Exploring the role of neuropeptides and their effects on sperm function

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Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Signed Ana-Maria Tomova

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List of Abbreviations

8-bromo-cAMP	8-bromoadenosine 3', 5'-cyclic monophosphate
AC	Adenylyl Cyclase
AKT	Protein Kinase B
AMP	Adenosine Monophosphate
APS	Ammonium Persulphate
Arg	Arginine
Asn	Asparagine
ATP	Adenosine Triphosphate
AVPR2	Vasopressin Receptor 2
BCA	Bicinchoninic Acid
cAMP	Cyclic Adenosine Monophosphate
CASA	Computer Assisted Sperm Analysis
cDNA	Complimentary Deoxyribonucleic Acid
CO_2	Carbon Dioxide
Cys	Cysteine
DAG	Diacyl Glycerol
DAPI	4',6-diamindino-2-phenylindole
dH_2O	Deionised Water
DNA	Deoxyribonucleic Acid
ECL	Electrochemiluminescence
FITC	Fluorescein Isothiocyanate
Gln	Glutamine
Gly	Glycine
HK2	Human Kidney 2 cells
HRP	Horse Radish Peroxidase
Ile	Isoleucine
IP_3	Inositol 1,4,5-Triphosphate
JAK	Janus Kinase
Leu	Leucine
MCF-7	Michigan Cancer Foundation 7 cells
$MgCl_2$	Magnesium Chloride
mTOR	Mechanistic Target of Rapamycin
NH_2	Amidogen
OXTR	Oxytocin Receptor
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
PI_3K	Phosphoinositide 3-Kinase
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PRLR	Prolactin Receptor
Pro	Proline
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Ser	Serine
SH2	Src Homology 2
SPM	Sperm Preparation Medium
STAT	Signal Transducers and Activators of Transcription
TBE	Tris Borate EDTA
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween
TEMED	N, N, N', N'-Tetramethylethylenediamine
Thr	Threonine
Tyr	Tyrosine

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Abstract

Neuropeptides are small peptides that act as signalling molecules. These have a wide range of important roles in numerous physiological, metabolic and behaviour functions. For example, oxytocin and vasopressin have essential roles in salt balance, social behaviour, and stress regulation. The role of neuropeptides in reproduction is well documented: it has been demonstrated they are involved in sexual behaviour, maternal-infant bonding, pair-bonding, lactation, pregnancy, penile erection, ejaculation, uterus contraction, and sperm transfer. The receptors for neuropeptides are found throughout both the male and female reproductive tracts. Several neuropeptides are found in both seminal plasma and follicular fluid indicating some involvement directly in fertilisation. Neuropeptide levels are altered in mood disorders and these altered levels could potentially have implications in infertility. However, very little is currently known about the effects of neuropeptides on sperm function, this study explored this area further.

Considering the overarching link that neuropeptides have between pair bonding, mental health and fertility it was hypothesised that any perturbations in their action may impede successful reproduction. This study investigated how neuropeptides could regulate sperm function. These data may lead to future therapies in assisted reproduction.

Semen samples were produced by masturbation, separated from the seminal plasma and probed for neuropeptide receptors via immunocytochemistry. This is the first time, to knowledge, that vasopressin receptor 2 has been localised on human sperm. As no other receptors were localised this study focused on any effect vasopressin may have on sperm function by incubation with a vasopressin agonist and measuring any modulations in motility and PKA phosphorylation activity.

No significant differences were found in motility or PKA phosphorylation activity in sperm treated with vasopressin agonist, further optimisation of the assays is required.

1.0 Introduction

The neuroendocrine system is responsible for regulating a vast number of functions; homeostasis, metabolism, thirst, hunger, sleep, behaviour, mood and reproduction. It comprises of the hypothalamus as a link from the nervous system to the endocrine system through the pituitary.

Mood has been implicated in infertility. Stress, depression and anxiety have been shown to negatively impact both male and female fertility. Neuropeptides and their receptors are found throughout the limbic system such as amygdala and hypothalamus and are important in mood regulation; differing levels of neuropeptides in the blood have been implicated in mood disorders such as postpartum depression providing an important link between neuropeptides, reproduction and mood.

Neuropeptides are involved throughout all phases of reproduction, from sexual behaviour, to conception, to lactation and parent-infant bonding and the receptors are found throughout both the male and female reproductive tracts. Several neuropeptides are found in both seminal plasma and follicular fluid indicating some involvement directly in fertilisation. Neuropeptide levels are altered in mood disorders and these altered levels could potentially have implications in infertility.

1.1 Neuropeptides

Neuropeptides are short chain polypeptides. They are peptides that function as neurotransmitters and some can also function in the periphery as peptide hormones for example, oxytocin, vasopressin, prolactin corticotropin-releasing hormone and ghrelin. However, unlike neurotransmitters they are not re-absorbed by the cell and hence once secreted typically have more prolonged actions. They influence a variety of biological processes such as, reward, metabolism, analgesia, memory, appetite, behaviour and also reproduction (Mac E Hadley, 1996).

The majority of neuropeptides function through G protein-coupled receptors for instance both oxytocin and vasopressin, these are a group of cell surface receptors, when activated by the binding of the neuropeptide, activate the heterotrimeric G

proteins inducing an intracellular signalling cascade resulting in a cellular response (Gomes *et al*., 2013).

1.2 Neuropeptides in reproduction

Neuropeptides are important in all aspects of reproduction, from pair bonding, sexual behaviour, penile erection, both male and female orgasm and sperm transfer to pregnancy and parturition to lactation and maternal-infant bonding (Filippi *et al*., 2002; Fuchs *et al*., 1990).

The involvement of neuropeptides in reproduction is most evident in the female where, for instance both oxytocin and prolactin are vital in pregnancy and during birth (Nissen *et al*., 1996; Uvnäs-Moberg *et al*., 1990), and neuropeptide Y has been implicated in fertility regulation and has been shown to suppress fertility in female fats (Toufexis *et al*., 2002, Roa and Herbison, 2012). Other neuropeptides have also been shown to be related to female fertility, pituitary adenylate cyclase-activating polypeptide (PACAP) has been shown to reduce fertility in female mice via reduced maternal behaviour and smaller little sizes (Shintani *et al*., 2002).

Murgatroyd and Nephew, (2013) demonstrated that early life chronic stress has longitudinal effects and reduced maternal behaviour and nursing efficiency in adult female rats. This was specifically associated with attenuated gene expression for the neuropeptides vasopressin, oxytocin and prolactin in the brain.

Depression and chronic anxiety not only negatively affect the mother but also their adult offspring, vasopressin, oxytocin and prolactin were implicated in the aetiology of both stress related mood disorders and maternal behaviour for both humans and rodents. Low levels of oxytocin and prolactin have been associated with postpartum depression (Abou-Saleh *et al*., 1998; Skrundz *et al*., 2011) and the opposite is true for vasopressin, high levels of vasopressin are associated with depression in humans and in animal models of depression (Murgatroyd *et al*., 2004, Rotzinger *et al*., 2010, Surget and Belzung, 2008).

In regards to sperm function, Gonzales (1989) found prolactin released from the seminal vesicles directly stimulate sperm motility and negatively impacted concentration. In animal studies vasopressin has been found to negatively affect

sperm motility and overall function (Kwon *et al*., 2013). Oxytocin levels have been found to elevate significantly post ejaculation however, no correlation was found between oxytocin levels and sperm motility and quality and it was postulated that in humans it is primarily involved in the precipitation of ejaculation (Ogawa *et al*., 1980). In domestic animal breeding studies oxytocin has been shown to aid sperm transport when added to semen preparations during artificial insemination (Okazaki et al., 2014).

Although there are several neuropeptides involved in reproduction there were three of particular interest. Oxytocin, prolactin and vasopressin are well documented in literature for their integral roles in mood, sexual behaviour and sperm transport and reproduction, and their receptors are found throughout male and female reproductive tracts and tissues (Filippi *et al*., 2002; Fuchs *et al*., 1990; Ouhtit *et al*., 1993).

1.3. Oxytocin

Oxytocin is a nonapeptide (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2), which is synthesised in the hypothalamus and secreted from posterior pituitary (Norman and Henry, 2015). Oxytocin is associated with pregnancy and parturition – stimulating uterine contractions, and is involved in lactation. Studies have shown , that knockout mice have no ability to lactate (Zingg and Laporte, 2003). Oxytocin has been found to increase maternal behaviour (mother-offspring bonding), female-male pair-bonding and sexual behaviour, i.e. lordosis behaviour and increases duration and frequency of mating. In other studies, oxytocin was shown to increases maternal aggression in mice. However, there are conflicting reports for the effects on male aggression (Caldwell and Young III, 2006). In female mice progesterone has been found to directly inhibit oxytocin by having a high affinity for the oxytocin receptor, resulting in uterine quiescence (low myometrium contractibility) (Gimpl and Fahrenholz, 2001).

The oxytocin receptor is a type I G protein-coupled receptor, primarily G_q that activates the phospholipase C pathway (**Figure 1**). Oxytocin binds to its receptor, activating phospholipase C (PLC) which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3).

IP$_3$ binds to its receptors causing an influx of calcium into the cell while DAG remains membrane bound, together IP$_3$ and DAG activate protein kinase C (PKC) which goes on to phosphorylate target proteins. In the central nervous system oxytocin and its receptor is widely distributed in a variety of areas in the brain, hypothalamus, ventral hippocampus, amygdala, olfactory bulbs. The neuropeptide and its receptor is found throughout both male and female reproductive tracts, for example in the pregnant uterus, placenta, ovaries, corpus luteum, testis, leydig cells, epididymis, prostate, mammary tissues, for review see (Gimpl and Fahrenholz, 2001).

Oxytocin use in intrauterine insemination in farm animals has been shown to aid sperm transport (Gibson *et al.*, 2004; Langendijk *et al.*, 2005). However, there have been conflicting findings in other animal studies using intrauterine insemination, where the use of oxytocin had no effect on fertility and fertilisation rates (Sayre and Lewis, 1997). In humans it has been suggested that oxytocin also aids sperm transport via contractility of the uterus acting as a peristaltic pump that aids the transport of sperm towards the ovary with the dominant follicle (Wildt *et al.*, 1998).
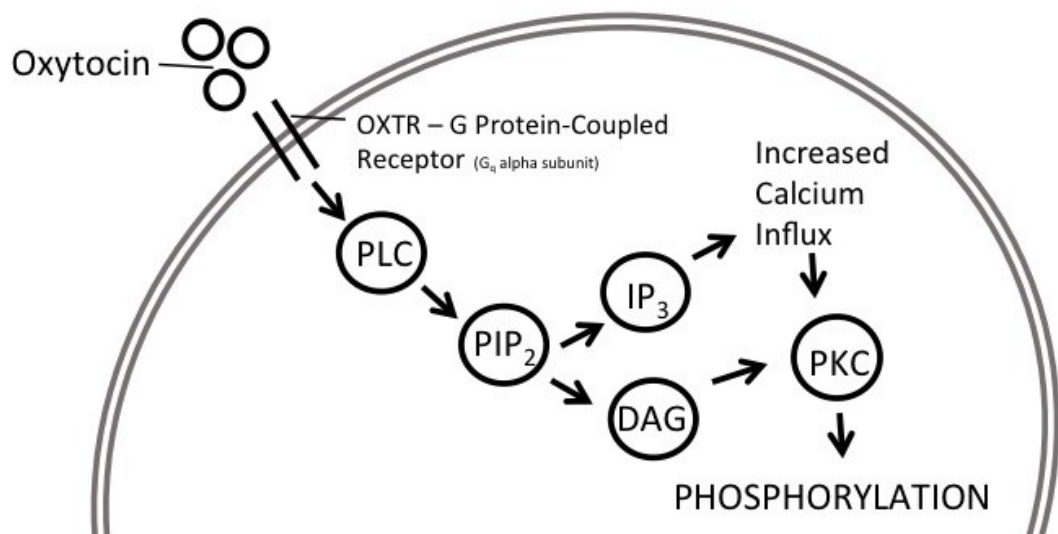
Figure 1. Oxytocin signalling cascade via G protein-coupled receptor, G$_q$ alpha subunit.

<u>1.3.2 Prolactin</u>

Prolactin has a wide range of biological actions, such as: metabolism, immunoprotection, growth, water and electrolyte balance, behaviour and reproduction. The receptor is a member of a subfamily of hematopoietic cytokine receptors (Norman and Henry, 2015). The prolactin receptor has been localised in a variety of tissues and cells; mammary gland, throughout the reproductive organs, the central nervous system, pituitary, skin, lungs, adrenal cortex, liver, heart, gastrointestinal tract, bone, pancreas, lymphoid tissues, kidney and sperm as well as a variety of cancer cells (Tsai-Morris, Dufau, 2004; Pujianto *et al., 2010)*. Prolactin plays a vital role in cell proliferation and by working synergistically with growth hormone to stimulate this proliferation and differentiation. In animals with seasonal reproduction it has been shown that there are seasonal fluctuations in prolactin, peaking during breeding season (Curlewis, 1992).

The prolactin receptor is a type 1 cytokine receptor that activates the JAK/STAT, PI3K/AKT/mTOR (cell cycle regulation) pathway (Brooks *et al*., 2014, Helmer *et al*., 2010). The JAK/STAT pathway is involved in myriad cell signalling and is highly conserved, it also regulates gene expression via ligand-specific signal pathways and mediating chromatin modifications and communicating with the core promotors. The relevant cytokine binding to the cell surface receptor which activates the relevant JAKs, these phosphorylate tyrosine residues on the cytokine receptor and form a binding site for molecules with SH2 domains, the SH2 domains that possess STATs are also phosphorylated by JAKs and form hetero/homodimers which translocate to the nucleus and activate transcription of target genes (Aaronson and Horvath, 2002). The PI3K/AKT/mTOR pathway is directly related to cell proliferation, quiescence and cancers, commonly in cancers this pathway is overactive and leads to inhibition of apoptosis. This pathway, in short, functions by activating phosphoinositide 3-kinase (PI_3K) which activates protein kinase B (AKT) which in turn activates mechanistic target of rapamycin (mTOR), this regulates processes such as autophagy, protein synthesis, cell survival/growth and cell motility (Clevenger *et al*., 2003, Hay and Sonenberg, 2004).
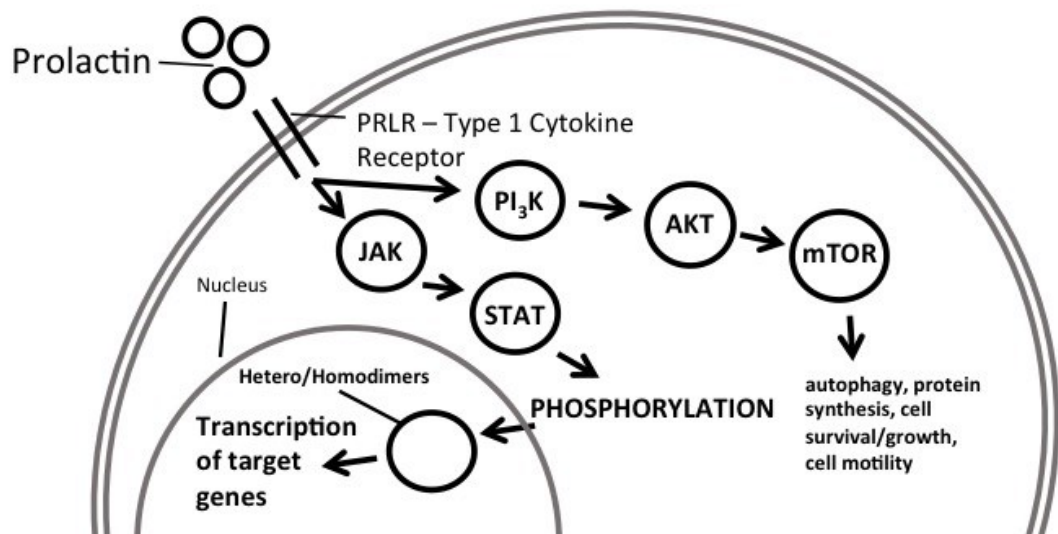
Figure 2. Prolactin signalling cascade via type 1 cytokine receptor.

1.3.3 Vasopressin

A nonapeptide (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH$_2$) that is synthesised in the hypothalamus and secreted from posterior pituitary. Vasopressin is structurally very similar to oxytocin. Both are constituted of 9 amino acids, only 2 of these 9 differ between oxytocin and vasopressin (Norman and Henry, 2015).

Vasopressin has been implicated in corpus luteum function in conjunction with oxytocin in animal studies, porcine corpora lutea were collected at a variety of stages through the oestrous cycle and fluctuations of vasopressin, oxytocin and progesterone were measured. It was observed that concentrations of oxytocin and vasopressin were essentially similar until the end of the cycle where a significant increase of vasopressin was detected (Choy and Watkins, 1988). Injections of vasopressin into the cauda epididymis of rats showed potential as a male contraceptive, where all doses used caused oligozoospermic (low sperm concentration) ejaculation with the higher dose producing teratozoospermia (abnormal sperm morphology) ejaculation, while sexual behaviour remained unaffected (Ratnasooriya and Jayakody, 2005).

The vasopressin receptor 2 is a G protein-coupled receptor (G$_s$ alpha subunit - the cAMP dependent pathway), which activates adenylyl cyclases converting ATP to

cyclic AMP (cAMP) thus activating protein kinase A which leads to phosphorylation of target proteins (Holmes *et al*., 2003).
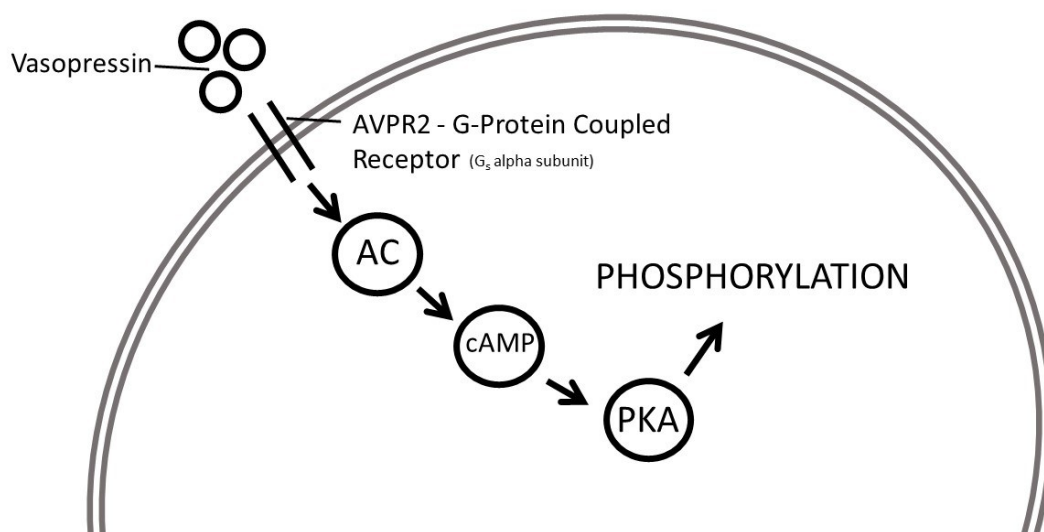
Figure 3. Vasopressin signalling cascade via vasopressin receptor 2.

Although the role of neuropeptides in reproduction is very well documented, there is little known about the role of neuropeptides on sperm function.

