MODULATION OF CALCIUM SIGNALLING IN HUMAN SPERM BY NITRIC OXIDE

by

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A thesis submitted to
The University of Birmingham
For the degree of
DOCTOR OF PHILOSOPHY

School of Biosciences
The University of Birmingham
November 2008

ABSTRACT

Nitric oxide (NO) generation by nitric oxide synthase (NOS) is implicated in gamete interaction and fertilization. *In vitro* studies were undertaken to assess the ability of human sperm and cumulus cells (surrounding the oocyte) to generate NO, investigate the mechanism of action of NO, the NO-mediated $[Ca^{2+}]_i$ signalling pathways, the possible interaction of NO with progesterone (a product of cumulus) and its impact in the regulation of human sperm functions.

Immunofluorescent staining revealed constitutive NOS in human cumulus. DAF-FM diacetate staining demonstrated NO production by cumulus cells. Human sperm exposure to NO donors caused mobilization of stored Ca^{2+} by a mechanism not requiring guanylate cyclase activation but mimicked by S-nitrosoglutathione (GSNO; an S-nitrosylating agent). Dithiothreitol application, to reduce protein –SNO groups, rapidly reversed the actions of NO and GSNO on $[Ca^{2+}]_i$. The effects of NO, GSNO and dithiothreitol on protein S-nitrosylation, assessed using the biotin-switch assay, closely paralleled their actions on $[Ca^{2+}]_i$. Progesterone mobilizes stored Ca^{2+} in human sperm, by a mechanism involving ryanodine receptor (RyR) activation. Pre-treatment with NO reduced the amplitude of the Ca^{2+} response to ryanodine (a RyR agonist), suggesting convergence of the actions of NO and ryanodine. Sperm pre-treatment with NO greatly enhanced the progesterone effect on $[Ca^{2+}]_i$, causing a prolonged increase in flagellar excursion. We conclude that NO regulates mobilization of stored Ca^{2+} in human sperm by protein (possibly RyRs) S-nitrosylation, that this action is synergistic with that of progesterone and that this synergism is potentially highly significant in gamete interactions leading to fertilization.

ACKNOWLEDGEMENTS

I would like to express my enormous gratitude to my supervisor Dr. S.J. Publicover of the School of Biosciences at the University of Birmingham, for his unconditional support, encouragement, and expert critical advice and comments during my PhD studies.

Thanks to the staff at the Assisted Conception Unit, Birmingham Women's Hospital for providing donors, particularly Dr. Kirkman-Brown for his help with my work. I would also like to thank the donors who provided the samples, which were essential to carry out my research.

I really appreciate the friendship and support I received from other members of our research group, particularly Sarah Costello and Aduén Morales Garcia for the laughs.

Most importantly, I would like to specially thank my long-term boyfriend, João Facucho Oliveira, for his love and encouragement and for keeping me sane during the crazy times. Words cannot express the incredible support João has been throughout my PhD studies.

I am forever grateful to my mother, father, sister and grand-parents for their continuous support, tolerance and love. They were simply always amazing. I would also like to thank João's father and my Birmingham friends for their support during my studies.

I would like to thank “Fundação para a Ciência e a Tecnologia” (FCT) Portugal for providing financial support during my PhD studies and the School of Biosciences for allowing me to carry out my research.

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LIST OF ABBREVIATIONS

AKAP - A-Kinase anchor protein

ALH - Amplitude of lateral head displacement

ANGS - Angeli's salt

ART - Assisted reproductive techniques

BCF - Beat cross frequency

Biotin-HPDP - Biotin-N-[6-(biotinamido)hexyl]-3-(2-pyridyldithio)propionamide

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

BSA - Bovine serum albumin

BWW - Biggers, Whitten and Whittingham

Ca^{2+} - Calcium ions

cADPR - Cyclic adenosine diphosphate-ribose

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

CaM - Calmodulin

cAMP - Cyclic adenosine monophosphate

CASA - Computer-assisted semen analysis

CCCP - Carbonyl cyanide m-chlorophenylhydrazone

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

cGMP - Cyclic guanosine monophosphate

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

Cl^- - Chloride ions

CNG - Cyclic nucleotide-gated

COC - Cumulus oocyte complex

CSNO - S-Nitrosocysteine

DAF-FM - 4-amino-5-methylamino-2',7'-difluorofluorescein

DAG - Diacylglycerol

DMSO - Dimethyl sulfoxide

DTT - Dithiothreitol

EDRF - Endothelium derived relaxing factor

EDTA - Ethylenediaminetetraacetic acid

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

eNOS - Endothelial nitric oxide synthase

ERKs - Extra cellular signal regulated kinases

FAD - Flavin adenine dinucleotide

FMN - Flavin mononucleotide

FS - Fibrous sheath

FSH - Follicle stimulating hormone

$GABA_A$ - 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

HNO - Nitroxyl

H_2O_2 - Hydrogen peroxide

Hyp - Hyperactive

ICSI - Intracytoplasmic sperm injection

iNOS - Inducible nitric oxyde synthase

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

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

IVF - *In vitro* fertilization

LH - Luteinizing hormone

L_NAME - N(G)-nitro-L-arginine-methyl ester

mAC - Membrane-associated adenylyl cyclase

MAPK - Mitogen-activated protein kinases

MDA - Malondialdehyde

MMTS - Methyl methanethiosulfonate

mPR - Membrane progesterone receptor

MS - Mitochondrial sheath

Na^+ - Sodium ions

NADPH - Nicotinamide adenine dinucleotide phosphate

NO - Nitric oxide

^+NO - Nitrosonium

NO_2^- - Nitrite ion

NOS - Nitric oxide synthase

nNOS - Neuronal nitric oxide synthase

$O_2^{\bullet-}$ - Superoxide anion

ODFs - Outer dense fibers

ODQ - 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

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

$ONOO^-$ - Peroxynitrite

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

PBS - Phosphate buffered saline

PC12 - Pheocromocytoma cell line

PDL - Poly-D-lysine

PGRMC1 - Progesterone membrane receptor component 1

PGRMC2 - Progesterone membrane receptor component 2

PIP_2 - Phosphatidylinositol 4,5-biphosphate

PKA - Protein kinase A

PKC - Protein kinase C

PKG - Protein kinase G

PLC - Phospholipase C

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

R^2 - Coefficient of determination

RNE - Redundant nuclear envelope

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RSNO - S-Nitrosothiols

RT-PCR - Reverse transcription-polymerase chain reaction

RyRs - Ryanodine receptors

sAC - Soluble adenylyl cyclase

SDS – Sodium dodecyl sulphate

SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

sEBSS - Supplemented Earle's balanced salt solution

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

sGC - Soluble guanylyl cyclase

-SH - Thiol / sulfhydryl

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

SNP - Sodium nitroprosside

SOAF - Sperm oocyte activating factors

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

SOD - Superoxide dismutase

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

Spermine NONOate - N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine

SS - Disulfide

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

CHAPTER ONE

SIGNAL TRANSDUCTION IN HUMAN SPERMATOZOA

Foreword to Chapter One

This chapter summarises the recent progress that has been made in understanding the complex series of activities that spermatozoa must perform to fertilize the oocyte. Emphasis is placed on regulation of these actions by calcium ions (Ca^{2+}), nitric oxide (NO) and progesterone.

1.1 Semen

Ejaculated spermatozoa are suspended in seminal fluid and spermatozoa constitute only a small part of the semen, from 1% to 5% of the total volume (Mortimer, 1994). Seminal fluid is a mixture of components generated by distinct glands. These components are incompletely mixed during ejaculation and therefore the initial ejaculate is not a completely homogeneous mixture. Approximately 5% of the ejaculate, the first portion, is made up of secretions from the Cowper (bulbourethral) and Littre glands. The second portion of the ejaculate, from 15% to 30% of it, originates from the prostate. Small contributions of the ampulla and epididymis then follow and, at last, of the seminal vesicles that contribute to the remainder and majority of the ejaculate (Polakoski *et al.*, 1976; Mann and Lutwak-Mann, 1981; Coffey, 1995). The acid phosphatase, citric acid, inositol, Ca^{2+}, zinc and magnesium present in the ejaculate are mainly contributed by the prostate. The contribution of the seminal vesicles is rich in fructose, ascorbic acid, and prostaglandins, whereas the L-carnitine and neutral alpha-glucosidase concentrations are indications of epididymal function (WHO Manual, 1999). A minute portion of the fructose found in the ejaculate derives from the ampulla of the ductus deferens (Owen and Katz, 2005).

1.2 Mammalian spermatogenesis

The purpose of spermatogenesis is to establish and maintain the production of totally differentiated sperm, which in eutherian mammals ranges from >200 million per ejaculate in humans to 2-3 billion per ejaculate in bulls (Sutovsky and Manandhar, 2006). This huge sperm production requires the unique architecture of the seminiferous epithelium, a self-contained system, well isolated from the blood stream, permitting only selective uptake of

paracrine factors. Also exclusive to the reproductive system and germ line is that it is relatively quiescent and not fully differentiated until the onset of puberty (Sutovsky and Manandhar, 2006). At birth, the primordial germ cells in the seminiferous tubules became the non-proliferative A-type spermatogonia that are mitotically quiescent until the peri-pubertal period. The augmented production of gonadotropic hormones then stimulates a huge mitotic proliferation of A-type spermatogonia, paralleled by testicular descent and differentiation of A-type into B-type spermatogonia able to enter meiosis. Spermatogenic stem cells undergo perpectual renewal to maintain sperm production. An A-type spermatogonium can commit either to differentiation or to renewal. Continuous sperm production is sustained throughout the reproductive lifespan of eutherian mammals around the rate of hundreds of millions per day. That enormous proliferation and differentiation needs a certain level of quality control that is achieved by programmed cell death or apoptosis (Sinha Hikim and Swerdloff, 1999).

The unique architecture of the seminiferous tubule affords the seminiferous epithelium with the supply of required nutrients combined with a relative autonomy from the body's immune defence, maintained by blood-testis barrier. The tubule is effectively divided into basal and adluminal compartments. The basal compartment is bounded on its outer side by the basement membrane with A-type spermatogonia directly adjacent to this membrane and B-type spermatogonia which may only poorly contact the basement membrane. The basal pole of Sertoli cells extends into this compartment. The blood-testis barrier, formed by intricate cell-cell junctions between adjacent cytoplasmic projections of Sertoli cells (Johnson and Boekelheide, 2002a; b), forms the upper limit of the basal compartment. Primary spermatocytes penetrate this barrier and enter the adluminal compartment where meiosis and spermiogenesis occur (Sutovsky and Manandhar, 2006).

Continuous differentiation and renewal of male germ cells is required for spermatogenesis. Renewal is assured by the successive mitotic divisions of the spermatogenic stem cells and spermatogonia, referred to as proliferative phase. This implies two different fates for a germ cell, either it undergoes mitosis to duplicate itself or it enters meiosis to differentiate into a sperm cell. This later stage is referred to as meiotic phase. Temporally, these two phases can either be viewed as concomitant or sequential. Spatially, the proliferative phase occurs in the basal compartment rich in nutrients, whereas the differentiating cells in the adluminal compartment are protected from immune defence by the blood-testis barrier (Sutovsky and Manandhar, 2006).

The haploid phase stands out within the context of spermatogenesis as this is when the germ cells acquire unique sperm accessory structures required for the detachment of these cells from the seminiferous tubules, acquisition of fertilizing potential, transit through the female reproductive tract and oocyte fertilization. This last part of spermatogenesis can be divided into spermiogenesis, during which the haploid spermatid generates sperm accessory structures, and spermiation, a process during which the remnants of germ cell cytoplasm are rejected and the totally differentiated sperm detach from the seminiferous epithelium (Sutovsky and Manandhar, 2006).

During spermiogenesis, a somatic cell-like, but haploid, round spermatid transforms into a highly specialized sperm cell capable of acquiring progressive motility and fertilizing potential. Such an extensive remodelling process needs the biogenesis of novel, transient or permanent, accessory structures to be synchronized with the remodelling, reduction or total degradation of most spermatid organelles. This requires the transcription, translation and pos-translational modification of numerous constitutive and germ cell-specific gene products during the early steps of spermiogenesis (Dadoune *et al.*, 2004; Eddy, 2002; Kleene, 1993).

At the organelle level, formation of sperm accessory structures includes the derivation of acrosomal cap/acrosome from the Golgi (Moreno *et al.*, 2000), the incorporation of spermatid cytosol into sperm head skeleton, the perinuclear theca (Oko, 1995) and the development of the sperm axoneme with outer dense fibers (ODFs) and fibrous sheath (FS; Oko, 1998). Shaping of the sperm head and nuclear hypercondensation are accomplished by removal of histones, which includes their sequential displacement by transitional proteins and replacement by protamines (Meistrich *et al.*, 2003). The spermatid centrosome, composed of two perpendicularly opposed centrioles and a halo of microtubule-nucleating pericentriolar material, is reduced to a single, proximal centriole of a mature sperm cell, embedded in a dense mass of capitulum (Sutovsky *et al.*, 1999a), which is achieved by the degradation of the distal centriole once it assists growing the axonemal microtubule doublets (Manandhar *et al.*, 1998). About a half of spermatid mitochondria are rejected during spermiogenesis and the other half acquire a reinforced outer mitochondrial membrane, the mitochondrial capsule (Cataldo *et al.*, 1996), being rearranged into a helical mitochondrial sheath (MS). The sperm nuclear envelope is reduced but not totally removed during spermiogenesis, as its nuclear pores complexes are removed during elongation of spermatids (Sutovsky *et al.*, 1999b).

Throughout spermiogenesis, the germ cells remain associated with Sertoli cells by cell-cell junctions and with each other by cytoplasmic bridges. Spermiogenesis is terminated at spermiation, when totally differentiated sperm detach from each other and from the apical surface of seminiferous epithelium and go through the seminiferous tubule lumen to the rete testis (Fig. 1.1; Guraya, 1995).

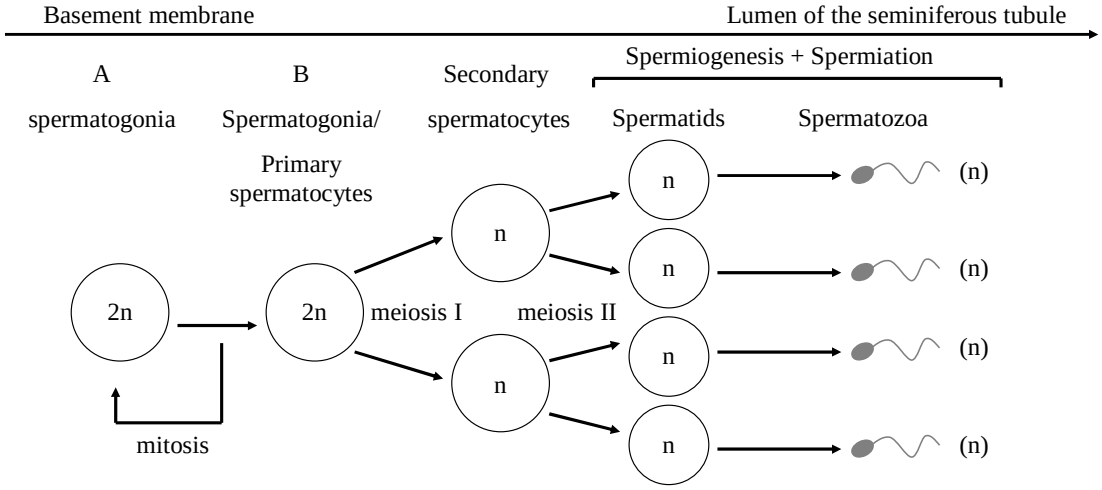

Fig. 1.1. Schematic representation of mammalian spermatogenesis.

1.2.1 Regulation of spermatogenesis

Spermatogenesis is regulated by the secretion of hypothalamic gonadotropin releasing hormone (GnRH) that stimulates the pituitary gland to secrete the follicle stimulating hormone (FSH) and luteinizing hormone (LH; McLachlan, 2000). LH is believed to be primarily responsible for stimulating testosterone secretion by Leydig cells localized in the testicular stroma (Sutovsky and Manandhar, 2006). It is likely that testosterone and FSH are both required to initiate spermatogenesis (Weinbauer and Nieschlag, 1998) and that FSH stimulates spermatogenesis by interacting with receptors present in Sertoli cells (Baird, 1999).

1.3 The sperm cell

Mature sperm are fully differentiated, specialized and compartmentalized cells (reviewed by Naz and Rajesh, 2004). The spermatozoon is composed of a sperm head and a tail or flagellum, covered by a plasma membrane (see Fig. 1.2). The rodent sperm head is hook-shaped, whereas ungulate, carnivore and primate sperm heads are spatula-shaped. A major distinction between ungulate and rodent sperm is the total absence of a sperm centrosome and centrioles in the rodents, as opposed to a reduced form of centrosome with a single proximal centriole in the ungulates. All eutherian mammals share the other general features of the sperm head and tail, as described below (Sutovsky and Manandhar, 2006).

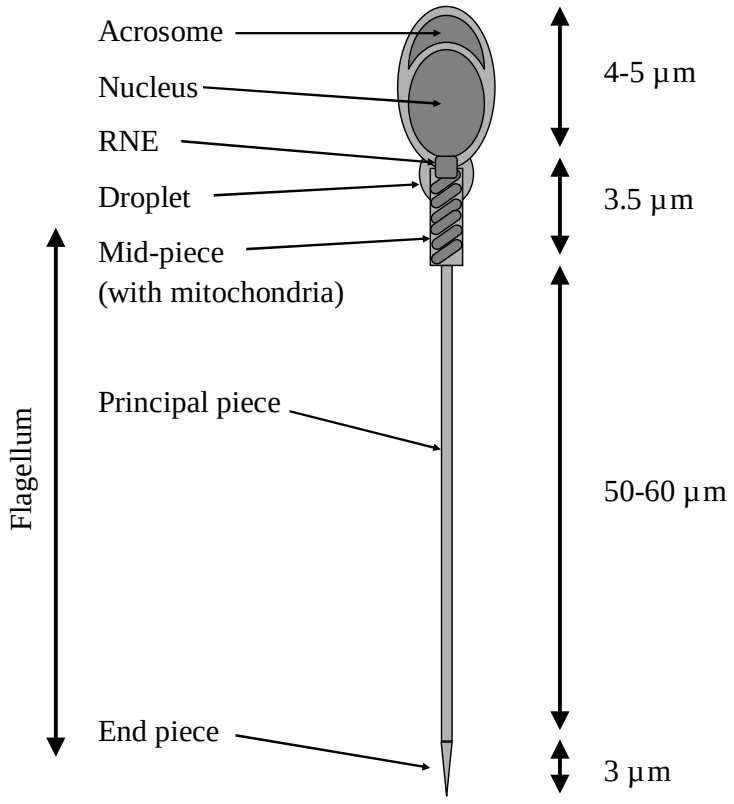

Fig. 1.2. Sperm cell structure.

From the inside-out, the sperm head is constituted by a nucleus, in which the DNA condensing core and linker histones have been partially replaced during spermiogenesis by protamines, positively charged DNA proteins that enable nuclear hypercondensation into a compact, hydrodynamic shape permissive of sperm motility and penetration through the egg vestments (Brewer *et al.*, 2002; Dadoune, 2003). The nucleus is covered by a reduced nuclear envelope, from which the nuclear pore complexes have been removed during spermiogenesis, except for the redundant nuclear envelopes (RNEs) found in some species at the base of the sperm nucleus (Ho and Suarez, 2003). Sperm nucleus is protected by the perinuclear theca, forming a rigid shell composed of disulfide (SS) bond-stabilised structural proteins amalgamated with various other proteins (Oko, 1995). Sperm perinuclear theca can be divided into three segments that mirror three segments of the sperm head and serve unique functions during fertilization. The subacrosomal layer underlies the acrosomal segment of the sperm head, and functions to anchor the acrosome, a Golgi-derived vesicle that forms a cap on the proximal hemisphere of the sperm head and have proteases and receptors needed for sperm interaction with the oocyte zona pellucida. The inner and outer acrosomal membranes hold inside a dense acrosomal matrix containing those proteases that are believed to digest a fertilization slit in the zona (reviewed by Gerton, 2002; Yoshinaga and Toshimori, 2003). The equatorial segment is a folded-over complex of perinuclear theca, inner and outer acrosomal membranes, which carries receptors involved in the initial binding of the sperm to the egg plasma membrane, once the fertilizing sperm cell penetrates through the zona and reaches the perivitelline space. The postacrosomal sheath is thought to have a complex of signalling proteins collectively named SOAF, or the sperm borne, oocyte activating factors (reviewed by Sutovsky *et al.* (2003)).

The four major ultrastructural subdivisions of the flagellum have been well described (see Fig.1.3). These are the connecting piece, the mid-piece, the principal piece and the end-piece (Fawcett, 1975). The connecting piece is the short, most proximal portion of the flagellum that attaches to the implantation fossa of the nucleus in the sperm head. From the remnant of the centriole at this point, the axoneme extends throughout the length of all subdivisions of the sperm flagellum. The axoneme consists of a ring of nine microtubule doublets surrounding a pair of microtubules in the central region. Inner and outer dynein arms project from each of the outer doublets, these arms being responsible for generating the motive force of the flagellum. Nine radial spokes, each deriving from one of the outer microtubular doublet pairs, extend inwards towards the central pair in a helical fashion (Clermont *et al.*, 1990; Fawcett, 1975).

The mid-piece is the next most proximal flagellar portion. The mid-piece presents nine ODFs surrounding each of the nine outer axonemal microtubule doublets, and a MS that encloses the ODFs and the axoneme. The ODFs extend throughout the mid-piece and into the principal piece of the flagellum. The MS is restricted to the mid-piece. The end of the mid-piece and the start of the next flagellar portion, the principal piece, are marked by the annulus (Turner, 2006).

At the start of the principal piece, the MS terminates and two of the ODFs are replaced by the two longitudinal columns of the FS. The FS columns run the length of the principal piece and are stabilised by circumferential ribs that surround the ODFs. The FS is the only structure that is restricted to the principal piece. The ODFs and FS taper and eventually end, as the principal piece nears its termination. The end-piece is the short terminal portion of the flagellum and has only the axoneme surrounded by the plasma membrane (Turner, 2006).

Axonemes are highly conserved in all ciliated and flagellated eukaryotic cells. The ODFs, MS and FS are accessory structures that are exclusive to the mammalian spermatozoa flagellum (Turner, 2006).

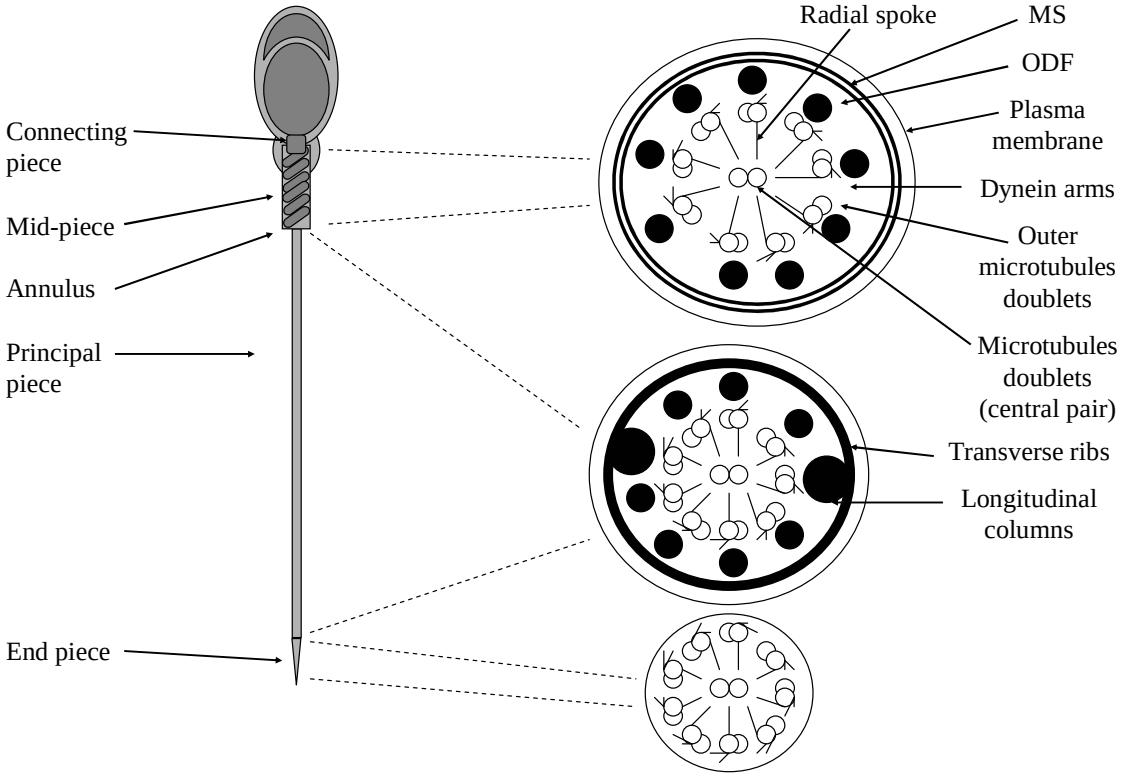

Fig. 1.3. Sperm flagellum structure.

The sperm nucleus is highly condensed. Spermatozoa are generally believed to be devoid of any major transcriptional and translational activity, such that post-translational modification events are central in regulating sperm functions (Naz and Rajesh, 2004). Sperm DNA is highly packed and consequently not accessible for transcription and sperm are generally considered not to have the necessary equipment for protein translation (Luconi *et al.*, 2004). Moreover, sperm undergo alterations in their functional ability during maturation in the epididymis, capacitation (see section 1.7) in the female reproductive tract and fertilization

(see section 1.6). Those alterations must be achieved by modifications of already existing proteins rather than modification in gene expression, and can be regulated by signals from the sperm environment or may arise spontaneously (Ford, 2004).

However, it has been reported that translation of nuclear-encoded mRNAs by the mitochondrial ribosomes is a phenomenon that occurs in mature sperm, opening up the possibility for an alternative translation pathway in other cells. Further research is needed to understand the cellular mechanisms involved if the translation is intramitochondrial. Though, the possibility for extramitochondrial translation cannot be excluded. A number of articles on somatic cells and sperm indicate that mitochondrial translation machinery components exist outside the mitochondria (reviewed by Gur and Breitbart (2008)).

1.4 The oocyte

The ovulated oocyte (Fig. 1.4) is surrounded by a hyaluronic acid-rich layer of cumulus cells. Though the cumulus is the first egg vestment to be penetrated by the spermatozoon, remarkably little is known about this process. The cumulus oophorus comprises two distinct structures, an outer cell layer/mass (cumulus cells) and an inner cell layer (corona radiata), both of which secrete steroids (Laufer *et al.*, 1984; Osman *et al.*, 1989), and proteins (Tesarik *et al.*, 1988). The cells of the human corona radiata are contiguous with the zona pellucida and pass through it. Corona cells have many gap junctions with each other and the oocyte, allowing propagation of Ca^{2+} signals and molecules. These gap junctions are known to be important in signalling during oocyte growth (Mattioli and Barboni, 2000; Motta *et al.*, 1994; 1995). The links between the oocyte and the cumulus are believed to be reduced or totally lost

at ovulation (Gregory *et al.*, 1994; Motta *et al.*, 1994; 1995), but the cumulus cells still have projections running throughout the zona pellucida to near the oolemmal surface (Motta *et al.*, 1994). It appears likely that passage through the cumulus mediates or prepares sperm for acrosome reaction (section 1.8; Tesarik *et al.*, 1988). Another function of the cumulus-corona may be selection or removal of sperm. Corona cells and leukocytes, which are present throughout the cumulus, appear to phagocytose abnormal and/or supernumerary sperm (Nottola *et al.*, 1998). Moreover, the cumulus oophorus and follicular fluid have been demonstrated to influence and maintain the movement characteristics in subpopulations of human spermatozoa in ways that may be significant for penetration of the cumulus (Mendoza and Tesarik, 1990; Tesarik *et al.*, 1990). Upon penetration of the cumulus mass, sperm bind to the zona pellucida, a thick, highly glycosylated protein matrix, secreted during oogenesis, which surrounds the oocyte. Zona pellucida functions include prevention of polyspermy, embryo protection prior to implantation, blockade of heterospecific fertilization, and regulation of endocrine profiles during folliculogenesis (Yanagimachi, 1994a).

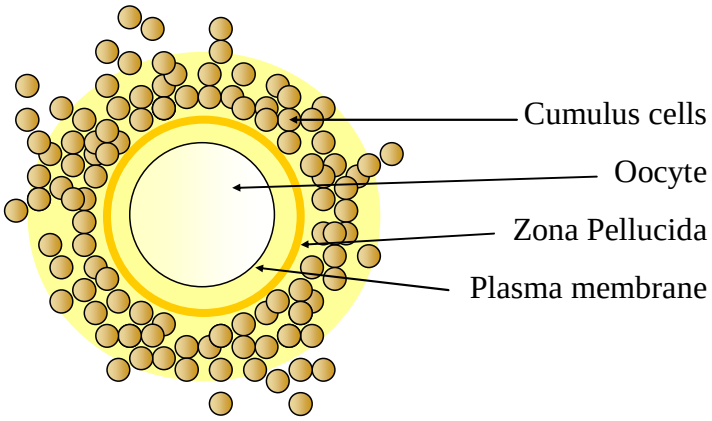

Fig. 1.4. Oocyte structure.

1.5 Transport of sperm

Passage of spermatozoa through the female reproductive tract is regulated to maximize the chance of fertilization and ensure that sperm with vigorous motility and normal morphology will be the ones to succeed (Suarez and Pacey, 2006).

Oocytes are generally fertilized within hours of ovulation (Austin, 1957; Harper, 1994). In some species, spermatozoa may be inseminated days (pigs, cattle and horses) or months (some bat species) prior to the oocyte arrival. In humans, there is evidence that fertilization takes place when intercourse occurs up to five days prior to ovulation (Wilcox *et al.,* 1995). Spermatozoa are exposed to physical stresses during ejaculation and contractions of the female tract, and they may suffer oxidative damage. Also, since sperm are allogenic to the female, they may meet the female immune system defences, intended for infectious organisms (Menge and Edwards, 1993). Sperm must in some way use their restricted resources to preserve their fertility. Of the millions of spermatozoa inseminated at coitus in humans, only a few thousand reach the Fallopian tubes and, ordinarily, only a single spermatozoon fertilizes an oocyte (Suarez and Pacey, 2006). For a review see Suarez and Pacey (2006).

In humans, contractions of the uterine muscle may draw spermatozoa from the cervix into the uterus. Most likely, sperm should pass through the uterine cavity before significant numbers of leukocytes arrive in order to ensure fertilization (Suarez and Pacey, 2006). Sperm are apparently stored in the initial segment of the Fallopian tubes, which may serve to prevent polyspermic fertilization, by allowing only a few sperm at a time to reach the oocyte in the ampulla (Suarez and Pacey, 2006). Data of human sperm distribution in the Fallopian tubes have not totally elucidated the events of sperm transport. However, human sperm must be stored somewhere in the female tract, the Fallopian tubes being strong candidates for storage

sites, as the human endosalpingeal epithelium prolongs sperm survival *in vitro* (Suarez and Pacey, 2006). It is uncertain whether sperm are stored in the human cervix (Suarez and Pacey, 2006).

1.6 Fertilization

Fertilization is the process of union of the male and female gametes, sperm and egg, initiating the development of a new individual (Wassarman, 1999). Following release from the seminiferous epithelium in the testis, spermatozoa are transported through the epididymis, where other biochemical and functional modifications take place. Sperm are subsequently stored in the cauda epididymidis, where they are in a functionally inactive status, immotile and unable to interact with eggs (Wassarman and Florman, 1997). Upon deposition into and migration up the female reproductive tract, spermatozoa undergo capacitation, a process that enables sperm to bind to the egg and acrosome react (Darszon *et al.*, 1996; Visconti and Kopf, 1998). Hyperactivated motility is characteristic of the swimming patterns of capacitated sperm and appears to be vital for fertilization *in vivo*, allowing sperm to reach the oocyte through the mucus-filled oviduct and helping sperm penetrating the cumulus oophorus and zona pellucida (Ho and Suarez, 2001a). Once ovulated eggs and spermatozoa are present in the oviduct, fertilization in mammals includes a series of steps in a mandatory order (Fig. 1.5). Firstly, mammalian spermatozoa may be guided to the egg by a chemoattractant generated by the cumulus cells that surround the egg, or the oocyte itself. Sperm must then bind, in a species-specific manner, to the extracellular coat of the egg, the zona pellucida, and undergo the acrosome reaction, enabling sperm to penetrate the zona pellucida. Upon reaching the perivitelline space between the egg zona pellucida and the plasmalemma, sperm

must bind and fuse with the plasma membrane. The fusion of a single spermatozoon with the egg plasma membrane stops further sperm, which have penetrated the zona pellucida, from fusing with it. A zygote is formed upon egg fertilization, and sperm become unable to bind the zona pellucida (Wassarman *et al.*, 2001).

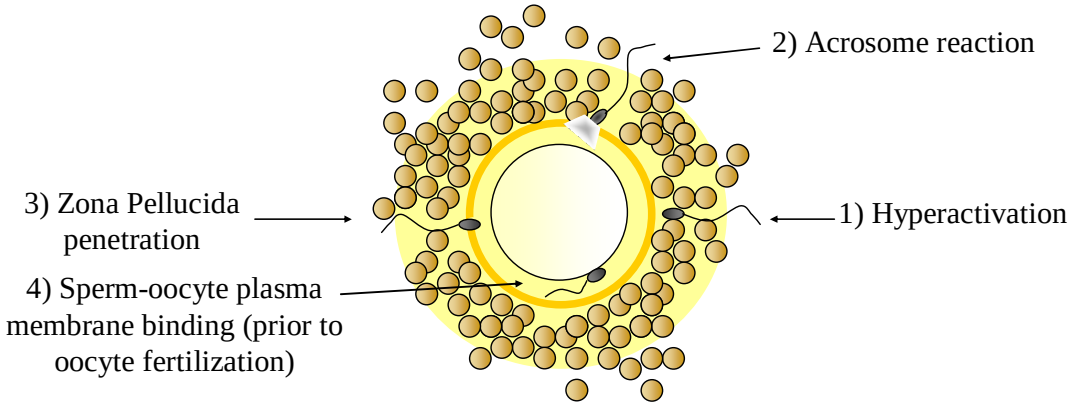

Fig. 1.5. Schematic representation of fertilization.

1.7 Capacitation

After spermatogenesis and spermiogenesis, mammalian sperm appear to be morphologically mature but functionally immature, as they have not acquired progressive motility or the capacity to fertilize an egg. Although signalling pathways mature during sperm transit through the epididymis, total fertilization ability *in vivo* is gained only upon residence in the female reproductive tract, a process termed capacitation. Similar observations have been made using various *in vitro* assays, suggesting that a series of events, some triggered by environment cues, give sperm the capacity to fertilize the egg (Travis and Kopf, 2002). Capacitated spermatozoa have the ability to exhibit hyperactivated motility and to undergo a physiological acrosome reaction (Yanagimachi, 1994b). Capacitation is associated with several biochemical events, most notably an increase in protein tyrosine phosphorylation (Baldi *et al.*, 2000; 2002; Breitbart, 2003; Guraya, 2000; Visconti *et al.*, 1998; Visconti and Kopf, 1998), but also include an increase in plasma membrane fluidity due to cholesterol efflux, a redistribution of membrane phospholipid content, changes in intracellular ionic and metabolic contents and membrane hyperpolarization (Patrat *et al.*, 2000).

Understanding of capacitation mostly derives from *in vitro* studies, under defined incubation conditions that initiate capacitation events, including cholesterol removal from the sperm membranes, mediated by sterol binding proteins (Visconti *et al.*, 1998). Some sperm proteins are phosphorylated at tyrosine residues, through a cyclic adenosine monophosphate (cAMP)-dependent pathway (Visconti *et al.*, 1998; 2002). Mammalian sperm express a bicarbonate-sensitive soluble adenylyl cyclase (sAC; Chen *et al.*, 2000) that may regulate protein tyrosine phosphorylation (Visconti *et al.*, 2002), and probably also accounts for the need of extracellular bicarbonate for capacitation to occur (Evans and Florman, 2002). A rise in

intracellular bicarbonate levels and pH, and the related cAMP synthesis, may stimulate the cyclic nucleotide-gated (CNG) channels in the flagellum, which regulate flagellar motility (Wiesner *et al.*, 1998) and may contribute to the hyperactivated motility exhibited by capacitated sperm (Ho and Suarez, 2001a). Plasma membrane hyperpolarization is caused, at least partly, by an increased contribution of potassium channels in setting sperm membrane potential (Florman *et al.*, 1999).

De Jonge (2005) has recently reviewed the biological basis for human capacitation. The female genital tract "filters" the sperm cells; many sperm will not pass into the cervical mucus. Passage through and into the uterus will only be possible for those sperm with vigorous progressive motility, normal morphology and a plasma membrane functioning appropriately in response to environmental conditions. The uterus acts to further filter spermatozoa, such that sperm may undergo premature acrosome reaction, be affected by the deleterious effects of reactive oxygen molecules, or experience motility failure. The cells that enter in the oviducts attach to the oviductal epithelium. Some of them may fail to detach, decreasing even more the number of cells that reach the cumulus oocyte complex (COC). The COC and the zona pellucida are the last "filters" (De Jonge, 2005).

1.8 Acrosome reaction

The acrosome is a large secretory vesicle that overlies the nucleus in the apical region of the sperm head (Wassarman, 1999; Yanagimachi, 1994b). The acrosomal membrane that underlies the plasma membrane is called "outer" acrosomal membrane and the one that overlies the nucleus is called "inner" acrosomal membrane (Wassarman, 2002). The

mammalian acrosome reaction is the fusion, at multiple points, between the outer acrosomal membrane and the overlying plasma membrane, resulting in the release of acrosomal contents and exposure of the inner acrosomal membrane (Fig. 1.6; reviewed by Kirkman-Brown *et al.* (2002)). The exocytotic process is confined to the anterior region of the sperm head and is not extended beyond the equatorial region. Only acrosome reacted spermatozoa have the capacity to pass through the zona pellucida, bind and fuse with the oocyte. The acrosome reaction results in the release of hydrolytic and proteolytic enzymes, which are thought to be necessary for spermatozoa penetration through the oocyte vestments (Yanagimachi, 1994b). Capacitation is required for sperm to be receptive to oocyte vestments, to bind to the zona pellucida and to acrosome react (Saling and Storey, 1979). Spontaneous acrosome reaction occurs at an extremely low level (Yanagimachi, 1994b), and may be caused by the self aggregation of sperm receptor for the zona pellucida (Saling, 1989). A different hypothesis is that sodium (Na^+) and/or the Ca^{2+} pumping mechanism loose efficiency along time, leading to a gradual elevation in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and intracellular pH, resulting in spontaneous acrosome reaction (Yanagimachi, 1994b). Although the fusion of the outer acrosomal membrane and the overlying plasma membrane in human sperm appears to progress in a distinct way from that of other mammals, the final result is the same (Nagae *et al.*, 1986; Yanagimachi, 1994a). It has been proposed that release of acrosomal enzymes combined with vigorous sperm motility enable sperm penetration through the zona, allowing binding of the sperm to the oocyte plasma membrane (Talbot, 1985).

The egg specific extracellular matrix, the zona pellucida, stimulates acrosomal exocytosis in mammalian spermatozoa, *in vitro* and under physiological conditions (Yanagimachi, 1994b; Saling, 1989). Progesterone and its analogue 17 α-OH-progesterone, a constituent of the follicular fluid, have been shown to stimulate the acrosome reaction in mammalian

spermatozoa (Meizel, 1995; Garcia and Meizel, 1999; Osman *et al.*, 1989). The acrosome reaction can also be stimulated by ionophores, which exchange extracellular Ca^{2+} ions for other ions like Na^{+} and hydrogen (H^{+}; Florman, 1994).

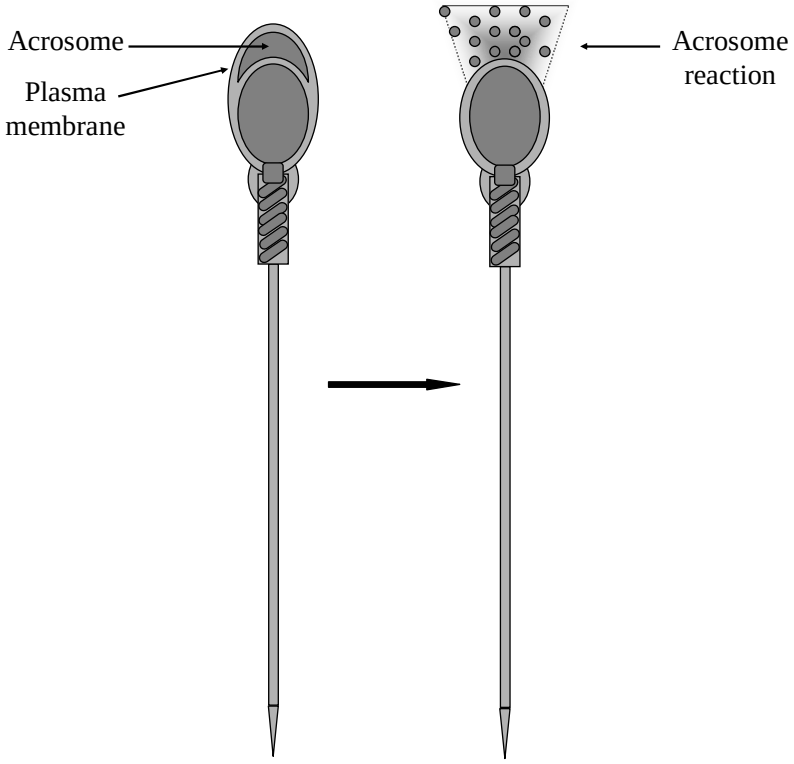

Fig. 1.6. Schematic representation of the acrosome reaction.

1.8.1 Induction of the acrosome reaction by zona pellucida

The complex process of fertilization requires a number of steps in order to be successful. Vital early events include the binding of spermatozoa to the zona pellucida and consequent stimulation of the acrosome reaction (Lefièvre *et al.*, 2004). Experiments, mainly in mouse, indicated that the zona pellucida is composed of three proteins (ZP1, ZP2 and ZP3). The murine zona pellucida is the accepted model for zona pellucida structure in higher vertebrates

and is formed by repeating units of ZP2-ZP3 heterodimers organized in filaments cross-linked by ZP1 dimers (Greve and Wassarman, 1985). Experiments in mouse revealed that ZP3 was the primary sperm ligand (Bleil and Wassarman, 1980a; 1983) and ZP2 was the secondary ligand binding to acrosome-reacted spermatozoa and eliciting important events for the prevention of polyspermy (Bleil *et al.*, 1981; Bleil and Wassarman, 1980b). ZP1 is proposed to contribute to the structural integrity of the zona pellucida matrix (Green, 1997; Greve and Wassarman, 1985; Wassarman, 1999). Recent studies revealed that four glycoproteins (ZP1, ZP2, ZP3 and ZPB) are expressed and present in the human and other zona pellucidae. In the human zona pellucida glycoproteins, ZPB and ZP3 are thought to be required for sperm-zona pellucida binding. Therefore, although the mouse zona pellucida contains three proteins of which ZP3 is the primary spermatozoa receptor, it may be inadequate as a model for sperm-zona interaction in other species (Lefièvre *et al.*, 2004).

ZP3 receptors are localized over the anterior region of the sperm head. A number of proteins have been reported as potential receptors for the zona pellucida. Many putative zona pellucida receptors have already been described (Topfer-Petersen *et al.*, 2000). A number of cellular pathways appear to be involved in the induction of acrosome reaction by zona pellucida. Sperm-ZP3 binding causes the opening of T-type, low voltage-operated Ca^{2+} channels (VOCCs; Arnoult *et al.*, 1996a) leading to a transient Ca^{2+} influx and stimulation of heterotrimeric G proteins, G_{i1} and G_{i2} (Ward *et al.*, 1994). These initial events lead to the activation of phospholipase C (PLC; Fukami *et al.*, 2001; Roldan *et al.*, 1994; Tomes *et al.*, 1996) and a rise in intracellular pH (Arnoult *et al.*, 1996b; Florman *et al.*, 1989), resulting in a sustained Ca^{2+} influx that drives exocytosis (Florman, 1994; Florman *et al.*, 1989). The identity of the channel that mediates the sustained Ca^{2+} influx induced by ZP3 has recently

been investigated. The *Drosophila melanogaster transient receptor potential (TRP)* gene encodes a light-activated cation channel in photoreceptor cells. The members of the classical mammalian TRP (TRPC) family are homologues of this gene and are candidate subunits of PLC-dependent Ca^{2+} entry channels (Minke and Cook, 2002). The linkage mechanisms between PLC action and the opening of TRPC channels are still to be determined. Various TRPC genes are expressed in the mammalian male germ line (Jungnickel *et al.*, 2001; Trevino *et al.*, 2001; Wissenbach *et al.*, 1998) and TRPC2 has been revealed to be a subunit of the sustained Ca^{2+} entry channel in mouse spermatozoa that is activated by ZP3 (Jungnickel *et al.*, 2001). Inositol-1,4,5-triphosphate receptors ($InsP_3Rs$) are present in the sperm acrosome (Walensky and Snyder, 1995) and may contribute to the activation of TRPC2 channels by ZP3. TRPC2 is a pseudogene in humans (Vannier *et al.*, 1999; Wes *et al.*, 1995) and probably in bovine systems (Wissenbach *et al.*, 1998). Another ion channel must assume this function in ZP3 signal transduction in the sperm of those species. Several soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins occur in the acrosomal region of mammalian and sea urchin sperm (Hutt *et al.*, 2002; Michaut *et al.*, 2000; Schulz *et al.*, 1997), and these may couple Ca^{2+} influx to exocytosis (Evans and Florman, 2002). The acrosome reaction is a vital functional event. Prior to exocytosis, capacitated spermatozoa are able to penetrate the cumulus oophorus and adhere selectively to the zona pellucida (Cummins and Yanagimachi, 1986) but the acrosome reaction is an essential step to enable sperm to fuse with the egg plasma membrane (Yanagimachi, 1994b; Florman *et al.*, 1999).

