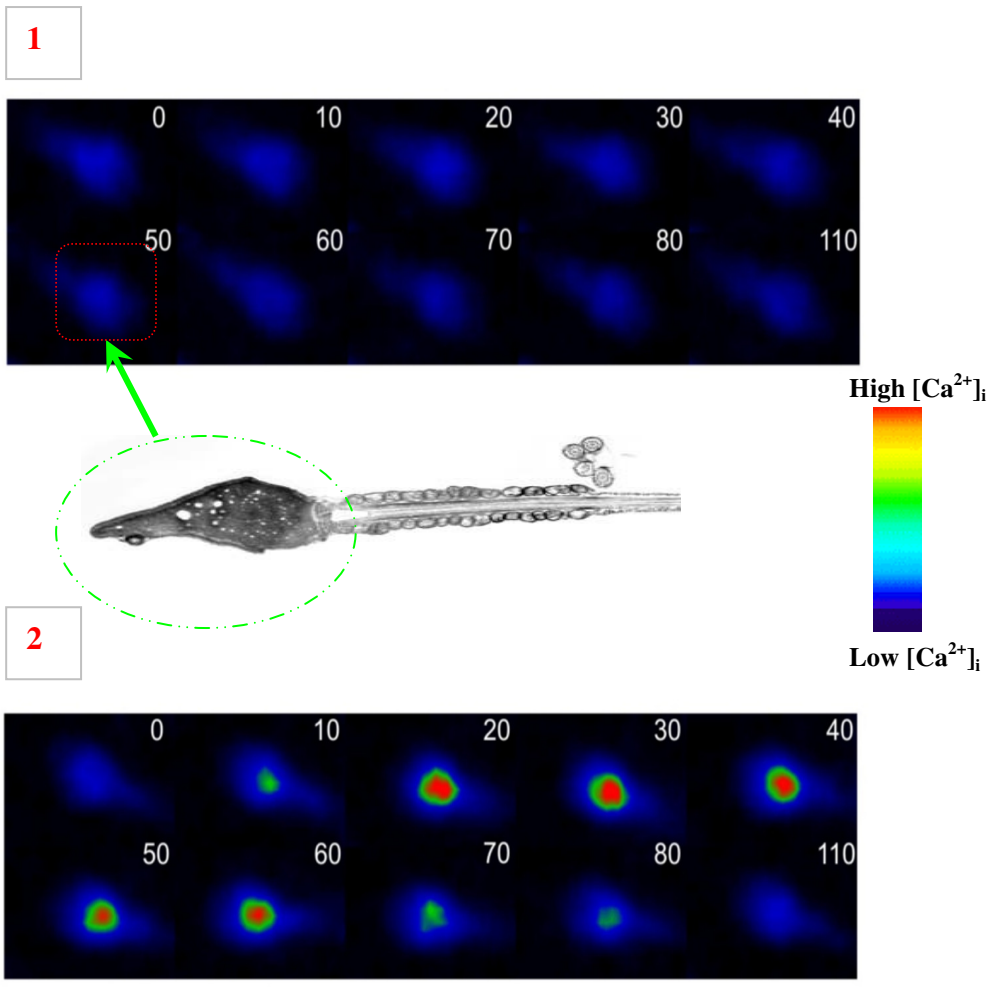

Figure.8. (B). Pseudocolour image series (warm colours show high $[Ca^{2+}]_i$) of : **(1)** untreated capacitated human spermatozoa during resting control period and **(2)** capacitated human spermatozoa confronted with a P gradient resulting in a single $[Ca^{2+}]_i$ oscillation. Numbers in grey indicates time course in seconds.

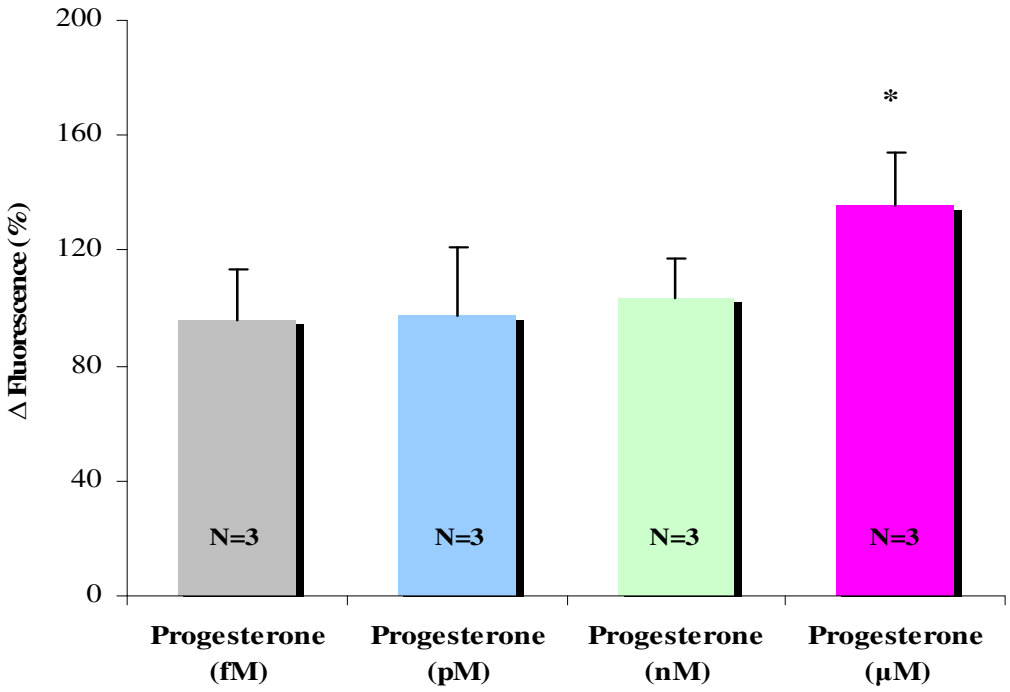

Figure.8. (C). Mean normalized fluorescence response of capacitated human spermatozoa response to P gradient in sEBSS (cells were first incubated in sEBSS for >5hour). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Asterisks (*) show differences in significance (P<0.05%).

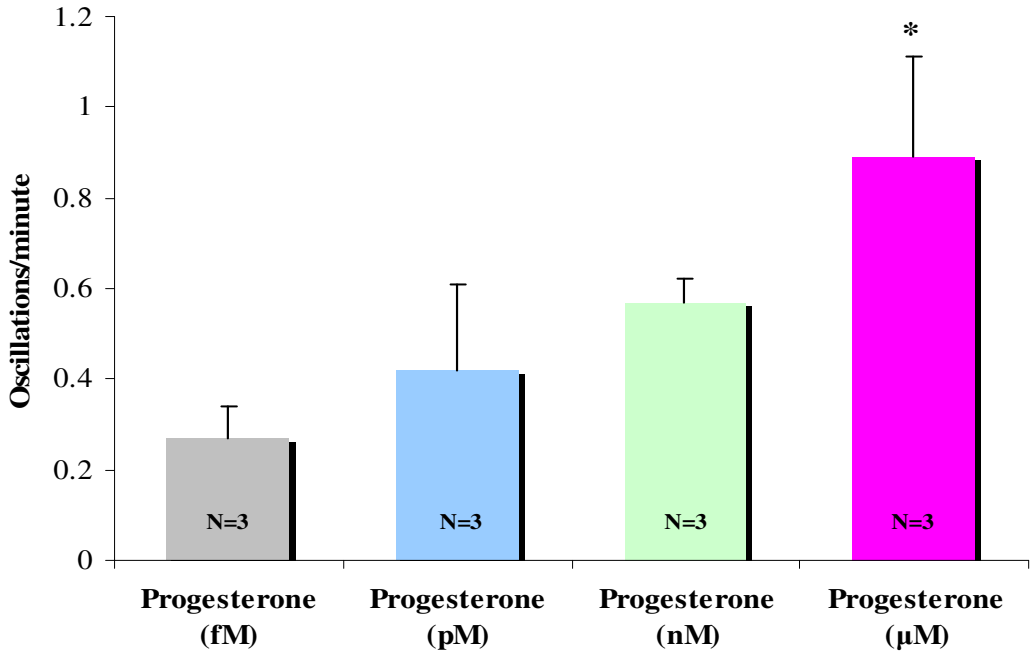

Figure.8. (D). Bar graph indicating the frequency of stimulated oscillations in capacitated spermatozoa confronted with a P gradient in sEBSS (>5 hour incubation on sEBSS). The error bars represent the SEM, and the number (N) of experimental replicates is indicated in each bar. Asterisks (*) show differences in significance (P<0.05%).

5.3 Discussion

In the work described in this chapter attempts to address the following questions:

i) Does bourgeonal exert a chemotactic effect in capacitated and non-capacitated spermatozoa?

ii) If so, does the chemotactic response depend on $[Ca^{2+}]_o$?

iii) Are intracellular Ca^{2+} stores involved in the chemotactic response to bourgeonal?

iv) Do Ca_v play a role in chemotaxis response to bourgeonal ?

v) Does the concentration of bourgeonal that result in chemotaxis stimulate cAMP production or AR?

vi) Do human spermatozoa show clear $[Ca^{2+}]_i$ elevations when exposed to series of steps of bourgeonal (1fM to 1mM) or progesterone (1fM to 1μM) ?

When capacitated human spermatozoa where exposed to a range of bourgeonal concentrations (10^{-5} to 10^{-14} M) in the Zigmond chamber, assessment of chemotactic responses showed a clear typical bell-shaped dose-response curve characteristic of chemotaxis (Adler et al., 1973). Peak chemotactic response was observed at 10^{-9} M bourgeonal (1nM), the response at this concentration resembling that seen with 10 pM progesterone (Teves et al, 2006). Thus a 1nM bourgeonal gradient in Ca^{2+} containing media exerts a chemotactic effect in capacitated human spermatozoa. These findings corroborate the conclusions of Spehr et al. (2003), though the method of assessment used in that study was less specific for chemotaxis. Furthermore experimental data provided by Spehr et al (2003) suggests that spermatozoa chemotactically respond to

bourgeonal at $\geq 10^{-6}$ M; in contrast to 10^{-9}M bourgeonal acquired with our discriminating experimental assay. The difference in chemotactic response might be due to the experimental protocol/technique to determine chemotaxis and cell preparation used by Spehr's group (rather uncertain whether cells were capacitated or not). Spehr's group determined chemotaxis to bourgeonal with microcapillaries with ascending, uniform and descending chemical gradients (bourgeonal). With this technique spermatozoa cells response did not result in bell-shaped response curve; typical of responses in all cellular models (Adler et al., 1973), spermatozoa seemed to further respond to the elevating bourgeonal concentrations. Adler et al. (1973) using the same technique with *E.coli*, demonstrated the cells responded to certain sugars with a typical bell-shaped response curve. Therefore the data provided by Spehr show resemblance to data from spermatozoa exposed to temporal gradients. In temporal responses the fraction of responding cells could elevate further than 80%, a process that is not restricted to capacitated spermatozoa (Gakamsky et al., 2009). Having established a clear chemotactic response to bourgeonal in capacitated spermatozoa, it was crucial to examine whether non-capacitated spermatozoa were also able to chemotactically respond to a bourgeonal gradient. It is clear in the literature that capacitated spermatozoa are a subpopulation (≈10%) of cells that are able to undergo chemotaxis. To prevent capacitation, spermatozoa were bathed in HAM F-10 medium in the absence of albumin for <30 minutes, required for capacitation (**see Chapter 1**). Cells prepared in this way showed no significant chemotactic response to bourgeonal. Parallel experiments showed that, if prepared under capacitating conditions, cells from the same samples were capable of responding chemotactically to bourgeonal. Teves et al. (2006)

showed similarly that non-capacitated spermatozoa were unable to chemotactically respond to a P gradient. Since the chemotactic response to bourgeonal is dependent on capacitation, it was examined whether a chemotactically active dose is also capable of inducing AR; a process which also requires capacitation. Assessment of AR demonstrated that 1nM bourgeonal does not significantly stimulate AR

Using the most effective dose of bourgeonal (1nM), the role of $[Ca^{2+}]_i$ signaling in the chemotactic response to bourgeonal was studied. When capacitated spermatozoa were confronted with a 1nM bourgeonal gradient in low-Ca^{2+} (NCFsEBSS; <5μM/L Ca^{2+}; Harper et al., 2004) the chemotactic response was eliminated. Additionally, when spermatozoa were confronted with a 1pM P gradient in low-Ca^{2+} the chemottactic response was suppressed; as previously reported by Teves et al. (2009) using the same bathing medium (NCFsEBSS); which did not significantly alter spermatozoa motility. This is consistent with the findings of Gakamsky et al. (2009), who showed that 'behavioral' responses of human sperm to both bourgeonal and progesterone exhibited dependence on external Ca^{2+}, requiring $>10^{-4}$ M. When capacitated spermatozoa were pretreated with nifedipine (1, 10, 100 μM), the chemotactic response to bourgeonal was inhibited in a dose dependent manner. Hence L-type (Ca_v1) and T-type (Ca_v3) channels, which are inhibited by nifedipine, might play a role in the chemotactic response to bourgeonal.

To examine possible participation of Ca^{2+} stores, capacitated spermatozoa were pretreated with TMB-8, an inhibitor of Ca^{2+} store release channels. This inhibited the chemotactic response of capacitated spermatozoa to P but the chemotactic response to a

1nM bourgeonal gradient was unaltered. Thus the chemotactic response to bourgeonal requires extracellular Ca^{2+} which presumably enters the cell though membrane channels, but stored Ca^{2+} appears not to play a role.

Spehr et al. (2003, 2004, 2006) suggested that the response of spermatozoa to bourgeonal occurs through induction by the agonist of mAC, leading to the production of cAMP. Hence it was logical to examine whether treatment of human sperm with the maximally chemotactic dose (1 nM) would increase production of cAMP. When cells were incubated with IBMX (to inhibit cAMP phosphodiesterase) there was a clear increase in [cAMP] compared with parallel non-bourgeonal treated incubations. However, these results cannot be taken as evidence that generation of cAMP is involved the chemotactic signaling pathway activated by a bourgeonal gradient. The temporal nature of the chemotactic response is such that only techniques that provide high temporal resolution (such as rapid mixing; Kaupp et al., 2003, 2008) will allow determination of the participation of cAMP, but these data are consistent with the model of Spehr et al (2003, 2004, 2006).

When capacitated spermatozoa were exposed to a series of stepped increased in bourgeonal concentration (fM to mM) >15% of the cells responded to each concentration with an elevation in OGB fluorescence. A small proportion of spermatozoa, after responding at very low concentrations, showed no response to the succeeding increases in bourgeonal concentration. Thus spermatozoa may undergo an

adaptation phase, possibly involving temporal desensitization of the receptor, as suggested by Gakamsky et al. (2009). In the model proposed by Gakamsky and colleagues (2009) the response characteristics include an excitation phase, composed of a delay (i.e., not including a change in the motility parameter) and a subsequent turn and an adaptation phase during which the cell ceases to respond to the chemoattractant even if the attractant is still present (Gakamsky et al., 2009). When sperm were exposed to P gradient composed of incremented steps (as for bourgeonal), Ca^{2+} oscillations were elicited in >20% of the sperm population. These increased in amplitude and frequency with the elevating P concentration. Here too a small proportion of spermatozoa showed an adaptation phase. These preliminary results suggest that the P gradient levels that induce specific sperm processes (e.g. chemotaxis, acrosome reaction priming, and hyperactivation) also trigger differential calcium oscillations that might be crucial as spermatozoa swim *in vivo* towards the attractant source. Consequently these oscillation increasing amplitude and frequency might significantly influence mammalian spermatozoa motility, not only by stimulation of chemotaxis, but hyperactivation of spermatozoa *in vivo* when required.

Many of the experiments reported in this chapter were performed in collaboration with **Dr Teves**, **Dr Giojalas**, **Dr Gidobaldi** and **Dr Uñates** from the Cebicem. Cordoba Argentina. All of the experiments both in Argentina and Birmingham were carried out by Aduén Andrés Morales García (author of this thesis). Additionally, one of the experiments in this chapter was done with the contribution of project students **Jack Lewis** and **Danielle Breen** (University of Birmingham, School of Bioscience). Additionally a few sections of this Chapter were published (with Dr Teves as main author) & other section will be published with Dr Uñates as first author and Aduén Morales García as second (or co-author, details yet to be confirmed).

VI

CHAPTER ~SIX~

Chapter Six

Chapter Six

General Discussion

The aim of this project was to examine the $[Ca^{2+}]_i$ signal response of capacitated and non-capacitated spermatozoa to the floral scent bourgeonal and to investigate further the signal cascade that leads to the chemotactic response. The work of Spehr's group provided a break-through in the identification of ectopic ORs in mammalian spermatozoa (hOR17-4) and in demonstration of their putative role in mammalian sperm chemotaxis. However, the technique of sperm preparation used in these experiments was puzzling regarding whether the spermatozoa were capacitated or not. All of the experiments reported here employed clearly defined media with characterised effects on capacitation to distinguish between capacitating and non-capacitating conditions (WHO, 1999). The data published by Spehr et al. (2003, 2004, 2006) proposed that the response of bourgeonal is dependent on $[Ca^{2+}]_o$ and that the opening of the Ca^{2+} channel(s) is due to the direct or indirect effect of both cAMP and/or PKA. Additionally that the effect of the floral scent does not induce AR and has a chemottactic effect on mammalian spermatozoa, however this was proposed without the use of discriminating technique(s) to distinguish between chemotaxis and other processes that result in sperm accumulation (chemokinesis, trapping, hyperactivation) (Eisenbach & Giojalas, 2006).