1.4 Sperm biology

1.4.1 Sperm structure

The structure of sperm is a highly specialised cell with the function of delivering the haploid paternal genome to the oocyte. The sperm can be apportioned into three major sections; the head, the midpiece and the tail. The head of the sperm contains the haploid paternal genome and is surrounded by the acrosome that contains vital enzymes such as acrosin that digest the zona pellucida allowing sperm to enter the perivitelline space and enable sperm-oocyte fusion. The midpiece contains mitochondria for ATP production and the energy source for motility. The tail executes the movement propelling the sperm through the female reproductive tract towards the oocyte.
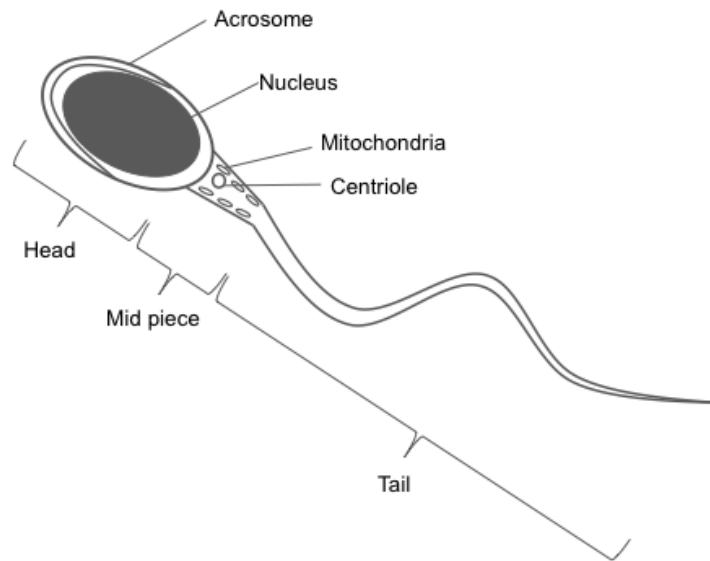
Figure 4. Sperm structure. Representation of main components of the sperm.

1.4.2 Spermatogenesis / maturation

Sperm are produced in the testis during the process of spermatogenesis; this process starts at puberty and normally continues throughout the male's life (**Figure 5**).

The whole process of spermatogenesis itself is a lengthy process that takes just over 70 days. Beginning with spermatogonia, the initial cells in this process, replicate at first via mitosis into primary spermatocytes and then via meiosis I to produce secondary spermatocytes and these divide again via meiosis II the haploid round cell spermatids. Next, the spermatids undergo spermiogenesis; this process alters the cell from round cell into flagellated cell, development of the acrosome and the tail as well as an increase in nuclear condensation to produce spermatozoa. However, these sperm lack motility and must undergo further maturation in the epididymis (Coward and Wells, 2013; Moore, 1998; Ruwanpura *et al*., 2010).

In brief, the epididymis is a coiled tube responsible for storage, maturation and transportation of sperm between the testes and the vas deferens. It consists of three different sections, the caput or head, corpus or body and cauda or tail. It takes approximately two weeks for sperm to pass through the epididymis and undergo maturation. Sperm undergo many essential morphological and functional changes

during their passage through the epididymis including, modification of the chromatin, acrosome and gain the ability to become motile and fertilise the oocyte (Clulow *et al.*, 1994; Hamilton *et al.*, 1969; Hess, 2000; Overstreet and Cooper, 1978).
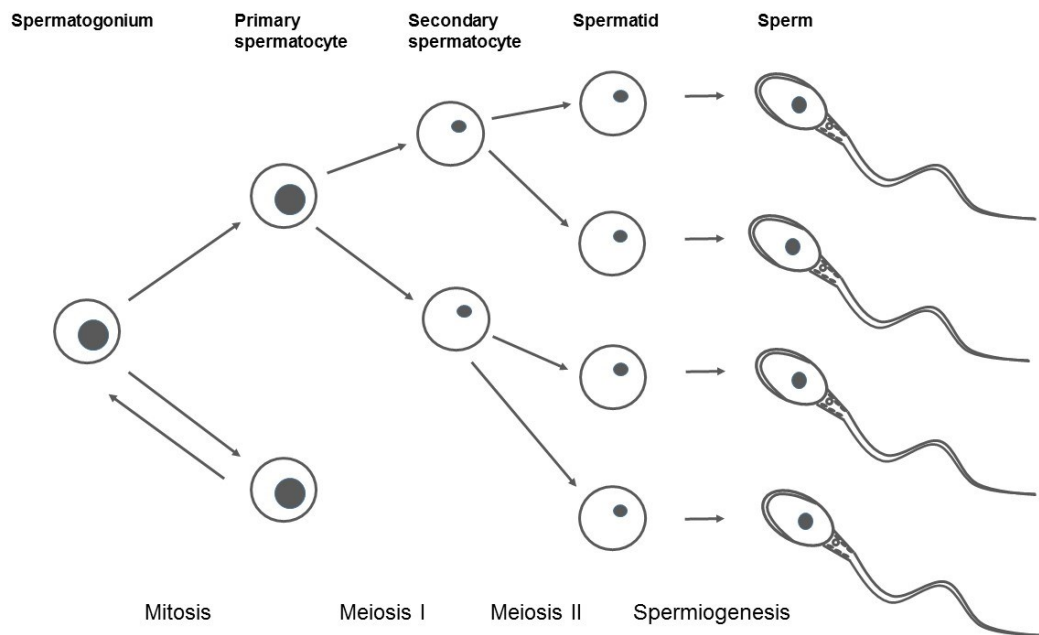
Figure 5. Spermatogenesis. Basic representation of the process of spermatogenesis.

1.4.3 Sperm function

Sperm must undergo biochemical and physical changes in order to successfully fertilise an oocyte. One important process is capacitation.

Capacitation comprises a reversible set of physiological changes that involve the removal of cholesterol by albumin in the female reproductive tract and a prerequisite for hyperactivation (see below) and the acrosome reaction. *In vivo*, it involves the removal of inhibitory factors from seminal plasma and the interaction of the sperm with components in the female reproductive tract (Jaiswal and Eisenbach, 2002). The removal of cholesterol permits the influx of bicarbonate and Ca^{2+} ions, which in turn activate adenylate cyclase, thereby elevating cAMP concentrations. The increased cAMP levels activate protein kinase A, which then phosphorylates several tyrosine kinases, including chaperone proteins and those important for the acrosome reaction. These migrate to the sperm head where they are phosphorylated (Aitken and Nixon, 2013, Yoshida *et al.*, 2008).

Hyperactivated sperm penetrate a layer of cells surrounding the oocyte (the cumulus oophorous) and undergo the acrosome reaction (Jin *et al*, 2011). The sperm then penetrate the zona pellucida enabling sperm-oocyte fusion and the completion of fertilisation.

1.4.4 Sperm parameters

Sperm motility is an easy measure of function and one of the most vital parameters, motile sperm are required for fertilisation otherwise the sperm cannot fertilise the oocyte.

Human sperm motility is categorised into progressive (A+B), where the sperm are moving linearly or in large circles, non-progressive (C), sperm displaying any other patterns of motility and immotile (D), no movement whatsoever (World Health Organization, 2010a). When using computer assisted sperm analysis (CASA) progressive motility can be separated into fast progressive (A) (> 25 µm/sec) and slow progressive (B) (**Figure 6**).
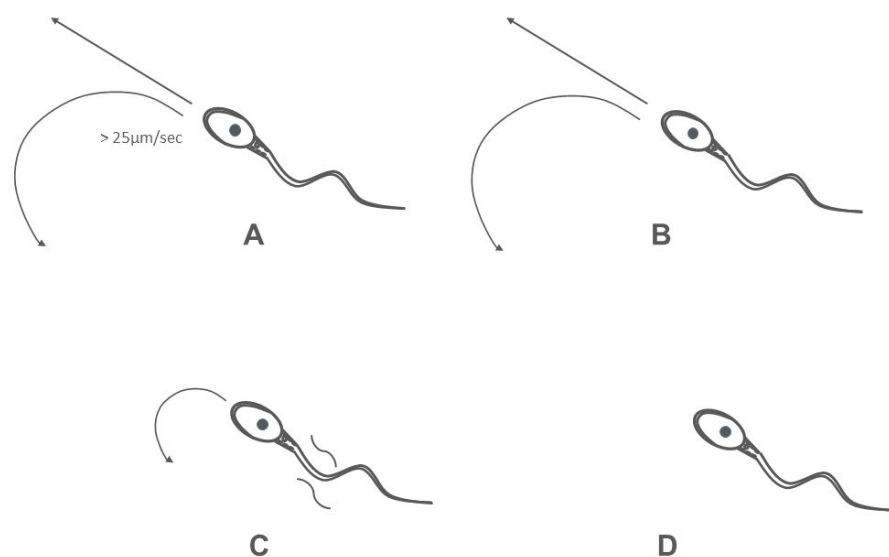
Figure 6. Sperm motility groups. A – fast progressive sperm, moves linearly or in large circles quicker than 25 µm/sec. B – slow progressive sperm, moves linearly or in large circles slower than 25 µm/sec. C – non-progressive sperm, moves in small circles or 'twitches'. D – immotile sperm, no movement.

1.4.5 Sperm chemotaxis

Human sperm have been shown to respond to chemotactic factors in human follicular fluid (Ralt *et al*., 1994). In rabbits, progesterone has been identified as a chemotactic factor and is released from the oocyte-cumulus complex. Progesterone forms a concentration gradient as it diffuses away from the oocyte-cumulus complex. The sperm swim towards the more concentrated chemoattractant gradient (Guidobaldi *et al*., 2008). It has been observed that sperm chemotactic responsiveness is acquired during capacitation and it has been postulated that this is a means of selecting for capacitated sperm only (Cohen-Dayag *et al*., 1995).

The role of neuropeptides in modulating sperm behaviour has not been explored in depth. It can be postulated that female stress and mood may influence post-coital fertility by altering sperm function. Furthermore, as neuropeptides have been detected in follicular fluid and semen they are likely to play an essential role in sperm physiology in both the male and female reproductive tracts (**Figure 7**).
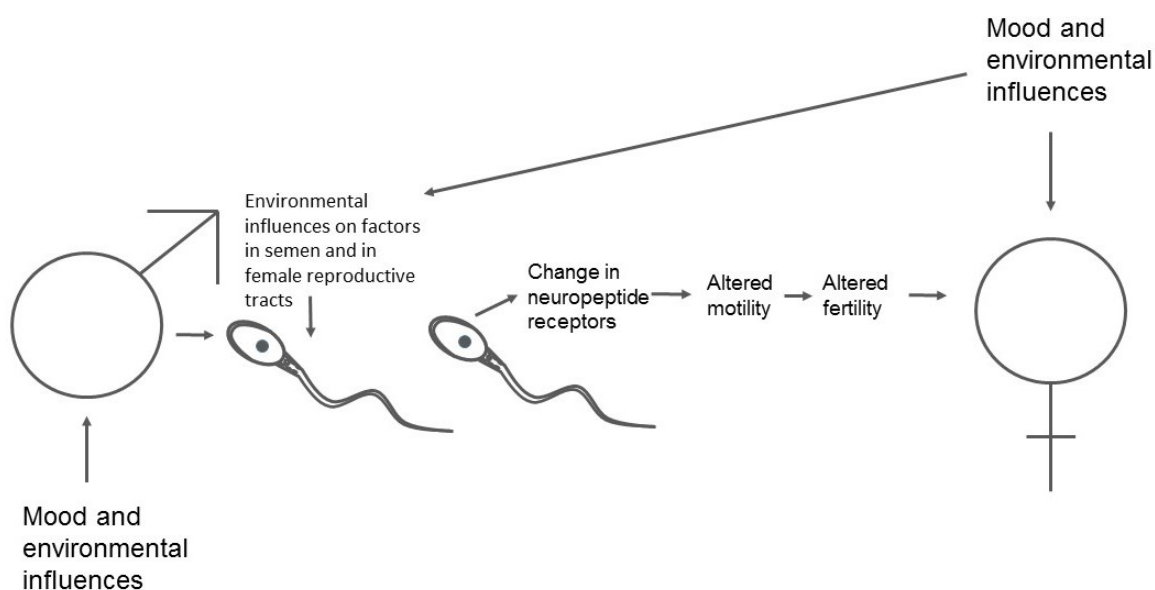
Figure 7. Neuropeptides on human fertility. Proposed hypothesis of the effects that neuropeptides may have on sperm and fertility.

1.5 Aims and Objectives

Aim:

(i) To investigate the presence of oxytocin, vasopressin and prolactin receptors on human sperm.

(ii) To examine the function of oxytocin, vasopressin receptors on sperm through *in vitro* assays measuring intracellular responses to oxytocin, vasopressin treatment.

Objectives: to investigate potential links between neuropeptides and sperm function via the following specific objectives:

(i)   Human sperm obtained from volunteers will be treated with oxytocin, vasopressin and prolactin
(ii)   Modulations in sperm motility will be assessed via CASA
(iii)   Immunolocation of neuropeptide receptors on human sperm.
(iv)   Cell signalling regulation after neuropeptide treatment will be investigated.

2.0 Materials and Methods

Materials Used:

Table 1. Equipment used.

Equipment
Agilent Technologies SureCycler 8800 – Thermal cycler
Atto AE-6220 Duel Slab Chamber – SDS-PAGE gel tank
Bio-Rad ChemiDoc^TM Touch Imaging System – Western blot and agarose gel imaging system
Bio-Rad PowerPac^TM Basic Power Supply - power supply for gel tanks and transfer system
Bio-Rad Sub-Cell® GT Cell – Agarose gel electrophoresis tank
Bio-Rad Trans-Blot® SD Semi-Dry Transfer System – Western blot transfer system
BioTek® SYNERGY HT Microplate Reader
Leica DFC 365 FX – Fluorescent microscope
Millipore Amplicon® Ultra 0.5ml centrifugal filters Ultracel® 3K – Protein concentration filters
NUAIRE^TM Autoflow Direct Heat CO_2 Incubator – Cell culture incubator
NUAIRE^TM Biological Safety Cabinets – Class II safety cabinet
Olympus CX41 – Phase contrast microscope (CASA)
Scarstedt Falcon Tubes
Sigma 3-16PK centrifuge
SLS lab basics vortexer
TECHNE Dri-Block® DB-2D – Heat block
Thermo scientific NanoDrop 2000c Spectrophotometer
Thermofisher Glassware
Tomy Capsulefuge PMC-860 - Micro centrifuge
Zeiss Axio Imager Z1 – Fluorescent microscope
Gilson Pipettes
Plasticware from Starstedt or Fisher Scientific
Microplates – Grenia
Cell culture plates and T75 Flasks – Nunc by Fisher Scientific
Eppendorf tubes – Starlabs
Pipette tips – Starlabs
Sample collection pots – Sterilin UK
CASA counting chamber 20 micron – Cell Vision

Table 2. Software used.

Software
AxioVision version 4.8.2.0
Bio-Rad Image Lab™ software
Gen 5 version 2.05.5 BioTek®
Microsoft® Excel® 2013 version (15.0.4841.1000)
Sperminator® Pro Creative Diagnostics 1.0
Leica LAS X Suite microscope imaging software

Table 3. Antibodies used.

Antibodies	Supplier	Catalogue #
Alexa fluor® 488 F(ab') 2 goat anti-mouse IgG, IgM (H+L)	Thermo Fisher Scientific	A-10684
Alexa fluor® 488 goat anti-rabbit IgG (H+L)	Invitrogen	A-11008
Anti-goat HRP	Sigma	A5420
Anti-goat IgG-FITC	Santa cruz biotechnology	
Anti-oxytocin receptor	Abcam	ab87312
Anti-oxytocin receptor	Abcam	ab115664
Anti-prolactin receptor	Abcam	ab2773
Goat anti-rabbit HRP	Bio-Rad	170-6515
Monoclonal Anti-β-Tubulin antibody produced in mouse	Sigma	T4026
Phospho(Ser/Thr) PKA substrate antibody	Cell signalling	9621S
Anti-AVPR V2 antibody	Abcam	Ab188748
Primers	Invitrogen	A15612

Table 4. Reagents used.

Reagents	Supplier	Catalogue #
[Arg^8] Vasopressin acetate salt	Sigma	V9879
2-Mercaptoethanol	Sigma	M7154
5x DNA Loading Buffer Blue	Bioline	BIO-37045
8-bromo-cAMP	Abcam	ab141448
Acrylamide/Bis 30% solution	Bio-Rad	161-0158
Agarose	Fisher scientific	M-12198

Amersham™ Protran® Western blotting membranes, nitrocellulose	Amersham™	GE10600001
Ammonium persulphate	Sigma	A3678
Bovine serum albumin	Sigma	A2153
Bromophenol blue	Sigma	B8026
Chloroform	Sigma	C7559
DAPI	Sigma	D9542
Dulbecco's Modified Eagle's Medium - high glucose	Sigma	D1145
Ethanol	Sigma	51976
EZ-ECL	Biological industries	20-500-120
Foetal bovine serum	Sigma	F0804
Glycerol	Sigma	G5516
Glycine	Sigma	G8898
GoTaq® 2-step RT-qPCR system	Promega	A6010
Hydrochloric acid	Sigma	H1758
HyperLadder™ 25bp	Bioline	BIO-33057
HyperLadder™ 50bp	Bioline	BIO-33054
ImmEdge Hydrophobic Barrier Pen (PAP pen)	Vector	H-4000
Isopropanol	Sigma	I9516
L-Glutamine	Sigma	G5792
MagicMark™ XP western protein standard	Life technologies	LC5603
Marvel dried milk powder	Sainsbury's	2051857
Methanol	Sigma	494437
MyTaq™ DNA Polymerase	Bioline	BIO-21105
N,N,N',N'-Tetramethylethylenediamine	Sigma	T9281
Normal goat serum	Vector	S-1000
Penicillin-Streptomycin	Sigma	P4333
Phosphate Buffered Saline	Oxoid	BR0014G
Phosphotase inhibitor cocktail set III	Millipore	524627
Pierce® BCA protein assay kit	Pierce	23225
Ponceau S	Sigma	P3504
Precision plus protein™ standards	Bio-Rad	161-0376
Protease inhibitor cocktail	Sigma	P8340
Bicarbonate	Sigma	S3817
Sodium chloride	Sigma	S3014
Sodium deoxycholate	Sigma	S1827
Sodium dodecyl sulphate	Sigma	L3771
Sperm freezing medium	Origio	10670010A
Sperm preparation medium with Phenol Red	Origio	10705060A
SupraSperm® System	Origio	10922060A
Tris base	Fisher scientific	BP152-1
Triton™ X-100	Sigma	T8787
TRIzol® Reagent	Ambion life technologies	15596018

Trypsin	Sigma	T2600000
Tween® 20	Sigma	P9416
Vectashield® mounting medium for fluorescence with DAPI	Vector	H-1200
Whatman® gel blotting papers	Whatman®	WHA10426981

Methods

[Note: further recipes in Appendix]

2.1 Sample procurement

Semen samples were produced by masturbation after 2-5 days of abstinence from healthy donors aged 18-38 years old (n = 5). Donors were given a participant information sheet with details of the study and completed a medical screening form and consent form, full ethical approval (see appendix 2). Semen was produced on site in a designated room and participants were instructed how to produce the sample in to a sample collection pot.

All semen samples were assessed according to the WHO 5th edition (2010). Liquefaction of semen was carried out a 37°C, which was followed by measurement of semen volume, sperm concentration and motility assessment.

To assess sperm motility and concentration, 5 µl of sample was applied to a counting slid and analysed using computer assisted sperm analysis (CASA) (Sperminator$^{®}$) (Olympus CX41). The CASA measures sperm concentration (million/ml), velocity and grades sperm into A (fast progressive), B (slow progressive), C (non-progressive) and D (immotile) providing a percentage of these grades as an output (**Figure 6**).

2.2 Sperm Isolation
Sperm was separated from seminal plasma using density gradient centrifugation unless indicated otherwise, where the swim-up technique was employed.