A neuronal glycine receptor/chloride (Cl^-) channel (GlyR) has been identified on mammalian sperm plasma membranes, and there is indication that a GlyR has a role in the initial zona pellucida-evoked response (Llanos *et al.*, 2001). The relationship of the GlyR to the model

involving TRP proteins is uncertain, but a possibility is that the Cl^- efflux mechanism provided by the channel, leads to depolarization and activation of VOCCs (Garcia and Meizel, 1999).

Also, the zona pellucida could act on sperm acrosome reaction through a tyrosine kinase-dependent mechanism (Bailey and Storey, 1994). ZP3 increases the phosphorylation on a tyrosine residue of a 95 kDa protein, which has a tyrosine kinase activity itself, and this has been proposed to be involved in the zona pellucida action on human sperm acrosomal exocytosis (Brewis *et al.*, 1998; Burks *et al.*, 1995).

1.8.2 Induction of the acrosome reaction by progesterone

In mammalian sperm, the steroid hormone progesterone has been shown to stimulate Ca^{2+} influx and acrosome reaction (Aitken, 1997; Osman *et al.*, 1989). This effect is seen at micromolar doses of progesterone, similar to those in the cumulus oophorus (Osman *et al.*, 1989), and is thus expected to have significance during fertilization *in vivo* (Fisher *et al.*, 1998; Garcia and Meizel, 1999). Progesterone-stimulated $[Ca^{2+}]_i$ signal has been well studied in human spermatozoa. Application of micromolar doses of progesterone leads to a rapid transient rise in $[Ca^{2+}]_i$ (Aitken *et al.*, 1996; Blackmore *et al.*, 1990; Foresta *et al.*, 1993; Kirkman-Brown *et al.*, 2000; Meizel and Turner, 1991; Plant *et al.*, 1995; Tesarik *et al.*, 1996). A role for VOCCs in this effect is unclear (Blackmore and Eisoldt, 1999; Fraire-Zamora and Gonzalez-Martinez, 2004; Garcia and Meizel, 1999; Kirkman-Brown *et al.*, 2003) though it seems likely that receptor-operated mechanisms and opening of a nonspecific cation channel are responsible for the majority of this initial signal. Recent results suggest that

mobilization of stored Ca^{2+} contributes to the later slow component of the initial $[Ca^{2+}]_i$ transient (Bedu-Addo *et al.*, 2007). In addition to the initial transient, a subsequent sustained rise in $[Ca^{2+}]_i$ occurs (Baldi *et al.*, 1991; Bonaccorsi *et al.*, 1995; Kirkman-Brown *et al.*, 2000; Tesarik *et al.*, 1996; Yang *et al.*, 1994). These studies suggested that store-operated Ca^{2+} channels (SOCs) may play a role in generating this influx, but this is still not established (Blackmore and Eisoldt, 1999; Harper *et al.*, 2004). Progesterone withdrawal results in immediate abolition of many of the responses; for example, if progesterone is removed as the $[Ca^{2+}]_i$ transient peaks, an almost mirrored reduction in Ca^{2+} below baseline occurs, possibly due to increased activity of Ca^{2+} removal systems (Kirkman-Brown and Connolly, unpublished data).

As a consequence of this $[Ca^{2+}]_i$ increase, hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) to the second messengers inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG) occurs (Thomas and Meizel, 1989). $InsP_3$ can subsequently trigger Ca^{2+} release from intracellular stores by acting on an $InsP_3R$/ Ca^{2+} channel (Fukami *et al.*, 2003). Type 1 $InsP_3Rs$ are primarily localized on the outer acrosomal membrane of human sperm (Kuroda *et al.*, 1999b), thus $[Ca^{2+}]_i$ release takes place into the cytoplasmic compartment between the acrosomal membrane and the plasma membrane. DAG may play various roles, including activation and regulation of plasma membrane channels as those of the TRP family (Clapham *et al.*, 2005). Studies indicate that acrosome reaction requires emptying of an acrosomal Ca^{2+} store (De Blas *et al.*, 2002; Herrick *et al.*, 2005). In murine systems, this will be a response to $InsP_3$ generated due to zona binding (Evans and Florman, 2002; O'Toole *et al.*, 2000; Roldan *et al.*, 1994; Walensky and Snyder, 1995). It is not clear whether this operates to generate progesterone-induced acrosome reaction in humans. Sperm from $PLC\delta4^{-/-}$ mice exposed to zona pellucida or progesterone do not show the sustained $[Ca^{2+}]_i$ increase, which is essential

for acrosome reaction (Fukami *et al.*, 2003). Simultaneous activation of phospholipase A2 is also vital to murine acrosome reaction, and is accomplished through mutual G-protein regulation (Pietrobon *et al.*, 2005). Moreover, control results from wild-type sperm with zona pellucida or progesterone showed that the initial $[Ca^{2+}]_i$ response initiated from the acrosomal region for zona pellucida and from the neck region for progesterone, suggesting acrosome reaction stimulation via distinct propagation mechanisms (Fukami *et al.*, 2003). It is not yet known whether this also happens in humans and how this links to the proposed RNE Ca^{2+} storage site (Correia *et al.*, 2007).

Subsequent signalling processes in human spermatozoa seem to involve protein kinase C (PKC)-mediated phosphorylation (Roggero *et al.*, 2005), guanine triphosphate-binding proteins and cAMP (Branham *et al.*, 2006). For more information, see Tomes *et al.* (2002; 2004).

As in other cells presenting rapid activation in response to steroids, the identity of the membrane progesterone receptor (mPR) in sperm is uncertain. Whether these membrane receptors correspond to truncated forms of previously characterized classical intracellular steroid receptors or are a totally novel class of receptors is disputed. Although the nature of mPR on sperm remains ambiguous, a number of possible candidates have been proposed (Correia *et al.*, 2007). Losel *et al.* (2004) have recently localized the progesterone membrane receptor component 1 (PGRMC1) to the inner leaflet of the plasma membrane of porcine sperm, with only a minor part of the protein protruding to the outside. As a highly lipophilic molecule, progesterone could access this site. PGRMC1, progesterone membrane receptor component 2 (PGRMC2), and the classical nuclear progesterone receptor can be detected in human spermatozoa (Losel *et al.*, 2005).

A number of laboratories argue for the binding of progesterone to a form of the neuronal gamma-aminobutyric acid type A ($GABA_A$) receptor (Roldan *et al.*, 1994; Shi and Roldan, 1995; Turner and Meizel, 1995; Wistrom and Meizel, 1993). Picrotoxin, a blocker of the $GABA_A/Cl^-$ channel, significantly reduced the magnitude of the Ca^{2+} influx, abolished the Ca^{2+} wave and inhibited the acrosome reaction (Calogero *et al.*, 1999; Meizel *et al.*, 1997). Bicuculline, an antagonist of the $GABA_A$ receptor, blocks the acrosome reaction (Calogero *et al.*, 1999). However, not every study strongly supports the action of progesterone via a $GABA_A$ receptor. Baldi *et al.* (1991) reported that bicuculline had no effect on the progesterone-induced Ca^{2+} influx or acrosome reaction. The classical $GABA_A$ receptor does not present a clear binding site for progesterone, thus if this is a candidate for the receptor, then either the binding activity is closely associated with the receptor or a novel form of the $GABA_A$ receptor exists in sperm (Correia *et al.*, 2007).

A family of G protein-coupled mPRs were identified, cloned and characterized from humans and other vertebrates. This was believed to be a major step forward, with a gene family comprised of three different groups classified α, β and γ, with forms through *Xenopus* and *Fungu* to human (Zhu *et al.*, 2003). Particularly, mPRα was abundantly found in the testis, suggesting that it was a sperm progesterone receptor. Although it was initially shown that the human α, β and γ mPR had high affinity for progesterone when expressed in *Escherichia coli*, recent studies with expression in mammalian cell systems showed no progesterone-binding activity. These studies also revealed that mPRs failed to localize to the membrane, activate cAMP generation or mitogen-activated protein kinase (MAPK) as proposed, and had no effect on $[Ca^{2+}]_i$ mobilization. Opposing data on human myometrial cells appear to demonstrate that mPRα and mPRβ signal and couple to G proteins (Krietsch *et al.*, 2006).

As there are various candidates for the sperm progesterone receptor and the signalling pathways reported to be activated by the action of progesterone are diverse, the presence of multiple, structurally and functionally discrete species of progesterone receptors within or on spermatozoa can not be excluded. On the contrary, the possibility that sperm progesterone membrane receptors may have attributes of two or more of the candidate receptors, within a common molecule or complex should not be excluded (Correia *et al.*, 2007).

The most commonly observed effect of progesterone-stimulated Ca^{2+} influx, the induction of acrosome reaction, is debatably not an adaptive response. Human sperm respond with a rise in $[Ca^{2+}]_i$ and acrosome react to doses much lower than the 1-10 µmol/l that is thought to occur adjacent to the oocyte (Baldi *et al.*, 1991; Harper *et al.*, 2003; Osman *et al.*, 1989). Thus, many cells should undergo acrosome reaction before they enter the cumulus, possibly compromising their capacity to subsequently penetrate the zona pellucida (Harper and Publicover, 2005).

1.9 Motility

Most mammalian sperm exhibit two forms of physiological motility: activated motility as is observed in ejaculated sperm, and hyperactivated motility as is observed in most sperm recovered from the fertilization site (Katz and Yanagimachi, 1980; Suarez and Osman, 1987). The flagellum of an activated sperm generates a symmetrical, lower-amplitude waveform that drives spermatozoa in a relatively straight line in moderately non-viscous media, such as seminal plasma or semen extender. It is probable that activated motility helps in the propulsion of the sperm through the female reproductive tract (Turner, 2006).

Some time after sperm reach the oviduct, the pattern of the flagellar beat is altered, becoming asymmetric and of a higher amplitude. This is called 'hyperactivated motility' and results in a circular or figure-8 trajectory in spermatozoa suspended in seminal plasma or extender (Yanagimachi, 1970; 1994b; Ishijima *et al.*, 2002). It was showed that hyperactivated sperm swim in a relatively straight line when placed in the more viscous environment of the oviduct. The flagellar beat of activated spermatozoa is insufficient to propel the sperm progressively through the oviduct. It has been suggested that the physiological role of hyperactivated motility is to enable sperm to detach from the oviductal epithelium and move progressively through the oviductal environment to reach the fertilization site. Also, sperm hyperactivated motility may aid to penetrate the egg vestments (Ho and Suarez, 2001a; Stauss *et al.*, 1995; Suarez *et al.*, 1991). In rodents, and possibly other species, hyperactivated sperm motility is correlated with the sperm capacity to fertilize an oocyte *in vitro* (Boatman and Robbins, 1991; Fraser and Quinn, 1981). The available data suggest that a normal sperm must be capable to acquire activated motility in the caudal female reproductive tract and uterus, and hyperactivated motility in the oviduct so that it has a reasonable chance of fertilizing the egg. Male fertility will be significantly reduced if these events do not occur at the proper times and in the proper places. Hyperactivation is regularly seen in association with the onset of capacitation, but it has been shown that these pathways are divergent as hyperactivation and capacitation can also occur independently (Marquez and Suarez, 2004; Olds-Clarke, 1989).

1.9.1 Mechanism of flagellar bending and energy requirements

Dyneins are the 'motor' proteins that project from the outer microtubular doublets of the axoneme. There are several dyneins that are all part of a protein family (Holzbaur and Vallee, 1994; Milisav, 1998; Porter and Johnson, 1989). Activation of the dynein ATPase causes

sliding of adjacent outer axonemal microtubular doublets. Because the doublets slide along one another, the flagellum bends (Gibbons and Rowe, 1965; Tash and Means, 1982).

In spermatozoa, mitochondria exist only in the MS of the mid-piece. As in other cells, sperm mitochondria generate ATP through aerobic respiration. Sperm mitochondria have a number of unique proteins or protein isoforms that are not found in somatic mitochondria. In mice, these include sperm-specific isoforms of lactate dehydrogenase and hexokinase (Bunch *et al.*, 1998; Burgos *et al.*, 1995; Mori *et al.*, 1998; Travis *et al.*, 1998). Therefore, defects in these sperm-specific proteins may cause problems only in sperm mitochondrial function, rather than mitochondrial dysfunction in every cell (Turner, 2006).

Spermatozoa axonemal dyneins have a high requirement for ATP as an energy source for flagellar motility. Since sperm mitochondria are exclusive to the mid-piece while axonemal motor extends throughout the length of the flagellum, mitochondrial ATP would have to diffuse some distance to effectively supply the full length of the axoneme. Mathematical models have been developed using the diffusion constant of ATP and a morphometric estimate of the volume of the mouse sperm flagellum (Du *et al.*, 1994), and suggest that the ATP generated in the mid-piece is not enough to diffuse efficiently along the length of the tail to satisfy the energy requirements of the axonemal dynein ATPase (see Turner (2006)). It has been shown in mice that if mitochondrial oxidative phosphorylation is defective, fertilization can still occur, spermatozoa generate ATP, but at lower levels than wild-type sperm, and motility is present, though reduced (Narisawa *et al.*, 2002). These results suggest that mitochondrial oxidative phosphorylation is not the principal ATP source, which supports flagellar motility (Turner, 2006).

It seems that glycolysis is accomplished along the length of the principal piece and that this is the most significant source of ATP for the tail. A number of glycolytic enzymes such as hexokinase, lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase have been identified in the FS/principal piece of an increasing number of mammalian species (Bradley *et al.*, 1996; Bunch *et al.*, 1998; Mori *et al.*, 1998; Travis *et al.*, 1998; Westhoff and Kamp, 1997). All the glycolytic enzymes downstream of glyceraldehyde-3-phosphate dehydrogenase stay attached to the cytoskeleton, even after membrane removal, suggesting they are components of either the FS or the ODFs. Also, mammalian spermatozoa produce lactate from glucose under aerobic conditions (Storey and Kayne, 1975). ATP production through glycolysis is needed for hyperactivated sperm motility (Hoshi *et al.*, 1991; Urner and Sakkas, 1996). Fertilization is not blocked by inhibition of oxidative phosphorylation (Fraser and Quinn, 1981). Studies of knockout mice demonstrated the importance of FS/principal piece glycolysis. Targeted deletion of the sperm-specific glyceraldehyde-3-phosphate dehydrogenase, an enzyme that localises to the FS, causes male sterility in association with severe aberrations in sperm motility (Miki *et al.*, 2004). Therefore, glycolysis in the principal piece, but not necessarily oxidative phosphorylation in the mid-piece, is needed for normal mammalian flagellar motility (Turner, 2006).

The dynein ATPase is activated and the hydrolysis of ATP is converted into force, when the axonemal dynein arms become phosphorylated (Tash, 1989). The dynein arms subsequently interact with their adjacent microtubular doublets and produce a power stroke, causing the microtubules to slide past one another (Brokaw, 1972; 1989; Satir, 1968; Shingyoji *et al.*, 1977; Summers and Gibbons, 1971). As the axoneme is connected to the base of the sperm head, the sliding force results in the bending of the flagellum. Dynein dephosphorylation by

the calmodulin (CaM)-dependent protein phosphatase calcineurin reverses this process. Dynein generates force in just one direction (Sale and Satir, 1977). Therefore, the occurrence of a normal axonemal bend needs that phosphorylation/dephosphorylation and the associated activation/inactivation of the dynein arms take place in an asynchronous way both around the circumference and along the length of the axoneme (Wargo and Smith, 2003). Each dynein arm interacts with its adjacent doublet, generates a stroke to force that doublet to bend, and subsequently liberates the doublet for the axoneme to return to its initial position and to bend again in the opposite direction (Turner, 2006).

1.9.2 Regulation of motility

The cAMP-dependent phosphorylation of flagellar proteins is, at least partly, responsible for the initiation and maintenance of activated sperm motility in mammals (San Agustin and Witman, 1994; Tash and Means, 1982; 1983). Male infertility, which is highly correlated with poor sperm motility, is one of the phenotypes seen in mice with a targeted deletion of the sperm-specific isoform of the catalytic subunit of the cAMP-dependent serine/threonine kinase, protein kinase A (PKA; Skalhegg *et al.*, 2002). Therefore, it is likely that one mechanism of cAMP action is to have an effect on sperm motility through PKA activation (Turner, 2006). PKA may work through multiple pathways to regulate flagellar function, but one likely mechanism of its action is that serine/threonine phosphorylation of PKA target proteins causes activation of downstream, yet unidentified, tyrosine kinase or kinases whose targets are primarily located in the flagellum (Lawson *et al.*, 2008; Leclerc *et al.*, 1996; Si and Olds-Clarke, 2000). Tyrosine phosphorylation of a specific subset of proteins in the flagellum results in motility. Only a few of the protein targets for PKA phosphorylation in spermatozoa have been identified (Tash and Bracho, 1998). A known target is axonemal dynein and its

phosphorylation seems to be a key regulatory point in the initiation of flagellar motility (Tash, 1989).

cAMP may also activate other signalling pathways in spermatozoa and testes, comprising a CNG channel and/or cAMP-mediated guanine nucleotide exchange factors (Burton *et al.*, 1999).

In addition, tyrosine phosphorylation is strongly associated with the onset of sperm motility (Tash and Bracho, 1998) and is likely to be downstream of serine/threonine phosphorylation in the motility regulation pathway. Various specific protein targets of tyrosine phosphorylation in spermatozoa have been suggested and phosphorylation of these proteins has been strongly related to the onset of motility in bovine sperm (Vijayaraghavan *et al.*, 1997; 2000).

Tyrosine phosphorylation and dephosphorylation of proteins in the flagellum has been respectively related to the onset and ending of hyperactivated sperm motility in primates and rodents (Chan *et al.*, 1998; Mahony and Gwathmey, 1999; Si and Okuno, 1999). The A-kinase anchor protein (AKAP) 4 is one of the phosphotyrosine containing proteins (Si, 1999). The AKAPs form a family of proteins that target PKA and other proteins to specific subcellular locations (Scott *et al.*, 2000). These results suggest that alterations in AKAP-mediated protein targeting are involved in the control of hyperactivated sperm motility (Turner, 2006).

Extracellular Ca^{2+} is generally needed for motility in epididymal sperm samples and Ca^{2+} appears to regulate activated and hyperactivated motility (Ho *et al.*, 2002; Lindemann and Goltz, 1988; Suarez *et al.*, 1987; Tash and Means, 1987; White and Aitken, 1989;

Yanagimachi, 1994b). One mechanism by which Ca^{2+} is related to flagellar function is through its control of the sAC, which produces cAMP to activate PKA. Sperm motility requires the sAC, which differs molecularly and biochemically from the transmembrane adenylyl cyclases (tmACs), partly because sAC is uniquely sensitive to bicarbonate and Ca^{2+} (Buck *et al.*, 1999; Chen *et al.*, 2000; Esposito *et al.*, 2004; Hess *et al.*, 2005b; Jaiswal and Conti, 2003; Liguori *et al.*, 2004; Litvin *et al.*, 2003; Wuttke *et al.*, 2001).

How spermatozoa effect a Ca^{2+} increase within the flagellum is not totally elucidated. Intracellullar Ca^{2+} stores may be involved, particularly in hyperactivated motility regulation. In addition to the acrosome, the RNE of the sperm neck has been implicated as a resource of intracellullar flagellar Ca^{2+} (Ho and Suarez, 2003).

There is significant evidence to propose that another way in which sperm affect changes in intracellular Ca^{2+} levels is the passage of extracellular Ca^{2+} through one or more channels in the membrane (Quill *et al.*, 2003; Ren *et al.*, 2001; Sakata *et al.*, 2002; Wennemuth *et al.*, 2000; Westenbroek and Babcock, 1999; Wiesner *et al.*, 1998). A number of VOCC α1-subunits have been identified in spermatozoa (Lievano *et al.*, 1996; Wennemuth *et al.*, 2000; Westenbroek and Babcock, 1999) and Ca^{2+} channel activity has been discovered in late-stage spermatogenic cells and mature spermatozoa (Arnoult *et al.*, 1996a; 1998; 1999; Benoff, 1998; Wennemuth *et al.*, 2000). VOCCs are present on the sperm membrane, being involved in the control of the acrosome reaction (Evans and Florman, 2002). Various Ca^{2+} channel subunits specifically localise to the principal piece of the flagellum, consistent with their playing roles in the control of sperm motility (Ren *et al.*, 2001; Westenbroek and Babcock, 1999).

CNG Ca^{2+} channels exist on the sperm flagellum and developing spermatogenic cells. Distinct subunits of CNG Ca^{2+} channels exist in distinct spatial and temporal patterns in spermatozoa.

Spermatozoa CNG channels respond to cAMP and cyclic guanosine monophoshate (cGMP; Wiesner *et al.*, 1998). Sperm CNG channels seem most sensitive to cGMP, but their response to cAMP suggests that this second messenger could be related to both of the key motility regulating pathways, PKA and Ca^{2+} signalling (Turner, 2006).

There is other evidence supporting a role for extracellular Ca^{2+} in mammalian sperm motility. Flagellar wave asymmetry in permeabilised sperm is increased by Ca^{2+}, which eventually, at sufficiently high levels, inhibits motility (Tash and Means, 1982). The decline in motility is linked with a decrease in protein phosphorylation. CaM and the Ca^{2+}/CaM–dependent phosphatase calcineurin mediate this decrease in phosphorylation (Tash *et al.*, 1988; Tash and Means, 1987).

The CatSper1 gated cation channel specifically localises to the principal piece of the flagellum of mature spermatozoa and is needed for cAMP-stimulated Ca^{2+} influx into sperm. Targeted deletion of the *CatSper1* gene causes ablation of the cAMP-induced increase in intracellular Ca^{2+} and causes male infertility associated with poor sperm motility (Ren *et al.*, 2001). CatSper2, a related voltage-gated putative Ca^{2+} channel has been described in the sperm flagellum (Quill *et al.*, 2001). Targeted deletion of the *CatSper2* gene causes male infertility associated with an absence of hyperactivated sperm motility (Quill *et al.*, 2003). Therefore, both forms of motility require channel-mediated movement of extracellular Ca^{2+} into the flagellum (Turner, 2006).

Mice with a targeted deletion of an unrelated voltage-dependent Ca^{2+} channel, $Ca_v2.3$ (α_{1E}), were fertile but demonstrated abnormalities in sperm intracellular Ca^{2+} transients and differences in sperm linearity in comparison with wild-type cells, further indicating that membrane channels are significant in the control of flagellar function (Sakata *et al.*, 2002).

Interestingly, a progesterone gradient simulating the stimulus met by the spermatozoa during approach to the egg stimulated a response different to any previously described in human or other mammalian spermatozoa. Cells responded with novel and complex changes in $[Ca^{2+}]_i$ and flagellar activity, generated by an atypical mechanism. Sperm responded with a $[Ca^{2+}]_i$ ramp that triggered slow $[Ca^{2+}]_i$ oscillations (Harper *et al.*, 2004). These studies led to observations compatible with a role for cyclical store emptying/refilling in the generation of $[Ca^{2+}]_i$ oscillations in human sperm, but depending on Ca^{2+} influx to maintain the minimum $[Ca^{2+}]_i$ needed to refill the store, as is regularly the case in somatic cell $[Ca^{2+}]_i$ oscillations (Gregory and Barritt, 2003; Shuttleworth and Mignen, 2003). There was no evidence that $InsP_3Rs$ participate in the generation of $[Ca^{2+}]_i$ oscillations in human spermatozoa, but pharmacological manipulations that affect ryanodine receptors (RyRs) were clearly effective. Although RyRs were not detected in bovine sperm (Ho and Suarez, 2001b), RyR 3 has been detected in mouse spermatozoa and RyR 1 in immature cells (Trevino *et al.*, 1998). Immunolocalisation using specific polyclonal antibodies for RyR 1 and RyR 2 revealed the presence of these receptors in the neck and mid-piece region of human sperm (Lefièvre, unpublished data). A temporary progesterone stimulus is able to switch cells to a prolonged and apparently irreversible pattern of $[Ca^{2+}]_i$ oscillations, consistent with a model where Ca^{2+}-induced Ca^{2+} release (CICR), mediated by RyRs, is enough for repetitive store mobilization resulting in $[Ca^{2+}]_i$ oscillations in human spermatozoa (Harper *et al.*, 2004). During spermiogenesis in many species, including humans (Kerr, 1991; Westbrook *et al.*, 2001), a membrane complex named the RNE originates in the sperm neck region. The RNE is thought to function as a Ca^{2+} store in bovine sperm and to regulate the flagellar beat (Ho and Suarez, 2001b; 2003). Both the binding of BODIPY-FL-X-ryanodine (a membrane-permeant fluorescein-tagged derivative; Coussin *et al.*, 2000; Holz *et al.*, 1999) and $[Ca^{2+}]_i$ oscillations

in human sperm appear to occur primarily in the postacrosome region /mid-piece junction. $[Ca^{2+}]_i$ oscillations were not dependent upon mitochondrial Ca^{2+} uptake, and thus it is probable that the RNE functions as a Ca^{2+} store in human sperm, contributing to $[Ca^{2+}]_i$ oscillations. These oscillations were synchronized with movements of the sperm head driven by enhancement of flagellar activity during the periods of high $[Ca^{2+}]_i$. This study led to the conclusion that the progesterone-induced $[Ca^{2+}]_i$ oscillations modulate the flagellar beat, an effect potentially of enormous significance in the penetration of the egg vestments before fertilization (Harper *et al.*, 2004). Apparently, Ca^{2+} re-uptake during oscillations is, at least partly, dependent on the activity of the secretory pathway Ca^{2+} ATPase 1 (SPCA1; Harper *et al.*, 2005).

1.10 Sperm guidance mechanisms

For mammalian fertilization to occur, ejaculated sperm must reach the oocyte, which has moved from the ovary into the Fallopian tube after ovulation. Only tiny numbers of sperm enter the oviduct (Fallopian tube), attach to the oviductal epithelium at the isthmus (part nearer to the uterus), creating there a sperm storage site (Suarez, 2002). At this site, sperm, a few at a time (Cohen-Dayag *et al.*, 1995; Eisenbach, 1999), are believed to undergo capacitation, which is to gain the status of readiness to fertilize the oocyte. These few sperm are released from the storage site and have to be guided to make the remaining long and obstructed way to the oocyte (see Eisenbach and Giojalas (2006) for a recent review). Two active mechanisms of sperm guidance have been revealed in mammals: chemotaxis, which is the cell movement up a concentration gradient of chemoattractant (for an extensive review see Eisenbach (2004)), and thermotaxis, the directed cell movement along a temperature gradient

(Bahat *et al.*, 2003). Another potential guidance mechanism might be the pull of sperm by muscular contractions in the female genital tract (Wildt *et al.*, 1998).

Recent studies indicate that sperm chemoattractants are secreted both before ovulation within the follicle and after egg maturation outside the follicle, and that the sources of chemoattractants are both the mature egg and the surrounding cumulus cells (Sun *et al.*, 2005).

The chemoattractant that is secreted from the egg is not known. One chemoattractant that is secreted by the cumulus cells is possibly progesterone. It is not known whether the cumulus cells secrete chemoattractants other than progesterone (Eisenbach and Giojalas, 2006). Two odorants, bourgeonal (Spehr *et al.*, 2003) and lyral (Fukuda *et al.*, 2004), were found to be chemoattractants for human and mouse spermatozoa, respectively. Because these chemoattractants are possibly not secreted in the female genital tract, it is likely that they are not the physiological sperm chemoattractants and that their cognate receptors identify other, structurally similar chemoattractants (Eisenbach and Giojalas, 2006).

The finding of olfactory receptors coupled to G proteins in mammalian sperm (Asai *et al.*, 1996; Branscomb *et al.*, 2000; Defer *et al.*, 1998; Gautier-Courteille *et al.*, 1998; Parmentier *et al.*, 1992; Vanderhaeghen *et al.*, 1993; 1997; Walensky *et al.*, 1995; 1998), and their localisation to the mid-piece of the tail of mature sperm (Spehr *et al.*, 2004; Walensky *et al.*, 1995), raised the possibility that some of these proteins might be chemotaxis receptors (Parmentier *et al.*, 1992; Vanderhaeghen *et al.*, 1997). Two olfactory receptors, OR17-4 on the human sperm flagella (Spehr *et al.*, 2003), and OR23 in mouse round spermatids (Fukuda *et al.*, 2004), were identified and their respective ligands, bourgeonal and lyral, were found to be sperm chemoattractants. Human sperm stimulated with bourgeonal show an intracellular Ca^{2+} elevation (Spehr *et al.*, 2003), which is initiated in the mid-piece and extends to the

sperm head. The discovery of membrane-associated adenylyl cyclase (mAC) isoforms on the sperm flagellum and the observation that a specific antagonist of this enzyme inhibited the chemotactic response to bourgeonal raised the possibility that the chemotactic response to bourgeonal might be mediated by mAC (Spehr *et al.,* 2004).

Progesterone at doses in the pM range is a chemoattractant for human and rabbit sperm (Teves *et al.*, 2006). One of the progesterone receptors is located at the sperm head, but it is not known whether this is the chemotaxis receptor or whether the progesterone chemotaxis receptor is a distinct protein located on the flagellum, similar to OR17-4. The signal transduction pathway that occurs during chemotaxis to progesterone has not been clarified (Eisenbach and Giojalas, 2006).

The fertilization site in the oviduct has been revealed to be 1-2°C warmer than the storage site for sperm in rabbits (David *et al.*, 1972) and in pigs (Hunter and Nichol, 1986). Though, data for the temperature in the human Fallopian tube are not available. A study in rabbits revealed that this temperature difference is time dependent and that the difference increases from 0.8 ± 0.2°C prior to ovulation to 1.6 ± 0.1°C following ovulation (Bahat *et al.*, 2005). Rabbit and human sperm sense a temperature difference and respond to it by thermotaxis, swimming from the cooler to the warmer temperature (Bahat *et al.*, 2003). The thermotactic response is as strong at a temperature difference of 0.5°C as at a difference of 2°C, indicating that sperm can sense very small differences in temperature. Though, as the distance between the storage and the fertilization sites is much bigger than the distance employed in the *in vitro* thermotaxis assay (Bahat *et al.*, 2003), the gradient of temperature that is sensed by sperm *in vivo* is possibly shallower. It is still unknown whether sperm can sense as shallow a temperature gradient as the one that exists in the oviduct. As for mammalian chemotaxis, only

capacitated sperm are thermotactically responsive (Bahat *et al.*, 2003). The sperm thermotaxis molecular mechanism is still unknown (Eisenbach and Giojalas, 2006).

In vivo, thermotaxis may be complementary to chemotaxis, each mechanism being functional where the other mechanism is not effective. Between the isthmic sperm reservoir and the fertilization site, a gradient of temperature exists at ovulation (Bahat *et al.,* 2005) and long-range chemotaxis may not occur because of a mixing effect of the peristaltic movements of the oviduct. In this region, it is reasonable that thermotaxis is the main guidance mechanism, because those movements are not expected to affect the temperature gradient. In the immediate vicinity of the oocyte, including the viscous milieu of the cumulus cells surrounding the oocyte, the contrary seems to occur: a measurable temperature gradient can not possibly be maintained, and a chemoattractant gradient appears to be very efficient due to the relatively small distance and the viscoelastic milieu of the cumulus that resists the movements of the oviduct (Cohen-Dayag *et al.*, 1994). Thus, it is sensible that, *in vivo*, capacitated sperm are guided by thermotaxis from the reservoir to the warmer fertilization site. The oviductal contractions, which move liquid from the isthmus to the ampulla (Battalia and Yanagimachi, 1979), may assist the swimming sperm. The guidance is likely accomplished by chemotaxis at close proximity to the oocyte and within the cumulus mass (Bahat and Eisenbach, 2006).

1.11 Ca^{2+} signalling in sperm

Though there is little doubt that sperm use a range of cell messengers, it has been revealed that $[Ca^{2+}]_i$ also plays a key role in all important sperm functions that occur following ejaculation (Jimenez-Gonzalez *et al.,* 2006). Based on some of the data summarized by

Conner *et al.* (2007), an intricate model for sperm Ca^{2+}-homeostasis involving a number of types of Ca^{2+} permeable channels in the plasma membrane and at least two stores is suitable (Conner *et al.*, 2007). In addition to a range of VOCCs, which are clearly located to sperm regions, the CatSpers, which are crucial for activation of hyperactivated motility, are specifically expressed in the principal piece of the tail, as is the plasma membrane Ca^{2+}-ATPase (PMCA) 4. It seems that the acrosome functions as an $InsP_3$-releasable store, activated by agonists associated with PLC. Recent studies suggest mobilization of acrosomal Ca^{2+} is involved in acrosome reaction activation (De Blas *et al.*, 2002; Herrick *et al.*, 2005). A distinct store, possibly the RNE, is present in the sperm neck region and has a crucial role in the regulation of flagellar beat mode. However, more work needs to be done to further understand the interrelatedness of these mechanisms (Conner *et al.*, 2007).

1.11.1 VOCCs

VOCCs, similar to those of somatic cells, have been discovered in mature and immature spermatozoa (Arnoult *et al.*, 1996a; Jimenez-Gonzalez *et al.*, 2006). Patch clamping of immature male germ cells (rodent and human) has revealed that these cells exhibit typical low-voltage activated, fast-inactivating (T-type) currents (Arnoult *et al.*, 1996a; Jagannathan *et al.*, 2002). Transcripts for α 1C [Cav1.2], α 1B [Cav2.2], α 1E [Cav2.3], α 1G [Cav3.1], α 1H [Cav3.2] and α 1I [Cav3.3] Ca^{2+} channels are present in motile human sperm (Park *et al.*, 2003). Immunostaining experiments using specific antibodies revealed that in human sperm, α 1H [Cav3.2] is located to the principal piece of the flagellum and in the postacrosome region, and α 1I [Cav3.3] is limited to the mid-piece region (Trevino *et al.*, 2004). High voltage activated channels show similar localization in mouse and human spermatozoa (Trevino *et al.*, 1998; 2004; Wennemuth *et al.*, 2000; Westenbroek and Babcock, 1999). L-type channels

have been detected in every study using reverse transcription-polymerase chain reaction (RT-PCR) and/or immunostaining techniques (Goodwin *et al.*, 2000).

1.11.2 SOCs

Mobilization of intracellular Ca^{2+} stores is thought to activate Ca^{2+}-permeable ion channels (SOCs) in the plasma membrane – capacitative Ca^{2+} entry – in both non-excitable and excitable cells (Putney, 1990). Pharmacological mobilization of stored Ca^{2+} activates Ca^{2+} influx in non-capacitated human spermatozoa (Blackmore, 1993). This Ca^{2+} influx generates a sustained $[Ca^{2+}]_i$ elevation that is believed to cause acrosome reaction in both mammalian and non-mammalian sperm (Gonzalez-Martinez *et al.*, 2001; Hirohashi and Vacquier, 2003; O'Toole *et al.*, 2000) and regulates chemotaxis in ascidian sperm (Yoshida *et al.*, 2003). In mouse sperm, TRP2 may be a required component of the channel responsible for the sustained $[Ca^{2+}]_i$ elevation (triggered by ZP3) that causes acrosome reaction, which could be regulated either by the depletion of intracellular Ca^{2+} stores (O'Toole *et al.*, 2000) or through receptor activation (Harteneck *et al.*, 2000).

1.11.3 CNG channels and CatSpers

Cyclic nucleotide signalling is very important in the functioning of all sperm. In invertebrate cells, cGMP is of significance in the control of motility and acrosome reaction (Kaupp *et al.*, 2003). In mammalian sperm (including humans), cGMP levels are a lot lower and cAMP seems to be of greater significance (Ain *et al.*, 1999; Lefièvre *et al.*, 2000). Manipulation of cAMP and cGMP levels in mouse sperm, stimulated a transient $[Ca^{2+}]_i$ increase, lasting 20-60 seconds, which was apparently caused by Ca^{2+} influx through CNG channels (Kobori *et al.*,

2000). A CNG Ca^{2+} permeable channel is expressed in sperm, existing in the principal piece of the flagellum, being more sensitive to cGMP than to cAMP (Weyand *et al.*, 1994; Wiesner *et al.*, 1998). Olfactory receptors, which activate a cyclic nucleotide-mediated Ca^{2+} influx and control chemotactic activity, have been described in human spermatozoa (Spehr *et al.*, 2003).

CatSpers form a novel family of ion channels, expressed solely in spermatozoa. Four distinct subunits have been identified: CatSper 1 (Ren *et al.*, 2001), CatSper 2 (Quill *et al.*, 2001) and CatSpers 3 and 4 (Lobley *et al.*, 2003). In mature cells, CatSper 2 is located to the sperm tail (Quill *et al.*, 2001) and CatSper 1 to the principal piece of the tail (Ren *et al.*, 2001), suggesting that these channels may be involved in the regulation of sperm motility. Initial studies suggest that the expression of CatSper is reduced in human sperm with poor motility (Nikpoor *et al.*, 2004). CatSper knockout mice studies revealed that in mutant sperm lacking CatSper 1, motility was severely reduced and consequently, sperm were not able to fertilize (Ren *et al.*, 2001). Moreover, there was no increase in flagellar $[Ca^{2+}]_i$ upon addition of cell-permeant cAMP/cGMP in CatSper 1-/- cells, suggesting that the channel might be cAMP-gated. Carlson *et al.* (2003) and Quill *et al.* (2003) subsequently confirmed the significance of CatSper 1 and CatSper 2 in sperm motility (more specifically in the hyperactivated motility needed to penetrate the zona). It was also revealed that CatSper 1 is needed for depolarisation-evoked Ca^{2+} entry, suggesting that CatSper 1 functions as a VOCC (assisted by cyclic nucleotides) that regulates hyperactivation (Carlson *et al.*, 2003).

1.11.4 Ca^{2+} clearance mechanisms

In most cells, Ca^{2+} clearance is carried out primarily by Na^{+}-Ca^{2+} exchangers that extrude Ca^{2+} out of the cell or into intracellular Ca^{2+} stores, or ATP requiring Ca^{2+} pumps (Ca^{2+}-

ATPases; Michelangeli *et al.*, 2005). Analysis of the Ca^{2+} clearance in mouse spermatozoa suggests that both Ca^{2+} exchangers and Ca^{2+} pumps are important contributors to Ca^{2+} clearance in mammalian sperm, but the relative importance of each is still to be established (reviewed by Jimenez-Gonzalez *et al.* (2006)). Three types of ATP requiring Ca^{2+} pumps have been identified: 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; Toyoshima and Inesi, 2004).

1.11.5 $InsP_3Rs$

The $InsP_3$-sensitive Ca^{2+} channel has been well studied in numerous types of cells, including sperm (Jimenez-Gonzalez *et al.*, 2006). A number of studies have revealed that sperm from various mammals, including humans, have proteins that cross-react with various $InsP_3R$-specific antibodies (Ho and Suarez, 2003; Kuroda *et al.*, 1999a; Minelli *et al.*, 2000). The use of isoform-specific $InsP_3R$ antibodies revealed that $InsP_3R1$ was predominantly located to the anterior acrosome (Ho and Suarez, 2003; Kuroda *et al.*, 1999a). The $InsP_3R$ labelling was reduced or lost once sperm had acrosome reacted further confirming that the $InsP_3Rs$ are located on the outer acrosomal membrane (Kuroda *et al.*, 1999a). It has been shown that the posterior region of the head, the mid-piece and part of the flagellum were specifically labelled with an $InsP_3R3$-specific antibody in human sperm, whereas the $InsP_3R2$ was not detected. In bull sperm, the RNE was stained by an $InsP_3R1$-specific antibody (Ho and Suarez, 2003). Anti-$InsP_3R$ staining also localises to the RNE in approximately half of human sperm, but the strongest staining was localised in the acrosomal region (Naaby-Hansen *et al.*, 2001). Thimerosal, an $InsP_3R$ activator, has been used to assess whether Ca^{2+} mobilization from $InsP_3R$ containing Ca^{2+} stores contribute to regulate sperm function. Thimerosal induces the

acrosome reaction, confirming a role for $InsP_3R$ in sperm physiology (Herrick *et al.*, 2005). Two $InsP_3R$ containing Ca^{2+} stores exist within sperm, one in the acrosome and the other a much smaller Ca^{2+} store located within the RNE. Studies by Naaby-Hansen *et al.* (2001) and by Ho and Suarez (2003) revealed that both regions contain calreticulin (a low affinity, high capacity Ca^{2+} buffering protein) that is always associated with $InsP_3R$ containing Ca^{2+} stores in somatic cells, further supporting the notion that both of these compartments are real Ca^{2+} stores. It seems that Ca^{2+} stored in the RNE of human sperm is mobilized upon progesterone stimulation by CICR, in an $InsP_3$-independent way (Harper *et al.*, 2004).

1.11.6 RyRs

Studies employing *in situ* hybridization methods using ribo-probes specific for the different RyR isoforms and immuno-histochemical methods using RyR isoform specific antibodies, provided initial evidence for the presence of RyRs in spermatozoa. Analysis of mouse testis sections led to the conclusion that germ cells such as spermatocytes and spermatids expressed RyR1 and RyR3 but not RyR2 (Giannini *et al.*, 1995). Several later studies revealed that developing mouse spermatocytes and spermatids expressed RyR1 and RyR3 (Chiarella *et al.*, 2004; Trevino *et al.*, 1998). It was also shown that only RyR3 could be found in mature sperm and that similar staining patterns were seen in intact and acrosome-reacted sperm, indicating it was unlikely that RyR3 was localised on the acrosome (Trevino *et al.*, 1998). Human spermatozoa can be specifically labelled with a fluorescence analogue of ryanodine (BODIPY-FL-X-ryanodine). This labelling appears to be mostly concentrated to the posterior head and mid-piece junction, with only low levels of labelling around the acrosome (Harper *et al.*, 2004), and co-localises with the SPCA1 and with the $[Ca^{2+}]_i$ oscillations that occur in response to stimulation with progesterone (Harper *et al.*, 2004; 2005).

The presence and functionality of RyRs in mature spermatozoa have also been indicated by several pharmacological based studies. A study using digitonin permeabilised bovine spermatozoa revealed that caffeine and ryanodine decreased Ca^{2+} accumulation within the cells in a similar way to $InsP_3$, indicating the activation of a Ca^{2+} efflux channel with RyR-like properties (Minelli *et al.*, 2000). In intact human spermatozoa, the progesterone-stimulated $[Ca^{2+}]_i$ oscillations are $InsP_3$-independent but could be altered by ryanodine, with higher concentrations reducing the frequency of these oscillations and lower concentrations increasing the frequency. Moreover, tetracaine, a RyR inhibitor, could abolish these $[Ca^{2+}]_i$ oscillations at once (Harper *et al.*, 2004). Spermatozoa contain a functional RyR that not only plays an important physiological role in regulating agonist-stimulated Ca^{2+} changes but also in spermatozoa development (Chiarella *et al.*, 2004).

1.12 Reactive oxygen species (ROS)

What is known as the oxygen paradox, the vital requirement of oxygen for life but the inherent toxicity of oxygen derivatives, the ROS, has led to the acquisition of major knowledge on pathology and the development of many aspects of medicine. ROS are known for their detrimental effects on almost all cells and tissues and have been assigned triggering or related roles in cancer, vascular problems, aging, diabetes, neuropathy, etc (Halliwell and Gutteridge, 1999). Only in the 1990s, researchers discovered that very low doses of ROS, generated at the time of cell activation, could have the role of second messengers and thus have positive roles in signal transduction cascades (Droge, 2002; Filomeni *et al.*, 2005).