The first approach of this study was to examine the Ca^{2+} response of capacitated spermatozoa to a stepped increase in bourgeonal concentration and whether the

response to the ligand was reversible. Experimental data demonstrated that spermatozoa showed a clear sustained response when constantly stimulated with bourgeonal; **[Figure.1.],** as previously reported by Spehr et al. (2003). Additionally it was also observed that a small subpopulation of spermatozoa showed a single transient response, halting a further elevation in OGB fluorescence, even when the ligand was still present. This strongly suggesting that a subpopulation of spermatozoa activated a negative feedback mechanism that terminated a further response to bourgeonal with the closure of the membrane channel(s). Bourgeonal had a dose-response effect and here too all of the cells responded with a single transient response, terminating any further elevation in OGB fluorescence. Furthermore, experimental data acquired in this study demonstrated that the response of capaciated human spermatozoa to bourgeonal is reversible. When spermatozoa were stimulated with bourgeonal, resulting in an elevation in OGB fluorescence, when ligand was washed-off the OGB fluorescence dropped to control levels, and upon the reintroduction of bourgeonal the cells once again showed a clear response to the agonist. Hence demonstrating whilst the agonist is bound to hOR17-4 located in the midpiece of mammalian spermatozoa (Spehr et al., 2003, 2004) the consequent signal cascade is stimulated, consequently the removal of the ligand from the bathing media results in the termination of the response (ligand is no longer bound to G-protein coupled receptor).

In this study it was demonstrated that capacitated human spermatozoa show no significant response when exposed to bourgeonal in low-Ca^{2+} (<5μM/L Ca^{2+}), clearly showing that like many physiological process in sperm, the response is dependent

primarily on Ca^{2+} and in the case bourgeonal Ca^{2+}-influx. This was further demonstrated when spermatozoa where stimulated with the ligand in Ca^{2+}-containing media buffered with EGTA; resulting in no significant response **[Figure.1.]**. This strongly correlates with publications by Spehr et al. (2003, 2004, 2006) and Gakamsky et al. (2009), both reporting that the response to bourgeonal is dependent on $[Ca^{2+}]_o$ and that the $[Ca^{2+}]_i$ elevation is due to Ca^{2+}-influx from the extracellular medium. This hypothesis was corroborated by the observation that bis-phenol pre-treated capacitated spermatozoa showed a clear response to bourgeonal when exposed to the ligand in Ca^{2+}-containing medium. Intracellular Ca^{2+} stores in human spermatozoa do not play a role in the response to bourgeonal and the $[Ca^{2+}]_i$ elevation is due to Ca^{2+}-influx.

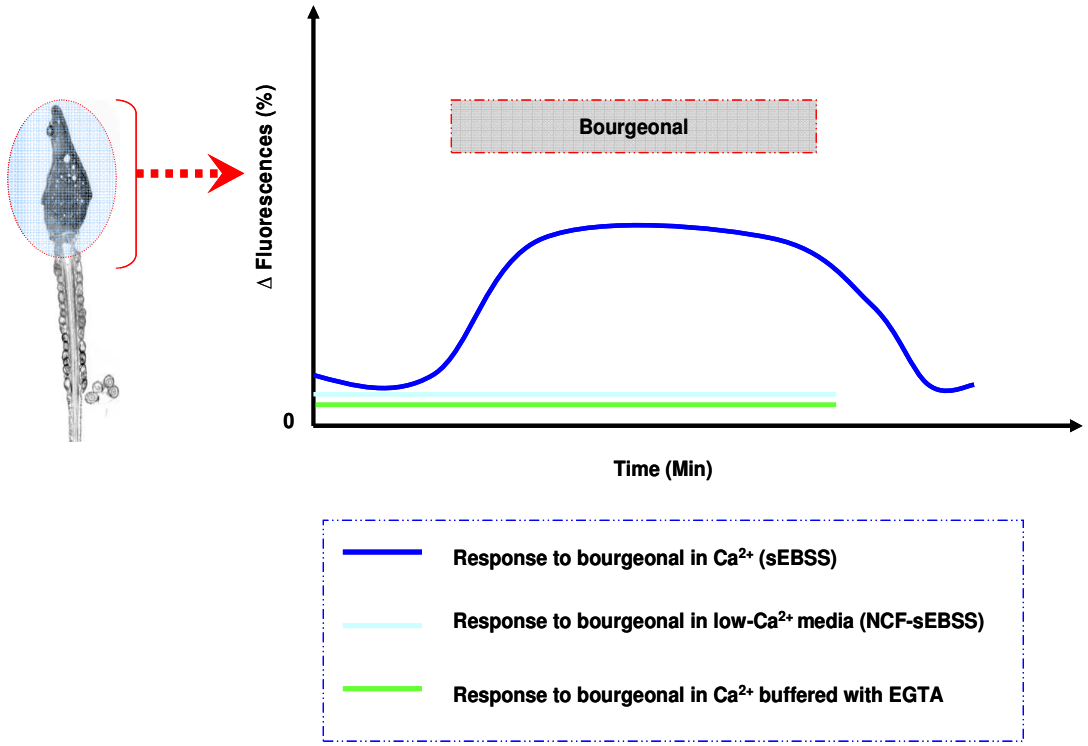

Figure.1. Summary of responses recorded from the head of capacitated human spermatozoa, when confronted with bourgeonal in varying Ca^{2+} conditions or media buffered with Ca^{2+} chelator (Spermatozoa Image from: Costello et al., 2008).

When similar experiments were carried out on cells not incubated under capacitating conditions clearly demonstrated that capacitation is required for the cells to generate a response to bourgeonal. However, whether this effect was absolute or was due to an increase in sensitivity in capacitated cells was not investigated. Thus the responses of non-capacitated spermatozoa to higher concentrations (>100 μM) might reflect a difference in bourgeonal sensitivity (Gakamsky et al. 2009). For example, the elevation in cAMP and stimulation of PKA, which take place when mammalian spermatozoa undergo capacitation (Parinaud and Milhet, 1996; Chen et al., 2000; Lefièvre et al., 2002) are believed to be key components in the signal transduction pathways in

response to bourgeonal (Spehr et al., 2003, 2004, 2006) and thus might modulate OR-mediated responses. **[Figure.2.]**.

In this research study it was possible to demonstrate that capacitated spermatozoa do not result in AR when incubated with bourgeonal in Ca^{2+} (nM to µM). Spehr et al. (2003, 2004) demonstrated that at higher concentrations than the ones used in this studies, bourgeonal had no significant effect on the acrosomal status of human spermatozoa.

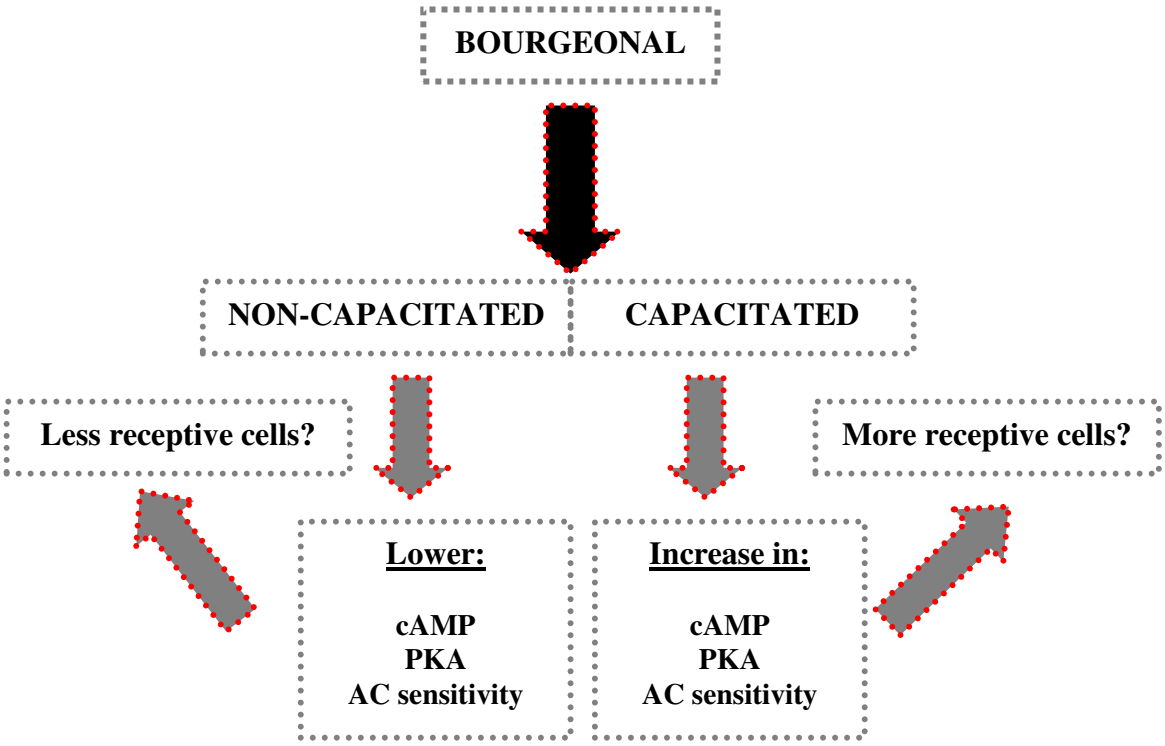

Figure.2. Proposed model exploring intracellular variations that suggest the why capacitated spermatozoa might be more responsive to bourgeonal than non-capacitated.

When bourgeonal preatreated cells in low-Ca^{2+} were introduced to high-Ca^{2+} a single transient $[Ca^{2+}]_i$ response occurred, rapidly terminating any further response to bourgeonal. Thus when spermatozoa were treated with bourgeonal in low-Ca^{2+} saline this had a priming effect. Thus whilst bourgeonal is bound to its receptor the signal transduction pathway results in the opening of the membrane Ca^{2+} channel(s) but in the absence of Ca^{2+} no response was induced through Ca^{2+}-influx. Consequently upon the introduction of Ca^{2+} the capacitated spermatozoa respond with the activation of the negative feedback mechanism halting any further elevation in $[Ca^{2+}]_i$, resulting in a single transient elevation in OGB fluorescence. This mechanism may be of great significance in the chemotactic action of bourgeonal, providing the temporal fidelity required for sampling a concentration gradient.

When spermatozoa were incubated with bourgeonal and IBMX in Ca^{2+} there was a significant elevation in cAMP. Hence, it was therefore investigated whether the activation of Ca^{2+}-influx by bourgeonal and the termination of this influx could be elicited by dbcAMP. When spermatozoa were exposed to dbcAMP in Ca^{2+}-containing medium a clear sustained elevation of $[Ca^{2+}]_i$ occurred. No response was detected when cells were exposed to dbcAMP in low-Ca^{2+}, suggesting that, as with bourgeonal, cAMP opens a membrane Ca^{2+} channel. Similarly to treatment with bourgeonal, a small subpopulation of sperm responded to dbcAMP with a single transient elevation in OGB fluorescence, consistent with the presence of a feedback mechanism **[Figure.3.]**. When the effects of dbcAMP were investigated using the Ca^{2+}-readmission protocol to investigate feedback, the kinetics were as with bourgeonal. Furthermore, pretreatment

with IBMX to prevent hydrolysis of cAMP generated in response to bourgeonal did not affect kinetics of the transient **[Figure.3.]**. Thus, though cAMP may open a Ca^{2+}-channel(s) in bourgeonal-treated cells, either directly or indirectly (via PKA; Spehr et al., 2003, 2004, 2006), (cAMP) concentration does not play a part in termination of the response. A direct effect of Ca^{2+} (or Ca^{2+}/CaM) on the membrane channel is thus the likely mechanism of feedback. CNG channels, which are present in sperm (Jimenez-Gonzalez et al., 2006; Darszon et al., 1999) activated by cAMP and modulated by CaM (Molday, 1996, Kaupp et al., 2002) are thus a candidate for the bourgeonal-activated channel **[Figure.5.]**.

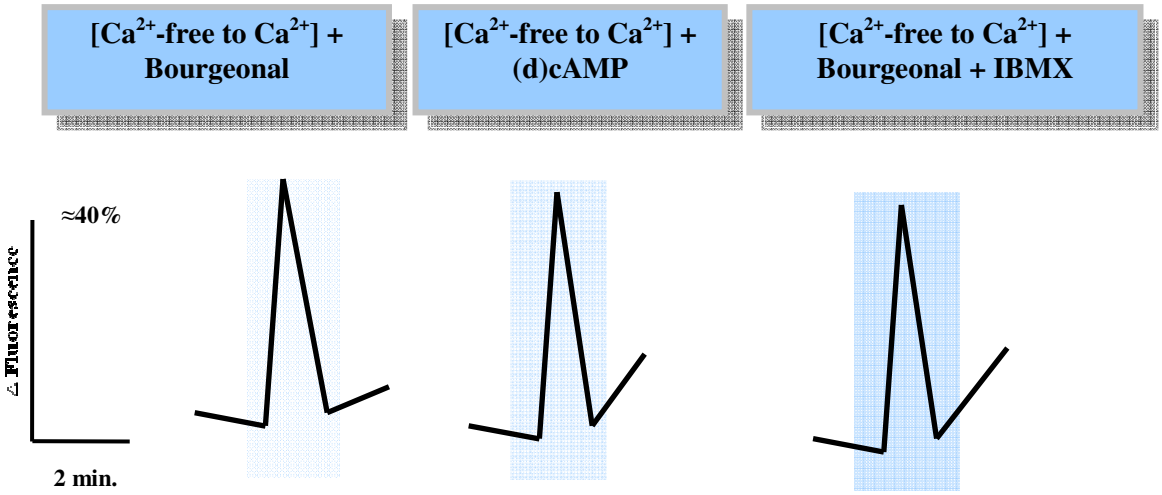

Figure.3. Summary of responses recorded from the head of capacitated human spermatozoa when investigating the negative feedback response.

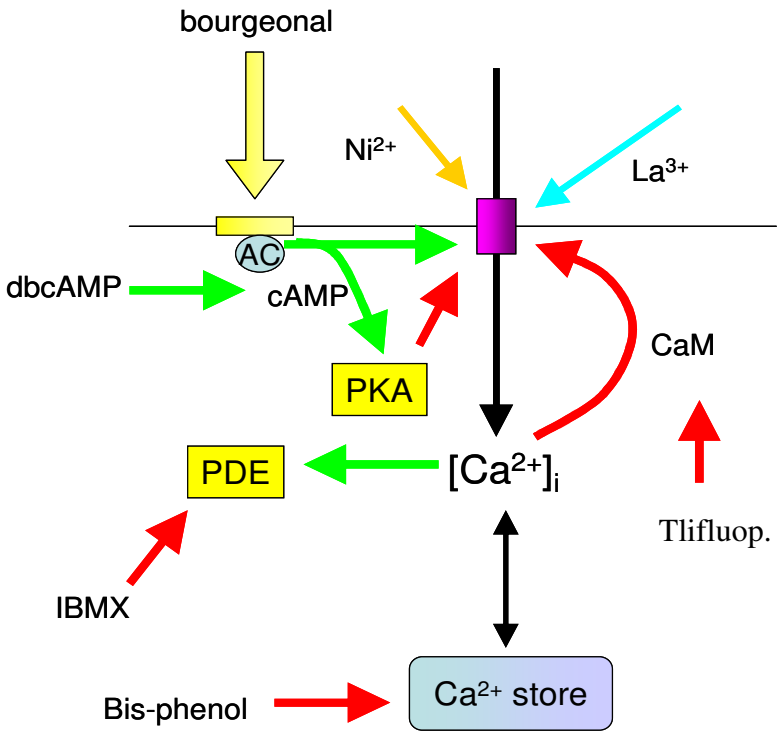

Figure.4. Summary of signaling components that potentially contribute to modulation of the membrane channel(s) activated upon binding of bourgeonal. Drugs and analogues used during my investigation and their probable sites of action are also shown

To investigate whether CNG channels might indeed be involved, the potential involvement of CaM in modulation of the bourgeonal-activated channel (CNG channels ?) was examined, by using trifluoperazine a CaM antagonist (Cheung, 1980). Inhibition of CaM would result in the loss of the Ca^{2+}-mediated negative feedback such that the Ca^{2+} transient that occurs upon readmission of Ca^{2+} to bourgeonal treated cells in low-Ca^{2+} medium should be followed by a plateau. However, though the drug did prolong the decay of the transient, control experiments showed that this effect was probably due mainly to an effect on the rate of Ca^{2+} clearance rather than gating of Ca^{2+} channels.

Plasma membrane Ca^{2+} ATPases are activated by CaM (Luthra et al., 1982) and inhibition of this process may have been responsible for my observations.

However, a potential role of CNG channels in the response to bourgeonal was demonstrated in this study with the use of Ni^{2+} and to a less extent with La^{3+}. Pretreated with these ions, at doses that block CNG channels, did suppress the $[Ca^{2+}]_i$ response to the ligand. Thus CNG channels remain a putative pathway for bourgeonal-activated Ca^{2+} influx **[Figure.5.]**.