2.2.1 Density Gradient

Sperm cells were separated from seminal plasma via density gradient centrifugation. Semen (1 ml) was layered on top of SupraSperm$^{®}$ gradient (55% - 2 ml and 80% - 2 ml) and centrifuged at 300G for 20 minutes (**Figure 8**). Supernatant was discarded, 3 ml of sperm preparation media (SPM) was added and the pellet was resuspended. The sample was centrifuged at 300G for 10 minutes twice,

removing the supernatant each time. The remaining pellet was resuspended in 1 ml SPM, and the concentration and motility of sperm measured.
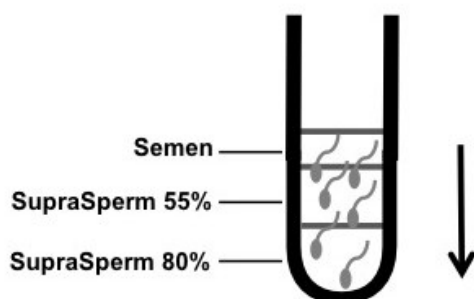
Figure 8. Density gradient.

2.2.2 Swim-Up Method

For assessing PKA activity in sperm, the swim–up technique was used so minimise spontaneous capacitation *in vitro*. Sperm was placed beneath 2 ml of PBS and incubated at an angle of approximately 45° at 37°C for 30-60 minutes (**Figure 9**). Top layer of PBS was then pipetted into a falcon tube and washed with 3 ml PBS and pelleted via centrifugation at 300G twice. Pellet was resuspended in PBS (World Health Organization, 2010).
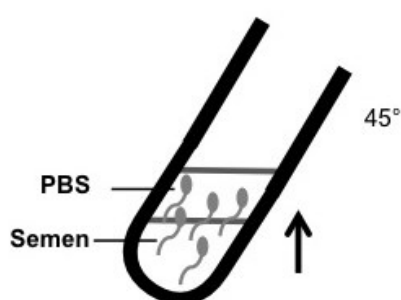
Figure 9. Swim-up method.

2.3 Immunocytochemistry

2.3.1 Fixing sample on slides

The sample (10 µl) was smeared onto a slide. The slide was immersed in ice-cold methanol, 4% formaldehyde or 10% neutral buffered formalin (appendix 1) for 10 minutes. The slide was air-dried and either used immediately or stored at -20°C until required.

2.3.2 Immunolocalisation of neuropeptide receptors

Slides taken from -20°C were left to thaw at room temperature for five minutes. The perimeter of the slides were drawn around with a PAP pen then rehydrated in phosphate-buffered saline (PBS) for 10 minutes and permeabilised in PBS-Tween 0.1% (v/v) for 10 minutes. Blocking buffer [500 µl] was added to each slide, which was covered with the lid of the humidity chamber and left for 1 hour. Slides were washed in PBS-Tween 0.1% 3 times for 5 minutes (appendix 1).

Primary antibody was diluted in blocking buffer (1:50, 1:100, 1:200) where 200 µl was added to the slides followed by overnight incubation at 4°C. A no primary antibody control was prepared by adding 200 µl of blocking buffer to a slide and stored as above.

After incubation, all slides were washed in PBS-Tween 0.1% 3 times for five minutes. At low light levels, 250 µl of the appropriate fluorescent-conjugated secondary antibody (1:2000 dilution with blocking buffer unless otherwise stated) was added to each slide. Slides were covered and left for 1 hour. Slides were washed 3 times in PBS-Tween 0.1% for five minutes in darkness. A drop of VECTASHIELD® mounting medium with DAPI was added to each slide, a coverslip was placed on top and sealed with an enamel sealant. Slides were left in darkness for half an hour to develop before imaging using fluorescent microscopy (Zeiss Axio Imager Z1/Leica DFC 365 FX).

2.4 Cell culture and *in vitro* assays.

All cells were cultured in class II safety cabinets and incubated in direct heat 5% CO_2 incubators at 37°C.

All cells, excluding sperm, were cultured using standard culture media (appendix 1) and standard cell culture technique in either 6 well cell culture plates or T75 flasks.

Sperm cells were prepared via density gradient/swim-up and washed twice in sperm preparation media (SPM), SPM was the media used in all experiments unless otherwise specified.

2.4.1 Incubations with agonists

All cells were incubated with neuropeptide (10 pM, 10 nM, 10 µM) or positive control 8-bromo-cAMP (100 µM) agonists in their appropriate media, either SPM or standard culture media (appendix 1), at 37°C for 5-90 minutes.

2.5 Protein extraction and quantification

2.5.1 Radioimmunoprecipitation assay cell/tissue lysis

Radioimmunoprecipitation assay (RIPA) lysis buffer (appendix 1) was used for protein extraction (Six and Kasel, 1978). 1 ml of RIPA with 1:100 (v/v) protease inhibitor cocktail and 1:100 (v/v) phosphotase inhibitor cocktail was used for 10 million cells or 0.1 g of tissue. Sample and lysis buffer were incubated at 4°C under constant agitation for 30-60 minutes dependant on whether it was cells (30 minutes) or tissue (60 minutes).

Samples were centrifuged at 12,000G for 20 minutes at 4°C, the supernatant was aspirated and stored at -80°C and the pellet discarded.

2.5.2 Protein quantification – Bicinchonic acid assay (BCA assay)

Bicinchonic acid (BCA) assay was used (Pierce™ BCA protein assay kit) to quantify all proteins. The BCA assay is a colourmetric assay that determines total protein concentration due to colour changes in the samples when compared to known standards (Smith *et al*., 1985). Protein standards were made to the manufacturer's instructions. Reagent A was mixed with reagent B at 50:1 (v/v) to make the working reagent, 25 µl of protein standard or sample was pipetted into a 96 well microplate and 200 µl of working reagent was added to each protein sample. The microplate was incubated at 37°C for 30 minutes and then absorbance was measured at 562 nm on a microplate reader. Standard curve was calculated using Microsoft® Excel software and equation from graph (y=mx+c) was used to quantify unknown proteins.

2.6 Western blotting

2.6.1 SDS-Page

Table 5. SDS-Page gel recipe.

Reagent	Separating Gel	Stacking Gel
dH_2O	3.2 ml	5.6 ml
Separating buffer	2.5 ml	-
Stacking buffer	-	2.5 ml
Bis/Acrylamide 30%	4.3 ml	1.9 ml
APS 10%	100 µl	100µl
TEMED (add last)	10 µl	10 µl

Separating gel was prepared in a universal tube as per recipe in **Table 5**. Of the separating gel, 5 ml was pipetted into the gel cast, 100 µl of isopropanol was pipetted on top of the gel to ensure it sets evenly and the gel was left to polymerise for 15 minutes. The isopropanol was decanted and gel rinsed with dH_2O. Stacking gel was prepared in a universal as per recipe in table 5, this was then pipetted on top of the separating gel until the gel cast was full, the gel comb was then inserted and the gel was left to polymerise for a further 15 minutes. Once the gel was set the gel casts were placed in the gel tank and it was filled with electrode buffer to the marker and gel combs removed.

Protein samples were diluted laemmli buffer 4X (appendix 1) and then heat denatured at 100°C for 15 minutes. 2.5 ng/µl of protein sample was loaded into the gel (unless otherwise stated) along with protein ladder at manufacturer's recommendation. Gel tank was connected to power pack and ran at 60V for 60 minutes, then the voltage was increased to 120V and left to run until the dye front reached the end of the gel.

<u>2.6.2 Blotting</u>

Stacking gel was removed and discarded. Blotting paper was cut into 8 pieces per gel and soaked in towbin buffer (appendix 1) along with the nitrocellulose membrane for approximately 2 minutes. A sandwich of 4 pieces of blotting paper, the nitrocellulose membrane, the gel and a further 4 pieces of blotting paper, in that order, were laid out onto the transfer system. Any air bubbles were eliminated from this 'sandwich' by rolling a clean test tube (or something similar) over the components. The lid was placed on the transfer system and connected to the power pack, this was then ran at 100V for 1 hour (unless otherwise stated).

<u>2.6.3 Blocking</u>

The membrane was then incubated for 1 hour in blocking buffer (5% bovine serum albumin in 0.01% TBS-T v/v, unless otherwise stated, appendix 1) at room temperature on a shaker. Blocking buffer was then discarded and primary antibody solution (1:1000 antibody to blocking buffer v/v) was added to membrane and the membrane was incubated overnight at 4°C on a shaker.

Primary antibody was discarded and the membrane was washed for 5 minutes in triplicate with PBS-T at room temperature on a shaker. The appropriate secondary HRP conjugated antibody was then added in solution (1:1000 antibody to TBS-T v/v, unless otherwise stated) to the membrane and left to incubate on a shaker at room temperature. Secondary antibody solution was discarded and membrane was once again washed 3 times for 5 minutes with TBS-T at room temperature on a shaker.

2.6.4 Developing

Membranes were kept in TBS-T to prevent them from dehydrating. Cling film was laid out on a flat surface and membrane was placed onto the cling film protein side up. Electrochemiluminescence (ECL) solution was used to develop the membrane. In the dark, ECL solution A and B were mixed in equal parts and then 1ml of ECL solution was added to the membrane and left to develop for approximately 5 minutes. The ECL was drained off and the membrane was imaged using ChemiDoc™ Touch Gel and Western Blot Imaging System.

Membranes were then washed 3 times with TBS-T for five minutes and incubated with loading control antibody overnight at 4°C on a shaker and the process from then on was repeated.

2.7 RNA extraction (Trizol & Chloroform)

Suspension cells were pelleted via centrifugation at 300G for 10 minutes and the supernatant removed prior to extraction. RNA was extracted from adherent cells directly from the cell culture flask as follows; culture media was removed and the TRIzol® reagent was added at the appropriate volume for the amount of cells, usually either 1 or 2 ml (usually 1 ml for sperm, 2 ml for HeLa/other adherent cells in a T75 flask) and left for 15 minutes at room temperature. TRIzol® reagent was added. The TRIzol® cell suspension was transferred to 1.5 ml Eppendorf tubes (as many as appropriate) and 200 µl of chloroform was added to each Eppendorf tubes and vigorously shaken. These were then left for a further 15 minutes at room temperature. The Eppendorf tubes were centrifuged at 12,000G for 15 minutes at 4°C. The aqueous phase, which is clear and containing the RNA, was pipetted into a new 1.5 ml Eppendorf tubes and 500 µl of isopropanol was added. This was then vortexed and left at room temperature for 10 minutes. The suspension was centrifuged at 12,000G for 10 minutes at 4°C and the supernatant was aspirated and discarded. After, 1 ml of 75% ethanol was added, vortexed and centrifuged at 8000G for 5 minutes at 4°C. The supernatant was aspirated and discarded and the pellet was dissolved in 30 µl of nuclease free water. RNA quantity and purity was

investigated using NanoDrop 2000C Spectrophotometer. After quantification RNA was immediately reverse transcribed into cDNA to avoid degradation during storage, any remainder of RNA was stored at -80°C for up to 6 months.

2.8 Polymerase Chain Reaction (PCR)

2.8.1 Primer design

All primers were designed using Primer3 Plus hosted by NCBI for use with BLAST.

2.8.2 Reverse transcription PCR:

All reverse transcription was performed using Agilent's SureCycler 8800 thermal cycler and a reverse transcription kit (GoTaq® 2-Step RT-qPCR System by Promega). Following manufacturer's instructions, on ice, RNA and reverse transcription primer was combined appropriately using up to a maximum of 5 μg per reaction and to a final volume of 10 μl. This mixture was denatured at 70°C for 5 minutes and chilled at 4°C for 5 minutes. The GoScript™ reaction mix was prepared as follows, Nuclease-Free Water - 1.5 μl, GoScript™ 5X Reaction Buffer – 4 μl, $MgCl_2$ 25 mM – 2 μl, PCR Nucleotide Mix 10 mM – 1 μl, Recombinant RNasin® Ribonuclease Inhibitor - 0.5 μl, GoScript™ Reverse Transcriptase – 1 μl. The reaction mix was combined with the RNA and reverse transcription primer in the reaction tube and cDNA was synthesised under the following cycle conditions:

Table 6. cDNA synthesis cycle conditions for GoTaq 2-Step RT-qPCR system by Promega.

Step	Temperature	Time
Anneal	25°C	5 minutes
Extend	42°C	1 hour
Inactivate	70°C	15 minutes

cDNA was stored at -20°C until use.

2.8.3 Endpoint PCR

Primers were optimised using a thermal cycler and a DNA polymerase kit. Following the manufacturer's instructions, on ice, in the reaction tube the reaction was set up as follows:

5x MyTaq Reaction Buffer – 10 µl
cDNA - as required
Primers 20 µM each – 1 µl
MyTaq DNA Polymerase – 1 µl
ddH2O - up to 50 µl

The following cycling conditions were used to amplify the target:

Table 7. Endpoint PCR cycle conditions for primer optimisation.

Step	Temperature	Time
Initial denaturation	*95°C*	*1 minute*
Denaturation	95°C	30 seconds
Annealing	60°C	30 seconds
Extension	72°C	30 seconds
		x35 cycles
Final Extension	*72°C*	*10 seconds*

PCR product was immediately used in agarose gel electrophoresis to ensure appropriate amplification of the target sequence (**Table 6**).

2.8.4 Agarose Gel Electrophoresis

All gels were composed of 2% agarose in 1X TBE buffer (appendix 1) with midori green as the DNA intercalating agent. Bioline 5x DNA loading buffer blue was mixed appropriately with the PCR product prior to loading, Bioline HyperLadder™ 25bp or 50bp were used appropriately dependant on expected amplicon size and the gel ran at 80V for 60 minutes, or until the dye front reached the end of the gel.

Gels were imaged using ChemiDoc™ Touch Gel and Western Blot Imaging System.

2.9 Statistical analysis

Statistical analysis of progressive sperm motility was performed in Microsoft Excel® 2013 software using a standard paired T-Test. Analysis of western blot band intensity was performed in Bio-Rad Image Lab™ software.

3.0 Results

3.1 Immunolocalisation of neuropeptide receptors on human sperm

Immunocytochemistry was employed to investigate the presence of neuropeptides receptors on sperm cells. Sperm separated from seminal plasma was fixed on slides and probed for vasopressin receptor 2, oxytocin receptor and prolactin receptor.

3.1.1 Immunolocalisation of vasopressin receptor 2

Human kidney cells (HK2) express the vasopressin receptor 2 (Thibonnier *et al*., 1998) and was used as a positive control for vasopressin receptor 2 immunolocalisation showing positive cell surface staining of the receptor in all cells (Figure 10).
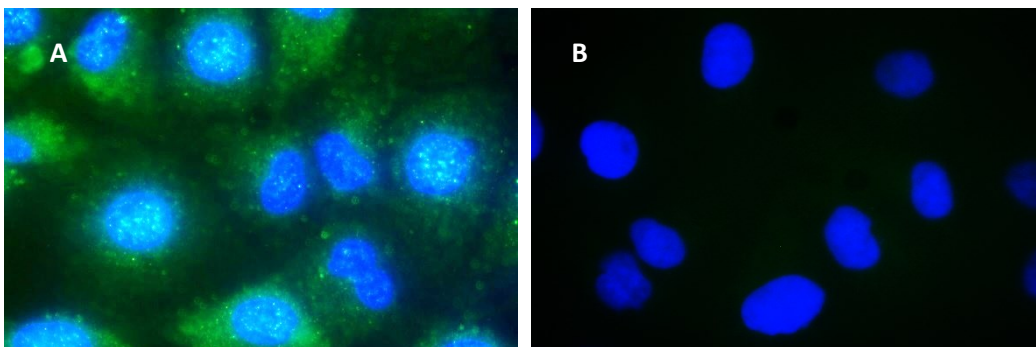
Figure 10. Immunolocalisation of vasopressin receptor 2 on human kidney cells (HK2). HK2 cells stained for vasopressin receptor 2 (green) localized on the cell surface membrane (A). No primary antibody control (B). Nuclei were stained with DAPI (blue). Magnification x640. Representative images from N = 3.

Immunolocalisation of vasopressin receptor 2 displayed strong staining on the acrosome region of human sperm, staining was seen on most sperm cells however a few did not show staining for the vasopressin receptor 2 and it is postulated that this is due to its localisation on the acrosome, some sperm may have undergone spontaneous acrosome reaction and therefore no longer have the receptor present (Figure 11).
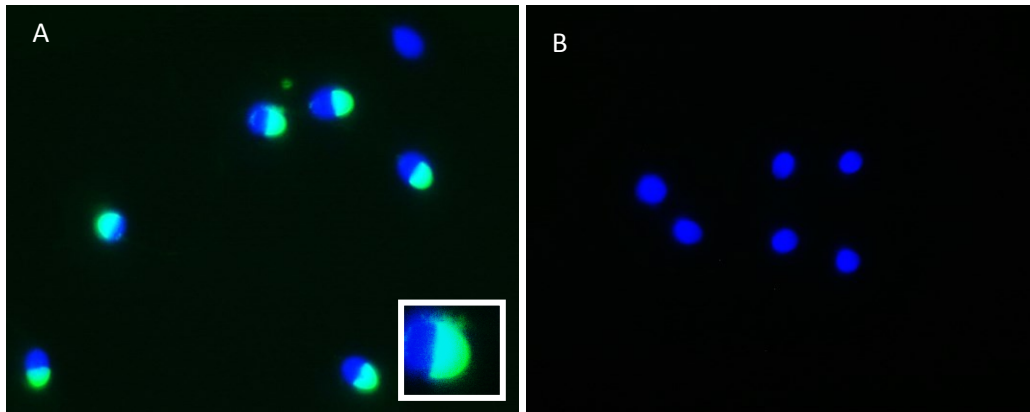
Figure 11. Immunolocalisation of vasopressin receptor 2 in human sperm. Vasopressin receptor 2 (green) was localized to the acrosome region of sperm (A and insert). No primary antibody control (B). Nuclei were stained with DAPI (blue). Magnification x1000. Representative images from N = 5.

3.1.2 Immunolocalisation of oxytocin receptor

MCF-7 cells, a human breast cancer cell line known to express the oxytocin receptor (Cassoni *et al*., 1994), was used as a positive control. Positive staining for oxytocin receptor on the cell surface membrane on all cells is shown in Figure 12.

No immunolocalisation of oxytocin receptor was detected on human sperm (Figure 13).
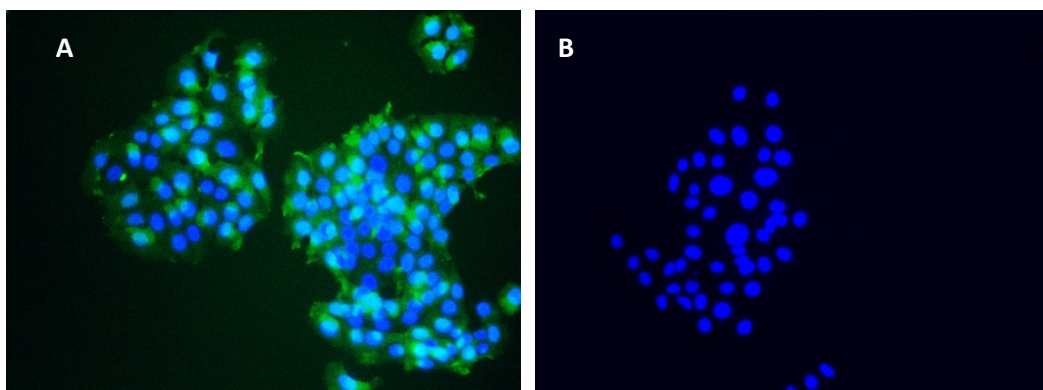
Figure 12. Immunolocalisation of oxytocin receptor in MCF-7 cells (human breast cancer cell line). MCF-7 cells stained for oxytocin receptor (green) localized on the cell surface membrane (A). No primary antibody control (B). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 3.
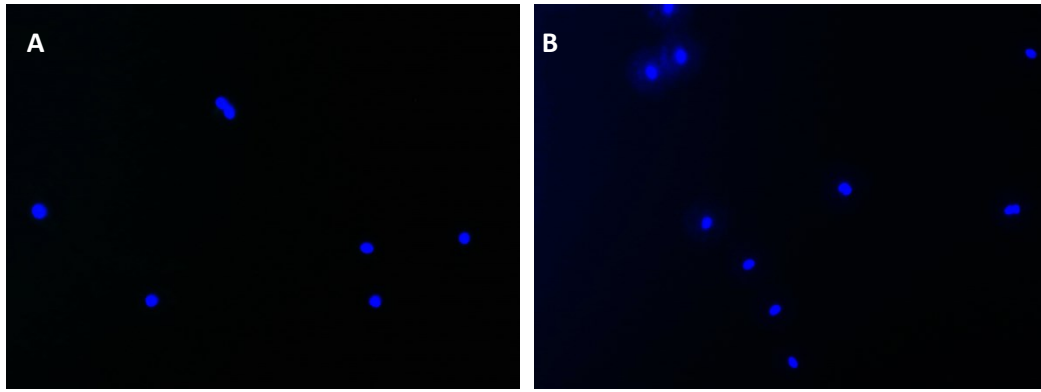
Figure 13. Immunolocalisation of oxytocin receptor in human sperm. Oxytocin receptor was not detected on human sperm (A). No primary antibody control (B). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 3.