1.12.1 Sources of ROS in semen

ROS represent a broad class of molecules including radical (hydroxyl ion, superoxide, nitric oxide (NO), peroxyl, etc) and non-radical (ozone, singlet oxygen, lipid peroxide, hydrogen peroxide) and oxygen derivatives (Agarwal and Prabakaran, 2005). Reactive nitrogen species (RNS; NO, peroxynitrite, nitroxyl ion, etc) are free nitrogen radicals and considered a ROS subclass (Darley-Usmar *et al.*, 1995; Sikka, 2001).

Nearly every human ejaculate is considered to be contaminated with potential sources of ROS (Aitken, 1995). Human semen contains different types of cells such as immature and mature sperm, round cells from distinct stages of spermatogenesis, leukocytes and epithelial cells. Among these different types of cells, leukocytes and sperm have been shown to be the main sources of ROS (Garrido *et al.*, 2004).

The missing link between poor sperm quality and increased generation of ROS is explained by the cytoplasmic droplets (excess residual cytoplasm). The cytoplasmic droplets result from defective spermiogenesis and are a major source of ROS (Gomez *et al.*, 1996). Studies indicate that retention of residual cytoplasm by sperm is positively correlated with generation of ROS via mechanisms that may be mediated by the cytosolic enzyme glucose-6-phosphate dehydrogenase (Aitken, 1999).

ROS generation by sperm has been proposed to occur through two ways: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the sperm plasma membrane (Aitken *et al.*, 1992) and NADPH-dependent oxidoreductase (diphorase) at the mitochondria (Gavella and Lipovac, 1992).

Leukocytospermia (increased leukocyte infiltration in semen) is defined by the World Health Organization (WHO) as the presence of peroxidase-positive leukocytes in concentrations of

>1 million/ml of semen. There is controversy over the clinical significance of leukocytospermia (Thomas *et al.*, 1997). Sperm parameters such as poor quality, decreased hyperactivation and defective sperm function (Wolff, 1995) have been attributed to leukocytospermia. In contrast, no correlation was reported between seminal leukocyte concentrations and impaired sperm quality (Tomlinson *et al.*, 1993) or defective sperm function (Aitken *et al.*, 1994). Other studies (Athayde *et al.*, 2007; Sharma *et al.*, 2001) led to the conclusion that oxidative stress occurs even in patients with a very low seminal leukocyte count and increases with an increase in leukocyte count.

Peroxidase-positive leukocytes in the human ejaculate include polymorphonuclear leukocytes and macrophages (Thomas *et al.*, 1997). These cells are derived principally from the prostate gland and the seminal vesicles (Wolff, 1995). Leukocytes may be activated by inflammation and infection (Pasqualotto *et al.*, 2000), and can generate up to 100-fold higher amounts of ROS in comparison to non-activated leukocytes (Plante *et al.*, 1994; Shekarriz *et al.*, 1995).

1.12.2 Oxidative stress

Of the many causes of male infertility, oxidative stress has been recognized to affect the fertility status and therefore, it has been extensively studied in the recent past. Like any other aerobic cell, sperm are constantly facing the 'oxygen-paradox' (Sies, 1993). Oxygen is crucial to maintain life as physiological levels of ROS are needed to maintain normal cell function. In opposition, its breakdown products such as ROS can be detrimental for cell function and survival (de Lamirande and Gagnon, 1995b).

Every cellular component including lipids, proteins, nucleic acids and sugars are potential targets of oxidative stress. The level of oxidative stress-induced damage depends on the

nature and amount of ROS involved, as well as on the extent of exposure to ROS and extracellular factors like oxygen tension and the composition of the surrounding environment, including ROS scavengers (Agarwal *et al.*, 2008).

Lipids exist in the sperm plasma membranes in the form of polyunsaturated fatty acids, fatty acids containing more than two carbon-carbon double bonds. These lipids are attacked by ROS leading to a cascade of chemical reactions called peroxidation. Malondialdehyde (MDA) is one of the by-products of lipid peroxidation, and has been used in various biochemical assays to monitor the level of peroxidative damage sustained by sperm (Aitken *et al.*, 1989; 1994). Data from these assays show an excellent correlation when examining the relationship between impaired sperm function, discussed in terms of motility, and the capacity for sperm-oocyte fusion (Aitken *et al.*, 1993).

Decreased sperm motility has been correlated with increased ROS levels (Agarwal *et al.*, 1994; Armstrong *et al.*, 1999; Lenzi *et al.*, 1993), though the exact mechanism through which this occurs is not understood. One hypothesis suggests that hydrogen peroxide (H_2O_2) diffuses across the membranes into the cells and inhibits the activity of some crucial enzymes such as glucose-6-phosphate dehydrogenase. This modifies the intracellular availability of NADPH, which is subsequently used as a source of electrons by sperm to fuel the production of ROS by NADPH oxidase (Aitken *et al.*, 1997). Another hypothesis involves a series of interrelated events causing a reduction in axonemal protein phosphorylation and sperm immobilization, both of which are linked to a decrease in membrane fluidity that is required for sperm-oocyte fusion (de Lamirande and Gagnon, 1992). When sperm are incubated overnight, the observed loss of motility is highly correlated with the lipid peroxidation status of the sperm (Gomez *et al.*, 1998). Moreover, antioxidants revive sperm motility *in vivo* and *in vitro*, providing

evidence that lipid peroxidation is a major cause for motility loss in sperm (Suleiman *et al.*, 1996).

In the male germ line cells, DNA damage is related to poor fertilization rates following *in vitro* fertilization (IVF), defective pre-implantation embryonic development, high rates of miscarriage, and morbidity in the offspring. Causes of DNA damage include defective spermatogenesis, oxidative stress, infection and abortive apoptosis (Aitken *et al.*, 2007). DNA damage is regularly induced by oxidative stress, rather than being the outcome of other processes such as defective apoptosis (Barroso *et al.*, 2000; Kemal Duru *et al.*, 2000; Kodama *et al.*, 1997).

Oxidative stress-induced DNA damage may have significant clinical implications in the context of assisted reproductive techniques (ART). Studies have showed that ROS are generated at significantly high levels in response to repeated cycles of centrifugation involved in conventional sperm preparation techniques used for ART (Agarwal *et al.*, 1994). A high percentage of the sperm selected for ART may have damaged DNA as they usually derive from the environment experiencing oxidative stress (Kodama *et al.*, 1997).

Clinical or subclinical varicocele (Gonda *et al.*, 1987) has been revealed to cause male infertility in approximately 15% of infertile couples (Schoor *et al.*, 2001). These patients have increased ROS in serum, testes and semen (Koksal *et al.*, 2003). Increased NO has been demonstrated in the spermatic veins of patients with varicocele (Mitropoulos *et al.*, 1996; Romeo *et al.*, 2001), which could cause spermatozoal dysfunction (Ozbek *et al.*, 2000).

1.12.3 Defences against ROS

Oxidative stress occurs when the generation of ROS overwhelms the antioxidant defence mechanisms resulting in cellular damage. Sperm and seminal plasma are well endowed with protective antioxidants (Fujii *et al.*, 2003; Garrido *et al.*, 2004). Superoxide dismutase (SOD) and catalase are enzymatic antioxidants which convert the superoxide anion ($O_2^{\bullet-}$) and H_2O_2 into water and oxygen, causing their inactivation. SOD is present in spermatozoa and seminal plasma (Mennella and Jones, 1980; Zini *et al.*, 1993). The final member of the seminal enzymatic antioxidant triad is glutathione peroxidase. Glutathione peroxidase forms a family of antioxidants that are involved in the reduction of hydroperoxides using glutathione as an electron donor. These enzymes are localized in the testis, prostate, seminal vesicles, vas deferens, epididymis, seminal plasma and sperm (reviewed by Vernet *et al.* (2004)).

The non-enzymatic antioxidants present in the semen comprise ascorbic acid (vitamin C), urate, α-tocopherol (vitamin E), glutathione, albumin, amino acids (taurine and hypotaurine), carnitine, carotenoids, flavenoids and prostasomes. These antioxidants mainly act by directly neutralizing free radical activity chemically. They also protect against free radical attack via two other mechanisms. Albumin can intercept free radicals by becoming oxidized itself, thus sparing sperm from attack (Twigg *et al.*, 1998). Alternatively, prostasomes secreted by the prostate have been revealed to fuse with leukocytes in the semen and diminish their free radical generation (Saez *et al.*, 1998; Skibinski *et al.*, 1992).

The seminal plasma antioxidants are clearly helpful in preventing sperm oxidative attack after ejaculation. Though, during spermatogenesis and epididymal storage, spermatozoa are not in contact with the seminal plasma antioxidants, and must rely on epididymal/testicular antioxidants and their own intrinsic antioxidant ability for protection. Testicular biopsies from men with varicocele-related oxidative stress have revealed an increase in oxidative DNA

damage in spermatogonia and spermatocytes (Ishikawa *et al.*, 2007). Thus, although seminal plasma antioxidants may assist minimizes ejaculated sperm oxidative stress, they are not capable of preventing oxidative damage initiated at the level of the testis and epididymis (Tremellen, 2008).

1.12.4 NO and spermatozoa

NO is produced from L-arginine by the NO-synthase (NOS) enzymes (Palmer *et al.*, 1988). NO synthesis requires various co-factors, specifically NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin, CaM and Ca^{2+} (Moncada and Higgs, 1995). Molecular oxygen is used in NO synthesis, which proceeds via the generation of the intermediate N^{ω}-hydroxyarginine and results in the formation of L-citrulline additionally to NO (reviewed by Herrero and Gagnon (2001)).

$$O_2 + \text{L-arginine} \xrightarrow[\text{Co-factors}]{\text{NOS}} \text{N-O}^{\bullet} + \text{L-citrulline} \quad \text{(reviewed by Herrero and Gagnon (2001))}$$

Two forms of NOS have been described, the constitutive and the inducible NOS (iNOS). Constitutive NOS was initially located in the nervous system (nNOS) and vascular endothelium (eNOS), but it is now known to be expressed in a number of cells in distinct organs (Knowles and Moncada, 1994). In the vasculature, NO induces vasodilatation, inhibits platelet aggregation and controls programmed cell death amongst other functions, whereas NO generated by neurons acts as a neurotransmitter (reviewed by Herrero and Gagnon (2001)).

Constitutive NOS (nNOS and eNOS) are Ca^{2+}- and CaM- dependent and produce small quantities of NO that perform an array of physiological functions (reviewed by Herrero and Gagnon (2001)). Although iNOS is Ca^{2+}- and CaM-dependent, it is different from the constitutive NOS as it requires very low levels of Ca^{2+} to be totally activated, and it appears to bind CaM very tightly so that CaM forms a constitutive subunit to this isoform (Nathan and Xie, 1994). Although iNOS was initially thought to be induced only in pathological conditions, now it has been also identified in physiological settings such as ovulation, pregnancy and labour (Bansal *et al.*, 1997; Buhimschi *et al.*, 1996; Shukovski and Tsafriri, 1994). Studies using antibodies against nNOS, eNOS and iNOS have showed that these enzymes exist in almost every tissue studied, including the reproductive system (Burnett *et al.*, 1995; Mara Suburo *et al.*, 1995; Rosselli *et al.*, 1996).

Herrero *et al.* (1996) and Lewis *et al.* (1996) demonstrated that NOS was present in mouse and human sperm. In mouse, NOS was seen on the acrosome and tail of non-capacitated sperm. NOS localisation to the head disappeared with time when sperm were incubated under capacitating conditions, suggesting that NOS has a role in capacitation (Herrero *et al.*, 1996). In human sperm, specific labelling with nNOS and eNOS was seen on the postacrosomal and equatorial regions, but not on the flagellum. Sperm samples from normozoospermic individuals showed more intense immunoreactivities to eNOS and nNOS antibodies than those from asthenozoospermic patients, which had little or no NOS immunoreactivity (Lewis *et al.*, 1996). O'Bryan *et al.* (1998) classified human sperm eNOS immunostaining into four patterns: faint postacrosomal and equatorial staining, intense postacrosomal and equatorial staining, diffuse sperm head staining and sperm mid-piece staining with or without diffuse head staining. The first two patterns were always seen in normal sperm, while the last two

patterns were regularly related with morphologically abnormal sperm and with a decrease in sperm motility, suggesting that NO may be associated with normal sperm physiology (O'Bryan *et al.,* 1998).

The presence of NOS in sperm was confirmed by Western blot analysis. A band corresponding to a human sperm protein of 145 kd was recognized after immunoblotting with the eNOS antiserum, under nonreducing conditions (Revelli *et al.,* 1999). Recent studies provided further evidence for the presence NOS in human sperm (Willipinski-Stapelfeldt *et al.*, 2004; Zhang *et al.*, 2006). A 140 kd protein was observed for sea urchin (Kuo *et al.*, 2000) and mouse sperm NOS (Herrero *et al.*, 1997), though it is intriguing that in the mouse, the three NOS antibodies tested (anti-nNOS, anti-eNOS and anti-iNOS) could recognize a single band. This may be explained by the facts that the antibodies used were polyclonal and because there is a high degree of homology between the neuronal, endothelial and inducible isoforms (Herrero *et al.*, 1997). Also, it was recently reported that one-by-one deletion of the eNOS, nNOS and iNOS in knockout mice had no visible effect on sperm function and male fertility (Yang *et al.,* 2005).

1.12.5 Nitroxyl (HNO)

Studies describing potentially important biological and /or pharmacological activity of HNO have ignited increased interest in this one-electron reduced and protonated congener of NO. Amongst all regularly studied nitrogen species, HNO is the most misunderstood and enigmatic, which is probable due to its high reactivity and ephemeral nature (Paolocci *et al.*, 2007).

Recent studies have shown that HNO is a poor acid (pK_a>11; Bartberger *et al.*, 2002; Shafirovich and Lymar, 2002), suggesting that HNO is basically the exclusive species at physiological pH for all intents and purposes (reviewed by Paolocci *et al.* (2007)).

Endogenous production of HNO has yet to be demonstrated *in vivo* (Donzelli *et al.*, 2008).

It appears that HNO is very reactive toward thiols (-SH). Reaction of HNO with a –SH results in the initial formation of a putative *N*-hydroxysulfenamide by attack of a nucleophilic sulphur atom on the electrophilic nitrogen atom of HNO. The *N*-hydroxysulfenamide can further react with excess -SH (or a vicinal protein -SH) to provide the corresponding SS and hydroxylamine (Doyle *et al.*, 1988). The result of the associaton of HNO with -SH may depend upon the fate of the intermediate adduct. If the *N*-hydroxysulfenamide interacts with a second -SH, the SS product can be readily converted to the starting –SH under biological conditions. This pathway would result in a reversible modification of –SH structure and function by HNO (Paolocci *et al.*, 2007).

Either directly or together with other reactive oxygen and nitrogen species, HNO can affect the oxidation of various biomolecules. HNO is readily capable of –SH oxidation, a process thought to occur without free radical intermediates (Paolocci *et al.*, 2007).

The most well-studied, used and convenient donor of HNO is sodium trioxodinitrate or Angeli's salt (ANGS; $Na_2N_2O_3$; Bonner and Ravid, 1975; Bonner and Hughes, 1988; Angeli, 1903). ANGS has been pivotal in the discovery of the biology and pharmacology of HNO. ANGS spontaneously decomposes to generate 1 equivalent each of HNO and nitrite ion

(NO_2^-), at pHs above 4. The rate of the thermal decay of ANGS is pH-independent from pH 4-8 and then successively decreases with increased pH. This decay profile is very useful as for

practical purposes, stock solutions of ANGS are relatively stable at high pH whereas the release profile is pH insensitive near physiological conditions (Paolocci *et al.*, 2007).

The potency by which HNO elicits many of its pharmacological actions and the apparent selectivity of HNO towards numerous of its established biological targets would suggest that the actions of pharmacologically administered HNO are the result of signalling pathways that already exist to respond to HNO. Also, other nitrogen oxides are already known to be generated and used as signalling molecules, suggesting that Nature would also exploit the distinctive and selective HNO chemistry (Paolocci *et al.*, 2007). Among the various pharmacological effects of HNO (reviewed by Paolocci *et al.* (2007)), recent findings about its effects in the cardiovascular system are particularly interesting. *In vitro* experiments have shown that HNO elicits a rapid Ca^{2+} release from the sarcoplasmic reticulum in the cardiac and skeletal muscle via activation of cardiac RyRs (RyR2) and skeletal Ca^{2+} release channels (RyR1), respectively (Tocchetti *et al.*, 2005; Cheong *et al.*, 2005). In both circumstances, the effects were reversible upon addition of dithiothreitol (DTT), a –SH reducing agent. HNO increased the single channel open probability of both purified RyR2 (Tocchetti *et al.*, 2005) and RyR1 (Cheong *et al.*, 2005). These effects were also reversed upon addition of DTT. It has been shown that HNO activates RyR2 and stimulates sarcoplasmic reticulum Ca^{2+}-ATPase, increasing Ca^{2+} re-uptake into the sarcoplasmic reticulum. It is likely that the combination of these effects will induce and sustain positive ionotropy and accelerate relaxation in murine myocytes (Tocchetti *et al.*, 2005). Thus, at the cellular level, the HNO generated by ANGS enhances Ca^{2+} cycling to increase contractility, which occurs in a cGMP- and cAMP-independent way. Other data show that HNO is also able of enhancing myofilament responsiveness to Ca^{2+}, increasing myocardial contractility in rat cardiac trabeculae (Dai *et al.*, 2005). Dai and coworkers proposed that HNO additionally acts as a

Ca^{2+} sensitizing agent, as the increase in force development with HNO is greater than the rise in Ca^{2+} transients. Changing intracellular redox conditions with DTT prevented HNO-stimulated augmentation in muscle force development, consistent with the idea of HNO affinity for strategically localised -SH (Dai *et al.,* 2005).

1.12.6 Regulation of sperm functions by ROS

There is substantial evidence to suggest that small amounts of ROS are required for sperm to acquire fertilizing abilities (Aitken, 1997; 1999; Gagnon *et al.*, 1991). It has been shown that low levels of ROS are essential for fertilization, acrosome reaction, hyperactivation, motility and capacitation (Agarwal *et al.*, 2004; Griveau and Le Lannou, 1997). It has also been shown that co-incubation of sperm with low doses of H_2O_2 stimulate capacitation, hyperactivation, acrosome reaction and oocyte fusion (Aitken, 1995; de Lamirande *et al.*, 1993; Griveau *et al.*, 1994; Kodama *et al.*, 1996). ROS such as NO and the $O_2^{-\bullet}$ have also shown to promote capacitation and the acrosome reaction (Griveau *et al.*, 1995). In addition, ROS have been implicated in sperm oocyte interaction (Agarwal *et al.*, 2007).

Sperm capacitation is dependent on an early increase of intracellular cAMP levels and PKA activation (De Jonge, 2005; de Lamirande *et al.*, 1997; Lefièvre *et al.*, 2002; Olds-Clarke, 2003; Yanagimachi, 1994b). This increase in cAMP/PKA regulates the phosphorylation of tyrosine residues, principally of two insoluble FS proteins of 80 and 105 kDa, which are antigenetically related to AKAPs (Carrera *et al.*, 1996; Leclerc *et al.*, 1996; 1997; Visconti *et al.*, 1995). The role of ROS became clear when it was observed that ROS scavengers and NOS inhibitors block, and that exogenous addition of ROS promotes capacitation and

increases protein tyrosine phosphorylation (de Lamirande and Gagnon, 1993a; 1993b; de Lamirande *et al.*, 1997; Herrero *et al.*, 2003; Leclerc *et al.*, 1997; O'Flaherty *et al.*, 1999; 2004b; 2006a; 2006b; Thundathil *et al.*, 2003). Sperm generate, over hours, the controlled and low amounts of $O_2^{-\bullet}$ (de Lamirande and Gagnon, 1995a) and NO (de Lamirande and Gagnon, 1993a; Herrero *et al.*, 2003; Lewis *et al.*, 1996) they require for capacitation. Almost all inhibitors tested prevented the late tyrosine phosphorylation of FS proteins (De Jonge, 2005; de Lamirande and Gagnon, 2002; de Lamirande *et al.*, 1997; Nauc *et al.*, 2004; O'Flaherty *et al.*, 2006a; 2006b; Thundathil *et al.*, 2003; Visconti *et al.*, 1995), but the generation of ROS is not affected by any of these chemicals and appears to precede all known transduction event of capacitation (de Lamirande and O'Flaherty, 2008).

In seminal plasma, sperm swim in relatively linear manner, but acquire a very special type of movement during capacitation when they progress in the female genital tract and get close to the oocyte (de Lamirande and Gagnon, 1995b; de Lamirande *et al.*, 1997; Yanagimachi, 1994b; Gagnon and de Lamirande, 2006). This hyperactivated motility is non-progressive, flagellar activity being high amplitude and whiplash-like. Hyperactivation is crucial for sperm to penetrate the zona pellucida viscous environment that surrounds the oocyte (de Lamirande *et al.*, 1997; Stauss *et al.*, 1995; Suarez and Dai, 1992; Gagnon and de Lamirande, 2006). Sperm hyperactivated motility is also controlled by ROS, being prevented by SOD and catalase and promoted by exogenous addition of ROS (de Lamirande and Gagnon, 1993a; 1993b). The mechanism by which ROS act is currently unknown but could be linked to the increased phsphorylations of flagellar proteins (de Lamirande *et al.*, 1997; Harrison, 2004; Leclerc *et al.*, 1997; O'Flaherty *et al.*, 2004a; 2005; 2006a; Thundathil *et al.*, 2003).

The initial reports on sperm motility and NO evaluated the effects of NO-releasing compounds on sperm motion and viability. It has been demonstrated that low doses of sodium nitroprosside (SNP), a NO-releasing compound, was beneficial to the maintenance of post-thaw human sperm motility and viability (Hellstrom *et al.*, 1994). A parallel study suggested that NO could stimulate mouse sperm hyperactivation (Herrero *et al.*, 1994). It was also demonstrated that low doses of SNP enhanced sperm motility in hamsters and teleosts (Creech *et al.*, 1998; Yeoman *et al.*, 1998). On the contrary, it has been shown that sperm motility is decreased in the presence of different doses of SNP (Tomlinson *et al.*, 1992; Herrero *et al.*, 1994; Rosselli *et al.*, 1995; Weinberg *et al.*, 1995). Sperm motility inhibition was observed with high doses of the NO-releasing compound and this effect was probably caused by an inhibition of sperm respiration (Weinberg *et al.*, 1995). Although some of these cited reports appear to be contradictory, the data may be totally compatible and the variance attributable to the dose of NO in the sample and the time frame over which the experiments were performed. Thus, high doses of NO reduce sperm motility while low doses of NO enhance it (Herrero and Gagnon, 2001). In semen collected from distinct donors, a negative correlation between the doses of NO and the percentage of motile cells was observed, supporting the debate that low doses of NO enhance motility (Nobunaga *et al.*, 1996). Also, Schaad *et al.* (1996) reported that nNOS activity was inhibited by human seminal plasma. The inhibitor existent in the seminal plasma was a heat-stable, noncompetitive inhibitor of nNOS, but the physiological significance of the existence of this inhibitor in the seminal plasma is uncertain. It is appealing to think that the function of this inhibitor may be to prevent the development of hyperactivation and capacitation or to maintain NO at low doses to protect sperm from toxic damage. Consistent with this hypothesis is the fact that high levels of NO have been found in semen of infertile men with reduced sperm motility, and it is in these

clinical cases that NOS inhibitors have succeeded in enhancing motility (Nobunaga *et al.*, 1996; Perera *et al.*, 1996; Rosselli *et al.*, 1995). SNP addition to sperm of infertile patients further decreased motility, possibly reflecting the fact that oxidative stress was already high. Thus, it is plausible that excessive generation of NO, principally by leukocytes, could lead to spontaneous hyperactivation that impairs sperm transport along the lower female reproductive tract or result in premature capacitation and thus cause infertility (Herrero and Gagnon, 2001). In conclusion, the effect of NO on sperm motility and viability will be determined by its concentration. This bimodal motility response to different doses of NO-releasing compounds is not unexpected since the dual nature of NO as a transduction molecule at low doses and as a cytotoxic effector at high doses is distinguished in other systems (Herrero and Gagnon, 2001). Considering that sperm can synthesize NO, it is sensible to think that endogenous NO generated by sperm regulates sperm motility. Human and hamster sperm motility were depressed by the addition of the NOS inhibitor N(G)-nitro-L-arginine-methyl ester (L_NAME) to the incubation medium (Donnelly *et al.*, 1997; Yeoman *et al.*, 1998). Furthermore, concentrations of nitrite generated by sperm were observed to be lower in samples from asthenozoospermic men than in those from normozoospermic donors, suggesting that the reduction of endogenous NO may influence sperm motility and therefore fertilization (Lewis *et al.*, 1996).

The acrosome reaction is also a redox-regulated process. Catalase, SOD and NOS inhibitors block, and exogenous addition of any ROS triggers, the acrosome reaction (de Lamirande *et al.*, 1998; Herrero *et al.*, 2003; O'Flaherty *et al.*, 1999; 2004b; Revelli *et al.*, 1999). Moreover, capacitated sperm induced to acrosome react by exposure to the Ca^{2+} ionophore A23187 or lysophosphatidylcholine generate $O_2^{-\bullet}$. The increase in protein tyrosine phosphorylation

associated with the acrosome reaction is partially reversed by SOD (in the case of lysophosphatidylcholine and A23187) or catalase (in the case of A23187), and abolished in the presence of these two antioxidants (de Lamirande *et al.*, 1998). The mechanisms by which ROS influence the acrosome reaction and linked phosphorylations are still unknown, though, the time courses of these effects and their modulation by kinases suggest that they are distinct and complex (de Lamirande and O'Flaherty, 2008).

Redox modulation of the –SH / SS pair on proteins (particularly -SH groups that have a lower pk_a; Kim *et al.*, 2000) is one of the major mechanisms by which ROS act on transduction elements and pathways (de Lamirande and Gagnon, 1998; 2003; Filomeni *et al.*, 2005; Sen, 2000). This form of modification is reversible, specific, easy and economic (Kim *et al.*, 2000). The effects of NO were initially thought to be exclusively mediated via activation of soluble guanylyl cyclase (sGC; Murad, 1994a), but recent studies indicated that NO can also induce its biological effects via non-cGMP-dependent pathways (Stamler, 1994; Pfeiffer *et al.*, 1999). Notably, NO can act via the S-nitrosylation of -SH, a process which has been implicated in the regulation of key enzymes, including PKC (Gopalakrishna *et al.*, 1993) and glyceraldehyde-3-phosphate dehydrogenase (Clancy *et al.*, 1994). A further reaction of NO in biological media is a direct bimolecular reaction with $O_2^{\bullet-}$, yielding peroxynitrite ($ONOO^-$; Beckman and Koppenol, 1996). $ONOO^-$ is a potent oxidant and has been shown to react with nearly all classes of biomolecules *in vitro*, such as myeloperoxidase, glutathione peroxidase, cytochrome *c*, ascorbate, etc (Pryor and Squadritto, 1995).

RESEARCH AIMS

To:

Assess the ability of human (and mouse) cumulus cells, which surround the oocyte, and human spermatozoa to generate NO.

Investigate the effects of NO on $[Ca^{2+}]_i$ in capacitated human spermatozoa and elucidate its target(s) and mechanism of action. Examine the effects of HNO on $[Ca^{2+}]_i$ in capacitated human spermatozoa.

Assess the contribution of NO-induced effects to the regulation of human sperm functions.

Evaluate the interaction of NO and progesterone (a product of the cumulus cells) in the regulation of $[Ca^{2+}]_i$ and consequent effects on human sperm functions.

CHAPTER TWO

NITRIC OXIDE (NO) AND NITROXYL (HNO) ELEVATE $[Ca^{2+}]_i$ IN CAPACITATED HUMAN SPERMATOZOA

2.1 Abstract

Endothelial and neuronal nitric oxide synthase (eNOS and nNOS) were immunolocalised in human cumulus oophorus, the cellular layer that invests the oocyte. 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate staining revealed NO generation in human and mouse cumulus, which was blocked by cell pre-treatment with the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L_NAME). Detectable levels of NO synthesis in human sperm were not found. The effects of the NO donor N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate) and the HNO donor, Angeli's salt (ANGS), on $[Ca^{2+}]_i$ in capacitated human sperm were determined. NONOate increased $[Ca^{2+}]_i$ in both the presence or absence of added Ca^{2+}, the low-Ca^{2+} conditions not altering the NO effects. The rise in $[Ca^{2+}]_i$ was primarily localised to the neck/mid-piece region, spreading into the posterior of the sperm head. The data suggest that NO elevates $[Ca^{2+}]_i$ by mobilization of an intracellular store rather than by Ca^{2+} influx at the plasmalemma. NONOate treated cells showed an abrupt decrease in $[Ca^{2+}]_i$ upon NONOate washout, responding subsequently with a $[Ca^{2+}]_i$ rise, usually oscillations, in the neck/mid-piece region, when re-stimulated with the NO donor. The data indicate that the NO effects are reversible. ANGS induced a sustained $[Ca^{2+}]_i$ increase, under both standard and low-Ca^{2+} conditions. The data suggest that HNO mobilizes stored Ca^{2+}.

2.2 Introduction

Sperm capacitation, acrosome reaction and motility are essential processes for fertilization (Baldi *et al.*, 2000; Darszon *et al.*, 2001; Ho and Suarez, 2001a; Visconti *et al.*, 2002). Changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) mediate the effects of certain physiological agonists of these processes (Baldi *et al.*, 2000; Darszon *et al.*, 2001; Ho and Suarez, 2001a; Visconti *et al.*, 2002; Wassarman *et al.*, 2001).

NO, produced by nitric oxide synthases (endothelial, neuronal and inducible types; eNOS, nNOS and iNOS), acts as a rapid paracrine (and possibly autocrine) cellular messenger, usually by activation of soluble guanylate cyclase (sGC; Ahern *et al.*, 2002; Arnold *et al.*, 1977; Braughler *et al.*, 1979; Miki *et al.*, 1977). The action of NO is of particular relevance in the cardiovascular tissue (where it was originally named endothelium derived relaxing factor [EDRF]) and in nervous system (Calabrese *et al.*, 2007; Li and Moore, 2007; Rastaldo *et al.*, 2007) but it is clear that NO exerts effects in most tissue and cell types (Moncada and Higgs, 1991).

Mammalian sperm (including human) undergo extensive redox regulated signalling (Baker and Aitken, 2004; Ford, 2004) and many studies have shown a significant positive effect of exogenous NO on sperm function (Herrero *et al.*, 2003; Herrero and Gagnon, 2001; Rosselli *et al.*, 1998). Generation of NO endogenously and/or by cells of the female tract may contribute to capacitation, inducing tyrosine phosphorylation by mechanisms involving and/or independent of the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway (Funahashi, 2002; O'Flaherty *et al.*, 2004a; 2005; 2006b; Roy and Atreja, 2008;

Thundathil *et al.*, 2003). Moreover, NO stimulates or contributes to stimulation of the acrosome reaction (Funahashi, 2002; Herrero *et al.*, 2003; O'Flaherty *et al.*, 2004b; Revelli *et al.*, 2001; Yang *et al.*, 2005). With regard to effects of NO on motility of mammalian sperm, a number of studies have revealed that application of NO *in vitro* has functional effects, but the data here are complex. Treatment with high concentrations of NO donors, or prolonged exposure to NO, suppresses motility, possibly reflecting cytotoxic effects that may occur in the testis or in spermatozoa held in semen (Calabrese, 2001; Herrero *et al.*, 1994; Joo *et al.*, 1999; Weinberg *et al.*, 1995; Wu *et al.*, 2004; Zhang and Zheng, 1996). Low doses of NO and short-term exposure may stimulate motility (Calabrese, 2001; Herrero *et al.*, 1994; Zhang and Zheng, 1996).

Recently, there has been augmented interest in the one-electron reduction product of NO, nitroxyl (HNO; Miranda *et al.*, 2003). The physiological effects of HNO donors suggest potential use as a new treatment for cardiac failure, raising the significance of better understanding its molecular and cellular mechanisms (Dai *et al.*, 2007). HNO donors enhance myocardial contractility in normal (Paolocci *et al.*, 2001) and failing hearts (Paolocci *et al.*, 2003). HNO potentially induces release of Ca^{2+} from ryanodine receptors (RyRs) in skeletal (Cheong *et al.*, 2005) and cardiac muscles (Cheong *et al.*, 2005; Tocchetti *et al.*, 2007). HNO also increases sarcoplasmic reticular Ca^{2+} uptake to maintain the net diastolic calcium low. These effects seem to occur independently of both cyclic guanosine monophosphate (cGMP)- and cAMP-coupled signalling (Tocchetti *et al.*, 2007).

The aim of this study was to assess the ability of human and mouse cumulus cells and human sperm to synthesise NO, and to examine the effects of NO and HNO on $[Ca^{2+}]_i$ in capacitated human spermatozoa, using the NO donor N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate) and the HNO donor Angeli's salt (ANGS).

Part of this work has been published in the journal "Development".

2.3 Materials and methods

2.3.1 Materials

Primary antibodies (rabbit polyclonal anti-eNOS and anti-nNOS) and secondary antibody (donkey anti-rabbit Texas red) were purchased from Santa Cruz Biotechnologies Inc, (California, USA). SYTOX Green was obtained from Molecular Probes, Oregon, USA. DakoCytomation fluorescence mounting medium was supplied by Dako UK Ltd. (Ely, Cambridgeshire, UK). Salts used to prepare supplemented Earle's balanced salt solution (sEBSS) and Ca^{2+}-free sEBSS (CFsEBSS), chemicals used to prepare Biggers, Whitten and Whittingham (BWW) medium (see Appendix I for details), pluronic F-127, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), N(G)-nitro-L-arginine methyl ester (L_NAME) and Triton-X-100 were purchased from Sigma Aldrich (Poole, Dorset, UK). Fatty-acid free bovine serum albumin (BSA) was purchased from SAFC Biosciences (Lenexa, KS, USA). 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). Spermine NONOate and ANGS were obtained from Merck Chemicals Ltd. (Beeston, Nottingham, UK). All chemicals were cell culture-tested grade where available.

2.3.2 Detection of NO synthesis in human and mouse cumulus cells

Surplus human cumulus cells were obtained during intracytoplasmic sperm injection (ICSI) cycles carried out at The Assisted Conception Unit, Birmingham Women's Hospital [Human Fertilization and Embriology Authority (HFEA) Centre 0199]. Human cumulus masses were washed in sEBSS and subsequently incubated in the dark at 37ºC and 6% CO_2 with 5 µM

DAF-FM diacetate for 30 minutes. Excess DAF-FM diacetate was eliminated by three washes in sEBSS. The cumulus was then transferred to microscope slides under a coverslip supported on vaccum grease spots to gently compress it. The slides were observed under a Nikon inverted fluorescence microscope (488 nm excitation/ 540 nm emission). Control incubations were performed with 1 mM L_NAME present since the start of the incubation, using similar exposures to those in the absence of L_NAME.

Immature female mice (Balb-c) were superovulated by injection of pregnant mares with serum gonadotrophin (10 iu) and human chorionic gonadotrophin (hCG; 10 iu) approximately 36 hours later. Mice were killed by cervical dislocation 12 to 16 hours after hCG was injected. The oviducts were removed and ruptured under BWW medium to liberate the cumulus masses. The cumulus masses were washed in three changes of BWW medium and subsequently processed as for human, but in BWW and not sEBSS, and at room temperature.

2.3.3 Detection of NOS in human cumulus cells

Loose human cumulus from oocyte retrieval was stored in PBS at 4ºC. Cells were subsequently smeared onto standard microscope slides, air-dried and then fixed with 100% methanol (-20ºC, 6 minutes). The slides were exposed to 50% (v/v) methanol in PBS (20ºC, 5 minutes), washed three times in 0.1% (v/v) Triton X-100 in PBS and then re-hydrated with PBS (20ºC, 15 minutes). Slides were blocked in 1% (w/v) BSA and 5% (v/v) goat serum in PBS (30 minutes, 37ºC in 5% CO_2 in air), and then incubated with rabbit polyclonal anti-eNOS or anti-nNOS (1:50 dilution in 1% (w/v) BSA in PBS, 37ºC in 5% CO_2 in air, 60 minutes). Slides were washed with PBS and then the secondary antibody (donkey anti-rabbit

Texas red – 1:200 dilution in 1% (w/v) BSA in PBS) was applied (37ºC in 5% CO_2 in air, 60 minutes). Slides were washed with PBS to eliminate excess of secondary antibody prior to incubation with SYTOX Green (1:30 dilution in 1% (w/v) BSA in PBS; 20ºC, 15 minutes). Slides were then washed and coverslips were mounted using DakoCytomation fluorescence mounting medium. Finally, slides were observed under a Zeiss 410 Axiovert 100 microsystems LSM confocal microscope and images were taken using MrGrab 1.0 (Carl Zeiss Vision GmbH, Germany).

2.3.4 Spermatozoa preparation and capacitation

Donors were recruited at the Birmingham Women's Hospital (HFEA Centre 0119), in accordance with the Human Embriology 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°, the top layer of each tube, containing the motile cells, was collected into a 15 ml polystyrene Falcon tube (Becton Dickinson, USA). Sperm concentration was determined using a Neubauer counting chamber, in accordance with the World Health Organisation 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 6 hours, at 37°C and 5% CO_2, for imaging experiments.

2.3.5 Single cell imaging

Sperm density was reduced to 4 million cells/ ml, using the same medium, immediately before cell labelling and chamber preparation. 200 µl aliquots of cells were then loaded with 12 µM OGB-1AM (0.6% DMSO, 0.12% pluronic F-127) for 40 minutes, after which the entire aliquot was transferred to a perfusable imaging chamber (180 µl volume) for 20 minutes, at 37°C and 5% CO_2. The chamber lower surface was a 1% PDL coated coverslip, allowing cells to adhere. 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±1°C, in a constant flow of medium, with a perfusion rate of approximately 0.4 ml/minute. Cells were imaged with a Nikon TE200 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 Hamamatsu Orca 1 cooled CCD camera controlled by iQ software (Andor Technology, Belfast, UK).

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

Sperm were then superfused with spermine NONOate (100 µM) dissolved in the same medium.

Spermine NONOate washout experiments were also carried out. The initial NONOate stimulation in complete sEBSS was followed by NONOate washout, using the same saline, and reintroduction of the drug at the same concentration to the recording chamber.

Cells were also exposed to ANGS (60 µM) in sEBSS (or CFsEBSS) + 0.3% BSA.

Control experiments consisted in cell superfusion either with sEBSS + 0.3% BSA or CFsEBSS + 0.3% BSA, in which case cells were stabilised in low-Ca^{2+} saline before recording. It is noteworthy that, it has been attempted to start recording in standard saline and then superfuse with low-Ca^{2+} saline before stimulating cells with NONOate in low-Ca^{2+} medium. However, cells tended to become strongly destabilised when changing media, compromising the possibility to obtain clear results. To overcome this obstacle and to avoid excessive cell illumination (Morgan and Thomas, 2006), sperm capacitation in standard medium was followed by superfusion with low-Ca^{2+} saline for enough time to allow sperm to stabilise in it, the recording being performed in low-Ca^{2+} medium.

2.3.6 Imaging Data Processing

Data were processed offline using iQ software as described previously by Kirkman-Brown *et al.* (2000). 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. 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 normalised to pre-stimulus values with the equation

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

where R is normalised 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. Cell responses were observed from time-fluorescence intensity plots.

2.3.7 Imaging Data Statistical Analysis

For each cell, Microsoft Excel was used to calculate the mean and 95% confidence interval of fluorescence intensity for 14 images during the control period $(Con \pm con)$, 14 images from minute 3 after treatment $(A \pm a)$ and, 14 images from minute 6 after treatment $(B \pm b)$. An increase in fluorescence was considered significant at each sampling group of points if

$$X - x > Con + con$$

A decrease in fluorescence was considered significant at each sampling group of points if

$$X + x < Con - con$$

where *'X'* and *'x'* are the mean and 95% confidence interval for the sampling group of points and *'Con'* and *'con'* are the mean and 95% confidence interval for the control period. Cells were categorized as non responders when fluorescence remained stable. In experiments with two or more treatments, the same procedure was followed, by defining additional 'control' periods. Cells were sorted into those showing increase, decrease or no change in fluorescence after treatment. Observation of fluorescence-time plots for the sorted cells confirmed that this procedure provided an accurate sorting of cell responses. More complex $[Ca^{2+}]_i$ responses (such as oscillations) were quantifified by direct examination of time-fluorescence intensity plots only. Data from each type of experiment were pooled to calculate the frequency of each type of response, which is stated in the text as mean ± SEM. Microsoft Excel was used to perform t-tests for unpaired data. Statistical significance was set at $P<0.05$.

Often, the number of experimental replicates considered to calculate the frequency of responses, does not coincide with the number of experimental replicates to perform further statistical analysis, for the same experiments. Though all experiments of a certain type clearly show the same patterns of response, only the ones with stable control periods and steady

responses were considered for further statistical analysis. Moreover, in some of these cases, experiments are not long enough to perform statistical analysis at a certain point in time.

2.3.8 Evaluation of $[Ca^{2+}]_i$ oscillations

Spermatozoa were categorized as oscillators in accordance with Harper *et al.* (2004); sperm cells displaying $[Ca^{2+}]_i$ oscillations were directly identified from time/fluorescence intensity plots (Harper *et al.*, 2004).

2.4 Results

2.4.1 Expression of NOS and NO synthesis by cumulus cells

To confirm that human spermatozoa encounter increased NO concentrations upon approaching the oocyte, the expression of NOS in isolated fragments of human cumulus was assessed. Antibodies for eNOS revealed the presence of this isoform in almost every cell of human cumulus fragments (Fig. 2.1). Similar results were obtained with antibodies specific for nNOS (data not shown). To investigate whether human cumulus fragments synthesised NO, cells were loaded with the NO probe DAF-FM diacetate (5 µM). A rapid increase in fluorescence was observed upon cell loading, indicative of NO synthesis (Fig. 2.1.D). Cell incubation with L_NAME (1 mM) completely blocked the increase in fluorescence (data not shown). Human sperm incubation with DAF-FM diacetate did not generate a detectable fluorescent signal (data not shown), indicating that NOS activity in spermatozoa is much lower than in the cumulus. Loading of mouse cumulus with DAF-FM diacetate also revealed NO synthesis by the cumulus cells, although only 10-15% of cells became stained (data not shown). The immunocytochemistry and DAF-FM diacetate staining reactions were performed by Dr. L. Lefièvre and Dr. C. Ford.

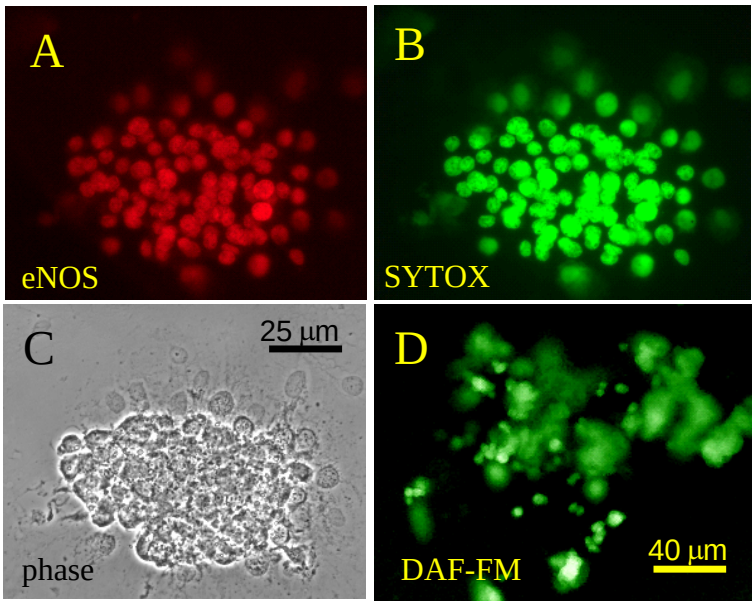

Fig. 2.1. NOS detection and NO synthesis in human cumulus cells. **A.** Staining of human cumulus fragments sample for eNOS. **B.** Staining of same sample with SYTOX Green to reveal all cumulus cells. **C.** Phase image of same cumulus sample. **D.** DAF-FM diacetate staining (green fluorescence) of human cumulus.