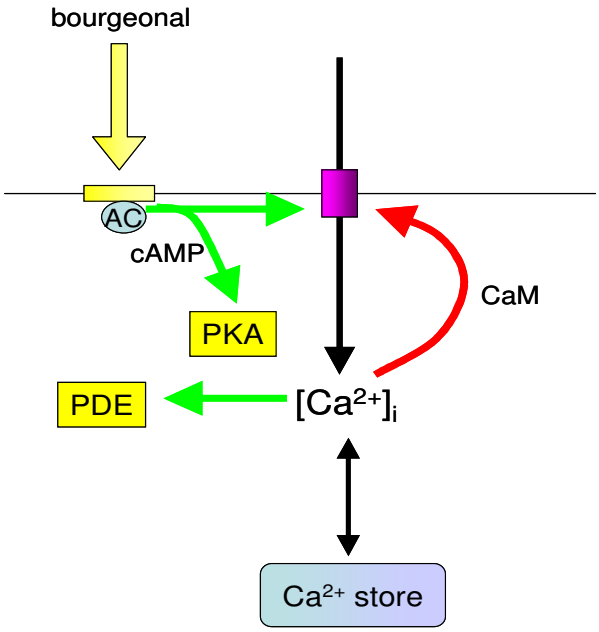

Figure.5. Summary of signaling components that may underlie activation of Ca^{2+} influx by bourgeonal and negative feedback terminating the response. Red arrow (→) indicates the modulatory effect of Ca^{2+} or Ca^{2+}/CaM on the Ca^{2+} channel (CNG?).

The acquired data throughout this thesis suggest the potential role of CNG channels, located in the flagellar midpiece (Publicover et al., 2007, Molday et al., 1996), in response to bourgoenal. The binding of bourgeonal to the hOR17-4, located in the flagellar midpiece (Spehr et al., 2003, 2004, 2006), could result in an elevation in cAMP and PKA, consequently opening of the CNG channels (or other channels). The opening results in a elevation in $[Ca^{2+}]_i$, which could then result in the activation of a feedback mechanism resulting in the closure of the CNG channel by the modulatory effect of CaM (Ca^{2+}/CaM) (Molday et al., 1996, Kaupp et al., 2002) **[Figure.5.]**. This model suggests part of intracellular response and the temporal resolution of the chemotactic response and a potential directional change. However, this is not an absolute model of the signalling events in response to bourgeonal, other Ca^{2+} channels (CatSpers, TRPc ??) and other signal cascade(s) could be involved too. The described events, hypothesised from the data acquired **[Figure.5.],** explains in an elegant (and simple) way the occurrence of the feedback mechanism, terminating the response. However, it is possible that other events could occur in response to bourgeonal; the extensive data acquired and further research would shed light on this possibility.

"Behavioral" assessment of capacitated human spermatozoa showed that the cells respond chemotactically to a bourgeonal gradient. Maximum response occurred at 10^{-9} M bourgeonal (1nM). These data are largely consistent with the report of Spehr et al. (2003, 2004, 2006), who observed a chemotactic response at a concentration $\geq 10^{-6}$ M. Moreover it was also demonstrated that the chemotactic response to bourgeonal was dependent on Ca^{2+} but that the Ca^{2+} stores do not participate. Significantly, it was

possible to show that non-capacitated spermatozoa showed no chemottactic response to a bourgeonal gradient, consistent with previously published findings that only capacitated spermatozoa are the chemotactically responding cells (Cohen-Dayag et al., 1995; Eisenbach, 1999; Fabro et al., 2002; Teves et al., 2006).

Positive control experiments, using 10pM P were in agreement with data published by Teves et al. (2006), confirming the sensitivity of the assay. To test the Ca^{2+} response to chemotactic doses of P, a range of concentrations (fM to μM) were used to examine the response of capacitated spermatozoa to these treatments. A subpopulation of spermatozoa (≈20%) responded with Ca^{2+} oscillations, which increase in amplitude and frequency with the increasing P concentration. Thus the very low concentrations that stimulate chemotaxis in human spermatozoa are also capable of triggering Ca^{2+} oscillations (Uñates D.R. & Morales-Garcia A.A, 2008). Similarly, when treated with a very low bourgeonal concentrations (fM to mM) a subpopulation of spermatozoa (≈15%) responded with a series of peaks, starting at fM. Thus when human sperm encounter a chemoattractant gradient *in vivo*, the cells may respond in this way in the presence of an increasing chemoattractant concentration. Capacitated spermatozoa would detect the lower concentrations of the attractant, possibly inducing a turn (Ca^{2+} spike?). At high concentrations, inducing increased frequency and magnitude of Ca^{2+} spikes, hyperactivation may occur **[Figure.6.]**.

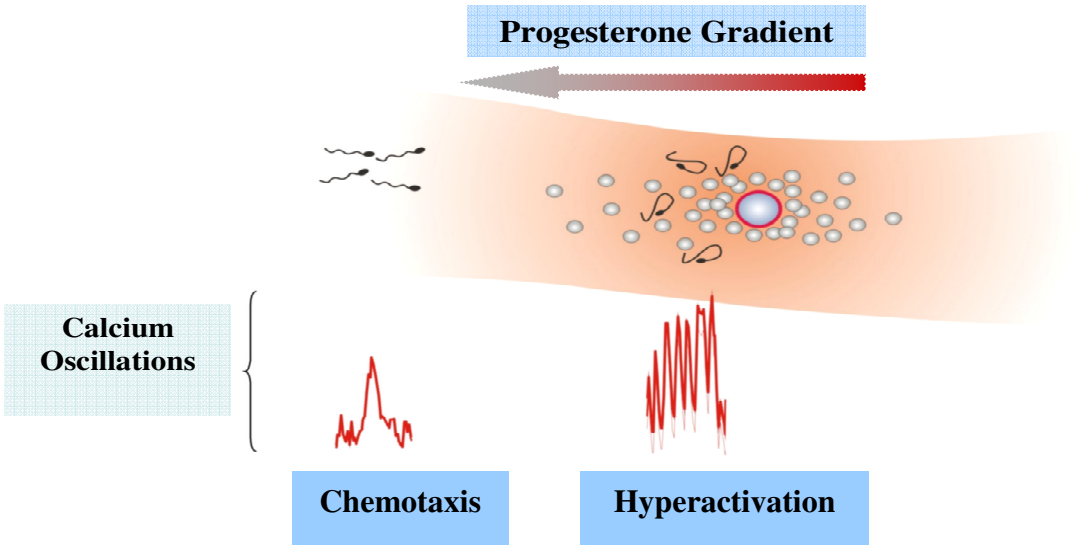

Figure.6. Diagramatic representation of proposed effects of an increasing P gradient. Progesterone at pM concentrations guides spermatozoa towards the occyte via chemotaxis. This process could be regulated by oscillations of low frequency and magnitude. In the proximity of the oocyte, where P concentration would be at µM levels. P stimulates hyperactivated motility enabling spermatozoa to travel through the cumulus cells, a process that might be regulated by oscillations of greater magnitude and frequency (DR Uñates & Morales-Garcia A.A, 2008).

7.1 Future Research

It is clear that Ca^{2+} is a critical element in all the processes that spermatozoa undergo, including the response to bourgeonal and derivatives. It's also clear that bourgeonal exerts a chemotactic response in capacitated spermatozoa but not uncapacitated cells. However, a number of aspects of the model described above are currently only speculative. Future work must address these.

- The involvement of cAMP in activating the Ca^{2+} channel is probable but not proven. It will be necessary in the future directly to quantify the kinetics of [cAMP] in cells stimulated with bourgeonal and other chemo-attractants.

- It is still uncertain whether the CNG channels are involved in the response to bourgeonal, and if not, what Ca^{2+} channels are involved? A major limitation of work on human sperm is the difficulty of genetic manipulation, such that a pharmacological approach must be used, accepting its potential for secondary effects on other processes.

- The mechanism that enables capacitated spermatozoa to respond chemotactically, whereas uncapacitated cells cannot, must be established. It has been postulated that non-capacitated spermatozoa are capable of responding to a ligand, however higher concentrations must be needed to see a response, due to lower receptivity of the cells

(Gakamsky et al., 2008). If this is the case, what are the changes that enable the capacitated spermatozoa to be more “sensitive” than non-capacitated.

The work reported here is another step forward towards the understanding of the effects of chemo-attractants on human sperm and specifically the role of the OR hOR17-4. However, we are a long way from being able to provide a functional explanation of chemotaxis in human (or other mammalian) sperm equivalent to the models that have been proposed for echinoderm spermatozoa.

APPENDIX I

MEDIA

Supplemented Earle's Balanced Salt Solution (sEBSS)

Composition:

Sodium Dihyd. Phosphate 0.122g/l (1.0167 mM)
Potassium Chloride 0.4g/l (5.4 mM)
Magnesium Sulphate.7H2O 0.2g/l (0.811 mM)
Dextrose Anhydrous 1.0g/l (5.5 mM)
Sodium Pyruvate 0.3g/l (2.5 mM)
DL-Lactic Acid, Sodium 4.68g/l (19.0 mM)
Calcium Chloride.2H2O 0.264g/l (1.8 mM)
Sodium Bicarbonate 2.2g/l (25.0 mM)
Sodium Chloride 6.8g/l (116.4 mM)

The composition of this medium is based upon Supplemented Earle's Balanced Salt Solution (with/out Phenol Red recipe). The osmolarity of the medium was adjusted with sodium chloride to 285-295 mOsm, and checked using an Advanced Micro Osmometer (Vitech Scientific Ltd, West Sussex, UK) [pre-calibrated using a 50 mOsm/Kg H2O and a 850 mOsm/Kg H2O calibration standards]. The pH was adjusted to 7.25-7.4 with 1M hydrochloric acid (HCl) and 1M sodium hydroxide (NaOH). sEBSS was stored in 100ml glass beakers at 4°C until use. Thus upon use, the pH was rechecked and adjusted if necessary. When experiments involved capaciated spermatozoa 0.3% Bovine serum albumin (BSA) was introduced into the medium and filtered, to remove any possible residues.

HEPES-buffered saline

Composition:

Potassium Chloride 0.336g/l (4.5 mM)
Magnesium Chloride. 6H2O 0.9ml from aM stock 0.9g/l (1.0mM)
Dextrose Anhydrous 1.621g/l (9.0 mM)
Calcium Chloride.2H2O 1.8ml from a 1M stock 1.8g/l (2.0 mM)
Sodium Chloride ≈7.889g/l (135.0 mM)
Hepes 2.147g/l (135.0mM)

Identical protocol as for standard sEBSS; however NCFsEBSS. When experiments involved capaciated spermatozoa 0.3% Bovine serum albumin (BSA) was introduced into the medium and steryle-filtered to remove any possible residues.

Ca^{2+}-free sEBSS (NCFsEBSS)

Composition:

Sodium Dihyd. Phosphate 0.122g/l (1.0167 mM)
Potassium Chloride 0.4g/l (5.4 mM)
Magnesium Sulphate.7H2O 0.2g/l (0.811 mM)
Dextrose Anhydrous 1.0g/l (5.5 mM)
Sodium Pyruvate 0.3g/l (2.5 mM)
DL-Lactic Acid, Sodium 4.68g/l (19.0 mM)
Sodium Bicarbonate 2.2g/l (25.0 mM)
Sodium Chloride 6.8g/l (118.4 mM)

Identical protocol as for standard sEBSS; however NCFsEBSS was stored at 4°C, in a 100 ml volume polystyrene bottles until use (recommendation by Dr Kirkman-Brown).When experiments involved capaciated spermatozoa 0.3% Bovine serum albumin (BSA) was introduced into the medium and steryle-filtered to remove any possible residues.

HAM-F10 Medium Composition

COMPONENTS	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75	7.5	0.1
L-Alanine	89	9	0.101
L-Arginine hydrochloride	211	211	1
L-Asparagine-H2O	150	15	0.1
L-Aspartic acid	133	13	0.0977
L-Cysteine	121	25	0.207
L-Glutamic Acid	147	14.7	0.1
L-Glutamine	146	146	1
L-Histidine hydrochloride-H2O	210	23	0.11
L-Isoleucine	131	2.6	0.0198
L-Leucine	131	13	0.0992
L-Lysine hydrochloride	183	29	0.158
L-Methionine	149	4.5	0.0302
L-Phenylalanine	165	5	0.0303
L-Proline	115	11.5	0.1
L-Serine	105	10.5	0.1
L-Threonine	119	3.6	0.0303
L-Tryptophan	204	0.6	0.00294
L-Tyrosine disodium salt dihydrate	261	2.62	0.01
L-Valine	117	3.5	0.0299
Vitamins			
Biotin	244	0.024	0.0000984
Choline chloride	140	0.7	0.005
D-Calcium pantothenate	477	0.7	0.00147
Folic Acid	441	1.3	0.00295
Niacinamide	122	0.6	0.00492
Pyridoxine hydrochloride	206	0.2	0.000971
Riboflavin	376	0.4	0.00106
Thiamine hydrochloride	337	1	0.00297
Vitamin B12	1355	1.4	0.00103
i-Inositol	180	0.5	0.00278
Inorganic Salts			
Calcium Chloride (CaCl2) (anhyd.)	111	33.29	0.3
Cupric sulfate (CuSO4-5H2O)	250	0.0025	0.00001
Ferric sulfate (FeSO4-7H2O)	278	0.834	0.003
Magnesium Sulfate (MgSO4) (anhyd.)	120	74.62	0.622
Potassium Chloride (KCl)	75	285	3.8
Potassium Phosphate monobasic (KH2PO4)	136	83	0.61
Sodium Bicarbonate (NaHCO3)	84	1200	14.29
Sodium Chloride (NaCl)	58	6900	118.97
Sodium Phosphate dibasic (Na2HPO4) anhydrous	142	153.7	1.08
Zinc sulfate (ZnSO4-7H2O)	288	0.0288	0.0001
Other Components			
D-Glucose (Dextrose)	180	1100	6.11
HEPES	238	5958	25.03
Hypoxanthine Na	159	4.7	0.0296
Lipoic Acid	206	0.2	0.000971
Phenol Red	376.4	1.2	0.00319
Sodium Pyruvate	110	110	1
Thymidine	242	0.7	0.00289

APPENDIX II

PUBLICATIONS AND PRESENTATIONS OF RESEARCH

Machado-Oliveira Gisela; Lefièvre Linda; Ford Christopher; Herrero M Belen; Barratt Christopher; Connolly Thomas J; Nash Katherine; **Morales-Garcia Aduen**; Kirkman-Brown Jackson; Publicover Steve, (2008). Mobilisation of Ca^{2+} stores and flagellar regulation in human sperm by S-nitrosylation: a role for NO synthesised in the female reproductive tract. Development (Cambridge, England) 2008;135(22):3677-86.

Publicover, S. J., Giojalas, L. C., Teves, M. E., Oliveira, G. S., **Garcia, A. A**., Barratt, C. L.and Harper, C. V. (2008). Calcium signalling in the control of motility and guidance in mammalian sperm. *Front Biosci* 13, 5623-37.

Teves M., Guidobaldi H., Uñates D., Sanchez R., Miska W., Publicover S., **Morales García A.**, Giojalas L.(2009). Molecular Mechanism for Human Sperm Chemotaxis Mediated by Progesterone. PLoS ONE 4(12): e8211

CONFERENCE POSTERS & ABSTRACTS

Gordon Conference, Fertilization & Activation Of Development, USA (2009)

DOES HUMAN SPERMATOZOA CONTAIN RYANODINE RECEPTORS? Linda Lefièvre, Katherine Nash, Gisela Machado-Oliveira, **Aduen Morales Garcia**, Frank Michelangeli and Stephen Publicover

INTRACELLULAR CALCIUM STORES & STORE-OPERATED CALCIUM INFLUX IN HUMAN SPERMATOZOA. Linda Lefièvre, Katherine Nash, **Aduen Morales Garcia**, Frank Michelangeli and Stephen Publicover

44th Annual meeting, Argentine Society for Biochemistry and Molecular Biology Research. November 2008. Villa Carlos Paz, Cordoba. Argentina.

INTRACELLULAR CALCIUM OSCILLATION IN SPERMATOZOA EXPOSED TO A CONCENTRATION GRADIENT OF PROGESTERONE. Uñates DR, **Morales-Garcia AA.**, Publicover Sj, Giojalas LC.

Society for Reproduction and Fertility (SRF), Scotland (2008)

INTRACELLULAR CALCIUM SIGNALLING AND MAMMALIAN SPERM CHEMOTAXIS. **Morales-Garcia A.A**, JC Kirkman-Brown, CL Barratt, SJ Publicover

University of Birmingham, Bioscience Simposium (2007 & 2008)

INTRACELLULAR CALCIUM SIGNALLING AND MAMMALIAN SPERM CHEMOTAXIS**. Morales-Garcia A.A**, JC Kirkman-Brown, CL Barratt, SJ Publicover

References

Abou-Haila, Tulsini D.R.P., (2009), Signal transduction pathways that regulate sperm capacitation and acrosome reaction.Archives of biochemistry and biophysics 485; 72-81.

Adeoya-Osiguwa, S. & L.R. Fraser: (1996). Evidence for Ca^{2+}-dependent ATPase activity, stimulated by depcapacitation factor calmodulin, in mouse sperm. Mol Reprod. Dev 44, 111-120

Adler J (1973) A method for measuring chemotaxis and use of the method to determine optimun conditions for chemotaxis by Escherichia coli. Journal of Genetics and Microbiology 74: 77–91.

Adolf-Friedrich H., Wolfgang S. & Michail D., (2003). Understanding spermatogenesis is a prerequesite for treatment. Review. Reproductive Biology of Endocrinology.

Afzelius, B.A. (1959). Electron microscopy of the sperm tail. Results obtained with a new fixative. J. Biophys. Biochem. Cytol., 5, 269–278.

Afzelius, B.A. (1988). On the numbering of peripheral doublets in cilia and flagella. Tiss. Cell, 20, 473–476.

Ain R., Uma Devi K., Shivaji S., Seshagiri P.B. (1999). Pentoxifylline-stimulated capacitation and acrosome reaction in hamster spermatozoa: involvement of intracellular signalling molecules. Mol Hum Reprod 5,618-626

Aitken R.J., Clarkson J.S. (1988). Significance of reactive oxygen species and antioxidants in defining the efficacy of preparation techniques. Journal of Andrology 9: 367–376.