3.1.3 Immunolocalisation of prolactin receptor

MCF-7 express prolactin receptors (Melck *et al*., 2000) and were used as a positive control in these studies. Immunolocalisation with clear labelling observed on the cell surface of each cell (Figure 14). However, prolactin receptor was not detected on human sperm in the present study (Figure 15).
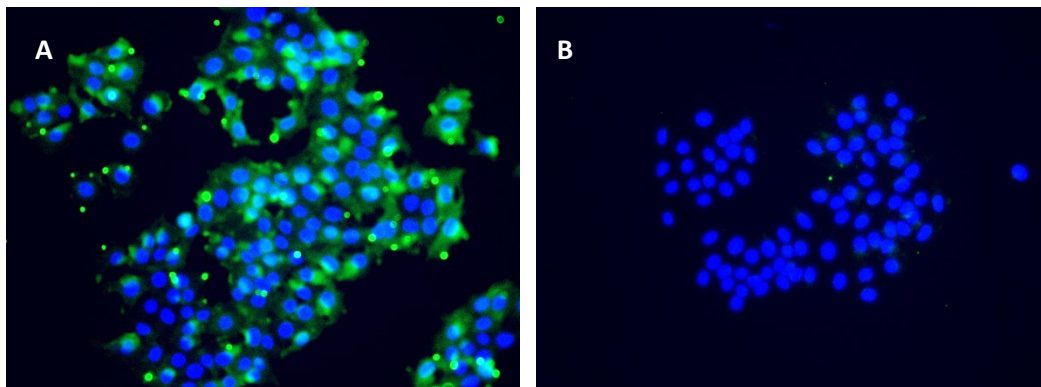
Figure 14. Immunolocalisation of prolactin receptor in MCF-7 cells. MCF-7 cells stained for prolactin receptor (green) localized on the cell surface membrane (A). No primary antibody control (B). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 3.
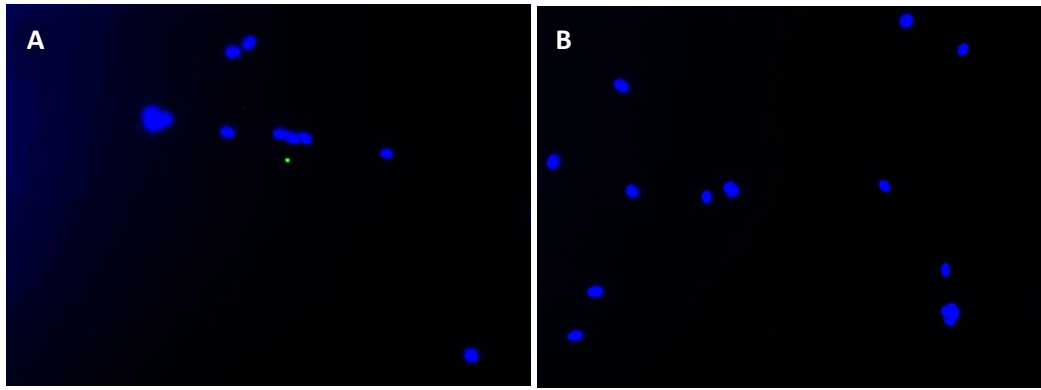
Figure 15. Immunolocalisation of prolactin receptor in human sperm. Prolactin receptor was not detected on human sperm (A). No primary antibody control (B). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 3.

3.2 Neuropeptides and sperm function

This study has localised vasopressin receptor 2 on human sperm. However, oxytocin and prolactin were not localised on sperm, therefore the role of vasopressin on human sperm was further investigated.

3.2.1 Vasopressin and sperm motility

Human sperm were separated from seminal plasma using density gradient centrifugation (as outlined in section 2). Washed sperm were incubated with vasopressin agonist at concentrations (10 pM, 10 nM and 10 µM) for 30 and 90 minutes at 37°C. Sperm motility was measured using on the CASA. Progressive motility was calculated by adding motility grade A and B (WHO, 2010). No significant difference was observed between the control and treatment progressive motility after incubation with vasopressin (Figures 14 and 15) [statistical analysis: 2-tailed, paired t-test p = > 0.05. N = 4].
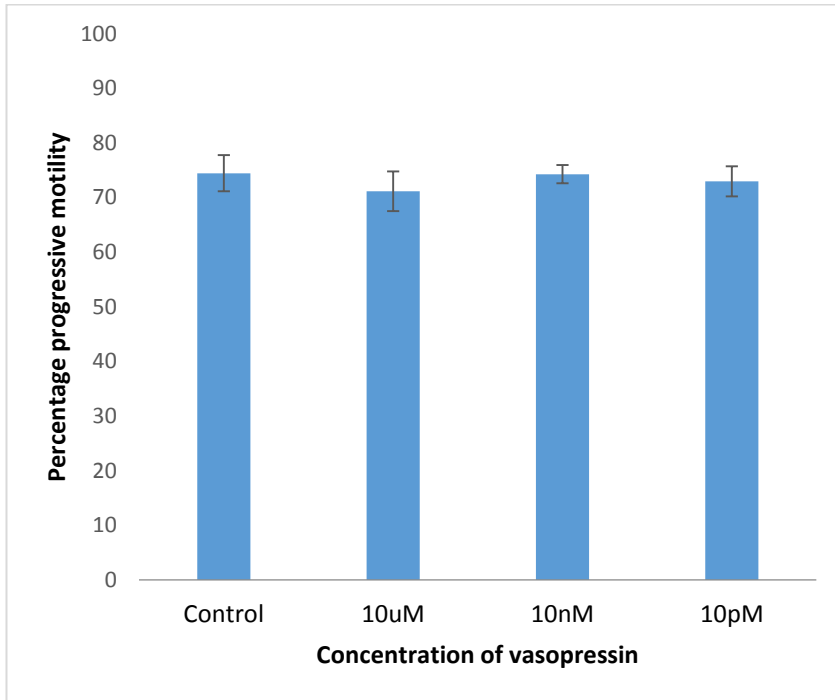
Figure 16. Percentage progressive motility after 30 minutes incubation with vasopressin at varying concentrations and no treatment control. No difference in percentage progressive motility was observed at any concentration, statistical analysis (*p = > 0.05*. N = 4.)
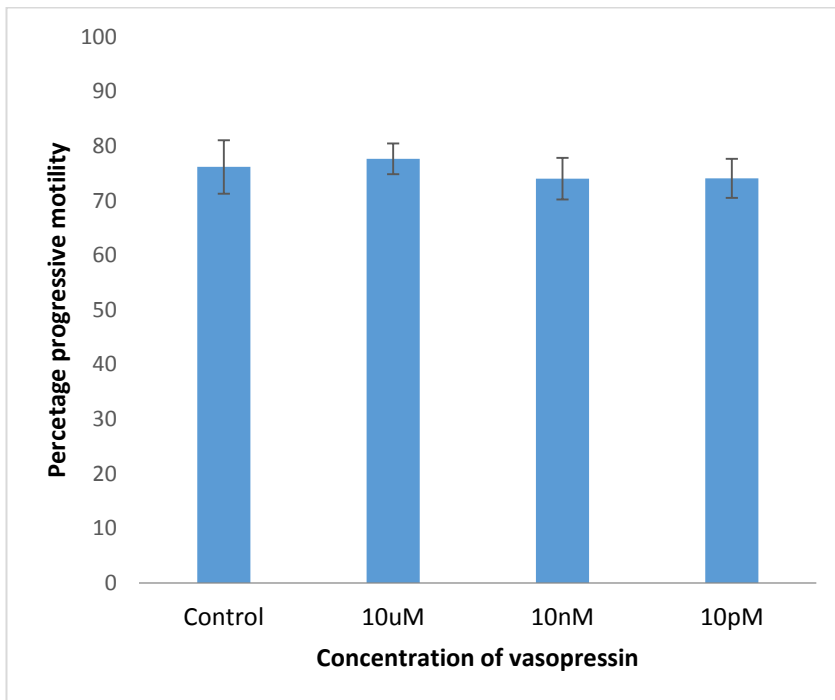
Figure 17. Percentage progressive motility after 90 minutes incubation with vasopressin at varying concentrations and no treatment control. No difference in percentage progressive motility was observed at any concentration (*p = > 0.05*. N = 4).

3.3 PKA activation

To further investigate the effects of vasopressin on the cellular function of human sperm, the activation of PKA was examined through immunolocalisation of phosphorylated serine and threonine substrates using the phospho-PKA substrates antibody. These substrates are phosphorylated by PKA and can be used as a measure of PKA activity within the cell (Kwon *et al*., 2013).

There were no visible signal differences between all treatments and controls (Figures 18, 19, 20, 21 and 23) except for the sperm cells incubated with the PKA activator 8-bromo-cAMP. PKA was localised in HeLa throughout the cell (Figures 18 and 19), in sperm it was localised to the tail except when incubated with 8-bromo-cAMP in which there was also localisation on the equatorial region of the head of the sperm (Figure 22 and arrow in A).

3.3.1 Phospho-PKA substrates immunocytochemistry

As a positive control and for assay optimisation HeLa cells were utilised. HeLa were treated with PKA activator 8-bromo-cAMP and immunolocalisation of phospho-PKA substrates in HeLa cells with and without treatment positive staining for phospho-PKA substrates evenly throughout the cells and in all cells with no visible signal differences between the two samples (Figures 18 and 19).
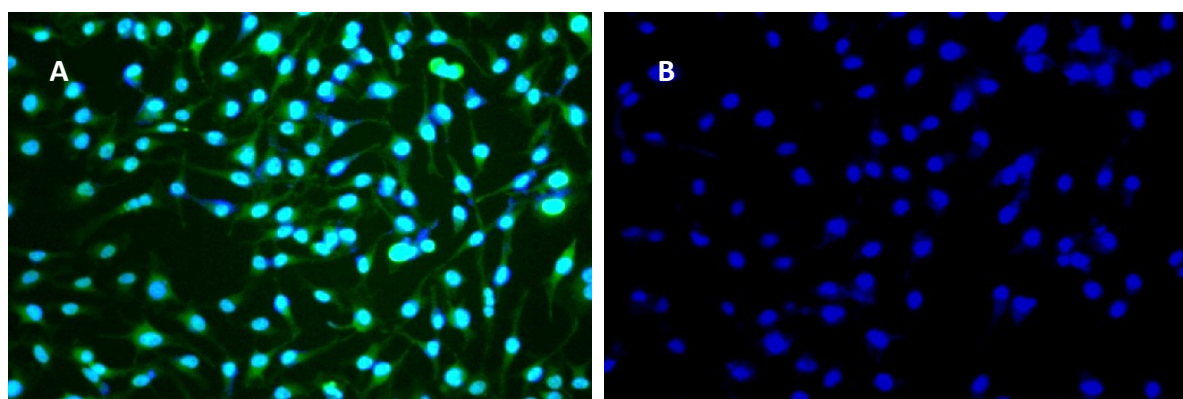
Figure 18. Immunolocalisation of Phospho-PKA Substrates in HeLa cells induced with 8-bromo-cAMP. Phospho-PKA substrates (green) detected on HeLa cells induced with 8-Bromo-cAMP (A). No primary antibody control (B). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 3.

**Figure 19. Immunolocalisation of Phospho-PKA Substrates in HeLa cells.** Phospho PKA substrates (green) detected on HeLa cells (A). No primary antibody control (B). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 3.

Immunolocalisation of phospho-PKA substrates in human sperm showed localisation primarily in the tail of the sperm (Figures 20, 21, 22, 23). All sperm samples were separated from the seminal plasma via swim-up method in PBS (methods section 2.2.2). In Figure 20, a no treatment control, positive staining for phospho-PKA substrates was observed on the tail of the sperm, no staining was observed elsewhere (Figure 20, A). In Figure 21 sperm were treated with 25mM bicarbonate to capacitate the sperm (Da Ros *et al*., 2004, Stauss *et al*., 1995). This was done as sperm that have undergone capacitation have been observed to have a much higher rate of PKA activity than non-capacitated sperm (Lefievre *et al*., 2002). Localisation of phospho-PKA substrates was once again observed along the tail of the sperm (Figure 21, A). In Figure 22 sperm were treated with 100 µM of the PKA activator 8-bromo-cAMP for 5 minutes and showed increased signal differences for phospho-PKA substrates from the other treated and control samples, including positive staining for the phospho-PKA substrates along the equatorial region of the head of the sperm (Figure 22, A, arrow). This indicated that the PKA activator 8-bromo-cAMP could successfully be used to increase PKA activity *in vitro*. Figure 23 showed sperm incubated with 10 µM of vasopressin agonist, positive immunolocalisation of the phospho-PKA substrates was once again observed along the tail of the sperm (Figure 23, A).

All no primary controls showed no staining indicating there was no non-specific binding of the antibody (C in Figures 20, 21, 22 and 23).

Phase contrast images of both stained and no primary controls showed the full sperm morphology (B and D in Figures 20, 21, 22 and 23)