2.4.2 NO effects on $[Ca^{2+}]_i$ in human sperm

Expression of active NOS in human cumulus cells indicates that spermatozoa will encounter NO as they approach the oocyte. To investigate the possible effects of this stimulus on $[Ca^{2+}]_i$ in capacitated human sperm, the NO donor spermine NONOate was applied to sperm previously loaded with OGB-1AM. Spermine NONOate (100 µM) induced a $[Ca^{2+}]_i$ increase (Fig. 2.2) in 73±5% of cells (8 experiments). After treatment with spermine NONOate, $[Ca^{2+}]_i$ tended to gradually rise (Fig. 2.2). The latency of the rise was 0-2 minutes and fluorescence typically stabilised at approximately 20% above control levels after 10 minutes (Fig. 2.2 and 2.4.A). In 19% of cells, oscillations were superimposed on the NO-stimulated $[Ca^{2+}]_i$ increase (Fig. 2.2; yellow trace). Controls in which untreated sperm were continuously superfused with sEBSS, showed a small

upward drift in $[Ca^{2+}]_i$ (Fig. 2.2 insert) in most cells (5 experiments), fluorescence increasing by approximately 3% during the 10 minutes equivalent to the period of NONOate exposure (Fig. 2.2 insert and 2.4.B). NONOate-induced $[Ca^{2+}]_i$ rise in sperm bathed in sEBSS was significantly greater ($P<0.001$; unpaired-t) than that seen in untreated sperm bathed in sEBSS. To establish whether the spermine NONOate-induced increase in $[Ca^{2+}]_i$ was primarily due to Ca^{2+} influx at the plasma membrane, the same experiments were repeated in medium to which no Ca^{2+} had been added (CFsEBSS), where $[Ca^{2+}]_o$ is ≤ 5 μM (Harper *et al.*, 2004). Under low-Ca^{2+} conditions, spermine NONOate (100 μM) stimulated a $[Ca^{2+}]_i$ rise (Fig. 2.3) in 64±12% of cells (4 experiments). The latency of the rise was 0-2 minutes and fluorescence tended to stabilise at approximately 24% above control levels after 10 minutes (Fig. 2.3 and 2.4.A). $[Ca^{2+}]_i$ oscillations were rarely observed (Fig. 2.3). In controls in which untreated sperm were continuously superfused with CFsEBSS, $[Ca^{2+}]_i$ tended to remain approximately at baseline levels in most cells (Fig. 2.3 insert and 2.4.B; 3 experiments; $P<0.001$, compared to sEBSS controls). Thus, NONOate induced a highly significant $[Ca^{2+}]_i$ rise under low-Ca^{2+} conditions ($P<0.001$; unpaired-t). Spermine NONOate-stimulated rise in fluorescence under low-Ca^{2+} conditions was significantly ($P<0.05$; unpaired-t) higher than the NONOate-stimulated increase in fluorescence under normal Ca^{2+} conditions (Fig. 2.4.A). These results indicate that NO increases $[Ca^{2+}]_i$ in human sperm by mobilization of an intracellular store rather than by Ca^{2+} influx at the plasmalemma. The $[Ca^{2+}]_i$ rise in both the presence and absence of added Ca^{2+} was localised primarily to the neck/mid-piece region, spreading into the posterior region of the sperm head (Fig. 2.4.C).

Spermine NONOate washout experiments were performed to investigate whether the NO effects were reversible. Spermine NONOate (100 µM) induced $[Ca^{2+}]_i$ rise was followed by an abrupt decrease in $[Ca^{2+}]_i$ (71±9% of cells; 3 experiments), reaching levels slightly above those seen before treatment. Many cells then showed a spontaneous $[Ca^{2+}]_i$ elevation during the washout period. Upon reintroduction of spermine NONOate (100 µM) to the imaging chamber, most cells again responded (51±6% of cells; 3 experiments) with a marked $[Ca^{2+}]_i$ rise, regularly occurring as a series of oscillations in the neck/mid-piece region (Fig. 2.5).

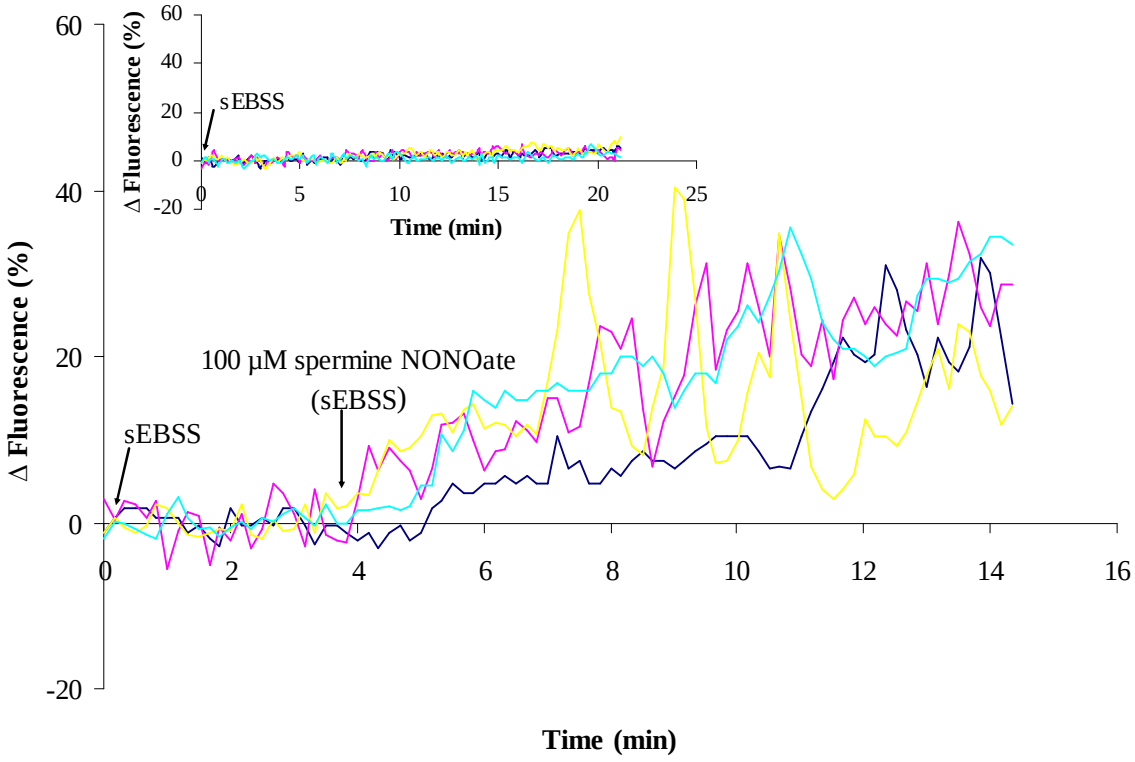

Fig. 2.2. The effects of spermine NONOate on $[Ca^{2+}]_i$ in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 4 minutes, and then superfused with 100 µM spermine NONOate in the same medium. Traces represent 4 individual cell responses. **Insert:** Control in which sperm prepared as for the NONOate experiment were superfused with sEBSS in the absence of any treatment. Traces show 4 single cell responses.

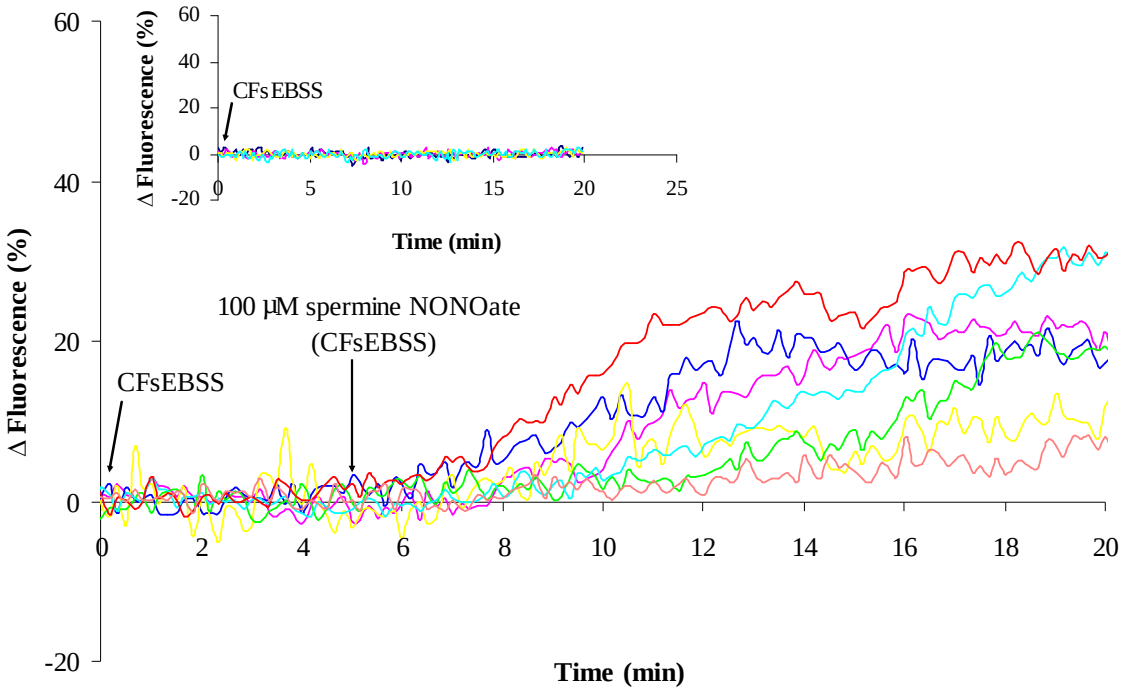

Fig. 2.3. The effects of spermine NONOate on $[Ca^{2+}]_i$ in capacitated human sperm bathed in CFsEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with CFsEBSS for 5 minutes (after being stabilised in CFsEBSS), and then superfused with 100 µM spermine NONOate in the same medium. Traces show 7 single cell responses. **Insert:** Control in which sperm prepared as for the NONOate experiment were superfused with CFsEBSS in the absence of any treatment. Traces show 4 single cell responses.

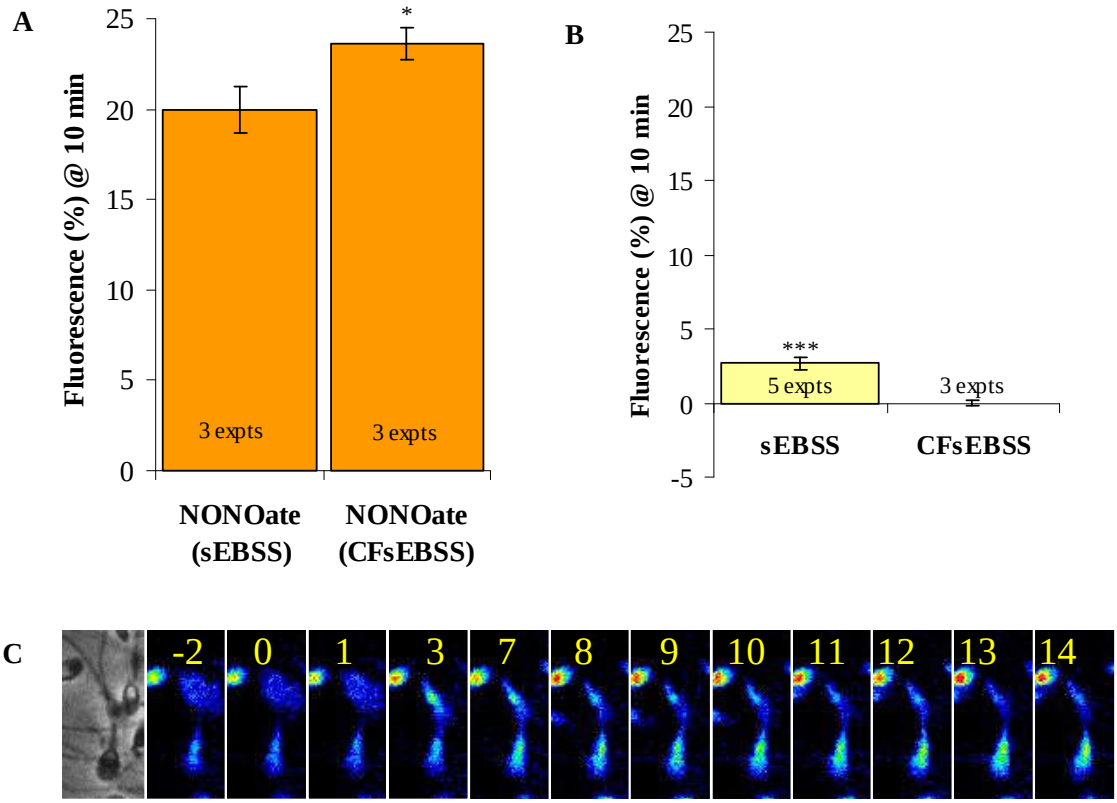

Fig. 2.4.A. Mean NONOate-induced $[Ca^{2+}]_i$ increase in standard sEBSS and in CFsEBSS in capacitated human sperm. Bar chart shows the mean normalised increase in fluorescence 10 minutes after NONOate was added to sperm (sEBSS = 271 cells; CFsEBSS = 214 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance ($P<0.05$). **B.** Drift of $[Ca^{2+}]_i$ (fluorescence) in control cells bathed in sEBSS and CFsEBSS. Bar chart shows the mean normalised increase in fluorescence over a 10 minute period measured as in the experimental protocol (sEBSS = 215 cells; CFsEBSS = 169 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance ($P<0.001$) **C.** Pseudocolour image series (warm colours show high $[Ca^{2+}]_i$) shows spermine NONOate-stimulated increase in $[Ca^{2+}]_i$ in sperm neck/mid-piece region. Numbers represent time in minutes following exposure to 100 µM spermine NONOate.

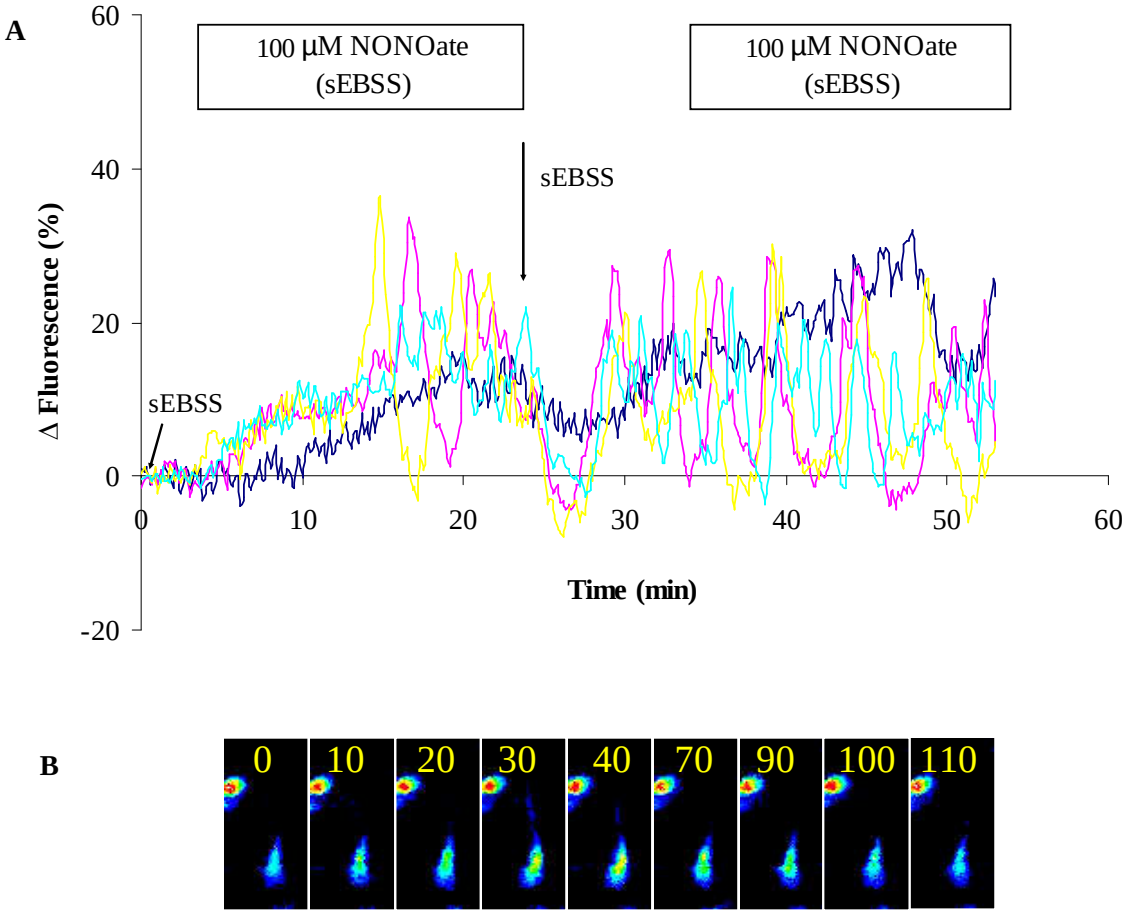

Fig. 2.5.A. The effects of spermine NONOate washout in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 4 minutes, then superfused with 100 µM NONOate, which was washed out and finally NONOate was re-applied, at the same dose. Traces show 4 single cell responses. **B.** Pseudocolour image series (warm colours show high $[Ca^{2+}]_i$) of a single $[Ca^{2+}]_i$ oscillation. Numbers show time in seconds.

2.4.3 HNO effects on $[Ca^{2+}]_i$ in human sperm

Having examined the effects of NO on $[Ca^{2+}]_i$, the effects of the NO redox sibling HNO on $[Ca^{2+}]_i$ in capacitated human sperm were also determined. The HNO donor ANGS was used to study these effects. ANGS (60 µM) stimulated a $[Ca^{2+}]_i$ elevation (Fig. 2.6) in 71±10% of cells (5 experiments). OGB-1AM loaded cells exposed to ANGS tended to show a slow rise in $[Ca^{2+}]_i$. The latency of the $[Ca^{2+}]_i$ elevation was 0-5 minutes and there was a tendency for the fluorescence continuously to rise while stimulus was present (Fig. 2.6). In 16% of cells, oscillations were superimposed on the HNO-induced $[Ca^{2+}]_i$ increase (Fig. 2.6; pink trace). Controls in which untreated sperm were continuously superfused with sEBSS, showed a small upward drift in $[Ca^{2+}]_i$ (Fig. 2.6 insert) in most cells, fluorescence increasing by approximately 3% during the 10 minutes equivalent to the period of ANGS exposure (5 experiments; Fig. 2.6 insert and 2.8.B). ANGS-induced $[Ca^{2+}]_i$ elevation in sEBSS was significantly greater ($P<0.001$; unpaired-t) than that observed in controls. To determine whether the increase in $[Ca^{2+}]_i$ was primarily due to Ca^{2+} influx at the plasma membrane, the same experiments were repeated in saline to which no Ca^{2+} had been added (CFsEBSS), where $[Ca^{2+}]_o$ is $\leq$ 5µM (Harper *et al.*, 2004). ANGS induced a $[Ca^{2+}]_i$ rise under low-Ca^{2+} conditions (Fig. 2.7) in 64±11% of cells (4 experiments). Although some cells responded similarly as in standard medium, the general tendency was for fluorescence to stabilise at approximately 20%, 5 minutes after ANGS was added, and the latency of the $[Ca^{2+}]_i$ rise was 0-5 minutes (Fig. 2.7 and 2.8.A). In 8% of cells, oscillations were superimposed on the HNO-induced $[Ca^{2+}]_i$ elevation under low-Ca^{2+} conditions. In controls in which untreated sperm were continuously superfused with CFsEBSS, fluorescence remained at baseline levels in most cells (Fig. 2.7 insert and 2.8.B; 3 experiments). Therefore, ANGS stimulated a highly significant $[Ca^{2+}]_i$ rise under low-Ca^{2+} conditions ($P<0.001$; unpaired-t). ANGS-induced

increase in fluorescence under low-Ca^{2+} conditions was significantly greater ($P<0.001$; unpaired-t) than that occurred under normal Ca^{2+} conditions, 10 minutes after the drug was added to the cells (Fig. 2.8.A).

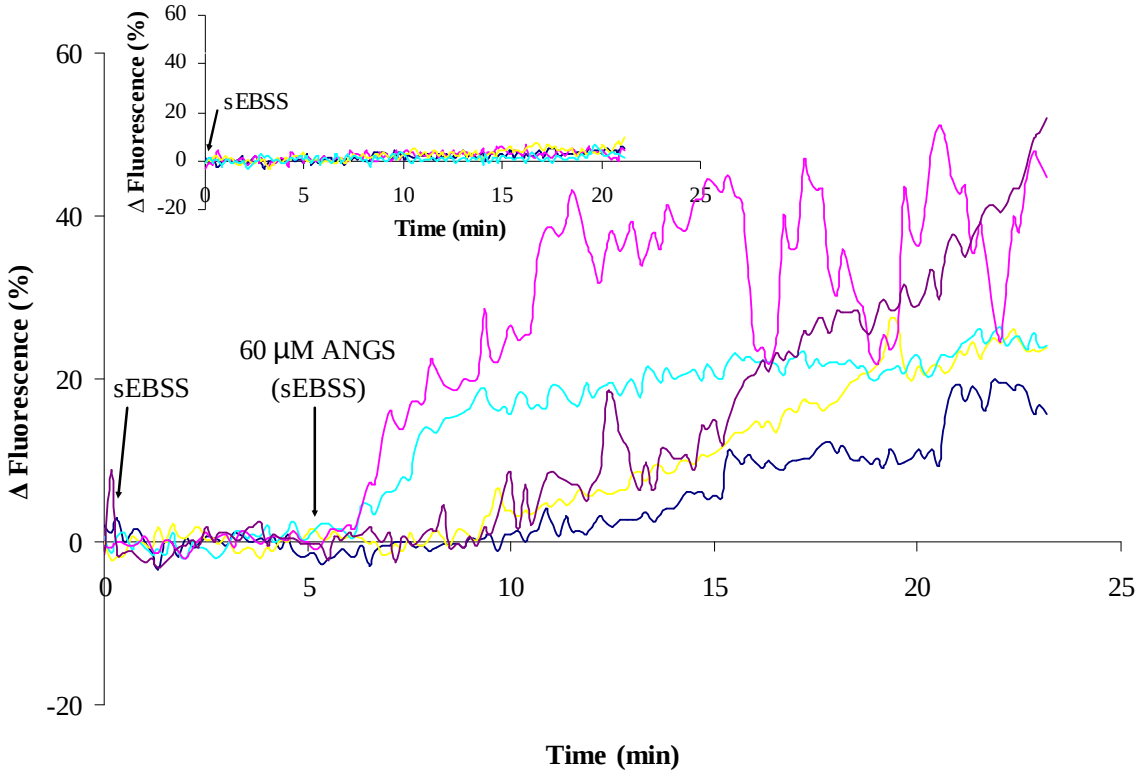

Fig. 2.6. The effects of HNO on $[Ca^{2+}]_i$ in capacitated human sperm bathed in sEBSS. Cells were incubated in sEBSS for 6 hours, superfused with sEBSS for 5 minutes and then superfused with 60 μM ANGS in sEBSS. Traces show 5 individual cell responses. **Insert:** Control in which sperm prepared as for the ANGS experiment were superfused with sEBSS in the absence of any treatment. Traces show 4 single cell responses.

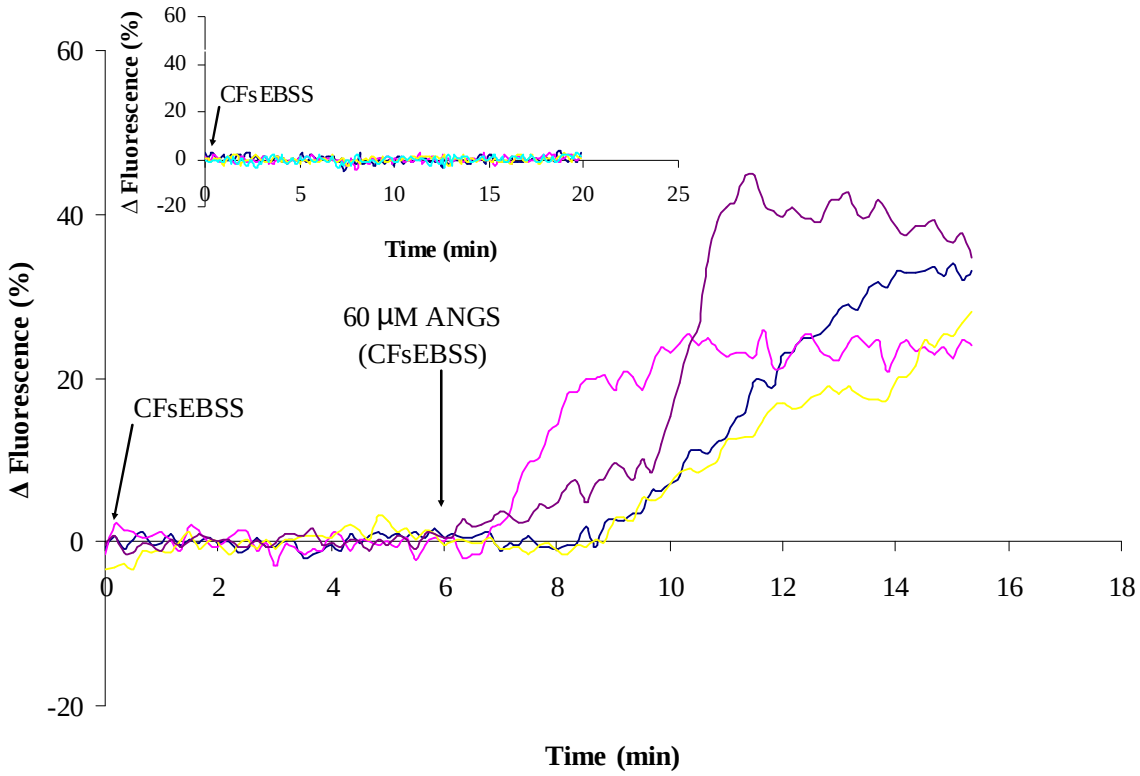

Fig. 2.7. The effects of HNO on $[Ca^{2+}]_i$ in capacitated human sperm bathed in CFsEBSS. Cells were incubated in sEBSS for 6 hours, superfused with CFsEBSS for 6 minutes (after being stabilised in low-Ca^{2+} saline) and then superfused with 60 µM ANGS in CFsEBSS. Traces show 4 single cell responses. **Insert:** Control in which sperm prepared as for the ANGS experiment were superfused with CFsEBSS in the absence of any treatment. Traces show 4 single cell responses.

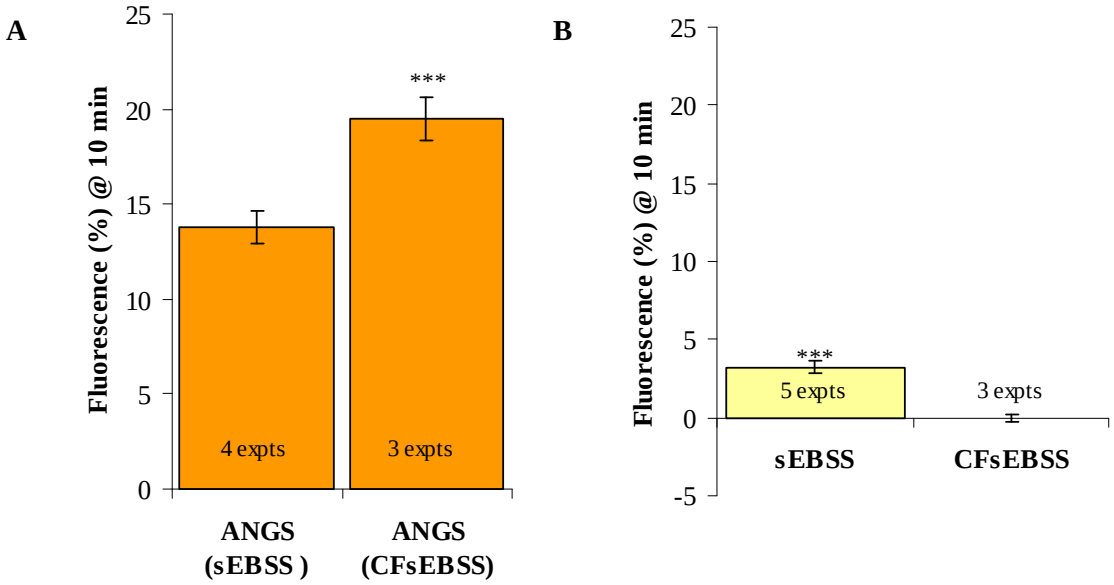

Fig. 2.8.A. Mean ANGS-induced $[Ca^{2+}]_i$ increase in standard sEBSS and in CFsEBSS in capacitated human sperm. Bar chart shows the mean normalised increase in fluorescence 10 minutes after 60 µM ANGS was added to sperm (sEBSS = 288 cells; CFsEBSS = 164 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks indicate differences in significance ($P<0.001$). **B.** Drift of $[Ca^{2+}]_i$ (fluorescence) in control cells bathed in sEBSS and CFsEBSS. Bar chart shows the mean normalised increase in fluorescence over a 10 minute period measured as in the experimental protocol (sEBSS = 215 cells; CFsEBSS = 169 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance ($P<0.001$).

2.5 Discussion

The expression of NOS has been revealed in cells of the male and female mammalian reproductive tracts and also in gametes of vertebrates and invertebrates, suggesting that NO may play important physiological roles in fertilization (Creech *et al.*, 1998; Kim *et al.*, 2004; Rosselli *et al.*, 1998; Thaler and Epel, 2003). It has been reported that NOS is present in the mammalian oviduct (Rosselli *et al.*, 1996; Ekerhovd *et al.*, 1999; Lapointe *et al.*, 2006) and also in the oocyte and in the cumulus and corona cells that surround it (Hattori *et al.*, 2001; Reyes *et al.*, 2004; Tao *et al.*, 2004). Numerous cell types in the mammalian female reproductive tract, including oviductal cells, synthesise significant levels of NO (Rosselli *et al.*, 1998).

Immunocytochemistry revealed that constitutive NOS (eNOS and nNOS) are expressed in human cumulus cells. Comparison of NOS staining and SYTOX showed that nearly every cell in the human cumulus samples used stained positively for constitutive NOS, and DAF-FM diacetate staining showed that the majority were synthesising NO (Fig. 2.1). Although cumulus invested-mouse oocytes were also generating NO, only 10-15% of the cumulus cells strongly stained by DAF-FM diacetate (data not shown). Nevertheless, a radius of influence of up to 200 µm for each individual cell (Lancaster, 1997) may be enough to avoid a 'patchy' distribution of NO within the cumulus. eNOS and nNOS are expressed in human sperm (Herrero *et al.*, 1996; Lewis *et al.*, 1996; O'Bryan *et al.*, 1998; Revelli *et al.*, 1999; Sengoku *et al.*, 1998; Willipinski-Stapelfeldt *et al.*, 2004; Zhang *et al.*, 2006). NOS inhibitors reduce synthesis of NO in human sperm suspensions (Donnelly *et al.*, 1997; Revelli *et al.*, 1999), at concentrations that are reported to decrease sperm motility (Lewis *et al.*, 1996), serine-

threonine and tyrosine phosphorylation (Herrero *et al.*, 1999; O'Flaherty *et al.*, 2004a), acrosome reaction (Herrero *et al.*, 1999; Revelli *et al.*, 1999) and sperm-oocyte fusion (Francavilla *et al.*, 2000). Although evidence has been found that NO can be produced by human sperm, endogenous generation of NO was not detectable under the incubation conditions in our assay. This finding is consistent with that of Zini *et al.* (1995) whose data showed that human spermatozoa do not have detectable NOS activity. The assay we used is not as sensitive as other assays used in different laboratories (Revelli *et al.*, 1999), which may explain the failure to detect NOS activity in human sperm. Thus, although there is evidence that sperm synthesise NO, it is not clear whether this synthesis is physiologically significant (Lefièvre *et al.*, 2007). Therefore, autocrine effects of NO generated by sperm are likely to be modest compared to NO doses generated by sources within the female tract. Potential sources of NO within the female tract include endothelial cells, smooth muscle and leukocytes. As the action of NO on Ca^{2+} signalling is reversible, the modulation effect of this action will be dynamic, being turned on and off as 'required' in response to sources of NO.

Treatment of capacitated human sperm with spermine NONOate (100 µM) caused a clear rise in $[Ca^{2+}]_i$ in the neck/mid-piece region of most cells, regularly spreading into the posterior of the sperm head (Fig. 2.2, 2.3 and 2.4.C). This effect of NO was not altered by omission of Ca^{2+} from the saline (≈ 1000-fold reduction in $[Ca^{2+}]_o$; Fig. 2.3 and 2.4.A), showing that the effect of NO primarily reflects store mobilization. Interestingly, the $[Ca^{2+}]_i$ elevation that occurred under low-Ca^{2+} conditions was significantly ($P<0.05\%$) higher than that occurred under normal Ca^{2+} conditions (Fig. 2.4.A), possibly because the Ca^{2+} mobilizing effects of NO are enhanced by the low levels of Ca^{2+} in the extracellular medium. In a number of cell types (e.g. macrophages, gut interstitial cells, pancreatic β-cells), NO raises $[Ca^{2+}]_i$, via the

activation of a ryanodine receptor (RyR), mobilizing inositol-1,4,5-triphosphate ($InsP_3$)-insensitive Ca^{2+} pools (Willmott *et al.*, 1996). The distinct rise in $[Ca^{2+}]_i$ seen under control conditions (sEBSS but not CFsEBSS; Fig. 2.2 insert, 2.3 insert and 2.4.B), is consistent with the report that long-wavelength laser irradiation of mouse sperm stimulates a rise in $[Ca^{2+}]_i$ and consequently the ability to fertilize, both events being abrogated by the absence of Ca^{2+} in the incubation medium during irradiation. The effect of the irradiation may be mediated by the generation of hydrogen peroxide (H_2O_2) by sperm (Cohen *et al.*, 1998).

A subset of responsive cells presented $[Ca^{2+}]_i$ oscillations, superimposed on the NONOate-stimulated $[Ca^{2+}]_i$ rise (Fig. 2.2; yellow trace), resembling those stimulated by progesterone (Harper *et al.*, 2004; Kirkman-Brown *et al.*, 2004). Washout of NONOate induced an immediate decrease in $[Ca^{2+}]_i$, reaching levels similar to those seen prior to NONOate exposure. Most cells then showed a slow $[Ca^{2+}]_i$ rise and spontaneous resumption of oscillation (Fig. 2.5), once more resembling the effect of progesterone (Harper *et al.*, 2004).

Although studies have recently been carried out to understand the potential effects of the NO redox sibling HNO, the participation of HNO in the modulation of sperm functions has not yet been investigated. Exposure of capacitated human sperm to ANGS led to a progressive rise in $[Ca^{2+}]_i$ (Fig. 2.6). This effect of ANGS was not modified by omission of Ca^{2+} from the saline (Fig. 2.7), indicating that the effect of HNO may also reveal primarily store mobilization. Moreover, the HNO-induced $[Ca^{2+}]_i$ rise under low-Ca^{2+} conditions occurred faster (then fluorescence tended to stabilise) than in standard Ca^{2+} conditions, which may explain the difference in significance ($P<0.001$) when comparing the variation in OGB-1AM fluorescence 10 minutes after applying ANGS under both conditions (Fig. 2.8.A). The low levels of Ca^{2+} in the extracellular medium may lead to the faster Ca^{2+} mobilizing ability of

HNO under these conditions. These findings are consistent with the previous reports that HNO potentially stimulate Ca^{2+} release from RyRs in skeletal (Cheong *et al.*, 2005) and cardiac muscles (Cheong *et al.*, 2005; Tocchetti *et al.*, 2007), suggesting that store mobilization via activation of RyRs could underlie our observations.

The data presented in this study show that NO is generated in the cumulus cells that surround the oocyte. The results indicate that the ability of NO and that of HNO to raise $[Ca^{2+}]_i$ reflect primarily intracellular store mobilization, and that the NO effects on $[Ca^{2+}]_i$ are reversible.

CHAPTER THREE

NITRIC OXIDE (NO) ACTS THROUGH AN ALTERNATIVE PATHWAY TO THE SOLUBLE GUANYLATE CYCLASE (sGC) IN CAPACITATED HUMAN SPERMATOZOA

3.1 Abstract

The potential significance of sGC and generation of cyclic guanosine monophosphate (cGMP) in the action of NO on $[Ca^{2+}]_i$ in capacitated human spermatozoa was investigated. 8-bromoguanosine-3'-5'-cyclophosphate sodium salt (8-bromo cGMP) caused a sustained elevation of $[Ca^{2+}]_i$ (mean increase in fluorescence >11%). This effect was greatly reduced in low-Ca^{2+} conditions. These results demonstrate that, unlike the NO effects on $[Ca^{2+}]_i$, cGMP exerts its effects mainly through Ca^{2+} influx at the plasma membrane. Inhibition of sGC with saturating doses of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a sGC inhibitor, did not modify the amplitude of the response to N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate). The data show that NO acts through an alternative pathway to the sGC-cGMP in human spermatozoa.

3.2 Introduction

Cyclic nucleotide signalling is fundamental in the functioning of all sperm (Jimenez-Gonzalez *et al.*, 2006). CNG (cyclic nucleotide-gated) channels are directly activated by cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) and are permeable to Ca^{2+} (Finn *et al.*, 1996; Kaupp, 1995). Due to their high Ca^{2+} permeability (Frings *et al.*, 1995), CNG channels are the principal candidates for mediating Ca^{2+} influx in sperm that is regulated by cyclic nucleotides (Wiesner *et al.*, 1998). In invertebrate cells, cGMP regulates motility and the acrosome reaction (Kaupp *et al.*, 2003). In mammalian sperm, cGMP levels are very low and cAMP seems to have greater significance (Ain *et al.*, 1999; Lefièvre *et al.*, 2000). Cyclic nucleotides stimulate Ca^{2+} entry in mouse spermatozoa (Kobori *et al.*, 2000). A CNG channel, of the type found in vertebrate photoreceptors and olfactory neurons, was cloned from bovine testis (Weyand *et al.*, 1994). The channel is expressed in sperm, occurs in the principal piece of the flagellum, and is more sensitive to cGMP than cAMP (Weyand *et al.*, 1994; Wiesner *et al.*, 1998). Olfactory receptors, which activate cyclic nucleotide-mediated Ca^{2+} influx and regulate chemotaxis, have been described in human spermatozoa (Spehr *et al.*, 2003).

It is well established that a number of NO actions on smooth muscle cells, endothelial cells and platelets involve activation of sGC and production of cGMP (Murad, 1994b), but the mechanisms by which NO influences sperm function are still to be elucidated (Revelli *et al.*, 2001). sGC stimulation by NO and the significance of cGMP signalling in the sperm of marine invertebrates are well established (Revelli *et al.*, 2002). However, cGMP concentration and cGMP-specific phosphodiesterase (type 5) and protein kinase G (PKG)

actions are low or undetectable in human sperm (Lefièvre *et al.*, 2000; Willipinski-Stapelfeldt *et al.*, 2004). Stimulation of the acrosome reaction is the primary role of cGMP in human sperm (Bielfeld *et al.*, 1994; Revelli *et al.*, 2002). Furthermore, studies on capacitation have demonstrated that NO alters activities of other kinases. Addition of exogenous NO is associated with a rise in cAMP levels, causing tyrosine phosphorylation (Belen Herrero *et al.*, 2000) and contributes to the activation of protein extra cellular signal regulated kinases (ERKs; O'Flaherty *et al.*, 2006b; Thundathil *et al.*, 2003). It has been observed that intracellular cGMP increases during NO-dependent stimulation of acrosome reaction in capacitated bull sperm (Zamir *et al.*, 1995). A cGMP-independent action of NO is the covalent modification of proteins via S-nitrosylation, the creation of S-NO bond by cysteine thiol (-SH) nitrosylation to create a nitrosothiol (Hess *et al.*, 2005a; Stamler *et al.*, 1992).

The aim of this study was to elucidate whether the sGC transduction pathway is involved in the NO-induced increase in $[Ca^{2+}]_i$ in human sperm, using the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and the cGMP analogue 8-bromoguanosine-3'-5'-cyclophosphate sodium salt (8-bromo cGMP).

Part of this work has been published in the journal "Development".

3.3 Materials and methods

3.3.1 Materials

Salts used to prepare supplemented Earle's balanced salt solution (sEBSS) and Ca^{2+}-free sEBSS (CFsEBSS; see Appendix I for details), pluronic F-127, dimethyl sulfoxide (DMSO), ODQ, 8-bromo cGMP were all from Sigma-Aldrich (Poole, Dorset, UK). Fatty-acid free bovine serum albumin (BSA) was purchased from SAFC Biosciences (Lenexa, KS, USA). Oregon Green 488 BAPTA 1-acetoxymethyl (OGB-1AM) was obtained from Invitrogen Molecular Probes (Paisley, UK). Poly-D-lysine (PDL) was acquired from BD Biosciences (Oxford, UK). N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate) was purchased from Merck Chemicals Ltd. (Beeston, Nottingham, UK). All chemicals were cell-culture-tested grade where available.

3.3.2 Spermatozoa preparation and capacitation

As in Chapter Two, page 70.

3.3.3 Single cell imaging

As described in Chapter Two, page 71, with the following specifications to this chapter:

OGB-1AM loaded sperm were superfused with sEBSS (or CFsEBSS) + 0.3% BSA for an initial control period, then stimulated with 8-bromo cGMP (100 μM) dissolved in the same medium. In control experiments, cells were continuously superfused either with sEBSS (or CFsEBSS) + 0.3% BSA, in the absence of any treatment. In experiments using CFsEBSS,

cells were stabilised in low-Ca^{2+} saline before start recording, the recording being performed in CFsEBSS.

Additional experiments were performed using sEBSS + 0.3% BSA. After an initial control period in which sperm were bathed with standard medium, cells were exposed to ODQ (10 or 100 μM) for approximately 10 minutes. Spermine NONOate (100 μM) was then added in the continuous presence of ODQ.

3.3.4 Imaging Data Processing

As in Chapter Two, page 72.

3.3.5 Imaging Data Statistical Analysis

As in Chapter Two, page 73.

3.3.6 Evaluation of $[Ca^{2+}]_i$ oscillations

As in Chapter Two, page 74.

3.4 Results

3.4.1 Effects of cGMP and sGC inhibition on the NO-induced $[Ca^{2+}]_i$ signal

sGC is the 'classic target' for NO in its role as a messenger. Although cGMP (and thus sGC activity) seems to be low in mammalian spermatozoa, there is evidence that effects on acrosome reaction and probably other sperm functions are exerted through this pathway (Herrero *et al.*, 1998; Revelli *et al.*, 2001). The membrane-permeant analogue 8-bromo cGMP was used to investigate whether generation of cGMP accounts for the NO-stimulated $[Ca^{2+}]_i$ rise. 100 μM 8-bromo cGMP caused a rapid $[Ca^{2+}]_i$ elevation (77±9% of cells; 4 experiments), reaching a plateau after 2-3 minutes (Fig. 3.1.A) and fluorescence stabilised 5-20% above control levels (mean ≈ 11%; Fig. 3.1). Controls in which untreated sperm were continuously superfused with sEBSS, showed a small upward drift in $[Ca^{2+}]_i$ (Fig. 3.1.A insert). The $[Ca^{2+}]_i$ elevation induced by 8-bromo cGMP was significantly greater ($P<0.001$; unpaired-t) than the small drift seen under control conditions (Fig. 3.1). 8-bromo cGMP also increased $[Ca^{2+}]_i$ under low-Ca^{2+} conditions (Fig. 3.2.A; 63±9% of cells; 3 experiments). The $[Ca^{2+}]_i$ rise stimulated by 8-bromo cGMP under low-Ca^{2+} conditions, though significantly greater ($P<0.001$; unpaired-t) than the negligible drift observed in control cells (Fig. 3.2), was much smaller and developed more slowly than under standard conditions (Fig. 3.1, 3.2 and 3.3; $P<0.001$, unpaired-t). Therefore, unlike NO, cGMP raises $[Ca^{2+}]_i$ in human spermatozoa by increased Ca^{2+} influx rather than by mobilization of intracellular stores.

To ensure that the response to NO was not due to sGC activation, experiments were performed using the sGC inhibitor ODQ. 10 minutes pre-treatment with 10 μM ODQ, a concentration in excess of that required to inactivate sGC (Garthwaite *et al.*, 1995; Schrammel

et al., 1996), did not affect the amplitude of the spermine NONOate (100 µM) stimulated increase in $[Ca^{2+}]_i$ (P>0.05; unpaired-t; Fig. 3.4 and 3.5.B). 61% of ODQ pre-treated cells showed a $[Ca^{2+}]_i$ increase in response to NONOate (P>0.05; unpaired-t, compared with NONOate without ODQ pre-treatment) and >27% of cells presented superimposed oscillations on the NONOate-induced elevation of $[Ca^{2+}]_i$ (Fig. 3.4; blue, yellow and green traces). The experiments with ODQ were repeated using 100 µM of the inhibitor, more than five times the saturating concentration for blockade of sGC. 79% of cells showed a $[Ca^{2+}]_i$ rise in response to spermine NONOate (P>0.05; unpaired-t, compared to NONOate without and with 10 µM ODQ pre-treatment) and 36% of cells showed superimposed oscillations on the NONOate-induced $[Ca^{2+}]_i$ rise (Fig. 3.5.A; blue and brown traces). Although the amplitude of the increase in fluorescence recorded 10 minutes after application of NONOate was reduced in the presence of 100 µM ODQ (by >40%; P<0.001, unpaired-t; compared to NONOate without and with 10 µM ODQ), a response to NONOate was still clearly visible. Furthermore, in many cells the response to NONOate was more rapid under these conditions, occurring as a peak 1-2 minutes after NONOate application (Fig. 3.5.A).