Aitken R.J., (2006).Sperm function tests and fertility. International Journal of Andrology. ISSN 0105-6263.

Al-Anzi, B. & Chandler, D. E. A .(1998).Sperm chemoattractant is released from Xenopus egg jelly during spawning. Dev. Biol. 198, 366–375

Alam R., Stafford S.,Forsythe P.,Harrison R., Faubion D., Lett-Brown M.A. and Grant J.A**.** (1993).RANTES is a chemotactic and activating factor for human eosinophils. J. Immunol. 150, 3442–3448

Anant B.P. & James W.P., (2005). Store-operated calcium channels. Physiol. Rev 85: 757-810.

Anderson R.A., Feathergill K.A., Rawlins R.G., Mack S.R. and Zaneveld L.J.D. (1995). Atrial natriuretic peptide: a chemoattractant of human spermatozoa by a guanylate cyclase-dependent pathway Molecular Reproduction and Development 40 371–378

Arnoult C., Cardullo R.A., Lemos J.R. and Florman H.M. (1996a). Activation of mouse sperm T-type Ca2+ channels by adhesion to the egg zona pellucida. Proc Natl Acad Sci USA 93(23), 13004-13009.

Arnoult C, Zeng Y and Florman HM (1996b). ZP3-dependent activation of sperm cation channels regulates acrosomal secretion during mammalian fertilization. J Cell Biol 134(3), 637-645.

Arnoult C., Villaz M. and Florman H.M. (1998). Pharmacological properties of the T20 type Ca2+ current of mouse spermatogenic cells. Mol Pharmacol 53(6), 1104-1111.

Arnoult C., Kazam I.G., Visconti P.E., Kopf G.S., Villaz M. and Florman H.M. (1999). Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proc Natl Acad Sci U S A 96(12), 6757-6762.

Austin C.R. (1951). Observations on the penetration of the sperm into the mammalian egg. Aust J of Sci Res,Series B 4:581

Austin C.R. (1952).The "capacitation" of the mammalian sperm. Nature 170: 326

Babcock D.F., First N.L., Lardy H.A. (1975). Transport mechanism for succinate and phosphate localized in the plasma membrane of bovine spermatozoa. J Biol Chem. 250,6488-6495.

Baccetti, B., Pallini, V. and Burrini, A.G. (1976). The accessory fibers of the sperm tail. III High sulfur and low sulfur components in mammals and cephalopods. J. Ultrastruct. Res., 57, 289–308.

Baccetti, B., Porter, K.R. and Ulrich, M. (1985). High voltage electron microscopy of sperm axoneme. J. Submicrosc. Cytol., 17, 171–176.

Bahat A., Tur-Kaspa I., Gakamsky A., Giojalas L.C., Breitbart H.,Eisenbach M. (2003). Thermotaxis of mammalian sperm cells: a potential navigation mechanism in the female genital tract. Nat Med 2003;9:149 – 50.

Bahat, A., Eisenbach M. & Tur-Kaspa, I. (2005). Periovulatory increase in temperature difference within the rabbit oviduct. Hum. Reprod. 20, 2118–2121

Baldi E., Casano R., Falsetti C., Krausz C., Maggi M. and Forti G.(1991). Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J Androl 12,323–330.

Baldi E.R. Cassano, C. Falsetti C.s. Krausz M. Maggi & G. Fortu: (1991). Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J Androl 12, 323-330.

Baldi E.M. Luconi L. Bonaccorsi & G. Forti: (1998). Nongenomic effects of progesterone on spermatozoa: mechanisms of signal transduction and clinical implications. Front Biosci 3, d1051-1059

Ballesteros L.M., Delgado N.M., Rosado A., Correa C. and Hernandez-Perez O. (1988). Binding of chemotactic peptide to the outer surface and to whole human spermatozoa with different affinity states Gamete Research 20 233–239

Baltes P, Sánchez R., Peña P., Villegas J, Turley H. (1998). Evidence for the synthesis and secretion of a CBG-like serpin by human cumulus oophorus and fallopian tubes. Andrologia 30: 249–253.

Bartram S. & Boland W. (2003). The Arduous Way to the Egg: Follow the Nose, Angewandte Chemie. Int. Ed. 42, 4729-4731.

Barratt C.L.R. and Cooke I.D. (1991). Sperm transport in the human female reproductive tract – a dynamic interaction International Journal of Andrology 14 394–411

Battalia, D. E. & Yanagimachi, R. (1993). Enhanced and co-ordinated movement of the hamster Bedford, J. M. & Kim, H. H. Cumulus oophorus as a sperm sequestering device, in vivo. J. Exp. Zool. 265, 321–328.

Baumann A. & Frings S. (1994). Matthias Godde, Reinhard Seifert and U.Benjamin KauppPrimary structure and functional expression of a Drosophila cyclic nucleotide-gated channel present in eyes and antennae. The EMBO Journal vol.13 no.21 pp.5040-5050.

Baxendale, R.W., Fraser, L.R., (2003a). Immunolocalization of multiple Galpha subunits in mammalian spermatozoa and additional evidence for G alphas. Mol. Reprod. Dev. 65, 104–113.

Baxendale, R.W., Fraser, L.R., (2003b). Evidence for multiple distinctly localized adenylyl cyclase isoforms in mammalian spermatozoa. Mol. Reprod. Dev. 66, 181–189.

Beavo J.A. (1995). Cyclic nucleotide phosphodiesterase: functional implications of multiple isoforms. Am J Physiol; 75:725–748.

Bedford, J. M. in Germ Cells and Fertilization, (1982). Vol. 1.eds Austin, C. R. & Short, R. V.) 128–163 Cambridge University Press, Cambridge, England.

Bedford J.M. and Kim H.H. (1993). Cumulus oophorus as a sperm sequestering device, in vivo. Journal of Experimental Zoology 265 321–328

Bedu-Addo K., (2005). Calcium signalling in human spermatozoa. PhD Thesis, Univeristy of Birmingham, School of Biocience.

Bedu-Addo K., Lefievre L., Moseley F.L., Barratt C.L. & Publicover S.J. (2005). Bicarbonate and bovine serum albumin reversibly 'switch' capacitation-induced events in human spermatozoa. Molecular Human Reproduction 11 683-691

Bedu-Addo K.; Barratt C.L.R; Kirkman-Brown J.C.; Publicover S.J. (2007). Patterns of [Ca2+](i) mobilization and cell response in human spermatozoa exposed to progesterone. Developmental biology;302(1):324-32.

Beltrán C.; Vacquier V.D; Moy G.; Chen Y.; Buck J.; Levin L. R; Darszon A. (2007). Particulate and soluble adenylyl cyclases participate in the sperm acrosome reaction.Biochemical and biophysical research communications.358(4):1128-35.

Benoff S. (1998). Voltage dependent calcium channels in mammalian spermatozoa. Front Biosci. 3:D1220-1240

Berrios J., Osses N., Opazo C., Arenas G., Mercado L., Benos D.J., Reyes J.G. (1998). Intracellular Ca2+ homeostasis in rat round spermatids. Biol Cell. 90,391-398.

Bern R.M. & Levy R.M.(2000, Third edition) Principales of physiology. Mosby Inc.USA.

Berridge M.J., Lipp P, Bootman MD. (2000). The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol. 1, 11-21.

Berridge M.J., Bootman M. D. And Roderick L.H, (2003). Calcium signalling: Dynamics, homeostasis and remodelling. Nature Review. Vol.4.

Billington R.A; Harper C.; Bellomo E.A; Publicover S.; Barratt C.L.R; Genazzani A.A. (2006). Characterization of cyclic adenine dinucleotide phosphate ribose levels in human spermatozoa. Fertility and sterility;86(4):891-8.

Blackmore P.F., Beebe S.J., Danforth D.R., Alexander N. (1990). Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. J Biol Chem. 265,1376-1380

Blackmore, P.F.: (1992).Thapsigargin elevates and potentiates the ability of progesterone to increase intracellular free calcium in human sperm: possible role of prinuclear calcium. Cell Calcium 14, 53-60

Blackmore P.F. (1999). Extragenomic actions of progesterone in human sperm and progesterone metabolites in human platelets. Steroids. 64,149-156.

Blackmore P.F, Eisoldt S. (1999). The neoglycoprotein 1 mannose-bovine serum albumin, but not progesterone, activates T-type calcium channels in human spermatozoa. Mol Hum Reprod. 5,498-506.

Blaustein M.P. and Lederer W.J. (1999). Sodium/calcium exchange: its physiological implications.Physiol Rev 79(3), 763-854.

Branscomb A., Seger J. & White R.L. (2000). Evolution of odorant receptors expressed in mammalian testes. Genetics 156, 785–797.

Braun R.E., Behringer R.R., Peschon J.J., Brinster R.L., Palmiter R.D., (1989).Genetically haploid spermatids are phenotypically diploid. Nature.337(6205):373-6.

Brenner B.M., Ballerman B.J, Gunning M.E. and Zeidel M.L. (1990). Diverse biological actions of atrial natriuretic peptide Physiological Reviews 70 665–699

Brini M. (2004). Ryanodine receptor defects in muscle genetic diseases. Biochem Biophys Res Commun 322,1245-1255

Böhmer M.,Van Q.,Weyand I., Hagen V., Beyermann M., Matsumoto M., Hoshi M., Hildebrand E. & Kaupp U.B. (2005). Ca2+ Spikes in the Flagellum Control Chemotactic Behaviour of Sperm, The EMB Journal, Vol. 24, Nº 15.

Bozkurt H.H. and Woolley D.M. (1993). Morphology of nexin links in relation to interdoublet sliding in the sperm flagellum. Cell Motility Cytoskel., 24, 109–118.

Brokaw C.J. (1958). Chemotaxis of bracken spermatozoids. The role of bimalate ions Journal of Experimental Zoology 35 192–196

Brokaw C.J., Josslin R., Bobrow L. (1974). Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa.Biochem Biophys Res Commun 58: 795–800

Brokaw C.J. (1980). Elastase digestion of demembranated sperm flagella. Science (NY), 207, 1365–1367.

Brokaw C.J. (1996). Microtubule sliding, bend initiation, and bend propagation parameters of Ciona sperm flagella altered by viscous load. Cell Motil. Cytoskeleton, 33, 6–21.

Bronson R. and Hamada Y. (1977). Gamete interactions in vitro. Fertility and Sterility 28 570–576

Brown G.R., Benyon S.L., Kirk C.J., Wictome M., East J.M., Lee A.G., Michelangeli F. (1994). Characterisation of a novel Ca2+ pump inhibitor (bis-phenol) and its effects on intracellular Ca2+ mobilization. Biochim Biophys Acta.1195,252-258

Brucker C., Lipford G.B. (1995).The human sperm acrosome reaction: physiological and regulatory mechanisms, An update. In Edwards RG (ed): “Human Reproduction Update.” Oxford: Oxford University Press 51-62

Buck L.B. and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65, 175-187

Bultynck G., Sienaert I., Parys J.B., Callewaert G., De Smedt H., Boens N., Dehaen W.,Missiaen L. Related Articles, (2003).Links Pharmacology of inositol trisphosphate receptors. Pflugers Arch. Mar;445(6):629-42.

Burkman L.J. (1990). Hyperactivated motility of human spermatozoa during in vitro capacitation and implications for fertility. In Controls of Sperm Motility: Biological and Clinical Aspects pp 303–329 Ed. C Gagnon. CRC Press, Boca Raton

Calogero A.E., Burrello N., Palermo I., Grasso U., D'Agata R. (2000). Effects of progesterone on sperm function: mechanisms of action. Hum Reprod.15 Suppl 1:128–45.

Campell N.A. & Reece J.B. (2002, sixth edition) Biology. World Student Series.

Carafoli E., Brini M. R.(2000).Calcium pumps: structural basis for and mechanism of calcium transmembrane transport. Curr Opin Chem Biol.4,152-161

Carlson A.E., Westenbroek R.E., Quill T., Ren D., Clapham D.E., Hille B., Garbers D.L. and Babcock D.F. (2003). CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm. Proc Natl Acad Sci USA 100(25), 14864-14868.

Carrera A., Moos J., Ning X., Gerton G., Tesarik J., Kopf G., Moss S. (1996). Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: identification of a kinase anchor proteins as major substrates for tyrosine phosphorylation. Dev Biol; 180:284-296

Castellano L.E., Trevino C.L., Rodriguez D., Serrano C.J., Pacheco J., Tsutsumi V., Felix R. and Darszon A. (2003). Transient receptor potential (TRPC) channels in human sperm: expression, cellular localization and involvement in the regulation of flagellar motility. FEBS Lett 541, 69-74.

Catterall W.A. (2000). Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 16, 521-555.

Catterall W.A., Streissnig J, Snutch T and Perez-Reyes E (2003). International Union of Pharmacology. XL. Compendium of voltage-gated ion channels: Calcium channels. Pharmacol Rev 55, 579-581.

Chang M.C. (1951).Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168:997-998

Chang M.C., Austin CR, Bedford JM, Brackett BG, Hunter RHF, Yanagimachi R (1977): Capacitation of spermatozoa and fertilization in mammals. In Greep RO, Koblinsky MA (eds): "Frontiers in reproduction and fertility control." Cambridge, MIT Press 434-451

Chaudhry P.S., Casillas ER.(1988).Calmodulin-stimulated cyclic nucleotide phosphodiesterases in plasma membranes of bovine epididymal spermatozoa. Arch Biochem Biophys; 262:439–444.

Chen Y., Cann M.J., Litvin T.N., Iourgenko V., Sinclair M.L., Levin L.R. and Buck J. (2000). Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science 289,625–628.

Chiarella P., Puglisi R., Sorrentino V., Boitani C., Stefanini M. (2004) Ryanodine receptors are expressed and functionally active in mouse spermatogenic cells and their inhibition interferes with spermatogonial differentiation. J Cell Sci 117,4127-4134.

Cohen-Dayag A., Ralt D., Tur-Kaspa I., Manor M., Makler A., Dor J., Mashiach S. and Eisenbach M. (1994). Sequential acquisition of chemotactic responsiveness by human spermatozoa.Biol. Reprod. 50, 786–790

Cohen-Dayag A., Tur-Kaspa I., Dor J., Mashiach S. and Eisenbach M. (1995). Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors Proceedings of National Academy of Sciences USA 92 11 039–11 043

Coll J.C. and Miller R.L. (1992). The nature of sperm chemo-attractants in coral and starfish. In Comparative Spermatology: 20 Years After pp 129–134 Ed. B Baccetti. Raven Press, New York

Conner S.J.; Lefièvre L., Hughes D.C., Barratt C.L.R. (2005). Cracking the egg: increased complexity in the zona pellucida. Human reproduction (Oxford, England);20(5):1148-52.

Conner SJ, Lefievre, L., Kirkman-Brown, J, Michelangeli, F, Jimenez-Gonzalez, C, Machado-Oliveira, GSM, Pixton, KL, Brewis, IA, Barratt, CLR, Publicover SJ (2007) Understanding the physiology of pre-fertilsation events in human spermatozoa – a necessary prerequisite to developing rational therapy. Soc Reprod Fertil Suppl. 63:237-55.

Cross N.L. (1998). Role of cholesterol in sperm capacitation. Biol Reprod 59,7–11.

Cook S.P., Babcock D.F. (1993). Selective modulation by cGMP of the K+ channel activated by speract. J Biol Chem 268: 22402–22407

Cook S.P., Babcock D.F. (1993).Activation of Ca2+ permeability by cAMP is coordinated through the pHi increase induced by speract. J Biol Chem 268: 22408 -22413

Cook S.P., Brokaw C.J., Muller C.H. and Babcock D.F. (1994). Sperm chemotaxis:egg peptides control cytosolic calcium to regulate flagellar responses. Developmental Biology 165 10–19

Cosson, M. P. (1990). In. Controls of Sperm Motility: Biological and Clinical Aspects (ed. Gagnon, C.) 103–135 (CRCPress, Boca Raton, Florida).

Costello S.; Michelangeli F.; Nash K.; Lefievre L.; Morris J.; Machado-Oliveira G.; Barratt C.; Kirkman-Brown J.; Publicover S., (2009). Ca2+-stores in sperm: their identities and functions.Reproduction (Cambridge, England) 2009;138(3):425-37.

Cross, N.L. (1996).Effect of cholesterol and other sterols on human sperm acrosomal responsiveness. Mol Reprod Dev 45, 212-217.

Cross N.L, Razy-Faulkner P. (1997). Control of human sperm intracellular pH by cholesterol and its relationship to the response of the acrosome to progesterone. Biol Reprod 56: 1169-1174

Curry, A.M. and Rosenbaum, J.L. (1993) Flagellar radial spoke: a model molecular genetic system for studying organelle assembly. Cell Motility Cytoskel., 24, 224–232.

Curry, M.R. and Watson, P.F. (1995). Sperm structure and function. In Grudzinskas, J.G. and Yovich, J.L. (eds), Gametes–The Spermatozoon. Cambridge University Press, Cambridge, UK, pp. 45–69.

da Fonseca P.C., Morris S.A., Nerou E.P., Taylor C.W., Morris E.P. (2003). Domain organization of the type 1 inositol 1,4,5-trisphosphate receptor as revealed by single particle analysis. Proc Natl Acad Sci U S A 100,3936-3941.