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There were no visible signal differences for phospho-PKA substrates between the control, capacitated sperm and sperm incubated with vasopressin (Figure 20, A , Figure 21, A and Figure 23, A).
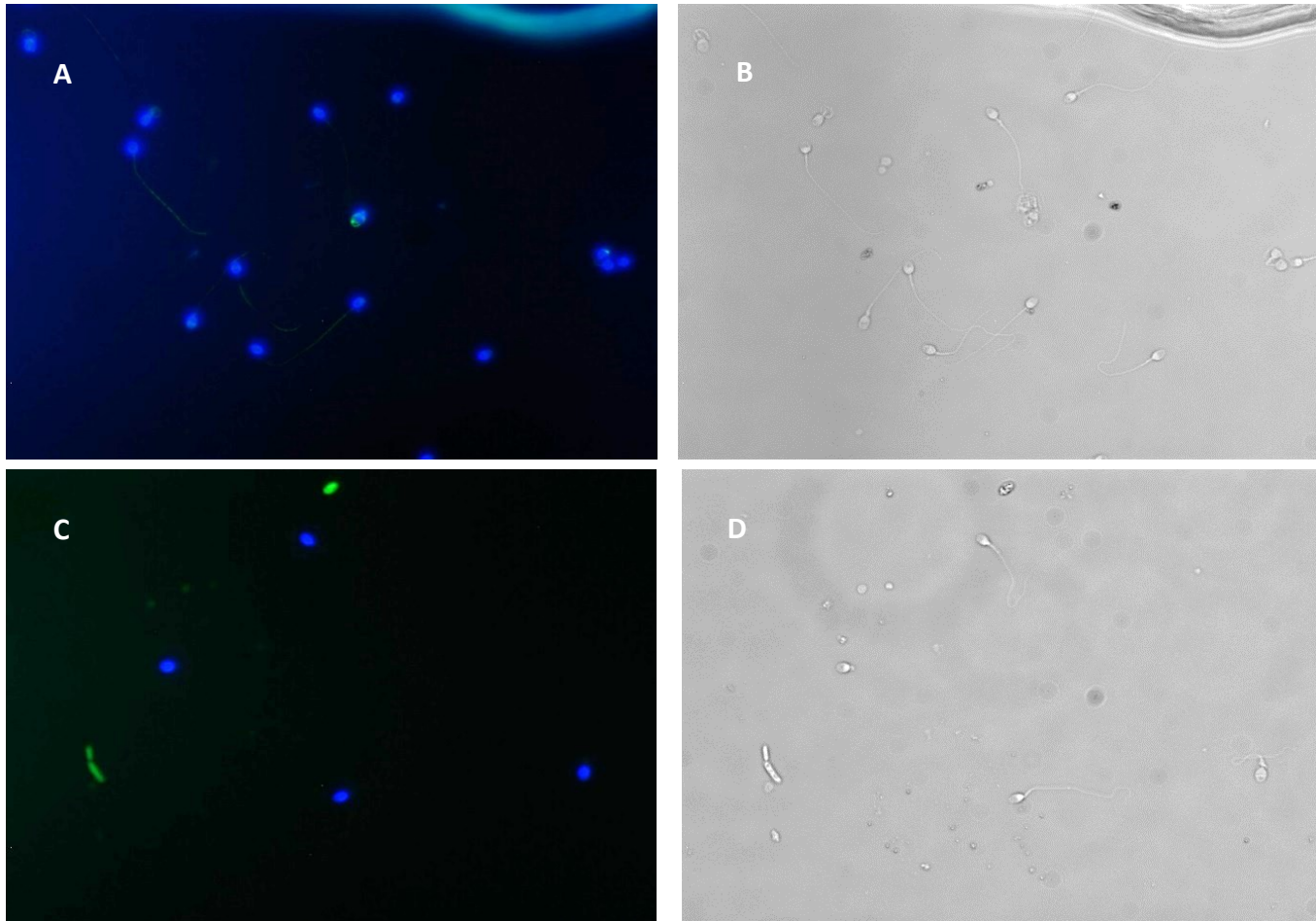
Figure 20. Immunolocalisation of Phospho-PKA Substrates in human sperm cells. Phospho PKA substrates (green) detected on human sperm washed and incubated with PBS (A). Phase contrast image of A (B). No primary control (C) and phase contrast of no primary control (D). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 1.
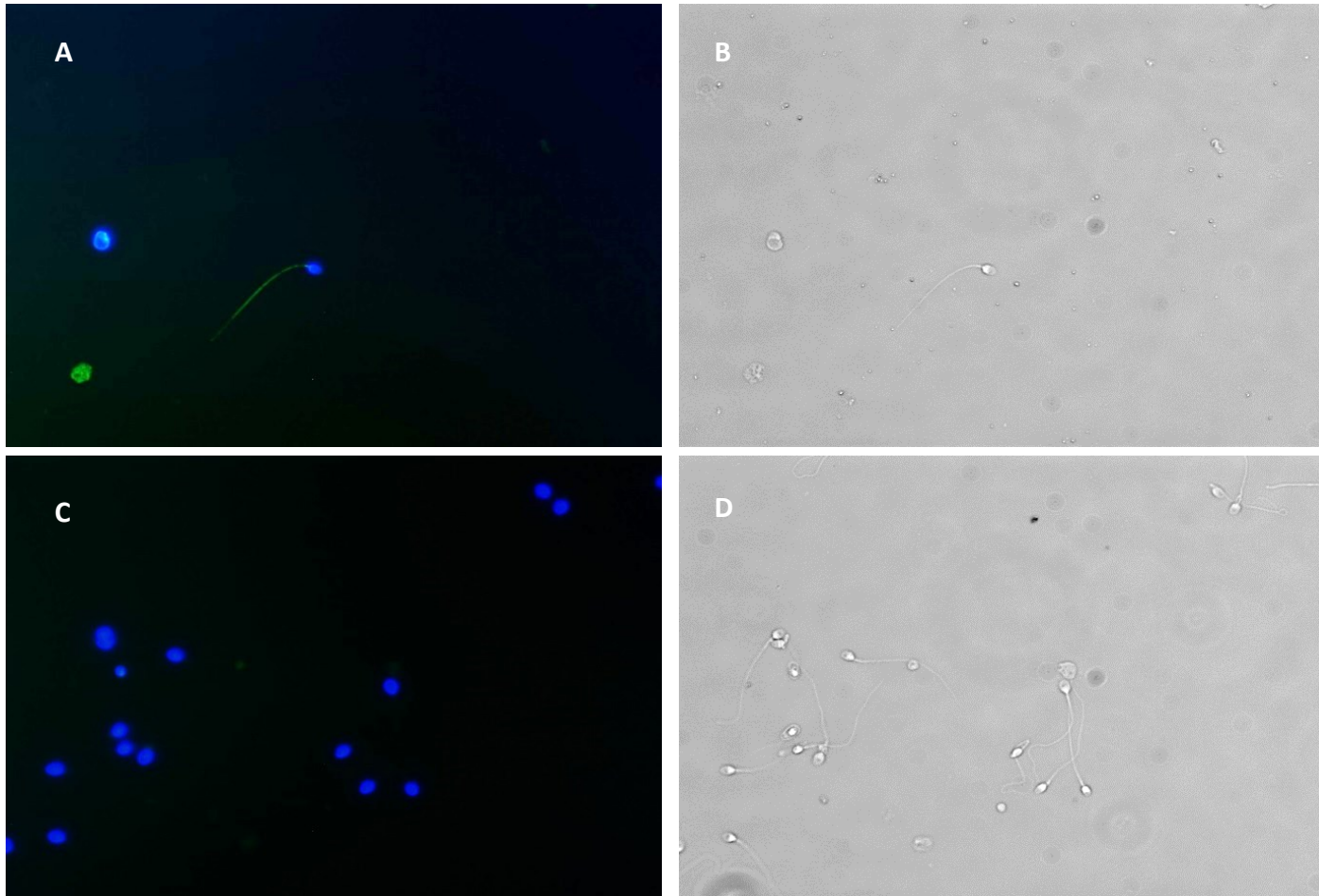
Figure 21. Detection of Phospho-PKA Substrates in capacitated human sperm cells. Phospho-PKA substrates (green) detected on human sperm washed in PBS and incubated with 25mM HCO3. (A). Phase contrast image of A (B). No primary control (C) and phase contrast of no primary control (D). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 1.
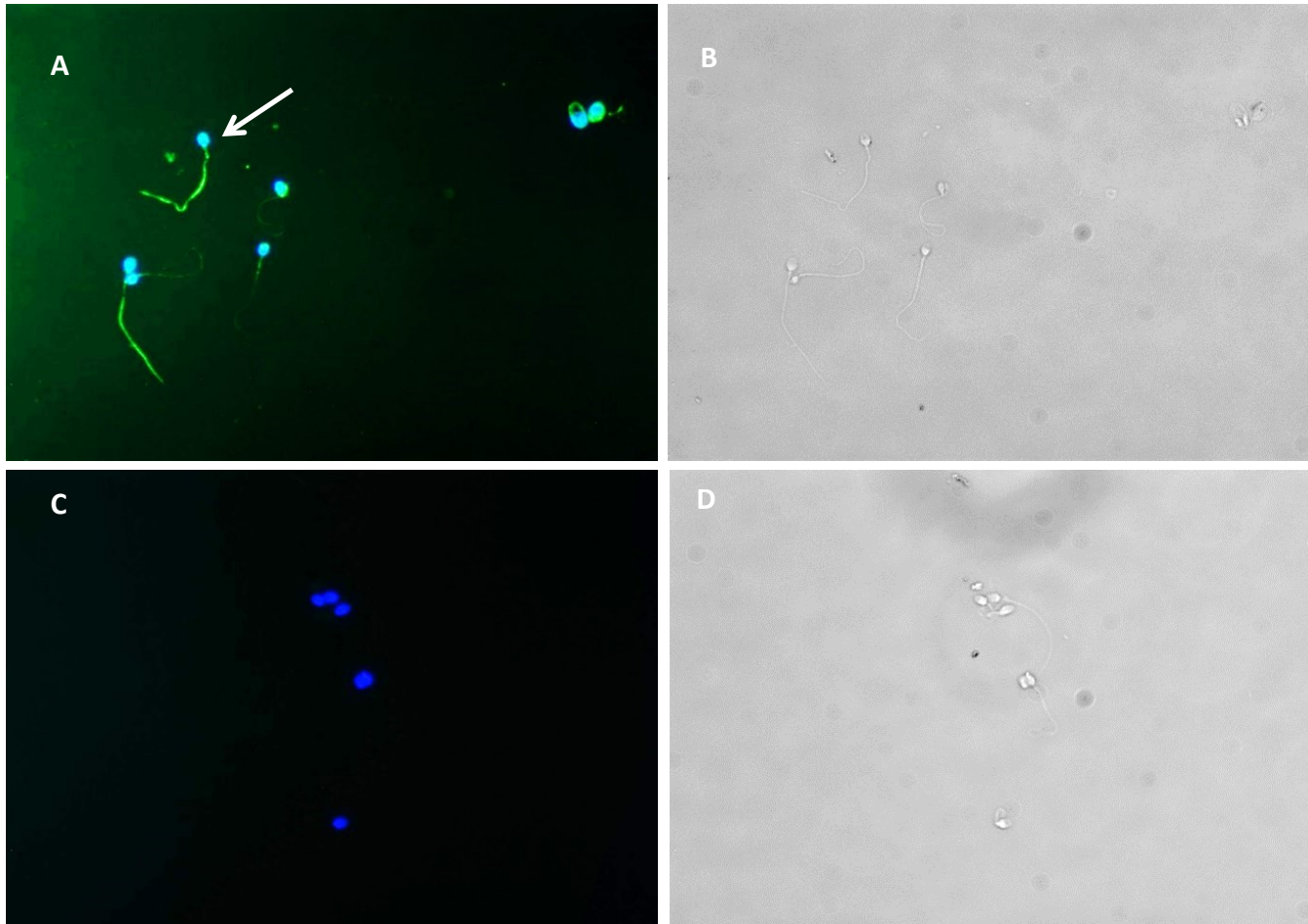
Figure 22. Detection of Phospho-PKA Substrates in human sperm cells washed in PBS and incubated with 8-Bromo-cAMP. Phospho-PKA substrates (green) detected on human sperm washed in PBS and incubated with 8-Bromo-cAMP. (A). Phase contrast image of A (B). No primary control (C) and phase contrast of no primary control (D). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 1.

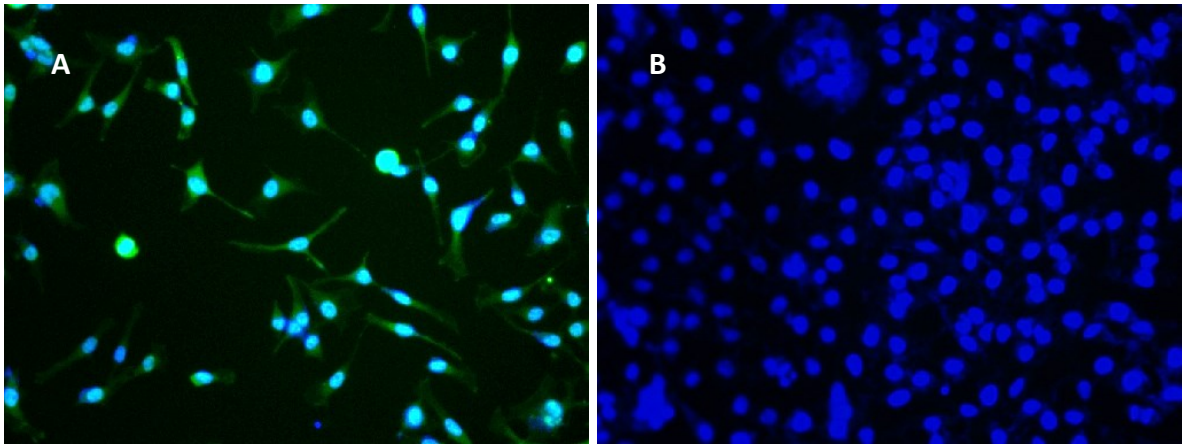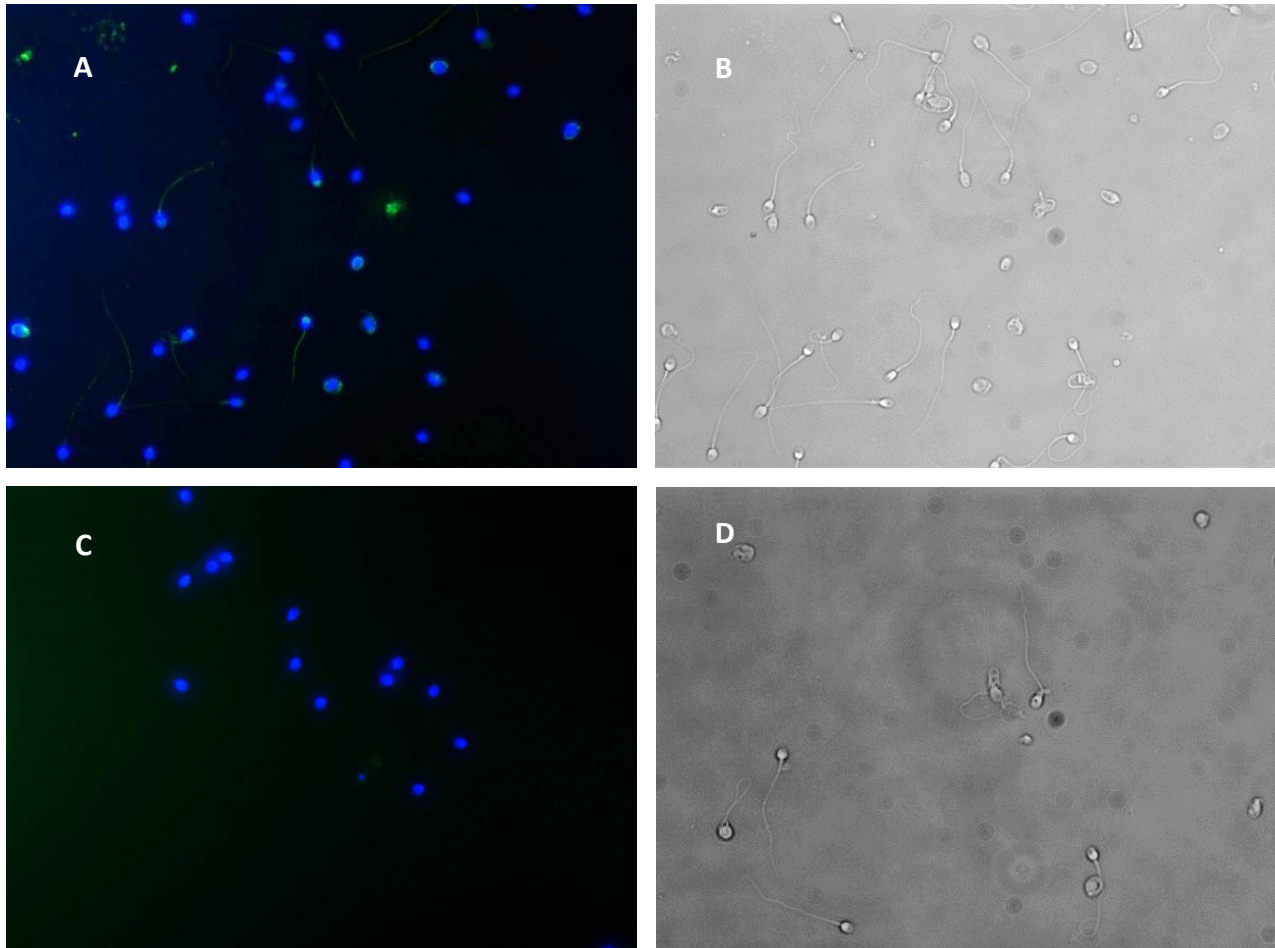**Figure 23. Detection of Phospho-PKA Substrates in human sperm cells washed in PBS and incubated with 10µM vasopressin.** Phospho-PKA substrates (green) detected on human sperm washed in PBS and incubated with 10µM vasopressin. (A). Phase contrast image of A (B). No primary control (C) and phase contrast of no primary control (D). Nuclei were stained with DAPI (blue). Magnification x400. Representative images from N = 1.

3.3.2 Phospho PKA substrates western blots

To investigate the regulation of PKA, HeLa and sperm cells were incubated with 8-bromo-cAMP to induce PKA phosphorylation activity as a positive control for PKA phosphorylation activity.

Western blot analysis for phospho-PKA substrates showed, in HeLa, the PKA activator 8-bromo-cAMP upregulated phospho-PKA activity after both 5 and 30 minutes in bands at 25 kDa and 30 kDa when corrected for Actin using Image Lab™ software. No observable difference between the 5 and 30 minute incubations with 8-bromo-cAMP (Figures 24a and 24b). Western blots using sperm did not show any conclusive data, which may be due to poor protein yields from the human sperm samples. Further optimisation is required in order to obtain results from sperm samples (Figure 25).
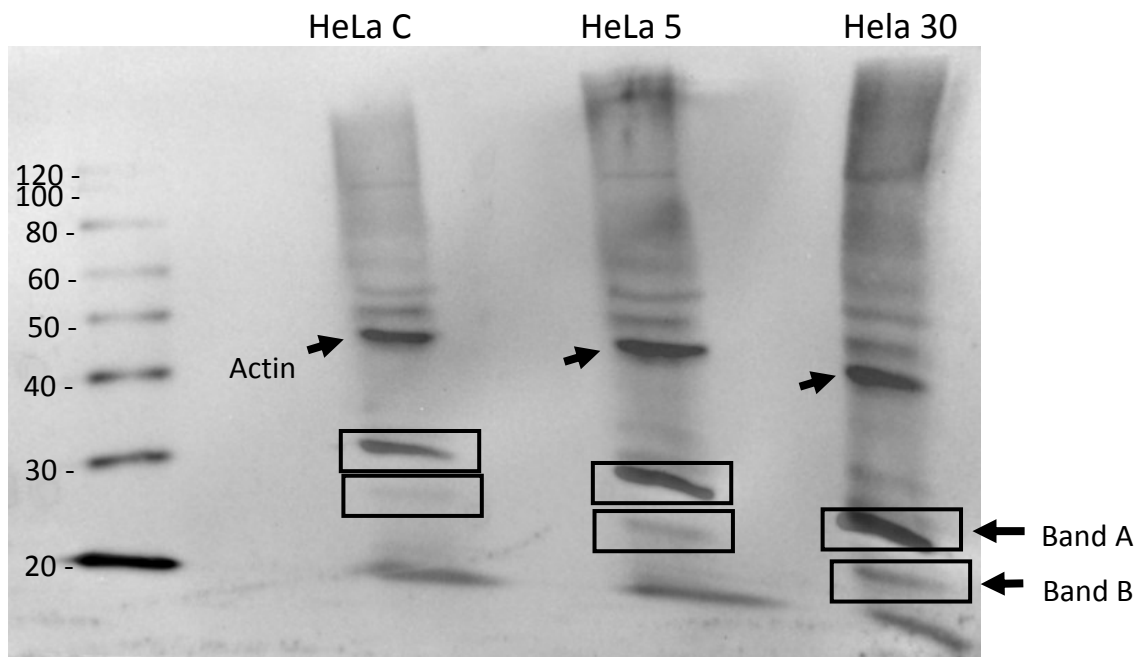
Figure 24. Overlay of representative western blot of Phospho-PKA substrates and Actin protein extracted from untreated and treated HeLa cells. HeLa cells were untreated (HeLa C) and treated with 8-bromo-cAMP for 5 minutes (HeLa 5) and 30 minutes (HeLa 30). Representative images from N = 9.
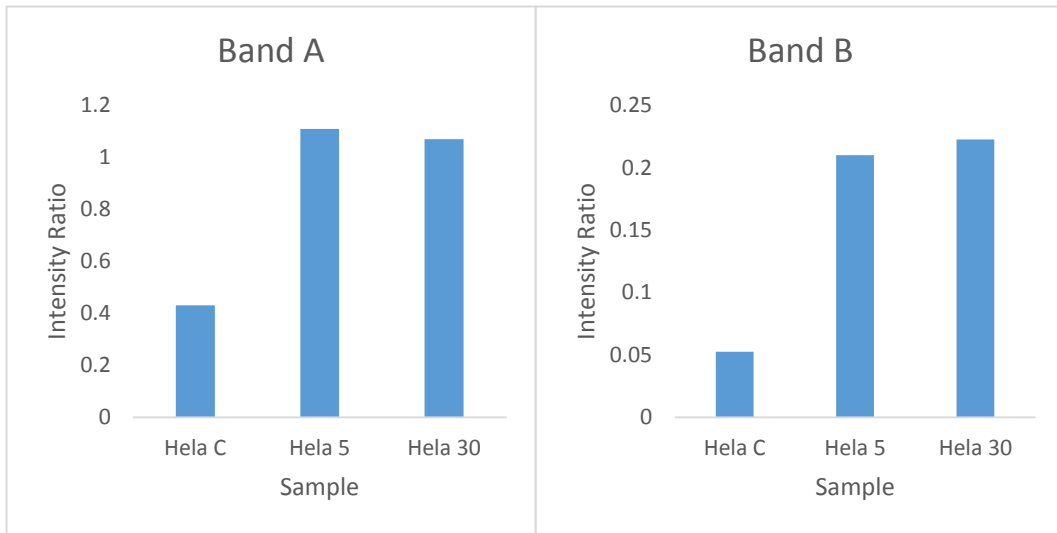
Figure 24b. Band intensity ratio for band A and band B of representative western blot 24a. HeLa Actin band compared to HeLa band A and B treated for 5 minutes with PKA activator 8-bromo-cAMP (HeLa 5) and treated for 30 minutes with PKA activator 8-bromo-cAMP (HeLa 30). Representative of N = 1.
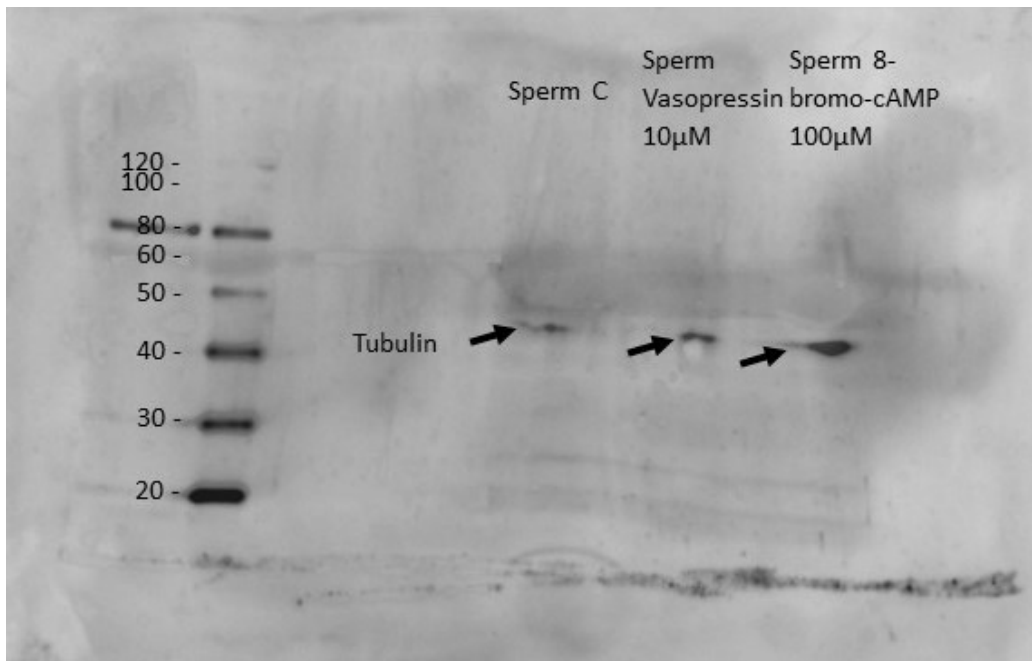
Figure 25. Overlay of representative western blot of Phopho PKA substrates and β-Tubulin protein extracted from untreated and treated human sperm cells. Human sperm were washed and untreated (Sperm C) and treated with vasopressin for 5 minutes (sperm VP) and for 5 minutes PKA activator 8-bromo-cAMP (sperm 8-bromo-cAMP). Representative images from N = 1.