The doses of ODQ used in the 10 µM and 100 µM experiments resulted in addition of DMSO (vehicle) at 0.04% and 0.07%, respectively. Controls (0.04% DMSO) showed an upward drift of fluorescence (9±1%, 10 minutes after addition of DMSO). ODQ also stimulated a $[Ca^{2+}]_i$ drift (7±1%, 10 minutes after addition of ODQ) but it was not significant (P>0.05%) compared to that seen in solvent control experiments, suggesting that the small rise in fluorescence observed during sperm pre-treatment with ODQ may be due primarily to presence of the solvent.

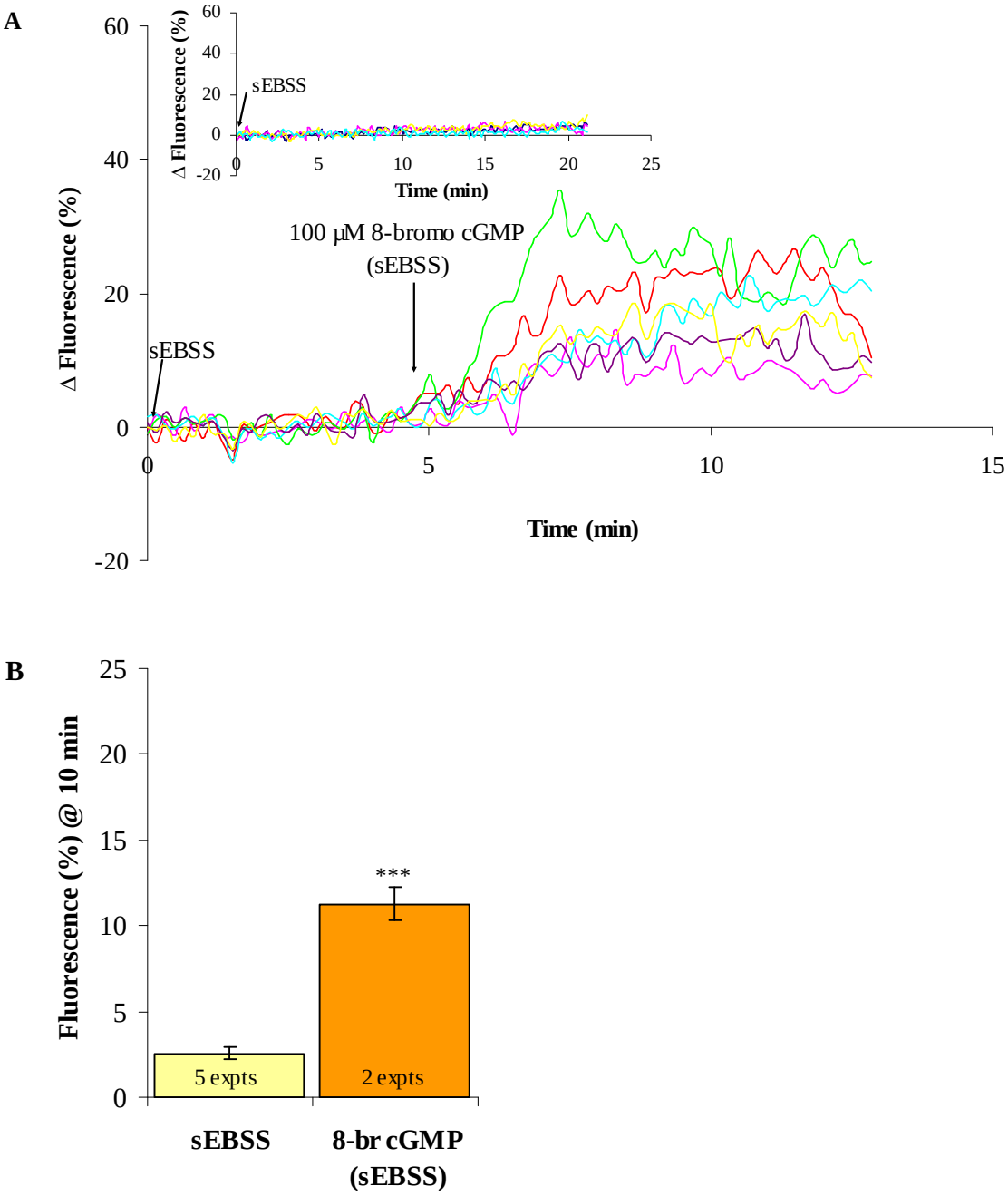

Fig. 3.1.A. The effects of 8-bromo cGMP on $[Ca^{2+}]_i$ in capacitated human sperm bathed in sEBSS. Sperm incubated in sEBSS for 6 hours, then superfused with sEBSS for 5 minutes and stimulated with 100 µM 8-bromo cGMP in the same medium. Traces show 6 individual cell responses. **Insert:** Control in which sperm prepared as for the 8-bromo cGMP experiment were superfused with sEBSS in the absence of any treatment.Traces show 4 single cell responses. **B.** Mean drift in $[Ca^{2+}]_i$ (

fluorescence) occurring under control conditions (sEBSS) and 8-bromo cGMP-induced $[Ca^{2+}]_i$ rise in capacitated human sperm bathed in sEBSS. Bar chart shows the mean normalised increase in fluorescence 10 minutes after adding 8-bromo cGMP to sperm bathed in sEBSS (72 cells) and over a 10 minute period measured as in the experimental protocol (215 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance (P<0.001).

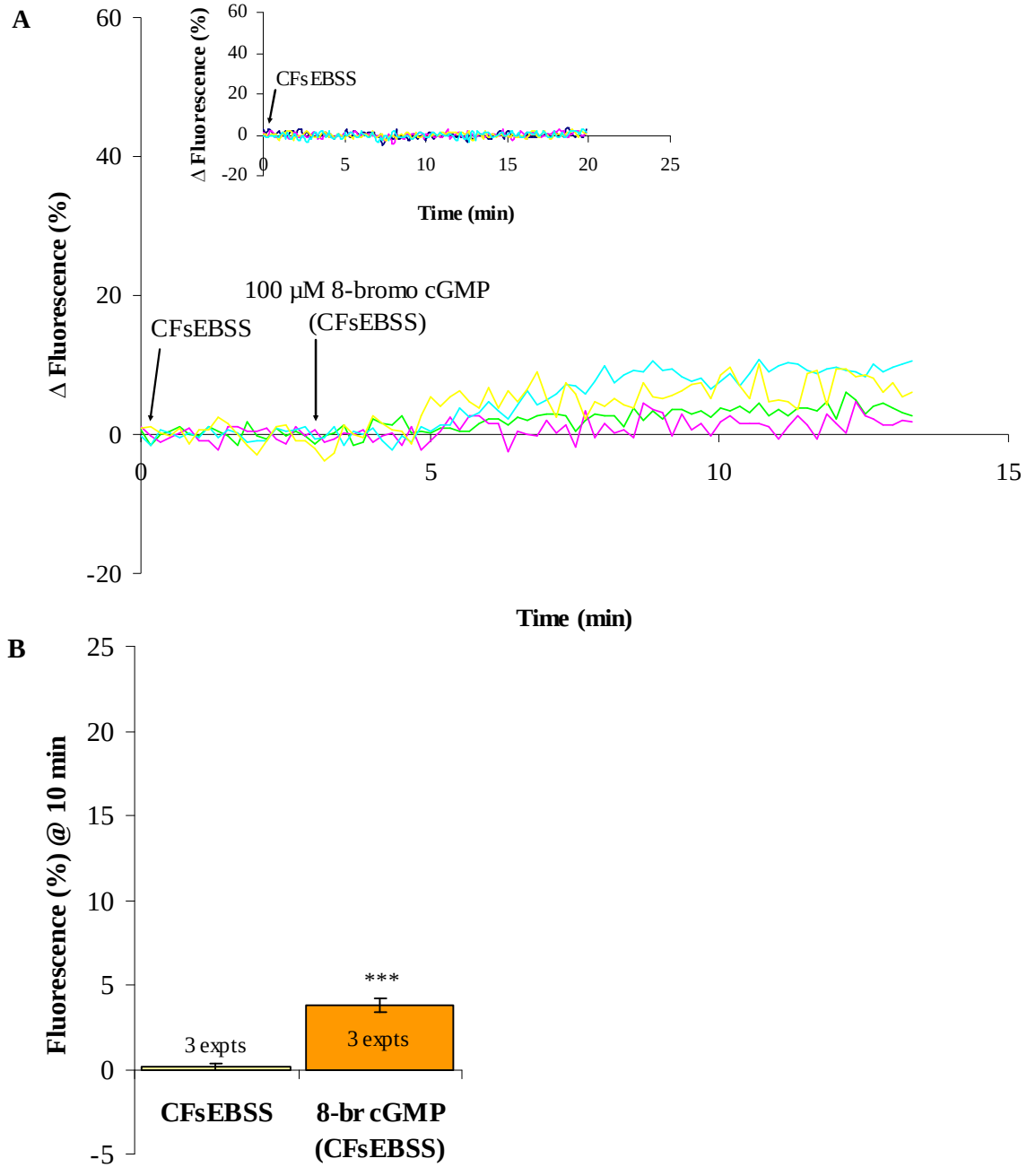

Fig. 3.2.A. The effects of 8-bromo cGMP on $[Ca^{2+}]_i$ in capacitated human sperm bathed in CFsEBSS. Sperm were incubated in sEBSS for 6 hours, then superfused with CFsEBSS for 3 minutes (after being

stabilised in CFsEBSS) and stimulated with 100 µM 8-bromo cGMP in the same saline. Traces represent 4 single cell responses. **Insert:** Control in which sperm prepared as for the 8-bromo cGMP experiment were superfused with CFsEBSS in the absence of any treatment. Traces show 4 cell responses. **B.** Mean drift in $[Ca^{2+}]_i$ (fluorescence) occurring under control conditions (CFsEBSS) and 8-bromo cGMP-induced $[Ca^{2+}]_i$ rise in capacitated human sperm bathed in CFsEBSS. Bar chart shows the mean normalised increase in fluorescence 10 minutes after adding 8-bromo cGMP to sperm bathed in CFsEBSS (122 cells) and over a 10 minute period measured as in the experimental protocol (169 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance (P<0.001).

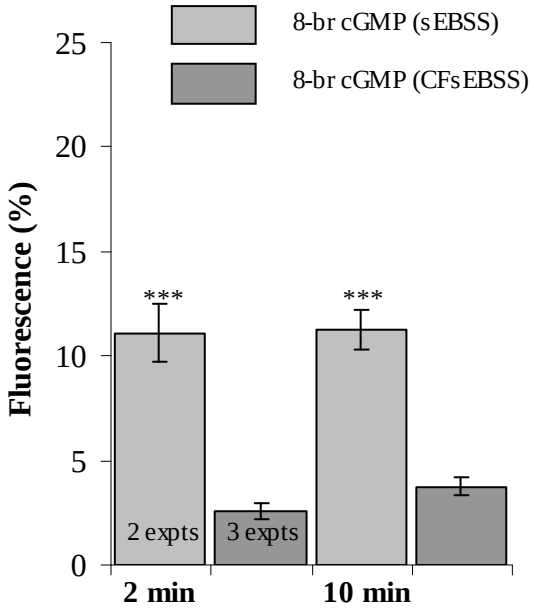

Fig. 3.3. Mean $[Ca^{2+}]_i$ rise induced by 8-bromo cGMP in capacitated human sperm bathed in sEBSS or CFsEBSS. Bar chart shows the mean normalised increase in fluorescence 2 and 10 minutes after 100 µM 8-bromo cGMP was added to sperm (sEBSS = 72 cells; CFsEBSS = 122 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance (P<0.001).

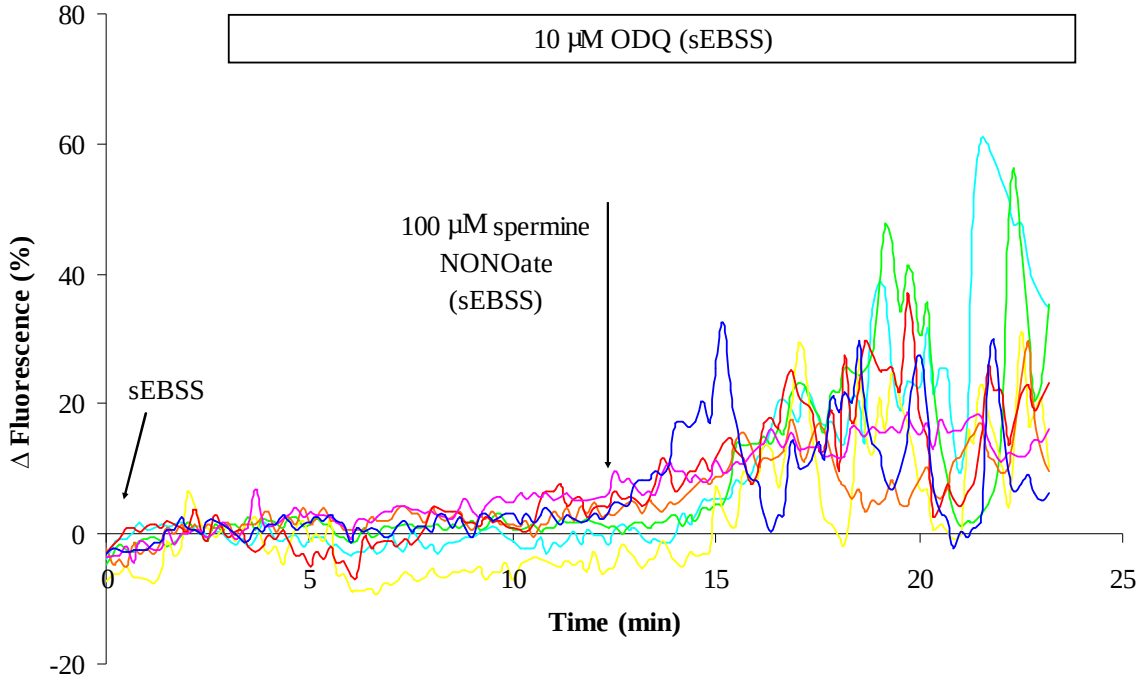

Fig. 3.4. The effects of 10 µM ODQ on spermine NONOate-induced $[Ca^{2+}]_i$ increase in capacitated human sperm bathed in sEBSS. Cells were incubated in sEBSS for 6 hours, superfused with sEBSS for 3 minutes, then pre-treated with ODQ for approximately 10 minutes and finally stimulated with 100 µM spermine NONOate, in the continued presence of ODQ. Traces show 7 single cell responses.

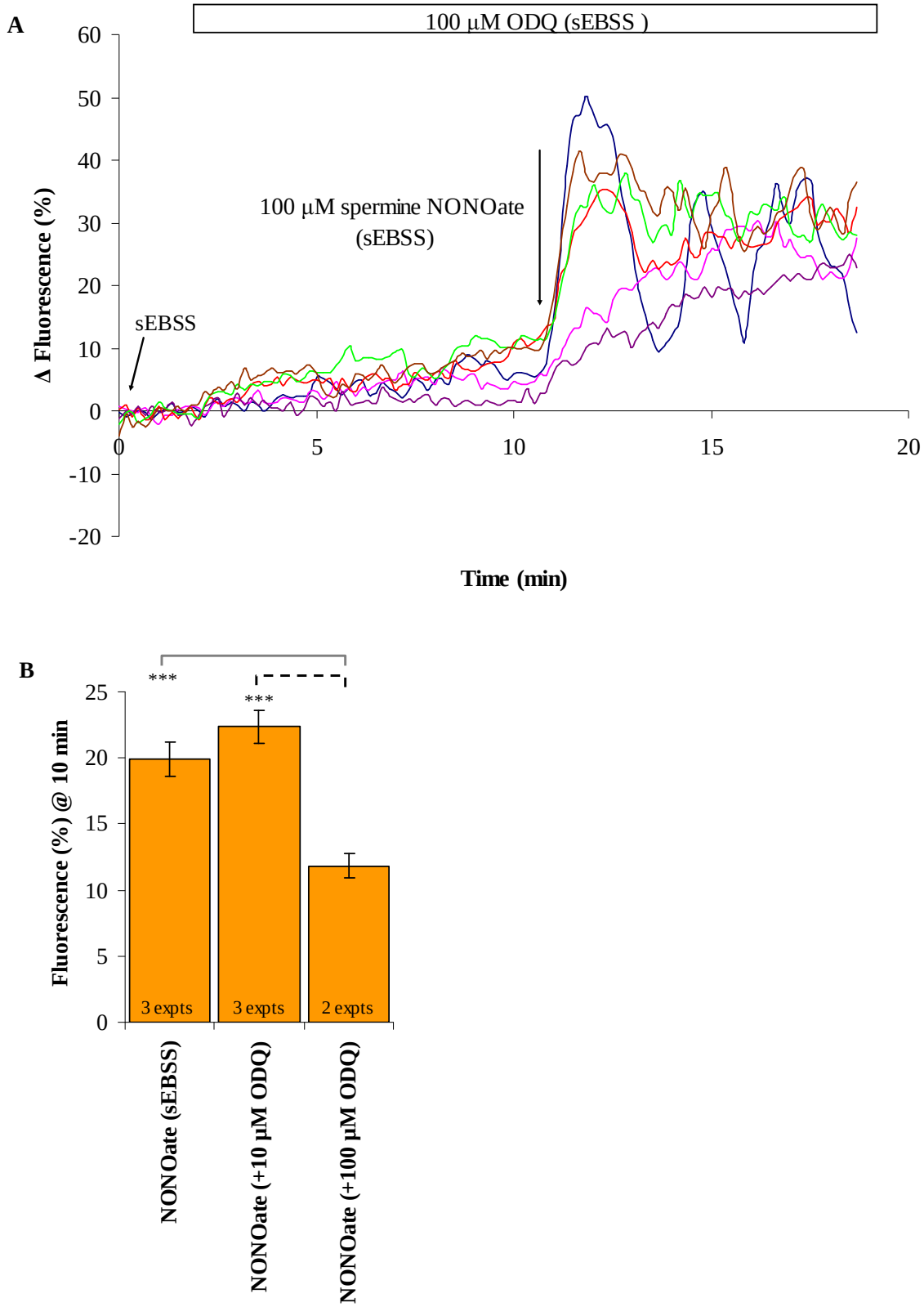

Fig. 3.5.A. The effects of 100 µM ODQ on spermine NONOate-induced $[Ca^{2+}]_i$ increase in capacitated human sperm bathed in sEBSS. Cells were incubated in sEBSS for 6 hours, superfused with sEBSS for 2.5 minutes, then exposed to ODQ for approximately 10 minutes and finally exposed to 100 µM

NONOate, in the presence of ODQ. Traces show 6 single cell responses. **B.** Differences between the spermine NONOate-induced $[Ca^{2+}]_i$ increase in the absence or presence of ODQ in capacitated human sperm bathed in sEBSS. Bar chart shows the mean normalised increase in fluorescence 10 minutes after adding 100 µM spermine NONOate to sperm (sEBSS = 271 cells; 10 µM ODQ in sEBSS = 208 cells; 100 µM ODQ in sEBSS = 137 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance ($P<0.001$).

3.5 Discussion

Although data regarding the effects of NO on human sperm function have been accumulating in the recent past, the intracellular mechanisms by which NO exerts these effects have not yet been clarified (Revelli *et al.*, 2001). The primary actions of NO in target tissues include activation of sGC, causing an increase in cGMP concentration and actions mediated through PKG or through direct action on CNG channels, and direct modulation of protein function by S-nitrosylation of exposed cysteine residues (Ahern *et al.*, 2002; Davis *et al.*, 2001). It has been suggested that NO is a chemoattractant for human spermatozoa, acting through stimulation of sGC (Miraglia *et al.*, 2007), and when we exposed human sperm to cGMP we observed a sustained rise in $[Ca^{2+}]_i$ similar to that observed with NO (Fig. 3.1.A). However, this effect was clearly dependent on $[Ca^{2+}]_o$ (Fig. 3.2 and 3.3), consistent with generation primarily by Ca^{2+} influx rather than store mobilization. These findings are consistent with those of Kobori *et al.* (2000) whose data showed that manipulation of cGMP and cAMP levels in mouse sperm induced a transient elevation of $[Ca^{2+}]_i$, which was significantly reduced in low-Ca^{2+} medium or in the presence of Ca^{2+}-channel blockers, cGMP being more effective than cAMP in rising $[Ca^{2+}]_i$. Moreover, sperm treatment with saturating concentrations of ODQ (10 µM), an effective inhibitor of sGC, did not modify the response to NO (Fig. 3.4 and 3.5.B). Sperm treatment with even higher doses of the inhibitor (100 µM), more than five times the saturating dose for blockade of sGC, still did not prevent sperm responding to NO, although the increment in fluorescence recorded 10 minutes after application of NO was significantly smaller than in control experiments (Fig. 3.5.B). Two factors may have contributed to this. Firstly, the increase in resting $[Ca^{2+}]_i$ due to pre-treatment with this very high dose (100 µM) of ODQ was such that a similar increment in $[Ca^{2+}]_i$ would produce a smaller percentage change in fluorescence. Secondly, the kinetics of

the response to NONOate under these conditions were different, fluorescence rising rapidly to a peak and then falling during the first 2-3 minutes after application of NONOate. Thus, it is concluded that NO-induced mobilization of Ca^{2+} shows little, if any sensitivity to ODQ even at 100 μM. In contrast, stimulation of acrosome reaction by NO was significantly blocked by 10 μM ODQ (Ford, W. C., unpublished data).

It has been reported that NO mobilizes Ca^{2+} from intracellular stores in the sea urchin egg via a pathway involving cGMP, activating the cyclic adenine dinucleotide phosphate ribose (cADPR)-sensitive Ca^{2+} release mechanism (Willmott *et al.*, 1996). Willmott *et al.* (1996) proposed that NO accounts for cADPR synthesis via a cGMP-dependent mechanism. cADPR binds to its receptor (Walseth *et al.*, 1993), opening a ryanodine receptor (RyR)-like Ca^{2+} channel in the endoplasmic reticulum (Galione *et al.*, 1991), elevating $[Ca^{2+}]_i$ (Willmott *et al.*, 1996). Conversely, it has been found that high doses of cADPR are present in human sperm and that these cells generate the molecule, but it does not appear to contribute to the regulation of the sperm themselves (Billington *et al.*, 2006). This mechanism of action of NO does not appear to underlie our observations.

It has been recently shown that NO, at doses used in this study, causes S-nitrosylation of numerous sperm proteins (Lefièvre *et al.*, 2007), suggesting that this alternative effect of NO could underlie our observations.

The results of this study indicate that the NO-induced increase in $[Ca^{2+}]_i$ via mobilization of intracellular Ca^{2+} stores in capacitated human sperm does not involve the activation of sGC.

CHAPTER FOUR

REGULATION OF PROTEIN THIOLS (–SH) REDOX STATE AND THE EFFECTS OF NITRIC OXIDE (NO) AND NITROXYL (HNO) IN CAPACITATED HUMAN SPERMATOZOA

4.1 Abstract

The –SH reducing agent dithiothreitol (DTT) was used to investigate the reversibility of the effects of NO on $[Ca^{2+}]_i$ by -SH group reduction. DTT reversed N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate)-induced elevation in $[Ca^{2+}]_i$. To determine whether reduction of mitochondrial -SH groups, leading to enhanced Ca^{2+} accumulation, might account for the effects of DTT on NONOate-induced increase of $[Ca^{2+}]_i$, NONOate treated cells were exposed to carbonyl cyanide m-chlorophenylhydrazone (CCCP), to disrupt mitochondrial membrane potential and inhibit Ca^{2+} uptake by mitochondria, prior to treatment with DTT. DTT was effective in decreasing $[Ca^{2+}]_i$ in CCCP treated cells. The data indicate that the mitochondria are not involved in Ca^{2+} uptake induced by -SH reducing agents. The effects of the protein S-nitrosylating agent S-nitrosoglutathione (GSNO) on $[Ca^{2+}]_i$ in capacitated human sperm were assessed. GSNO elevated $[Ca^{2+}]_i$, its effects being rapid in comparison to those of spermine NONOate (see Chapter Two). DTT and glutathione (GSH) were used to study the reversibility of the effects of GSNO on $[Ca^{2+}]_i$ by -SH group reduction or by prevention of protein S-nitrosylation, respectively. Both DTT and GSH reversed GSNO-induced elevation of $[Ca^{2+}]_i$. These results show that reversal or impediment of protein S-nitrosylation reverses NO-stimulated elevation of $[Ca^{2+}]_i$ and indicate that NO acts through S-nitrosylation of protein -SH groups. GSNO-induced protein S-nitrosylation in human sperm was reversed by DTT or simply by removal of the S-nitrosylating agent. The effect of DTT on Angeli's salt (ANGS)-treated cells was also investigated. ANGS-induced elevation of $[Ca^{2+}]_i$ was reversed by DTT. This result shows that the effect of HNO on $[Ca^{2+}]_i$ is reversed by -SH reducing agents.

4.2 Introduction

The mature mammalian spermatozoon is a 'minimalist' cell, specialised for its exclusive function of delivering the haploid nucleus and activating factors to the female gamete. To achieve this purpose, 'surplus' cytoplasm, including all endoplasmic reticulum is jettisoned during the last stage of differentiation, spermiogenesis. Though Gur and Breitbart (2008) have recently outlined reported findings that suggest that protein translation in mature spermatozoa may occur, differentiated sperm lack much of the apparatus which enables somatic cells to perform DNA transcription and protein expression. Differentiated sperm must undergo functional modifications during maturation in the epididymis and upon interaction with the female reproductive tract (Suarez and Pacey, 2006). These events, which are crucial for fertilization to occur, depend primarily on post-translational modification of proteins (e.g. tyrosine phosphorylation; Ficarro *et al.*, 2003) in mature cells (Lefièvre *et al.*, 2007).

NO can act independently of cyclic guanosine monophosphate (cGMP), via protein S-nitrosylation, the creation of S-NO bond by cysteine –SH nitrosylation to create a nitrosothiol (Hess *et al.*, 2005a; Stamler *et al.*, 1992). S-nitrosylation is a regulated post-translational protein modification that controls physiological cellular signalling similarly to phosphorylation and acetylation (Foster and Stamler, 2004; Hess *et al.*, 2005a). More than 120 proteins from animal and plant cells have been reported to undergo S-nitrosylation (Greco *et al.*, 2006; Hao *et al.*, 2006; Hess *et al.*, 2005a; Rhee *et al.*, 2005; Wang *et al.*, 2006) and a growing number have been revealed to be functionally regulated by S-nitrosylation, including the estrogen receptor (Garban *et al.*, 2005), connexin 43 hemichannels (Retamal *et al.*, 2006) and dynamin (Wang *et al.*, 2006). S-nitrosylation is still unexplored in spermatozoa (Lefièvre *et al.*, 2007).

In biological systems, NO participates in a equilibrium with S-nitrosothiols (RSNO), HNO and also metal complexes that can liberate nitrosonium ($^{+}$NO; Hughes, 1999). NO and its donors oxidize -SH through S-nitrosylation and HNO converts -SH to unstable S-derived hydroxylamines that interact with another -SH to form a disulfide (SS) bond and hydroxylamine (Arnelle and Stamler, 1995; Doyle et al., 1988). It is sensible to predict that activation of ryanodine receptors (RyRs) by HNO occurs via an oxidation reaction through the formation of SS bonds (Cheong *et al.*, 2005). Low molecular weight RSNO, like S-nitrosoglutathione (GSNO), S-nitrosocysteine (CSNO) and S-nitrosohomocysteine are thought to be signalling molecules that regulate the action of -SH-containing enzymes via trans-S-nitrosylation (Stamler *et al.*, 2001). An example of such modification is the S-nitrosylation of RyR types 1 and 2 (Stoyanovsky *et al.*, 1997). RSNO have been thought to have a role in many NO-mediated biological effects, but the mechanisms underlying RSNO or their S-nitroso groups' uptake into cells are poorly defined (Zhang and Hogg, 2004).

The purposes of this study were to assess the effects of nitrosylating conditions on $[Ca^{2+}]_i$ and sperm proteins using the nitrosylating agents GSNO and CSNO, and the subsequent -SH reducing agent (dithiothreitol or DTT) and S-nitrosylation blocking agent (glutathione or GSH) effects in those cells to determine the reversibility of the nitrosylating effects on $[Ca^{2+}]_i$ and proteins by reversal or prevention of protein S-nitrosylation. The same approach was used in cells exposed to the NO donor N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate), and upon exposure to the mitochondria uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP), to determine whether the reduction of mitochondrial -SH groups accounts for the effects of DTT on NO-induced

elevation of $[Ca^{2+}]_i$. The reducing agent DTT was also used to evaluate the reversibility of HNO (donated by the HNO donor Angeli's salt (ANGS)) effects on $[Ca^{2+}]_i$.

Part of this work has been published in the journal "Development".

4.3 Materials and methods

4.3.1 Materials

Salts used to prepare supplemented Earle's balanced salt solution (sEBSS) and Ca^{2+}-free sEBSS (CFsEBSS; see Appendix I for details), pluronic F-127, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), Percoll, sodium dodecyl sulphate (SDS), methyl methanethiosulfonate (MMTS), L-cysteine to prepare CSNO and exhausted CSNO and chemicals to prepare M medium, lysis buffer, HEN medium, TBS and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (see Appendix I for details) were all from Sigma-Aldrich (Poole, Dorset, UK). Fatty-acid free bovine serum albumin (BSA) was purchased from SAFC Biosciences (Lenexa, KS, USA). Oregon Green 488 BAPTA 1-acetoxymethyl (OGB-1AM) was supplied by Invitrogen Molecular Probes (Paisley, UK). Poly-D-lysine (PDL) was acquired from BD Biosciences (Oxford, UK). Spermine NONOate, ANGS, DTT, GSH, CCCP and GSNO were purchased from Merck Chemicals Ltd. (Beeston, Nottingham, UK). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics Ltd. (Lewes, East Sussex, UK). EZ-Link Biotin-N-[6-(biotinamido)hexyl]-3-(2-pyridyldithio)propionamide (EZ-Link Biotin-HPDP) was obtained from Perbio Science UK Ltd. (Cramlington, Northumberland, UK). Nitrocellulose membrane was supplied by GE Healthcare UK Ltd. (St. Giles, Bucks, UK). IgG fraction monoclonal mouse antibiotin and secondary antibody conjugated with horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories (Stratech Scientific, Soham, Cambridgeshire, UK). Lumi-GLO, an enhanced chemiluminescence kit, was purchased from Insight Biotechnology Ltd. (Wembley, Middlesex, UK). The Silver Stain Plus was supplied by Bio-Rad Laboratories Ltd. (Hempstead, Hertfordshire, UK). All chemicals were cell-culture-tested grade where available.

4.3.2 Spermatozoa preparation and capacitation

As in Chapter Two, page 70, with the following specifications to this chapter:

To perform the biotin-switch assay, semen was layered over 1 ml fractions of 45% and 90% Percoll (made isotonic with M medium; see Appendix I for details). Samples were then centrifuged at 2000 g for 20 minutes. Concentration of spermatozoa was calculated using an improved Neubauer haemocytometer and at least 400 cells were scored (Kvist and Björndahl, 2002). Percoll-washed sperm were further washed with PBS to eliminate Percoll. Cells were then diluted and incubated in PBS.

4.3.3 Single cell imaging

As in Chapter Two, page 71, with the following specifications to this chapter:

All experiments were carried out using sEBSS + 0.3% BSA. Initial control periods were obtained using the same medium.

Spermatozoa were stimulated with spermine NONOate (100 μM) after which DTT (1 mM) was applied to the cells, in the continued presence of the NONOate.

Additionally, upon sperm treatment with spermine NONOate (100 μM), CCCP (10 μM) was applied in the presence of NONOate. DTT (1 mM) was then introduced to the imaging chamber, in the continuous presence of NONOate and CCCP.

Cells were also stimulated with GSNO (100 μM), after which either DTT (1 mM) or GSH (100 μM) was added to the perfusing tube in the continued presence of GSNO.

Also, cells were superfused with standard medium, and then exposed to DTT (1 mM) dissolved in the same saline.

Finally, cells were superfused with ANGS (60 µM). DTT (1 mM) was subsequently added in the continued presence of ANGS.

4.3.4 Imaging Data Processing

As in Chapter Two, page 72.

4.3.5 Imaging Data Statistical Analysis

Categorization and quantification of different types of responses were performed using the statistical logics described in Chapter Two, page 73.

A scattergram was plotted to show the level of correlation between the size of the response to spermine NONOate and the size of the response to DTT. Linear regression line and coefficient of determination (R^2) values were considered to further evaluate the correlation between those responses.

4.3.6 Biotin-switch assay and immunodetection of proteins by Western blotting

The biotin-switch assay was used to detect S-nitrosylated proteins in human sperm, in accordance with Lefièvre *et al.* (2007). Briefly intact sperm, at 50 million cells/ml, were incubated with the nitrosylating agents GSNO and L-nitrosocysteine (CSNO), alone or in the presence of 1mM DTT, the control compounds GSH and exhausted CSNO, or no treatment, at 37ºC for up to 60 minutes. Sperm were then centrifuged at 2000 g for 5 minutes and the supernatant was removed unless stated otherwise. Upon centrifugation, sperm were resupended in lysis buffer (see Appendix I for details), and treated with protease inhibitors.

Alternatively after supernatant removal, cells were washed twice with 500 µl PBS, immediately centrifuged at 2000 g for 5 minutes and finally resuspended in the lysis buffer. Sperm were incubated for 5-15 minutes at room temperature and centrifuged at 2000 g for 5 minutes. Protein concentration was determined and then adjusted to < 0.5 mg/ml. Proteins were precipitated using four volumes of ice-cold acetone at -20ºC for 20 minutes, centrifuged at 2000 g for 5 minutes at 4ºC, washed twice with 70% acetone and finally dried. Protein precipitate was resuspended in HEN medium (see Appendix I for details) containing 2.5% SDS. Blockage of free -SH was performed using MMTS (20 mM), a rapidly -SH-reactive agent, for 30 minutes at 50ºC. Following free -SH blockage, extracts were precipitated with acetone as described above, to eliminate MMTS, and then resuspended in HEN medium containing 1% SDS. Biotinylation was achieved following 1 hour incubation with 1 mM biotin-HPDP and 1 mM ascorbate, at 25ºC. Samples were further acetone precipitated as described above. Pellets were resuspended in 1x SDS-PAGE loading buffer (see Appendix I for details) in the absence of reducing agents and the samples were resolved by SDS-PAGE (10%) and transferred for biotin immunoblotting. All experimental steps preceding SDS-PAGE were performed in the dark. A single ejaculate was used each time and different donors were used on each occasion.

Proteins were separated by electrophoresis on SDS-PAGE (10%) gels and electrotransferred onto nitrocellulose membrane. Non-specific binding sites on membranes were blocked with 5% w/v dry skimmed milk in TBS supplemented with 0.1% Tween-20 (TTBS) for the detection of biotinylated proteins. The membranes were incubated at room temperature for 1 hour with the antibiotin antibody (1/10000). The nitrocellulose membranes were then extensively washed with TTBS, incubated with the secondary antibody conjugated with

horseradish peroxidase for 1 hour and again washed with TTBS. Positive immunoreactive bands were detected by chemiluminescence using Lumi-GLO, in accordance to the manufacturer's instructions. Silver staining of the proteins transferred on the membranes was carried out following the detection to confirm equal protein loading for all samples (Jacobson and Karsnäs, 1990).

4.4 Results

4.4.1 The effects of -SH reduction and protein S-nitrosylation blockage on NO-induced $[Ca^{2+}]_i$ signal

As detailed in Chapter Three, the effects of NO on $[Ca^{2+}]_i$ in human sperm are not mediated by the activation of soluble guanylate cyclase (sGS) and production of cGMP. An alternative biological action of NO is directly to alter protein function by S-nitrosylation of -SH in specific target motifs (Hess *et al.*, 2005a; Stamler *et al.*, 1992), an action of NO that we recently detected in human sperm (Lefièvre *et al.*, 2007). DTT is a cell-permeant -SH reducing agent which, even at low concentrations (1 mM), reverses biological effects stimulated by protein S-nitrosylation (Stoyanovsky *et al.*, 1997). The effects of DTT on spermine NONOate-induced $[Ca^{2+}]_i$ rise in capacitated human sperm were thus determined. DTT (1 mM) caused an abrupt decrease in $[Ca^{2+}]_i$ (78±7% of cells; 5 experiments), sometimes preceded by a brief elevation in $[Ca^{2+}]_i$ (possibly reflecting non-specific effects of DTT), in cells where spermine NONOate (100 μM) effects were well established (Fig. 4.1.A). Fluorescence reached approximately 3% above control levels and fell, in some cells, to levels observed before NONOate treatment. Many cells showed 5-10% fluorescence recovery during the following 10 minutes (Fig. 4.1.A). The amplitude of the spermine NONOate-stimulated rise in fluorescence was correlated with the size of the drop in fluorescence upon DTT treatment (Fig. 4.1.B).

Oxidation of protein -SH can lead to Ca^{2+} mobilization from mitochondria, an effect that is reversed by DTT (Halestrap *et al.*, 1997; Pariente *et al.*, 2001; McStay *et al.*, 2002), and might therefore contribute to the observed $[Ca^{2+}]_i$ responses. In order to determine whether

reduction of mitochondria -SH groups account for the effects of DTT on NO-stimulated elevation of $[Ca^{2+}]_i$, spermine NONOate (100 μM) treated cells were stimulated with CCCP (10 μM), prior to DTT (1 mM) exposure, to collapse the mitochondrial inner membrane potential and mobilize mitochondrial Ca^{2+} (Konji *et al.*, 1985). Most cells showed a rise in $[Ca^{2+}]_i$ after CCCP addition, consistent with mitochondria participation in $[Ca^{2+}]_i$ buffering (Wennemuth *et al.*, 2003), this mobilization of mitochondrial Ca^{2+} activating $[Ca^{2+}]_i$ oscillations in many cells (Fig. 4.2, red trace). Cells were then exposed to DTT, responding with a clear drop in $[Ca^{2+}]_i$ (96±3% of cells; 4 experiments), despite the inability of mitochondria to accumulate Ca^{2+}. Fluorescence reached approximately 5% above control levels, returning, in some cells, to levels observed before application of NONOate. Fluorescence recovered approximately 10% over the following 5 minutes (Fig. 4.2).

GSNO, a membrane-impermeant protein S-nitrosylating agent (Ji *et al.*, 1999; Zaman *et al.*, 2006) can act at intracellular targets, probably by generation of the membrane permeant product cys-NO (Zhang and Hogg, 2004). The effects of GSNO on $[Ca^{2+}]_i$ in capacitated human sperm were thus investigated. GSNO (100 μM) induced a $[Ca^{2+}]_i$ rise (Fig. 4.3.A) in 70±5% of cells (7 experiments). GSNO-induced increase in $[Ca^{2+}]_i$ was rapid in comparison to the action of spermine NONOate (100 μM), reaching a plateau in approximately 3 minutes in most cells (Fig. 4.3.A). Fluorescence tended to stabilise at approximately 12% (Fig. 4.3.B). In 18% of cells, oscillations were superimposed on the GSNO-induced rise in $[Ca^{2+}]_i$. In control experiments, fluorescence increased by approximately 3% during the period corresponding to the application of GSNO (Fig. 4.3.A insert and 4.3.B). GSNO-induced $[Ca^{2+}]_i$ increase in cells bathed in sEBSS was significantly greater ($P<0.001$; unpaired-t) than the $[Ca^{2+}]_i$ rise observed under control conditions (Fig. 4.3.B).

DTT (1 mM) generated a rapid fall in fluorescence, sometimes preceded by a brief rise in $[Ca^{2+}]_i$, in sperm previously exposed to GSNO (100 μM; 86±8% of cells; 4 experiments; Fig. 4.4). Levels of fluorescence fell to approximately 3% above control levels (Fig. 4.4 and 4.5.B), returning, in some cells, to levels observed before exposure to GSNO (Fig. 4.4).

Decomposition of GSNO can lead to release of NO (Singh *et al.*, 1996). To test the possibility that GSNO was acting as an NO donor rather than an S-nitrosylating agent, we co-applied 100 μM GSH. Decomposition of GSNO to generate NO is accelerated in the presence of an equal dose of GSH (Singh *et al.*, 1996), but formation of membrane permeant product cys-NO, leading to direct S-nitrosylation of intracellular proteins is suppressed (Zhang and Hogg, 2004). GSNO (100 μM) treated cells responded with a decrease in $[Ca^{2+}]_i$, sometimes preceded by a small rise, when GSH (100 μM) was introduced to the recording chamber, in the continued presence of GSNO (100 μM; 81±14% of cells; 3 experiments; Fig. 4.5.A). Fluorescence levels reached approximately 4% below control levels and tended to stabilise at a lower level of fluorescence (Fig. 4.5) although some cells showed fluorescence recovery of approximately 5% over the following 5 minutes. Fluorescence levels observed upon application of GSH to sperm exposed to GSNO were significantly ($P<0.001$; unpaired-t) lower than those seen upon application of DTT to GSNO-treated cells (Fig. 4.5.B). These data indicate that mobilization of Ca^{2+} by GSNO is by a "direct" action to nitrosylate target proteins in spermatozoa.

As DTT strongly promoted Ca^{2+} uptake, further investigation was carried out to determine whether DTT affected the $[Ca^{2+}]_i$ in NO untreated spermatozoa. DTT caused a reduction in $[Ca^{2+}]_i$ in the majority of control cells (3 experiments; Fig. 4.6). However, the amplitude of the DTT effect was small, mean fluorescence levels reaching only approximately 3% below control levels. In most of these cells, there was then a slight recovery of fluorescence (≈2%) over the following 5 minutes (Fig. 4.6). The amplitude of the rise in fluorescence occurred during the control period was correlated with the size of the drop in fluorescence following DTT treatment.

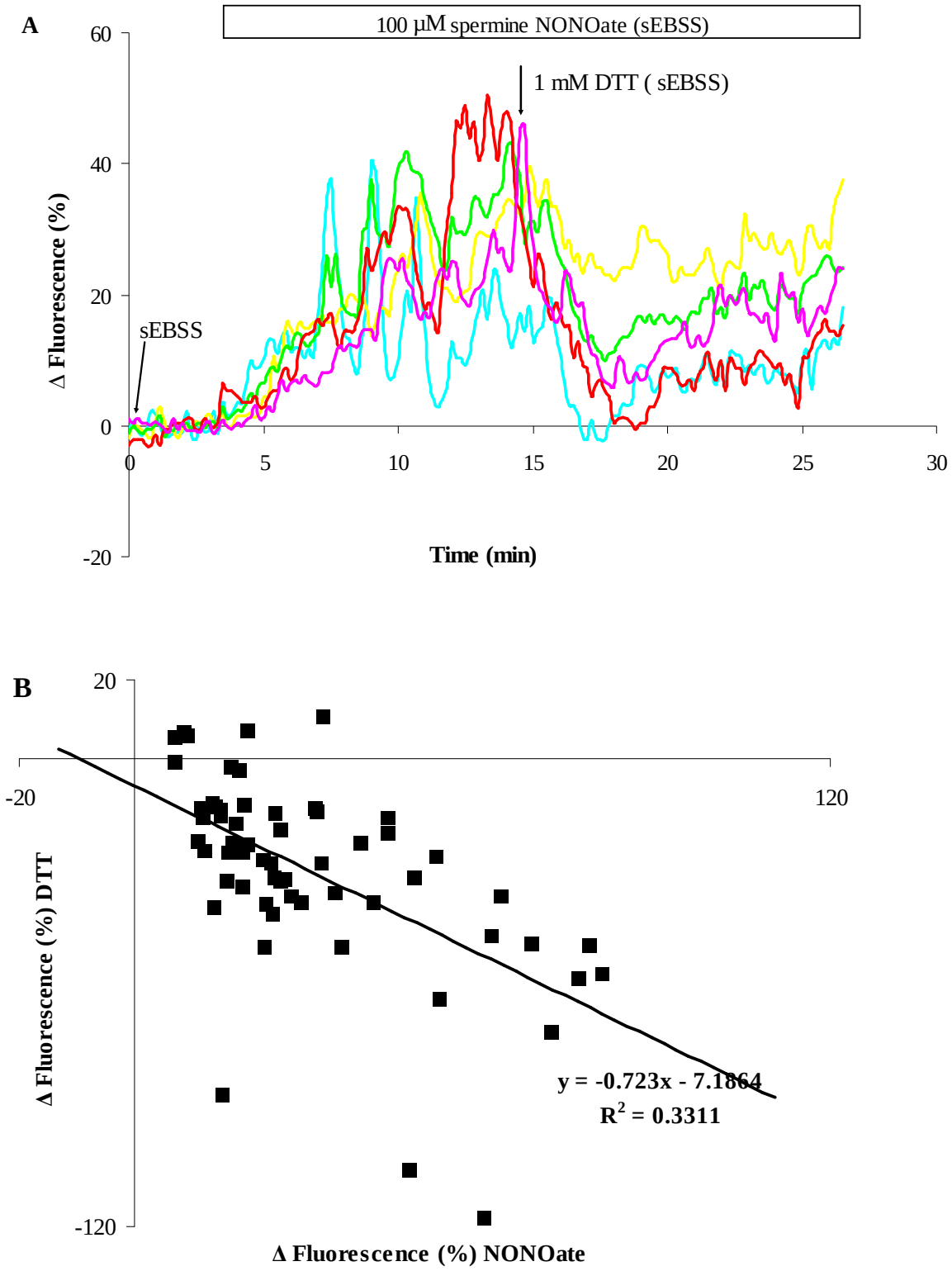

Fig. 4.1.A. The effects of DTT on spermine NONOate-induced $[Ca^{2+}]_i$ increase in capacitated human sperm bathed in sEBSS. Spermatozoa were incubated in sEBSS for 6 hours, superfused with sEBSS for 3 minutes then exposed to 100 µM spermine NONOate and finally 1 mM DTT, in the presence of

NONOate. Traces show 5 single cell responses. **B.** Scattergram shows the negative correlation between the spermine NONOate-induced rise in fluorescence and the following DTT-stimulated decrease in fluorescence. A linear regression line is plotted with the correspondent equation and R^2. Scattergram shows results of one experiment and is representative of five repeats. Each black square represents a cell.