De Jonge C.J., Han H.L., Lawrie H., Mack S.R., Zaneveld L.J. (1991). Modulation of the human sperm acrosome reaction by effectors of the adenylate cyclase/cyclic AMP second-messenger pathway. J Exp Zool;258:113–125.

de Lamirande E. and Gagnon C. (1995). Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Hum Reprod 10(Suppl. 1),15–21.

de Lamirande E., Leclerc P. and Gagnon C. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 3,175–194.

de Lamirande E. and Gagnon C. (2002). The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. Mol Hum Reprod 8,124–135.

Darszon A., Pedro Labarca, Takuya Nishigak & Felipe Espinosa, (1999). Ion Channels in Sperm Physiology. Physiological Review. 79, 481–510.

Darszon, A., Beltran, C., Felix, R., Nishigaki, T. & Trevino, C. L. (2001).Ion transport in sperm signaling. Develop. Biol. 240, 1–14

Darszon, A., Wood, C.D., Beltran, C., Sanchez, D., Rodriguez, E., Gorelik,J., Korchev, Y.E., Nishigaki, T., (2004). Measuring ion fluxes in sperm. Methods Cell Biol. 74, 545–576.

Darszon,A. Nishigaki,T. Wood,C. Trevino,C.L. Felix,R. Beltran,C. (2005). Calcium channels and Ca2+ fluctuations in sperm physiology.Int. Rev. Cytol.243, 79-172 .

Darszon A., Juan J. Acevedo, Blanca E. Galindo, Enrique O Hernández-González Takuya Nishigaki, Claudia L Treviño, Chris Wood and Carmen Beltrán (2006). Sperm channel diversity and functional multiplicity.Reproduction. 131 977–988

David, A., Vilensky, A. & Nathan, H. (1972). Temperature changes in the different parts of the rabbit's oviduct. Int. J. Gynaec. Obstet. 10, 52–56

David L. Garbers (2001).Swimming with sperm. Nature. Vol 413.

Defer, N., Marinx, O., Poyard, M., Lienard, M.O., Jegou, B., Hanoune, J., (1998). The olfactory adenylyl cyclase type 3 is expressed in male germ cells. FEBS Lett. 424, 216–220.

DeMott R.P., Suarez S.S. (1993). Hyperactivated sperm progression in the mouse oviduct. Biol Reprod 46:779-785

Dermot M. F. COOPER.(2003).Regulation and organization of adenylyl cyclases and cAMP. 375, 517–529 517 Biochem. J.

Dickman Z. (1963). Chemotaxis of rabbit spermatozoa. Journal of Experimental Biology 1–5

Dragileva E., Rubinstein S. and Breitbart H. (1999). Intracellular Ca2+-Mg2+-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. Biol Reprod 61(5), 1226-1234.

Dym M., Fawcett D.W., (1971). Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod.4(2):195-215.

Dym, M. (1977). The male reproductive system. In L. Weiss and R. O. Greep (eds.), Histology, 4th Ed. McGraw-Hill, New York, pp. 979–1038.

Dym M., (1994). Spermatogonial stem cells of the testis. Proc Natl Acad Sci U S A.22;91(24):11287-9.

Eisenbach M. and Ralt D. (1992). Precontact mammalian sperm–egg communication and role in fertilization American Journal of Physiology 262 (CellPhysiology 31) C1095–C1101

Eisenbach M., & Tur-Kaspa, I. (1994). Human sperm chemotaxis is not enigmatic anymore. Fertil. Steril., 62, 233-235.

Eisenbach M. (1995). Sperm changes enabling fertilization in mammals Current Opinion in Endocrinology and Diabetes 2 468–475

Eisenbach, M. & Tur-Kaspa, I. (1999). Do human eggs attract spermatozoa? BioEssays 21, 203–210

Eisenbach M. (1999). Sperm Chemotaxis. Reviews of Reproduction, Journals of Reproduction & Fertility, 4, 56-66.

Eisenbach M., (1999). Mammalian Sperm Chemotaxis & Its Association with Capacitation, Review Article, Developmental Genetics 25:87-94

Eisenbach M., (2004). Towards Understanding the Molecular Mechanism of Sperm Chemotaxis, J. Gen. Physiol. Vol. 124, 105-108.

Eisenbach M. & Giojalas L.C., (2006). Sperm guidance in mammals-an unpaved road to the egg. Review. Vol. 7. Nature.

Elisabetta B., Michaela L., Lorella B., Csilla K. and Gianni F., (1996). Human sperm activation during capacitaron and acrosome reaction: Role of calcium, protein phosphorylation and lipid remodelling pathways. Frontiers in Bioscience 1, d189-205.

Ertel E.A., Campbell K.P., Harpold M.M., Hofmann F., Mori Y., Perez-Reyes E., Schwartz A., Snutch T.P., Tanabe T., Birnbaumer L. (2000). Nomenclature of voltage-gated calcium channels. Neuron 25(3), 533-535.

Evans JP, Florman HM. (2002). The state of the union: the cell biology of fertilization. 30 Nat Cell Biol Suppl s57-s63.

Fabro G., Rovasio R.A., Civalero S., Frenkel A., Caplan R., Eisenbach M. (1999).Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. Biol Reprod;67:1565–71.

Fabro G., Rovasio R.A., Civalero S., Frenkel A., Caplan R. (2002). Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. Biology of Reproduction 67: 1565–1571.

Fawcett D.W. (1965). The anatomy of the mammalian spermatozoon with particular reference to the guinea pig. Z Zellforsch, 67, 279–296.

Fawcett D.W. (1975). The mammalian spermatozoon. Dev. Biol., 44, 394–436.

Fill M., Copello J.A. (2002). Ryanodine receptor 1 tor calcium release channels. 2 Physiol Rev 82,893-922.

Firestein, S., (2001). How the olfactory system makes sense of scents. Nature 413, 211–218.

Fisch J.D., Behr B., Conti M. (1998). Enhancement of motility and acrosome reaction in human spermatozoa: differential activation by type-specific phosphodiesterase inhibitors. Hum Reprod; 13:1248–1254.

Flesch F.M., Brouwers J.F.H.M., Nievelstein P.F.E.M., Verkleij A.J., van Golde L.M.G., Colenbrander B., Gadella B.M. (2001). Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. J Cell Sci 114 (in press)

Flechon, J.E. & Hunter, R. H.F. (1981). Distribution of spermatozoa in the utero-tubal junction and isthmus of pigs, and their relationship with the luminal epithelium after mating: a scanning electron microscope study. Tissue Cell 13, 127–139

Florman, H.M., & N. L. First. (1988).The regulation of acrosomal exocytosis, II. The zona pellucida-induced acrosome reaction of bovine spermatozoa is controlled by extrinsic positive regulatory elements. Dev. Biol. 128: 464-473.

Florman, H. M.: (1994).Sequential focal and global elevations of sperm intracellular Ca^{2+} are initiated by zona pellucida during acrosomal exocytosis. Dev Biol 165, 152-164.

Fomina A.F. and Nowicky M.C. (1999). A current activated on depletion of intracellular Ca2+ stores can regulate exocytosis in adrenal chromaffin cells. J Neurosci 19, 3711-3722.

Friend, D.S. and Fawcett, D.W. (1974). Membrane differentiations in freeze-fractured mammalian sperm. J. Cell Biol., 63, 641–664.

Fukami K.,Yoshida M., Inoue T., Kurokawa M., Fissore R.A., Yoshida N.,Mikoshiba K. and Takenawa T.(2003). Phospholipase C§4 is required for Ca2+ mobilization essential for acrosome reaction in sperm.J cell Biol. 161, 79-88.

Fukuda, N., Yomogida, K., Okabe, M. & Touhara, K. (2004).Functional characterization of a mouse testicular olfactory receptor and its role in chemosensing and in regulation of sperm motility. J. Cell Sci. 117, 5835–5845.

Futnahashi H., A. Asano, T. Fujiwara, T, T. Nagai, K. Niwa & L.R. Fraser: (2000). Both Fertilization promoting peptide and adenosinestimulate capacitaion but inibit spontaneous acrosome loss in ejaculated boar spermatozoa in vitro. Mol Reprod Dev 55, 117-24

Fraser LR.(1981). Dibutyryl cyclic AMP decreases capacitation time in vitro in mouse spermatozoa. Journals of Reproduction & Fertility, 423

Fraser L.R. & C. A. McDermott. (1992). Ca^{2+}-related changes in the mouse sperm capacitation state: a posible role for Ca^{2+}-ATPase. J Reprod Fertil 96, 363-377

Fraser L.R.: Mechanisms regulating capacitation and the acrosome reaction. In: (1995). Human sperm acrosome reaction.Eds: Fenichel P., Parinaud J. Colloque/INSERIM John Libbey Eurotext Ltd, Montrouge, France, Vol. 236, 17-33.

Fraser, L.R.: (1998). Role of fertilization promoting peptide (FPP) in modulating mammalian sperm function, Front Biosci 3, D 1187-91.

Fraser L.R. (1998). Sperm capacitation and the acrosome reaction. Human Reproduction (supl.) 13:9-19

Fraser, L.R. (1999). New insights into possible causes of male infertility. Human. Repro 14, 38-46.

Fraser L.R., Sussan A., Rhona W.B., Samra M. and Olufunmilayo O.O., (2005). First messenger regulation of mammalian sperm function via adenylyl cyclase/cAMP. Journal of reproductive & development Vol. 51, Nº 1.

Francis S.H., Turko I.V., Corbin J.D. (2001). Cyclic nucleotide phosphodiesterases: relating structure and function. Prog Nucleic Acid Res Mol Biol; 65:1–52.

Fukuda,N., Yomogida,K., Okabe,M., and Touhara,K. (2004). Functional characterization of a mouse testicular olfactory receptor and its role in chemosensing and in regulation of sperm motility. J. Cell Sci. 117, 5835-5845.

Gagnon, C. (1995). Regulation of sperm motility at the axonemal level. Reprod. Fertil. Dev., 7, 847–855.

Gagnon C. & de Lamirande E. (2006). Urology Research lab., Royal Hospital and faculty of Medicine, McGill Uni. Montréal, Canada.

Gakamsky A.; Pinchasov M.; Eisenbach M.(2008). The sperm chemoattractant secreted from human cumulus cells is progesterone. Human reproduction (Oxford, England);23(10):2339-45.

Gakamsky Anna; Armon Leah; Eisenbach Michael (2009). Behavioral response of human spermatozoa to a concentration jump of chemoattractants or intracellular cyclic nucleotides. Human reproduction (Oxford, England);24(5):1152-63.

Garcia M.A., Meizel S. (1999). Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. Biol Reprod. 60, 102-109

Garcia R.L. and Schilling W.P. (1997). Differential expression of mammalian TRP homologues across tissue and cell lines. Biochem biophys Res Commun 239, 279-283.

Gautier-Courteille, C., Salanova, M. & Conti, M. (1998). The olfactory adenylyl cyclase III is expressed in rat germ cells during spermiogenesis. Endocrinology 139, 2588–2599

Gibbons, I.R. (1965). Chemical dissection of cilia. Arch. Biol. (Liege), 76, 317–352.

Gibbons, I.R. (1974). Mechanisms of flagellar motility. In Afzelius, B.A. (ed.), The Functional Anatomy of the Spermatozoon. Pergamon Press, Oxford and New York, pp. 127–140.

Gibbons, I.R. and Grimstone, A.V. (1960). On flagellar structure in certain flagellates. J. Biophys. Biochem. Cytol., 7, 697–716.

Gibbons, I.R. and Gibbons, B.H. (1980). Transient flagellar waveforms during intermittent swimming in sea urchin sperm. I. Wave parameters. J. Muscle Res. Cell Motility, 1, 31–59.

Giojalas L.C. and Rovasio R.A. (1998). Mouse spermatozoa modify their dynamic parameters and chemotactic response to factors from the oocyte microenvironment International Journal of Andrology 21 201–206

Giojalas L.C., Rovasio R.A., Fabro G., Gakamsky A., Eisenbach M. (2004). Timing of sperm capacitation appears to be programmed according to egg availability in the female genital tract. Fertility and Sterility 82: 247–249.

Glusman, G., Yanai, I., Rubin, I., Lancet, D., (2001). The complete human olfactory subgenome. Genome Res. 11, 685–702.

Gnessi L., Ruff M.R., Fraioli F. and Pert C.B. (1985). Demonstration of receptormediated chemotaxis by human spermatozoa. A novel quantitative bioassay Experimental Cell Research 161 219–230

Gnessi L., Fabbri A., Silvestroni L., Moretti C., Fraioli F., Pert C.B. and Isidori A. (1986). Evidence for the presence of specific receptors for N-Formyl chemotactic peptides on human spermatozoa Journal of Clinical Endocrinology and Metabolism 63 841–846

Griffin J.E. & Ojeda.S.R. (2000, Fourth edition) Textbook of endocrine physiology. Oxford University press. Inc.

Guidobaldi H.A., Teves M.E., Uñaates D.R., Anastasía A., Giojalas L.C. (2008). Progesterone from the Cumulus Cells Is the Sperm Chemoattractant Secreted by the Rabbit Oocyte Cumulus Complex. PLoS ONE 3(8): e3040.

Gonzalez-Martinez M.T., Galindo B.E., de De La Torre L., Zapata O., Rodriguez E., Florman H.M., Darszon A. (2001). A sustained increase in intracellular Ca(2+) is required for the acrosome reaction in sea urchin sperm. Dev Biol.236,220-229

Gomendio, M., Harcourt, A.H. & Roldán, E. R. S. In. (1998). Sperm Competition and Sexual Selection (eds Birkhead, T. R. & Moller, A. P.) 667–751 (Academic Press, London, 1998).

Goodwin L.O., Leeds N.B., Hurley I., Mandel F.S., Pergolizzi R.G. and Benoff S. (1997). Isolation and characterization of the primary structure of testis-specific L-type calcium channel: implications for contraception. Mol Hum Reprod 3(3), 255-268.

Goodwin L.O., Leeds N.B., Hurley I., Cooper G.W., Pergolizzi R.G. and Benoff S. (1998). Alternative splicing of exons in the alpha1 subunit of the rat testis L-type voltage27 dependent calcium channel generates germ line-specific dihydropyridine binding sites. Mol Hum Reprod 4(3), 215-226.

Goodwin L.O., Karabinus D.S., Pergolizzi R.G. and Benoff S. (2000). L-type voltage31 dependent calcium channel α1C subunit mRNA is present in ejaculated human spermatozoa. Mol Hum Reprod 6(2), pp.127-136.

Gould, J. E., Overstreet, J. W. & Hanson, F. W. (1984).Assessment of human sperm function after recovery from the female reproductive tract. Biol. Reprod. 31, 888–894

Hagiwara S., Kawa K. (1984). Calcium and potassium currents in spermatogenic cells dissociated from rat seminiferous tubules. J Physiol. (Lond) 356, 135-149.

Hansen C, Srikandakumar A. and Downey B.R. (1991). Presence of follicular fluid in the porcine oviduct and its contribution to the acrosome reaction Molecular Reproduction and Development 30 148–153

Harper M.J.K. (1970). Factors influencing sperm penetration of rabbit eggs in vivo. Journal of Experimental Zoology 173: 47–62.

Harper M.J.K. (1973). Stimulation of sperm movement from the isthmus to the site of fertilization in the rabbit oviduct Biology of Reproduction 8 369–377

Harper M.J.K. (1982).Sperm and egg transport. In: Austin CR, Short RV, eds. Germ cells and fertilization. second ed. Cambridge: Cambridge University Press. pp 102–127.

Harper, M. J. K. (1994). The *Physiology of Reproduction* (*E. Knobil* and *JD Neill*, *Eds.*), 2nd *ed*., pp. *123-187. Raven Press*, *New*

Harper, C. V., Barratt, C. L. & Publicover, S. J. (2004). Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of [Ca2+]i oscillations and cyclical transitions in flagellar beating. J. Biol. Chem. 279,46315–46325

Harper C., Wootton L., Michelangeli F., Lefievre L., Barratt C., Publicover S. (2005). Secretory pathway Ca2+-ATPase (SPCA1) Ca2+ pumps, not SERCAs, regulate complex [Ca2+]i signals in human spermatozoa. J Cell Sci.118,1673-1185

Harper C.V.; Barratt C.L.R.; Publicover S.J.; Kirkman-Brown J.C. (2006). Kinetics of the progesterone-induced acrosome reaction and its relation to intracellular calcium responses in individual human spermatozoa. Biology of reproduction;75(6):933-9.Fertility and sterility;86(4):891-8.

Harper C.V.; Cummerson J.A.; White M.R.H.; Publicover S.J.; Johnson P.M. (2008). Dynamic resolution of acrosomal exocytosis in human sperm. Journal of cell science;121(Pt 13):2130-5.

Harteneck C., Plant T.D., and Schultz G. (2000). From worm to man: three subfamilies of TRP. channels. Trends Neurosci 23(4), 159-166.

Hart N.H. (1990). Fertilization in teleost fish: mechanism of sperm–egg interactions. International Review of Cytology 121 1–66

Herrick S.B., Schweissinger D.L., Soo-Woo K., Bayan K.R., Mann S. and Cardullo R.A. (2005). The acrosomal vesicle of mouse sperm is a calcium store. J Cell Physiology 202, 663-671.

Hirohashi N. and Vacquier D. (2003). Store-operated calcium channels trigger exocytosis of the sea urchin sperm acrosomal vesicle. Biochem Biophys Res Commun 32 304, 285-292.

Ho H.C. & Suarez S.S. (2001). An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca2+ store is involved in sperm hyperactivated motility. Biology of Reproduction 65 1606-1615

Ho H.C. & Suarez S.S. (2003). Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. Biology of Reproduction 68 1590-1596

Hornung. D., Ryan, L.P., Chao. V.A., Vigne, J.L. Schriock. E.D and Taylor, R.N. (1997). Immuno-localization and regulation of the chemokine RANTES in human endometrial and endometriosis tissue and cells. J.Clin. Endocrinol. Metab., 85, 1621-1628.