3.4 Exploring Neuropeptide transcripts in human sperm.

To further investigate the presence of neuropeptide receptors in human sperm transcripts for vasopressin receptor 2, prolactin receptor and oxytocin receptor were explored.

Sperm cells are transcriptionally quiescent (Johnson *et al*., 2011), however the neuropeptide receptor transcripts may be present in mature sperm (carried on from spermatids and earlier cells from spermatogenesis) regardless of whether the receptor itself is present on mature human sperm. Should the transcripts be present this could indicate a possible role during spermatogenesis and sperm maturation that may be vital for mature sperm function. Oxytocin receptor primers 2,3 and 5 were optimized using cDNA from MCF-7 cells (appendix 3). However, for sperm samples optimization was not yet possible during this study and further work is required.

4.0 Discussion

This study set out to investigate the role of neuropeptides on human sperm, by localising the receptors on human sperm and examining of any physiological role.

The oxytocin and vasopressin receptors have been found throughout both male and female reproductive tissues, the epididymis, vas deferens, leydig cells, prostate, testis (Filippi *et al*., 2002) and the ovaries (Fuchs *et al*., 1990). Vasopressin, oxytocin and prolactin are found in both seminal plasma and follicular fluid (Brotherton, 1990; Schaeffer et al., 1984; Goverde et al., 1998; Laufer et al., 1984; Schoenfeld et al., 1979) indicating a possible role in sperm function and fertilisation.

In the present study, the vasopressin receptor 2 was immunolocalised on the acrosome region of the human sperm (Figure 11). However, both the oxytocin and prolactin receptors were not immunolocalised (Figures 13 and 15).

To date, the oxytocin receptor has not been localised on sperm. Oxytocin, in high levels, is known to have an affinity for the vasopressin receptor 2 (Joo *et al*., 2004, Li *et al*., 2008). Though the oxytocin was not localised in human sperm, in high levels, oxytocin may function through the vasopressin receptor 2 on sperm. Kwon *et al*. (2013) showed that when the vasopressin receptor 2 is activated in sperm it has negative effects on sperm function (motility, capacitation and acrosome reaction). One study showed that the use of an oxytocin antagonist improved the fertility of a woman who suffered 15 years of infertility and 7 failed *in vitro* fertilisation/embryo transfer attempts (Pierzynski *et al*., 2007). Oxytocin is known to be present in high levels in pregnant and breastfeeding women (Kumaresan *et al*., 1974; Nissen *et al*., 1996; Otsuki *et al*., 1983; Uvnäs-Moberg *et al*., 1990). It could therefore be postulated that oxytocin at high levels may modulate sperm function locally in the tubal milieu.

In this study the prolactin receptor was not localised on human sperm, however Pujianto *et al*. (2010) showed expression of the prolactin receptor on human sperm in particular in the tail region. They demonstrated that prolactin has prosurvival effects on human sperm via the inhibition of apoptosis, supports prolonged motility, suppresses capacitation and prevents the formation of spontaneous DNA fragmentation via caspase suppression.

Vasopressin receptor 2 was the only neuropeptide receptor localised on human sperm in this study. Therefore, the role of vasopressin in sperm function was further investigated. An easy and effective measure of function is progressive sperm motility. Motile sperm is vital for fertilisation to occur and easy to measure *in vitro* either manually or using CASA. Capacitation causes sperm to be hypermotile, an influx of intracellular calcium leads to an upregulation of PKA, all essential for acrosome reaction and fertilisation. Should time have permitted this study would have benefitted from investigating capacitation status and acrosome reaction in order to have a more complete idea of any effect that vasopressin may be having on human sperm function. Sperm incubated at various concentrations of vasopressin for 30 and 60 minutes showed no significant difference in motility between control and treated samples (Figures 16 and 17). However, Kwon *et al*. (2013) found in mouse sperm there was a dose dependant negative effect on percentage progressive sperm motility when incubated with vasopressin, but only a significant difference at the highest concentration (10 µM). They found that high quantities of vasopressin strongly inhibited the acrosome reaction and capacitation and suggested that vasopressin has strong negative effects on normal sperm function and the potential use of vasopressin as a male contraception. If the vasopressin receptor 2 has inhibitory effects on sperm function and high levels of vasopressin are present in the Fallopian tube during acute stress, sperm may be negatively affected therefore lowering fertility. This could be a method of post-coital sexual selection, whereby females may influence the behaviour of sperm locally through increasing levels of vasopressin during times of duress (Nakanishi *et al*., 2004; Sakkas *et al*., 2015).

The findings of Kwon *et al*. (2013) and the present study have revealed some differences in the localisation of the vasopressin receptor 2 on sperm. In mouse, the receptor was localised along the tail and midpiece. Furthermore, Kwon *et al*. (2013) used sperm from the caput epididymis, which are not mature and do not have the ability to be motile or fertilise an oocyte (Jones, 1999). They also used sperm isolated from the cauda epididymis and showed localisation on the acrosome, indicating that the vasopressin receptor 2 develops on the acrosome during sperm maturation (Kwon *et al*., 2013). In the present study, mature human sperm produced via masturbation and the vasopressin receptor 2 was localised exclusively on the acrosomal region (Figure 11). Further investigation into immunolocalising the

vasopressin receptor 2 on immature human sperm would support the idea that the vasopressin receptor 2 develops on sperm during maturation.

No significant effect on human sperm progressive motility was observed after treatment with vasopressin, this could be due to the small sample size used in this study with limited donors and further investigation is required.

To examine any intracellular signalling activity of vasopressin on sperm, the activity of PKA was analysed. The vasopressin receptor 2 is a G protein-coupled receptor (G_s alpha subunit) that activates the cAMP dependant pathway. The increases in cAMP which activates PKA, thus phosphorylating serine and threonine residues on target proteins (Neves *et al*., 2002).

To optimise the assay, HeLa cells were used. After treatment with 8-bromo-cAMP with PKA activator and then probed for any PKA activity via immunocytochemistry. Both control and treated samples showed strong labelling for phospho-PKA substrates throughout the cell, though no visible difference in fluorescent signal was observed (Figures 18 and 19).

PKA is activated during capacitation (Lefievre *et al*., 2002) and capacitation can be induced *in vitro* using bicarbonate. To induce sperm PKA activity in this study, sperm were treating with bicarbonate (25mM) (Da Ros *et al*., 2004; Lefievre *et al*., 2002) . The capacitated sperm showed positive labelling for phospho-PKA substrates along the tail (Figure 20) but no visible differences in fluorescence were observed between this and the control sample (Figure 20). This may be due to sperm undergoing spontaneous capacitation.

The sperm sample incubated with PKA activator 8-bromo-cAMP showed positive staining for phospho-PKA substrates along the tail and the equatorial region of the head (Figure 22). There was an increased immunofluorescence in treated sperm compared to untreated sperm, which may indicate increased PKA activity. This showed that 8-bromo-cAMP induces increased phosphorylation of on target proteins via PKA in human sperm, allowing for its use as a positive control in further assays.

After treatment with vasopressin, sperm showed positive staining for phospho-PKA substrates along the tail (Figure 23), though no visible difference in PKA activity was observed between the treated and control sample. Though it may not be a visible difference via immunofluorescence, Kwon *et al*. (2013) showed that vasopressin

decreases phospho-PKA substrates in sperm in a dose dependant manner via western blot analysis.

To assess PKA activity cells were treated with PKA activator 8-bromo-cAMP. To optimise the assay – HeLa cells were used. Extracted protein was used for western blot analysis and probed for phospho-PKA substrates. HeLa cells treated with 8-bromo-cAMP (after 5 and 30 minutes incubations) showed upregulation of PKA activity through increased phosphorylation of target proteins (Figures 24a). Further analysis showed upregulation of substrates phosphorylation around 25 and 30 kDa (Figures 24b). These data demonstrate that PKA activity can be detected after stimulation of cells *in vitro* using 8-bromo-cAMP. The next step was to examine PKA activity in sperm. Sperm were treated with 8-bromo-cAMP and vasopressin for 5 minutes. However, when western blotting was attempted with human sperm protein extract after treatment with 8-bromo-cAMP and vasopressin no conclusive data were obtained. This assay requires further optimisation and due to time restraints, this was not possible during this study.

Although sperm are transcriptionally quiescent and the oxytocin and prolactin receptors may not be present on mature human sperm, if the transcripts are present this could indicate a role for the neuropeptide and its receptor earlier in spermatogenesis. Kwon *et al*. (2013) found when immunolocalising vasopressin receptor 2, the receptor was not present on the acrosome of the caput mouse sperm (immature) until the mouse sperm was further matured in the cauda. The presence of transcripts for neuropeptide receptors in sperm was explored.

Primers for the human oxytocin receptor were optimised using cDNA from MCF-7 cells, known to express oxytocin receptor. Oxytocin receptor primer sets 2, 3 and 5 amplified as expected and the no template controls were clear (appendix 3). No conclusive data was generated for in the present study.

4.1 Limitations and future work

The major limitations of the present study were mostly technical, which include optimisation of Western blots and immunocytochemistry. Given the time scale of this study, it was not possible to acquire data that are more conclusive.

To further investigate the role of neuropeptides on sperm function, other neuropeptides that have been implicated in reproduction such as neuropeptide Y, leptin, PACAP, ghrelin and others (Brann *et al*., 2002; García *et al*., 2007; Shintani *et al*., 2002; Toufexis *et al*., 2002) should be explored via immunocytochemistry for their receptors in human sperm. If the receptor is successfully located to then explore any modulation in motility after incubation with a specific receptor agonist and antagonist.

Using proteomics to fully profile any neuropeptides found in follicular fluid and semen would give direction to further exploration of neuropeptides involved in reproduction and potentially sperm function. Any chemotactic effects of these neuropeptides could be investigated via a simple assay using a microfluidic device (Koyama *et al*., 2006).

As the vasopressin receptor 2 was localised on the acrosome region on the head of the human sperm in order to investigate the effects this receptor has when activated the experiments mentioned in this study require further optimisation and larger sample sizes in order to negate for both inherent differences in between donors and samples themselves. To further explore vasopressin's effect on human sperm function both capacitation status and acrosome reaction post-incubation with vasopressin agonist and antagonist should be explored. Percentage of positively labelled sperm should be measured in order to obtain more quantitative data. As the vasopressin receptor 2 is found on the acrosome this suggests that it may play a part in the acrosome reaction. In renal cells, the vasopressin receptor 2 triggers the signalling cascade, activating adenylyl cyclases, which activate cAMP, activating PKA resulting in the translocation of aquaporin 2 channels from the intracellular vesicle to the cell membrane (Dibas *et al*., 1998; Nielsen *et al*., 1995), aquaporin 2 has been localised in the ampulla of the vas deferens of the rat and is suggested that it is involved in sperm concentration via fluid removal from the lumen. However, the aquaporin 2 channels found in the vas deferens have been shown to not be regulated by vasopressin unlike in renal cells (Matsuzaki *et al*., 2002; Stevens *et al*., 2000). Vasopressin could potentially be important in the acrosomal shedding during acrosome reaction via increased osmosis via this pathway.

5.0 Conclusion

The presence of the receptors for neuropeptides oxytocin, vasopressin and prolactin was explored. The vasopressin receptor 2 was the only receptor localised on human sperm and any functional role was examined though results were limited. Sperm exposed to varying concentrations of vasopressin *in vitro* did not show any effect on progressive sperm motility. Sperm PKA activation by vasopressin was investigated – however, no conclusive data was obtained.

The vasopressin receptor 2 was localised to the acrosomal region. It is therefore likely that vasopressin may play a role in the acrosome reaction. Further work is required to establish any role vasopressin has on human sperm. Additionally, investigating the possible roles of other neuropeptides and sperm function is an area worth investigating, linking neuroendocrinology and sperm biology.

6.0 References

Aaronson, D.S., Horvath, C.M., 2002. A road map for those who don't know JAK-STAT. Science 296, 1653–1655.

Abou-Saleh, M.T., Ghubash, R., Karim, L., Krymski, M., Bhai, I., 1998. Hormonal aspects of postpartum depression. Psychoneuroendocrinology 23, 465–475.

Aitken, R.J., Nixon, B., 2013. Sperm capacitation: a distant landscape glimpsed but unexplored. Mol. Hum. Reprod. gat067.

Anthony W Norman, Helen L Henry, 2015. Hormones, Third. ed. Academic Press.

Brann, D.W., Wade, M.F., Dhandapani, K.M., Mahesh, V.B., Buchanan, C.D., 2002. Leptin and reproduction. Steroids 67, 95–104.

Brooks, A.J., Dai, W., O'Mara, M.L., Abankwa, D., Chhabra, Y., Pelekanos, R.A., Gardon, O., Tunny, K.A., Blucher, K.M., Morton, C.J., 2014. Mechanism of activation of protein kinase JAK2 by the growth hormone receptor. Science 344, 1249783.

Brotherton, J., 1990. Vasopressin: another pregnancy protein in human seminal plasma. Andrologia 22, 305–307.

Caldwell, H., Young III, W., 2006. Oxytocin and vasopressin: genetics and behavioral implications, in: Handbook of Neurochemistry and Molecular Neurobiology. Springer, pp. 573–607.

Cassoni, P., Sapino, A., Negro, F., Bussolati, G., 1994. Oxytocin inhibits proliferation of human breast cancer cell lines. Virchows Arch. 425, 467–472.

CH Tsai-Morris, ML Dufau, 2004. PRLR (prolactin receptor). Atlas Genet Cytogenet Oncol Haematol 8, 321–324.

Choy, V., Watkins, W., 1988. Arginine vasopressin and oxytocin in the porcine corpus luteum. Neuropeptides 11, 119–123.

Clevenger, C.V., Furth, P.A., Hankinson, S.E., Schuler, L.A., 2003. The role of prolactin in mammary carcinoma. Endocr. Rev. 24, 1–27.

Clulow, J., Jones, R., Hansen, L., 1994. Micropuncture and cannulation studies of fluid composition and transport in the ductuli efferentes testis of the rat: comparisons with the homologous metanephric proximal tubule. Exp. Physiol. 79, 915–928.

Cohen-Dayag, A., Tur-Kaspa, I., Dor, J., Mashiach, S., Eisenbach, M., 1995. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. Proc. Natl. Acad. Sci. 92, 11039–11043.

Coward, K., Wells, D., 2013. Textbook of clinical embryology. Cambridge University Press.

Curlewis, J., 1992. Seasonal prolactin secretion and its role in seasonal reproduction: a review. Reprod. Fertil. Dev. 4, 1–23.

Da Ros, V.G., Munuce, M.J., Cohen, D.J., Marín-Briggiler, C.I., Busso, D., Visconti, P.E., Cuasnicú, P.S., 2004. Bicarbonate is required for migration of sperm epididymal protein DE (CRISP-1) to the equatorial segment and expression of rat sperm fusion ability. Biol. Reprod. 70, 1325–1332.

Dibas, A.I., Mia, A.J., Yorio, T., 1998. Aquaporins (Water Channels) Role in Vasopressin-Activated Water Transport. Exp. Biol. Med. 219, 183–199.

Filippi, S., Vannelli, G., Granchi, S., Luconi, M., Crescioli, C., Mancina, R., Natali, A., Brocchi, S., Vignozzi, L., Bencini, E., 2002. Identification, localization and functional activity of oxytocin receptors in epididymis. Mol. Cell. Endocrinol. 193, 89–100.

Fuchs, A.-R., Behrens, O., Helmer, H., Vangsted, A., Ivanisevic, M., Grifo, J., Barros, C., Fields, M., 1990. Oxytocin and vasopressin binding sites in human and bovine ovaries. Am. J. Obstet. Gynecol. 163, 1961–1967.

García, M.C., Lopez, M., Alvarez, C.V., Casanueva, F., Tena-Sempere, M., Diéguez, C., 2007. Role of ghrelin in reproduction. Reproduction 133, 531–540.

Gibson, S., Tempelman, R.J., Kirkwood, R.N., 2004. Effect of oxytocin-supplemented semen on fertility of sows bred by intrauterine insemination. J. Swine Health Prod. 12, 182–185.

Gimpl, G., Fahrenholz, F., 2001. The oxytocin receptor system: structure, function, and regulation. Physiol. Rev. 81, 629–683.

Gomes, I., Wardman, J.H., Stockton Jr, S.D., Devi, L.A., 2013. Neuropeptide Receptors. Presented at the Colloquium Series on Neuropeptides, Morgan & Claypool Life Sciences, pp. 1–167.

Gonzales, G.F., 1989. Functional Structure and Ultrastructure of Seminal Vesicles. Arch. Androl. 22, 1–13. doi:10.3109/01485018908986745

Goverde, H., Bisseling, J., Wetzels, A., Braat, D., Pesman, G., Sweep, F., Meuleman, E., 1998. A neuropeptide in human semen: oxytocin. Arch. Androl. 41, 17–22.

Guidobaldi, H.A., Teves, M.E., Uñates, D.R., Anastasía, A., Giojalas, L.C., 2008. Progesterone from the cumulus cells is the sperm chemoattractant secreted by the rabbit oocyte cumulus complex. PLoS One 3, e3040.

Hamilton, D.W., Jones, A.L., Fawcett, D.W., 1969. Cholesterol biosynthesis in the mouse epididymis and ductus deferens: a biochemical and morphological study. Biol. Reprod. 1, 167–184.

Hay, N., Sonenberg, N., 2004. Upstream and downstream of mTOR. Genes Dev. 18, 1926–1945.

Helmer, R.A., Panchoo, M., Dertien, J.S., Bhakta, S.M., Hewetson, A., Chilton, B.S., 2010. Prolactin-induced Jak2 phosphorylation of RUSH: A key element in Jak/RUSH signaling. Mol. Cell. Endocrinol. 325, 143–149.

Hess, R.A., 2000. Oestrogen in fluid transport in efferent ducts of the male reproductive tract. Rev. Reprod. 5, 84–92.

Holmes, C.L., Landry, D.W., Granton, J.T., 2003. Science review: vasopressin and the cardiovascular system part 1–receptor physiology. Crit. Care 7, 1.

Jaiswal, B., Eisenbach, M., 2002. Capacitation.

Johnson, G.D., Lalancette, C., Linnemann, A.K., Leduc, F., Boissonneault, G., Krawetz, S.A., 2011. The sperm nucleus: chromatin, RNA, and the nuclear matrix. Reproduction 141, 21–36.

Jones, R., 1999. To store or mature spermatozoa? The primary role of the epididymis. Int. J. Androl. 22, 57–66.

Joo, K.W., Jeon, U.S., Kim, G.-H., Park, J., Oh, Y.K., Kim, Y.S., Ahn, C., Kim, S., Kim, S.Y., Lee, J.S., 2004. Antidiuretic action of oxytocin is associated with increased urinary excretion of aquaporin-2. Nephrol. Dial. Transplant. 19, 2480–2486.

Koyama, S., Amarie, D., Soini, H.A., Novotny, M.V., Jacobson, S.C., 2006. Chemotaxis assays of mouse sperm on microfluidic devices. Anal. Chem. 78, 3354–3359.