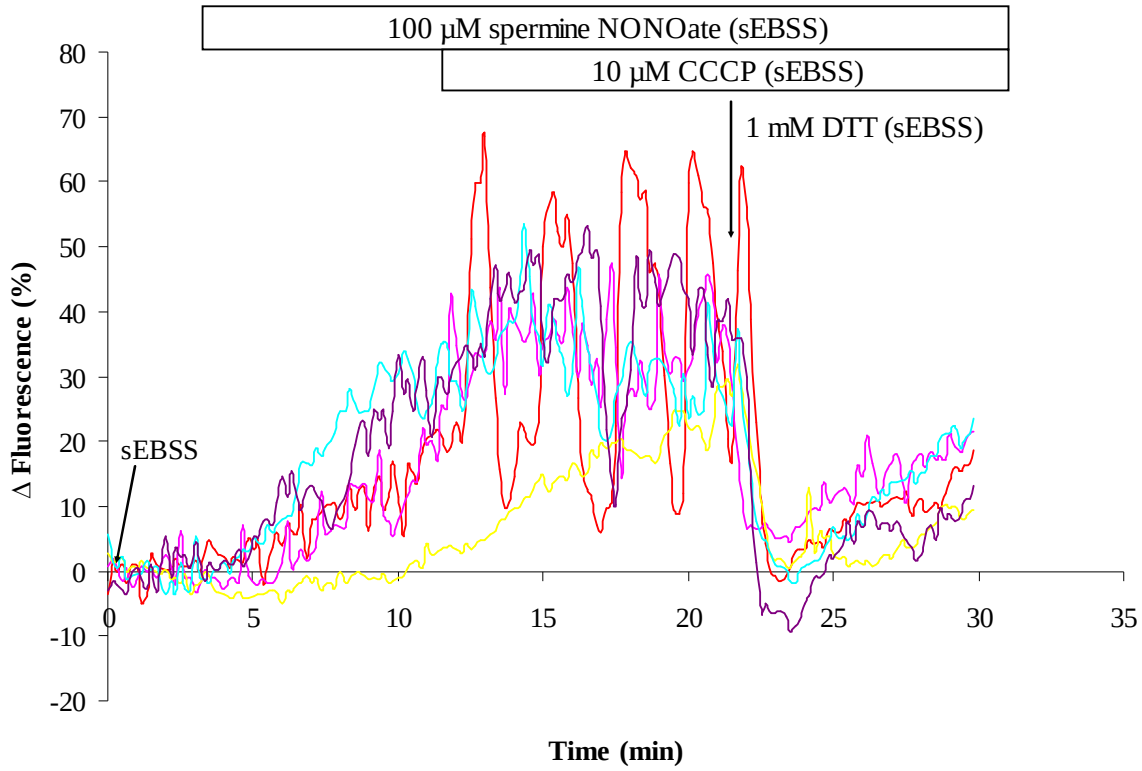

Fig. 4.2. The effects of DTT on spermine NONOate-induced $[Ca^{2+}]_i$ increase in capacitated human sperm pre-treated with CCCP. Cells were incubated in sEBSS for 6 hours, superfused with sEBSS for 3 minutes, then sequentially exposed to 100 µM spermine NONOate, spermine NONOate and 10 µM CCCP and finally spermine NONOate, CCCP and 1 mM DTT in the same saline. Traces show 5 single cell responses.

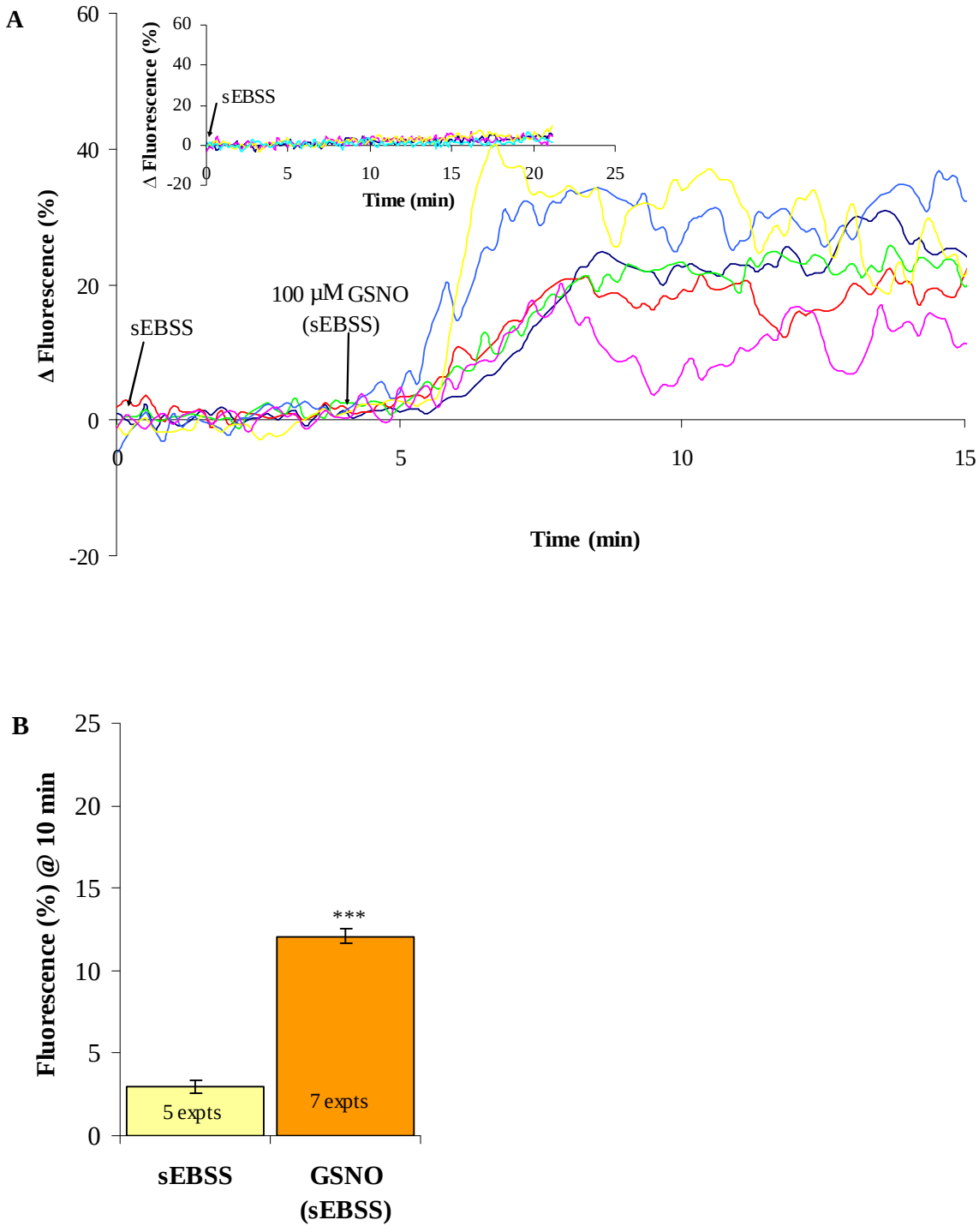

Fig. 4.3.A. The effects of GSNO on $[Ca^{2+}]_i$ in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 4 minutes and then exposed to 100 µM GSNO in the same saline. Traces represent 6 single cell responses. **Insert:** Control in which sperm prepared as for the GSNO experiment were superfused with sEBSS in the absence of any treatment.Traces show 4 single cell responses. **B.** Differences between the drift in $[Ca^{2+}]_i$

(fluorescence) occurring under control conditions (sEBSS) and GSNO-induced $[Ca^{2+}]_i$ rise in capacitated human sperm bathed in sEBSS. Bar chart represents the mean normalised increase in fluorescence 10 minutes after application of GSNO to sperm bathed in sEBSS (401 cells) and over a 10 minute period measured as in the experimental protocol (215 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance (P<0.001).

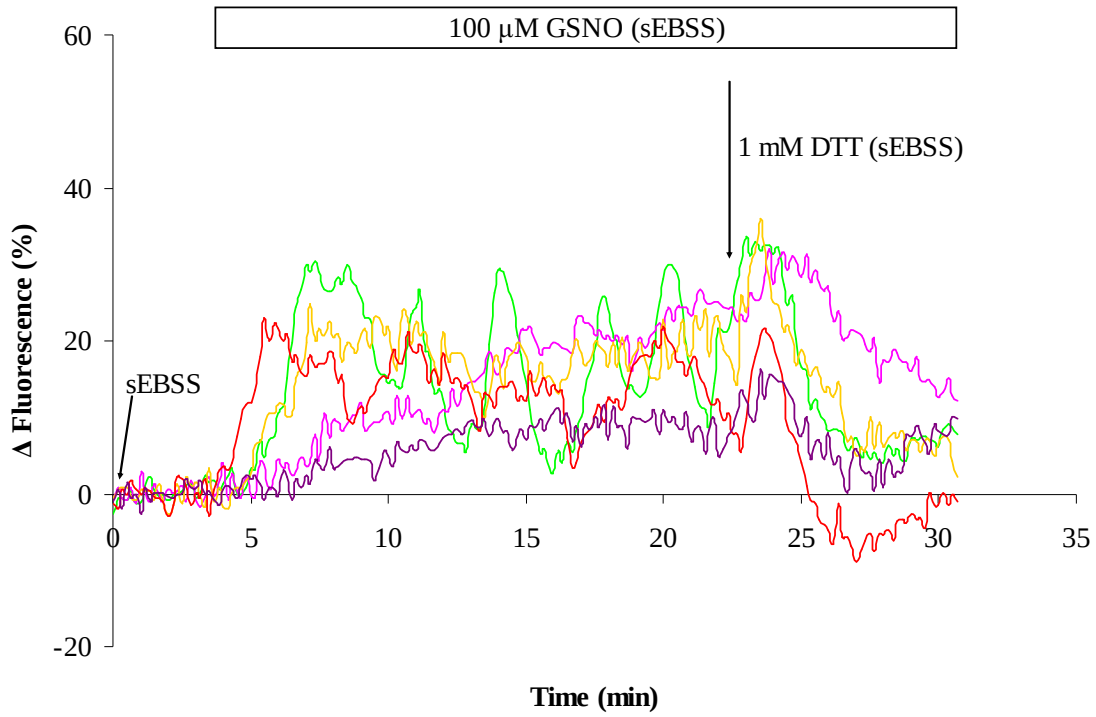

Fig. 4.4. The effects of DTT on GSNO-induced $[Ca^{2+}]_i$ increase in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 4 minutes, exposed to 100 µM GSNO and then 1 mM DTT, in the presence of GSNO, in the same saline. Traces show 5 individual cell responses.

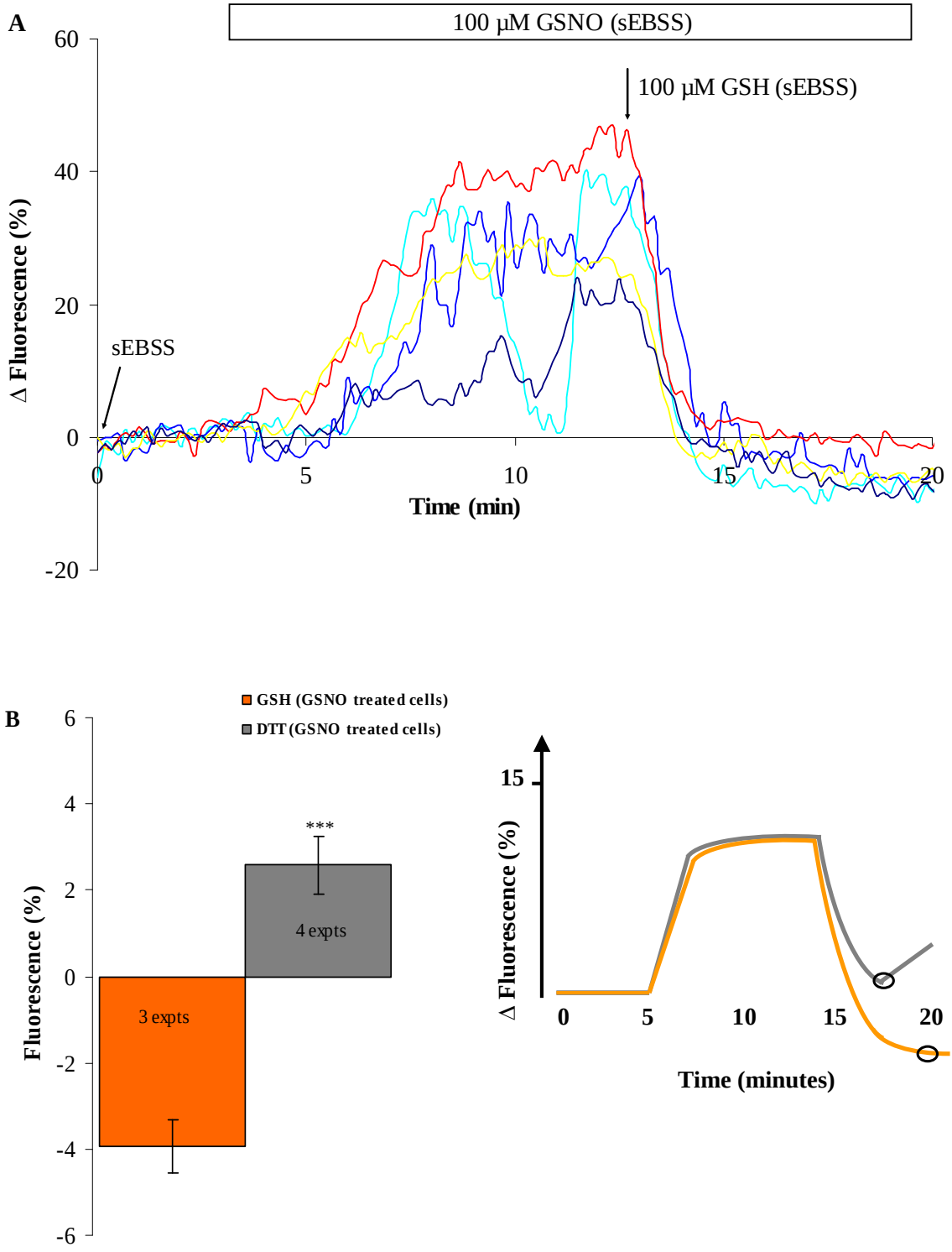

Fig. 4.5.A. The effects of GSH on GSNO-induced $[Ca^{2+}]_i$ rise in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 3 minutes then exposed to 100 µM GSNO and finally 100 µM GSH, in the presence of GSNO. Traces show 5 individual cell responses. **B.** The effects of GSH and DTT on $[Ca^{2+}]_i$ in GSNO treated and capacitated

human sperm bathed in sEBSS. **Left panel:** Bar chart represents the mean normalised fluorescence at the lowest point over 5 minutes immediately after GSH or DTT was added to GSNO treated sperm bathed in sEBSS (GSH = 187 cells; DTT = 214 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance ($P<0.001$). **Right panel:** Schematic representation of the sperm responses to GSH or DTT after exposure to GSNO. Black circles indicate the lowest fluorescence values over 5 minutes immediately after GSH or DTT were applied to sperm.

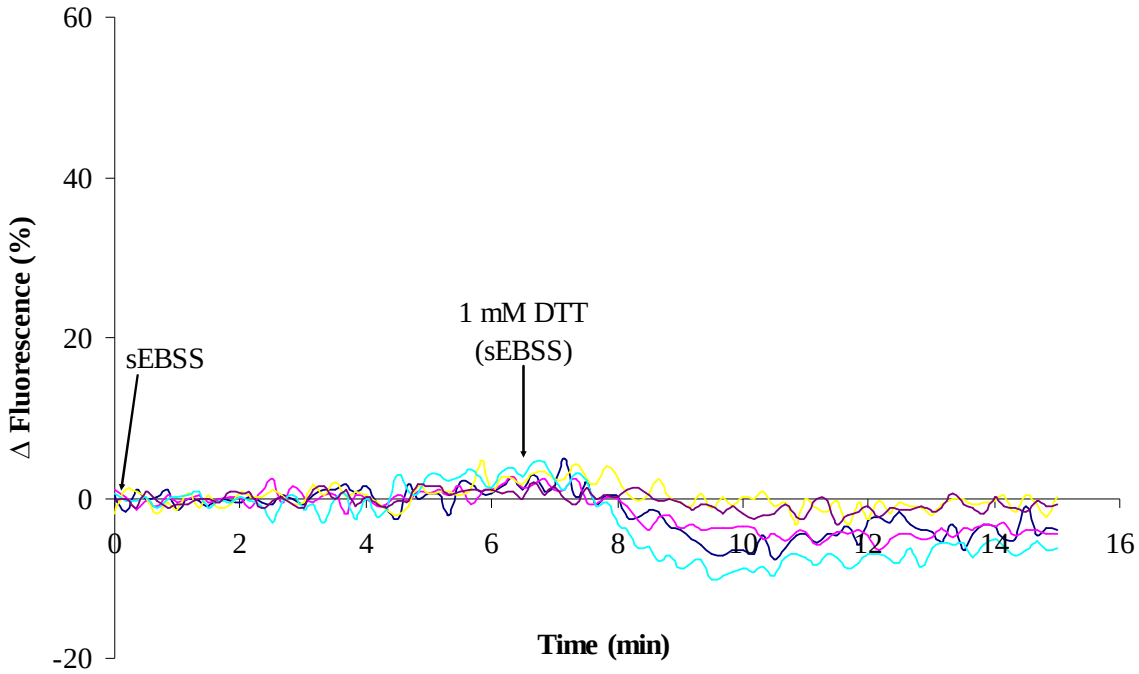

Fig. 4.6. The effects of DTT on $[Ca^{2+}]_i$ in untreated and capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 6.5 minutes, and then exposed to 1 mM DTT in the same saline. Traces show 5 individual cell responses.

4.4.2 Protein S-nitrosylation and its reversibility by -SH reducing agents and S-nitrosylation blocking agents

GSNO effects on $[Ca^{2+}]_i$ were rapid (≈ 3 minutes for peak elevation of fluorescence; Fig. 4.3.A), and were rapidly reversed in the presence of DTT (fig. 4.4) and GSH (Fig. 4.5). The $[Ca^{2+}]_i$ reduction observed upon washout of spermine NONOate (see Chapter Two, Fig. 2.5.A) and upon application of DTT to NONOate-treated cells (Fig. 4.1.A) were similarly rapid. The kinetics and reversibility of S-nitrosylation of sperm proteins after stimulation with GSNO were therefore assessed. Immediately after cell exposure to GSNO (50 µM; ≈ 5 minutes for preliminary centrifugation; see methods for details) sperm were lysed and processed for the biotin-switch assay. At this stage, protein S-nitrosylation had already reached its steady-state level, and prolonged incubation (up to 60 minutes) had a very small effect (Fig. 4.7). Moreover, after 60 minutes of incubation under S-nitrosylating conditions, subsequent washing with PBS was sufficient to reverse protein S-nitrosylation almost totally, within minutes (Fig. 4.8). Treatment with 1 mM DTT for 5 minutes, after 60 minutes of stimulation with GSNO (1 mM), completely reversed the GSNO-induced protein S-nitrosylation (Fig. 4.9). The biotin-switch assay and immunodetection of proteins by Western blotting were performed by Dr. L. Lefièvre.

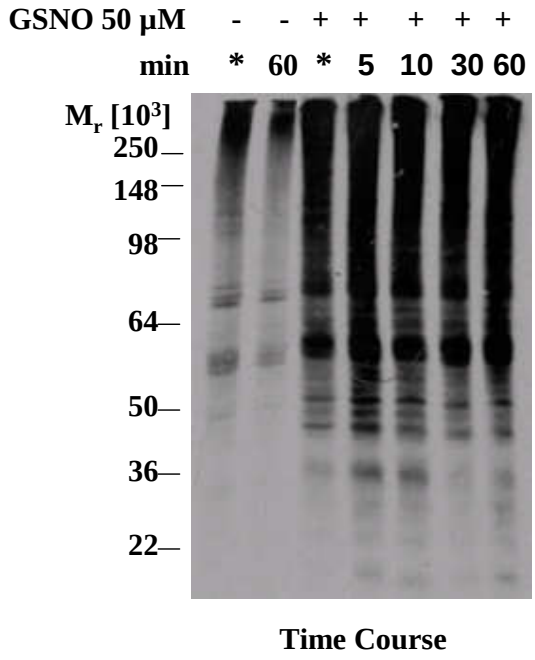

Fig. 4.7. GSNO and protein S-nitrosylation. Cells were exposed to 50 µM GSNO and processed for the biotin-switch assay. Lane 1 shows background levels of S-nitrosylation in sperm processed immediately for the assay (indicated by *). Lane 2 shows levels of S-nitrosylation after 60 minutes of incubation of sperm in sEBSS. Lanes 3, 4, 5, 6 and 7 show increased protein S-nitrosylation in sperm processed for the assay immediately after treatment with GSNO (*) and in sperm incubated with GSNO for 5, 10, 30 and 60 minutes respectively.

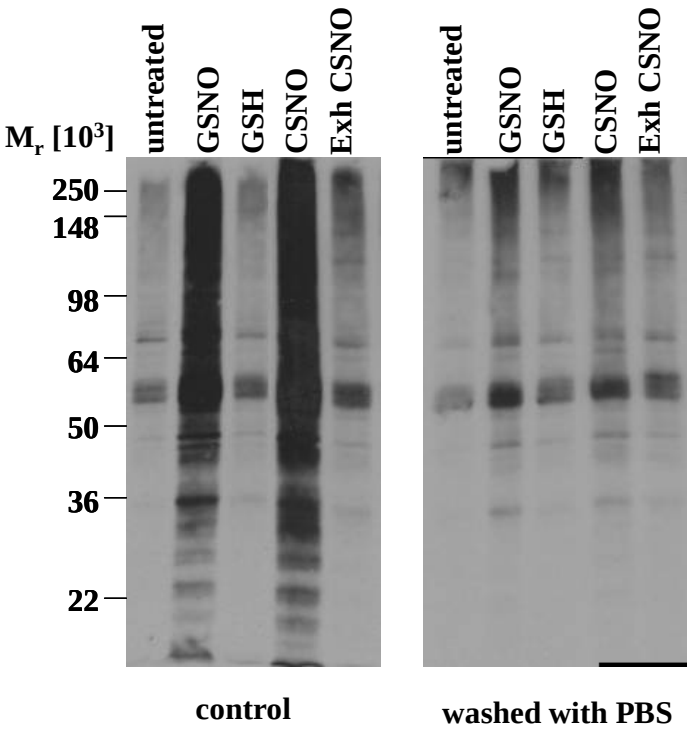

Fig. 4.8. Reversibility of GSNO-induced protein S-nitrosylation. Left panel shows protein S-nitrosylation levels in untreated sperm incubated for 10 minutes (lane 1), in GSNO and CSNO-treated sperm (lanes 2 and 4 respectively) and in GSH and exhausted CSNO-treated sperm (lanes 3 and 5 respectively; controls). Right panel presents same treatments in sperm washed in PBS immediately before processing for the biotin-switch assay.

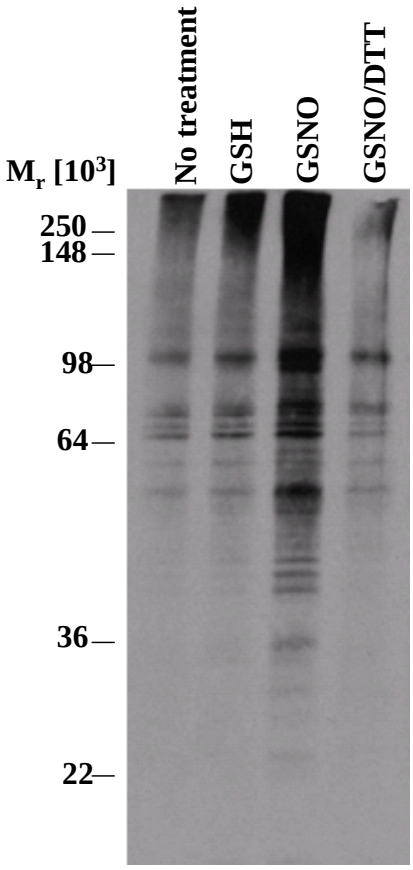

Fig. 4.9. Reversibility of GSNO-induced protein S-nitrosylation. Lane 1 shows endogenous levels of protein S-nitrosylation in sperm incubated in sEBSS for 60 minutes. Lanes 2 and 3 show protein S-nitrosylation levels in GSH (1 mM) treated cells (control) and in GSNO (1 mM) treated cells, respectively. Lane 4 shows protein S-nitrosylation in cells incubated as for lane 3 but cells were exposed to 1 mM DTT 5 minutes before processing for the assay.

4.4.3 The effects of -SH reducing agents on HNO-induced $[Ca^{2+}]_i$ signal

Recent studies have revealed the redox nature of the HNO effects in the cardiovascular system (Cheong *et al.*, 2005; Dai *et al.*, 2007; Tocchetti *et al.*, 2007). The effects of -SH reducing agents on the HNO-induced $[Ca^{2+}]_i$ rise in human sperm were therefore evaluated. When ANGS (60 µM) treated spermatozoa were exposed to DTT (1 mM), there was a rapid decrease in fluorescence (66±15% of cells; 3 experiments), sometimes preceded by a short elevation in fluorescence (Fig. 4.10). In most cells, fluorescence returned to levels slightly

above (≈ 4%) those seen during the control period, and then recovered approximately 10% over the following 5 minutes (Fig. 4.10).

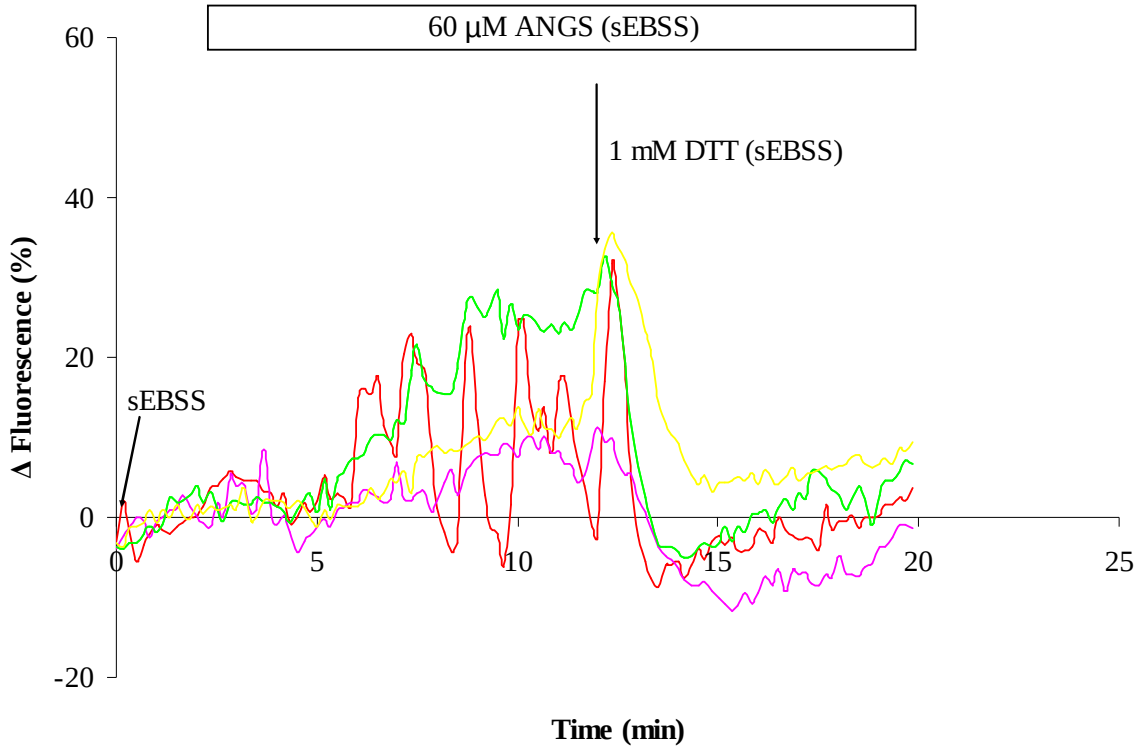

Fig. 4.10. The effects of DTT on HNO-induced $[Ca^{2+}]_i$ increase in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 2.5 minutes then exposed to 60 µM ANGS and finally 1 mM DTT was applied in the presence of ANGS. Traces show 4 individual cell responses.

4.5 Discussion

The action of NO was rapidly reversed by exposure to 1 mM DTT (Fig. 4.1.A), an effect that was not sensitive to pre-treatment with CCCP (Fig. 4.2), and thus not a reflection of effects on mitochondrial Ca^{2+} accumulation. The relationship between NO-induced Ca^{2+} mobilization (increase in OGB-1AM fluorescence) and the reduction in $[Ca^{2+}]_i$ caused by subsequent exposure to DTT (decrease in OGB-1AM fluorescence) is of particular significance. In every case (5 experiments) there was a negative correlation (Fig. 4.1.B). The characteristics of non-ratiometric fluorescence $[Ca^{2+}]$ measurements are such that, as $[Ca^{2+}]_i$ rises, the change in fluorescence for a given increment in $[Ca^{2+}]$ is reduced. If the action of DTT on $[Ca^{2+}]_i$ was by a mechanism separate to reversal of the action of NO, the DTT-stimulated decrease in absolute fluorescence would be smaller in those cells which had presented the greatest NO-stimulated elevation in $[Ca^{2+}]_i$. A positive correlation would result instead of the observed negative correlation. We therefore conclude that DTT reverses the action of NO and that NO-induced mobilization of stored Ca^{2+} reflects direct modulation of protein function by S-nitrosylation. It is relevant that, when spermine NONOate was washed off and reintroduced after 5-10 minutes, the effect of NO was apparently enhanced, particularly the generation of $[Ca^{2+}]_i$ oscillations (see Chapter Two, Fig. 2.5). Protein S-nitrosylation reverses rapidly upon washout of NONOate, so this persistence of effect may reflect increased Ca^{2+} leak at the plasma membrane (and consequent filling of the Ca^{2+} store), perhaps due to an increase in cGMP concentration. Furthermore, sperm exposure to GSNO, which can cause direct transnitrosylation independent of generation of NO, acted similarly to NO but more rapidly (Fig. 4.3.A), consistent with a direct action (nitrosylation by NO is believed to require a number of intermediate reactions and therefore has slower kinetics (Ahern *et al.*, 2002)). The effects of GSNO must be carefully interpreted as this compound can decay to liberate NO,

which is far more effective in crossing biological membranes. However, direct measurement of NO liberation by GSNO demonstrates that this compound is a very poor generator of NO (Jarboe *et al.*, 2008). Moreover, the effect of GSNO on $[Ca^{2+}]_i$ was reversed by co-application of GSH (Fig. 4.5.A). Previous reports indicate that GSH enhances breakdown of GSNO to liberate NO (Singh *et al.*, 1996) but will inhibit generation of membrane permeant cys-NO, which is required for protein S-nitrosylation by GSNO in intact cells (Zhang and Hogg, 2004). A mechanism for RSNO uptake into cells has been proposed and involves transnitrosation reaction between GSNO and L-cysteine to form L-CysNO, which is taken up into the cell via an amino acid transport system. Once inside the cell, CysNO undergo transnitrosation reactions with cellular -SH to from GSNO and S-nitrosated proteins. Reactions between -SH and RSNO may contribute to the loss of extracellular RSNO (Zhang and Hogg, 2004). GSNO-induced $[Ca^{2+}]_i$ rise was also reversed by the -SH reducing agent DTT (Fig. 4.4). Donors of NO or RSNO mobilize Ca^{2+} from skeletal and cardiac microsomes, an effect that is reversed by –SH reducing agents (Stoyanovsky *et al.*, 1997). Interestingly, when GSH was added to GSNO-treated sperm, $[Ca^{2+}]_i$ tended to stabilise at a reduced level, whereas upon DTT addition it often showed a partial recovery (Fig. 4.5.B). These data are consistent with the inability of GSNO to further transnitrosylate proteins (Ca^{2+}-channels) as GSH blocks the formation of CysNO, abrogating further Ca^{2+} mobilization, whereas DTT reverses protein S-nitrosylation, leading to channels closure and Ca^{2+} uptake, but does not prevent further nitrosylation and consequent Ca^{2+} mobilization by NO. Evaluation of sperm protein S-nitrosylation, using the biotin-switch method (Foster and Stamler, 2004; Lefièvre *et al.*, 2007) showed that, similar to the effect of GSNO on $[Ca^{2+}]_i$, GSNO-stimulated protein S-nitrosylation was rapid, occurring in $\leq$5 minutes (the minimum period that could be assessed using the assay; Fig. 4.7) and was immediately reversed by washout of GSNO (Fig. 4.8) or

co-application of DTT (Fig. 4.9). These data demonstrate the redox nature of these processes and are consistent with the recent report that a large number of human sperm proteins are potential targets for S-nitrosylation (Lefièvre *et al.*, 2007). Lefièvre *et al.* (2007) identified 260 proteins present in sperm from normozoospermic men, which became S-nitrosylated at cysteine residues in the presence of NO donors. Comparison with the only publicly available human sperm proteome found only 30 proteins (12%) in common (Martinez-Heredia *et al.*, 2006). A previous study provided an estimation of at least 1760 proteins in the human sperm proteome (Johnston *et al.*, 2005). These findings indicate that protein S-nitrosylation upon exposure to NO is not a ubiquitous process, a crucial characteristic of a modification if it is to exert specific downstream effects (Lefièvre *et al.*, 2007). Functional analysis of the identified proteins showed that proteins associated with metabolism, energy production and cell movement were abundant (18%), suggesting that S-nitrosylation has a role in sperm motility. Proteins involved in signal transduction were also found to be abundant (10%), suggesting that S-nitrosylation has a role in the modulation of sperm function (Lefièvre *et al.*, 2007). Therefore, these results demonstrate the potential significance of protein S-nitrosylation, regarding the regulation of human sperm functions.

DTT decreased $[Ca^{2+}]_i$ in untreated cells (Fig. 4.6) and in all these experiments there was a negative correlation between the elevation in $[Ca^{2+}]_i$ (increase in OGB-1AM fluorescence) observed during the control period and the following drop in $[Ca^{2+}]_i$ (decrease in OGB-1AM fluorescence) upon application of DTT. These observations suggest that the rise in resting $[Ca^{2+}]_i$ which occurs in some cells may be related to changes in protein -SH status.

This study also showed that the action of HNO on $[Ca^{2+}]_i$ was rapidly reversed upon application of DTT (Fig. 4.10), again suggesting the redox nature of the process. It has been reported that HNO produced by ANGS leads to Ca^{2+} release from skeletal and cardiac sarcoplasmic reticulum vesicles, potentially via activation of RyRs, an effect that is reversed by the -SH reducing agent DTT, causing re-uptake of Ca^{2+} by the sarcoplasmic reticulum vesicles (Cheong *et al.*, 2005). Similarly, HNO-induced Ca^{2+} release from murine myocytes sarcoplasmic reticulum vesicles is also completely reversed by DTT (Tocchetti *et al.*, 2007).

In summary, these data indicate that mobilization of stored Ca^{2+} by NO or GSNO occurs via modulation of protein function by S-nitrosylation and that mitochondria are not the stores targeted by NO for it to exert its effects. Moreover, these results suggest that mobilization of stored Ca^{2+} by HNO occurs via S-oxidation of proteins.

CHAPTER FIVE

RYANODINE AND 4-CHLORO-m-CRESOL (4-CmC) ELEVATE $[Ca^{2+}]_i$ IN CAPACITATED HUMAN SPERMATOZOA

5.1 Abstract

Human sperm exhibit ryanodine receptors (RyRs) primarily in the posterior region of the head /mid-piece junction. RyRs contain thiol (-SH) groups, which are prone to undergo S-nitrosylation, and are therefore potential targets for nitric oxide (NO). The effects of ryanodine and 4-CmC, agonists of RyRs, on sperm $[Ca^{2+}]_i$ were therefore investigated. Ryanodine and 4-CmC stimulated a rise in $[Ca^{2+}]_{i,}$ but the kinetics of responses were distinct. Ryanodine elicited a $[Ca^{2+}]_i$ plateau whereas 4-CmC typically induced a $[Ca^{2+}]_i$ transient. Although 4-CmC increased $[Ca^{2+}]_i$ even in low-Ca^{2+} conditions, the amplitude of the responses was reduced. 4-CmC effects on $[Ca^{2+}]_i$ were abolished when experiments were performed in sEBSS-ethylene glycol-bis (β-amino-ethylether)-N,N,N'N'-tetraacetic acid (EGTA) medium. These data suggest that these agonists mobilize Ca^{2+} from an intracellular store, via activation of RyRs, although 4-CmC effects appear to be partially dependent on a pathway mediated by Ca^{2+} influx in sperm and the store may be very rapidly depleted upon exposure to EGTA. N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate) pre-treated cells responded differently to ryanodine in the continued presence of NONOate, as the amplitude of the $[Ca^{2+}]_i$ responses became smaller and a small $[Ca^{2+}]_i$ peak was often discernible. These data suggest convergence of the NO and ryanodine actions.

5.2 Introduction

Ryanodine receptors (RyRs) are intracellular Ca^{2+} channels that play a crucial role in $[Ca^{2+}]_i$ signalling, principally amplification of signal by Ca^{2+}-induced Ca^{2+} release (CICR). These proteins have a large number of sulfhydryl (–SH) groups and are therefore prone to be S-nitrosylated (Otsu *et al.*, 1990; Takeshima *et al.*, 1989). RyRs are believed to be activated by alterations in $[Ca^{2+}]$, the putative second messenger cyclic adenosine diphosphate-ribose (cADPR) and also via conformational coupling with associated proteins (Zucchi and Ronca-Testoni, 1997). Pharmacological modulators of RyRs comprise caffeine and low doses of ryanodine as activators and local anaesthetics, ruthenium red and high doses of ryanodine that close the Ca^{2+} channel (Zucchi and Ronca-Testoni, 1997). The three isoforms of the RyRs are designated as RyR 1, originally identified in skeletal muscle; RyR 2, originally identified in cardiac muscle and RyR 3, identified in brain but with a wider tissue distribution in general (Brini, 2004). Analysis of mouse testis sections revealed that cells believed to be germ cells, like spermatocytes and spermatids expressed RyR 1 and RyR 3 but not RyR 2 (Giannini *et al.*, 1995). Mouse developing spermatocytes and spermatids express RyR 1 and RyR 3 (Chiarella *et al.*, 2004; Trevino *et al.*, 1998). Moreover, Trevino *et al.* (1998) demonstrated that RyR 3 was present in mature fully developed spermatozoa, and that RyR 3 localisation was unlikely to be on the acrosome. Human sperm can be specifically labelled with a fluorescent analogue of ryanodine (BODIPY-FL-X-ryanodine), binding primarily to the posterior region of the head and mid-piece (Harper *et al.*, 2004). Immunolocalisation studies confirmed the presence of RyR 1 and RyR 2 in the neck and mid-piece region of human sperm (Lefièvre *et al.*, unpublished data). RyRs are present in human sperm but the low incidence of detection indicates that these receptors may be present in low abundance (Lefièvre *et al.*, 2007), which is consistent with their restricted cellular localisation (Harper *et al.*, 2004) and may be

expected for the presence of a high conductance ion channel in cells with such a small cytoplasmic volume (Lefièvre *et al.*, 2007). Pharmacological-based studies have also suggested the presence of the RyR in mature bovine sperm (Minelli *et al.*, 2000). In later studies, RyRs were not detected in bovine sperm (Ho and Suarez, 2001b). Furthermore, high concentrations of ryanodine reduced spermatogonial proliferation and increased cell meiosis, stressing that sperm have functional RyRs that not only play a physiological role in regulating agonist-induced Ca^{2+} changes but also in sperm development (Chiarella *et al.*, 2004).

4-CmC is a potent agonist of RyR 1 and RyR 2 (Choisy *et al.*, 1999; Matyash *et al.*, 2002; Zorzato *et al.*, 1993), but is a poor activator of the RyR 3 (Fessenden *et al.*, 2000). 4-CmC comprises a single benzene ring with hydroxyl, methyl, and chloro groups at the 1, 3 and 4 positions. The 1-hydroxyl group is required to activate RyR 1 and the hydrophobic groups at the 3- and/or 4-positions improve activity (Jacobson *et al.*, 2006). 4-CmC activates RyR 1 via modulation of the Ca^{2+} sensitivity of the channel (Herrmann-Frank *et al.*, 1996).

Recently it has been reported that exposure of human spermatozoa to nitric oxide (NO) results in S-nitrosylation of RyRs (Lefièvre *et al.*, 2007). The aim of this study was therefore to assess whether treatment of human sperm with ryanodine and 4-CmC interacts with (or occludes) the ability of NO to elevate $[Ca^{2+}]_i$.

5.3 Materials and methods

5.3.1 Materials

Salts used to prepare supplemented Earle's balanced salt solution (sEBSS), Ca^{2+}-free sEBSS (CFsEBSS) and sEBSS- Ethylene glycol-bis (β-amino-ethylether)-N,N,N'N'-tetraacetic acid (EGTA; see Appendix I for details), pluronic F-127, dimethyl sulfoxide (DMSO), EGTA and 4-CmC were purchased from Sigma-Aldrich (Poole, Dorset, UK). Fatty-acid free bovine serum albumin (BSA) was obtained from SAFC Biosciences (Lenexa, KS, USA). Oregon Green 488 BAPTA 1-acetoxymethyl (OGB-1AM) was acquired from Invitrogen Molecular Probes (Paisley, UK). Poly-D-lysine (PDL) was purchased from BD Biosciences (Oxford, UK). N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate) was obtained from Merck Chemicals Ltd. (Beeston, Nottingham, UK). Ryanodine was acquired from Biomol International, L.P. (Exeter, UK). All chemicals were cell-culture-tested grade where available.

5.3.2 Spermatozoa preparation and capacitation

As in Chapter Two, page 70.

5.3.3 Single cell imaging

As described in Chapter Two, page 71, with the following specifications to this chapter:

OGB-1AM loaded sperm were superfused with sEBSS + 0.3% BSA for an initial control period. Sperm were then stimulated with ryanodine (100 μM) dissolved in the same medium. In addition, and upon an initial control period in which sperm were bathed with standard

sEBSS + 0.3% BSA, cells were pre-treated with spermine NONOate (100 μM), during sufficient time for the NO effects to become well established, and then stimulated with ryanodine (100 μM) in the continued presence of NONOate, the drugs being dissolved in the same saline.

In a different set of experiments, cells were superfused with sEBSS (or CFsEBSS) + 0.3% BSA for an initial control period and then exposed to 4-CmC (1 mM) dissolved in the same saline. These experiments were repeated in sEBSS-EGTA (sEBSS containing 6 mM EGTA and 5 mM Ca^{2+}), in which case cells were superfused with standard sEBSS for an initial control period and then briefly superfused with sEBSS-EGTA prior to application of 4-CmC dissolved in sEBSS-EGTA.

5.3.4 Imaging Data Processing

As in Chapter Two, page 72.

5.3.5 Imaging Data Statistical Analysis

As in Chapter Two, page 73.

5.3.6 Evaluation of $[Ca^{2+}]_i$ oscillations

As in Chapter Two, page 74.