Hornong. D., Bentzien, F. Wallwiener, D., Kiesel. K. and Taylor, R.N. (2001). Chemokine bioactivity of RANTES in endometriotic and normal endometrial stromal cells and peritoneal fluid. Mol. Hum. Reprod., 7. 163-168.

Hunter, R. H. F. & Nichol, R. (1986).A preovulatory temperature gradient between the isthmus and the ampulla of pig oviducts during the phase of sperm storage. J. Reprod. Fert. 77, 599–606

Hunter, R. H. F. (1987).Human fertilization in vivo, with special reference to progression, storage and release of competent spermatozoa. Hum. Reprod. 2, 329–332

Hunter R.H.F (1993). Sperm:egg ratios and putative molecular signals to modulate gamete interactions in polytocous mammals Molecular Reproduction and Development 35 324–327

Iqbal M., Shivaji S., Vijayasarathy S. and Balaram P. (1980). Synthetic peptides as chemoattractants for bull spermatozoa: structure activity correlations Biochemical and Biophysical Research Communications 96 235–242

Ishijima, S. & Mohri, H. (1990). In. Controls of Sperm Motility: Biological and Clinical Aspects (ed. Gagnon, C.) 29–42 (CRC Press, Boca Raton, Florida,).

Isobe T., Minoura H., Tanaka K., Shibahara T., Hayashi N., Toyoda N. (2002). The effect of RANTES on human sperm chemotaxis. Hum Reprod;17:1441– 6.

Ito M., Smith T.T., and Yanagimachi R. (1991). Effect of ovulation on sperm transport in the hamster oviduct Journal of Reproduction and Fertility 93 157–163

Jaiswal, B.S., Eisenbach, M., Tur-Kaspa, I., (1999a). Detection of partial and complete acrosome reaction in human spermatozoa: which inducers and probes to use? Mol. Hum. Reprod. 5, 214–219.

Jaiswal, B.S., Tur-Kaspa, I., Dor, J., Mashiach, S., Eisenbach, M., (1999b). Human sperm chemotaxis: is progesterone a chemoattractant? Biol. Reprod. 60, 1314–1319.

Jaiswal, B.S. and Eisenbach, M. (2002). Capacitation. In: Fertilization (Hardy, D.M., ed.) pp. 57-117. Academic Press, San Diego.

Janica P.E. & Harvey M.F., (2004). The state of the union: The cell biology of fertilization. Review. Nature Cell Biology & Nature Medicine.

Jeon B.G., Moon J.S., Kim K.C., Lee H.J., Choe S.Y., Rho G.J. (2001). Follicularfluid enhances sperm attraction and its motility in human. J AssistReprod Gen;18:407–12.

Jimenez-Gonzalez, Michelangeli F., Harper C.V., Barratt C.L.R., Publicover S.J., (2004). Calcium Signalling in Human Spermatozoa: a Specialised "Toolkit" of Channels, Transporters and Stores, University of Birmingham.

Jimenez-Gonzalez C.; Michelangeli F.; Harper C.V.; Barratt C.L.R.; Publicover S.J. (2006). Calcium signalling in human spermatozoa: a specialized 'toolkit' of channels, transporters and stores. Human reproduction update;12(3):253-67.

Jiménez-González M. C.; Gu Y.; Kirkman-Brown J.; Barratt C.L.R.; Publicover S. (2007). Patch-clamp 'mapping' of ion channel activity in human sperm reveals regionalisation and co-localisation into mixed clusters. Journal of cellular physiology;213(3):801-8.

Johson M.H., Everitt B.J. (2000; fith edition). Essential Reproduction. Backwell Science Ltd. London.

Jungnickel M.K., Marrero H., Birnbaumer L., Lemos J.R. and Florman H.M. (2001). Trp2 regulates entry of Ca2+ into mouse sperm triggered by egg ZP3. Nature Cell Biol 3, 28 499-502.

Karstrom-Encrantz, L., Runesson, E. Bostrom, E.K. and Brannstrom M. (1998). Selective presence of the chemokine growth-regulated oncogene alpha (GRO alpha) in the human follicle and secretion from cultured granulosa-jutein cells at ovulation. Mol. Hum. Reprod., 4. 1077-1083.

Katz D.F., Yanagimachi R., Dresdner R.D. (1978). Movement characteristics and power output of guinea-pigand hamster spermatozoa in relation to activation. J Reprod Fertil 52:167-172

Kaupp B.U., & Reinhard Seifert. (2002).Cyclic Nucleotide-Gated Ion Channels. *Physiol Rev,* 82: 769–824, 2002; 10.1152

Kaupp B.U, Johannes Solzin, Eilo Hildebrand, Joel E. Brown, Annika Helbig, Volker Hagen, Michael Beyermann, Francesco Pampaloni & Ingo Weyand, (2003).The Signal Flow & Motor Response Controlling Chemotaxis of Sea Urchin Sperm, Nature Cell Biology, Vol. 5.

Kaupp, U.B., Hildebrand, E. and Weyand, I. (2006). Sperm chemotaxis in marine invertebrates - molecules and mechanism. J. Cell. Physiol. 208, 487-494.

Kaupp B., Kashikar U., Weyand I. (2008). Mechanisms of Sperm Chemotaxis. Annual Review of Physiology, March 2008, Vol. 70, Pages 93-117

Kazuo Inaba, (2003). Molecular architecture of the sperm flagella: Molecules for motility and signalling. Zoological Science. 20: 1043-1056.

Khorram. O., Taylor. R.N., Ryan. L.P, Schall. T.J. and Landers. D.V. (1993). Peritoneal fluid concentration of cytokine RANTES correlate with severity of endometriosis. Am. J. Obstet. Gynecol., 169. 1545-1549.

Khorasani A.M., A.P. Cheung & C.Y. Lee: (2000).Cholesterol inhibitory effects on human sperm-induced acrosome reaction. J Androl 21, 586-94

Kirkman-Brown J.C., Sutton KA., Florman H.M. (2003). How to attract sperm. Nat Cell Biol 5: 93–96

Kobori H., Miyazaki S., Kuwabara Y. (2000). Characterization of intracellular Ca2+ increase in response to progesterone and cyclic nucleotides in mouse spermatozoa. Biol Reprod.63,113-1.20

Koehler, J.K. (1983). Structural heterogeneity of the mammalian sperm flagellar membrane. J. Submicrosc. Cytol., 15, 247–253.

Kopf G.S., Ning X.P., Visconti P.E., Purdon M., Galantino-Homer H., Fornés M. (1999). Signalling mechanisms controlling mammalian sperm fertilization competence and activation. In Gagnon C (ed): "The male gamete:From basic science to clinical applications." Vienna IL (USA): Cache River Press, pp105-118

Kraev A., Quednau B.D., Leach S., Li X.F., Dong H., Winkfein R., Perizzolo M., Cai X., Yang R., Philipson K.D. (2001). Molecular cloning of a third member of the potassium-dependent sodium-calcium exchanger gene family, NCKX3. J Biol Chem 276(25), 23161-23172.

Krannich S. and Monika S. (2008). Cyclic Nucleotide-Activated Currents in Cultured Olfactory Receptor Neurons of the Hawkmoth *Manduca sexta*. Neurophysiol 100: 2866-2877

Krannich S. (2008). Electrophysiological and pharmacological characterization of ion channels involved in moth olfactory transduction cascades. Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften. Vom Fachbereich Biologie der Philipps-Universität Marburg.

Kuroda Y., Kaneko S., Yoshimura Y., Nozawa S., Mikoshiba K. (1999). Are there inositol 1,4,5-triphosphate (IP3) receptors in human sperm? Life Sci 65,135-143.

Lawson C., Dorval V., Goupil S. and Pierre Leclerc. (2007). Identification and localisation of SERCA 2 isoforms in mammalian sperm. Molecular Human Reproduction pp. 1–10

Leclerc, P. and Kopf, G.S. (1995) Mouse sperm adenylyl cyclase: general properties and regulation by the zona pellucida.Biol. Reprod. 52, 1227-1233

Leclerc P, de Lamirande E, Gagnon C. (1996). Cyclic adenosine 39, 59 monophosphate- dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. Biol Reprod; 55:684–692.

Leclerc, P. and Kopf, G.S. (1999) Evidence for the role of heterotrimetric guanine nucleotide-binding regulatory proteins in the regulation of the mouse sperm adenylyl cyclase by the egg's zona pellucida. J Androl 20: 126-134

Lee S-L., Kao C-C. and Wei Y-H. (1994). Antithrombin III enhances the motility and chemotaxis of boar sperm Comparative Biochemistry and Physiology 107A 277-282

Lefebvre R. and Suarez S.S. (1996). Effect of capacitation on bull sperm binding to homologous oviductal epithelium Biology of Reproduction 54 575–582

Lefièvre L., de Lamirande E. and Gagnon C. (2000). The cyclic GMP-specific phosphodiesterase inhibitor, sildenafil, stimulates human sperm motility and capacitation but not acrosome reaction. J Androl 21,929–937.

Lefièvre L., Jha K.N., de Lamirande E., Visconti P.E. and Gagnon C. (2002). Activation of protein kinase a during human sperm capacitation and acrosome reaction. J Androl 23,709–716.

Lefièvre L., Eve de Lamirande and Claude Gagnon, (2002). Presence of Cyclic Nucleotide Phosphodiesterases PDE1A, Existing as a Stable Complex with Calmodulin, and PDE3A in Human Spermatozoa. *Biology of Reproduction* , vol. 67 no. 2 423-430

Lefièvre L.; Conner S.J; Salpekar A.; Olufowobi O.; Ashton P.; Pavlovic B.; Lenton W.; Afnan M.; Brewis I.A.; Monk M.; Hughes D.C.; Barratt C.L.R. (2004). Four zona pellucida glycoproteins are expressed in the human. Human reproduction (Oxford, England);19(7):1580-6.

Lefièvre L.; Chen Yongjian; Conner S.J.; Scott Joanna L.; Publicover S.J.; Ford W. C.L.; Barratt C.L.R. (2007). Human spermatozoa contain multiple targets for protein S-nitrosylation: an alternative mechanism of the modulation of sperm function by nitric oxide? Proteomics;7(17):3066-84.

Lefievre L, Bedu-Addo K, Conner SJ, Machado-Oliveira GS, Chen Y, Kirkman-Brown JC, Afnan MA, Publicover SJ, Ford WC, Barratt CL. (2007) Counting sperm does not add up any more: time for a new equation? Reproduction. 133:675-84

Lefièvre L.; Machado-Oliveira G.; Ford C.; Kirkman-Brown J.; Barratt C.; Publicover S. (2009). Communication between female tract and sperm: Saying NO* when you mean yes. Communicative & integrative biology 2009;2(2):82-5.

Leslie B. Vosshall, (2004). Olfaction: Attracting Both Sperm & Dispatch the Nose, Current Biology, Vol. 14.

Lievano A., Santi C.M., Serrano C.J., Trevino C.L., Bellve A.R., Hernandez-Cruz A., Darszon A. (1996). T-type Ca2+ channels and alpha1E expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. FEBS Lett.; 388:150-154

Li H.S., Xu X.Z.S. and Montell C. (1999). Activation of a TRPC3-dependent cation current through the neurotrophin BDNF. Neuron 24(1), 261-273.

Linck, R.W. (1979). Advances in the ultrastructural analysis of the sperm flagellar axoneme. In Fawcett, D.W. and Bedford, J.M. (eds), The Spermatozoon. Urban and Schwarzenberg Inc. Baltimore, Munich, pp.99–115.

Linda Buck and Richard Axel, (1991). A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell, Volume 65, Issue 1, Pages 175-187*

Liman E.R., Corey D.P. and Dulac C. (1999). TRP2: a candidate transduction channel for mammalian pheromone sensory signalling. Proc Natl Acad Sci USA 96, 5791-5796.

Lobley A., Pierron V., Reynolds L., Allen L. and Michalovich D. (2003). Identification of human and mouse CatSper3 and CatSper4 genes: characterisation of a common interaction domain and evidence for expression in testis. Reprod Biol Endocrinol 1(1), 11 53.

Luck M.R., Griffiths S., Gregson K., Watson E., Nutley M. and Cooper A. (2001). Follicular fluid responds endothermically to aqueous dilution. Hum. Reprod. 16, 2508–2514

Luconi M., Bonaccorsi L., Maggi M., Pecchioli P., Krausz C., Forti G. (1998). Identification and characterization of functional nongenomic progesterone receptors on humans sperm membrane. J Clin Endocrilnol Metab;83:877– 85.

Luconi, M., M. Muratori, M. Maggi, P. Pecchioli, A. Peri, M. Mancini, E. Filimberti, G. Forti & E. Baldi: (2000).Uteroglobin and transglutaminase modúlate human sperm functions. J Androl 21, 676-688

Luconi M.., Porazzi I., Ferruzzi P., Marchiani S., Forti G. and Baldi E. (2005). Tyrosine phosphorylation of the A kinase anchoring protein 3 (AKAP3) and soluble adenylate cyclase are involved in the increase of human sperm motility by bicarbonate. Biol Reprod 72,22–32.

Machado-Oliveira Gisela; Lefièvre Linda; Ford Christopher; Herrero M. Belen; Barratt Christopher; Connolly Thomas J.; Nash Katherine; Morales-Garcia Aduen; Kirkman-Brown Jackson; Publicover Steve (2008). Mobilisation of Ca2+ stores and flagellar regulation in human sperm by S-nitrosylation: a role for NO synthesised in the female reproductive tract. Development (Cambridge, England) 135(22):3677-86.

Machelon, V., Nome, F. and Emilie.D. (2000). Regulated on activation normal T expressed and secreted chemokine is induced by tumor necrosis factor alpha in granulosa cells from human preovulatory follicle. J. Clin Endocrinol. Metab., 57, 1066-1074.

Madan G. Luthra. (1982). Trifluoperazine inhibition of calmodulin-sensitive Ca^{2+} ATPase and calmodulin insensitive (Na^{+} + K^{+})- and Mg^{2+}-ATPase activities of human and rat red blood cells. Biochimica et Biophysica Acta (BBA) – Biomembranes Volume 692, Issue 2

Maier I. and Müller D.G. (1986). Sexual pheromones in algae Biological Bulletin 170 145–175

Manor M. (1994). Identification and Purification of Female-Originated Chemotactic Factors .PhD Thesis, Weizmann Institute of Science, Rehovot

Martini F.H., Ober C.W., Garrison C.W., Wech K., Hutchings R.T., (2001; Fith edition). Fundamentalls of Anatomy and physiology. Publish. Prentice-Hall Inc.

Makler A., Reichler A., Stoller J. and Feigin P.D. (1992). A new model for investigating in real time the existence of chemotaxis in human spermatozoa Fertility and Sterility 57 1066 1074

Makler A., Stoller J., Reichler A., Blumenfeld Z. and Yoffe N. (1995). Inability of human sperm to change their orientation in response to external chemical stimuli Fertility and Sterility 63 1077–1082

Manor, M. (1994). Identification and Purification of Female-Originated Chemotactic Factors. Ph.D. Thesis, The Weizmann Institute of Science

Marquez B., Ignotz G. & Suarez S.S. (2007). Contributions of extracellular and intracellular Ca2+ to regulation of sperm motility: Release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm. Developmental Biology 303 214-221

Mashukova A. (2006). Investigation of olfactory receptor desensitization; Deparment of cell Physiology: Inter. Graduate school of neurosciences (IGSN) Ruhr Uni. Bochum, Germany

Mayora L.S., Tomes C.N. & Belmonte S.A.(2007). Acrosomal exocytosis, a special type of regulated secretion. IUBMB Life 59 286-292

Meizel, S. & Turner, K. O. (1991). Progesterone acts at the plasma membrane of human sperm. Mol. Cell. Endocrinol. 77, R1–R5

Meizel S. & Turner K.O.(1993). Initiation of the human sperm acrosome reaction by thapsigargin. The Journal of Experimental Zoology 267 350-355

Meizel, S., (2004). The sperm, a neuron with a tail: 'neuronal' receptors in mammalian sperm. Biol. Rev. Camb. Philos. Soc. 79, 713–732.

Mendoza C, Carreras A, Moos J and Tesarik J (1992). Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using Pisum sativum agglutinin. J Reprod Fertil 95,755–763.

Michelangeli F., Mezna M., Tovey S., Sayers L.G. (1995). Pharmacological modulators of the inositol 1,4,5-trisphosphate receptor. Neuropharmacology 34 1111-1122.

Michelangeli F., Ogunbayo O.A., Wootton L.L. (2005). A plethora of interacting organelle Ca2+ stores. Curr Opin Cell Biol. 17(2), pp.135-40.

Miller R.L. (1966). Chemotaxis during fertilization in the hydroid Campanularia. Journal of Experimental Zoology 162 23–44

Miller R.L. (1982). Synthetic peptides are not chemoattractants for bull sperm Gamete Research 5 395–401

Miller R.L. and King K.R. (1983). Sperm chemotaxis in Oikopleura dioica FOL, 1872 (Urochordata: Larvacea) Biological Bulletin 165 419–428

Miller R.L. (1985). Sperm chemo-orientation in the metazoa. In Biology of Fertilization, Vol. 2: Biology of the Sperm, Metz CB, Monroy A (eds) pp 275–337. New York: Academic Press

Miller, R.L. (1985). in The Biology of Fertilization, eds. Metz, C.B. and Monroy, A.(Academic, New York), Vol. 2, pp. 275-337

Miller R.D. and Vogt R. (1996). An N-terminal partial sequence of the 13 kDa Pycnopodia helianthoides sperm chemoattractant 'startrak' possesses spermattracting activity Journal of Experimental Biology 199 311–318

Miller R.L. (1997). Specificity of sperm chemotaxis among great barrier reef shallow-water holothurians and ophiuroids Journal of Experimental Zoology 279 189–200

Missiaen L., Raeymaekers L., Dode L., Vanoevelen J., Van Baelen K., Parys J.B., Callewaert G., De Smedt H., Segaert S., Wuytack F. (2004). SPCA1 pumps and Hailey Hailey disease. Biochem Biophys Res Commun. 322,1204-1213.