Kumaresan, P., Anandarangam, P., Dianzon, W., Vasicka, A., 1974. Plasma oxytocin levels during human pregnancy and labor as determined by radioimmunoassay. Am. J. Obstet. Gynecol. 119, 215–223.

Kwon, W.-S., Park, Y.-J., Kim, Y.-H., You, Y.-A., Kim, I.C., Pang, M.-G., 2013. Vasopressin effectively suppresses male fertility. PloS One 8, e54192.

Langendijk, P., Soede, N., Kemp, B., 2005. Uterine activity, sperm transport, and the role of boar stimuli around insemination in sows. Theriogenology 63, 500–513.

Laufer, N., Botero-Ruiz, W., DeCherney, A., Haseltine, F., Polan, M., Behrman, H., 1984. Gonadotropin and prolactin levels in follicular fluid of human ova successfully fertilized in vitro. J. Clin. Endocrinol. Metab. 58, 430–434.

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

Li, C., Wang, W., Summer, S.N., Westfall, T.D., Brooks, D.P., Falk, S., Schrier, R.W., 2008. Molecular mechanisms of antidiuretic effect of oxytocin. J. Am. Soc. Nephrol. 19, 225–232.

Mac E Hadley, 1996. Endocrinology, Fourth. ed.

Matsuzaki, T., Tajika, Y., Tserentsoodol, N., Suzuki, T., Aoki, T., Hagiwara, H., Takata, K., 2002. Aquaporins: a water channel family. Anat. Sci. Int. 77, 85–93.

Melck, D., De Petrocellis, L., Orlando, P., Bisogno, T., Laezza, C., Bifulco, M., Di Marzo, V., 2000. Suppression of Nerve Growth Factor Trk Receptors and Prolactin Receptors by Endocannabinoids Leads to Inhibition of Human Breast and Prostate Cancer Cell Proliferation 1. Endocrinology 141, 118–126.

Moore, H., 1998. Contribution of epididymal factors to sperm maturation and storage. Andrologia 30, 233–239.

Murgatroyd, C.A., Nephew, B.C., 2013. Effects of early life social stress on maternal behavior and neuroendocrinology. Psychoneuroendocrinology 38, 219–228.

Murgatroyd, C., Wigger, A., Frank, E., Singewald, N., Bunck, M., Holsboer, F., Landgraf, R., Spengler, D., 2004. Impaired repression at a vasopressin promoter polymorphism underlies overexpression of vasopressin in a rat model of trait anxiety. J. Neurosci. 24, 7762–7770.

Nakanishi, T., Isotani, A., Yamaguchi, R., Ikawa, M., Baba, T., Suarez, S.S., Okabe, M., 2004. Selective passage through the uterotubal junction of sperm from a mixed population produced by chimeras of calmegin-knockout and wild-type male mice. Biol. Reprod. 71, 959–965.

Neves, S.R., Ram, P.T., Iyengar, R., 2002. G protein pathways. Science 296, 1636–1639.

Nielsen, S., Chou, C.-L., Marples, D., Christensen, E.I., Kishore, B.K., Knepper, M.A., 1995. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. Proc. Natl. Acad. Sci. 92, 1013–1017.

Nissen, E., Uvnäs-Moberg, K., Svensson, K., Stock, S., Widström, A.-M., Winberg, J., 1996. Different patterns of oxytocin, prolactin but not cortisol release during breastfeeding in women delivered by caesarean section or by the vaginal route. Early Hum. Dev. 45, 103–118.

Ogawa, S., Kudo, S., Kitsunai, Y., Fukuchi, S., 1980. Increase in oxytocin secretion at ejaculation in male. Clin. Endocrinol. (Oxf.) 13, 95–97.

Okazaki, T., Ikoma, E., Tinen, T., Akiyoshi, T., Mori, M., Teshima, H., 2014. Addition of oxytocin to semen extender improves both sperm transport to the oviduct and conception rates in pigs following AI. Anim. Sci. J. 85, 8–14.

Otsuki, Y., Yamaji, K., Fujita, M., Takagi, T., Tanizawa, O., 1983. Serial plasma oxytocin levels during pregnancy and labor. Acta Obstet. Gynecol. Scand. 62, 15–18.

Ouhtit, A., Morel, G., Kelly, P.A., 1993. Visualization of gene expression of short and long forms of prolactin receptor in rat reproductive tissues. Biol. Reprod. 49, 528–536.

Overstreet, J., Cooper, G., 1978. Sperm transport in the reproductive tract of the female rabbit: I. The rapid transit phase of transport. Biol. Reprod. 19, 101–114.

Pierzynski, P., Reinheimer, T.M., Kuczynski, W., 2007. Oxytocin antagonists may improve infertility treatment. Fertil. Steril. 88, 213.e19-213.e22. doi:10.1016/j.fertnstert.2006.09.017

Pujianto, D.A., Curry, B.J., Aitken, R.J., 2010. Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. Endocrinology 151, 1269–1279.

Ralt, D., Manor, M., Cohen-Dayag, A., Tur-Kaspa, I., Ben-Shlomo, I., Makler, A., Yuli, I., Dor, J., Blumberg, S., Mashiach, S., 1994. Chemotaxis and chemokinesis of human spermatozoa to follicular factors. Biol. Reprod. 50, 774–785.

Ratnasooriya, W., Jayakody, J., 2005. Effect of vasopressin on fertility of male rats.

Roa, J., Herbison, A.E., 2012. Direct regulation of GnRH neuron excitability by arcuate nucleus POMC and NPY neuron neuropeptides in female mice. Endocrinology 153, 5587–5599.

Rotzinger, S., Lovejoy, D.A., Tan, L.A., 2010. Behavioral effects of neuropeptides in rodent models of depression and anxiety. Peptides 31, 736–756.

Ruwanpura, S.M., McLachlan, R.I., Meachem, S.J., 2010. Hormonal regulation of male germ cell development. J. Endocrinol. 205, 117–131.

Sakkas, D., Ramalingam, M., Garrido, N., Barratt, C.L., 2015. Sperm selection in natural conception: what can we learn from Mother Nature to improve assisted reproduction outcomes? Hum. Reprod. Update 21, 711–726.

Sayre, B., Lewis, G., 1997. Fertility and ovum fertilization rate after laparoscopic or transcervical intrauterine artificial insemination of oxytocin-treated ewes. Theriogenology 48, 267–275.

Schaeffer, J.M., Liu, J., Hsueh, A.J., Yen, S.S., 1984. Presence of Oxytocin and Arginine Vasopressin in Human Ovary, Oviduct, and Follicular Fluid*. J. Clin. Endocrinol. Metab. 59, 970–973.

Schoenfeld, C., Amelar, R.D., Dubin, L., Numeroff, M., 1979. Prolactin, fructose, and zinc levels found in human seminal plasma. Fertil. Steril. 32, 206–208.

Shintani, N., Mori, W., Hashimoto, H., Imai, M., Tanaka, K., Tomimoto, S., Hirose, M., Kawaguchi, C., Baba, A., 2002. Defects in reproductive functions in PACAP-deficient female mice. Regul. Pept. 109, 45–48.

Six, H.R., Kasel, J.A., 1978. Radioimmunoprecipitation assay for quantitation of serum antibody to the hemagglutinin of type A influenza virus. J. Clin. Microbiol. 7, 165–171.

Skrundz, M., Bolten, M., Nast, I., Hellhammer, D.H., Meinlschmidt, G., 2011. Plasma oxytocin concentration during pregnancy is associated with

development of postpartum depression. Neuropsychopharmacology 36, 1886–1893.

Smith, P., Krohn, R.I., Hermanson, G., Mallia, A., Gartner, F., Provenzano, Md., Fujimoto, E., Goeke, N., Olson, B., Klenk, D., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Stauss, C.R., Votta, T.J., Suarez, S.S., 1995. Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. Biol. Reprod. 53, 1280–1285.

Stevens, A.L., Breton, S., Gustafson, C.E., Bouley, R., Nelson, R.D., Kohan, D.E., Brown, D., 2000. Aquaporin 2 is a vasopressin-independent, constitutive apical membrane protein in rat vas deferens. Am. J. Physiol.-Cell Physiol. 278, C791–C802.

Surget, A., Belzung, C., 2008. Involvement of vasopressin in affective disorders. Eur. J. Pharmacol. 583, 340–349.

Thibonnier, M., Conarty, D.M., Preston, J.A., Wilkins, P.L., Berti-Mattera, L.N., Mattera, R., 1998. Molecular pharmacology of human vasopressin receptors, in: Vasopressin and Oxytocin. Springer, pp. 251–276.

Toufexis, D., Kyriazis, D., Woodside, B., 2002. Chronic neuropeptide Y Y5 receptor stimulation suppresses reproduction in virgin female and lactating rats. J. Neuroendocrinol. 14, 492–497.

Uvnäs-Moberg, K., Widström, A.-M., Werner, S., Matthiesen, A.-S., Winberg, J., 1990. Oxytocin and prolactin levels in breast-feeding women. Correlation with milk yield and duration of breast-feeding. Acta Obstet. Gynecol. Scand. 69, 301–306.

Wildt, L., Kissler, S., Licht, P., Becker, W., 1998. Sperm transport in the human female genital tract and its modulation by oxytocin as assessed by hysterosalpingoscintigraphy, hysterotonography, electrohysterography and Doppler sonography. Hum. Reprod. Update 4, 655–666.

World Health Organization, 2010a. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 5th Edition.

World Health Organization, 2010b. WHO laboratory manual for the examination and processing of human semen.

Yoshida, M., Kawano, N., Yoshida, K., 2008. Control of sperm motility and fertility: diverse factors and common mechanisms. Cell. Mol. Life Sci. 65, 3446–3457.

Zingg, H.H., Laporte, S.A., 2003. The oxytocin receptor. Trends Endocrinol. Metab. 14, 222–227.

7.0 Appendices

Recipes

<u>Western blotting recipes</u>

Separating buffer

1 g SDS
45.4 g Tris Base
Make up to 250 ml with dH_2O
Adjust to pH 8.8
Can be stored at room temperature for a few months

Stacking Buffer

1 g SDS
15 g Tris Base
Make up to 250 ml with dH_2O
Adjust to pH 6.8
Can be stored at room temperature for a few months

APS

10% solution
(e.g. 100 mg in 1 ml dH_2O)
Can be stored in the fridge for a week

Running buffer/Electrode buffer

12.02 g Tris Base
4 g SDS
57.68 g Glycine
Made up to 2 L in dH_2O
Can be stored at room temp – will run out before it goes out of date

Sample buffer/Laemmli buffer (X4)

1.51g Tris Base
20ml Glycerol
Add 25 ml dH20 and adjust to pH 6.8
Then add 4 g SDS
10 ml 2-mercaptoethanol
0.004 g bromophenol blue
Make up to 100 ml with dH_2O
Filter and store in -20 in aliquots

Towbin Buffer

1.51 g Tris Base
7.2 g Glycine
0.167 g SDS

75 ml Methanol
Make up to 500 ml with dH$_2$O
Adjust pH to 8.3 and store at room temperature

Bovine Serum Albumin (for Blocking and Antibody solutions)

1% solution – add 1 g BSA to 100 ml PBS-T
Store in fridge for approx. a week

Milk (for Blocking and Antibody solutions)

5% solution – add 5 g milk powder to 100 ml PBS-T
Store in fridge for approx. a week

Stripping Buffer

0.9 g Tris-HCL
2 g SDS
Add 100 ml of dH$_2$O and adjust pH to 6.7
Then add 800 uL 2-mercaptoethanol

TBS-T

2.422 g Tris base
16.36 g NaCL
2 ml Tween
In 2 L dH$_2$O
Adjust to pH 7.4 and store at room temperature for 7-10 days or at 4°C for a month.

RIPA Lysis Buffer

50 mM Tris base adjusted to pH 8 (0.607g)
150 mM NaCL (0.292g)
0.5% Sodium deoxycholate (0.5g)
0.1% SDS (0.1g)
1% Triton X-100 (1ml)
Make up to 100ml with dH$_2$O

Immunocytochemistry recipes

Blocking buffer

5% goat serum
Made up in PBS-T

Blocking buffer with additional ingredients

10% horse serum
0.3 M glycine
1% bovine serum albumin

In PBS-T (1%)
Other

Tris-Borate-EDTA buffer (TBE)

89 mM Tris (pH 7.6)
89 mM boric acid
2 mM EDTA
In dH_2O

Standard cell culture media

440 ml DMEM high glucose
5 ml L-glutamine
50 ml heat inactivated foetal bovine serum
5 ml penicillin-streptomycin
Filtered through 0.22 μm filter

Neutral buffered formalin (10%)

50 ml formaldehyde (37% solution)
3.25 g dibasic sodium phosphate
2 g monobasic sodium phosphate
In 500 ml dH_2O

Application Number...SE111229AØ3

Date...01/04/2014..............

**Manchester
Metropolitan
University**

**MANCHESTER METROPOLITAN UNIVERSITY
FACULTY OF (INSERT RELEVANT FACULTY HERE)**

APPLICATION FOR ETHICAL APPROVAL

Introduction
All university activity must be reviewed for ethical approval. In particular, all undergraduate, postgraduate and staff research work, projects and taught programmes must obtain approval from their Faculty Academic Ethics committee (or delegated Departmental Ethics Committee).

APPLICATION PROCEDURE

The form should be completed legibly (preferably typed) and, so far as possible, in a way, which would enable a layperson to understand the aims and methods of the research. Every relevant section should be completed. Applicants should also include a copy of any proposed advert, information sheet, consent form and, if relevant, any questionnaire being used. The Principal Investigator should sign the application form. Supporting documents, together with one copy of the full protocol should be sent to the Administrator of the appropriate Faculty Academic Ethics Committee. **(Insert contact details)**

**Your application will require external ethical approval by an NHS Research Ethics
Committee if your research involves staff, patients or premises of the NHS (see guidance notes)**

Work with children and vulnerable adults
You will be required to have a Criminal Disclosure, if your work involves children or vulnerable adults.

The Faculty Academic Ethics Committee meets every (insert period) and will respond as soon as possible, and where appropriate, will operate a process of expedited review. Applications that require approval by an NHS Research Ethics Committee or a Criminal Disclosure will take longer - perhaps 3 months.

1. DETAILS OF APPLICANT (S)
1.1 Principal Investigator: (Member of staff or student responsible for work)
   Name, qualifications, post held, tel. no, e-mail

Dr Michael Carroll, Lecturer in Reproductive Science
0161 247 1231 (michael.carroll@mmu.ac.uk)

1.2 Co-Workers and their role in the project: (e.g. students, external collaborators, etc)
Details (Name, tel. no, email)

Dr Nesser Ahmed, co-supervisor (CN)
(0161 247 1163; N.Ahmed@mmu.ac.uk)

Dr Christopher Murgatroyd, co-supervisor (CN and MA)
(01610247 1213; c.murgatroyd@mmu.ac.uk)

Dr Jamie McPhee, Co. supervisor (MA)
(J.S.McPhee@mmu.ac.uk)

Ms Clare Nevin, PhD student (clare.nevin@stu.mmu.ac.uk)

Ana-Maria Tomova (ana-maria.tomova@stu.mmu.ac.uk)

Dr Oliver Sutcliffe (O.Sutcliffe@mmu.ac.uk)

Mrs Maha Kalid Abdulkareem, PhD student

Dr Andrew Povey, External collaborator, Reader in Molecular Epigenetics, University of Manchester.
(andy.povey@manchester.ac.uk)

Prof. Daniel Brison, - Scientific Director, Dept. Reproductive Medicine, CMFT
(Daniel.brison@manchester.ac.uk)

1.3 University Department/Research Institute/Other Unit:

This research will be completed in the School of Healthcare Science, and at St. Mary's IVF Unit, Dept. Reproductive Medicine, CMFT

2. DETAILS OF THE PROJECT
2.1 Title:

Investigating the effect of lifestyle and environment on human sperm.

2.2 Description of Project: (please **outline** the background and the purpose of the research project, 250 words max.).

It is estimated that one in six couples in the UK display some level of subfertility/infertility. This accounts for approximately 3.5 million people. Many factors are responsible for both male and female infertility, including; endocrine disorders, congenital abnormalities, infection-induced pathologies and environmental toxicity. Male factor infertility accounts for ~40% of couples attending Artificial Reproductive Technology (ART) clinics. Sperm is produced continuously from a niche of stem cells (spermatogonial stem cells) residing in the testis via a process (spermatogenesis), which takes approximately 64-74 days. The exposure to various environmental compounds and products of metabolism are believed to impair spermatogenesis and sperm function.

We will investigate the nature of how these compounds damage sperm. In particular, we will, initially, focus on the role advanced glycation end products (AGEs) have on sperm integrity. AGEs are known to accumulate during aging and in patients with diabetes, autoimmune diseases, or those who smoke. The aim of this study is to examine the effects of AGE compounds on the quality and developmental potential of sperm. This study will highlight how exposure to various environmental compounds, chemical compounds (such as legal highs) and lifestyle choices can influence male reproductive potential and further our understanding of issues concerning infertility. Additionally, we will examine the protective properties of aged garlic extract and other antioxidants.

We will also investigate aberrant epigenetic profiles in sperm by measuring methylation of sperm DNA and micro-RNA expression.

In addition we will explore the role of neuropeptides on sperm function.

Describe what type of study this is (e.g. qualitative or quantitative; also indicate how the data will be collected and analysed). Additional sheets may be attached.

We will undertake quantitative laboratory-based research to measure the biological effects AGEs and other compounds have on sperm membrane structure and DNA / RNA stability. We will also investigate any Epigenetics and micro RNA alterations. The presence of various neuropeptide receptors will be examined on sperm, in addition to the effect of neuropeptides on sperm function.

Fresh human semen will be exposed to varying concentrations of aged garlic extracts - substances that have been shown to exhibit antioxidant properties that could potentially reverse the damaging effects of AGE compounds. The sperm will then be assessed for improvements in membrane integrity and DNA damage.

Semen samples will be provided from consented volunteers, who will produce the semen sample from home and bring it to the laboratory. When necessary, volunteers may be required to produce a sample on site. This will be conducted in a designated room (phlebotomy room, first floor, IRM).

2.3 Are you going to use a questionnaire?   YES
   (Please attach a copy)

   Participants will complete a general health questionnaire.

2.4 Start Date / Duration of project:

The study will begin in September 2012 and continue for 5 years.

2.5 Location of where the project and data collection will take place:

The data collection will take place in the cell and molecular science laboratories in the School of Healthcare Science and at the Dept. of Reproductive Medicine, Central Manchester Foundation Trust. .

2.6 Nature/Source of funding

Faculty of Science and Engineering PhD studentship.

2.7 Are there any regulatory requirements?   No
If yes, please give details, e.g., from relevant professional bodies

3. DETAILS OF PARTICIPANTS

3.1 How many?

100 Healthy males

3.2 Age: 18+

3.3 Sex: men

3.4 How will they be recruited?
(Attach a copy of any proposed advertisement)
They will be recruited from amongst the University staff and student, using posters, announcement boards (Screens) and verbal announcements.

3.5 Status of participants: (e.g. students, public, colleagues, children, hospital patients, prisoners, including young offenders, participants with mental illness or learning difficulties.)

General public, students and staff

3.6 Inclusion and exclusion from the project: (indicate the criteria to be applied).

Inclusion Criteria
- Age 18+
- Males
- Subjects with general good health

Exclusion Criteria
- Males who underwent vasectomies
- HIV + males
- Prepubescent males (including those under 18 years)

3.7 Payment to volunteers: (indicate any sums to be paid to volunteers).

None

3.8 Study information:
Have you provided a study information sheet for the participants?
YES
Please attach a copy of the information sheet, where appropriate

This has been appended.

3.9 Consent:

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(A written consent form for the study participants MUST be provided in all cases, unless the research is a questionnaire.)