5.4 Results

5.4.1 The effects of ryanodine and 4-CmC on $[Ca^{2+}]_i$

Upon treatment with ryanodine (100 μM), human sperm responded with a rise in $[Ca^{2+}]_i$ (83±5% of cells; 3 experiments). Fluorescence stabilised approximately 1 minute after application of ryanodine (mean increase ≈ 9%; Fig. 5.1). In 12% of cells, oscillations were superimposed on the ryanodine-induced elevation of $[Ca^{2+}]_i$. In controls in which untreated sperm were continuously superfused with sEBSS, fluorescence tended to remain at baseline levels over this period (Fig. 5.1.A insert and 5.1.B). Therefore, ryanodine induced increase in fluorescence was very highly significant ($P<0.001$; unpaired-t).

4-CmC (1 mM) induced an increase in $[Ca^{2+}]_i$ in 89% of cells. The typical response to 4-CmC was a Ca^{2+} transient (peak ≈ 12% in fluorescence), followed by a sustained Ca^{2+} elevation (Fig. 5.2). 9% of cells presented $[Ca^{2+}]_i$ spikes or oscillations during the sustained elevation of $[Ca^{2+}]_i$ (Fig. 5.2; brown trace). Controls in which untreated cells were continuously superfused with sEBSS, showed a negligible drift in $[Ca^{2+}]_i$ in most cells (5 experiments; Fig. 5.2 insert and 5.5.B). Thus, 4-CmC stimulated a very highly significant ($P<0.001$; unpaired-t) $[Ca^{2+}]_i$ rise in sperm bathed in sEBSS. To establish whether the 4-CmC-induced $[Ca^{2+}]_i$ elevation was due to Ca^{2+} influx at the plasma membrane, the same experiments were repeated in medium to which no Ca^{2+} had been added (CFsEBSS), where $[Ca^{2+}]_o$ is ≤ 5μM (Harper *et al.*, 2004). Most cells (94%) responded to 4-CmC under low-Ca^{2+} conditions. The amplitude of cell responses was reduced by > 50% (Fig. 5.3 and 5.5.A) but the effect was very highly significant ($P<0.001$; unpaired-t) compared to controls continuously superfused with CFsEBSS, which showed a negligible $[Ca^{2+}]_i$ drift (Fig. 5.3 insert and 5.5.B). Oscillations

were very rarely observed after treatment with 4-CmC under low-Ca^{2+} conditions (< 2% of cells). However, when cells were stimulated with 4-CmC in EGTA buffered sEBSS, responses to 4-CmC were abolished (Fig. 5.4). Under control conditions, in which untreated sperm were superfused with sEBSS for an initial control period and then superfused with sEBSS-EGTA, there was an immediate drop in $[Ca^{2+}]_i$ when sEBSS-EGTA was introduced to the imaging chamber followed (in most cells) but a slow, further decay (Fig. 5.4 insert). Application of 4-CmC during this period had little, if any effect and cell responses were not significantly ($P>0.05$; unpaired-t) different to those observed in control cells continuously superfused with sEBSS-EGTA. Thus, 4-CmC effects on $[Ca^{2+}]_i$ under normal Ca^{2+} conditions were significantly greater ($P<0.001$; unpaired-t) than its effects on $[Ca^{2+}]_i$ under low-Ca^{2+} conditions or in EGTA buffered sEBSS (Fig. 5.5.A). Also, 4-CmC effects on $[Ca^{2+}]_i$ in low-Ca^{2+} saline were significantly ($P<0.001$; unpaired-t) different than its effects in EGTA buffered sEBSS (Fig. 5.5.A). These results suggest that, in human spermatozoa, 4-CmC mobilizes Ca^{2+} from an intracellular store, via activation of RyRs, but also that a Ca^{2+} influx pathway is important for it to exert its effects and that exposure to EGTA may very rapidly empty the store.

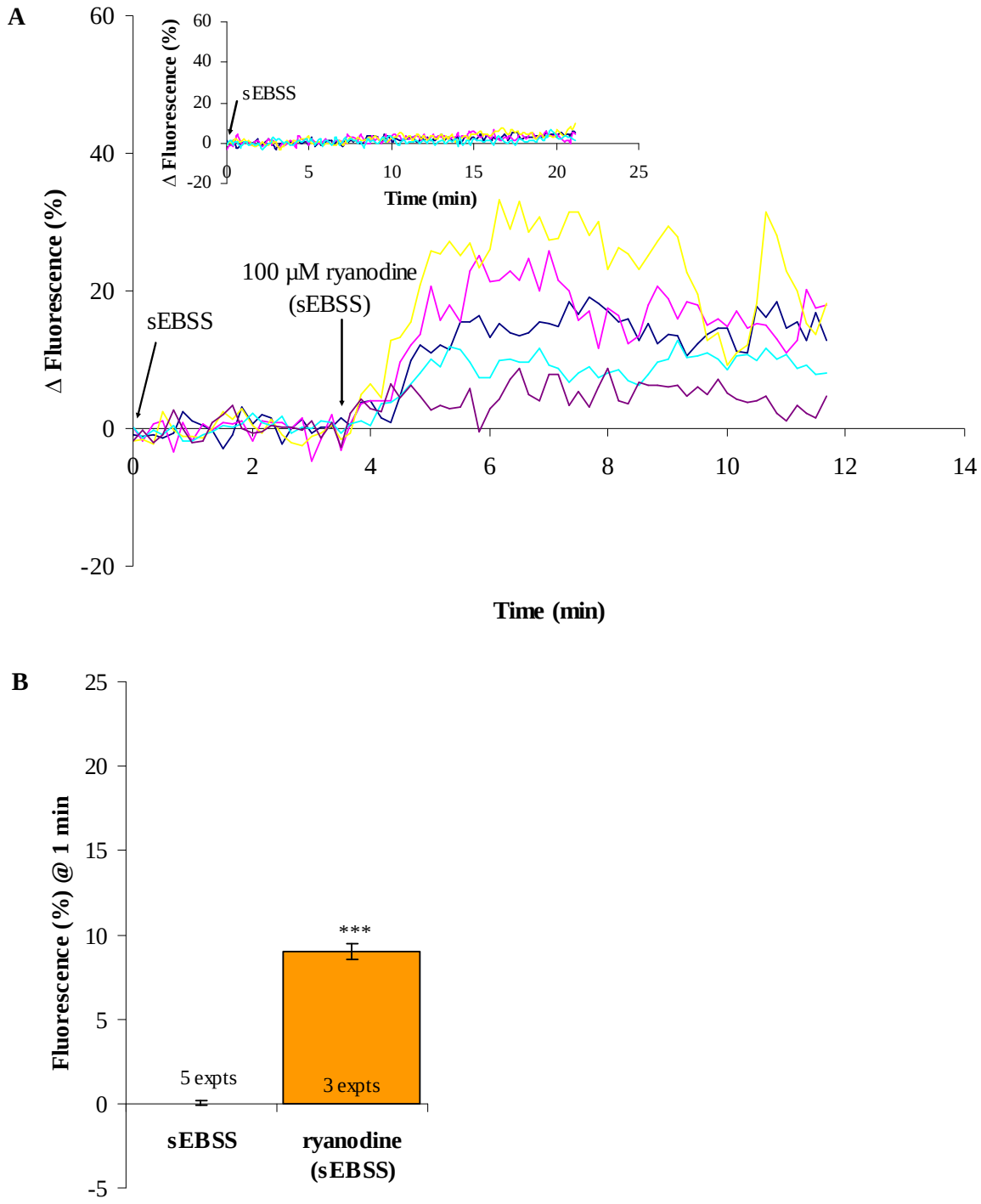

Fig. 5.1.A. The effects of ryanodine on $[Ca^{2+}]_i$ in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 3.5 minutes, and then superfused with 100 µM ryanodine in the same medium. Traces represent 5 individual cell responses. **Insert:** Control in which sperm prepared as for the ryanodine experiment were superfused with sEBSS in the absence of any treatment Traces show 4 single cell responses. **B.** Comparison between the increase in $[Ca^{2+}]_i$ (fluorescence) occurring after application of ryanodine and the levels of $[Ca^{2+}]_i$ (fluorescence)

under control conditions (sEBSS) in capacitated human sperm bathed in sEBSS. Bar chart shows the mean normalised fluorescence 1 minute after application of ryanodine and over a 1 minute period measured as in the experimental protocol (sEBSS = 215 cells; ryanodine = 113 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks indicate P<0.001.

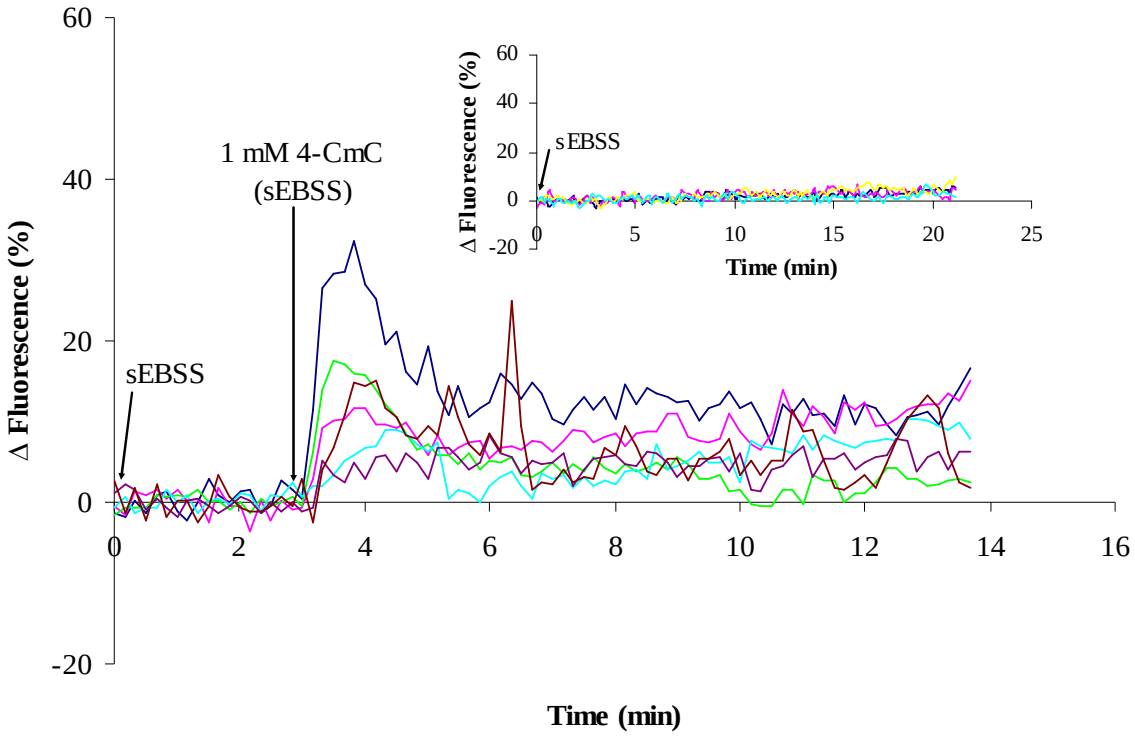

Fig. 5.2. The effects of 4-CmC on $[Ca^{2+}]_i$ in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 3 minutes, and then superfused with 1 mM 4-CmC in the same saline. Traces represent 6 single cell responses. **Insert:** Control in which sperm prepared as for the 4-CmC experiment were superfused with sEBSS in the absence of any treatment Traces show 4 single cell responses.

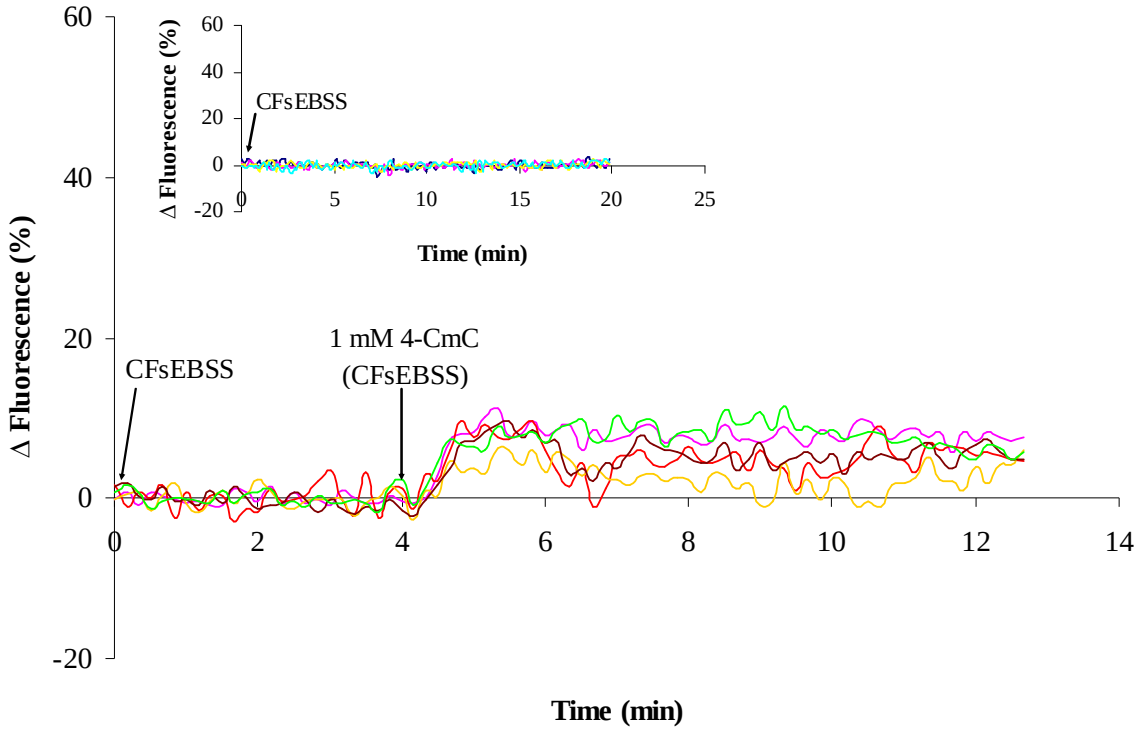

Fig. 5.3. The effects of 4-CmC on $[Ca^{2+}]_i$ in capacitated human sperm bathed in CFsEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with CFsEBSS for 4 minutes (after being stabilised in CFsEBSS), and then superfused with 1 mM 4-CmC in CFsEBSS. Traces show 5 individual cell responses. **Insert:** Control in which sperm prepared as for the 4-CmC experiment were superfused with CFsEBSS in the absence of any treatment Traces show 4 single cell responses.

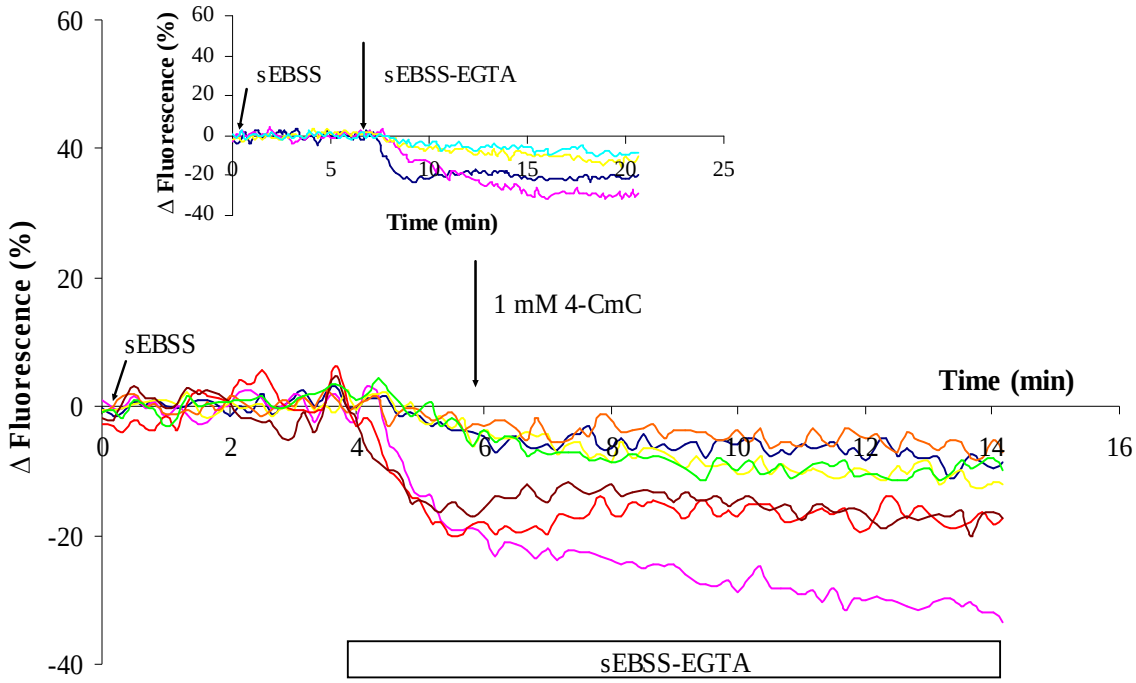

Fig. 5.4. The effects of 4-CmC on $[Ca^{2+}]_i$ in capacitated human sperm bathed in sEBSS-EGTA. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 4 minutes, then superfused with sEBSS-EGTA for 2 minutes, and finally exposed to 1 mM 4-CmC in sEBSS-EGTA. Traces show 7 single cell responses. **Insert:** Control in which sperm prepared as for the 4-CmC experiment were superfused with sEBSS-EGTA in the absence of any treatment Traces show 4 individual cell responses.

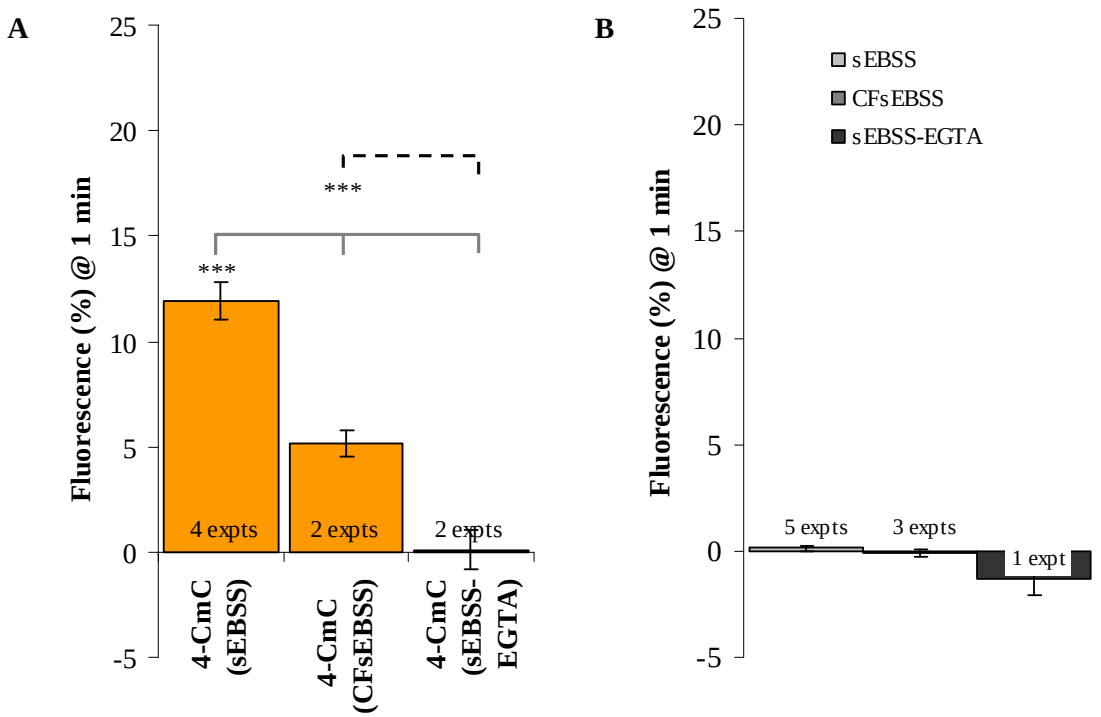

Fig. 5.5.A. Mean normalised increase in $[Ca^{2+}]_i$ (fluorescence) 1 minute after application of 1 mM 4-CmC to capacitated human sperm bathed in standard sEBSS, CFsEBSS or sEBSS-EGTA (sEBSS = 67 cells; CFsEBSS = 51 cells; sEBSS-EGTA = 58 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks indicate P<0.001. **B.** Mean drift in $[Ca^{2+}]_i$ (fluorescence) in untreated and capacitated human sperm bathed in standard sEBSS, CFsEBSS or sEBSS-EGTA. Bar chart represents the mean normalised drift in fluorescence over a 1 minute period measured as in the experimental protocol (sEBSS = 215 cells; CFsEBSS = 169 cells; sEBSS-EGTA = 92 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar.

5.4.2 The effects of NO on ryanodine-induced $[Ca^{2+}]_i$ signal

To assess whether the effects of ryanodine on $[Ca^{2+}]_i$ were modified by or interacted with those of NO, spermine NONOate pre-treated cells were exposed to ryanodine in the continued presence of the NO donor. There was an immediate increase in fluorescence upon application of ryanodine (Fig. 5.6.A) in 62% of cells. 27% of cells showed a transient $[Ca^{2+}]_i$ rise that then decayed to levels only 2-3% above those preceding stimulation. 22% of cells generated oscillations during this period (Fig. 5.6.A, brown and red traces). The amplitude of the ryanodine-induced $[Ca^{2+}]_i$ rise in NO pre-treated cells was significantly ($P<0.01$; unpaired-t) lower than the $[Ca^{2+}]_i$ rise observed in non-pre-treated cells (Fig. 5.6.B). Thus, sperm pre-treatment with NONOate partially occluded and truncated the sperm responses to ryanodine.

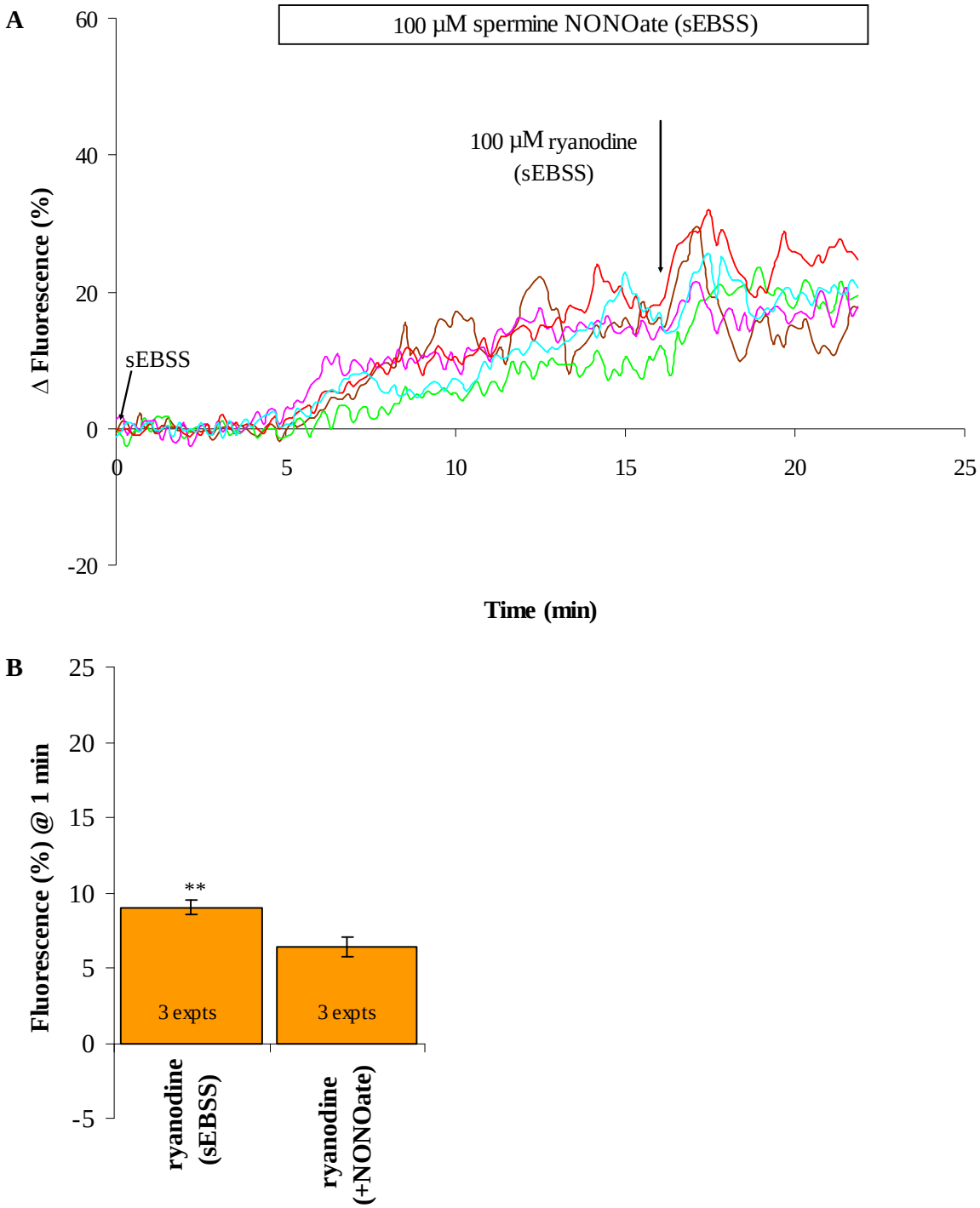

Fig. 5.6.A. The effects of spermine NONOate pre-treatment on ryanodine-induced $[Ca^{2+}]_i$ rise in capacitated human sperm bathed in sEBSS. Sperm were incubated in sEBSS for 6 hours, superfused with sEBSS for 5 minutes, pre-treated with 100 µM spermine NONOate and then exposed to 100 µM ryanodine in the continued presence of NONOate. Traces show 5 individual cell responses. **B.** Differences between the $[Ca^{2+}]_i$ elevation induced by ryanodine under control conditions (sEBSS) and ryanodine-induced $[Ca^{2+}]_i$ elevation in spermine NONOate pre-treated and capacitated human sperm

bathed in sEBSS. Bar chart represents the mean normalised increase in fluorescence 1 minute after ryanodine was applied to cells (sEBSS = 113 cells; NONOate pre-treated cells bathed in sEBSS = 129 cells). The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance ($P<0.01$).

5.5 Discussion

Application of 100 µM ryanodine to capacitated human sperm resulted in a rise in $[Ca^{2+}]_i$ (Fig. 5.1.A). This finding is consistent with the presence of RyRs in human sperm, which has previously been suggested by Harper *et al.* (2004) whose data showed human sperm staining with BODIPY-FL-X-ryanodine, a fluorescent analogue of ryanodine, in the posterior region of the head and mid-piece. This observation has been confirmed by Lefièvre *et al.* (unpublished data) whose data shows that RyR 1 and RyR 2 antibodies bind to the neck and mid-piece region of human sperm.

Some studies have previously demonstrated that chlorocresol is a powerful activator of RyRs. 4-CmC releases Ca^{2+} from a ruthenium red/caffeine-sensitive Ca^{2+} release channel in terminal cisternae (Zorzato *et al.*, 1993). Chlorocresol also releases Ca^{2+} from an inositol-1,4,5-triphosphate ($InsP_3$)-insensitive store in cerebellar microsomes. In pheocromocytoma cell line (PC12), chlorocresol releases Ca^{2+} from a caffeine and thapsigargin-sensitive intracellular store (Zorzato *et al.*, 1993). In our study, a $[Ca^{2+}]_i$ transient followed by a sustained $[Ca^{2+}]_i$ rise occurred upon sperm exposure to 4-CmC (Fig. 5.2). Our observations may therefore reflect mobilization of stored Ca^{2+} by 4-CmC via activation of RyRs. However, sperm responses to 4-CmC were greatly reduced in low-Ca^{2+} sEBSS (Fig. 5.3). Furthermore, 4-CmC application 2 minutes after sperm exposure to EGTA did not generate any detectable responses (Fig. 5.4). Zorzato *et al.* (1993) reported that chlorocresols induce a transient $[Ca^{2+}]_i$ rise in Ca^{2+}-free medium with EGTA in PC12 cells. Moreover, 4-CmC generated a transient followed by a sustained $[Ca^{2+}]_i$ rise in cultivated mouse myotubes, and replacement of extracellular Ca^{2+} by EGTA and use of channel blockers revealed that those responses reflected Ca^{2+} mobilization

from intracellular stores (Gschwend *et al.*, 1999). However, Ca^{2+} stores in mammalian sperm may be particularly labile and sensitive to EGTA-buffered medium. Exposure to EGTA results in an immediate drop in cytoplasmic [Ca^{2+}] and rapidly depletes intracellular Ca^{2+} stores in human sperm (Bedu-Addo *et al.*, 2007; Harper *et al.*, 2004). Therefore, our findings suggest that, in human sperm, 4-CmC effects on $[Ca^{2+}]_i$ reflect an action on the RyRs to mobilize stored Ca^{2+}, but Ca^{2+} influx may also be required as part of the response to 4-CmC. The absence of cell responses to 4-CmC in EGTA buffered sEBSS may reflect store depletion due to its sensitivity to EGTA.

NO or NO-related proteins modulate the activity of RyR 1 and RyR 2 (Sun *et al.*, 2001; 2003; Xu *et al.*, 1998; Zahradnikova *et al.*, 1997). Critical thiol (-SH) groups have been identified in the RyR 1 (Aracena-Parks *et al.*, 2006; Sun *et al.*, 2001) and donors of NO mobilize Ca^{2+} from skeletal and cardiac microsomes, via activation of RyRs, an effect that is reversed by -SH reducing agents (Stoyanovsky *et al.*, 1997). Treatment of human sperm with NO leads to S-nitrosylation of RyR 2 (Lefièvre *et al.*, 2007). Our present study revealed that sperm responses to 100 µM ryanodine were greatly reduced in the presence of NO (Fig. 5.6). This observation indicates that NO and ryanodine converge in their effects on sperm Ca^{2+} signalling. Whether this is at the RyR itself (the action of NO reducing sensitivity to ryanodine; Stoyanovsky *et al.*, 1997) or at the Ca^{2+} signal (NO activating the ryanodine-sensitive pathways, thus reducing the response to the drug) is not clear from these data. However, these results do emphasise the presence of RyRs in human spermatozoa and suggest that the Ca^{2+} mobilizing effects of NO and GSNO involve the same Ca^{2+} store as that mobilized by ryanodine.

CHAPTER SIX

NITRIC OXIDE (NO) INTERACTS SYNERGISTICALLY WITH PROGESTERONE IN CAPACITATED HUMAN SPERMATOZOA

6.1 Abstract

NO and progesterone, produced by the cumulus cells that surround the oocyte, mobilize stored Ca^{2+} in human spermatozoa. The effects of both agents on $[Ca^{2+}]_i$ are apparently mediated by ryanodine receptors (RyRs). NO and progesterone interaction effects on $[Ca^{2+}]_i$, flagellar activity and motility parameters in capacitated human spermatozoa were therefore examined. N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate) pre-treated spermatozoa showed prolonged and intensified Ca^{2+} responses to progesterone, in the continued presence of NO. Assessment of mid-piece displacement (as an indicator of flagellar beat mode) under the same conditions showed that an increase in flagellar activity occurred when progesterone was added. These data demonstrate that NO and progesterone interact to regulate human sperm motility. Computer-assisted semen analysis (CASA) immediately after application of progesterone, detected only marginal effects of co-stimulation, on free swimming cells, beat cross frequency (BCF) and straight-line velocity (VSL) being reduced. These data indicate that NO and progesterone contribute to the regulation of sperm motility although it appears that CASA is not a technique sensitive enough to detect changes in motility patterns under these experimental conditions.

6.2 Introduction

The zona pellucida, which surrounds the oocyte, is the best characterised agonist of $[Ca^{2+}]_i$ signalling in mammalian sperm. Upon sperm contact, the zona pellucida stimulates increases in $[Ca^{2+}]_{i,}$ leading to the acrosome reaction at the zona surface (Bailey and Storey, 1994; Florman, 1994). Progesterone, generated by and present in mammalian cumulus (Mingoti *et al.*, 2002; Osman *et al.*, 1989; Yamashita *et al.*, 2003), also leads to increases in $[Ca^{2+}]_i$ and is the best characterised agonist of human sperm. Similarly to the response to the zona pellucida, exposure to progesterone stimulates a "simple" signal by Ca^{2+} influx (Blackmore *et al.*, 1990; Plant *et al.*, 1995). The response to progesterone involves at least two influx pathways but their identity is not elucidated (Guzman-Grenfell and Gonzalez-Martinez, 2004). Although that responsiveness of human sperm to progesterone is associated with fertilization success *in vitro* (Krausz *et al.*, 1996) and demonstrates biological significance of this steroid, its role in fertilization is not elucidated. Micromolar concentrations of progesterone stimulate the acrosome reaction, but the significance of this response for fertilization *in vivo* is unclear (Guzman-Grenfell and Gonzalez-Martinez, 2004; Wassarman *et al.*, 2001). It has also been reported that progesterone stimulates hyperactivation, a dynamic swimming pattern caused by marked modifications in flagellar beat mode. Sperm become hyperactivated as they gain the capacity to fertilize. Hyperactivation is characteristic of sperm retrieved at the site and time of fertilization (Suarez and Ho, 2003).

Exposure of human sperm to gradients of progesterone originates a $[Ca^{2+}]_i$ ramp with superimposed slow oscillations, which do not induce acrosome reaction but modulate flagellar beat, an effect potentially significant in penetration of the oocyte vestments prior to

fertilization (Harper *et al.*, 2004). $[Ca^{2+}]_i$ oscillations can be generated upon application of progesterone as a concentration 'step' but the number of cells in which this signalling pattern occurs is low (Aitken and McLaughlin, 2007; Harper *et al.*, 2004; Kirkman-Brown *et al.*, 2004). Progesterone-induced Ca^{2+} influx appears to activate ryanodine receptors (RyRs) located in the sperm neck/mid-piece region, causing repetitive bursts of Ca^{2+}-induced Ca^{2+} release (CICR; Harper *et al.*, 2004). The identity of the Ca^{2+} store in the sperm neck/mid-piece is not yet established, but one of the strongest candidates is the redundant nuclear envelope (RNE), which appears to play a role in the regulation of the flagellum in murine sperm (Marquez *et al.*, 2007). During spermiogenesis in many species, including humans (Kerr, 1991; Westbrook *et al.*, 2001), a membrane complex called RNE originates in the neck region of spermatozoa. The RNE is believed to function as a Ca^{2+} store in bovine sperm and to control flagellar beat mode (Ho and Suarez, 2001b; Ho and Suarez, 2003). Ca^{2+} re-uptake during oscillations seems to be dependent, at least partly, on the activity of secretory pathway Ca^{2+}-ATPase (SPCA) 1 (Harper *et al.*, 2005). SPCA1 is present in mature human spermatozoa and is situated to the anterior mid-piece, extending into the posterior region of the head (Harper *et al.*, 2005), possibly reflecting expression in the putative Ca^{2+} store of the RNE (Ho and Suarez, 2003). For information about RyRs and their relation with NO see Chapter One (section 1.11.6) and Chapter Five, respectively.

The aim of this study was to examine the effects of co-stimulation with NO and progesterone on $[Ca^{2+}]_i$, flagellar activity and motility parameters in capacitated human spermatozoa.

Part of this work has been published in the journal "Development".

6.3 Materials and methods

6.3.1 Materials

Salts used to prepare supplemented Earle's balanced salt solution (sEBSS; see Appendix I for details), pluronic F-127, dimethyl sulfoxide (DMSO) and progesterone ([4-Pregnene-3,20-dione]) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Fatty-acid free bovine serum albumin (BSA) was obtained from SAFC Biosciences (Lenexa, KS, USA). Oregon Green 488 BAPTA 1-acetoxymethyl (OGB-1AM) was acquired from Invitrogen Molecular Probes (Paisley, UK). Poly-D-lysine (PDL) was purchased from BD Biosciences (Oxford, UK). N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate) was obtained from Merck Chemicals Ltd. (Beeston, Nottingham, UK). Computer-assisted semen analysis (CASA) slides (Microcells 50 μm depth with two chambers per slide) were from Conception Technologies (San Diego, CA, USA). All chemicals were cell-culture-tested grade where available.

6.3.2 Spermatozoa preparation and capacitation

Sperm were prepared and capacitated for live imaging and CASA as described in Chapter Two, page 70.

6.3.3 Single cell imaging

As in Chapter Two, page 71, with the following specifications to this chapter:

In all experiments, OGB-1AM loaded sperm were superfused with sEBSS + 0.3% BSA for an initial control period, and the drugs used were dissolved in the same saline.

In a set of experiments, cells were pre-treated with spermine NONOate (100 μM) for approximately 10 minutes, enough time for the NONOate to exert its effects. Progesterone (3 μM) was added in the continued presence of NONOate. In control experiments, NO untreated cells were superfused with progesterone (3 μM). In additional control experiments, NONOate pre-treated sperm were superfused with progesterone (3 μM) upon washout of NONOate. Finally, spermatozoa were exposed to spermine NONOate (100 μM) and solvent control (0.05% DMSO) was then added to the cells, in the continued presence of spermine NONOate.

6.3.4 Imaging data processing

As in Chapter Two, page 72.

6.3.5 Imaging data statistical analysis

More complex $[Ca^{2+}]_i$ responses (such as oscillations) were generated and were therefore quantifified by direct examination of individual time-fluorescence intensity plots. Data from each type of experiment were pooled to calculate the frequency of each type of response, stated in the text as mean ± SEM. Microsoft Excel was used to perform paired or unpaired-t tests, as appropriate, when comparing classes of responses. Statistical significance was set at $P<0.05$.

6.3.6 Evaluation of $[Ca^{2+}]_i$ oscillations

As in Chapter Two, page 74.

6.3.7 Flagellar activity assessment

Cells were observed under phase-contrast microscopy with an increased rate of image acquisition (1 Hz) to attempt to visualize changes in the mid-piece and thus flagellum displacement under experimental conditions. For this purpose, spermatozoa were not labelled and were directly introduced to the recording chamber for 5 minutes to allow cell attachment to the PDL coated slide. Fields of cells were preferentially selected in the boundary of the PDL coated area where sperm were loosely attached to the slide. Loosely-attached cells with a freely motile flagellum were chosen to assess the flagellar activity. Assessment of the mid-piece displacement and thus flagellar activity was adapted from that described previously (Harper *et al.*, 2004) using the ImageJ MTrackJ plugin. Using the mid-point of the mid-piece as a reference point, frame-to-frame displacement was measured throughout the experiments and subsequently plotted against time.

Cells were superfused with spermine NONOate (100 μM) for approximately 10 minutes, sufficient time for the NONOate effects to become well established. Progesterone (3 μM) was then added, in the continued presence of NONOate. In control experiments, sperm were stimulated with progesterone (3 μM) without spermine NONOate pre-treatment. In both cases, images started being taken before progesterone addition, to get an initial 1 minute control period, and continued to be taken for a further 4-5 minutes in the presence of progesterone.

6.3.8 CASA

100 μl sperm suspension aliquots, at 4 million cells/ ml, were prepared to assess motility parameters using the CASA machine (Hamilton Thorne running IVOS v.10). Cells were pre-

treated with spermine NONOate (100 μM) for approximately 10 minutes. Progesterone (3 μM) was then applied to the cells. Immediately after progesterone was added, 10 μl of sperm suspension was introduced into a 50 μm depth slide, and motility parameters were analysed by the CASA machine. In parallel, sperm were stimulated with spermine NONOate (100 μM) for approximately 10 minutes and motility assessed once cells loaded into the slide chamber. Additionally, sperm were directly incubated with progesterone (3 μM), in the absence of pre-incubation, and motility was assessed as previously, immediately after allowing cells to load well into the slide. Motility parameters were also analysed in untreated cells following the same procedure. Furthermore, cells were treated with spermine NONOate (100 μM) for about 10 minutes and solvent control (0.05% DMSO) was then added to the cells. Immediately after solvent control was added, 10 μl of sperm suspension was introduced in the slide chamber and motility was analysed. All CASA experiments were performed at 25°C to parallel the single cell imaging studies as closely as possible.

6.3.9 CASA data analysis

Percentages and mean ± SD correspondent to different motility patterns were automatically registered by the CASA machine while performing the experiments. Changes in motility parameters due to different treatments were compared in Microsoft Excel using paired t-tests. Statistical significance was set at $P<0.05$.

6.4 Results

6.4.1 NO and progesterone interaction effects on $[Ca^{2+}]_i$ signal

The effects of spermine NONOate and S-nitrosoglutathione (GSNO) on human sperm $[Ca^{2+}]_i$, stimulating sustained increase with superimposed oscillations, are similar to the effects of progesterone, which cyclically mobilizes Ca^{2+} stored in a membranous compartment in the sperm neck/mid-piece (Bedu-Addo *et al.*, 2007; Harper *et al.*, 2004; Harper and Publicover, 2005). These effects of progesterone involve activation of RyRs (Harper *et al.*, 2004; reviewed by Harper and Publicover, 2005), which are positively regulated by S-nitrosylation (Meissner, 2004; Stoyanovsky *et al.*, 1997) and RyR 2 was identified in the nitrosoproteome of human sperm (Lefièvre *et al.*, 2007). Since the action of NO on sperm $[Ca^{2+}]_i$ is by protein S-nitrosylation, leading to mobilization of stored Ca^{2+}, interaction or synergism between the actions of these two agonists, both of which will be encountered by sperm approaching the oocyte, might be anticipated. Spermatozoa were pre-treated with spermine NONOate (100 μM) for approximately 10 minutes, and subsequently exposed to progesterone (3 μM) in the continued presence of spermine NONOate. Response to progesterone was modified under these conditions (Fig. 6.1.A). In control cells, the usual duration of the initial progesterone-stimulated $[Ca^{2+}]_i$ transient is 2-2.5 minutes (Kirkman-Brown *et al.*, 2000; Fig. 6.1.A insert). Although some cells still responded as previously described (Fig. 6.1.A; brown and orange traces), most cells presented a longer initial response, emerging as a $[Ca^{2+}]_i$ plateau (Fig. 6.1.A; red and green traces) or the usual peak but with a distinct 'shoulder' on the tail of the response (Fig. 6.1.A; pink, yellow and blue traces). Transient duration analysis (from begin of rise to plateau or inflexion at the end of falling period) made clear that although prolonged responses (>2.5 minutes) occurred in spermine NONOate untreated spermatozoa (range 10-

50% of the population), NONOate pre-treatment increased the percentage of cells showing very prolonged transients whilst brief transients became uncommon (Fig. 6.1.B). Three sets of paired experiments (control and NONOate treated sperm from the same sample) showed that spermine NONOate pre-treatment increased the percentage of prolonged responses (≥2.5 minutes) from 42±8% to 92±3% ($P<0.025$; paired-t; Fig. 6.1.B). To investigate whether the effect of pre-treatment with NONOate would persist in the absence of the NO donor, we performed parallel experiments in which NONOate (100 μM) was washed off simultaneously with the introduction of progesterone (3 μM) to the imaging chamber. In these experiments, cell responses to progesterone resembled those of non-pre-treated cells, the proportion of transients of ≥2.5 minutes in two experiments being 40% and 45% ($P>0.05$, paired-t; non significant compared to parallel experiments without pre-treatment). Moreover, in solvent control experiments where progesterone was replaced by the correspondent dose of DMSO (0.05%), the shape of the responses was generally different, the transient $[Ca^{2+}]_i$ elevations being rare and the progressive $[Ca^{2+}]_i$ rises more frequent, consistent with sperm exposure to the NO stimulus in the absence of progesterone.