Molday R.S. (1996). Calmodulin regulation of cyclic-nucleotide-gated channels Calmodulin regulation of cyclic-nucleotide-gated channels. *Current Opinion in Neurobiology, Volume 6, Issue 4, Pages 445-452*.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., Axel, R., (1996). Visualizing an olfactory sensory map. Cell 87, 675–686.

Mombaerts, P. (2004b). Odorant receptor gene choice in olfactory sensory neurons: the one receptor–one neuron hypothesis revisited. Curr. Opin. Neurobiol. 14, 31–36.

Mombaerts, P.(2004a.). Genes and ligands for odorant, vomeronasal and taste receptors. Nat. Rev. Neurosci. 5, 263–278.

Moricard R. and Bossu J. (1951). Arrival of fertilizing sperm at the follicular cell of the secondary oocyte Fertility and Sterility 2 260–266

Morisawa, M. (1994).Cell signaling mechanisms for sperm motility. Zool. Sci. 11, 647–662

Mortimer D. (1986). An evaluation of different media for the zona-free hamster egg penetration test. Am J Obstet Gynecol 154,351–354.

Mortimer S.T. (1997). A critical review of the phisciological importance and analysis of sperm movement in mammals. Human Reproduction. Vol. 3. Nº 5 pp. 403-439.

Mortimer S.T. (2000). CASA-practical aspects. J Androl 21,515–524. O'Flaherty CO, de Lamirande E and Gagnon C (2004) Phosphorylation of the Arginine-X–X-(Serine/Threonine) motif in human sperm proteins during capacitation: modulation and protein kinase A dependency. Mol Hum Reprod 10,355–363.

Moseley F.L.C, K.N.Jha, Lars Björndahl, I.A.Brewis, S.J.Publicover, C.L.R.Barratt1, and L.Lefièvre. (2005). Protein tyrosine phosphorylation, hyperactivation and progesterone-induced acrosome reaction are enhanced in IVF media: an effect that is not associated with an increase in protein kinase A activation. Molecular Human Reproduction Vol.11, No.7 pp. 523–529

Navarro B., Kirichok Y., Chung J.J. & Clapham D.E. (2008). Ion channels that control fertility in mammalian spermatozoa. International Journal of Developmental Biology. 52 607-613.

Naz. P.K. and Leslie, M.H. (2000). Immunobiologic implication of RANTES is seminal plasma of fertile, infertile and immunoinfertile men. Am. J. Reprod. Inmmunol., 197-204.

Nelson, P.J. Kim, H.T., Manning. W.C., Goralski, T.J. and Krensky, A.M. (1993). Genomic organization and transcriptional regulation of RANTES cytokine gene. J. Inmmunol., 151, 2601-2612.

Newton S.C., Blaschuk O.W., Millette C.F. (1993). N-cadherin mediates Sertoli cell spermatogenic cell adhesion. Dev Dyn.197(1):1-13.

Nikpoor P., Mowla S.J., Movahedin M., Ziaee S.A. and Tiraihi T. (2004). CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility. Hum Reprod 19(1), 124-128.

Nussey, S.S. and Whitehead, S.A. (2001). Endocrinology: An Integrated Approach. London:Taylor & Francis.

O'Flaherty C.O., de Lamirande E. and Gagnon C. (2004) Phosphorylation of the Arginine-X–X-(Serine/Threonine) motif in human sperm proteins during capacitation: modulation and protein kinase A dependency. Mol Hum Reprod 10,355–363.

Oko, R. (1988). Comparative analysis of proteins from the fibrous sheath and outer dense fibres of rat spermatozoa. Biol. Reprod., 39, 169–182.

Oka, Y., Omura, M., Kataoka, H., Touhara, K. (2004). Olfactory receptor antagonism between odorants. EMBO J. 23, 120–126.

Oliphant G., Reynolds A.B., Thomas T.S. (1985). Sperm surface components involved in the control of the acrosome reaction. Am J Anat 174:269-283

Oliveira, R. G., Tomasi, L., Rovasio, R. A. & Giojalas, L. C. (1999).Increased velocity and induction of chemotactic response in mouse spermatozoa by follicular and oviductal fluids. J. Reprod. Fertil. 115, 23–27.

Osheroff J.E., Visconti P.E., Valenzuela J.P., Travis A.J., Alvarez J. and Kopf G.S. (1999). Regulation of human sperm capacitation by a cholesterol effluxstimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. Mol Hum Reprod 5,1017–1026.

O'Toole C.M., Arnoult C., Darzon A., Steinhardt R.A. & Florman H.M. (2000). Ca2+ entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Molecular Biology of the Cell 11 1571-1584

Orrenius S., Zhivotovsky B. & Nicotera P. (2003). Regulation of cell death: the calcium-apoptosis link. Natur Reviews in Molecular Cell Biology 47 552-565.

Osheroff J.E., Visconti P.E., Valenzuela J.P., Travis A.J., Alvarez J. and Kopf G.S. (1999). Regulation of human sperm capacitation by a cholesterol effluxstimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. Mol Hum Reprod 5,1017–1026.

Overstreet J.W. and Drobnis E.Z. (1991). Sperm transport in the female tract. In Advances in Donor Insemination pp 33–49 Eds CLR Barratt and ID Cooke. Cambridge University Press, Cambridge

Padinjat R., Andrews S. (2004). TRP channels at a glance. J Cell Sci 117,5707-5709

Park J.Y., Ahn H.J., Gu J.G., Lee K.H., Kim J.S., Kang H.W. and Lee J.H. (2003). Molecular identification of Ca2+ channels in human sperm. Exp Mol Med 35(4), 285-92.

Parekh A.B. & Putney J.W. Jr. (2005). Store-operated calcium channels. Physiological Reviews 85 757-810.

Parinaud J. and Milhet P. (1996). Progesterone induces Ca2+-dependent-3′,5′- cyclic adenosine monophosphate increase in human sperm. J Clin Endocrinol Metab 81,1357–1360.

Parmentier M., Libert F., Schurmans S., Schiffmann S., Lefort A., Eggerickx D., Ledent C., Mollereau C., Gérard C., Perret J. (1992). Expression of members of the putative olfactory receptor gene family in mammalian germ cells. Nature 355, 453–455

Parekh A.B. and Penner R. (1997). Store depletion and calcium influx. Physiol Rev 77, 27 901-930.

Philipson K.D. and Nicoll D.A. (2000). Sodium-calcium exchange: a molecular perspective. Annu Rev Physiol 62, 111-133.

Philipp S., Hambrecht S., Braslavski L., Schroth G., Freichel M., Murakami M., Cavalie A. and Flockerzi V. (1998). A novel capacitative calcium entry channel expressed in excitable cells. EMBO J 17, 4274-4282.

Pintado B., de la Fuente J., Roldan E.R. (2000). Permeability of boar and bull spermatozoa to the nucleic acid stains propidium iodide or Hoechst 33258, or to eosin: accuracy in the assessment of cell viability. Journal of Reproduction and Fertility 118: 145–152.

Pedersen, H. (1970). Observations on the axial filament complex of the human spermatozoon. J. Ultrastruct. Res., 33, 451–462.

Pfeffer W. (1884). Lokomotorische Richtungsbewegungen durch chemische Reize Untersuchungen aus d. Botan. Inst. Tübingen 1 363–482

Pratt S.A., Scully N.F., Shur B.D. (1993). Cell surface beta 1,4 galactosyltransferase on primary spermatocytes facilitates their initial adhesion to Sertoli cells in vitro. Biol Reprod. 49(3):470-82.

Publicover S., Harper C.V., Barratt C. (2007). [Ca2+]i signalling in sperm–making the most of what you've got. Nature Cell Biology 9: 235–242.

Publicover S.J. & Barratt C.L. (1999). Voltage-operated Ca2+ channels and the acrosome reaction: which channels are present and what do they do? Human Reproduction 14 873-879

Publicover S.; Harper C.V.; Barratt C. (2007). [Ca2+]i signalling in sperm- making the most of what you've got. Nature cell biology;9(3):235-42.

Publicover S.J.; Giojalas L.C.; Teves M.E.; de Oliveira G.S.Mendes-Machado; Garcia A.A. Morales; Barratt C.L.R.; Harper C.V. (2008). Ca2+ signalling in the control of motility and guidance in mammalian sperm. Frontiers in bioscience : a journal and virtual library;13:5623-37.

Purvis K., Rui H. (1988). High-affinity, calmodulin-dependent isoforms of cyclic nucleotide phosphodiesterase in rat testis. Methods Enzymol; 159:675–685

Putney J.W. (1990). Receptor-regulated calcium entry. Pharmacol Ther 48,427-434.

Putney J.W. (1999). TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium. *PNAS* 96 (26): 14669-14671

Quednau B.D., Nicoll D.A. and Philipson K.D. (1997). Tissue specificity and alternative splicing of the Na+/Ca2+ exchanger isoforms NCX1, NCX2, and NCX3 in rat. Am J Physiol 272, C1250-1261.

Quinn P., Micheal L.L., Minh H.o. Bastuba M., Hendee F. and Brody S.A. (1998). Confirmation of the beneficial effects of brief co-incubation of gametes in human in vitro fertilization. Fertil Steril 69,399–402.

Quill T.A., Ren D., Clapham D.E. and Garbers D.L. (2001). A voltage-gated ion channel expressed specifically in spermatozoa. Proc Natl Acad Sci USA 98(22), 12527-12531.

Quill T.A., Sugden S.A., Rossi K.L., Doolittle L.K., Hammer R.E. and Garbers D.L. (2003). Hyperactivated sperm motility driven by CatSper2 is required for fertilization. Proc Natl Acad Sci USA 100(25), 14869 14874.

Ralt D., Goldenberg M., Fetterolf P., Thompson D., Dor J., Mashiach S., Garbers D.L. and Eisenbach M. (1991). Sperm attraction of follicular factor(s) correlates with human egg fertilizability Proceedings of National Academy of Sciences USA 88 2840–2844

Ralt D., Manor M., Cohen-Dayag A., Tur-Kaspa I., Ben-Shlomo I., Makler A. (1994). Chemotaxis and chemokinesis of human spermatozoa to follicular factors. Biol Reprod;50:774–85.

Reinhardt T.A., Horst R.L, Waters W.R. (2004). Characterization of Cos-7 cells overexpressing the rat secretory pathway Ca2+-ATPase. Am J Physiol Cell Physiol 286,C164-9.

Ren D., Navarro B., Perez G., Jackson A.C., Hsu S., Shi Q., Tilly J.L. and Clapham D.E. (2001) A sperm ion channel required for sperm motility and male fertiliy. Nature 413, 603-609.

Riffell, J.A., Krug, P.J. and Zimmer, R.K. (2004). The ecological and evolutionary consequences of sperm chemoattraction. Proc. Natl. Acad. Sci. USA 101, 4501-4506

Rossato M., Di Virgilio F., Rizzuto R., Galeazzi C., Foresta C. (2001). Intracellular calcium store depletion and acrosome reaction in human spermatozoa: role of calcium and plasma membrane potential. Mol Hum Reprod. 7,119-128.

Rossi P., Pezzotti R., Conti M., Geremia R. (1985). Cyclic nucleotide phosphodiesterase in somatic and germ cells of mouse seminiferous tubules.J Reprod Fertil; 74:317–327.

Ruskoaho, H. (1992).Atrial natriuretic peptide: synthesis, release, and metabolism. Pharmacol. Rev. 44, 481–602

Sakkas D., Leppens-Luisier G., Lucas H., Chardonnens D., Campana A., Franken D.R. and Urner F. (2003). Localization of tyrosine phosphorylated proteins in human sperm and relation to capacitation and zona pellucida binding. Biol Reprod 68,1463–1469.

Serrano C.J., Trevino C.L., Felix R. and Darszon A. (1999) Voltage-dependent Ca(2+) channel subunit expression and immunolocalization in mouse spermatogenic cells and sperm. FEBS Lett 462(1-2), 171-176.

Serrano, H., Canchola, E. & García-Suárez, M. D. (2001).Sperm-attracting activity in follicular fluid associated to an 8.6-kDa protein. Biochem. Biophys. Res. Commun. 283, 782–784

Schall, T. J., Bacon, K., Toy, K. J. & Goeddel, D. V. (1990).Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 347, 669–671

Schiffmann E., Corcoran B.A. and Wahl S.M. (1975). N-Formyl methionyl peptides as chemoattractants for leucocytes Proceedings of National Academy of Sciences USA 72 1059–1062

Schuh. K., Cartwright E.J., Jankevics E., Bundschu K., Liebermann J., Williams .J.C., Armesilla A.L., Emerson M., Oceandy D., Knobeloch K.P., Neyses L.(2004). Plasma membrane Ca2+ ATPase 4 is required for sperm motility and male fertility.J Biol Chem. 279,28220-28226.

Sharona E. Gordon & William N. Zagotta. (1995). Subunit interactions in coordination of Ni^{2+} in cyclic nucleotide-gated channels Proc. Natl. Acad. Sci. USA Vol. 92, pp. 10222-10226, Neurobiology

Shiba K., Márián T., Kraszani Z., Baba A.S., Morisawa M. and Yoshida M. (2006). Na+/Ca2+ Exchanger modulates the flahellar wave pattern for the regulation of motilify action and chemotaxis in the ascidian spermatozoa. Cell motility and cytoskeleton, 63: 623-623.

Silvestroni, L., Palleschi, S., Guglielmi, R. & Croce, C. T. (1992).Identification and localization of atrial natriuretic factor receptors in human spermatozoa. Arch. Androl. 28, 75–82

Sliwa L. (1993a). Effect of heparin on human spermatozoa migration in vitro. Archives of Andrology 30 177–181

Sliwa L. (1993b). Heparin as a chemoattractant for mouse spermatozoa Archives of Andrology 31 149–152

Sliwa L. (1994). Chemotactic effect of hormones in mouse spermatozoa Archives of Andrology 32 83–88

Sliwa L. (1995). Chemotaction of mouse spermatozoa induced by certain hormones Archives of Andrology 35 105–110

Sliwa L. (1995).Effect of some sex steroid hormones on human spermatozoa migration in vitro. Eur J Obstet Gynecol Reprod Biol;58:173–5.

Sliwa L. (1999).Hyaluronic acid and chemoattractant substance from follicular fluid: in vitro effect of human sperm migration. Arch. Androl. 43, 73–76

Sliwa L. (2001). Substance P and beta-endorphin act as possible chemoattractants of mouse sperm. Arch.Androl. 46, 135–140

Soderling S.H., Beavo J.A.(2000). Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. Curr Opin Cell Biol; 12:174–179.

Song Y., Cygnar D.K., Sagullaev B., Valley M., Sarah H., Stephan A., Reisert J. And Zhao H., (2008). Olfactory CNG channel desensitization by Ca2+/CaM via the B1b subunit affects response termination but not sensitivity to recurring stimulation. Cell PressNeuron 58, 374-386.

Spehr, M., Wetzel, C.H., Hatt, H., Ache, B.W., (2002). 3-Phosphoinositides modulate cyclic nucleotide signaling in olfactory receptor neurons. Neuron 33, 731–739.

Spehr M., Gisselmann G., Poplawski A., Riffell J.A., Wetzel C.H., Zimmer R.K., Hatt H. (2003). Identification of a testicular odorant receptor mediating human sperm chemotaxis. Science;299:2054 – 8.

Spehr, M., Hatt, H., (2004). hOR17-4 as a potential therapeutic target. Drug News Perspect. 17, 165–171.

Spehr, M., Schwane, K., Heilmann, S., Gisselmann, G., Hummel, T., Hatt, H., (2004a). Dual capacity of a human olfactory receptor. Curr. Biol. 14, 832–833.

Spehr, M., Schwane, K., Riffell, J.A., Barbour, J., Zimmer, R.K., Neuhaus, E.M., Hatt, H., (2004b). Particulate adenylate cyclase plays a key role in human sperm olfactory receptor-mediated chemotaxis. J. Biol. Chem. 279, 40194–40203.

Spehr, M., Hatt, H., (2005). A potential role of odorant receptor agonists and antagonists in the treatment of infertility and contraception. Curr. Opin. Investig. Drugs 6, 364–368.

Spehr, M., Leinders-Zufall, T., (2005). One neuron-multiple receptors: increased complexity in olfactory coding? Sci. STKE 285, pe25.

Storey B.T., Keyhani E. (1973). Interaction of calcium 1 ion with the mitochondria of rabbit spermatozoa. FEBS Lett.37,33-36.

Storey B.T., Keyhani E. (1974). Energy metabolism of spermatozoa. II. Comparison of pyruvate and fatty acid oxidation by mitochondria of rabbit epididymal spermatozoa. Fertil Steril 10,857-864.

Strünker T.,Weyand I.,Bönigk W.,Van Q., Loogen A., Brown J.E., Kashikar N., Hagen V., Krause E. & Kaupp B. (2006).K^+-Selective cGMP-gated ion channel controls chemosensation of sperm.Nature Cell Biol. 8, 1149-1154

Suarez S.S., Katz D.F., Overstreet J.W. (1983). Movement characteristics and acrosomal status of rabbit spermatozoa revovered at the site and time of fertilization. Biol Reprod 29:1277-1287

Suarez S.S., Osman R.A.(1987). Initiation of hyperactivated flagellar bending in mouse sperm within the female reproductive tract. Biol Reprod. 36:1191-1198.