Have you produced a written consent form for the participants to sign for your records? *YES*

A copy has been appended

4. RISKS AND HAZARDS
Please respond to the following questions if applicable

4.1 Are there any risks to the researcher and/or participants?
(Give details of the procedures and processes to be undertaken, e.g., if the researcher is a lone-worker.)

Full risk assessments have been undertaken from both the protocols used at MMU and within the School of Healthcare Science. Each worker will be fully aware of risks associated.

Semen sample: There is a risk of infection when handling any human biological fluid and semen is no exception. Every precaution will be taken to minimise this risk with the use of sterile equipment and protective gloves.

4.2 State precautions to minimise the risks and possible adverse events:

Care will be taken when handling semen and spermatozoa. Through proper training, all techniques (molecular biology, cytology will be carried out to minimize any risk). Laboratory coats, disposable gloves (and eye protection where Risk assessment of procedure indicates this as a control measure)

4.3 What discomfort (physical or psychological) danger or interference with normal activities might be suffered by the researcher and/or participant(s)? State precautions which will be taken to minimise them:

None

5. PLEASE DESCRIBE ANY ETHICAL ISSUES RAISED AND HOW YOU INTEND TO ADDRESS THESE:

There are no lasting effects from participation in this study, so the ethical issues are minimal.

6. SAFEGUARDS /PROCEDURAL COMPLIANCE
6.1 Confidentiality:

(a) Indicate what steps will be taken to safeguard the confidentiality of participant records. If the data is to be computerised, it will be necessary to ensure compliance with the requirements of the Data Protection Act.

An identify number will be assigned to each participant upon entry to the study and this number used to label all study data. De-coding information (identity of subjects) will be securely backed-up and kept only by the study personnel. All completed questionnaires and information written on to paper or other non-computerised data will

be stored in a locked filing cabinet. Data that are stored on a computer will be password protected. Data will only be accessible by the named investigators.

> (b) If you are intending to make any kind of audio or visual recordings of the participants, please answer the following questions:

> a. How long will the recordings be retained and how will they be stored?

None

> b. How will they be destroyed at the end of the project?

None

> c. What further use, if any, do you intend to make of the recordings?
> None

6.2 Human Tissue Act:

The Human Tissue Act came into force in November 2004, and requires appropriate consent for, and regulates the removal, storage and use of all human tissue.

> a. Does your project involve taking tissue samples, e.g., blood, urine, hair, etc., from human subjects? YES – human semen

> b. Will this be discarded when the project is terminated? NO

> If NO – Explain how the samples will be placed into a tissue bank under the Human Tissue Act regulations:

All semen and spermatozoa will be stored according to our standard practice that conforms to the Human Tissue Act. Samples will be labelled with the participant ID number and stored in the -80 freezer until analysis of nucleic acids, seminal contents and spermatozoa are required. A digital or written record of the sample location will be kept alongside copies of the signed, informed consent. Sample will be retained for a period of no more than 10 years.

For sperm usage and storage used solely for research, and not for licensed treatment (IVF), the HFE Exemption Regulations (2009) are relevant. Effectively these regulations provide an exemption from the HFE Act for certain defined activities and purposes. Research only storage and use of sperm is one of these, hence research donated sperm is effectively considered as non-licensable material under the HFE Act and can be stored and used on unlicensed premises.

6.3 Insurance:

The University holds insurance policies that will cover claims for negligence arising from the conduct of the University's normal business, which includes research carried out by staff and by undergraduate and postgraduate students as part of their courses. This does **not** extend to clinical negligence. There are no

arrangements to provide indemnity and/or compensation in the event of claims for non-negligent harm.

Will the proposed project result in you undertaking any activity that would not be considered as normal University business? If so, please detail below:

Yes

6.4 Notification of Adverse Events (e.g., negative reaction, counsellor, etc):
(Indicate precautions taken to avoid adverse reactions.)

Please state the processes/procedures in place to respond to possible adverse reactions.

The procurement of semen carries very low adverse reactions. However, the procedures in place to deal with any adverse events have been explained where necessary above. We will also contact a University First Aid representative and emergency medical attention will be sought wherever necessary (e.g. in any instance where fainting may occur).
All adverse events will be reported to the Faculty Ethics Committee at the earliest opportunity.

In the case of clinical research, you will need to abide by specific guidance. This may include notification to GP and ethics committee. **Please seek guidance for up to date advice**, e.g., see the NRES website at http://www.nres.npsa.nhs.uk/

SIGNATURE OF PRINCIPAL INVESTIGATOR   DATE:

M. Carroll

....

SIGNATURE OF FACULTY ACADEMIC ETHICS   DATE:
COMMITTEE CHAIRPERSON:
.............William Graham..........................   5th Oct 2015

APPENDIX

Checklist of attachments needed:
1. Participant information sheet
2. Participant consent form
3. Semen procurement form
4. Medical
5. Study procedures
6. Risk assessment

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Appendix

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1.

Participant information sheet

Title of Study: *Investigating the effect of lifestyle and environment on human sperm.*

Study Background

Male factor infertility can account for 40% of infertility experienced by couples trying to conceive. As sperm is produced continuously in the testis through the process of spermatogenesis – the sperm cells are vulnerable to damage. This damage can occur in the sperm cell membrane and the DNA and RNA. In addition to damage to sperm nucleic acids, alteration in epigenetic status can occur, which include aberrant DNA methylation and microRNA expression.
We will investigate how environmental compounds, various drugs and lifestyle exposures can cause this damage. This information will offer potential therapeutic options that may improve male infertility.

Who can take part?

Any male aged over 18 years old.

What is involved?

You will be required to provide a semen sample either at home or within a secure room at the school of healthcare science. You will produce this semen via masturbation. A full sample is required. You will be provided with a sterile container from which to deposit your specimen.
The semen sample will be assessed for parameters such as motility (how well the sperm are moving), morphology (the shape of the sperm cells) and concentration (the number of sperm per millilitre of seminal fluid). The sperm will then undergo a variety of biochemical and molecular biology tests. The samples will be stored at -80°C for further analysis.
Your sperm sample will NOT, at any time, be used for any assisted reproductive techniques and will ONLY be used for research or teaching purposes.
You will also be asked to provide a blood sample (optional) and saliva. The blood will be taken by an experienced phlebotomist and is a quick and painless procedure. For the saliva sample, you will just spit in a sterile container Both blood and saliva will be prepared and stored at 80°C until required. These samples will be used for biochemical and molecular analysis.

Please note that this is not a diagnostic test and you will not be informed of the quality of your semen/sperm.

Are there any risks in taking part in the study?

There may be a slight risk of fainting due to the physical activity of semen production. If you are providing the sample on site, the secured room can be locked from the inside. After an allotted time has elapsed, a study team member will knock on the door to ensure you are OK. If there is no answer, they will enter the room using a key to establish your status.

2.

Participant informed consent.   ID code ....

Name: ....   Sex: Male / Female

Date of Birth: ....   Age: ....

Project title:

Investigating the effect of lifestyle and environment on human sperm.

Principal Investigator:
Dr Michael Carroll

Investigator/Collaborators:
Dr Nesser Ahmed
Dr Christopher Murgatroyd
Dr Oliver Sutcliffe
Prof. Daniel Brison (external collaborator)

Ethics approval number:

I have read and understood the information sheet and all verbal explanation outlining
the purpose of the study and the assessments involved.

Any questions I have about the study, or my participation in it, have been answered to
my satisfaction. I understand that I do not have to take part and that I may decide to
withdraw from the study at any point without having to give a reason. I understand that
my sperm will <u>not</u> be used, at any time, for any form of assisted reproductive technique,
such as fertilising human eggs or the creation of human embryos. I understand that
my semen may be also used for teaching purposes. My concerns regarding this study
have been answered and such further concerns as I have during the time of the study
will be responded to. It has been made clear to me that, should I feel that my rights are
being infringed or that my interests are otherwise being ignored, neglected or denied,
I should inform the Chair of the Ethics Committee of the School of Healthcare
Sciences, Manchester Metropolitan University, Oxford Road, Manchester, M1 5GD.

Signed _____   Date _____

I give my consent for semen collected from me during the course of this study to be
retained in the study biobank at MMU for retrospective biochemical and molecular biology
analysis.

                                                        Date

Signed _____   _____

Name (Print)....................................
3.
Witnessed _____ Date _____

Semen Procurement Form.

Introduction

Please follow the instructions concerning the production of a semen sample.

As a fresh sample is essential for this test, it must be brought to the laboratory within one hour of being passed at home.

NOTE: USING A PUBLIC TOILET TO PASS YOUR SAMPLE IS AGAINST THE LAW.

Instructions for collecting the semen sample

You should abstain from intercourse or masturbation for three to four days prior to providing the specimen.

The sample must be obtained by masturbation (manual stimulation) and should be collected directly into the specimen container provided. A condom or artificial lubrication must not be used for semen collection, as it will kill the sperm.

The complete specimen is needed for this study, so if any is spilt you must tell us, as a repeat specimen may be required.

Label the specimen container with your full name, date of birth and the date and time the specimen was passed.

Delivery of your sample

Deliver the sample to the School of Healthcare Science within **one hour** of passing the sample.

Keep the sample at body temperature while being transported to the laboratory, for example by carrying it in an inside pocket. Excessive cold or heat can damage the sperm.

For further information contact:
Dr M. Carroll (*michael.carroll@mmu.ac.uk*)
Phone: 0161 247 1231

Specimen details

Please complete this form and bring it with you to the laboratory along with your sample and the request form.

Name:_____

Date of Birth: _____

Date of specimen Time passed:_____

Time specimen passed: _____

Abstinence (days): _____

I confirm that this semen specimen is mine and I consent to its use for the purpose of research and education.

Signed

4.
Medical Screening Questionnaire

It is important that the investigators are aware of any health conditions before participation in this research study. This information will be kept strictly confidential.

Please answer the following questions as accurately as possible.

Are you currently taking any prescribed medication?	**YES/NO**
Are you currently attending your GP?	**YES/NO**
Have you ever suffered from a cardiovascular problem? i.e. *high blood pressure, anaemia, heart attack etc*	**YES/NO**
Have you ever suffered from a neurological disorder? i.e. *epilepsy, convulsions etc*	**YES/NO**
Have you ever suffered from an endocrine disorder? i.e. *diabetes etc*	**YES/NO**
Have you ever suffered from a chronic gastrointestinal disorder? i.e. *Crohn's disease, irritable bowel syndrome etc*	**YES/NO**
Have you ever suffered from a skin disorder? i.e. *eczema etc*	**YES/NO**
Do you suffer from any allergies? i.e. *any medications, foods etc*	**YES/NO**
Have you had a vasectomy or any urological surrey? i.e. *testicular surgery*	**YES/NO**
Have you had Mumps?	**YES/NO**
Have you had any testicular injuries / torsions?	**YES/NO**
Do you knowingly have, or had a Sexually Transmitted Infection?	**YES/NO**

If you have answered "yes" to any of these questions, please provide details below:

5.

Study Procedures

The following is a brief description of the procedures and techniques that will be employed during this study.

Semen procurement:

Semen will be produced by masturbation in to sterile containers provided. On occasions where participants cannot provide a specimen from home they will be asked to produce a sample on site in a dedicated, secured room. The specimen container will be placed in the plastic bag with the completed 'semen procurement form'.

Semen analysis:

Semen analysis is carried out within 30 minutes of specimen production. Volume, pH and other physical characteristics are noted. Sperm motility and concentration is measured and a sample of semen is smeared on to a glass slide for fixing and morphological and cytology analysis.

Blood analysis:
Blood will be taken and used for biochemical analysis. Blood hormones and biomolecules will be analysed.

Biochemical analysis:

Reaction Oxygen Species (ROS) are measured using the luminal assay and read by Luminescence. The effects of ROS on sperm membranes will be measured through quantification of the end-products of lipid peroxidation, malondialdehyde (MDA). The thiobarbituric acid reactive substances assay (TBARS Assay) will be used for this investigation. Thiobarbituric acid reacts with malondialdehyde to yield a fluorescent product. Hormone and other bioactive molecules will be measured in the semen.

DNA/RNA studies:

DNA and RNA will be isolated from both fresh and frozen sperm cells using commercial kits (QIAGEN) and examined for the expression levels of key genes responsible for sperm function. DNA methylation studies will be conducted also.

Chromatin assays such as chromatin dispersion assay and comet assays will be conducted to investigate the integrity of the sperm nucleus after exposure to various compounds.

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6.

The MANCHESTER METROPOLITAN UNIVERSITY
Faculty of Science and Engineering
RISK ASSESSMENT COVER SHEET

REFERENCE NUMBER (MC/01/10/12):   01 /10/2012 E202
(XYZ = initials of the originator; ddmmyy = date; LLn.nn = room number)

SCHOOL:
Healthcare Science (Faculty of Science and Engineering)

TITLE OF WORK:

Procurement of human semen

LOCATION OF WORK

Phlebotomy room (T1.19c), IRM, Ground floor, John Dalton Building.

INTENDED ACTIVITIES (attach methods sheets (e.g. standard operating practices) and work schedules to this form):

PERSONS AT RISK:

Participant producing sample

HAZARDS

1. HAZARD:

Slight risk of fainting due to the physical activity.

2. HAZARD:

Falling hazard and injury due to fainting

Are these hazards necessary in order to achieve the objectives of the activity?

Hazard Rating (delete as appropriate):   Very low

HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION (appropriate COSHH data sheets / risk assessments must be attached to this form):
ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR CORRECT HAZARD WARNING LABELS.

NAME OF MATERIAL *Please provide also approximate quantity and concentration if applicable.*	**HAZARD CLASS**	**HAZARD LABEL**	**DISPOSAL** *Hazardous materials must not be removed from laboratories. List disposal arrangements for* all materials listed below *in the location where* the work will be carried out:
N/A	N/A	N/A	N/A

RISK CONTROL METHODS (provide a summary of the hazards anticipated and attach detailed assessments with appropriate risk control methods to this form):

The room used for the procurement of semen will be maintained to minimize any clutter that may cause potential harm. The room has the capacity to be locked from the inside to ensure privacy. However, in the unlikely event of the participant fainting the door can be unlocked from the outside with a key.

Additionally, a member of the study team will be aware of the time the participant enters the room; should a stated time elapse, the study team member will knock on the door to knock to ensure the participant is OK. If there is no answer, the team member can enter the room using the key to evaluate the status of the participant.

Hazard Rating with Control Methods (delete as appropriate):

*Will any specific **training** be required (if YES give details)?*

NA

*Are there any specific **first aid** issues (if YES give details)?*

The appropriate first aider will be notified in the case of any injury obtained during the procedure.

PROCEDURE FOR EMERGENCY SHUT-DOWN (if applicable):

Not applicable

IF OFF-SITE INDICATE ANY OTHER ISSUES (e.g. associated with: individual's health and dietary requirements (obtain off-site health forms for all participating individuals and indicate where this information will be located); social activities, transportation, ID requirements; permissions for access and sampling).

	NAME	STAFF/STUDENT No.	DATE
Originator	Dr Michael Carroll	55039387	01/10/12
Supervisor (*XYZ*)			
Technical Manager			
Divisional / School Health and Safety Coordinator (p.p. HoS)	Prof. Mark Slevin		
DATE TO BE REVIEWED BY:			

Polymerase Chain Reaction

Table 8. Oxytocin receptor primers.

| Target Gene	Forward Primer	Reverse Primer	Amplicon Size
Oxytocin Receptor (OXTR 1)	CCGCACGGTCAAGA TGACTT	ATGAAGGCCGAGGCT TCCTTG	123
Oxytocin Receptor (OXTR 2)	CAAGCTCATCTCCAA GGCCA	GAAGGCCGAGGCTTC CTTG	145
Oxytocin Receptor (OXTR 3)	GTCTACATCGTGCC GGTCAT	TGAAGGCCGAGGCTT CCTTG	313
Oxytocin Receptor (OXTR 4)	TGCTGGACGCCTTT CTTCTT	GAAGGCCGAGGCTTC CTT	75
Oxytocin Receptor (OXTR 5)	CGCCCAAGGAAGCC TCG	GGCTCAGGACAAAGG AGGAC	213
Oxytocin Receptor (OXTR 6)	GACGCCTTTCTTCTT CGTGC	ATGAAGGCCGAGGCT TCCTTG	72
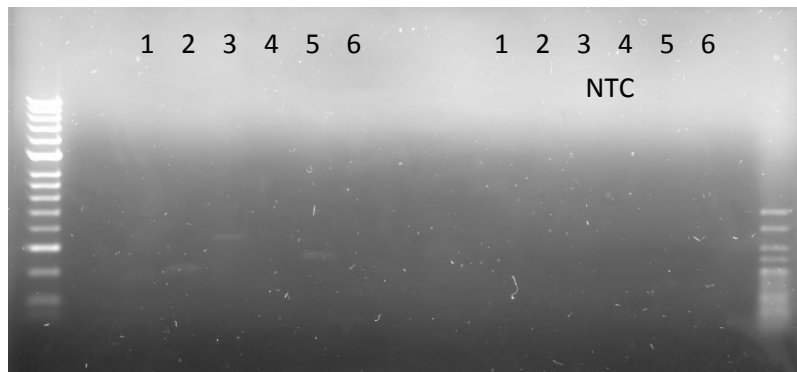
Figure 26. Agarose gel electrophoresis (2%) of end-point polymerase chain reaction optimising oxytocin receptor primers and no template controls. Primers 1-6 optimised using cDNA extracted from human breast cancer MCF-7 cells and primers (1-6) no template control (NTC). Representative images from N = 1.

Oxytocin receptor primers 1-6 were optimized using RNA extracted from human breast cancer MCF-7 cells cultured in standard media and extracted via Trizol method and converted to cDNA using Promega's GoTaq® 2-step RT qPCR kit (methods 2.whatever) as a positive control as they are known to express oxytocin

receptor. Oxytocin receptor primers 2,3 and 5 worked as expected, amplicon size was shown via gel electrophoresis, primers 1,4 and 6 showed no amplification. No template controls were clear showing no contamination.
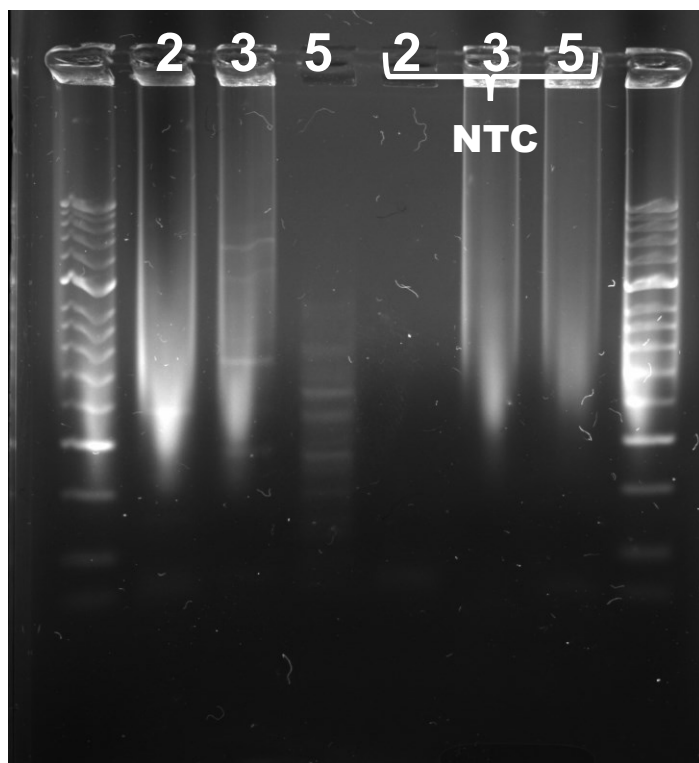

Figure 27. Agarose gel electrophoresis. Oxytocin primers on human sperm cDNA. Contamination present throughout. Primers 2, 3 and 5 optimised using cDNA extracted from human sperm cells and primers (2, 3, 5) no template control (NTC). Representative images from N = 1.