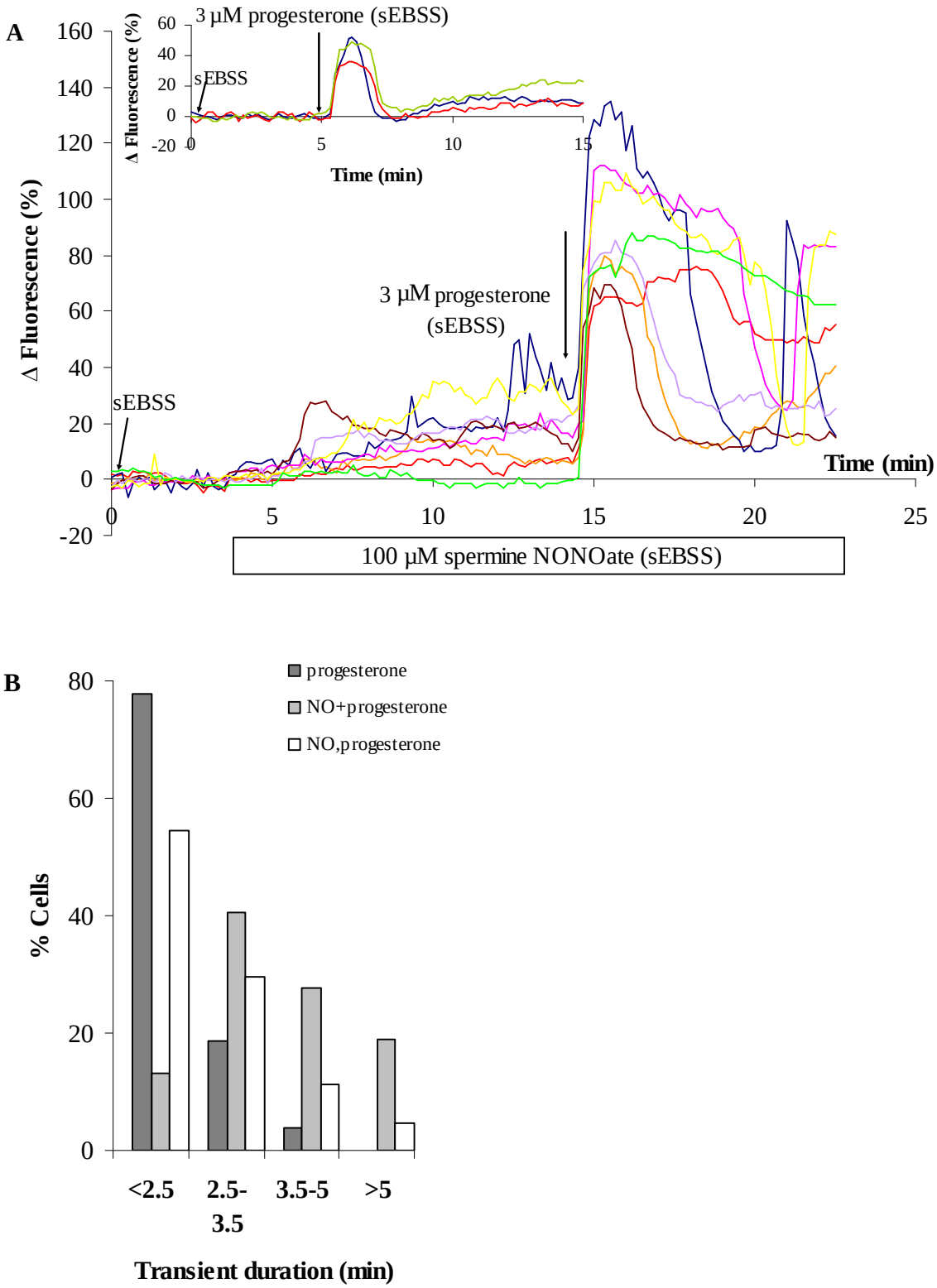

Fig. 6.1.A. The effects of spermine NONOate on progesterone induced-$[Ca^{2+}]_i$ signalling in capacitated human sperm bathed in sEBSS. Cells were incubated in sEBSS for 6 hours, superfused

with sEBSS for 4 minutes, pre-treated with 100 μM spermine NONOate for 10 minutes, and then stimulated with 3 μM progesterone, in the continued presence of NONOate. Traces show 8 single cell responses. **Insert:** Progesterone-induced $[Ca^{2+}]_i$ rise under control conditions (without NONOate pre-treatment). Cells were incubated in sEBSS for 6 hours, superfused with sEBSS for 5 minutes and then exposed to 3 μM progesterone. Traces show 3 single cell responses. **B.** Differences between the progesterone-induced $[Ca^{2+}]_i$ transient duration under control conditions (without NONOate pre-treatment), after spermine NONOate pre-treatment and upon washout of NONOate in capacitated human sperm bathed in sEBSS. Bar chart presents the progesterone-induced $[Ca^{2+}]_i$ transient duration plotted as the percentage of cells in each class, defined by the duration of $[Ca^{2+}]_i$ transient. Control cells (dark grey bars – 27 cells) were from the same sample as cells exposed to NONOate before and during progesterone stimulation (light grey bars – 69 cells) and cells in which NONOate was washed off as progesterone was applied (white bars – 44 cells).

6.4.2 NO and progesterone interaction effects on flagellar activity

Mobilization of stored Ca^{2+} in the neck/mid-piece region of human spermatozoa by progesterone or 4-aminopyridine clearly modifies the flagellar beat mode, comprising an increase in mid-piece bending and flagellar displacement, which is observed in loosely tethered cells (Bedu-Addo *et al.*, 2007; 2008; Harper *et al.*, 2004). Assessment of mid-piece (and thus flagellar) displacement was carried out to investigate whether co-stimulation with NO and progesterone modifies the flagellar beat mode. Frame-by-frame analysis showed that cells stimulated with progesterone (3 μM) responded with a brief (30-50 seconds) increase in flagellar displacement, in 70% of cells, consistent with increased flagellar activity (Fig. 6.2.A) during the initial $[Ca^{2+}]_i$ transient (Fig. 6.1.A insert). When cells were pre-treated with spermine NONOate (100 μM) for approximately 10 minutes, there was no significant effect on flagellar beat mode. However, when these cells were exposed to progesterone (3 μM) in the continued presence of NONOate, though the proportion of responsive cells was not altered (≈ 80%), the enhancement of flagellar activity (measured as an increase in frame-to-frame mid-piece displacement), was maintained for the duration of recording (≈ 4 minutes), including a series of peaks (Fig. 6.2.B). The kinetics of this increase in mid-piece

displacement were consistent with those of the enhanced $[Ca^{2+}]_i$ response to progesterone seen in cells previously exposed to spermine NONOate (Fig. 6.1).

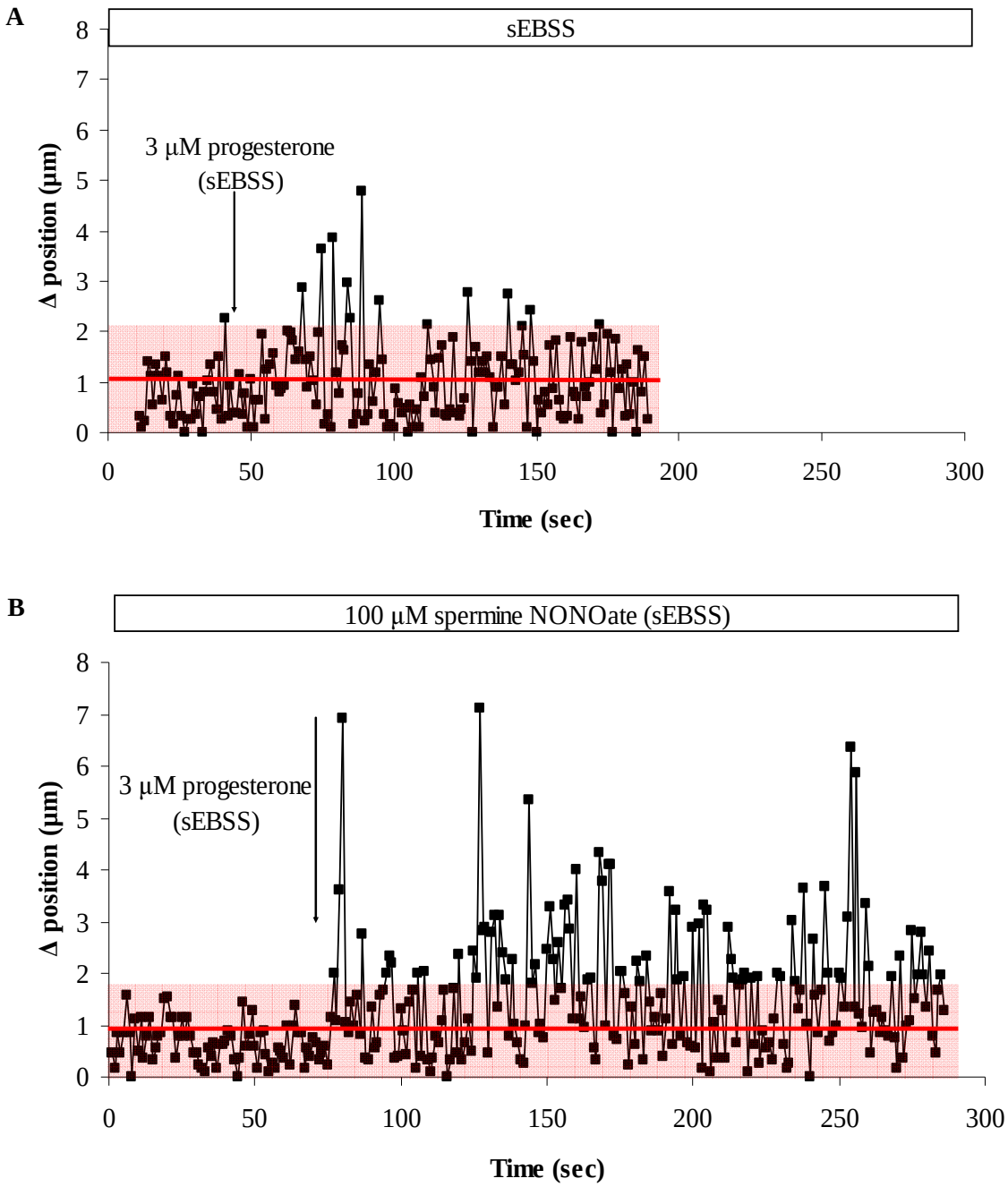

Fig. 6.2.A. Progesterone-induced increase in mid-piece displacement in capacitated human sperm bathed in sEBSS. Graph shows response of one cell (representative of > 100 cells in 2 experiments). Red line and shading represent the mean ± 2 SD of frame-to-frame mid-piece displacement during the

control period. **B.** Progesterone-induced increase in mid-piece displacement in spermine NONOate pre-treated and capacitated human sperm bathed in sEBSS. Graph shows response of one cell (representative of > 100 cells in 2 experiments). Red line and shading show the mean ± 2 SD of frame-to-frame mid-piece displacement during the control period.

6.4.3 NO and progesterone interaction effects on motility parameters

CASA (Hamilton-Thorn Research Inc., Beverly, MA, USA; Kay and Robertson, 1998) was performed to investigate whether sperm co-stimulation with NO and progesterone caused changes in motility parameters. The motility parameters that were used for detailed analysis are shown in Table 6.1. An effect of treatment was detected for BCF and VSL but not for the other motility parameters. BCF was significantly ($P<0.05\%$; paired-t) higher in untreated cells than when NONOate (100 µM) pre-treated cells were exposed to progesterone (3 µM) in the continued presence of NONOate (Fig. 6.3). Moreover, VSL was significantly ($P<0.01\%$; paired-t) higher in untreated cells than when NONOate (100 µM) pre-treated cells were stimulated with progesterone (3 µM) in the presence of NONOate (Fig. 6.4). These data are consistent with the prolonged and intensified $[Ca^{2+}]_i$ rise upon application of progesterone to NO pre-treated sperm and also with the increased flagellar excursion that occurs under exactly the same experimental conditions. No effect of treatment ($P>0.05$; paired-t) was detected on the motility parameters when comparing progesterone controls with progesterone and NONOate co-stimulated sperm, as exemplified for BCF and VSL in Fig. 6.3 and 6.4, respectively. These results may be due to the timing of the observations during experimental procedures (see below).

Item	**untreated**	3µM progesterone	**100µM NONOate+3µM progesterone**	100µM NONOate	100µM NONOate+0.05%DMSO
Donor A					
Motile(%)	64	39	35	68	52
VSL(µm/s)	**37.9 ± 13.4***	17.1 ± 11.0	**19.5 ± 9.6**	34.0 ± 15.7	28.6 ± 13.6
VCL(µm/s)	82.8 ± 23.1	48.3 ± 17.5	54.2 ± 15.9	67.8 ± 23.3	61.8 ± 22.2
BCF(Hz)	**19.6 ± 10.3**	14.9 ± 12.3	**14.4 ± 9.8**	16.4 ± 11.6	17.8 ± 10.8
Hyp(%)	0	0	0	0	0
VAP(µm/s)	45.0 ± 12.6	27.4 ± 10.8	29.6 ± 9.9	40.3 ± 15.6	34.9 ± 13.8
ALH(µm/s)	4.6 ± 1.7	3.9 ± 1.8	4.1 ± 1.5	4.1 ± 1.5	4.1 ± 1.4
Donor B					
Motile(%)	53	54	66	40	34
VSL(µm/s)	**36.3 ± 14.2**	31.1 ± 20.3	**34.9 ± 23.2**	33.1 ± 19.2	31.0 ± 18.3
VCL(µm/s)	80.1 ± 28.6	85.0 ± 31.9	98.2 ± 41.0	79.4 ± 38.5	73.6 ± 35.2
BCF(Hz)	**18.7 ± 10.5**	13.1 ± 10.6	**13.0 ± 12.5**	16.7 ± 12.3	17.7 ± 11.6
Hyp(%)	1	4	5	5	5
VAP(µm/s)	44.1 ± 14.7	47.7 ± 20.5	54.2 ± 27.2	42.4 ± 20.3	39.5 ± 18.9
ALH(µm/s)	4.5 ± 1.7	5.6 ± 2.9	5.5 ± 3.1	5.6 ± 2.2	5.6 ± 2.2
Donor C1					
Motile(%)	40	33	21	57	29
VSL(µm/s)	**34.6 ± 16.6**	30.6 ± 16.8	**26.9 ± 15.6**	27.6 ± 16.7	24.2 ± 13.2
VCL(µm/s)	73.7 ± 30.6	66.6 ± 29.4	58.9 ± 22.8	55.7 ± 25.3	53.6 ± 22.0
BCF(Hz)	**17.1 ± 10.6**	15.8 ± 11.1	**17.2 ± 11.4**	16.2 ± 10.2	15.4 ± 8.9
Hyp(%)	1	0	0	0	0
VAP(µm/s)	41.9 ± 17.3	38.9 ± 17.4	34.8 ± 15.6	34.0 ± 16.5	31.6 ± 15.0
ALH(µm/s)	4.3 ± 1.8	3.9 ± 1.7	4.0 ± 1.9	3.8 ± 1.8	4.1 ± 1.6
Donor C2					
Motile(%)	31	50	41	26	30
VSL(µm/s)	**34.9 ± 15.0**	33.5 ± 15.6	**26.1 ± 13.7**	31.7 ± 15.6	25.0 ± 11.3
VCL(µm/s)	70.7± 29.8	74.6 ± 28.3	59.8 ± 23.5	63.4 ± 23.2	55.0 ± 18.6
BCF(Hz)	**17.1 ± 8.8**	12.8 ± 9.8	**15.7 ± 9.9**	17.1 ± 7.5	15.8 ± 10.9
Hyp(%)	2	1	0	0	0
VAP(µm/s)	40.4 ± 14.9	41.8 ± 15.6	34.0 ± 15.2	38.3 ± 13.9	31.6 ± 11.7
ALH(µm/s)	4.2 ± 1.8	4.5 ± 2.1	4.6 ± 1.9	4.0 ± 1.6	3.8 ± 1.4
Donor D1					
Motile(%)	24	19	24	22	36
VSL(µm/s)	**35.3 ± 17.5**	28.5 ± 17.3	**23.5 ± 13.2**	31.5 ± 16.4	23.8 ± 14.0
VCL(µm/s)	70.6 ± 28.2	58.7 ± 29.8	55.7 ± 25.9	64.3 ± 27.6	52.8 ± 22.0
BCF(Hz)	**16.3 ± 8.7**	16.0 ± 10.7	**15.7 ± 9.9**	16.0 ± 8.5	15.4 ± 13.0
Hyp(%)	0	0	1	1	0
VAP(µm/s)	42.4 ± 17.8	34.5 ± 17.8	30.9 ± 14.1	38.4 ± 15.8	30.6 ± 14.3
ALH(µm/s)	4.0 ± 1.7	4.3 ± 1.8	4.7 ± 2.0	3.9 ± 1.8	3.2 ± 1.7
Donor D2					
Motile(%)	21	35	20	25	45
VSL(µm/s)	**33.3 ± 16.8**	30.6 ± 16.1	**23.9 ± 15.7**	30.7 ± 14.0	61.3 ± 96.1
VCL(µm/s)	67.9 ± 25.6	68.6 ± 28.0	57.3 ± 23.3	61.6 ± 20.6	80.3 ± 90.4
BCF(Hz)	**17.7 ± 9.7**	15.5 ± 10.3	**14.7 ± 9.1**	18.5 ± 12.3	18.8 ± 12.4
Hyp(%)	1	1	0	0	0
VAP(µm/s)	40.3 ± 15.7	39.3 ± 17.1	33.1 ± 16.7	37.0 ± 13.8	65.4 ± 94.6
ALH(µm/s)	4.0 ± 1.8	4.2 ± 1.9	4.4 ± 1.6	3.8 ± 1.5	2.9 ± 1.5

*Mean ± SD

Table 6.1. Summary of motility parameters obtained with CASA upon different treatments, and used for detailed analysis. Figures show motile and hyperactive cells as a percentage of all cells in those experiments, and the other figures are shown as mean ± SD. Column 1 shows the motility parameters; motile, VSL=straight-line velocity, VCL=curvilinear velocity, BCF=beat cross frequency, Hyp=hyperactive, VAP=average path velocity, ALH=amplitude of lateral head displacement. Row 1

shows treatments applied. 4 different donors were used (A, B, C and D; 1 and 2 indicate repeats using the same sample).

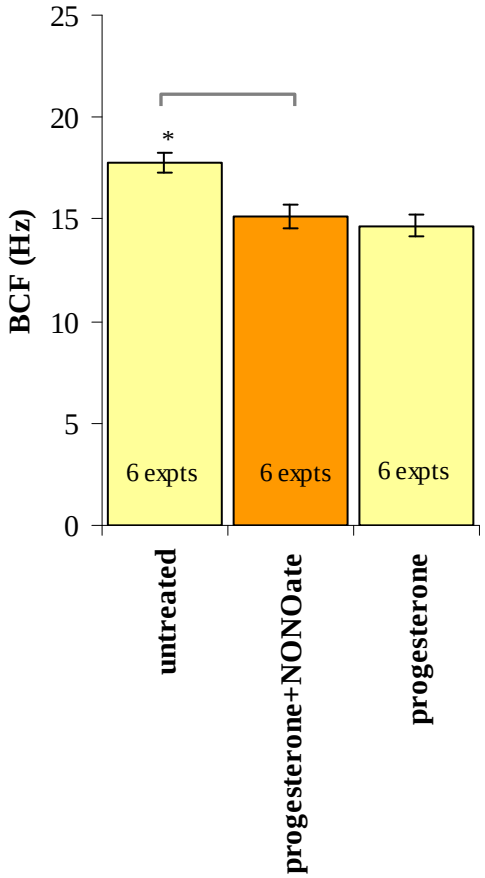

Fig. 6.3. Differences between the BCF in untreated, spermine NONOate and progesterone co-stimulated and progesterone stimulated and capacitated human sperm bathed in sEBSS. Bar chart presents the mean BCF in untreated sperm and that captured immediately after progesterone was added either to NONOate pre-treated sperm or to untreated sperm bathed in sEBSS. The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance ($P<0.05$).

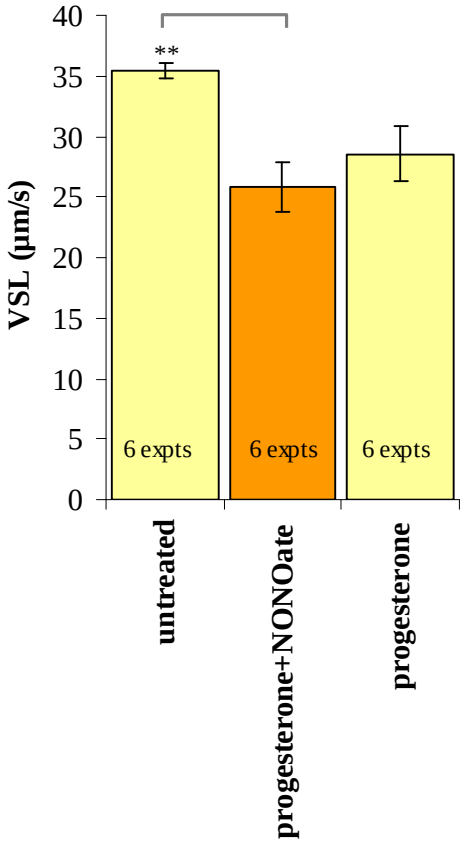

Fig. 6.4. Differences between the VSL in untreated, spermine NONOate and progesterone co-stimulated and progesterone stimulated and capacitated human sperm bathed in sEBSS. Bar chart shows the mean VSL in untreated sperm and that captured immediately after progesterone was added either to NONOate pre-treated sperm or to untreated sperm bathed in sEBSS. The error bars represent the SEM, and the number of experimental replicates is indicated in each bar. Asterisks show differences in significance ($P<0.01$).

6.5 Discussion

One of the targets for NO-induced S-nitrosylation in human sperm is RyR 2 (Lefièvre *et al.*, 2007). Immunofluorescence staining and Western blotting of human sperm with RyR antibodies has confirmed the presence of both RyR 1 and 2 in the neck/mid-piece region of human spermatozoa, RyR 2 appearing to be more prominent (Lefièvre *et al.,* in preparation). Since S-nitrosylation and S-oxidation (by nitroxyl or HNO) of RyRs increases open probability and allows mobilization of microsomal Ca^{2+} (Cheong *et al.,* 2005; Stoyanovsky *et al.*, 1997), we propose that an action on these receptors is the most likely cause of the Ca^{2+}-mobilizing abilities of NO and GSNO. Exposure of human sperm to progesterone stimulates a $[Ca^{2+}]_i$ plateau upon which oscillations, similar in kinetics, amplitude and localisation to those observed in NO-treated sperm, are superimposed. These oscillations are generated by a mechanism involving activation of RyRs, causing mobilization of stored Ca^{2+} localised to the sperm neck/mid-piece region (Harper *et al.*, 2004; 2005) and modulate flagellar beat (Harper *et al.*, 2004). In accordance with convergence of the actions of progesterone and NO, cell pre-treatment with spermine NONOate prolonged significantly the $[Ca^{2+}]_i$ transient induced by 3 μM progesterone (Fig. 6.1). This effect was totally dependent upon the continued presence of NO, no synergism being observed when the NO donor was washed off simultaneously with application of progesterone. In effect this means that the actions of NO are reversed within 2.5 minutes (duration of the "control" action of progesterone), consistent with the rapid reversibility of protein S-nitrosylation in spermatozoa.

Progesterone has been reported to have a weak hyperactivating effect on human spermatozoa (Jaiswal *et al.*, 1999; Uhler *et al.*, 1992; Yang *et al.*, 1994). In the presence of NO, this effect

might be expected to be enhanced, reflecting the increased duration of the $[Ca^{2+}]_i$ rise that occurs under these circumstances. Inspection of cells exposed to progesterone in the continued presence of NO confirmed that the action of progesterone was in fact transformed into a prolonged modulation of flagellar activity characterised by increased excursion of the mid-piece (Fig. 6.2.B), in contrast to the brief increased excursion of the mid-piece that occurred in controls, in which sperm were exposed to progesterone in the absence of NO (Fig. 6.2.A). Moreover, CASA revealed BCF and VSL differences in sperm pre-treated with NONOate and subsequently exposed to progesterone, when compared to untreated sperm. Both BCF and VSL decreased when progesterone was applied to NONOate-treated sperm (Fig. 6.3 and 6.4). These changes in motility parameters show consistency with an effect of progesterone and NO on sperm motility. Morphologically normal human sperm, incubated under capacitating conditions, exhibit a change in movement pattern, which resembles hyperactivation in other species, and was characterized by higher values of ALH, lower values of linearity, beat frequency and flagellar curvature ratio (Morales *et al.*, 1988). Therefore, reduced VSL and BCF might be expected in sperm displaying a more vigorous movement pattern. Our observations thus suggest that sperm co-stimulation with progesterone and NO result in a more dynamic motility pattern, which may be important to achieve fertilization *in vivo*.

There was no significant ($P>0.05$) difference between the motility parameters measured in samples exposed to progesterone in the absence or presence of NO, as showed for BCF and VSL in Fig. 6.3 and 6.4, respectively. In both cases, the measurements were obtained immediately after application of progesterone to the samples, which in the controls falls in the time window before the decay of the flagellar excursion amplitude. Therefore, this possibly accounts for the similarity between progesterone controls and NO and progesterone co-

stimulation experiments. It appears that CASA is not a technique sufficiently sensitive to detect modifications of motility patterns under our experimental conditions, as only two of the analysed motility parameters showed an effect of treatment.

We propose that NO-induced protein S-nitrosylation and progesterone act synergistically at the Ca^{2+} store of the sperm mid-piece/neck region, RyRs being the most likely site of coincidence-detection. The consequent prolonged elevation of $[Ca^{2+}]_i$ will modulate flagellar activity, particularly bending of the mid-piece (Bedu-Addo *et al.,* 2008), contributing to the hyperactivation that is crucial for penetration of the oocyte vestments.

CHAPTER SEVEN

GENERAL DISCUSSION

The aim of this thesis was to elucidate the NO effects on $[Ca^{2+}]_i$, its target and mechanism of action and to assess the interaction effects of NO and progesterone on $[Ca^{2+}]_i$ and its potential to regulate human sperm functions.

Our study revealed that constitutive NOS are expressed in human cumulus cells and that these cells generate NO. These findings are consistent with previous reports that NOS is present in the mammalian oocyte and in the cumulus and corona cells that surround it (Hattori *et al.*, 2001; Reyes *et al.*, 2004; Tao *et al.*, 2004) and that NO is synthesised in the mammalian female reproductive tract (Rosselli *et al.*, 1998). However, endogenous generation of NO by human sperm was not detectable under the incubation conditions of our assay. Although there is evidence that sperm is able to generate NO, there is controversy surrounding its physiological significance (Lefièvre *et al.*, 2007). Thus, it is likely that NO encountered by sperm upon approaching and entering the cumulus oophorus provides a particularly potent stimulus.

The effects of NO on $[Ca^{2+}]_i$ were therefore investigated. The NO donor spermine NONOate raised $[Ca^{2+}]_i$, even in low-Ca^{2+} conditions, in the neck/mid-piece region of most cells, often spreading into the posterior of the head, indicating that the effect of NO primarily reflects store mobilization. In various cell types (e.g. macrophages, gut interstitial cells, pancreatic β-cells), NO elevates $[Ca^{2+}]_i$, mobilizing Ca^{2+} pools (Willmott *et al.*, 1996). A subset of cells

generated $[Ca^{2+}]_i$ oscillations, superimposed on the NO-induced $[Ca^{2+}]_i$ rise, resembling those induced by progesterone (Harper *et al.*, 2004; Kirkman-Brown *et al.*, 2004). Washout of NONOate led to a drop in $[Ca^{2+}]_i$ and most cells then showed a slow $[Ca^{2+}]_i$ elevation and resumption of oscillation, again resembling the effect of progesterone (Harper *et al.,* 2004), and showing that the NO effect is reversible.

The effects of the NO redox sibling HNO on $[Ca^{2+}]_i$ were also examined. Sperm exposure to the HNO donor ANGS led to a rise in $[Ca^{2+}]_i$, an effect that was not altered by omission of Ca^{2+} from the saline, indicating that the effect of HNO on $[Ca^{2+}]_i$ may reflect primarily store mobilization. HNO potentially induces Ca^{2+} release from RyRs in skeletal (Cheong *et al.*, 2005) and cardiac muscles (Cheong *et al.*, 2005; Tocchetti *et al.*, 2007), suggesting that a similar mechanism of action could underlie our observations.

This study also investigated the NO target for it to exert its effects. The primary actions of NO in target tissues include activation of sGC, leading to a rise in cGMP concentration and PKG mediated actions, or through direct action on CNG channels, and direct modulation of protein function by S-nitrosylation of cysteine residues (Ahern *et al.*, 2002; Davis *et al.*, 2001). Sperm exposure to cGMP led to a rise in $[Ca^{2+}]_i$, similar to that observed with NO, but this time clearly dependent on $[Ca^{2+}]_o$, consistent with generation primarily by Ca^{2+} influx and not store mobilization. Our findings are consistent with those of Kobori *et al.* (2000) who showed that manipulation of cyclic nucleotide levels in mouse sperm is apparently associated with Ca^{2+} influx via CNG channels. Also, sperm treatment with saturating doses of ODQ, an effective inhibitor of sGC, did not alter the response to NO, reinforcing that the NO-induced

elevation of $[Ca^{2+}]_i$ does not involve the activation of sGC. Also, NO at doses used in our study, causes S-nitrosylation of various sperm proteins (Lefièvre *et al.*, 2007), suggesting that this alternative action of NO may underlie our observations. Previous studies indicate that NO stimulates human sperm acrosome reaction through activation of sGC (Ford, W. C., unpublished data), and may contribute to NO-mediated chemotaxis (Miraglia *et al.*, 2007), suggesting that NO can activate more than one signalling pathway in sperm.

Protein S-nitrosylation by NO was therefore investigated. NONOate-induced $[Ca^{2+}]$ rise was rapidly reversed with the -SH reducing agent DTT, the action of DTT being correlated with the extent of Ca^{2+} mobilization by NO, showing that the action of DTT on $[Ca^{2+}]_i$ was associated to the reversal of the action of NO. The effect of DTT on those cells was not sensitive to pre-treatment with the mitochondria uncoupling agent CCCP, and thus not a reflection of effects of -SH reduction on mitochondrial Ca^{2+} accumulation. The nitrosylating agent GSNO acted similarly to NO but more rapidly, consistent with a direct action. Nitrosylation by NO is thought to require several intermediate reactions and thus has slower kinetics (Ahern *et al.*, 2002). Also, the effect of GSNO on $[Ca^{2+}]_i$ was reversed by co-application of GSH, consistent with the reports that GSH inhibits generation of the membrane permeant cys-NO, which is necessary for protein S-nitrosylation by GSNO in intact cells (Zhang and Hogg, 2004). GSNO-induced $[Ca^{2+}]_i$ rise was also reversed by DTT. Our observations are consistent with those of Stoyanovsky *et al.* (1997) who found that donors of NO or RSNO mobilize Ca^{2+} from skeletal and cardiac microsomes, an effect reversed by –SH reducing agents. In accordance with the Ca^{2+} imaging data, GSNO-induced sperm protein S-nitrosylation was rapid, and was immediately reversed by washout of GSNO or co-application

of DTT. These observations are consistent with the report that a large number of human sperm proteins are potential targets for S-nitrosylation (Lefièvre *et al.*, 2007).

This study also investigated the reversibility of the HNO effects on $[Ca^{2+}]_i$ by -SH reducing agents. In agreement with other studies (Cheong *et al.*, 2005; Tocchetti *et al.*, 2007), it was observed that HNO-induced $[Ca^{2+}]_i$ elevation was rapidly reversed by DTT.

Our observations on the effect of protein S-nitrosylation indicate that identification of protein targets will be of great interest. Exposure of human sperm to NO leads to S-nitrosylation of a large number of proteins, including RyRs (Lefièvre *et al.*, 2007). Other studies have also indicated that NO modulates the activity of RyRs (Stoyanovsky *et al.*, 1997; Sun *et al.*, 2001; 2003; Xu *et al.*, 1998; Zahradnikova *et al.*, 1997). Therefore, ryanodine and 4-CmC, agonists of RyRs, as well as ryanodine and NO co-stimulation effects on $[Ca^{2+}]_i$ were examined. Sperm stimulation with ryanodine results in a rise in $[Ca^{2+}]_i$. This finding is consistent with the expression of RyRs in human sperm, which has previously been suggested by Harper *et al.* (2004) and confirmed by Lefièvre *et al.* (unpublished data). Moreover, a $[Ca^{2+}]_i$ increase occurred upon sperm treatment with 4-CmC. However, sperm responses to 4-CmC were reduced in low-Ca^{2+} sEBSS and were totally abolished in sEBSS-EGTA. These observations are contrary to reports by other investigators (Gschwend *et al.*, 1999; Zorzato *et al.*, 1993) showing that chlorocresols-induced rise in $[Ca^{2+}]_i$ remain in media containing EGTA. Our findings suggest that, in human sperm, 4-CmC effects on $[Ca^{2+}]_i$ are partially related with an action on the RyRs to mobilize stored Ca^{2+}, but a Ca^{2+} influx pathway should also be involved for 4-CmC to fulfil its effects, and that exposure to EGTA very rapidly empties the store.

Furthermore, sperm response to ryanodine was greatly reduced in the presence of NO, consistent with convergence of the effects of NO and ryanodine on sperm Ca^{2+} signalling, but it is not clear whether this is at the RyRs or at the Ca^{2+} signal. In human sperm, RyRs appear to exist in the area of the posterior head/neck/mid-piece junction (Harper *et al.*, 2004; Lefièvre *et al.*, unpublished data), and our studies revealed that NO-induced $[Ca^{2+}]_i$ rise is localised primarily to the neck/mid-piece, spreading into the posterior of the sperm head, stressing the expression of RyRs in human sperm and suggesting that the Ca^{2+} store mobilized by ryanodine is involved in the Ca^{2+} mobilizing effects of NO and GSNO.

NO-treated sperm generate oscillations similar in kinetics, amplitude and localisation to those generated upon stimulation with progesterone. Progesterone-induced oscillations are generated by a mechanism involving activation of RyRs, causing mobilization of stored Ca^{2+} localised to the sperm neck/mid-piece (Harper *et al.*, 2004; 2005) and modulate flagellar beat (Harper *et al.*, 2004). NO and progesterone interaction effects on $[Ca^{2+}]_i$, flagellar activity and motility patterns were therefore investigated. The $[Ca^{2+}]_i$ transient induced by progesterone was significantly prolonged in NO-pre-treated sperm, consistent with convergence of the actions of progesterone and NO. Also, the action of progesterone was transformed into a prolonged modulation of flagellar activity characterised by increased excursion of the mid-piece in NO-treated sperm, reflecting the increased duration of $[Ca^{2+}]_i$ rise under these conditions. These results may reflect enhancement of the weak hyperactivating effect of progesterone on human sperm (Jaiswal *et al.*, 1999; Uhler *et al.*, 1992; Yang *et al.*, 1994). Furthermore, a decrease in BCF and VSL was detected with CASA when progesterone was applied to NONOate-treated sperm (compared to untreated sperm), consistent with an interacting effect of progesterone and NO on sperm motility.

The data presented in this study has led to a proposed model for the interacting effects of NO and progesterone in human spermatozoa due to convergence at RyRs (Fig. 7.1).

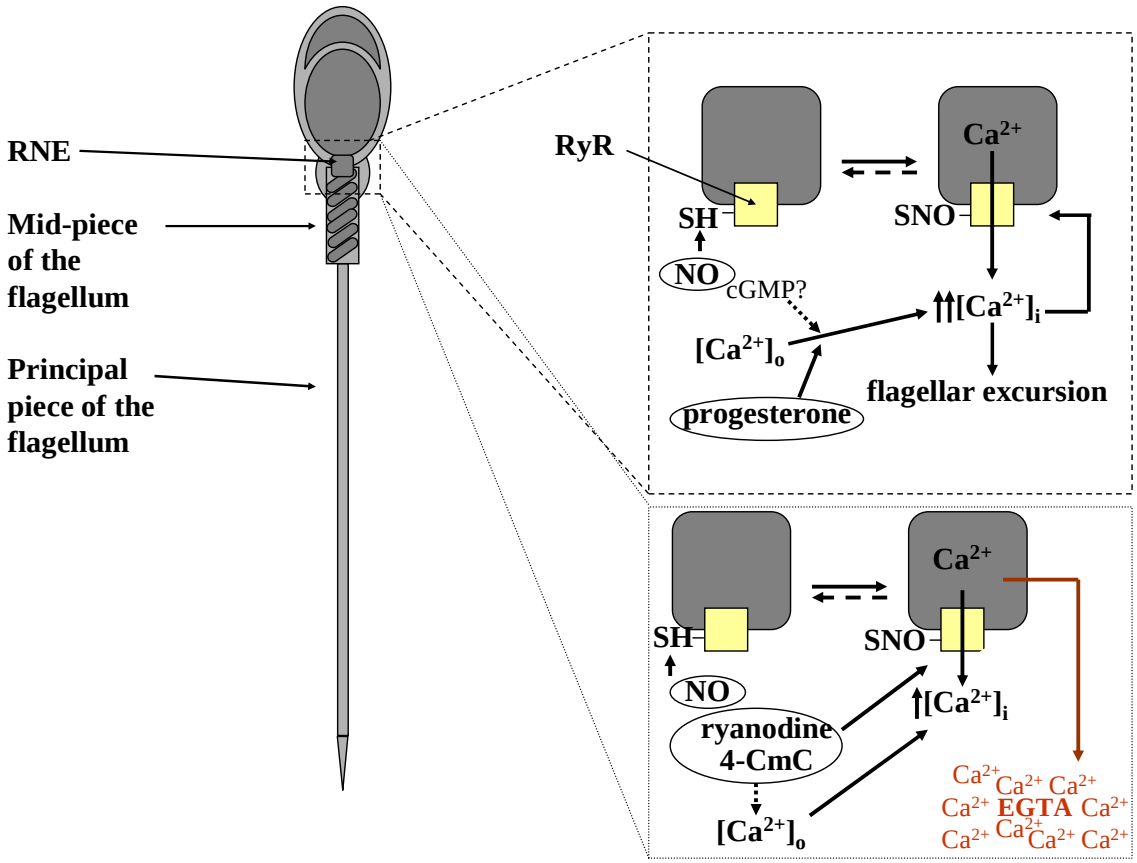

Fig. 7.1. Schematic representation of the proposed interacting effects of NO and progesterone, and the targetted receptors (RyRs), in the neck/mid-piece region of human sperm (compiled from the data from this study). NO induces S-nitrosylation of RyRs causing mobilization of stored Ca^{2+} and raising $[Ca^{2+}]_i$. NO-induced Ca^{2+} influx through generation of cGMP may also occur, enhancing this effect and causing CICR and $[Ca^{2+}]_i$ oscillations. Progesterone-induced Ca^{2+} influx mobilizes Ca^{2+} by CICR (Harper *et al.*, 2004) and becomes more effective in the presence of the RyR-sensitising effects of NO, modulating flagellar beat mode (upper panel). Ryanodine/4-CmC act on the RyRs, causing mobilization of stored Ca^{2+} and increasing $[Ca^{2+}]_i$. 4-CmC may also induce Ca^{2+} influx complementing its Ca^{2+} mobilization effect. Sperm superfusion with EGTA buffered sEBSS may rapidly empty the store, silencing 4-CmC effects on $[Ca^{2+}]_i$. NO actions on the RyRs converge with those of ryanodine/4-CmC, modulating the release of stored Ca^{2+} (reduced when compared with ryanodine stimulation only; lower panel).

7.1 Future research

A strict regulation of sperm functions is essential to achieve successful fertilization. Although *in vitro* studies have been carried out to understand the complex events that precede human fertilization, translation to what happens *in vivo* is far from being simple. Our investigations focus on the Ca^{2+} mobilizing effects of NO, a product of the cumulus cells that surround the oocyte, and its involvement in the control of sperm functions, namely motility. Progesterone is generated by the mammalian cumulus (Chian *et al.*, 1999; Mingoti *et al.*, 2002; Osman *et al.*, 1989; Yamashita *et al.*, 2003). Progesterone gradients lead to $[Ca^{2+}]_i$ mobilization which is associated with modulation of flagellar beat in human spermatozoa (Harper *et al.*, 2004). Our study has further elucidated this process giving light to the interacting effects of progesterone and NO. The flagellar activity modification upon mobilization of stored $[Ca^{2+}]_i$ may result from the direct action of the mobilized Ca^{2+} on the axoneme of the flagellum. However, further research is required to totally elucidate the downstream events involved in the alteration of sperm motility under these circumstances. This study has therefore contributed to clarify the complex set of events that sperm need to undergo to become able to fertilize the oocyte. Still, further research needs to be carried out to clarify the signalling pathways involved in the regulation of sperm functions, including motility and fertilization. More investigations need to be undertaken to elucidate the occurrence of these events *in vivo*, which may be helpful in the control of infertility in future.

APPENDIX I: MEDIA

Supplemented Earle's Balanced Salt Solution (sEBSS)

Sodium Dihyd. Phosphate 0.122g/l (1.0167 mM)
Potassium Chloride 0.4g/l (5.4 mM)
Magnesium Sulphate.$7H_2O$ 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.$2H_2O$ 0.264g/l (1.8 mM)
Sodium Bicarbonate 2.2g/l (25.0 mM)
Sodium Chloride ≈6.8g/l (116.4 mM)

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

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

Sodium Dihyd. Phosphate 0.122g/l (1.0167 mM)
Potassium Chloride 0.4g/l (5.4 mM)
Magnesium Sulphate.$7H_2O$ 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)

Prepared as for standard sEBSS but stored as 100 ml volumes in polystyrene bottles at 4°C until use. 0.3% BSA was added on experimental day.

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

Sodium Phosphate Monobasic 0.122g/l (1.018 mM)
Potassium Chloride 0.4g/l (5.366 mM)
Magnesium Sulphate.$7H_2O$ 0.2g/l (0.811 mM)
Dextrose Anhydrous 1.0g/l (5.551 mM)
Sodium Pyruvate 0.3g/l (2.724 mM)
DL-Lactic Acid, Sodium 4.68g/l (41.763 mM)

Calcium Chloride.$2H_2O$ 0.73g/l (4.966 mM)
Sodium Bicarbonate 2.2g/l (26.187 mM)
Sodium Chloride ≈5.0g/l (85.558 mM)
EGTA 2.28g/l (5.994 mM)

Prepared as for standard sEBSS. 0.3% BSA was added on experimental day.

Biggers, Whitten and Whittingham (BWW) medium

95 mM Sodium Chloride
4.8 mM Potassium Chloride
1.7 mM Calcium Chloride
1.2 mM Monopotassium Phosphate
1.2 mM Magnesium Sulphate
24.88 mM Sodium Bicarbonate
5.55 mM Glucose
0.27 mM Pyruvic Acid
0.25 mM Lactic Acid
0.5 units Penicillin ml^{-1}
0.5 µg Streptomycin ml^{-1}
20 mM Hepes buffer
0.3% BSA; 300 mOsmol l^{-1}

M medium

137 mM Sodium Chloride
2.5 mM Potassium Chloride
20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)
10 mM Glucose

Lysis buffer

250 mM Hepes (pH 7.7)
1 mM ethylenediaminetetraacetic acid (EDTA)
0.1 mM neocuproine
1% Triton
2.5% SDS

HEN medium

250 mM Hepes (pH 7.7)
1 mM EDTA
0.1 mM neocuproine

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer

2% SDS
10% Glycerol
62.5 mM Tris-HCl
(pH 6.8)

TBS

0.9% Sodium Chloride
20 mM Tris-HCl
(pH 7.8)

APPENDIX II: PUBLICATIONS AND PRESENTATIONS OF RESEARCH

Research articles

Machado-Oliveira, G., Lefièvre, L., Ford, C., Herrero, M. B., Barratt, C., Connolly, T. J., Nash, K., Morales-Garcia, A., Kirkman-Brown, J. and Publicover, S. (2008). Mobilization of calcium stores and flagellar regulation in human sperm by S-nitrosylation: a role for nitric oxide synthesised in the female tract. *Development* **135**, 3677-86.

Reviews

Lefièvre, L., Bedu-Addo, K., Conner, S. J., **Machado-Oliveira, G. S.**, Chen, Y., Kirkman-Brown, J. C., Afnan, M. A., Publicover, S. J., Ford, W. C. and Barratt, C. L. (2007). Counting sperm does not add up any more: time for a new equation? *Reproduction* **133**, 675-84.

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.

Bedu-Addo, K., Costello, S., Harper, C., **Machado-Oliveira, G.**, Lefièvre, L., Ford, C., Barratt, C. and Publicover, S. (2008). Mobilization of stored calcium in the neck region of human sperm – a mechanism for regulation of flagellar activity. *Int J Dev Biol* **52**, 615-26.

Book contributions

St. John, J. C., Amaral, A., Bowles, E., Oliveira, J. F., Lloyd, R., Freitas, M., Gray, H. L., Navara, C. S., **Oliveira, G.**, Schatten, G. P., Spikings, E. and Ramalho-Santos, J. (2006). The analysis of mitochondria and mitochondrial DNA in human embryonic stem cells. *Methods Mol Biol* **331**, 347-74.

Conner, S. J., Lefièvre, L., Kirkman-Brown, J., Michelangeli, F., Jimenez-Gonzalez, C., **Machado-Oliveira, G. S.**, Pixton, K. L., Brewis, I. A., Barratt, C. L. and Publicover, S. J. (2007). Understanding the physiology of pre-fertilization events in the human spermatozoa – a necessary prerequisite to developing rational therapy. *Soc Reprod Fertil* Suppl. 63, 237-55.

Abstracts

Nitric oxide mobilizes stored calcium in human spermatozoa: interaction with progesterone. American Society of Andrology: Annual Conference 2006.

Activation of human sperm calcium-signalling by NO and progesterone: a role in gamete interaction? Fertilization and Early Development: Gordon Annual Conference 2007.

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