Suarez S.S., Vincenti L., Ceglia M.W. (1987). Hyperactivated motility induced in mouse sperm by calcium ionophore A23187 is reversible. J Exp Zool. 244:331-336.

Suarez S.S., Katz D.F., Owen D.H., Andrew J.B., Powell R.L. (1991). Evidence for the function of hyperactivated motility in sperm. Biol Reprod. 44:375-381.

Suarez S.S. (1996). Hyperactivated motility in sperm Journal of Andrology 17 331–335

Suarez S.S. (1998). The oviductal sperm reservoir in mammals: mechanisms of formation Biology of Reproduction 58 1105–1107

Suarez, S.S., (1998). The oviductal sperm reservoir in mammals: mechanisms of formation. Biol. Reprod. 58, 1105–1107.

Suarez, S. S. (2002).In Fertilization (ed. Hardy, D. M.) 3–28 (Academic Press, San Diego.

Sun F., Giojalas L., Rovasio R., Tur-Kaspa I., Sanchez R., & Eisenbach M. (2003). Lack of species-specificity in mammalian sperm chemotaxis. Dev. Biol. 255, 423–427

Sun, F., Bahat, A., Gakamsky, A., Girsh, E., Katz, N., Giojalas, L.C., Tur-Kaspa, I., Eisenbach, M., (2005). Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants.Hum. Reprod. 20, 761–767.

Sundfjord J.A., Forsdahl F. and Thibault G. (1989). Physiological levels of immune reactive ANH-like peptides in human follicular fluid Acta Endocrinologica 121 578–580

Summers K.E., Gibbons I.R. (1971). Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. Proc Natl Acad Sci USA.68:3092-3096.

Suzuki N. (1995). Structure, function and biosynthesis of sperm-activating peptides and fucose sulfate glycoconjugate in the extracellular coat of sea urchin eggs Zoological Science 12 13–27

Tash J.S., Means A.R.(1982). Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol Reprod. 26:745-763

Tash J.S., Means A.R. (1983). Cyclic adenosine 3',5' monophosphate, calcium and protein phosphorylation in flagellar motility. Biol Reprod.28:75-104.

Tash J.S. (1987). Protein phosphorylation: the second messenger signal transducer of flagellar motility. Cell Motil Cytoskeleton. 14:332-339.

Tash J.S., Means A.R. (1987). Ca2+ regulation of sperm axonemal motility. Methods Enzymol; 139:808-823.

Tash J.S., Krinks M., Patel J., Means R.L., Klee C.B., Means A.R. (1988). Identification, characterization and functional correlation of calmodulin-dependent protein phosphatase in sperm. J Cell Biol.106:1626-1633.

Tash J.S. (1990). Role of cAMP, calcium and protein phosphorylation in sperm motility. In: Gagnon C (ed.), Controls of Sperm Motility: Biological and Clinical Aspects. Boca Raton, FL: CRC Press. 229–241

Tash J.S., Bracho G.E. (1994). Regulation of sperm motility: emerging evidence for a major role for protein phosphatases. J Androl. 15:505-509.

Tash J.S., Bracho G.E. (1998). Identification of phosphoproteins coupled to initiation of motility in live epididymal mouse sperm. Biochem Biophys Res Commun. 251:557-563.

Taylor C.W., Genazzani AA, Morris SA. (1999). Expression of inositol trisphosphate receptors. Cell Calcium 26,237-251.

Teves M.E., Barbano F., Guidobaldi H.A., Sanchez R., Miska W. (2006). Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. Fertility and Sterility 86: 745–749

Teves M., Guidobaldi H., Uñates D., Sanchez R., Miska W., Publicover S., Morales García A., Giojalas L.(2009). Molecular Mechanism for Human Sperm Chemotaxis Mediated by Progesterone. PLoS ONE 4(12): e8211

Trevino C.L., Santi C.M., Beltran C., Hernandez-Cruz A., Darszon A., Lomeli H. (1998). Localisation of inositol trisphosphate and ryanodine receptors during mouse spermatogenesis: possible functional implications. Zygote 6,159-172

Trevino C.L., Serrano C.J., Beltran C., Felix R. and Darszon A. (2001). Identification of mouse trp homologs and lipid rafts from spermatogenic cells and sperm. FEBS Lett 509(1), 119-125.

Trevino C.L., Felix R., Castellano L.E., Gutierrez C., Rodriguez D., Pacheco J., Lopez- Gonzalez I., Gomora J.C., Tsutsumi V., Hernandez-Cruz A., Fiordelisio T., Scaling A.L. and Darszon A. (2004) Expression and differential cell distribution of low-threshold Ca2+ channels in mammalian male germ cells and sperm. FEBS Lett 563(1-3), 87-92.

Toyoshima C. and Inesi G. (2004). Structural basis of ion pumping by Ca2+-ATPase of the sarcoplasmic reticulum. Annu Rev Biochem 73,269-292.

Tso W.W., Lee W.M. and Wong M.Y. (1979). Spermatozoa repellent as a contraceptive Contraception 19 207–211

Turner R.M., (2003). Tales from tail: What do we really know about sperm motility? Rev. Journal of Andrology, Vol. 24. Nº6.

Tur-Kaspa, I. (1992). Pathophysiology of the Fallopian tube. In Gleiber, N (ed.) *Tubal Cathetenzation.* Wiley-Liss Inc., NewYork, pp. 5-14

Uhler M.L., Leung A., Chan S.Y.W. and Wang C. (1992). Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction Fertility and Sterility 58 1191–1198

Vacquier, V.D. (1998). Evolution of Gamete Recognition Proteins. S*cience* 25. Vol. 281. no. 5385, pp. 1995 – 1998.

Vanderhaeghen, P., Schurmans, S., Vassart, G. & Parmentier, M. (1993). Olfactory receptors are displayed on dog mature sperm cells. J. Cell Biol. 123,1441–1452

Vanderhaeghen P., Schurmans S., Vassart G. and Parmentier M. (1997). Specific repertoire of olfactory receptor genes in the male germ cells of several mammalian species Genomics 39 239–246

Villanueva-Díaz C., Vadillo-Ortega F., Kably-Ambe A., Diaz-Perez M.A. and Krivitzky S.K. (1990). Evidence that human follicular fluid contains a chemoattractant for spermatozoa Fertility and Sterility 54 1180–1182

Villanueva-Díaz C., Arias-Martínez J., Bustos-López H. and Vadillo-Ortega F. (1992). Novel model for study of human sperm chemotaxis Fertility and Sterility 58 392–395

Villanueva-Díaz, C., Arias-Martínez, J., Bermejo-Martínez, L. & Vadillo-Ortega, F. (1995).Progesterone induces human sperm chemotaxis. Fertil. Steril. 64, 1183–1188

Villanueva, C. (1998).Crude mare follicular fluid exerts chemotactic effects on stallion spermatozoa. Reprod. Domest. Anim. 33, 321–324

Vermassen E., Parys J.B., Mauger J.P. (2004). Subcellular distribution of the inositol 13 1,4,5-trisphosphate receptors: functional relevance and molecular determinants. Biol 14 Cell. 96,3-17.

Visconti P.E., Moore G.D., Bailey J.L., Leclerc P., Connors S.A., Pan D., Olds-Clark P., Kopf G.S.(1995). Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation, are regulated by cAMP-dependent pathway. Development. 121:1139–1150.

Visconti P.E., Johnson L., Oyaski M., Fornes M., Moss S.B., Gerton G.L., Kopf G.S. (1997). Regulation, localization, and anchoring of protein kinase A subunits during mouse sperm capacitation. Dev Biol; 192:351–363

Visconti P.E., Westbrook V.A., Chertihin O., Demarco I., Sleight S. and Diekman A.B. (2002). Novel signalling pathways involved in sperm acquisition of fertilizing capacity. J Reprod Immunol 53,133–150

Vorup-Jensen T., T. Hjort, J.V. Abraham-Peskir, P. Gluttmann, J.C. Jensenious, E. Uggerhoj & R. Medenwaldt- (1999). X-ray miscroscopy of human spermatozoa shows change of mitochondria morphology after capacitation. Human Reprod 14 880-4

Walensky L.D., Roskams A.J., Lefkowitz R.J., Snyder S.H. and Ronnett G.V. (1995). Odorant receptors and desensitization proteins co-localize in mammalian sperm Molecular Medicine 1 130–141

Walensky, L.D., Snyder, S.H., (1995). Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosome of mammalian sperm. J. Cell Biol. 130, 857–869.

Walensky, L. D. (1998). Two novel odorant receptor families expressed in spermatids undergo 5′-splicing. J. Biol. Chem. 273, 9378–9387

Wang Y., Storeng R., Dale P.O., Abyholm T., Tanbo T. (2001). Effects of follicular fluid and steroid hormones on chemotaxis and motility ofhuman spermatozoa in vitro. GynecolEndocrinol.15:286 –92.

Ward G.E., Brokaw C.J., Garbers D.L., and Vacquier V.D. (1985). Chemotaxis of Arbacia punctulata spermatozoa to resact, a peptide from the egg jelly layer Journal of Cell Biology 101 2324–2329

Ward, G.E. and Kopf, G.S. (1993). Molecular Events Mediating Sperm Activation. Dev. Biol. 158, 9-3424

Waard M., Ronjat M. & Arnoult C. (2005). Junctate, an inositol 1,4,5-triphosphate receptor associated protein, is present in rodent sperm and binds TRPC2 and TRPC5 but not TRPC1 channels. Developmental Biology 286 326-37

Wang, F., Nemes, A., Mendelsohn, M., Axel, R., (1998). Odorant receptors govern the formation of a precise topographic map. Cell 93, 47–60.

Wang, Y., Storeng, R., Dale, P.O., Åbyholm, T. & Tanbo, T. (2001). Effects of follicular fluid and steroid hormones on chemotaxis and motility of human spermatozoa in vitro. Gynecol. Endocrinol. 15, 286–292

Wasco W.M., Orr G.A. (1984). Function of calmodulin in mammalian sperm: presence of a calmodulin-dependent cyclic nucleotide phosphodiesterase associated with demembranated rat caudal epididymal sperm. Biochem Biophys Res Commun; 118:636–642.

Wassarman, P. M., & E. S. Litscher. (1995). Sperm-egg recognition mechanism in mammals. Curr. Top. Dev. Biol. 30: 1-19.

Wassarmann P.M., (1999). Fertilization in Animals. Overview Article. Developmental Genetics 25:83-86.

Weber, M., Pehl, U., Breer, H., Strotmann, J., (2002). Olfactory receptor expressed in ganglia of the autonomic nervous system. J. Neurosci. Res. 68, 176–184.

Wellerdieck, C., Oles, M., Pott, L., Korsching, S., Gisselmann, G., Hatt, H.,(1997). Functional expression of odorant receptors of the zebrafish Daniorerio and of the nematode C. elegans in HEK293 cells. Chem. Senses22, 467–476.

Wells T.N.; Proudfoot A.E.; Power C.A. (1999). Chemokine Receptors & their role in leukocyte activation, Immunol Lett 65, 35-40

Wennemuth G., Westenbroek R.E., Xu T., Hille B. and Babcock D.F. (2000). CaV2.2 and CaV2.3 (N- and R-type) Ca2+ channels in depolarization-evoked entry of Ca2+ into mouse sperm. J Biol Chem 275(28), 21210-21217.

Wennemuth G., Babcock D.F. and Hille B. (2003). Calcium clearance mechanisms of mouse sperm. J Gen Physiol 122(1), 115-128. Erratum in: J Gen Physiol 29 (2003)122(3), 375.

Westenbroek R.E. and Babcock D.F. (1999).Discrete regional distributions suggest diverse functional roles of calcium channel alpha1 subunits in sperm. Dev Biol 207(2), 457-469.

Wetzel, C.H., Oles, M., Wellerdieck, C., Kuczkowiak, M., Gisselmann, G.,Hatt, H., (1999). Specificity and sensitivity of a human olfactory receptor functionally expressed in human embryonic kidney 293 cells and Xenopus Laevis oocytes. J. Neurosci. 19, 7426–7433.

Wetzel, C.H., Behrendt, H.J., Gisselmann, G., Stortkuhl, K.F., Hovemann, B.,Hatt, H., (2001). Functional expression and characterization of a Drosophila odorant receptor in a heterologous cell system. Proc. Natl. Acad. Sci.U.S.A. 98, 9377–9380.

Wictome M., Henderson I., Lee A.G., East J.M. (1992). Mechanism of inhibition of the calcium pump of sarcoplasmic reticulum by thapsigargin. Biochem J. 283,525-529

Wiesner B., Weiner J., Middendorff R., Hagen V., Kaupp U.B., Weyand I.(1998). Cyclic nucleotide-gated channels on the flagellum control Ca2+ entry into sperm. J Cell Biol.142:473-484

Wildt, L., Kissler, S., Licht, P. & Becker, W.(1998).Sperm transport in the human female genital tract and its modulation by oxytocin as assessed by hysterosalping oscintigraphy, hysterotonography, electro hysterography and Doppler sonography. Hum. Reprod. Update 4, 655–666

Williams, M. (1993).Sperm numbers and distributionwithin the human Fallopian tube around ovulation. *Human Reproduction*, Vol. *8*, No. 12, pp. *2019-2026*

Wojcikiewicz R.J., Luo S.G. (1998). Differences among type I, II, and III inositol-1,4,5- trisphosphate receptors in ligand-binding affinity influence the sensitivity of calcium stores to inositol-1,4,5-trisphosphate. Mol Pharmacol 53, 656-662.

Wood, C.D., Darszon, A. & Whitaker, M. (2003).Speract induces calcium oscillations in the sperm tail. J. Cell Biol. 161, 89-101

Wood C., Takuya Nisihigaki, Toshiaki Furuta, Shoji A. Baba, & Darszon A. (2005). Real-time Analysis of the Role of Ca2+ in Flagellar Movement & Motility in Single Sea Urchin Sperm, The Journal of Cell Biology, Vol. 169, Nº 5.

Woolley, D.M. and Nickels, S.N. (1985). Microtubule termination patterns in mammalian sperm flagella. J. Ultrastruct. Res., 90, 221–234.

Wootton L.L., Argent C.C., Wheatley M., Michelangeli F. (2004). The expression, 20 activity and localisation of the secretory pathway Ca2+ -ATPase (SPCA1) in different 21 mammalian tissues. Biochim Biophys Acta. 1664,189-197.

World Health Organization (1999). WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction, 4th edn. Cambridge University Press, Cambridge, UK.

Wuttke M.S., Buck J., Levin L.R. (2001). Bicarbonate-regulated soluble adenylyl cyclase. JOP. J. Pancreas (on line) 2001, *2*((4 Suppl)):154-158.

Xia J., Reigada D., Mitchell C.H. & Ren D. (2007). CATSPER channel-mediated Ca2+ entry into mouse sperm triggers a tail-to-head propagation. Biology of Reproduction 77 551-559.

Yan C., Zhao A.Z., Sonnenburg W.K., Beavo J.A.(2001). Stage and cell-specific expression of calmodulin-dependent phosphodiesterases in mouse testis. Biol Reprod; 64:1746–1754.

Yanagimachi R., Cherr G.N., Pillai M.C. and Baldwin J.D. (1992). Factors controlling sperm entry into the micropyles of salmonid and herring eggs Development Growth and Differentiation 34 447–461

Yanagimachi R. (1994). Mammalian fertilization. In Knobil E, Neill JD (eds): "The Physiology of Reproduction." New York: Raven Press Ltd., pp 189-317

Yanagimachi R. (1994). Mammalian fertilization. In The Physiology of Reproduction pp 189–317 Eds E Knobil and J Neill. Raven Press, New York

Yamashita Y., Shimada M., Okazaki T., Maeda T., Terada T. (2003). Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes. Biology of Reproduction 68: 1193–1198

Yoshida M., Inaba K. and Morisawa M. (1993). Sperm chemotaxis during the process of fertilization in the ascidians Ciona savignyi and Ciona intestinalis. Developmental Biology 157 497–506

Yoshida M., Inaba K., Ishida K and Morisawa M. (1994). Calcium and cyclic AMP mediate sperm activation, but Ca2+ alone contributes sperm chemotaxis in the ascidian, Ciona savignyi. Development Growth and Differentiation 36 589–595

Yoshida, M., Murata, M., Inaba, K. & Morisawa, M. (2002). A chemoattractant for ascidian spermatozoa is a sulfated steroid. Proc. Natl Acad. Sci. USA 99, 14831–14836

Yoshida M., Ishikawa M., Izumi H., De Santis R. and Morisawa M. (2003). Store operated calcium channel regulates the chemotactic behavior of ascidian sperm. Proc Natl Acad Sci U S A 100(1), 149-154

Yoshida M. (2004). Fertilization and sperm chemotaxis in ascidians.Methods Mol Biol.253,13-25

Zamir N., Riven-Kreitman R., Manor M., Makler A., Blumberg S., Ralt D. and Eisenbach M. (1993). Atrial natriuretic peptide attracts human spermatozoa in vitro. Biochemical and Biophysical Research Communications 197 116–122

Zhao G.Q., Deng K., Labosky P.A., Liaw L., Hogan B.L., (1996). The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. Genes Dev.1;10(13):1657-69.

Zucchi R., Ronca-Testoni S. (1997). The sarcoplasmic reticulum Ca2+channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. Pharmacol Rev 49,1-51